Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 1974–1978, April 1985
Biochemistry

Tumor-promoting phorbol diesters cause the phosphorylation of epidermal growth factor receptors in normal human fibroblasts at threonine-654

(sequence analysis/Ca^{2+}- and phospholipid-dependent protein kinase/receptor regulation/cell proliferation)

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Communicated by Robert L. Hill, November 29, 1984

ABSTRACT **The effect of tumor-promoting phorbol diesters to potentiate the action of epidermal growth factor (EGF) on cell proliferation is associated with phosphorylation of EGF receptors, acute depression of EGF binding, and inhibition of EGF receptor tyrosine kinase activity. In the present studies, normal human fibroblasts and A431 carcinoma cells were labeled with [^{32}P]phosphate and treated with and without 10 nM 4β-phorbol 12β-myristate 13α-acetate (PMA). The EGF receptors then were isolated by immunoprecipitation and digested with trypsin. Analysis of the labeled receptor phosphopeptides by reversed-phase HPLC revealed that PMA induces the phosphorylation of a unique phosphopeptide containing [^{32}P]phosphothreonine. Comparison of several chemical and physical properties of the ^{32}P-labeled phosphopeptide with the primary structure of the EGF receptor suggested the identity Lys-Arg-Thr(P)-Leu-Arg. This was confirmed by direct demonstration that a synthetic peptide of this structure comigrates during HPLC and electrophoresis with the ^{32}P-labeled phosphopeptide isolated from the EGF receptors of normal human fibroblasts. The phosphorylated site on the peptide corresponds to threonine-654 of the EGF receptor, which is located on the cytoplasmic side of the plasma membrane nine residues distant from the transmembrane domain. These data indicate that phosphorylation of the EGF receptor in human fibroblasts and A431 cells at threonine-654 may regulate the EGF receptor tyrosine kinase activity and the binding of EGF.**

The epidermal growth factor (EGF) receptor of cultured cells is acutely regulated by tumor-promoting phorbol diesters. These agents decrease the apparent affinity of the EGF receptor (1–3) and inhibit the tyrosine kinase activity (4, 5) within minutes of addition to A431 human epidermoid carcinoma cells. Associated with these effects is an increase in the phosphorylation state of the EGF receptor (5–7). Phosphoamino acid analysis indicates that phorbol diesters increase the level of phosphoserine and phosphothreonine. Phosphopeptide mapping demonstrates that the levels of all of the phosphopeptides present in control maps are increased in maps of the EGF receptor derived from phorbol diester-treated cells (5, 6). In addition to these phosphopeptides, some phosphopeptides are uniquely present in maps of the EGF receptor isolated from phorbol diester-treated cells. Phosphoamino acid analysis of these phosphopeptides, which are specifically induced by phorbol diesters, indicates the presence of phosphothreonine (5, 6). These peptides are phosphorylated by the putative phorbol diester receptor (5), Ca^{2+}- and phospholipid-dependent protein kinase (C kinase) (8–11). It is therefore possible that the effects of phorbol diesters on the EGF receptor are causally related to the specific phosphorylation of the receptor induced by phorbol diesters.

A problem that is associated with the studies described using A431 carcinoma cells is that these cells are transformed and overexpress the receptor for EGF. The physiological relevance of results obtained with A431 cells is unclear. This is because concentrations of EGF that are mitogenic when added to normal fibroblasts cause A431 carcinoma cell death. Furthermore, whereas 4β-phorbol 12β-myristate 13α-acetate (PMA) enhances the growth of fibroblasts synergistically with growth factors (12), PMA and EGF synergistically inhibit the growth of A431 cells (13).

We have investigated the effect of PMA to regulate the phosphorylation state of the EGF receptors of A431 cells and normal human fibroblasts, which, unlike A431 cells, respond to nanomolar concentrations of EGF with increased proliferation. PMA was observed to cause specific threonine and serine phosphorylation of the EGF receptor in both cell types. We report that a site on the EGF receptor uniquely phosphorylated in response to PMA is threonine-654. This residue is located nine amino acids away from the predicted transmembrane domain of the EGF receptor on the cytoplasmic side of the plasma membrane. It is therefore in a position that might be expected to play a role in receptor regulation.

