The major site of phosphorylation of the epidermal growth factor (EGF) receptor after treatment of cells with EGF is threonine 669. Phosphorylation of this site is also associated with the transmodulation of the EGF receptor caused by platelet-derived growth factor and phorbol ester. A distinctive feature of the primary sequence surrounding threonine 669 is the proximity of 2 proline residues (-Pro-Leu-Thr-Pro-). This site is not a substrate for phosphorylation by protein kinase C. To investigate the mechanism of the increased phosphorylation of the EGF receptor at threonine 669, in vitro assays were used to measure protein kinase and protein phosphatase activities present in homogenates prepared from cells treated with and without EGF. No evidence for the regulation of protein phosphatase activity was obtained in experiments using the [32P]phosphate-labeled EGF receptor as a substrate. A synthetic peptide corresponding to residues 663-681 of the EGF receptor was used as a substrate for protein kinase assays. Incubation of murine 3T3 L1 pre-adipocytes and human WI-38 fibroblasts with EGF caused a rapid increase (3-10-fold) in the level of threonine protein kinase activity detected in cell homogenates. Similar results were obtained after EGF treatment of Chinese hamster ovary cells expressing wild-type (Thr669) and mutated (Ala669) human EGF receptors. Activation of the threonine protein kinase activity was also observed in cells treated with platelet-derived growth factor, serum, and phorbol ester. Insulin-like growth factor-1 caused no significant change in protein kinase activity. Together these data indicate a role for the regulation of the activity of a threonine protein kinase in the control of the phosphorylation state of the EGF receptor at threonine 668. The significance of the identification of a growth factor-stimulated threonine protein kinase to the mechanism of signal transduction is discussed.

The cell surface receptor for epidermal growth factor (EGF) is a 170-kDa transmembrane glycoprotein. The binding of EGF to the extracellular domain of the receptor causes an increase in the tyrosine protein kinase activity of the receptor cytoplasmic domain (1). Treatment of cells with PDGF or with phorbol ester causes rapid alterations in the apparent affinity and tyrosine protein kinase activity of the EGF receptor. This process has been termed transmodulation (1). Treatment of human fibroblasts with EGF, PDGF, or with phorbol ester causes an increase in the phosphorylation state of the EGF receptor at several sites (2). One of these sites, threonine 654 (3, 4), is a substrate for protein kinase C. It has been proposed that the phosphorylation of the EGF receptor by protein kinase C at threonine 654 is mechanistically related to EGF receptor transmodulation (1, 2). Recently this hypothesis has been critically tested by site-directed mutagenesis of the EGF receptor cDNA and the investigation of the properties of wild-type and mutated receptors expressed in cultured cells. Substitution of threonine 654 with an alanine (5) or a tyrosine (6) residue does not significantly affect the regulation of EGF binding caused by PMA (but see Ref. 7). These data indicate that the phosphorylation of threonine 654 by protein kinase C is not required for the inhibition of EGF binding caused by PMA and that other mechanisms must account for this action. However, it is possible that the mechanism of receptor regulation does involve the phosphorylation of the EGF receptor. This is because multiple EGF receptor serine and threonine residues are phosphorylated in phorbol ester (3, 4, 8-11), diacylglycerol (12, 13), and PDGF (14, 15)-treated cells under conditions in which a decreased apparent affinity of the receptor is observed. These sites are also phosphorylated in phorbol ester-treated CHO cells that express [Ala654]EGF receptors (5). It is therefore possible that the phosphorylation of one or more of these sites is responsible for the regulation of the apparent affinity of the EGF receptor.

Recently the major site of regulated phosphorylation of the EGF receptor has been identified as threonine 669 (16). EGF receptor threonine 669 is not a substrate for phosphorylation by protein kinase C. However, threonine 669 is located close to the site phosphorylated by protein kinase C, threonine 654 (3, 4). The primary sequence surrounding threonine 654 contains several basic residues and is similar to the sequence of protein kinase C substrate sites on other proteins. In contrast, there are no basic residues in the primary sequence surrounding threonine 669. A distinctive feature of the location of threonine 669 is the proximity to 2 proline residues. The mechanism by which threonine 669 is phosphorylated in cells incubated with EGF, PDGF, or phorbol ester is not understood.

