The effect of autophosphorylation and protein kinase C-catalyzed phosphorylation on the tyrosine-protein kinase activity and ligand binding affinity of the epidermal growth factor (EGF) receptor has been studied. Kinetic parameters for the phosphorylation by the receptor kinase of synthetic peptide substrates having sequences related to the 3 in vitro receptor autophosphorylation sites (tyrosine residues 1173 (P1), 1148 (P2), and 1068 (P3)) were measured. The Kₚ of peptide P1 (residues 1164–1176) was significantly lower than that for peptides P2 (residues 1141–1151) or P3 (residues 1059–1072). The tyrosine residue 1173 was also the most rapidly autophosphorylated in purified receptor preparations, consistent with previous observations for the receptor in intact cells (Downward, J., Parker, P., and Waterfield, M. D. (1984) Nature 311, 483–485). Variation in the extent of receptor autophosphorylation from 0.1 to 2.8 mol of phosphate/mol of receptor did not influence kinase activity or EGF binding affinity either for purified receptor or receptor in membrane preparations.

Phosphorylation of the EGF receptor by protein kinase C was shown to cause a 3-fold decrease in the affinity of purified EGF receptor for EGF and to reduce the receptor kinase activity. In membrane preparations, phosphorylation of the EGF receptor by protein kinase C resulted in conversion of high affinity EGF binding sites to a low affinity state. This suggests that activation of protein kinase C by certain growth promoting agents and tumor promoters is directly responsible for modulation of the affinity of the EGF receptor for its ligand EGF. The regulation of the EGF receptor function by protein kinase C is discussed.

The binding of EGF to specific cell surface receptors initiates, in certain cells, a series of biochemical events which can induce cell proliferation by an as yet unclear mechanism (1, 2). A number of different experimental approaches, including the determination of the primary amino acid sequence, have been used to develop a working model for the structure of the receptor. In the model, the receptor polypeptide is divided into four distinct domains (3–6). Extracellular is an amino-terminal domain which binds EGF and this is linked through a putative single transmembrane domain to the cytoplasmic region of the receptor. This region has a domain with tyrosine-protein kinase activity which is able to autophosphorylate a carboxyl-terminal domain of the receptor. The amino acid sequence of the cytoplasmic receptor kinase domain is homologous to the conserved sequences of the src family of transforming proteins; the majority of which possess a protein-tyrosine kinase activity (4). It has been proposed that expression of a truncated EGF receptor by the v-erb B oncogene of avian erythroblastosis virus may induce transformation through generation of a ligand-independent receptor signal (3). The protein-tyrosine kinase activity (7), which is also associated with the v-erb-B protein (8–10), is the sole known intrinsic EGF receptor enzymatic activity and has consequently been the focus of much investigation with the goal of identifying physiological substrates that are important in signal transduction.

Autophosphorylation of the EGF receptor may represent a primary or regulatory event with respect to signal transduction. Investigations in our laboratory have shown that purified EGF receptor phosphorylates the receptor polypeptide at three distinct tyrosine residues which have been located close to the receptor carboxyl terminus at residues 1173, 1148, and 1068 (5). Tyrosine residue 1173 is the most extensively modified tyrosine residue in intact A431 cells treated with EGF (5). Additionally, in intact cells the EGF receptor is phosphorylated on serine and threonine residues as well as on tyrosine residues (5), indicating that other protein kinases can alter the phosphorylation state and presumably the function of this receptor. The only protein kinases that have been implicated in these phosphorylations are protein kinase C (12–15) and the cyclic AMP-dependent protein kinase (16, 17). While there is reason to doubt the physiological significance of the phosphorylation by the cyclic AMP-dependent protein kinase, there is good evidence for a functional role for the protein kinase C phosphorylation. The receptor site phosphorylated by protein kinase C has previously been identified by comparative peptide mapping and indirect sequencing (18, 19). Work employing phorbol esters and synthetic diacylglycerols indicates that not only may protein kinase C be involved in the phosphorylation of the receptor in vivo, but also provides circumstantial evidence that this may underlie the regulation of receptor affinity (transmodulation) (20).

In this study the site of the EGF receptor phosphorylated by protein kinase C has been directly sequenced. The effect of this phosphorylation and of the tyrosine autophosphorylations on the protein-tyrosine kinase activity and EGF bind-
ing affinity of the receptor in both purified and membrane systems has been studied to elucidate the role of these phosphorylation events in the control of cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Peptides P1, P2, P3, and RS were synthesized by Cambridge Research Biochemicals, and peptide RS was also obtained from Peninsula Laboratories. Human epidermoid carcinoma A431 cells were grown as described in Ref. 21 and plasma membranes from these cells were isolated by the method of Bianco et al. (22). The EGF receptor was purified from these cells using monoclonal antibody 9A as described previously (23). $[\gamma-^{32}P]ATP$ (>5000 Ci/mmol) was from Amersham. EGF was prepared from adult male mouse submaxillary glands (24) and iodinated using the glucose oxidase method (25). Protein kinase C was purified from bovine brains (26).