EXPERIMENTAL PROCEDURES

Materials. EGF was prepared as described (14, 15). [^{32}P]-Phosphate was from New England Nuclear. Carboxypeptidase B and L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (TPCK-treated trypsin) were obtained from Worthington. Trifluoroacetic acid and triethylamine were from Pierce. Acetonitrile was from Burdick and Jackson (Muskegon, MI). PMA was obtained from Sigma. Lys-Arg-Thr-Leu-Arg-Arg was obtained from Peninsula Laboratories (Belmont, CA). Purified C kinase was a gift from G. Johnson.

Methods. *Cell culture.* WI-38 fibroblasts and A431 cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum and Dulbecco's modified Eagle's medium supplemented with 5% calf serum, respectively.

Analysis of the phosphorylation state of the EGF receptor. Confluent WI-38 fibroblasts in 100-mm dishes were incubated with 5 ml of phosphate-free Dulbecco's modified Eagle's medium supplemented with 0.1% calf serum and 15 mCi (1 Ci = 37 GBq) of [^{32}P]phosphate for 24 hr. The cells were then treated with and without 10 nM PMA for 30 min. The monolayers were washed once and the cells were lysed with 4 ml of 1.5% Triton X-100/10% sodium deoxycholate/

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Abbreviations: PMA, 4β-phorbol 12β-myristate 13α-acetate; EGF, epidermal growth factor; TPCK-treated trypsin, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin; C kinase, Ca^{2+}- and phospholipid-dependent protein kinase.

0.1% $NaDodSO_4$/0.5 M NaCl/5 mM ethylenediamine tetraacetic acid/50 mM NaF/100 μM Na_3VO_5/1 mM phenylmethylsulfonyl fluoride/10 μg of leupeptin per ml/25 mM Hepes, pH 7.8. The lysate was clarified by centrifugation at 4°C for 30 min at 100,000 × g. The supernatant was then mixed with anti-EGF receptor antiserum (1:1000 dilution). After 60 min of incubation at 22°C on an end-over-end mixer, 20 μl of protein A-Sepharose CL-4B (Pharmacia) was added and the incubation was continued for a further 60 min. The immunoprecipitate was collected by centrifugation, extensively washed with lysis buffer, and finally washed with 0.1% $NaDodSO_4$/0.2% Triton X-100/25 mM Hepes, pH 7.8. The same procedure was used for A431 carcinoma cells except that the cells were seeded in 35-mm wells, grown to a density 2×10^5 cells per well, and labeled for 24 hr in 1 ml of phosphate-free Dulbecco's modified Eagle's medium supplemented with 0.1% calf serum and 1 mCi of [^{32}P]phosphate.

Phosphopeptide mapping. Immunoprecipitated EGF receptors were reduced, alkylated, and electrophoresed on a 7% polyacrylamide gel (16). The receptors were then digested at 37°C with 50 μg of TPCK-treated trypsin (Worthington) per ml in 3 ml of 50 mM NH_4HCO_3. After 5 hr, a second addition of trypsin was made (16). The digestion was allowed to proceed for a total of 24 or 48 hr. The phosphopeptides were resolved by reversed-phase HPLC as described in the legend to Fig. 2. Phosphoamino acid analysis was performed (7) by the method of Hunter and Sefton (17).

Automated NH_2-terminal sequence analysis. Sequence analysis of ^{32}P-labeled phosphopeptides was performed in the presence of 4 nmol of myoglobin by using a modified Beckman 890C liquid-phase Sequenator and a 0.1 M Quadrol program (Beckman 121078). The thiazolinones were converted to phenylthiohydantoins by reaction in 25% trifluoroacetic acid at 56°C and were identified and quantitated by a modification of the reversed-phase HPLC procedure described by Zimmerman *et al.* (18) using acetonitrile. The repetitive yield was estimated to be 94%. The radioactivity associated with the phenylthiohydantoins derived from the ^{32}P-labeled phosphopeptide released at each cycle was measured by Cerenkov counting.

