# Epidermal growth factor stimulates the phosphorylation of synthetic tyrosine-containing peptides by A431 cell membranes

(phosphotyrosine/pp60$^{src}$)

LINDA J. PIKE*, BYRON GALLIS†, JOHN E. CASNELLIE*, PAUL BORNSTEIN†, AND EDWIN G. KREBS*

*Howard Hughes Medical Institute Laboratories, Department of Pharmacology, University of Washington, Seattle, Washington 98195; and †Departments of Biochemistry and Medicine, University of Washington, Seattle, Washington 98195

**ABSTRACT** A431 cell membranes phosphorylate a synthetic peptide (Arg-Arg-Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Gly) in which residues 2–12 correspond to the sequence of the reported site of tyrosine phosphorylation in pp60$^{src}$. Epidermal growth factor stimulates the phosphorylation of this peptide 2-fold over basal levels in a dose-dependent fashion. Phosphorylation is linear for approximately 3 min at 30°C and occurs on the tyrosine residue. Kinetic analysis of the phosphorylation reaction indicates that epidermal growth factor increases the average $V_{max}$ from 3.8 to 7.5 nmol/min per mg and slightly decreases the average $K_m$ from 0.53 mM to 0.28 mM. A number of other peptides analogous to this tridecapeptide are also phosphorylated by A431 membranes. The data suggest that peptides with sequences similar to the site of tyrosine phosphorylation in pp60$^{src}$ are preferred substrates for the kinase in these membranes. Thus, the epidermal growth factor-stimulated protein kinase has the potential to interact with and phosphorylate pp60$^{src}$. However, the A431 membranes also phosphorylate a tyrosine-containing peptide of totally unrelated sequence, suggesting that the kinase possesses a broad specificity for peptide phosphorylation that may not reflect its specificity with protein substrates.

Epidermal growth factor (EGF) is a potent mitogen that stimulates the proliferation of a wide variety of cell types (1). This growth factor appears to work through a specific cell surface receptor that is found in high density in A431 cells, an epidermoid carcinoma cell line (2). Cohen and coworkers have recently shown that, in isolated A431 membrane vesicles, EGF stimulates the phosphorylation of tyrosine residues in the EGF receptor and in added proteins (3, 4). Based on the ability of highly purified preparations of EGF receptors to carry out similar phosphorylation reactions, the tyrosine protein kinase activity has tentatively been ascribed to the EGF receptor itself (5).

The tyrosine protein kinase activity associated with the EGF receptor is of particular interest because the transforming proteins from various RNA tumor viruses have been shown to be tyrosine-directed protein kinases (6–8). The Rous sarcoma virus-encoded transforming protein pp60$^{src}$ is a tyrosine kinase and is itself phosphorylated on a tyrosine residue (9–11) in what may (10) or may not (9) be an autophosphorylation reaction. Antibodies directed against pp60$^{src}$ are also phosphorylated on tyrosine when the antibody–pp60$^{src}$ complexes are incubated with [γ-$^{32}$P]ATP (9–11). Chinkers and Cohen (12) and Kudlow et al. (13) recently demonstrated the phosphorylation of anti-pp60$^{src}$ antibodies by preparations containing EGF receptors from A431 cells. This finding suggests some relationship between pp60$^{src}$ and the EGF receptor-associated kinase; however, the nature of this relationship is still unclear because an

tibodies to pp60$^{src}$ did not precipitate the EGF-stimulated kinase (12, 13).

To examine this question further, peptides containing the amino acid sequence around the site of tyrosine phosphorylation in pp60$^{src}$ (14–16) were synthesized. We report here that A431 membranes phosphorylate these synthetic peptides on their tyrosine residues and that EGF stimulates this reaction.

## MATERIALS AND METHODS

**Cells and Membranes.** A431 cells were grown as described (17). Membranes were isolated according to the procedure of Carpenter et al. (4) as modified by Brautigan et al. (17).