**Methods**

**Tyrosine Kinase Assay**—For measurement of tyrosine kinase activity in solubilized A431 cell plasma membranes, 10 μg of membrane protein was incubated with 50 nM Hepes, pH 7.4, 5% glycerol, 0.2% Triton X-100, 150 mM NaCl, 2 mM MnCl2, 12 mM MgCl2, 100 μM NaVO4, and 25 μM $[\gamma-^{32}P]ATP$ (5 Ci/mmol) in a total volume of 50 μl with varying concentrations of substrate peptides. Reactions were carried out for 2 min at 30 °C in the presence or absence of 200 nM EGF as stated. The reaction was stopped by the addition of 45 μl of 5% trichloroacetic acid followed by 5 μl of 30 mg/ml bovine serum albumin. The mixture was kept at 0 °C for 15 min then centrifuged at 10,000 × g for 15 min at 4 °C. 50 μl of each supernatant was spotted onto phosphocellulose paper squares (1.5 cm, Whatman P11) which were then washed in 30% acetic acid (3 × 10 min), and adsorbed radioactivity was measured. When purified receptor was used, incubations were carried out as above, except membranes were replaced by 2.5 ng of receptor protein. To determine the amount of $[^{32}P]$ phosphate incorporated into purified receptor in the course of a kinase assay, the trichloroacetic acid-precipitated protein pellet was redissolved in 50 μl of 1 M NaOH, and the suspension was heated at 60 °C for 3 min, vortexed vigorously, and spotted onto Whatman 3MM paper squares (1.5 cm). These were washed in 10% trichloroacetic acid (3 × 30 min), and adsorbed radioactivity was measured.

**Time Course of the Incorporation of Phosphoryluc into Each Auto-phosphorylation Site of the EGF Receptor**—100 μg of A431 Thom membranes (0.7 pmol of EGF receptor) were incubated in the phosphorylation buffer described above, without ATP but including 1 μg/ml EGF, for 30 min at 18 °C (total volume 100 μl). $[\gamma-^{32}P]ATP$ (100 Ci/mmol) was added (final concentration 5 μM) and the mixture was incubated at 0 °C between 0.5 and 5 min. The reaction was stopped by the addition of 50 μl of 4 × SDS gel sample buffer. Each mixture was run on a 7% SDS-polyacrylamide gel, and the EGF receptor-containing region of the gel was cut out and washed with 85% acetone, 5% acetic acid, 5% triethylamine, 5% water (3 × 30 min). The gel slice was then incubated with 500 μl of 100 mM NH4HCO3 containing 1 mg/ml DPC-treated trypsin (Sigma) for 48 h at 37 °C. The released peptides were applied to a Vydac C18 reverse phase HPLC column in 0.1% trifluoroacetic acid and eluted with an acetonitrile gradient (as described in Ref. 5).

**Preparation of Prephosphorylated EGF Receptors**—Receptor was purified in active form as described previously (23) except that the monoclonal antibody 8A2 column material carrying immobilized receptor was divided into two equal parts by mass. One part (EGFR-P) was incubated with 100 μg of $[^{32}P]ATP$ (0.05 Ci/mmol), 50 mM Hepes, pH 7.4, 5% glycerol, 0.2% Triton X-100, 150 mM NaCl, 2 mM MnCl2, 12 mM MgCl2, 100 μM NaVO4, for 2 h at 4 °C. The other part (EGFR-C) was incubated in a similar solution (as described in Ref. 5). The purified protein kinase C–phosphorylated receptor was fully reduced and alkylated, further purified by gel permeation HPLC, and cleaved with cyanogen bromide and the digest was fractionated by gel permeation HPLC as described previously (3). The major phosphate peak eluted was dialyzed against 50 mM NH4HCO3, lyophilized, and redisolved in 10 μl of 100 mM NH4HCO3, pH 7.6, which contained 10 μg/ml Staphylococcus aureus V8 protease. The digestion was continued for 48 h at 37 °C and then the digest was loaded onto a Vydac C18 reverse phase HPLC column equilibrated in 0.1% trifluoroacetic acid. A linear gradient from 0 to 40% acetonitrile was run in 40 min at a flow rate of 1 ml/min and 0.5-ml fractions were collected. The major peak from this separation was lyophilized and the amino-terminal sequence was determined using a gas phase sequencer assembled and operated as described by Hewick et al. (28). Phenylthiohydantoin (PTH) derivatives of amino acids were analyzed by HPLC as described in Ref. 3.

**Phosphoaminoo Acid Analysis**—The identity of the phosphoaminoo acid in phosphorylated peptides and proteins was determined by one-dimensional electrophoresis at pH 3.5 (7).

**Protein and Peptide Concentration Determinations**—The concentration of EGF receptor was determined both by radioimmunoassay (25) and by amino acid analysis using a Beckman System 630 High Performance Analyzer. Peptide and EGF concentrations were also determined by amino acid analysis.

**$[^{32}P]$-EGF Binding to Solubilized Receptor Phosphorylated by Protein Kinase C**—This was determined by radioimmunoassay (25): 0.46 pmol of purified EGF receptor was added to an incubation solution containing 10 mM MgCl2, 20 mM NaF, 100 μM ATP (where applicable), 50 mM Hepes, pH 7.4, 0.001% Triton X-100, 1 mM CaCl2, 100 μg/ml phosphatidylserine, 5 units of purified protein kinase C (where applicable), and 2 μg of monoclonal antibody R1 in a total volume of 0.2 ml, along with varying amounts of $[^{32}P]$-EGF (0.1–200 nM), added 15 min after the other reagents. This mixture was incubated at 18 °C for 1 h, then 10 μl of a 1:1 slurry of Protein A-Sepharose (Pharmacia) was added and the tube was tumbled for 20 min. The Protein A-Sepharose was washed by centrifugation and resuspension and counted as described previously (25). Backgrounds were measured by replacing the R1 antibody with 10 μl of 1:100 dilution of normal mouse serum. Each point was determined in duplicate with background subtracted.

