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Regulation of the Epidermal Growth Factor Receptor by Phosphorylation

Paul J. Bertics, Wolfgang Weber, Claude Cochet, and Gordon N. Gill
Department of Medicine, University of California, San Diego, La Jolla, California 92093

The receptor for epidermal growth factor (EGF) is a glycosylated transmembrane phosphoprotein that exhibits EGF-stimulable protein tyrosine kinase activity. On EGF stimulation, the receptor undergoes a self-phosphorylation reaction at tyrosine residues located primarily in the extreme carboxyl-terminal region of the protein. Using enzymatically active EGF receptor purified by immunoaffinity chromatography from A431 human epidermoid carcinoma cells, the self-phosphorylation reaction has been characterized as a rapid, intramolecular process which is maximal at 30–37°C and exhibits a very low Km for ATP (0.2 μM). When phosphorylation of exogenous peptide substrates was measured as a function of receptor self-phosphorylation, tyrosine kinase activity was found to be enhanced two to threefold at 1–2 mol of phosphate per mol of receptor. Analysis of the dependence of the tyrosine kinase activity on ATP concentration yielded hyperbolic kinetics when plotted in double-reciprocal fashion, indicating that ATP can serve as an activator of the enzyme. Higher concentrations of peptide substrates were found to inhibit both the self- and peptide phosphorylation, but this inhibition could be overcome by first self-phosphorylating the enzyme. These results suggest that self-phosphorylation can remove a competitive/inhibitory constraint so that certain exogenous susbtrates can have greater access to the enzyme active site. In addition to self-phosphorylation, the EGF receptor can be phosphorylated on threonine residues by the calcium- and phospholipid-dependent protein kinase C. The sites on the EGF receptor phosphorylated in vitro by protein kinase C are identical to the sites phosphorylated on the receptor isolated from A431 cells exposed to the tumor promoters 12-O-tetradecanoylphorbol 13-acetate or teleocidin. This phosphorylation of the EGF receptor results in a suppression of its tyrosine kinase and EGF binding activities both in vivo and in vitro. The EGF receptor can thus be variably regulated by phosphorylation: self-phosphorylation can enhance tyrosine kinase activity whereas protein kinase C-catalyzed phosphorylation can depress enzyme activity. Because these two phosphorylations account for only a fraction of the phosphate present in the EGF receptor in vivo, other protein kinases can apparently phosphorylate the receptor and these may exert additional controls on EGF receptor/kinase function.

Key words: epidermal growth factor receptor, protein-tyrosine kinase, self-phosphorylation, protein kinase C, oncogene, growth factor

W. Weber's present address is Institut fur Physiologische Chemie der Universitut Hamburg, Martinistr. 52, 2 Hamburg 2, West Germany.

C. Cochet's present address is Laboratoire de Biochimie Endocrinienne, Unite INSERM 224 CERMO, Domaiane Universitaire de st. Martin d'Heres, BP 68, 38402 Grenoble Cedex, France.

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Epidermal growth factor (EGF) is a small (6 KDa) peptide mitogen which can stimulate the proliferation of numerous epidermal and epithelial cell types [1,2]. The receptor for EGF is a 170-KDa transmembrane glycoprotein with intrinsic protein-tyrosine kinase activity [3–5]. The binding of EGF to the extracellular domain of the receptor results in an activation of its tyrosine kinase activity and increased intracellular substrate phosphorylation [4]. The cytoplasmic tyrosine kinase domain of the receptor is homologous to erb B, the transforming protein of the avian erythroblastosis virus [6], and also exhibits structural similarity to other cellular protein-tyrosine kinases which have been identified as the proto-oncogene products of several retroviruses [7–11]. These proteins appear important in the control of cell growth and their altered expression can lead to the abnormal proliferative state seen in transformed cells.

The EGF receptor is an 1,186-amino acid phosphoprotein in vivo and has been shown to contain 2.5–3.5 mol of phosphate per mol of receptor protein [8,12]. Various distinct phosphorylation sites have been detected using tryptic phosphopeptide mapping, including several phosphotyrosine-containing self-phosphorylation sites [4,13] and phosphothreonine-containing peptides which are phosphorylated both in vivo and in vitro by the calcium- and phospholipid-dependent protein kinase C [12,14,15]. In this report, we summarize the properties of the self-phosphorylation reaction and provide evidence that this process can serve as a mechanism for enhancing the receptor's tyrosine kinase activity toward certain exogenous substrates. In addition, we detail several experimental observations which demonstrate that the protein kinase C-catalyzed phosphorylation of the EGF receptor on threonine attenuates its protein-tyrosine kinase activity.

SELF-PHOSPHORYLATION OF THE EGF RECEPTOR

When A431 human epidermoid carcinoma cells are labeled with ^{32}P in vivo, the EGF receptor is constituitively phosphorylated at several serine and threonine residues [4,12]. Stimulation of these cells with EGF results in receptor self-phosphorylation on tyrosines located in the extreme carboxyl-terminus of the protein [13]. Tyrosine 1,173 is the major site of self-phosphorylation enhanced by EGF, although receptor self-phosphorylation also occurs on tyrosines 1,068 and 1,148 [13]. These identified sites of self-phosphorylation contain one or more acidic residues located amino-terminal to the phosphorylation site and at least one hydrophobic amino acid near to the phosphorylated tyrosine residue [13,16].