**Peptide Phosphorylation Assays.** Assays were performed in a total volume of 30 μl, and the mixture contained (final concentrations): 20 mM Hepes at pH 7.5, 2 mM MnCl$_2$, 0.2% Nonidet P-40, 10 μM zinc acetate, 50 μM [γ-$^{32}$P]ATP (2000–3000 cpm/pmol), and approximately 6–8 μg of A431 membrane protein. Unless otherwise noted, EGF and peptides were present at 0.3 μM and 2 mM, respectively. Assays were started by the addition of membranes and were terminated after 2 or 3 min by the addition of 50 μl of 5% trichloroacetic acid or 150 μl of 3.3% trichloroacetic acid plus 20 μl of bovine serum albumin (10 mg/ml). The larger volume of acid was used for peptides 4 and 5, which tended to precipitate in the smaller volume. The assay tubes were spun in a Beckman microfuge for 4 min to sediment the precipitated proteins.

For peptides 2–6, 40 μl of the small-volume supernatant or 50 μl of the large-volume supernatant was spotted on a square of phosphocellulose paper and washed in acetic acid as described (13). The phosphocellulose squares were dried, placed in vials with 6 ml of Aquasol, and assayed in a Beckman LS9000 liquid scintillation counter. For assay of peptide 1, the trichloroacetic acid supernatants were added to 1.5 ml of 4.8% phosphate pH 3.0 buffer and analyzed by high-pressure liquid chromatography as described (18). Fractions (1 ml) of the column eluate were collected and aliquots (100 μl) of the fractions containing phosphopeptide were assayed in 6 ml of Aquasol. For the paper assays, nonspecific phosphorylation was corrected for by subtracting the cpm bound to phosphocellulose paper in assays performed in the absence of peptide from those bound in assays performed in the presence of peptide. When the high-pressure liquid chromatography method was used, nonspecific phosphorylation was taken as that radioactivity eluting in the position of phosphopeptide and that was present in control assays not containing peptide.

**Phosphoamino Acid Analysis.** The trichloroacetic acid supernatant from a peptide phosphorylation assay was mixed with 1 ml of 5.7 M HCl to which was added approximately 50 μg each

Abbreviations: EGF, epidermal growth factor; pp60$^{src}$, phosphoprotein product of the Rous sarcoma virus transforming gene.

of phosphoserine, phosphothreonine, and phosphotyrosine. The tubes were evacuated, sealed, and heated for 2 hr at 110°C. The samples were then lyophilized and the residue was taken up to 25 $\mu$l in $H_2O$; 5 $\mu$l of this material was spotted on Whatman 3 MM paper and subjected to high-voltage paper electrophoresis (3000 V, 45 min, pH 3.6). $^{32}$P-Labeled phosphoamino acids were located by autoradiography and identified by comparison with the position of the marker amino acids visualized with ninhydrin.

**Materials.** Peptides were synthesized as described (18). Phosphoserine and phosphothreonine were purchased from Sigma. Phosphotyrosine was prepared according to the method of Rothberg *et al.* (19). EGF was purchased from Collaborative Research (Waltham, MA). [$\gamma$-$^{32}$P]ATP was from New England Nuclear.

## RESULTS

**Synthetic Peptides.** The sequences of the synthetic peptides used in these experiments are presented in Fig. 1. Peptide 1 corresponds to the reported site of tyrosine phosphorylation in the deduced sequence of pp60$^{src}$, residues 414–424 (14–16). This peptide can be phosphorylated by A431 membranes (see below). However, because the phosphorylated form of this peptide is difficult to separate from [$\gamma$-$^{32}$P]ATP, peptide 1 was modified by extending the sequence on the amino-terminal side to include a leucine and an arginine present in the natural sequence. In addition, a second arginine was added at the amino terminus and the carboxy-terminal sequence Gln-Gly was replaced by Gly to produce peptide 2 (18). Peptide 2 binds to phosphocellulose paper, thus providing a means whereby peptide phosphorylation can be readily measured by a simple assay procedure (18). Peptide 2 was used in the detailed studies of EGF-stimulated peptide phosphorylation. Peptide 3 lacks the threonine residue found in the first two peptides and thus contains tyrosine as the only phosphorylatable amino acid in the sequence. The asparagine at position 7 was also replaced with an alanine to prevent a change in sequence due to possible deamidation of asparagine. Peptides 4 and 5 are analogs of peptide 3 containing single amino acid substitutions: aspartic acid-6 and glutamic acid-5 each was replaced with an alanine to make peptides 4 and 5, respectively. Peptide 6 is an analog of a serine-containing peptide commonly used as a substrate for the cAMP-dependent protein kinase (20). The amino acid sequence of peptide 6 is similar to that found in pyruvate kinase and other substrates of the cyclic nucleotide-dependent kinase (21).