**$[^{32}P]$-EGF Binding to EGF Receptor in Membranes Phosphorylated by Protein Kinase C**—Due to the presence of phospholipid in the plasma membranes, proteolytically activated protein kinase C was used in these experiments in order that control incubations could be carried out in the presence of inactive protein kinase C. Protein kinase C (150 units/ml) was incubated in 10 mM Tris, pH 8.0, 0.5 mg/ml bovine serum albumin, 20 mM β-mercaptoethanol, and 4 μg/ml trypsin for 3 min at 30 °C before the reaction was stopped with a 50-fold excess of trypsin inhibitor. For control incubations trypsin inhibitor was added before the trypsin. The binding reaction was performed in a total volume of 200 μl of 10 mM MgCl2, 20 mM NaF, 100 μM ATP, 50 mM Hepes, pH 7.4, 3 units of purified protein kinase C with 50 μg of A431 plasma membrane protein (0.35 pmol of EGF receptor) and varying amounts of $[^{32}P]$-EGF (added 15 min after the other reagents). The mixture was incubated for 1 h at 18 °C, applied to glass fiber filters (Whatman GF/C), and washed with 3 × 20 ml of 0.3% m Hepes, pH 7.4, containing 0.1% Triton X-100 and 1% bovine serum albumin. The filters were then counted for γ radiation. Backgrounds were estimated by the inclusion of a 100-fold excess of unlabeled EGF in the incubation.

**RESULTS**

**Synthetic Autophosphorylation Site Peptides as Substrates for the Receptor Protein-Tyrosine Kinase**—Peptides contain-
Epidermal Growth Factor Receptor Phosphorylation

**TABLE I**

| Name | Sequence                  | Without EGF | With EGF |
|------|---------------------------|-------------|----------|
|      |                           | $K_{m}$  | $V_{max}$ | $K_{m}$  | $V_{max}$ |
|      |                           | mM        | units/mg  | mM        | units/mg  |
| P1   | RRKGSTAENAELYLRV          | 0.22 ± 0.05 | 2.3 ± 0.6  | 0.16 ± 0.05 | 9.4 ± 1.6  |
| P2   | RRISLDNPDYQDD             | 0.41 ± 0.05 | 3.8 ± 0.6  | 0.34 ± 0.07 | 9.8 ± 1.2  |
| P3   | RRDFTLPLVPEYNQS           | 0.45 ± 0.06 | 3.6 ± 0.8  | 0.41 ± 0.07 | 11.2 ± 2.6 |
| RS   | RRLIIADAFAARG             | 0.69 ± 0.08 | 3.3 ± 0.5  | 0.48 ± 0.05 | 8.1 ± 1.7  |

The figures represent the mean of three determinations each using duplicate points from 0.1 to 0.8 mM peptide with backgrounds (in the absence of peptide) subtracted. The standard deviation of the population is given.

**FIG. 1.** Time course of the incorporation of phosphate into each autophosphorylation site of the EGF receptor. The stoichiometry of $^{32}P$ label in each of the phosphopeptide peaks (defined in Ref. 5) P1 ( ), P2 ( ), and P3 ( ) and in the whole receptor ( ) is plotted against the time of incubation at 0 °C (see "Experimental Procedures").

**The Effect of Autophosphorylation on EGF Receptor Function**—Although the autophosphorylation of the EGF receptor has been studied in detail, the consequences of this modification on the kinase activity of the receptor and its EGF binding affinity have not been characterized. To investigate these parameters, EGF receptor was purified from A431 cells using the anti-carbohydrate monoclonal antibody 9A (23). Two preparations of receptor were made: a control preparation ("EGFR-C") which was not autophosphorylated and a phosphorylated preparation ("EGFR-P") which was extensively autophosphorylated while immobilized on the antibody matrix (see "Experimental Procedures"). Autophosphorylation of EGFR-P was carried out in the absence of EGF for 2 h; this has been shown to give a pattern of phosphorylation similar to that resulting from briefer incubations in the presence of EGF (5). The extent of autophosphorylation of EGFR-P was 1.6 mol of tyrosine phosphate/mol of receptor. The two preparations contained the same concentration of receptor as measured by radioimmunoassay (25) or by amino acid analysis. The kinetic parameters of these two preparations with respect to the phosphorylation of the synthetic peptide P1 were determined and are shown in Table II. Under all of the conditions used, the two preparations showed very similar enzymatic activities as judged by peptide $K_{m}$ and $V_{max}$ values.

Since significant levels of autophosphorylation of the receptor may be taking place during the time period used for the kinase assay, it was possible that the control "unphosphorylated" preparation (EGFR-C) had effectively become phosphorylated. In order to investigate this possibility, it was therefore necessary to measure the level of phosphorylation at the end of the assay. The rate of peptide P1 phosphorylation at varying concentrations of peptide for the preparations EGFR-P and EGFR-C in the presence or absence of EGF and at 30 or 0 °C is shown in Fig. 2. Also shown in Fig. 2 are the stoichiometries of phosphate newly incorporated into the receptor during the course of the assay. It is evident that significant levels of autophosphorylation can occur under these conditions and thus autophosphorylation is inhibited...
by high concentrations of peptide substrate.

To investigate the effect of the role of autophosphorylation on the tyrosine protein kinase activity of the EGF receptor, the data shown in Fig. 2 was used to calculate \( V_{\text{max}} \) values which are shown in Fig. 3 expressed as a function of receptor phosphorylation. Since the prephosphorylated receptor preparation EGFR-P had been shown to contain 1.6 mol of phosphate/mol of receptor, this value was added to that incorporated during the assay. Results shown in Fig. 3 demonstrate that over widely varying levels of phosphorylation, from 0.1 mol of phosphate/mol of receptor to greater than 2.8 mol of phosphate/mol of receptor there is no significant difference in the \( V_{\text{max}} \) for the phosphorylation of exogenous substrate either in the presence or absence of EGF. It therefore appears that the level of autophosphorylation does not influence this activity, at least in purified preparations of solubilized receptor.