Studies on the kinetics of self-phosphorylation utilized immunoaffinity-purified EGF receptor protein which was isolated from A431 cells and was fully saturated with EGF [17]. These investigations indicate that even at low temperatures (0°C) and modest ATP concentrations (5.0 μM), there is a very rapid ($<$ 15 sec) incorporation of 1 mol of phosphate per mol of receptor [17]. When the temperature dependence of self-phosphorylation was determined using 5 μM ATP, about two phosphates per receptor were incorporated after 10 min at 0°C, but the extent of self-phosphorylation rose with increasing temperature to a plateau of about four phosphates per receptor at 30–37°C [17]. Fig. 1A shows a double-reciprocal plot of the ATP dependence of the self-phosphorylation reaction. Under initial velocity conditions, a Km for ATP of about 0.2 μM is calculated. This value is in contrast to the Km for ATP of 1–3 μM determined for the phosphorylation of exogenous peptide substrates [18].

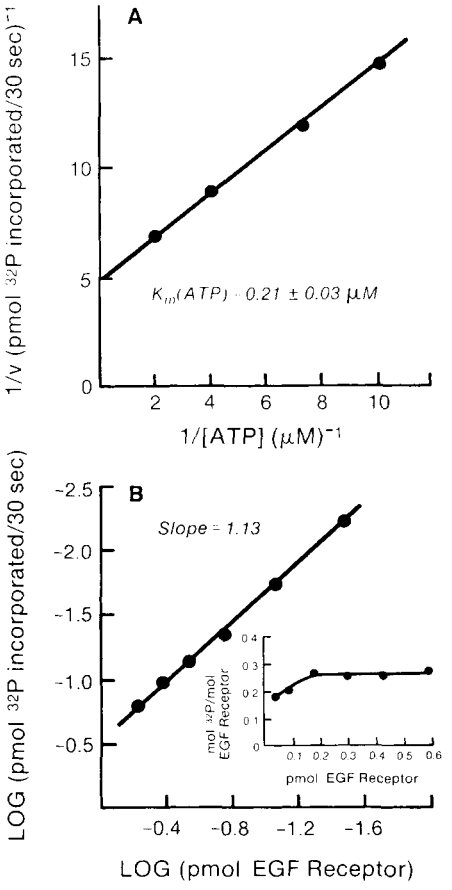


Fig. 1. The effect of ATP and enzyme concentration on EGF receptor self-phosphorylation. Panel A) determination of the Km for ATP of the self-phosphorylation process. Self-phosphorylation of 7.3 nM EGF receptor was measured at 0°C using a 30-sec assay time and an [γ-^{32}P]-ATP concentration range of 0.1–0.5 μM. The data points shown are the average of duplicate measurements and similar results were obtained in two other experiments. Panel B) Dependence of self-phosphorylation on enzyme concentration. Purified EGF receptor was assayed for self-phosphorylating activity in the presence of 0.3μM [γ-^{32}P]-ATP for 30 sec at 0°C. Enzyme concentration was varied from 0.54 to 9.7 nM. The data are plotted as the log of the initial velocity versus the log of enzyme amount (van't Hoff plot). Linear regression of the data points yielded a slope of 1.13 and a correlation coefficient 0.99. Inset, plot of the total amount of phosphate incorporated per receptor as a function of enzyme level. Reprinted from Weber et al [17].

Because the mechanism of EGF receptor self-phosphorylation could proceed by either an intramolecular or intermolecular process, the effect of enzyme concentration on the reaction rate was investigated [17]. At a constant ATP concentration, the initial velocity would be linear with enzyme concentration in an intramolecular (first-order) reaction whereas it would be hyperbolic (second order) in an intermolecular process. Furthermore, a plot of the log of the initial velocity versus the log of enzyme concentration (a van't Hoff plot) yields a slope equal to the enzyme's stoichiometry

in the reaction [19,20]. Figure 1B shows a van't Hoff plot of the initial velocity of EGF receptor self-phosphorylation over an 18-fold enzyme dilution range. The slope of the plot is one, indicating that a single EGF receptor is involved in the reaction; ie, the preferred self-phosphorylation occurs intramolecularly. Similar results were obtained using a 50-fold range of enzyme concentration and saturating ATP. Also consistent with an intramolecular model is the fact that the amount of phosphate incorporated per receptor varied by less than twofold even though receptor concentration varied by 18-fold (Fig. 1B inset), indicating that the self-phosphorylation reaction is relatively independent of enzyme concentration. This apparent intramolecular nature of the self-phosphorylation reaction does not appear to be an artifact of receptor aggregation because the purified receptor behaves as a monomeric 170–180-KDa protein upon gel filtration.