The purpose of the experiments reported here was to evaluate the role of the phosphorylation of the EGF receptor at threonine 669 and to determine the mechanism by which EGF, PDGF, and phorbol ester regulate the phosphorylation of the EGF receptor at this site. There are three potential mechanisms that could account for the regulation of the
phosphorylation state of threonine 669: 1) activation of a protein kinase; 2) inhibition of a protein phosphatase; and 3) EGF binding and receptor transmodulation may alter receptor conformation or change the receptor subcellular distribution. The approach that we used to examine these hypotheses was to measure the activities of protein kinases and protein phosphatases present in cell homogenates that utilize threonine 669 as a substrate. We report that no marked alteration in protein phosphatase activity was detected, but that EGF, PDGF, and phorbol ester markedly stimulated the activity of a protein kinase that phosphorylated threonine 669. The effect of EGF to stimulate this protein kinase activity was also observed in cells expressing a mutated [Ala69]EGF receptor. Together these data indicate a role for the regulation of protein kinase activity in the control of the phosphorylation state of the EGF receptor at threonine 669.

EXPERIMENTAL PROCEDURES

Materials

EGF was isolated from mouse submaxillary glands as described (17, 18) and iodinated by the immobilized lactoperoxidase procedure (19). [γ-32P]ATP and [32P]phosphate were from Du Pont-New England Nuclear. [35S]Methionine and [35S]A95 were from Amersham Corp. [3H]Thymidine was from ICN. The synthetic peptide T669 (NH2-Glu-Leu-Val-Glu-Pro-Leu-Thr-Pro-Ser-Gly-Glu-Ala-Pro-Ann-Gln-Ala-Leu-Leu-Ara-COOH) was prepared using an Applied Biosystems model 430A machine (Peptide Synthesis Core Facility, University of Massachusetts Medical School) and was purified by reverse-phase HPLC. Platelet-derived growth factor (porcine) was obtained from Bioprocessing Ltd. (Consett, Great Britain). Insulin-like growth factor-1 was from Amgen (Thousand Oaks, CA). Amethopterin and phorbol esters were from Sigma. Restriction enzymes, polynucleotide kinase, T4 DNA ligase, and Klenow were from Boehringer Mannheim.

Plasmid Construction

Oligonucleotide-directed mutagenesis of ThP+ (ACA), ThP- (ACC), and Thr- (ACA) to alanine (GCA or GCC) was carried out using 17-mer oligonucleotides according to Zoller and Smith (20) using methods described previously (21). The wild-type and mutated EGF receptor cDNAs were cloned as 4-kilobase XbaI-HindIII fragments into the expression vector pX (obtained from Dr. G. Johnson, Jewish National Center, Denver) which 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. The plasmids obtained were designated pXER, pXER(Ala69), pXER(Ala79), and pXER(Ala95).

Cell Culture

3T3-L1 and WI-38 fibroblasts were obtained from the American Type Culture Collection and were maintained in modified Eagle's medium supplemented with 5% calf serum. A431 cells were obtained from Dr. G. Todaro (Oncogene) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. A431 cells were obtained from Dr. G. Todaro (Oncogene) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. A431 cells were obtained from Dr. G. Todaro (Oncogene) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. A431 cells were obtained from Dr. G. Todaro (Oncogene) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. A431 cells were obtained from Dr. G. Todaro (Oncogene) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. A431 cells were obtained from Dr. G. Todaro (Oncogene) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. A431 cells were obtained from Dr. G. Todaro (Oncogene) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. A431 cells were obtained from Dr. G. Todaro (Oncogene) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum.

Phosphorylation of Synthetic Peptide T669 in Vitro

Cells were seeded in 35-mm wells and grown to a density of 2 × 10^6 cells/well. The monolayers were washed in serum free medium incubated for 30 min with 1 ml of 10 mM NaCl, 120 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 25 mM HEPES (pH 7.4), 30 μM bovine serum albumin at 37 °C. The cells were then treated without and with growth factors or phorbol ester for defined times. The medium was aspirated and the cells were collected by scraping in 0.5 ml of 25 mM HEPES (pH 7.4), 5 mM EGTA, 50 mM NaF, 10 μg/ml leupeptin (0 °C), and were homogenized by 10 passages through a 26-gauge needle. Phosphorylation reactions were performed at 22 °C using 5 μl of the cell extract, 25 μl of 50 mM HEPES (pH 7.4), 20 mM MgCl2, and 10 μl of synthetic peptide (5 mg/ml). The phosphorylation reaction was initiated by the addition of 10 μl of 50 μM [γ-32P]ATP (100 μCi/nmol) and was terminated by the addition of 10 μl of 90% formic acid. Two procedures were used to isolate the phosphorylated synthetic peptide from the reaction mixture.