Preparation of synthetic phosphopeptides. The synthetic hexapeptide Lys-Arg-Thr-Leu-Arg-Arg was phosphorylated by using a partially purified C kinase preparation. The buffer used contained 1 mM [γ-^{32}P]ATP, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 25 μg of phosphatidylserine per ml, and 25 mM Hepes (pH 7.4). The phosphorylated peptide was then purified by reversed-phase HPLC. The pentapeptide Lys-Arg-Thr(P)-Leu-Arg was prepared by incubation of 100 nmol of the hexapeptide in 10 mM NH_4HCO_3 with 1 unit of carboxypeptidase B per ml for 1 min at room temperature. The digestion products were separated by reversed-phase HPLC. The structure of the major product was confirmed to be Lys-Arg-Thr(P)-Leu-Arg by amino acid analysis.

RESULTS

The phosphorylation state of the EGF receptors of [^{32}P]-phosphate-labeled WI-38 fibroblasts was studied. Treatment of the cells with 10 nM PMA or 10 nM EGF for 30 min caused a 3-fold or 2-fold increase, respectively, in the amount of radioactivity associated with the EGF receptors (Fig. 1). Trypsin digestion of the EGF receptors yielded a complex mixture of ^{32}P-labeled phosphopeptides. To resolve these ^{32}P-labeled phosphopeptides we have employed reversed-phase HPLC. PMA treatment was observed to enhance the phosphorylation of a large number of peptides that contain phosphoserine or phosphothreonine and produce novel phosphopeptides that contain phosphothreonine (Fig. 2). Two such latter phosphopeptides (Fig. 2, peaks 1 and 2) were found to be eluted from the reversed-phase column at very low acetonitrile concentrations and were absent in preparations of EGF receptor isolated from control cells (Fig. 2). The two unique phosphopeptides were also observed after trypsin digestion of the EGF receptors isolated from PMA-treated A431 carcinoma cells.

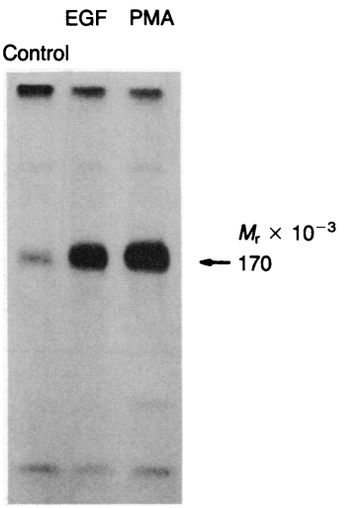

FIG. 1. Effect of PMA on the phosphorylation state of the EGF receptor of normal human fibroblasts. WI-38 cells were labeled for 24 hr with [^{32}P]phosphate and were treated with and without 10 nM PMA or 10 nM EGF for 30 min. The EGF receptors were then isolated by immunoprecipitation and polyacrylamide gel electrophoresis. An autoradiograph of the dried gel is presented.

Analysis of the time course of trypsin digestion of the EGF receptor demonstrated that, although both unique phosphopeptides (Fig. 2, peaks 1 and 2) were observed after prolonged (48 hr) trypsin digestion, only a single unique phosphopeptide (phosphopeptide 2) was found after trypsin digestion for a limited period (24 hr). Comparison of the ^{32}P-labeled phosphopeptide elution profiles of the EGF receptor digested under our conditions for 24 and 48 hr with trypsin suggested that there was a precursor–product relationship between the two unique phosphopeptides (Fig. 2).

The presence of incompletely digested EGF receptor phosphopeptides in phosphopeptide maps makes the interpretation of such maps difficult. We, therefore, further investigated the relationship between the two unique EGF receptor ^{32}P-labeled phosphopeptides produced by PMA treatment of intact cells. ^{32}P-labeled phosphopeptide 2 was prepared by HPLC, lyophilized, resuspended in 0.5 ml of 50 mM NH_4HCO_3, and incubated either with or without 5 μg of TPCK-treated trypsin for 24 hr at 37°C. The ^{32}P-labeled phosphopeptides present in each incubation were investigated by analytical HPLC. Trypsin digestion of phosphopeptide 2 caused the appearance of phosphopeptide 1 (Fig. 3). We conclude from this experiment that phosphopeptide 1 is formed after the prolonged digestion of phosphopeptide 2 with trypsin. Thus, phosphopeptides 1 and 2 contain the same phosphorylation site or sites on the EGF receptor. The presence of an inefficient protease-sensitive site on phosphopeptide 2 may be due to the possibility that the TPCK-treated trypsin obtained from Worthington possesses a very weak chymotrypsin-like activity. Alternatively, phosphopeptide 2 may possess inefficient cleavage sites for trypsin. An example of inefficient trypsin cleavage sites is a sequence containing several adjacent lysines or arginines.