The effect of self-phosphorylation on EGF receptor protein-tyrosine kinase activity was analyzed by measuring the ability of the immunoaffinity-purified receptor to phosphorylate exogenous substrates as a function if its self-phosphorylation state [21,22]. In these studies, the receptor was first incubated with ATP for various time periods to phosphorylate the receptor to differing degrees. After the first incubation, the src-peptide [23], an exogenous peptide substrate which corresponds to the site of self-phosphorylation of $p60^{V\text{-}src}$, was added to the reaction mixture and the tyrosine phosphorylation of this substrate was measured. Figure 2A shows that maximal src-peptide phosphorylation occurred when at least 1.5 mol of phosphate were incorporated per mol of receptor. Because ATP was present for substrate phosphorylation during the second incubation, self-phosphorylation occurred during this period resulting in a minimal phosphorylation state of about 0.6 mol of phosphate per mol of receptor protein by the end of the assay. Thus, the activity of the completely unphosphorylated receptor could not be measured, but increasing the self-phosphorylation state from 0.6 to 1.5 mol of phosphate per mol of receptor resulted in about a 50% increase in tyrosine kinase activity. Similar results were observed at various concentrations of the src-peptide as well as with another exogenous peptide substrate, angiotensin II [17,24].

It was reasoned that the magnitude of the observed effect of self-phosphorylation in these experiments was a minimal one because significant self-phosphorylation occurred under all assay conditions. However, if the enzyme was first incubated with saturating ATP to achieve a high level of self-phosphorylation and then peptide phosphorylation was measured using a very short incubation time and low levels of ATP, one observed little self-phosphorylation during the peptide assays. Increasing self-phosphorylation from a low to a higher level of phosphate per receptor increased enzyme activity two to threefold [22]. This stimulation does not appear to be due to ATP trapping on enzyme dilution with peptide substrates because the kinetic mechanism of the EGF receptor involves ordered addition of substrates wherein peptide binding precedes ATP binding [18]. It should be noted that this kinetic mechanism may also be valid for the self-phosphorylation reaction, except in this case the self-phosphorylation site would be considered the peptide/protein substrate which combines with the enzyme first.

To evaluate whether the self- and peptide phosphorylation sites competed for the same catalytic site, dose-response studies were carried out using the src-peptide as a putative inhibitor of self-phosphorylation. Figure 2B illustrates that less self-phosphorylation occurred at higher peptide concentrations, suggesting that the two

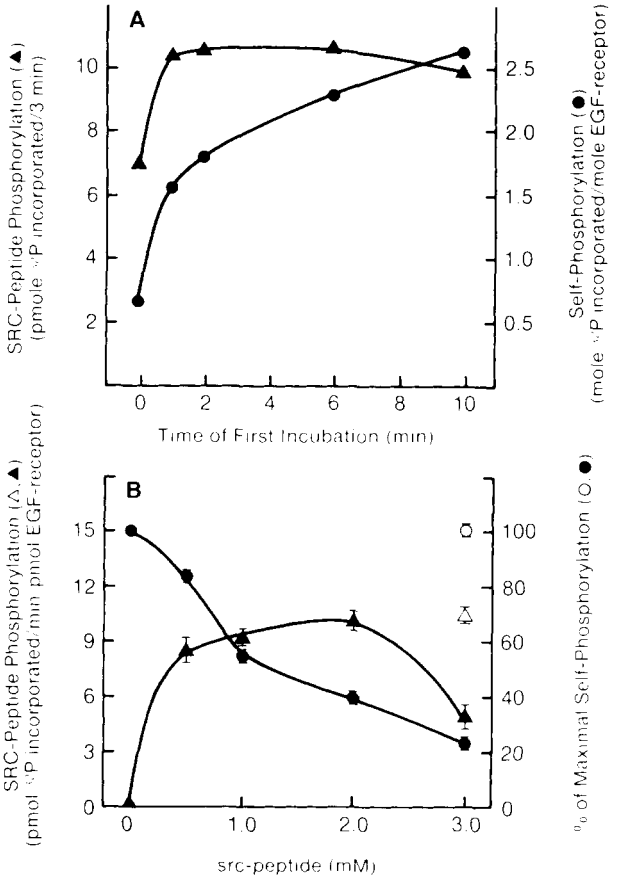


Fig. 2. Effect of EGF receptor self-phosphorylation on protein-tyrosine kinase activity. Panel A) EGF receptor phosphorylation of src-peptide as a function of its self-phosphorylation state. Immunoaffinity-purified EGF receptor (0.68 pmol/assay) was first incubated with 7.5 μM [γ-^{32}P]-ATP at 30°C for various time periods. After incubation with ATP, the src-peptide was added to the reaction mixture to give a final concentration of 1.5 mM and the reaction was carried out at 30°C for 3 min in the presence of 5 μm [γ-^{32}P]-ATP. The reactions were terminated by the addition of trichloroacetic acid. Peptide phosphorylation was measured using phosphocellulose paper and self-phosphorylation was measured using a filter binding assay [17]. Panel B) Inhibition of the EGF receptor self-phosphorylation reaction by the src-peptide. Purified EGF receptor (0.51 pmol/assay) was either combined with the indicated level of src-peptide before the reactions were initiated with ATP (●, ▲) or the EGF receptor was first incubated with 6.7 μM ATP for 1 min at 30°C before src-peptide addition (○, △). The ATP final concentration was 5.0 μM. The self- and peptide phosphorylation reactions were performed at 30°C for 30 sec. Each point is the mean of duplicate measurements ± ranges. Maximal self-phosphorylation was about 0.7 mol of phosphate per mol of receptor. Taken from Bertics and Gill [22].