High Pressure Liquid Chromatography—This purification procedure was used for experiments designed to investigate the physical and chemical properties of the phosphorylated synthetic peptide (Fig. 1). The synthetic peptide T669 phosphorylated in vitro (60 μl) was diluted with 1 ml of 0.1% trifluoroacetic acid (v/v) and applied to a C18 Sep-pak cartridge (Millipore). The Sep-pak was washed with 10 ml of 0.1% trifluoroacetic acid and subsequently with 6 ml of 5% formic acid. The phosphorylated peptide was eluted from the Sep-pak with 1 ml of 99.9% acetonitrile, 0.1% trifluoroacetic acid. The eluant was lyophilized, dissolved in 0.5 ml of 0.1% trifluoroacetic acid and injected onto a Vydac C4 reverse-phase HPLC column (0.46 × 25 cm) equilibrated with 0.1% trifluoroacetic acid. The column was eluted with a linear gradient of acetonitrile (1%/min) in 0.1% trifluoroacetic acid. The eluant was monitored using either detectors for both absorbance at 214 nm and Cerenkov radiation. The synthetic peptide T669 eluted from the column at 28.5% acetonitrile and was detected by absorbance at 214 nm. After phosphorylation with [γ-32P]ATP two peaks of UV absorbance were eluted from the column at 27 and 28.5% acetonitrile. A peak of Cerenkov radiation was detected at 27% acetonitrile, but not at 28.5% acetonitrile. The peak of UV absorbance and Cerenkov radiation eluted from the column at 27% acetonitrile was not observed if either the ATP or the synthetic peptide were omitted from the phosphorylation reaction. Analysis by thin layer electrophoresis demonstrated the presence of a single radioactive peptide which stained with ninhydrin and was detected by autoradiography. Phosphoamino acid analysis indicated the presence of [32P]Phosphothreonine. These observations suggest that the radioactive UV absorbance peak that eluted from the reverse-phase column at 27% acetonitrile was a phosphorylated form of the synthetic peptide T669.

Thin Layer Electrophoresis—This method was used for assays designed to measure the activity of protein kinases present in cell extracts using the peptide T669 as a substrate. The synthetic peptide was isolated from the phosphorylation reaction mixture by electrophoresis (4 °C) for 3 h at 500 V on a 100-μm cellulose thin layer plate using 30% (v/v) formic acid as solvent. The phosphorylated peptide was identified by autoradiography, and the incorporation of radioactivity into the peptide was quantitated by liquid scintillation counting.

Protein Phosphatase Assays

A431 cells were seeded in 35-mm wells and grown to a density of 1 × 10^6 cells/well. The cells were washed in phosphate-free Dulbecco's modified Eagle's medium and incubated in 1 ml of the same medium supplemented with 0.1% calf serum and 1 mCi/ml [32P]Phosphate. The cells were incubated for 4 h at 37 °C. EGF receptors were isolated by immunoprecipitation as described (4). The immunoprecipitates were washed and incubated in 45 μl of 25 mM HEPES (pH 7.4), 5 mM MgCl2, 10 μg/ml leupeptin at 22 °C with 5 μl of cell extract. The cell extracts were prepared as described for the protein kinase assays. After defined times the phosphorylation reaction was terminated by the addition of 120 μl of the radiolabeled UV absorbance peak with 5% dialyzed fetal bovine serum, 0.5 μM amethopterin, and 0.25 mg/ml G418. Stable colonies were isolated using cloning rings and screened for the expression of EGF receptors by measuring the cell surface binding of 125I-EGF at 0 °C. No specific binding of 125I-EGF was observed to the parental CHO cells.
Analysis of $^{125}$I-EGF Binding

CHO cells were seeded in 16-mm wells and grown to a density of 5 x 10⁴ cells/well. The cells were then incubated for 48 h in medium supplemented with 0.1% calf serum. Growth factors were added to the medium together with 5 µCi/ml $[^{3}H]$thymidine. After a further 24 h of incubation, the incorporation of radioactivity into acid-insoluble material was measured as described (12).

**Analysis of $[^{3}H]$Thymidine Incorporation**

CHO cells were seeded in 16-mm wells and grown to a density of 5 x 10⁴ cells/well. The cells were then incubated for 48 h in medium supplemented with 0.1% calf serum. Growth factors were added to the medium together with 5 µCi/ml $[^{3}H]$thymidine. After a further 24 h of incubation, the incorporation of radioactivity into acid-insoluble material was measured as described (12).