Since the affinity of receptor for EGF changes greatly upon solubilization (25), presumably as a result of conformational changes in the receptor, the kinase activity towards exogenous substrate peptide P1 was characterized for EGF receptor in A431 cell plasma membrane preparations. Membranes were preincubated with EGF for 20 min at 0 °C either in the presence or absence of 20 \( \mu \text{M} \) [\( \gamma \text{-}^{32}\text{P} \)]ATP. \( K_m \) and \( V_{\text{max}} \) values were determined in each case by a brief incubation with peptide and 200 \( \mu \text{M} \) [\( \gamma \text{-}^{32}\text{P} \)]ATP of the same specific activity. The incubations with peptide substrate were reduced to 30 s at 0 °C to minimize autophosphorylation of the control preparation. Results which are summarized in Table III show that the level of receptor phosphorylation did not effect the activity of the kinase whether the receptor was in membranes or purified in solution.

Receptor Phosphorylation by Protein Kinase C—The calcium- and phospholipid-dependent protein kinase, protein kinase C, has been shown to phosphorylate the EGF receptor (12, 18). This phosphorylation site has been located by comparative peptide mapping to threonine 654 (18, 19). In order

![Fig. 2. The effect of receptor autophosphorylation on the exogenous kinase activity. I. Purified EGF receptor was used to phosphorylate peptide P1 as described under "Experimental Procedures" under "Tyrosine Kinase Assay." Two batches of receptor were used: one was prephosphorylated (EGFR-P) as described under "Experimental Procedures" to a stoichiometry of 1.6 phosphotyrosine molecules/receptor molecule (represented here by \( \bigcirc \) and \( \bigtriangleup \)); the other was not prephosphorylated (EGFR-C) (represented here by \( \Delta \) and \( \bigtriangleup \)). \( \bigcirc \) and \( \bigtriangleup \) represent the rate of exogenous peptide phosphorylation during the assay; \( \bigcirc \) and \( \Delta \) represent the stoichiometry of [\( ^{32}\text{P} \)]phosphate incorporated into the receptor during the assay (measured as described under "Experimental Procedures"). Both plots against substrate peptide concentrations. A, receptor preincubated with 1 \( \mu \text{g/ml} \) EGF for 15 min at 30 °C, assay performed at 30 °C. B, receptor preincubated for 15 min at 30 °C, assay performed at 30 °C, no EGF present. C, receptor preincubated with 1 \( \mu \text{g/ml} \) EGF for 15 min at 30 °C, assay performed at 0 °C. D, receptor preincubated for 15 min at 30 °C, assay performed at 0 °C, no EGF present.](image)
peptide derived from purified phospho~lat~ by protein kinase
terminated the amino acid sequence of the phosphorylated
been used to predict the

rate from A431 cells was purified using
R1 aelnity column (3), and the immobilized receptor was
washed and then incubated with unlabeled ATP for 2 h at
4 °C to allow the tyrosine autophosphorylation to occur. ATP
was removed by washing and protein kinase C, calcium,
phosphotidylserine, and [γ-32P]ATP were added as described
under "Experimental Procedures." After 1 h at 18 °C, the
column was again washed, receptor eluted and purified as
previously described (3). Amino acid analysis showed that 210
pmol of pure EGF receptor were obtained. This material was
digested with cyanogen bromide and the resulting peptides
were collected and counted for 32P label; the

data presented in Fig. 4. Determination of the sequence of the site at
which protein kinase C phosphorylates the EGF receptor. Receptor
was purified and phosphorylated by protein kinase C and digested
with cyanogen bromide as described under "Experimental Proce-
dures." A, separation of cyanogen bromide peptides on a TSK 3,000
sizing column. Solid line, optical density profile of 280 nm of cyanogen
bromide peptides eluted from the TSK 3,000 column. 0.3-ml fractions
were collected and counted for 32P label; the broken line shows the
profile of 32P label/fraction. The positions at which standard molec-
ular weight marker proteins run on this system are marked with
arrows. B, reverse phase HPLC purification of a V8 digest of the
major phosphopeptide peak from A. The solid line shows the optical
density profile of the eluted peptides at 206 nm. The broken line shows
the profile of 32P label/fraction. C, fractions corresponding to the
major phosphopeptide peak from B were pooled, lyophilized, and
sequenced using a gas phase sequencer (see "Experimental Pro-
cedures"). The yield of PTH amino acid (PTH-oa) at each cycle is
plotted against residue number. The identity of the residue is written
above each peak in single letter code.

The stoichiometry of receptor phosphorylation.