substrates do in fact compete. It was also observed in these studies that high src-peptide concentrations inhibited peptide phosphorylation as well as self-phosphorylation. However, if the receptor was first incubated with ATP to reach a higher self-phosphorylation state (open circle and triangle in Fig. 2B), the inhibition of tyrosine kinase activity by high peptide levels was prevented. These results suggest that self-

phosphorylation removes an inhibitory constraint so that exogenous substrates can have greater access to the enzyme active site.

The effect of self-phosphorylation on EGF receptor tyrosine kinase activity has also been investigated by measuring the initial rates of phosphorylation of the substrate angiotensin II over a wide range of ATP concentrations [22]. In these studies, purified receptor was first incubated either in the presence or absence of ATP before peptide addition. The double-reciprocal plots of the reaction kinetics, shown in Figure 3, revealed significant differences between the two enzyme preparations. The prephosphorylated EGF receptor exhibited linear kinetics and an apparent Km for ATP of 0.73 $\pm$ 0.03 μM, and a Vmax of 13.2 $\pm$ 0.1 mol of phosphate incorporated per min per mol of receptor at 3 mM angiontensin II (Km = 0.8 mM) was calculated. The unphosphorylated receptor exhibited hyperbolic kinetics when plotted in double-reciprocal fashion and extrapolation of the curve at high ATP levels (the broken line in Fig. 3) yielded an apparent Km for ATP of 0.74 $\pm$ 0.05 μM and a Vmax of 10.2 $\pm$ 0.2 mol of phosphate incorporated per min per mol of enzyme. The observed hyperbolic kinetics suggest that ATP can serve to activate the enzyme [25]. At low ATP concentrations, the receptor may not be able to self-phosphorylate sufficiently to be fully active within the peptide assay time course, whereas at high ATP levels the enzyme can rapidly self-phosphorylate, allowing its kinetic behavior to approach that seen with the prephosphorylated enzyme.

PROTEIN KINASE C PHOSPHORYLATION OF THE EGF RECEPTOR

Earlier investigations by Sawyer and Cohen [26] on the biological effects of EGF on cellular function indicated that EGF can stimulate phosphatidylinositol turnover and Ca^{++} mobilization in A431 cells. The diacylglycerol formed during phosphatidylinositol metabolism has been postulated by Nishizuka [27,28] to be an endogenous activator of the calcium- and phospholipid-dependent protein kinase C, which can phosphorylate serine and threonine residues in a variety of protein substrates [29,30]. Tumor promoters such as 12-O-tetradecanoylphorbol 13-acetate (TPA) and teleocidin, which can have many profound effects on cell growth [31,32], can substitute for diacylglycerol to increase the affinity of protein kinase C for Ca^{++} and phospholipid in activating the enzyme [33]. Protein kinase C and the phorbol diester receptor of rat brain copurify, suggesting that the two activities may reside in the same molecule [34,35]. These observations, together with those indicating that tumor promoters can affect EGF receptor binding [36,37] and can elicit similar biological effects as certain growth factors, prompted several investigators to examine whether the EGF receptor can serve as a substrate for protein kinase C [12,14,15].

To activate protein kinase C in vivo, the tumor promoters TPA and teleocidin were added to A431 cells labeled to equilibrium with [^{32}P]-orthophosphate [12]. This treatment resulted in a two to threefold increase in the phosphorylation of the EGF receptor on both threonine and serine residues. As shown in Figure 4A–C, several new phosphopeptides were detected by tryptic mapping of the EGF receptor isolated from cells treated with tumor promoters. These phosphopeptides were not found in tryptic digests of the receptor obtained from control cells incubated with 4β-phorbol, which is inactive as a tumor promoter. Phospho-amino acid analysis of the peptides which were phosphorylated in response to tumor promoters revealed that they contained phosphothreonine (Fig. 4D). These tryptic peptides were well resolved from