**Purification of EGF Receptor Tryptic ([32P])Phosphopeptides**

A431 cells were labeled with $[^{32}P]$phosphate for 20 h by incubation with phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 0.1% calf serum and 2 µCi/ml $[^{3}P]$phosphate. EGF receptors were isolated from the cells by incubation of detergent extracts with a polyclonal anti-EGF receptor antibody as described (15). The samples were heated at 80 °C for 15 min in the presence of 80 µl of 10% sodium dodecyl sulfate, 14 µl diithiothreitol. After cooling, the EGF receptors were alkylated by adding 40 µl of 0.4 M iodoacetamide, 0.25 µl Tris-HCl (pH 8.8), and incubation at room temperature for 15 min. Subsequently, 80 µl of 75% glycerol, 220 µl 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 sodium dodecyl sulfate and precipitated with trichloroacetic acid as described (18). 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 C₈ column (0.46 x 25 cm) equilibrated with 10% acetonitrile (1% min) in 0.1% trifluoroacetic acid. Fractions were collected at 20-s intervals, and the $[^{32}P]$phosphopeptides were detected by measuring the Cerenkov radiation associated with each fraction. The peptide containing the major site of EGF receptor threonine phosphorylation eluted from the column at 27% acetonitrile and has been described previously (4, 5, 16).

**Two-dimensional Phosphopeptide Mapping**

$[^{32}P]$Phosphopeptides were analyzed by two-dimensional separation on 100-µm cellulose thin layer plates by electrophoresis in 30% formic acid (v/v) for 2 h at 400 V and ascending chromatography using water/butan-1-ol/pyridine/acetate acid (60:25:5:5:15) as solvent. The mobilities of the peptides was analyzed by autoradiography.

**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 Sefton (24) as described (4).

**Automated Amino-terminal Sequence Analysis**

Sequence analysis of $^{[32}P]$phosphate-labeled peptides was performed in the presence of 4 µl of adenylate kinase and 1.1 µM ATP using an automated Beckman 490C liquid-phase sequenator and a 0.1 M Quadrol Program (Beckman 121078). Two precycles were performed prior to the first cleavage. The anilino-thiazolines were converted to phenylthiobidanions by reaction in 25% trifluoroacetic acid at 50 °C and were identified and quantitated by a modification of the reverse-phase HPLC procedure described by Zimmerman et al. (25) using acetonitrile. The radioactivity associated with the phenylthiobidanions was determined with the postulated peak at the same position as the peak of interest, followed by an additional 20-s interval of reverse-phase HPLC elution from the column at 27% acetonitrile (4, 15).

**Characterization of Synthetic Peptide T669—A431 human epidermoid carcinoma cells were labeled with $[^{32}P]$phosphate, and the EGF receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis. The receptors were eluted from the gel and digested with trypsin. The $[^{32}P]$phosphopeptides obtained were separated by reverse-phase HPLC. The peptide containing the major site of EGF receptor phosphorylation in vitro was eluted from the column at 27% acetonitrile (4, 15) and collected. The radiochemical purity of the phosphopeptide was investigated by two-dimensional chromatography and electrophoresis on a 100-µm cellulose thin layer plate. Autoradiography of the thin layer plate demonstrated a single $[^{32}P]$phosphopeptide (Fig. 1). This $[^{32}P]$phosphopeptide was used for phosphoamino acid analysis and radiochemical sequencing. Fig. 1 shows that the peptide contained a $[^{32}P]$phosphothreonine residue that was released at the seventh cycle of automated Edman degradation. Inspection of the cDNA sequence of the EGF receptor (26) indicated three predicted receptor tryptic peptides that contained a threonine residue located at position 7 from the amino terminus. These threonine residues are located at positions 669, 759, and 969 in the predicted primary sequence of the EGF receptor (26). To identify which of these threonine residues is the phosphorylation site, oligonucleotide-directed mutagenesis of the receptor cDNA was used to replace each residue with alanine. The mutated cDNAs were cloned into expression vector and transfected into CHO cells. Stable clones expressing EGF receptors were isolated. The clones were labeled with $[^{32}P]$phosphate and the in vivo phosphorylation state of the EGF receptors was investigated. Similar $[^{32}P]$phosphopeptide maps were observed for wild-type, [Ala⁷]EGF, and [Ala⁸]EGF receptors. In contrast, the incorporation of $[^{32}P]$phosphate into the [Ala⁷]EGF receptor was observed to be very low (not shown). These data are consistent with the possibility that threonine 669 is the major site of EGF receptor phosphorylation in vivo. To confirm this hypothesis, a synthetic peptide was prepared that corresponds to residues 663-681 of the EGF receptor. This synthetic peptide was identical to the predicted EGF receptor tryptic peptide that contains threonine 669 and was designated T669. A phosphorylated derivative of the synthetic peptide was prepared by incubation of T669 with [γ-$^{32}$P]ATP as described under “Experimental Procedures.” The phosphorylated peptide was then purified by reverse-phase HPLC and was eluted from the column at 27% acetonitrile. The synthetic peptide phosphorylated in vitro and the receptor tryptic phosphate phosphorylated in vivo were compared by two-dimensional peptide mapping and by phosphoamino acid analysis. No significant difference between the properties of the two peptides was observed (Fig. 1). We conclude that the major site of phosphorylation of the EGF receptor in vivo is threonine 669. A similar conclusion has recently been reported by Heisermann and Gill (18).