Characterization of the Phosphopeptide Uniquely Induced by PMA. In preliminary experiments, we characterized some of the properties of phosphopeptide 2. Phosphoamino acid

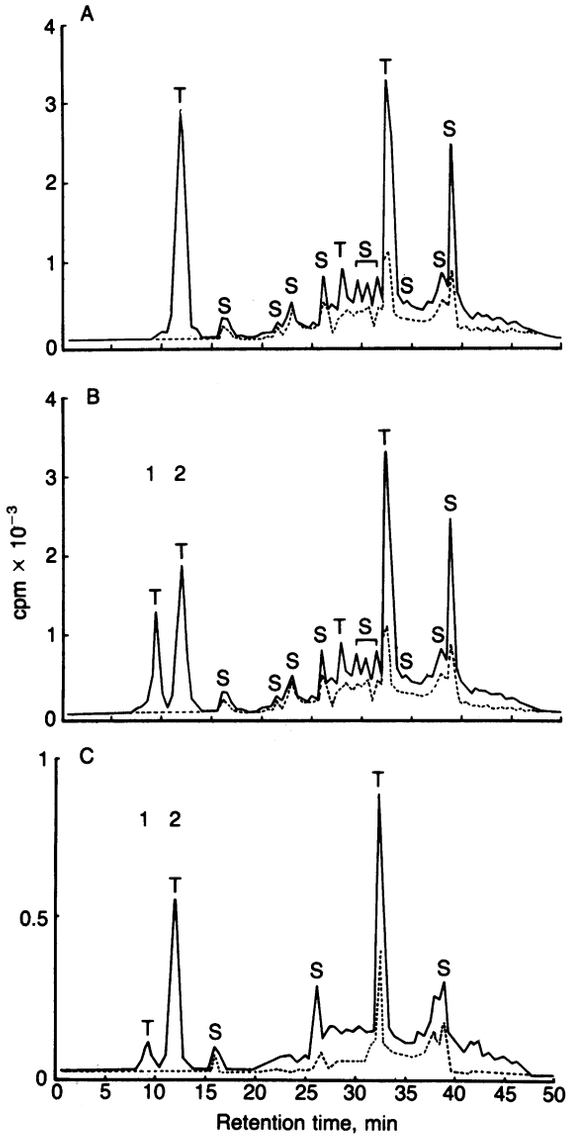

FIG. 2. Phosphopeptide maps of the EGF receptor isolated from A431 cells (*A* and *B*) and WI-38 fibroblasts. (*C*) EGF receptors derived from control cells (dashed line) and cells treated with 10 nM PMA for 30 min (solid line) were digested with trypsin for 24 hr (*A* and *C*) or 48 hr (*B*). The ^{32}P-labeled phosphopeptides were then lyophilized, dissolved in 0.5 ml of 1% trifluoroacetic acid, and resolved by reversed-phase HPLC using a Waters μBondapak C_{18} column equilibrated with 0.1% trifluoroacetic acid/0.05% triethylamine. The column was washed for 5 min and the ^{32}P-labeled phosphopeptides were then eluted with a linear gradient of acetonitrile (0–45%) over 45 min. The flow rate was 1 ml/min. ^{32}P-labeled phosphopeptides eluted were collected in fractions at 30-sec intervals. The ^{32}P-labeled phosphopeptides were detected by measuring the Cerenkov radiation with a β-counter. Peak fractions were analyzed for ^{32}P-labeled phosphoamino acids by partial acid hydrolysis and are identified as S (phosphoserine) and T (phosphothreonine). Phosphotyrosine was not detected in the absence of EGF. Phosphopeptides that are unique to samples derived from PMA-treated cells are labeled 1 and 2.