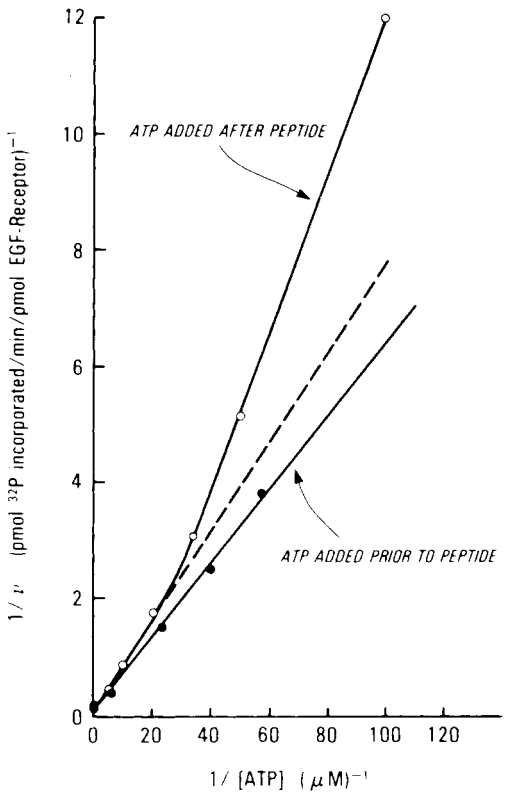
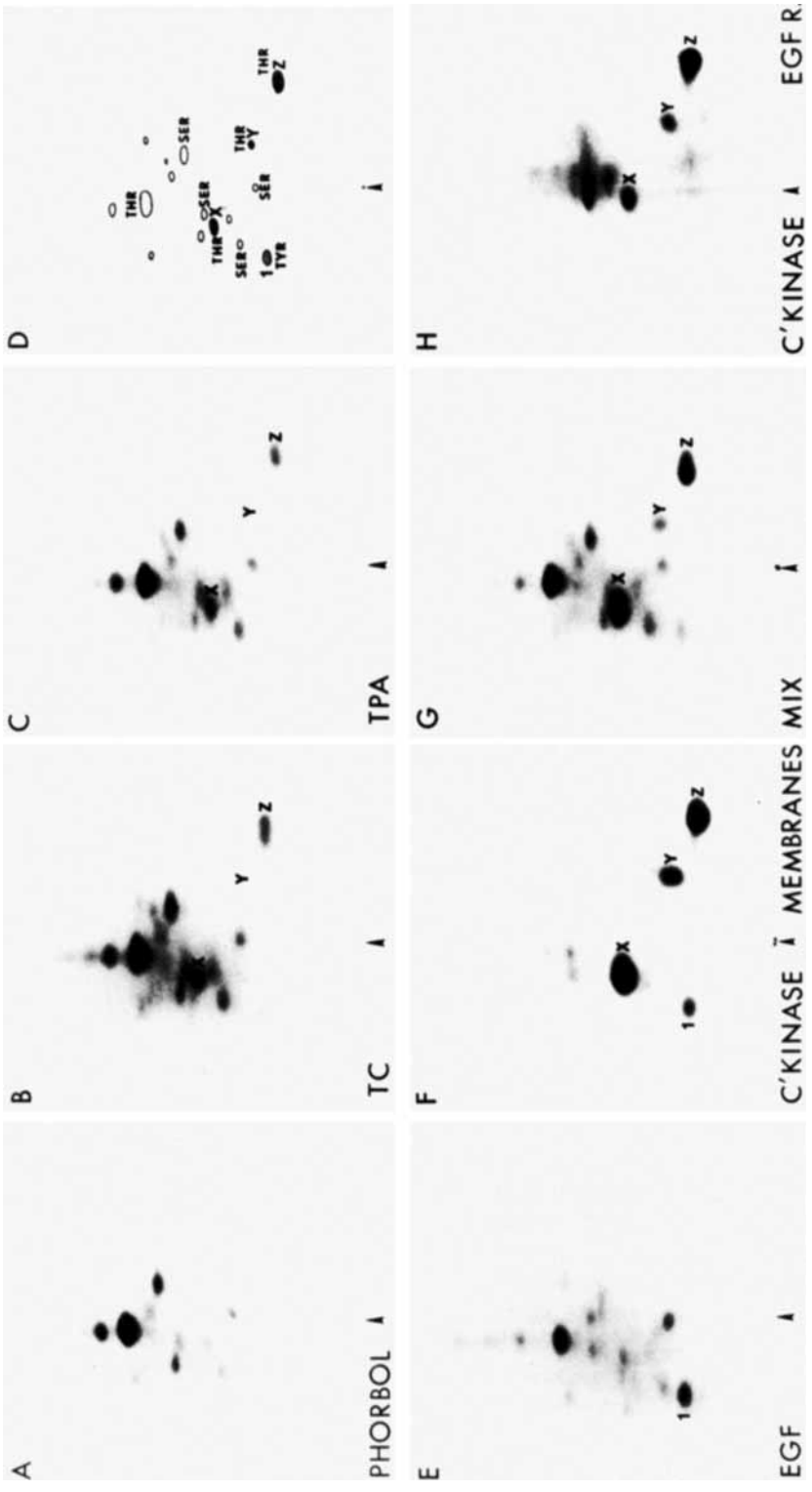


Fig. 3. ATP dependence of EGF receptor phosphorylation of angiotensin II. Purified EGF receptor (0.58 pmol/assay) was either first incubated with 1.5 times the indicated ATP concentration for 1 min at 30°C before angiotensin II addition (3 mM final concentration) (●) or the peptide was added before ATP addition (○). Final ATP concentration varied from 0.01 to 5.0 μM and each point is the mean of duplicate measurements. The peptide assays were run at 30°C for 30 sec and the final ATP concentration in those samples first incubated with ATP was determined by subtracting the amount of phosphate incorporated into the receptor during the first incubation from the initial ATP concentration. Taken from Bertics and Gill [22].

the phosphotyrosine-containing peptides obtained from the receptor isolated from EGF-treated cells (Fig. 4E) and from other phosphopeptides whose pattern was not affected by the addition of tumor promoters. These results suggested that protein kinase C activated by tumor promoters can phosphorylate the EGF receptor in vivo.