**Characterization of the [Ala⁷]EGF Receptor—Wild-type and [Ala⁷]EGF receptors were expressed in CHO cells. No specific binding of $^{125}$I-EGF was observed to the parental cells, but specific high affinity binding of $^{125}$I-EGF to the surface of the transfected cells was detected. The $^{125}$I-EGF binding isotherm was investigated (Fig. 2) and found to be curvilinear when plotted by the method of Scatchard (27). Analysis of the binding isotherm using the computer program LIGAND (28) showed that a two-site model provided a significantly better description of the experimental data than a one-site model (p > 0.97). The results of this analysis are summarized in Table I. No significant difference between the $^{125}$I-EGF binding properties of the wild-type and the [Ala⁷]EGF recep-
Threonine Protein Kinase Activation by EGF

I. **PEPTIDE MAPING**

![Peptide Mapping](image)

II. **PHOSPHOAMINO ACID ANALYSIS**

![Phosphoamino Acid Analysis](image)

III. **RADIOSEQUENCE ANALYSIS**

![Radiosequence Analysis](image)

**FIG. 1. Characterization of the synthetic peptide T669.** The synthetic peptide T669 phosphorylated in vitro and an EGF receptor tryptic peptide containing the major site of EGF receptor phosphorylation in vivo were purified and analyzed by two-dimensional [32P] phosphopeptide mapping (Panel A) and by phosphoamino acid analysis (Panel B). The radiosequence analysis of the EGF receptor peptide phosphorylated in vivo (32,000 cpm) is presented in Panel C.

... was observed. Exposure of the CHO cells to high concentrations of EGF caused the internalization and down-regulation of both the wild-type and the mutant receptors (not shown). Signal transduction by the EGF receptors was investigated by examining the effect of EGF to regulate the incorporation of [3H]thymidine into DNA. Fig. 3 shows that EGF caused an increase in the [3H]thymidine incorporation by CHO cells expressing wild-type and [Ala669]EGF receptors.

As the phosphorylation of threonine 669 (16) is associated with the transmodulation of the EGF receptor caused by PMA (4, 16, 11) and PDGF (14, 15), experiments were performed to investigate the regulation of the [Ala669]EGF receptor. Fig.

**TABLE I**

**Analysis of the 125I-EGF binding isotherm**

The 125I-EGF binding isotherm (Fig. 2) was analyzed by the computer program LIGAND (28). A significantly better fit of the experimental data to a two-site model than to a one-site model was obtained for control cells (p > 0.97). In contrast, after treatment with PMA the data were fitted to a one-site model better than a two-site model. The table summarizes the best fit of the data obtained (mean ± S.E.) for each condition.

| EGF receptor | Site 1 | Site 2 |
|--------------|--------|--------|
|              | $K_d$  | Sites/cell | $K_d$  | Sites/cell |
|              | nM     | $\times 10^{-3}$ | nM     | $\times 10^{-3}$ |
| Wild-type    |        |          |        |          |
| Control      | 7.2 ± 2.8 | 129 ± 23 | 0.64 ± 0.3 | 24 ± 6 |
| PMA          | 6.5 ± 1.8 | 132 ± 17 |          |          |
| [Ala669]     |        |          |        |          |
| Control      | 2.6 ± 0.3 | 84 ± 9  | 0.31 ± 0.02 | 14 ± 3 |
| PMA          | 4.0 ± 0.9 | 72 ± 14 |          |          |
Threonine Protein Kinase Activation by EGF

**FIG. 3. Effect of point mutation at threonine 669 on the regulation of threonine protein kinase activity and ([H]thymidine incorporation by EGF:** Panel A, CHO cells expressing wild-type EGF receptors and mutant [Ala<sup>669</sup>]EGF receptors were incubated without and with 100 nM EGF for 15 min at 37 °C. The cells were homogenized, and the extracts obtained were incubated with the peptide T669 and [γ-<sup>32</sup>P]ATP for 10 min at 22 °C as described under “Experimental Procedures.” The incorporation of radioactivity into the peptide is presented (mean ± S.D., n = 3). Panel B, the incorporation of [H]thymidine by CHO cells incubated for 24 h without and with 1 nM EGF is presented (mean ± S.D., n = 3). □, control; ■, EGF.