analysis indicated the sole presence of phosphothreonine. The peptide eluted at very low acetonitrile concentrations from a reversed-phase C_{18} HPLC column, indicating that it is probably a small, highly charged peptide. Thin-layer electrophoresis demonstrated that the peptide is extremely basic. At pH 8.9, the peptide migrates very strongly towards the cathode. Considering that the phosphothreonine residue in

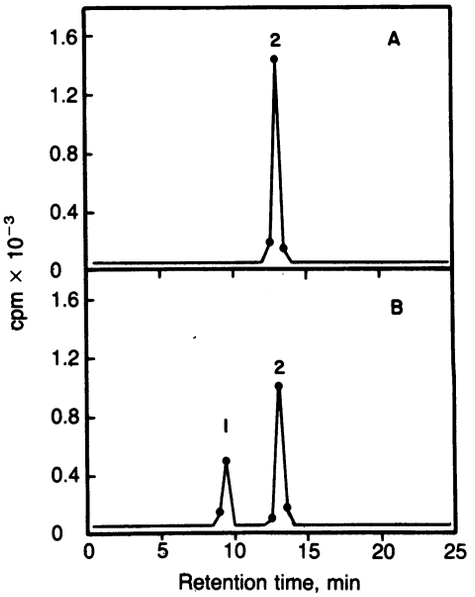

FIG. 3. Secondary digestion of the ^{32}P-labeled phosphopeptide induced by PMA. ^{32}P-labeled phosphopeptide 2 was prepared by HPLC. After lyophilization the phosphopeptide was resuspended in 0.5 ml of 0.5 M NH_4HCO_3 with (*B*) and without (*A*) 5 μg of TPCK-treated trypsin. After 24 hr of incubation at 37°C the ^{32}P-labeled phosphopeptides were analyzed by reversed-phase HPLC. Fractions were collected at 30-sec intervals and the Cerenkov radiation associated with each fraction was measured with a β-counter. The elution profile obtained during the first 25 min is presented. No peaks were observed after 25 min. Fractions containing >100 cpm are marked with filled circles.

the peptide will contribute two negative charges, it can be predicted that the peptide must contain many basic residues in order to have a net positive charge at pH 8.9. Secondary digestion of the phosphopeptide with *Staphylococcus aureus* V8 protease and α-chymotrypsin did not cause cleavage of the phosphopeptide when assayed by thin-layer electrophoresis and autoradiography (not shown). However, digestion of the phosphopeptide with TPCK-treated trypsin did cause a small amount of cleavage (Fig. 3). As α-chymotrypsin did not cleave the phosphopeptide, we conclude that this effect of trypsin is due to the presence of a poor trypsin cleavage site in the phosphopeptide rather than due to a chymotrypsin-like activity contaminating the trypsin.

NH_2-Terminal Sequence Analysis of the Phosphopeptide Induced by PMA. Automated NH_2-terminal sequence analysis of ^{32}P-labeled phosphopeptide 2 was carried out by using a Beckman 890C Sequenator. Insufficient material was available to identify the phenylthiohydantoins that were released at each cycle, but the Cerenkov radiation associated with the phenylthiohydantoins was measured (Fig. 4). A peak of radioactivity was observed at cycle 3. We conclude that the phosphothreonine present in the ^{32}P-labeled phosphopeptide is the third residue from the NH_2 terminus and that only a single labeled phosphate site is present.

COOH-Terminal Sequence Analysis of the Phosphopeptide Induced by PMA. The phosphopeptide was observed to be a substrate for carboxypeptidase B. The time course of digestion with carboxypeptidase B was analyzed by thin-layer electrophoresis at pH 8.9 (not shown). Short periods of digestion gave an initial cleavage product that migrated much more slowly towards the cathode compared with the original phosphopeptide. The large shift in the mobility of the phosphopeptide is consistent with the removal of a basic residue from the COOH terminus of the phosphopeptide. This is consistent with the specificity of trypsin to cleave at