The human monoclonal antibody
EGF, 20 μM ATP
EGF

14542

| Preincubation | Vmax | Kmax | r 
|---------------|------|------|-----
| EGF, 20 μM ATP| 0.60 | 0.17 | 0.991 |
| EGF | 0.54 | 0.19 | 0.992 |

Fig. 3. The effect of receptor autophosphorylation on the
exogenous kinase activity, II. The data presented in Fig. 2 has
been used to predict the Vmax values for the kinase reaction with
respect to peptide under the conditions described in the legend to
Fig. 2. The rate of the kinase reaction, V, at a given substrate
concentration, [S], and a known Kmax for peptide (see Table II) allow
the value of Vmax to be calculated from the Michaelis-Menten equation
Vmax = ([S] + Kmax V)/[S]. Predicted Vmax is plotted against the
stoichiometry of receptor phosphorylation. A, assays carried out at
30 °C. B, assays carried out at 0 °C. ○, prephosphorylated receptor
(EGFR-P) assayed in the presence of EGF. △, control nonphosphory-
lated receptor (EGFR-C) assayed in the presence of EGF. O,
prephosphorylated receptor (EGFR-P) assayed in the absence of
EGF. Δ, control nonphosphorylated receptor (EGFR-C) assayed in
the absence of EGF. In the case of the prephosphorylated preparation
(EGFR-P) the stoichiometry of phosphate groups/receptor includes
the 1.6 phosphates/receptor incorporated into the receptor prior to
the assay.

| TABLE III | Kmax and Vmax determinations for the substrate peptide P1 using
nonsolubilized A431 cell plasma membrane preparations |
|-----------|--------------------------------------------------------|
| Preincubation | Vmax | Kmax | r 
|---------------|------|------|-----
| EGF, 20 μM ATP| 0.60 | 0.17 | 0.991 |
| EGF | 0.54 | 0.19 | 0.992 |
was free phosphate (data not shown). Fractions corresponding to the phosphothreonine-containing peak were pooled, digested with V8 protease, and analyzed on a reverse phase HPLC column using 0.1% trifluoroacetic acid buffers and an acetonitrile gradient (Fig. 4B). Fractions containing the 32P-labeled peptide were pooled, and the amino-terminal sequence was determined on a gas phase sequencer (see “Experimental Procedures”). The yields and identities of phenylthiohydantoin derivatives of amino acids at each cycle are shown in Fig. 4C. The identification of 7 residues at the amino terminus of this peptide made it possible to unequivocally assign threonine 654 as the target for protein kinase C.

When EGF receptor from A431 cells metabolically labeled with [32P]orthophosphate was digested by this protocol, the only phosphopeptide found in receptor from tumor promoter (PMA) treated cells which was absent in receptor from control cells comigrated with the peptide sequenced here on reverse phase HPLC at pH 2.0 and 6.5 (data not shown).

Functional Effect of EGF Receptor Threonine 654 Phosphorylation—The effects of protein kinase C phosphorylation on ligand binding and protein-tyrosine kinase activity of the EGF receptor were studied. First, the exogenous kinase activity of protein kinase C phosphorylated and control receptor preparations at varying concentrations of EGF (see Fig. 5) were determined. Purified EGF receptor from A431 cells (23) was incubated with calcium, phosphatidylycerine, and MgATP with or without purified protein kinase C using concentrations of EGF from 0 to 160 nM (as described under “Experimental Procedures”). The kinase activity towards the pp60src-derived tyrosine kinase substrate peptide (RS in Table I) was measured in each case; this peptide was used because peptides P1, P2, and P3 acted as substrates for protein kinase C. It was found that the response of the EGF receptor protein-tyrosine kinase activity to EGF was attenuated following phosphorylation by protein kinase C. Thus, it required a 3-fold higher concentration of EGF to obtain a 50% activation of the EGF receptor protein-tyrosine kinase after phosphorylation of receptor by protein kinase C. The maximal kinase activity at saturating EGF concentrations was not, however, greatly altered: protein kinase C phosphorylation caused a reduction of only 19 ± 7% (mean and S.E., n = 3).

These results imply that phosphorylation of the EGF receptor at threonine 654 may directly decrease the affinity of the receptor for EGF. This was confirmed by Scatchard analysis (32) of the binding of 125I-labeled EGF to solubilized purified receptor using a radioimmunoassay (25). Scatchard plots for ligand binding to unphosphorylated EGF receptor, autophosphorylated receptor, and receptor phosphorylated by purified protein kinase C are shown in Fig. 6. While the affinity of the receptor for EGF was not changed by autophosphorylation ($K_d$ = 16.4 nM autophosphorylated, 14.9 nM unphosphorylated), phosphorylation by protein kinase C caused a significant decrease in the apparent affinity ($K_d$ = 46.6 nM). Thus, the affinity of the EGF receptor for its ligand in solution is decreased 3-fold when receptor is phosphorylated by protein kinase C, in agreement with the data presented in Fig. 5.

Since the affinity of the EGF receptor for its ligand is known to be greatly reduced upon solubilization (25), the effect of protein kinase C phosphorylation on the receptor in membrane preparations was studied. The binding of 125I-EGF to a plasma membrane preparation from A431 cells was investigated using a filter assay (see “Experimental Procedures”). Fig. 7 shows Scatchard plots generated for binding of EGF to nonphosphorylated receptor, autophosphorylated receptor, and for protein kinase C phosphorylated receptor (0.8 mol of phosphate/mol of receptor at Thr 654). The total number of receptor binding sites was unaffected by phosphorylation. The Scatchard plots for receptor in the absence of protein kinase C are curvilinear: this may indicate the presence of both low (Kd = 7 nM) and high affinity binding sites (Kd = 0.8 nM). For receptor which has been phosphorylated by protein kinase C, the Scatchard plot is virtually linear suggesting that the high affinity binding sites have been converted to low affinity sites by phosphorylation of the receptor; the total number of binding sites remained constant.