**FIG. 4. Measurement of phosphatase activity.** [<sup>32</sup>P]Phosphate-labeled EGF receptors isolated from A431 cells by immunoprecipitation were used as a substrate for the assay of protein phosphatase activity. The time course of dephosphorylation of the EGF receptor by extracts prepared from CHO cells expressing the wild-type EGF receptor is presented. The CHO cells were treated with and without 100 nM EGF for 15 min at 37 °C prior to homogenization. The data presented are normalized to the amount of radioactivity associated with the EGF receptor prior to incubation with the cell extract (22,314 cpm). Similar results were obtained in two separate experiments. O—O, control; ●—●, EGF.

2 shows that PMA caused the loss of the high affinity binding of <sup>125</sup>I-EGF to cells expressing wild-type and mutated [Ala<sup>669</sup>] EGF receptors. The CHO cells employed for these experiments express functional human PDGF receptors (B type). Treatment of the cells with PDGF caused an inhibition of the high affinity binding of <sup>125</sup>I-EGF to wild-type cell surface EGF receptors. 6 No significant difference between the results obtained for cells expressing wild-type and [Ala<sup>669</sup>]EGF receptors was observed (not shown).

**Measurement of Protein Phosphatase Activity**—The increase in the phosphorylation state of the EGF receptor at threonine 669 (16) caused by EGF (29) could be due to the inhibition of protein phosphatase activity. To test this hypothesis, the level of protein phosphatase activity present in homogenates prepared from CHO cells treated with and without EGF was measured. The substrate for the phosphatase assay used was the [<sup>32</sup>P]phosphate-labeled EGF receptor isolated from control A431 cells by immunoprecipitation using a rabbit polyclonal antibody directed against the extracellular domain of the receptor. Quantitative studies (15) of the phosphorylation of the EGF receptor in control A431 cells indicate that approximately 70% of the total [<sup>32</sup>P]phosphate incorporated into the receptor was at threonine 669 (16). Fig. 4 shows the time course of phosphatase activity using the [<sup>32</sup>P]phosphate-labeled EGF receptor as a substrate. Rapid dephosphorylation of the receptor was observed after the addition of a homogenate prepared from CHO cells expressing wild-type EGF receptors. Treatment of the cells with EGF before homogenization caused no significant change in the observed rate of dephosphorylation of the receptor (Fig. 4).

**Measurement of Protein Kinase Activity**—The increased phosphorylation state of the EGF receptor at threonine 669 (16) observed in EGF-treated cells (29) could be caused by the stimulation of the activity of a protein kinase that phosphorylates the EGF receptor at threonine 669. To test this hypothesis, the protein kinase activity in homogenates prepared from cells incubated with and without EGF was measured. The experimental strategy employed was to use the synthetic peptide T669 as a protein kinase substrate. CHO cells were homogenized and incubated with the peptide T669 and [γ-<sup>32</sup>P]ATP at 22 °C. The phosphorylated peptide was isolated and characterized in detail (Fig. 1). The radioactivity incorporated into the peptide was measured by liquid scintillation counting. Phosphoamino acid analysis indicated the presence of [<sup>32</sup>P]phosphothreonine (Fig. 1). As the synthetic peptide contains only a single threonine residue we conclude that the phosphorylated residue corresponds to EGF receptor threonine 669 (Fig. 1). Control experiments demonstrated that the rate of phosphorylation of the peptide under the standard assay conditions (see “Experimental Procedures”) was linear for 20 min at 22 °C.

CHO cells expressing the wild-type human EGF receptor were used to investigate the effect of EGF on the level of threonine protein kinase activity measured using the synthetic peptide T669 as a substrate. Treatment of the CHO cells with EGF caused a marked increase in the protein kinase activity detected in cell homogenates compared with control cells (Fig. 3). In five experiments the increase in protein

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2 J. L. Countaway, N. Girones, and R. J. Davis, submitted for publication.
The cells were homogenized and the extracts obtained were incubated with the peptide T669 and $[\gamma^{-32P}]$ATP as described under "Experimental Procedures." The figure presents the incorporation of radioactivity into the peptide observed after 10 min of incubation at $22^\circ C$ (mean ± S.D., $n = 3$). □ control; ■ EGF.