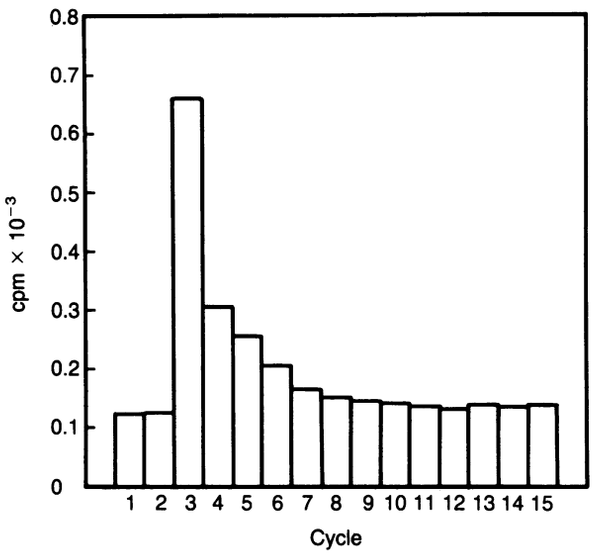

FIG. 4. NH_2-terminal sequence analysis of the ^{32}P-labeled phosphopeptide induced by PMA. The ^{32}P-labeled phosphopeptide (38,000 cpm; Cerenkov) was lyophilized, resuspended, and placed in a Beckman 890C Sequenator. The radioactivity released at each cycle associated with the phenylthiohydantoins was measured by Cerenkov counting. The yield obtained in cycle 3 was 1.7% of the input radioactivity. This yield is of the magnitude expected because the acid-catalyzed cyclization reaction of a phenylisothiocyanate-treated peptide that has an NH_2-terminal phosphothreonine causes the elimination of much of the phosphate during the cleavage rather than the formation of an anilinothiazolinone derivative. The phosphate released by elimination is not extracted from the spinning cup.

the COOH side of lysine and arginine residues in the original digestion. Longer periods of digestion led to the appearance of a third phosphopeptide that did not migrate during electrophoresis at pH 8.9. The level of this neutral phosphopeptide increased during the time course of the digestion and it was the major phosphopeptide present at the end of the digestion.

Identification of the Phosphorylation Site on the EGF Receptor Induced by PMA. The predicted primary structure of the EGF receptor (17) was used to identify the phosphorylation site on the EGF receptor induced by PMA. Several properties of the tryptic phosphopeptide containing this site have been determined. (*i*) NH_2-terminal sequence analysis demonstrated that there is a single phosphorylation site in the peptide that is at position 3 from the NH_2 terminus (Fig. 3). (*ii*) Phosphoamino acid analysis indicates that the phosphorylated residue is phosphothreonine. (*iii*) The phosphopeptide is extremely basic as it migrates towards the cathode during electrophoresis at pH 8.9. As the α-COOH group and the phosphothreonine residue will contribute a total of three negative charges at pH 8.9, it can be predicted that the phosphopeptide contains a minimum of three basic residues (lysine or arginine). (*iv*) The highly charged nature of the phosphopeptide is consistent with its elution profile from a reversed-phase HPLC column at very low acetonitrile concentrations. (*v*) The phosphopeptide is not cleaved by α-chymotrypsin or *S. aureus* V8 protease and thus does not contain phenylalanine, tyrosine, tryptophan, and glutamic acid residues. (*vi*) The carboxypeptidase digestion of the phosphopeptide demonstrated that the phosphopeptide markedly shifted its mobility towards the anode during electrophoresis at pH 8.9 when the first amino acid residue was cleaved. The presence of a basic residue (lysine or arginine) at the COOH terminus of the peptide would be consistent with these data. (*vii*) The second cleavage product of the carboxypeptidase digestion is neutral at pH 8.9.

The properties of phosphopeptide 2 were compared with the reported primary structure of the EGF receptor (19). Only one region of the EGF receptor is similar to the phosphopeptide. This is the sequence surrounding threonine-654: Arg-Lys-Arg-Thr-Leu-Arg-Arg.