![Fig. 5. Phosphorylation of the EGF receptor by protein kinase C: the effect on its activation by EGF.](image)

![Fig. 6. Scatchard plots for 125I-EGF binding to purified EGF receptor in solution: the effect of phosphorylation by protein kinase C.](image)
FIG. 7. Scatchard plots for $^{125}$I-EGF binding to EGF receptor in an A431 cell membrane preparation: effect of phosphorylation by protein kinase C. The binding of $^{125}$I-EGF to A431 cell plasma membrane preparations was measured as described under "Experimental Procedures." The specific activity of the ligand was $1.5 \times 10^{8}$ cpm/mol. The concentration of ligand was varied from 0.05 to 50 nM. Each point was determined in duplicate with background subtracted. ▲, binding assays performed in the presence of inactive protein kinase C and in the absence of ATP. ○, binding assays performed in the presence of inactive protein kinase C and in the presence of 100 μM ATP. ■, binding assays performed in the presence of activated protein kinase C and 100 μM ATP.

DISCUSSION

A detailed study of the effect of phosphorylation on the EGF receptor protein-tyrosine kinase and its ligand binding properties has been presented. Peptides containing sequences identical to the major in vitro autophosphorylation sites were synthesized and used as substrates for the EGF receptor protein-tyrosine kinase. The peptide containing the sequence surrounding tyrosine 1173 appeared to be the preferred substrate as deduced from the observed lower (2-fold) apparent $K_a$. This result was consistent with the observation that tyrosine 1173 is also the most rapidly autophosphorylated site on the intact purified receptor. In addition, tyrosine 1173 is the phosphorylation site that is most extensively phosphorylated in intact A431 cells in response to EGF (5). The apparent preference for the phosphorylation of receptor tyrosine 1173 in vitro thus appears to correlate with the situation observed for receptor in vivo.

Our study of the effects of autophosphorylation on protein-tyrosine kinase activity have shown that over a wide range of stoichiometry of phosphorylation (0.1–2.5 mol of phosphate/mol of receptor) there is no apparent change in either EGF binding to receptor or of receptor protein-tyrosine kinase activity. These results were obtained with both purified detergent-solubilized EGF receptor and also with receptor in A431 cell membrane preparations. This lack of response to autophosphorylation is consistent with previous published studies. For example, thiophosphorylation of the receptor does not greatly alter receptor kinase activity (33) and it has been shown that there is no lag phase in the phosphorylation of A431 cell membrane proteins in response to EGF (34), again indicating that autophosphorylation does not appear to activate the EGF-stimulated protein-tyrosine kinase activity.

The lack of effect of autophosphorylation on the activity of the EGF receptor protein-tyrosine kinase is interesting when the effects of autophosphorylation on certain protein-tyrosine kinases of the src family are considered. Thus, although the kinase activity of pp60src itself is thought to be activated by phosphorylation on tyrosine residues (35), removal of the major site of autophosphorylation (tyrosine 416) by mutagenesis does not alter the kinase activity of pp60src (36, 37), perhaps indicating that the regulatory autophosphorylation is occurring at a second site. Different results were reported in studies of P130 at its-kinase. In this case, deletion of the major autophosphorylation site, tyrosine 1073 (homologous to src tyrosine 416), caused a 5-fold decrease in kinase activity (38). In the case of the insulin receptor, autophosphorylation on tyrosine generates an active receptor protein-tyrosine kinase that is no longer dependent upon insulin (39, 40). The insulin receptor is phosphorylated at 2 or 3 tyrosines (40, 41), none of which have been assigned in the primary structure. In the insulin receptor, there is no domain analogous to the C-terminal domain which includes the autophosphorylation sites of the EGF receptor (42). Thus, it seems that there is no general or consistent response to autophosphorylation among these different protein-tyrosine kinases. In the case of the EGF receptor, one may suppose that autophosphorylation plays some as yet undefined role in the function of the receptor. One possible effect of autophosphorylation might be to alter the protein substrate specificity of the kinase. In this study, we have presented data based on the activity directed towards low molecular weight substrates and we cannot of course rule out an effect where autophosphorylation of the receptor may alter access of larger protein substrates to the catalytic site.

With respect to the role of the receptor as a protein kinase, the specific activity of the purified EGF receptor protein-tyrosine kinase is 1035 units/mg at saturating substrate concentrations. This value is similar to those observed for purified pp60src and Abeleon protein-tyrosine kinases and is similar to the specific activity of a number of serine/threonine-specific protein kinases. The turnover number for this protein relative to the catalytic subunit of the cAMP-dependent protein kinase (2500 units/mg) is 1.7. The well-characterized role of the cAMP-dependent protein kinase as a regulator of intracellular protein phosphorylation and the similar phosphorylation potential of the purified EGF receptor suggests that the EGF receptor may indeed function as a protein tyrosine kinase in vivo. In this respect, we have been unable to demonstrate the phosphorylation of phosphotyrosinolysitol employing purified EGF receptor and would conclude that this is not an activity intrinsic to the receptor.

We have determined the amino acid sequence of the site on the EGF receptor phosphorylated by protein kinase C (threonine 654). This direct observation confirms the conclusion of Hunter et al. (18) and Davis and Czech (19) where in vitro phosphorylated synthetic peptides based on the sequence around Thr 654 were compared with phosphopeptides from the receptor by peptide mapping. Our data show that phosphorylation of the EGF receptor at Thr 654 directly reduces receptor affinity for its ligand EGF, both in purified preparations and in membrane systems. For purified solubilized receptor, phosphorylation by protein kinase C induces an absolute change in ligand binding from high to low affinity, while for receptor in membranes the curvilinear Scatchard plot becomes straight, indicating that conversion of high affinity to low affinity sites takes place within a mixed population or, conceivably, indicating that a homogenous population of high affinity sites interacting with negative cooperativity has been converted to a population of noninteracting low affinity sites. This implies that the loss of high affinity

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*J. Downward, M. D. Waterfield, and P. Parker, unpublished observations.*
Epidermal Growth Factor Receptor Phosphorylation

EGF binding sites seen on treatment of A431 cells and fibroblasts with tumor promoters (14, 43) or diacylglycerol (15) is caused by the phosphorylation of the EGF receptor on Thr 654 by activated protein kinase C. Our experiments have not included tumor promoters or diacylglycerols as activators of protein kinase C and therefore indicate that these amphiphilic compounds do not directly contribute to the drop in affinity.