When EGF is added to A431 cells, there is increased phosphorylation of the EGF receptor on the same phosphothreonine-containing tryptic peptides phosphorylated in response to tumor promoters [12,14]. This is presumably a result of EGF stimulation to phospholipid turnover leading to an activation of protein kinase C. To determine whether protein kinase C could phosphorylate the EGF receptor in vitro, A431 cell membranes containing large amounts of EGF receptor were incubated with partially purified protein kinase C, ATP, Ca^{++}, and Mg^{++} [12]. Under these conditions, a 170-KDa membrane protein was phosphorylated and was identified as the EGF receptor based on its comigration with purified EGF receptor and on its specific interaction with a monoclonal anti-EGF receptor antibody. The phosphory-


A
B
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EGF R.
X
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Z
1
THR
SER
TYR

lation of this protein was strongly inhibited by chlorpromazine, a known inhibitor of protein kinase C [38], but was not affected by calmodulin. The receptor phosphorylated by protein kinase C was shown by tryptic phosphopeptide mapping (Fig. 4F,G) to contain primarily phosphothreonine, some phosphoserine, and a small amount of phosphotyrosine (apparently a result of some receptor self-phosphorylation), whereas the receptor incubated with ATP, EGF, and Mn^{++} (required for maximal tyrosine kinase activity) contained primarily phosphotyrosine (Fig. 4E).

To confirm the specificity of the apparent protein kinase C-catalyzed phosphorylation of the EGF-receptor, the latter was purified to homogeneity by immunoaffinity chromatography using an anti-EGF receptor monoclonal antibody which recognizes the EGF binding region of the receptor [17]. Protein kinase C catalyzed the phosphorylation of this purifed EGF receptor on the same threonine residues found previously (Fig. 4H), indicating that the EGF receptor can act as a substrate for protein kinase C.

As shown by the experiments in Figure 4, phosphotryptic peptide mapping of the EGF receptor phosphorylated in response to tumor promoters in vivo, or by protein kinase C in vitro, results in the production of two major and one minor new phosphothreonine-containing peptides. Subsequent work by Hunter et al [15] has revealed that these three peptides all contain the identical site of threonine phosphorylation. The generation of these three peptides is apparently due to the presence of successive basic amino acid residues located on either side of the protein kinase C-phosphorylated threonine. Trypsin is unable to cleave efficiently when there exist two or more basic residues sequentially; ie, trypsin does not function well as an exoprotease.

The effect of protein kinase C on EGF receptor protein-tyrosine kinase activity has also been examined in vivo and in vitro [7,12]. When A431 cell membranes containing EGF receptor were first incubated with protein kinase C, Mg^{++}, Ca^{++}, and ATP (unlabeled) followed by addition of EGF, Mn^{++}, and [γ-^{32}P]-ATP, there was a marked reduction in ^{32}P incorporation into the EGF receptor. This indicated that protein kinase C phosphorylation of the receptor decreases its ability to self-phosphorylate. The influence of tumor promoters on EGF-induced tyrosine phosphorylation of the receptor and other cellular proteins in intact A431 was also investigated [12]. Addition of EGF alone increased the relative phosphotyrosine content of total

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Fig. 4. Tryptic phosphopeptide maps of the EGF receptor phosphorylated in response to tumor promoters or directly by protein kinase C. Panels A–E) subconfluent 5-cm-diameter cultures of A431 cells were labeled with 5 mCi of ^{32}P in 2 ml of Dulbecco-Vogt-modified Eagle's medium lacking phosphate and supplemented with 4% complete calf serum. After 16 hr, 4β-phorbol, teleocidin, or TPA was added, and 1 hr later the cells were lysed and the EGF receptor was isolated by immunoprecipitation. In another experiment, EGF was added to ^{32}P-labeled cells for 1 hr before lysis and immunoprecipitation. The EGF receptor was digested with trypsin and an equal fraction of each sample was analyzed. Autoradiographs were exposed with the use of a fluorescent screen. Arrowheads point to the origin of sample application. A) 100 ng/ml 4β-phorbol; B) 100 ng/ml teleocidin (TC); C) 100 ng/ml TPA: D) diagram illustrating the positions of phosphopeptides of the EGF receptor with their component phosphoamino acid indicated (SER, phosphoserine, THR, phosphothreonine, TYR, phosphotyrosine); E) 60 ng/ml EGF. Panels F–H) phosphotryptic maps of the EGF receptor phosphorylated by protein kinase C. F) EGF receptor phosphorylated in A431 cell membranes by protein kinase C; G) mixture of sample analyzed in F with sample analyzed in B; H) immunoaffinity-purified EGF receptor phosphorylated by protein kinase C. Peptides X, Y, and Z are those whose phosphorylation is stimulated by tumor promoters. Reprinted from Cochet et al [12].