To identify unequivocally the phosphopeptide, we investigated the properties of synthetic phosphopeptides that correspond to predicted tryptic fragments of the sequence surrounding threonine-654. Two phosphopeptides were prepared: Lys-Arg-Thr(P)-Leu-Arg and Lys-Arg-Thr(P)-Leu-Arg-Arg. Comparison of the chromatographic properties of phosphopeptide 2 with the synthetic phosphopeptides by HPLC indicated that phosphopeptide 2 comigrated with the pentapeptide (not shown). Similarly, the pentapeptide comigrated with phosphopeptide 2 during thin-layer electrophoresis at pH 8.9 (Fig. 5) and pH 3.5 (not shown). We, therefore, conclude that the phosphopeptide isolated from the EGF receptor after trypsin digestion is Lys-Arg-Thr(P)-Leu-Arg and that the phosphorylated site is identified as threonine-654.

DISCUSSION

Treatment of A431 carcinoma cells and WI-38 fibroblasts with PMA causes a 3-fold increase in the phosphorylation state of the EGF receptor. Phosphopeptide mapping indicates that some of this increase is due to enhanced phosphorylation of sites that are phosphorylated in control cells. In addition, PMA causes the phosphorylation of the EGF receptor at a unique site that is not phosphorylated in control cells. The data presented in this paper indicate that the sequence around the unique site that becomes phosphorylated when PMA is added to normal fibroblasts and A431 cells is Val-Arg-Lys-Arg-Thr(P)-Leu-Arg-Arg-Leu based on the comparison of the known primary structure of the EGF

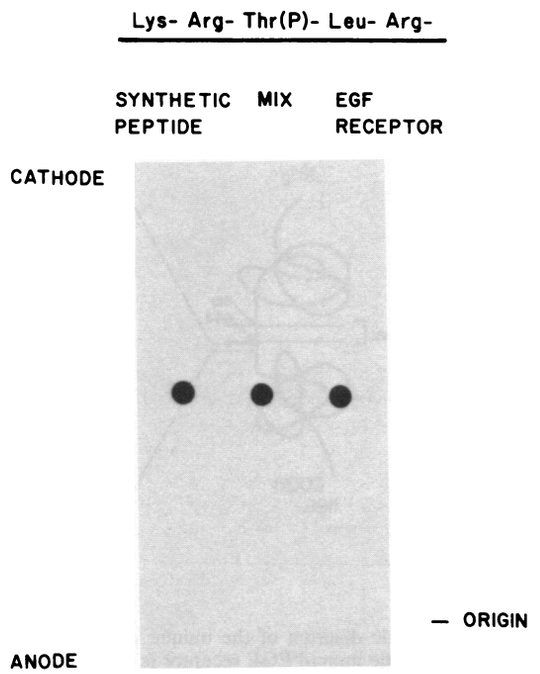

FIG. 5. Identification of phosphopeptide 2. Phosphopeptide 2 was compared with the synthetic peptide Lys-Arg-Thr(P)-Leu-Arg by thin-layer electrophoresis at pH 8.9 (1% ammonium carbonate) by using 100-μm cellulose thin-layer plates (Machery & Nagel). The electrophoresis was performed at 500 V for 3 hr. The samples were phosphopeptide 2 (400 cpm, Cerenkov), synthetic peptide (400 cpm), and phosphopeptide 2 (200 cpm) mixed with synthetic peptide (200 cpm). An autoradiograph (24-hr exposure) of the dried sheet is presented.

receptor (19) with the properties of a tryptic phosphopeptide we have characterized. The phosphorylated residue is threonine-654. Trypsin digestion of the EGF receptor phosphorylated in intact cells treated with PMA releases a major fragment, which we have isolated: Lys-Arg-Thr(P)-Leu-Arg. The identification of this fragment is based on sequence analysis of the labeled peptide indicating that the third amino acid residue is phosphothreonine, sequential carboxypeptidase digestion and charge analysis of the resulting phosphopeptides, resistance to digestion by other proteases, and comigration with the authentic synthetic peptide during HPLC and thin-layer electrophoresis.