**Phosphorylation of Peptide 2 by A431 Cell Membranes.** A431 membranes were capable of phosphorylating peptide 2 in both the absence and presence of EGF (Fig. 2). EGF stimulated the phosphorylation of the peptide approximately 2-fold. The phosphorylation was linear for approximately 3 min at 30°C in the absence or presence of EGF. After this time, phosphorylation of the peptide continued in a nonlinear fashion, the decline in rate most likely occurring as a result of ATP depletion by other ATP-utilizing enzymes in these membranes.

A431 membranes phosphorylated peptide 2 on a tyrosine residue (Fig. 3). Phosphorylation reactions were stopped by addition of trichloroacetic acid and the precipitated proteins were removed by centrifugation. The supernatants were hydrolyzed in HCl and analyzed for phosphoamino acids. Lanes 2 and 4 demonstrate that only labeled phosphotyrosine was synthesized in those reaction mixtures that contained A431 membranes and peptide 2. No phosphotyrosine was formed in the absence of added peptide (lanes 1 and 3). As expected, addition of EGF to the peptide phosphorylating assays led to an increase in the amount of detectable phosphotyrosine (lane 4 vs. lane 2).

The stimulation of peptide 2 phosphorylation by EGF exhibited a typical sigmoid dose–response curve with half-maximal stimulation occurring at approximately 30 nM (Fig. 4). This value is in good agreement with previously published data for the stimulation of endogenous protein phosphorylation by EGF in A431 membranes (5).

**Kinetics of Peptide 2 Phosphorylation.** Fig. 5 shows a Lineweaver–Burk plot for the phosphorylation of peptide 2 by A431 membranes. EGF caused an increase in the $V_{max}$ from 4.6 to 10.1 nmol/min per mg in this experiment. The mean effect of EGF based on several experiments (Table 1) was an increase from 3.8 to 7.5 nmol/min per mg. The $K_m$ for peptide 2 was
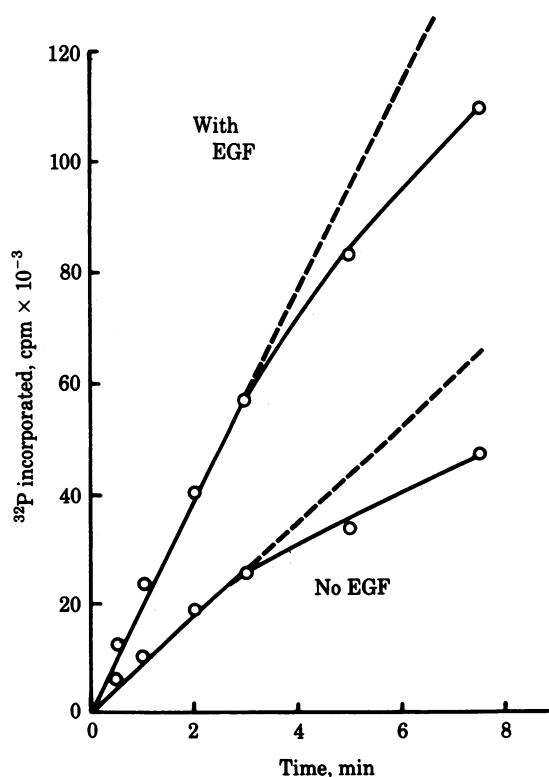
Peptide

| Number | Sequence |
|---|---|
| 1 | Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Gln-Gly |
| 2 | Arg-Arg-Leu-Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Gly |
| 3 | Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly |
| 4 | Arg-Arg-Leu-Ile-Glu-Ala-Ala-Glu-Tyr-Ala-Ala-Arg-Gly |
| 5 | Arg-Arg-Leu-Ile-Ala-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly |
| 6 | Leu-Arg-Arg-Ala-Tyr-Leu-Gly |

FIG. 1. Sequences of synthetic tyrosine-containing peptides.