TABLE I. Effect of TPA on EGF-Stimulable Protein-Tyrosine Kinase Activity In Vivo*

Additions	Percentage phosphotyrosine content	
	Total cell protein	EGF receptor protein
None	0.04	0
EGF	0.21	8
TPA	0.04	1
TPA + EGF	0.12	3

*In vivo labeling was performed for 16 hr as described in Figure 4. TPA (100 ng/ml) was then added, followed 50 min later by 60 ng/ml of EGF. The cells were harvested 10 min following this treatment. An aliquot of each lysate was extracted into phenol and the phosphoamino acid content of the hydrolyzed protein was then measured [4]. Using a second aliquot of the extract, the EGF receptor was isolated by immunoprecipitation and SDS-polyacrylamide gel electrophoresis and its phosphoamino acid content was quantitated following hydrolysis of the receptor protein. Authentic internal standards were included, and the results are expressed as the percentage of total phosphoamino acids released as phosphotyrosine. Taken from Cochet et al [12].

cellular protein from 0.04 to 0.21% (Table I). Treatment of the cells with TPA alone had no effect on cell phosphotyrosine levels, whereas TPA reduced the EGF-stimulated increase in total cellular phosphotyrosine content by about 45%. Furthermore, the EGF-stimulated increase in the EGF receptor phosphotyrosine content was reduced about 60% by TPA (Table I). Thus, protein kinase C phosphorylation of the EGF receptor results in decreased self-phosphorylation both in vivo and in vitro and in decreased EGF-stimulated tyrosine kinase activity in vivo.

DISCUSSION

The discovery that the transforming proteins of a number of retroviruses possess protein-tyrosine kinase activity pointed to the importance of this enzymatic activity in the regulation of cell proliferation and transformation [11]. The observation that the receptor for EGF possessed protein-tyrosine kinase activity and was homologous to the transforming proteins of certain retroviruses indicated that this receptor probably plays an important role in the control of cell growth [1–4,11,39]. Therefore, it is not too surprising that such a key metabolic enzyme/receptor would be subject to considerable regulation. In addition to activation of its tyrosine kinase activity by ligand binding, the EGF receptor can also be regulated by phosphorylation and gene amplification [7–9].

Human epidermoid carcinoma A431 cells contain a very large number of EGF receptors [40] and their growth can be inhibited, rather than stimulated, by relatively high EGF concentrations [41]. The selection of A431 clonal variants which were resistant to the growth inhibitory effects of EGF revealed that these clones possessed reduced levels of EGF receptor protein [7,42]. This suggested a quantitative relationship between cell proliferation and the number of activated EGF receptors. Cell growth increased with increasing EGF receptor activation up to a certain threshold level; excessive numbers of activated EGF receptors inhibited cell proliferation.

The differing EGF receptor number in various A431 clones also suggested that there was a selectable mechanism for regulating the level of receptor protein. Using EGF receptor cDNA clones, it was observed that gene amplification accounted for the varying EGF receptor protein in A431 clonal variants and thus their consequent growth responses to a given EGF concentration [7,43]. This gene amplification correlated directly with the translocation of chromosome 7, which contains the EGF receptor gene [43,44]. Because receptor amplification would be expected to confer a growth advantage under conditions of limiting EGF, it is interesting to note that several other epidermoid carcinomas have been recently described which possess amplified levels of EGF receptor [45]. Presumably this increase in receptor concentration is sufficient to profoundly alter cell proliferation and thereby provide a growth advantage to the transformed cells.

In this report we have summarized evidence indicating that in addition to gene amplification and ligand binding, phosphorylation may also serve to regulate EGF receptor function. Previous studies on the protein-tyrosine kinase activities of the insulin receptor [46,47] and the transforming proteins of the Rous sarcoma [48] and Fujinami sarcoma viruses [49] indicated that self-phosphorylation of the enzymes could activate their kinase activity. Self-phosphorylation of the EGF receptor, which occurs primarily on its carboxyl-terminus, also enhances the ability of the receptor to phosphorylate certain exogenous substrates. Whereas the exact mechanism by which this occurs is unknown, self-phosphorylation of the carboxyl-terminus may remove a competitive/inhibitory constraint so that various exogenous substrates can have greater access to the substrate binding region of the enzyme. This situation may be similar to the role of self-phosphorylation in regulating type II cAMP-dependent protein kinase [50]. The regulatory and catalytic subunits of the cAMP-dependent protein kinase interact in part through a substratelike region on the regulatory subunit [51]. This interaction is diminished by self-phosphorylation, and thus favors the formation of a free catalytic subunit which is enzymatically active [52].