Addition of tumor-promoting phorbol diesters to cultured cells causes an inhibition of the high-affinity binding of ^{125}I-labeled EGF (1–4, 7). This inhibition may be due to a decrease in the affinity of the EGF receptor or to a reduction in the number of high-affinity EGF receptors. Thus, phorbol diesters cause a perturbation of the EGF receptor function. Furthermore, treatment of cells with phorbol diesters inhibits the EGF-stimulated tyrosine kinase activity of the receptor (4, 5). The molecular basis for these actions of phorbol diesters is not understood. A possibility is that the specific phosphorylation of the EGF receptor at threonine-654 is mechanistically involved in these effects of phorbol diesters on the EGF receptor.

The unique phosphorylation site induced by the addition of PMA to WI-38 fibroblasts and A431 cells (threonine-654) is located in an interesting region of the EGF receptor (Fig. 6). The site is nine amino acids from the cytoplasmic side of the predicted transmembrane domain of the EGF receptor in a region that links the EGF binding domain to the protein kinase domain. If a conformational change occurs subsequent to the binding of EGF to the receptor, the sequence surrounding the transmembrane domain will be of great importance to the transmission of this signal. The very basic sequence around threonine-654 may be involved in the interaction of the receptor with other proteins or phospholipids by an electrostatic mechanism. The introduction of a phosphate group into this sequence could be expected to alter these interactions and may be sufficient to perturb the function of the EGF receptor.

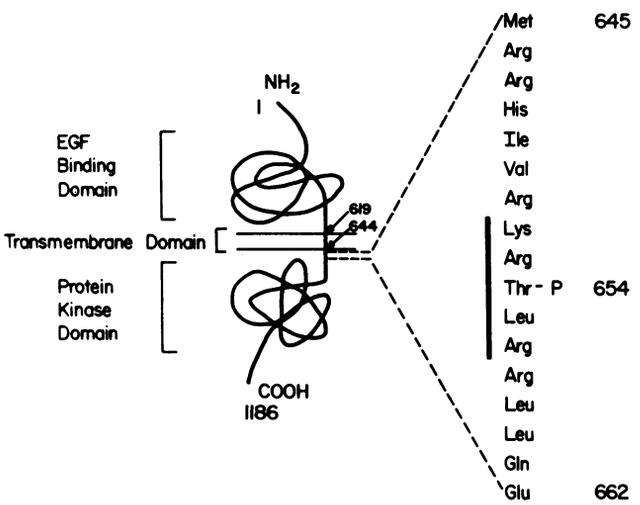

FIG. 6. Schematic diagram of the unique phosphorylation site induced by PMA. The human EGF receptor is presented schematically based on the results reported by Ullrich *et al.* (19). The predicted domains are marked: EGF binding; transmembrane; and the protein kinase. The region surrounding the proposed phosphorylation site (threonine-654), which is detectably labeled with ^{32}P only in the presence of PMA, is expanded to show the sequence at this site (residues 645–662). The deduced structure of the phosphopeptide isolated (phosphopeptide 2, Fig. 2) is indicated with a bar.

After the initial review of this manuscript. Hunter *et al.* (20) reported that purified C kinase will catalyze phosphorylation of a synthetic peptide that is based on the sequence predicted by v-*erb*B. This peptide corresponds to amino acids 87–110 of the v-*erb*B sequence and is homologous to the region comprising the amino acids 643–666 of the EGF receptor. This result indicates that the sequence surrounding the phosphorylation site on the EGF receptor induced by PMA in intact cells (Fig. 6) is phosphorylated *in vitro* by the putative receptor for phorbol diesters (C kinase). This provides strong support for our proposal that PMA induces the phosphorylation of the EGF receptor threonine-654 in human fibroblasts and A431 cells *in vivo*.

We thank Drs. J. E. Mole and J. K. Anderson (University of Massachusetts Medical Center, Protein Chemistry Core Facility) for amino acid and sequence analyses. Dr. G. Johnson is thanked for the gift of C kinase. John Cruz is thanked for expert assistance with cell culture. The excellent secretarial assistance of Mary Halley and Judith Kula is greatly appreciated. We thank Dr. T. Hunter for providing a manuscript prior to publication. This work was supported by Grant AM30648 from the National Institutes of Health. R.J.D. was the recipient of a postdoctoral fellowship from the Damon Runyon-Walter Winchell Cancer Fund.

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