**Fig. 5. Regulation of threonine protein kinase activity in human and murine fibroblasts by EGF.** WI-38 human fetal lung fibroblasts and murine 3T3-L1 pre-adipocytes were incubated with and without 100 nM EGF for 15 min at $37^\circ C$. The cells were homogenized and the extracts obtained were incubated with the peptide T669 and $[\gamma^{-32P}]$ATP as described under "Experimental Procedures." The figure presents the incorporation of radioactivity into the peptide observed after 10 min of incubation at $22^\circ C$ (mean ± S.D., $n = 3$).

**Fig. 6. Time course of EGF action.** Murine 3T3 L1 pre-adipocytes cells were incubated with and without 100 nM EGF for defined times at $37^\circ C$. The cells were homogenized, and the extracts obtained were incubated with the peptide T669 and $[\gamma^{-32P}]$ATP as described under "Experimental Procedures." The figure presents the incorporation of radioactivity into the peptide observed after 10 min of incubation at $22^\circ C$ (mean ± S.D., $n = 3$).

**Fig. 7. Effect of EGF concentration on threonine protein kinase activity.** Murine 3T3 L1 pre-adipocytes were incubated with different concentrations of EGF for 15 min at $37^\circ C$. The cells were homogenized and the extracts obtained were incubated with the peptide T669 and $[\gamma^{-32P}]$ATP as described under "Experimental Procedures." The figure presents the incorporation of radioactivity into the peptide observed after 10 min of incubation at $22^\circ C$ (mean ± S.D., $n = 3$).

**Fig. 8. Effect of growth factors on the rate of phosphorylation of synthetic peptide T669.** Murine 3T3 L1 pre-adipocytes were incubated for 15 min ($37^\circ C$) without and with 100 nM EGF, 100 nM IGF-1, 10 nM PDGF, 100 nM PMA, and 5% calf serum. The cells were homogenized and the extracts obtained were incubated with the peptide T669 and $[\gamma^{-32P}]$ATP as described under "Experimental Procedures." The figure presents the incorporation of radioactivity into the peptide observed after 10 min of incubation at $22^\circ C$ (mean ± S.D., $n = 3$).

**DISCUSSION**

**Mechanism of Phosphorylation of the EGF Receptor at Threonine 669**—There are three potential mechanisms by which EGF, PDGF, and phorbol ester could regulate the phosphorylation state of the EGF receptor at threonine 669: 1) activation of a threonine protein kinase; 2) inhibition of a protein phosphatase; and 3) alteration of the receptor conformation or subcellular distribution. The experiments reported here were designed to examine these hypotheses.

Measurement of protein phosphatase activity in cell homogenates using $[^{32}P]$phosphate-labeled EGF receptors as a substrate indicated that there was no significant effect of EGF treatment on the level of phosphatase activity observed (Fig. 4). As this experiment employs an *in vitro* assay for protein phosphatase activity, the data obtained do not allow the conclusion that protein phosphatase activity is not regulated.
by EGF in intact cells. Chan et al. (30) have reported that type I protein phosphatase is activated by treatment of cells with EGF. Together these data do not support the hypothesis that EGF increases the phosphorylation of the receptor at threonine 669 by inhibiting protein phosphatase activity.

To measure the activity of protein kinases that phosphorylate the EGF receptor at threonine 669 an in vitro assay was developed using a synthetic peptide substrate that corresponds to residues 663–681 of the EGF receptor. It was observed that the synthetic peptide (T669) was phosphorylated on a unique threonine residue, threonine 669 (Fig. 1).

Treatment of murine 3T3 L1 pre-adipocytes and human WI-38 fetal lung fibroblasts with EGF caused a marked increase in the rate of phosphorylation of the synthetic peptide T669 by homogenates prepared from these cells. Similar results were obtained for CHO cells expressing human EGF receptors. These data are consistent with the hypothesis that the mechanism of EGF action to increase the phosphorylation of the receptor at threonine 669 is the activation of a threonine protein kinase.

It is possible that the phosphorylation state of threonine 669 is regulated by the conformation or subcellular distribution of the receptor. No evidence was obtained from the studies reported here that excludes these mechanisms from playing a role in the regulation of the phosphorylation of threonine 669. However, the observation of a growth factor-stimulated threonine protein kinase (Figs. 3, 5–8) indicates that EGF could increase threonine 669 phosphorylation in the absence of changes in the conformation or subcellular distribution of the receptor. Consistent with this proposal, it was observed that EGF treatment of CHO cells expressing the [Ala<sup>669</sup>]-EGF receptor caused a stimulation of threonine protein kinase activity (Fig. 3).