The loss of high affinity EGF binding sites observed when Swiss 3T3 cells are treated with PDGF (20, 44), bombesin (45) or vasopressin (46) ("transmodulation") is therefore probably mediated by the direct action of protein kinase C on the EGF receptor polypeptide itself; all these agents will stimulate phosphatidylinositol turnover and may therefore activate protein kinase C.

On treatment of A431 cells with tumor promoters, there is a large reduction in the level of phosphotyrosine in the EGF receptor (50% at 0.1 ng/ml PMA) (43). However, protein kinase C phosphorylation of the EGF receptor in vitro reduces its kinase activity at saturating EGF levels by only 20%. This apparent contradiction could be explained by assuming either that additional constraints exist for the receptor in the cell membranes which are lost on solubilization, or that a finely balanced cycle of autophosphorylation and dephosphorylation exists that at subsaturating concentrations of EGF, the receptor-tyrosine kinase activity is suppressed by greater than 20%. Thus, at 14 nM EGF (K<sub>e</sub> for EGF) the effect of phosphorylation of threonine 654 is to decrease the receptor kinase activity by approximately 60% (see Fig. 5).

The protein kinase C phosphorylation site on the EGF receptor (threonine 654) lies in the cytoplasmic domain, 10 residues C-terminal to the putative transmembrane sequence, within a highly basic stretch of amino acids which contains 8 out of 13 basic residues. The proximity of this sequence to the transmembrane domain suggests that these positively charged residues may interact with negatively charged head groups in the phospholipid bilayer. This interaction could be significantly perturbed by the introduction of a phosphate group at threonine 654. Such an effect may underlie the functional changes associated with the phosphorylation of the receptor by protein kinase C.

While our results indicate that phosphorylation of the receptor at Thr 654 causes a reduction in EGF binding affinity, it does not completely explain the presence of apparently high and low affinity receptors in intact cells. If A431 cells are labeled to equilibrium with [32P]orthophosphate and the EGF receptor is purified in the presence of phosphatase inhibitors, there is no detectable phosphorylation at Thr 654. Nevertheless, the receptor on these cells displays both high (5-10%) and low (90-95%) affinity states. It is therefore necessary to propose that factors in addition to the phosphorylation state of threonine 654 are involved in the distribution between high and low affinity states of the EGF receptor.

There are many possible explanations for this situation; however, it should be emphasized that in vitro with purified receptor one can observe an affinity change on phosphorylation by protein kinase C (see Fig. 6) indicating that in this situation the change in affinity is due to an alteration in the intrinsic physical state of the receptor and not to interactions of the receptor with other proteins. Perhaps the simplest explanation for these observations would be that the EGF receptor exists in a dynamic equilibrium between different aggregated forms, for example monomer and dimer. These forms could exhibit differing affinities for EGF, such that monomeric receptor would bind with high affinity and dimeric receptor with low affinity. In this event one can calculate an equilibrium constant (K<sub>e</sub>) given the total number of receptors/cell and the relative amounts of high and low affinity sites. Thus, from Ref. 20 we would calculate K<sub>e</sub> = 4.4 × 10<sup>-5</sup> [concentration units]<sup>-1</sup> for Swiss 3T3 cells. This equilibrium constant has dimensions of 1/concentration; it is evident, therefore, that changes in the total concentration of receptor will affect the monomer/dimer ratio. To take the extreme case of A431 cells for example, where there are 3.3 × 10<sup>6</sup> receptors/cell (15), and using the equilibrium constant derived for 3T3 cells, one can calculate that the proportion of receptors in a high affinity state (monomeric) would be 6.5% (accounting for the larger surface area of Swiss 3T3 cells relative to A431 cells). This value is surprisingly close to that determined empirically and would be consistent with the hypothesis. Receptor-receptor interaction provides not only one of many possible solutions to the paradox outlined above but also a basis for the interactions between the intracellular and extracellular domains of the receptor. Elaborating this simple model, one might predict that EGF also induces changes in the receptor aggregation state. Thus, EGF would bind initially (although not exclusively) to its high affinity site, creating a conformational change in the extracellular domain that would promote interaction between such domains.

This draws into close proximity the intracellular domains and produces in the presence of bound EGF an active tyrosine kinase. This model is meant to provide a conceptual basis with which to probe the communication between extracellular and intracellular domains of the EGF receptor. If such interactions occur, it may transpire that autophosphorylation also plays a functional role at this quaternary level. Biophysical studies are at present being employed to elucidate these points.