Earlier investigations by Cohen et al [5] indicated that the 170-KDa EGF receptor could be converted to a 150-KDa protein by a Ca^{++}-dependent protease, apparently via the removal of a 20-KDa piece from the carboxyl-terminus. The 150-KDa kinase was more active against some exogenous substrates but exhibited reduced self-phosphorylation activity. These observations support the concept that the carboxyl-terminus, which contains the major sites of self-phosphorylation, imposes negative control over the receptor's tyrosine kinase activity. Changes in this regulatory region of the molecule, either by phosphorylation or by protease action, can result in enhanced kinase activity. It is conceivable that self-phosphorylation may also play a role in targeting the receptor for internalization or interactions with other receptors or protein kinases. Interestingly, the avian erythroblastosis virus-transforming protein erb B lacks the carboxyl-terminus self-phosphorylation sites of the proto-oncogene EGF receptor [53] and thus would not be subject to the influence of this regulatory domain.

A model incorporating the available evidence on the role of self-phosphorylation in enhancing the EGF receptor protein-tyrosine kinase activity is shown in Figure 5. In this proposed scheme, ligand binding results in an enzyme conformational change which increases the tyrosine-kinase's catalytic rate and/or substrate binding, eg, higher-affinity binding of ATP. The activated receptor can then self-phosphorylate on tyrosine residues located in its extreme carboxyl-terminus. This region of the mole-

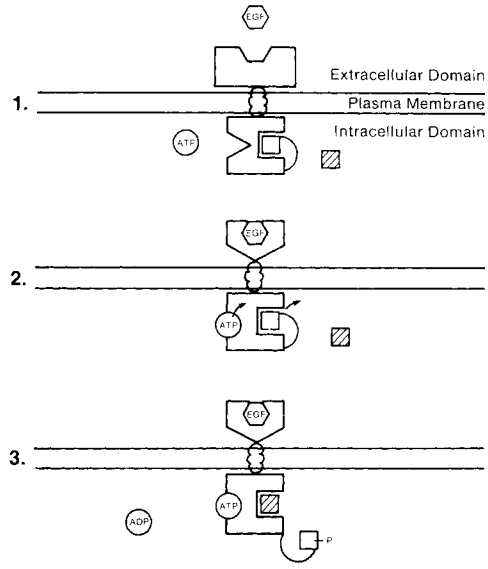

Fig. 5. Proposed model for the stimulation of EGF receptor protein-tyrosine kinase activity by self-phosphorylation.

cule can reversibly interact with the enzyme active site. This allows for the self-phosphorylation reaction to be competitively inhibited at sufficiently high concentrations of exogenous substrates. Phosphorylation of the carboxyl-terminal domain decreases its affinity for the enzyme active site, and/or results in an additional conformational change, thereby permitting exogenous substrates greater access to the substrate binding domain and enhanced tyrosine-kinase activity.

Besides undergoing self-phosphorylation, the EGF receptor can also serve as a substrate for protein kinase C, thereby providing a mechanism for interaction with other cellular pathways. Phosphorylation of the EGF receptor on threonine by protein kinase C results in an inhibition of the receptor's tyrosine kinase activity both in vivo and in vitro. Addition of tumor promoters to A431 cells, which stimulates protein kinase C, has been reported to inhibit high-affinity binding of ^{125}I-EGF, presumably through this same phosphorylation mechanism [36,37]. Earlier studies on the effects of tumor promoters on EGF receptor function had described both a decrease in receptor binding and number [54,55].

EGF has been reported to stimulate phosphatidylinositol turnover and Ca^{++} mobilization in A431 cells [12,14] and this process can lead to an activation of protein kinase C via diacylglycerol production [27,28]. Addition of EGF to A431 cells results in the phosphorylation of the same EGF receptor phosphothreonine-containing tryptic peptide as occurs with tumor promoters. The major site of protein kinase C-catalyzed phosphorylation of the receptor has recently been shown by Hunter et al [15] to be a threonine residue located about nine amino acids carboxyl-terminal to the transmembrane region. It is tempting to speculate that this phosphorylation creates a receptor/membrane interaction which uncouples the EGF binding domain from the intracellular tyrosine kinase domain, thereby reducing both EGF binding and protein-tyrosine kinase acitivity.

A model incorporating these observations would be as follows: protein kinase C, activated directly by tumor promoters or indirectly via phosphatidylinositol turnover and Ca^{++} mobilization stimulated by EGF or other hormones, phosphorylates the EGF receptor on a threonine residue near the intracellular plasma membrane surface, thereby attenuating EGF binding and protein-tyrosine kinase activity. This pathway would provide an important mechanism for desensitization of EGF receptor function, both in response to homologous signals, such as EGF or α-tumor growth factors, or to heterologous signals, such as fibroblast-derived growth factor [56].

Two different effects of phosphorylation on EGF receptor tyrosine kinase activity have now been described. Self-phosphorylation enhances protein-tyrosine kinase activity, whereas protein kinase C-catalyzed receptor phosphorylation inhibits kinase activity. These two phosphorylations account for a fraction of the total EGF receptor phosphorylation in vivo [12], and it would thus appear that other protein kinases can phosphorylate the receptor. These other phosphorylations may directly exert additional controls on receptor activity, or may function indirectly by targeting the receptor for entry into various intracellular pathways for internalization and turnover.

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