**Mechanism of Transmodulation of the EGF Receptor**—A working hypothesis that we have used to examine the mechanism of transmodulation of the EGF receptor proposes that receptor phosphorylation mediates the regulation of the receptor (for review see Ref. 2). Treatment of cells with phorbol ester (4, 10, 11) or PDGF (14, 15) causes the phosphorylation of the EGF receptor at several sites, including threonine 654 (3, 4) and threonine 669 (16). Previous studies have demonstrated that the phosphorylation of threonine 654 by protein kinase C does not fully account for the transmodulation of threonine 669. However, it is possible that the phosphorylation of threonine 669 by an alanine residue does not alter the regulation of the high affinity binding of 125I-EGF to the receptor by phorbol ester. However, the [Ala<sup>669</sup>]-EGF receptor was phosphorylated at threonine 669 (16) during transmodulation (5). Based on this result it is possible that the phosphorylation of the EGF receptor at threonine 669 may be mechanistically related to the process of transmodulation. We report here that the substitution of threonine 669 with an alanine residue does not alter the inhibition of high affinity binding of EGF caused by phorbol ester and PDGF (Fig. 2). These data demonstrate that replacement of either threonine 654 or threonine 669 with alanine does not alter the regulation of the high affinity binding of 125I-EGF to the receptor. The effect of simultaneous substitution of both threonine 654 and threonine 669 with alanine remains to be determined.

**Identity of the Growth Factor-stimulated Threonine Protein Kinase**—The protein kinase activity detected using in vitro assays with the synthetic peptide T669 is stimulated by the treatment of cells with EGF, PDGF, phorbol ester, and serum. The identity of this protein kinase is not known. It has been shown for many protein kinases that the primary sequence of a peptide substrate is a critical factor in determining substrate specificity (31). The proximity of 2 proline residues in the primary sequence of the EGF receptor surrounding threonine 669 is unusual for a site of protein phosphorylation (31). However, two protein kinases have been reported that exhibit a similar substrate specificity: glycogen synthase kinase-3 and the multifunctional protein kinase. Glycogen synthase kinase-3 (32) and multifunctional protein kinase (33, 34) have been reported to phosphorylate glycogen synthase and protein kinase II at sites located within a proline-rich primary sequence. It is possible that the growth factor-stimulated threonine protein kinase activity reported here may be accounted for by glycogen synthase kinase-3 or by multifunctional protein kinase, but previous studies of the properties of these enzymes do not support this hypothesis. 1) Activation of glycogen synthase kinase-3 and multifunctional protein kinase by growth factors has not been reported (31). 2) The phosphorylation state of a glycogen synthase kinase-3 substrate, the nerve growth factor receptor, is not regulated by treatment of cells with phorbol ester (35). 3) It has been proposed that the substrate specificity of glycogen synthase kinase-3 is serine-X-X-X-phosphoserine/threonine (36). The primary sequence surrounding EGF receptor threonine 669 does not conform to this proposed consensus primary sequence. 4) Treatment of adipocytes with insulin has been reported to cause a rapid inhibition of multifunctional protein kinase activity (37), but no effect of IGF-1 was observed on the threonine protein kinase activity present in homogenates of 3T3 L1 pre-adipocytes or CHO cells when the peptide T669 was used as a substrate (Fig. 8). Together, these data indicate that the growth factor-stimulated threonine protein kinase may be an enzyme that has not been previously described.

**Signal Transduction by the EGF Receptor**—Treatment of cultured cells with EGF (29), PDGF (14, 15), cell-permeable diacylglycerols (12, 13), or phorbol ester (4, 10, 11) causes the phosphorylation of the EGF receptor at threonine 669 (16). The data presented here indicate a role for the activation of a threonine protein kinase. It is likely that this growth factor-stimulated threonine protein kinase is able to phosphorylate protein substrates other than the EGF receptor in intact cells. Such phosphorylation of target proteins may be physiologically relevant for signal transduction by growth factor receptors (EGF and PDGF) and by protein kinase C (diacylglycerol and phorbol ester). Potential target proteins will probably share homology with the EGF receptor at the site of phosphorylation: Pro-Leu-Thr<sup>669</sup>-Pro. Recently Giugni et al. (38) reported that EGF activates a serine protein kinase in A431 human epidermoid carcinoma cells that phosphorylates the synthetic peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly. The identification of the substrates for these protein kinases and the elucidation of the mechanism by which EGF increases serine/threonine protein kinase activity is an important goal for future research.

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