REFERENCES
1. Carpenter, G. (1981) Handb. Exp. Pharmacol. 57, 89-132
2. Guroff, G. (1938) Growth and Maturation Factors, Vol. 1, Wiley, New York
3. Downward, J., Yarden, Y., Mayes, E., Sracce, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., and Waterfield, M. D. (1984) Nature 307, 521-527
4. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Lam, A. W., Lee, J., Yarden, Y., Liberman, T. A., Schlessinger, J., Downward, J., Mayes, E., Waterfield, M. D., Whittle, N., and Seeburg, P. H. (1984) Nature 309, 418-425
5. Downward, J., Parker, P., and Waterfield, M. D. (1984) Nature 311, 483-485
6. Hunter, T., and Cooper, J. A. (1985) Annu. Rev. Biochem. 54, 897-930
7. Uehiro, H., and Cohen, S. (1989) J. Biol. Chem. 255, 8363-8365
8. Kris, R. M., Lax, L, Gullick, W., Waterfield, M. D., Ullrich, A., Fridkin, M., and Schlessinger, J. (1985) Cell 40, 619-625
9. Gilmore, T., DeClue, J. E., and Martin, G. S. (1985) Cell 40, 609-618
10. Decker, S. J. (1985) J. Biol. Chem. 260, 2003-2006
11. Hunter, T., and Cooper, J. A. (1985) Cell 24, 741-752
12. Cochet, C., Gill, G. N., Meisenhelder, J., Cooper, J. A., and Hunter, T. (1984) J. Biol. Chem. 259, 2553-2558
13. Iwashita, S., and Fox, C. R. (1984) J. Biol. Chem. 259, 2559-2567
14. Davis, R. J., and Czech, M. P. (1984) J. Biol. Chem. 259, 8546-8549
15. McCaffrey, P. G., Friedman, B. A., and Rosner, M. R. (1984) J. Biol. Chem. 259, 12502-12507
16. Ghosh-Dastidar, P., and Fox, C. F. (1984) J. Biol. Chem. 259, 3864-3869
17. Ruckhoff, W. R., Rubin, R. A., and Earp, H. S. (1984) Mol. Cell. Endocrin. 34, 113-119
18. Hunter, T., Ling, N., and Cooper, J. A. (1984) Nature 311, 480-483
19. Davis, R. J., and Czech, M. P. (1986) Proc. Natl. Acad. Sci. U. S. A. 82, 1974-1978
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20. Collins, M. K. L., Sinnett-Smith, J. W., and Rozengurt, E. (1983) J. Biol. Chem. 258, 11689–11695
21. Lifshitz, A., Lazar, C. S., Buss, J. E., and Gill, G. N. (1983) J. Cell. Physiol. 115, 235–242
22. Thom, D., Powell, A. J., Lloyd, C. W., and Rees, D. A. (1977) Biochem. J. 168, 187–194
23. Parker, P. J., Young, S., Gullick, W. J., Mayes, E. L. V., Bennett, P., and Waterfield, M. D. (1984) J. Biol. Chem. 259, 9906–9912
24. Savage, C. R., Jr., and Cohen, S. (1972) J. Biol. Chem. 247, 7609–7611
25. Gullick, W. J., Downward, D. J. H., Marsden, J. J., and Waterfield, M. D. (1984) Anal. Biochem. 141, 253–261
26. Parker, P. J., Stabel, S., and Waterfield, M. D. (1984) EMBO J. 3, 953–969
27. Buss, J. E., and Stull, J. T. (1985) Methods Enzymol. 99, 7–14
28. Hewick, R. M., Hunkapiller, M. W., Hood, L. E., and Dreyer, W. J. (1981) J. Biol. Chem. 256, 7960–7967
29. Casnellie, J. E., Harrison, M. L., Pike, L. J., Hellstrom, K. E., and Krebs, E. G. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 282–286
30. Smart, J. E., Oppermann, H., Czernilofsky, A. P., Purchio, A. F., Erikson, R. L., and Bishop, J. M. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 6013–6017
31. Weber, W., Bertics, P. J., and Gill, G. N. (1984) J. Biol. Chem. 259, 14631–14636
32. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672
33. Cassel, D., Pike, L. J., Grant, G. A., Krebs, E. G., and Glaser, L. (1983) J. Biol. Chem. 258, 2945–2950
34. Carpenter, G., King, L., Jr., and Cohen, S. (1979) J. Biol. Chem. 254, 4884–4891
35. Purchio, A. F., Wells, S. K., and Collett, M. S. (1983) Mol. Cell. Biol. 3, 1589–1597
36. Snyder, M. A., Bishop, J. M., Colby, W. W., and Levinson, A. D. (1985) Cell 32, 891–901
37. Cross, F. R., and Hanafusa, H. (1983) Cell 34, 597–607
38. Weinmaster, G., Zoller, M. J., Smith, M., Hinze, E., and Pawson, T. (1984) Cell 37, 559–568
39. Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M., and Cobb, M. H. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3237–3240
40. Yu, K.-T., and Czech, M. P. (1984) J. Biol. Chem. 259, 5277–5286
41. Petruzzelli, L., Herrera, R., and Rosen, O. M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3327–3331
42. Ullrich, A., Bell, J. R., Chen, E. Y., Herbst, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liac, Y.-C., Tsukubawa, M., Mason, A., Seeburg, P. H., Grunfield, C., Rosen, O. M., and Ramochandran, J. (1985) Nature 313, 756–761
43. Friedman, B. A., Frackleton, A. R., Ross, A. H., Connors, J. M., Fujiki, H., Sugimura, T., and Rosner, M. R. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3034–3038
44. Bowen-Pope, D. F., DiCorleto, P. E., and Ross, R. (1983) J. Cell Biol. 96, 679–683
45. Brown, K. D., Blay, J., Irvine, R. F., Heslop, J. P., and Berridge, M. J. (1984) Biochem. Biophys. Res. Commun. 123, 377–384
46. Rozengurt, E., Brown, K. D., and Pettican, P. (1981) J. Biol. Chem. 256, 716–722