FIG. 2. Time course of peptide 2 phosphorylation by A431 cell membranes. The dashed lines represent the extrapolated linear time course.
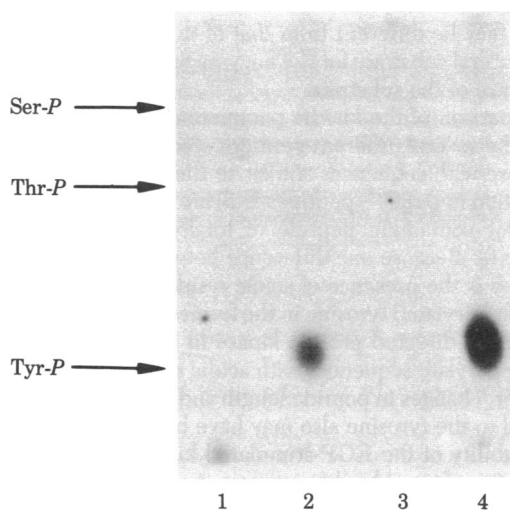
Biochemistry: Pike *et al.*

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FIG. 3. Phosphoamino acid analyses of phosphorylation reaction mixtures. A431 cell membranes were incubated for 3 min with [γ-$^{32}$P]ATP with or without peptide 2 and with or without EGF. Incubations were stopped by the addition of trichloroacetic acid. After pelleting of the precipitated proteins, the supernatants were hydrolyzed in HCl. After removal of the acid by lyophilization, the residue was dissolved in water and subjected to high-voltage paper electrophoresis. Lanes: 1, no EGF, no peptide 2; 2, no EGF, 2 mM peptide 2; 3, 0.3 μM EGF, no peptide 2; 4, 0.3 μM EGF, 2 mM peptide 2.
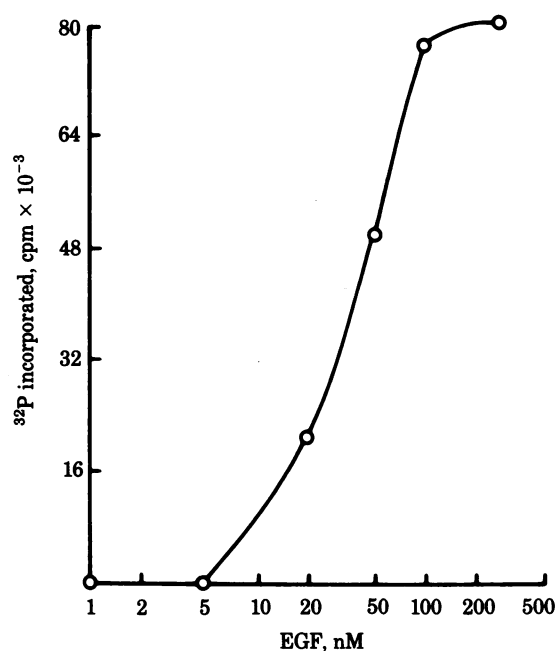


FIG. 4. Dose–response curve for stimulation of phosphorylation of peptide 2 by EGF. Peptide phosphorylation assays were for 3 min. Points represent the mean of duplicate determinations.

somewhat lower in the presence than in the absence of EGF (0.29 mM vs. 0.56 mM in this experiment); however, this effect was more variable than the increase in the $V_{max}$. On the average, the effect of EGF on $K_m$ was a decrease to 0.28 mM from 0.53 mM.

**Phosphorylation of Other Tyrosine-Containing Peptides.** A number of tyrosine-containing peptides of various sequences were tested for their ability to be phosphorylated by A431 membranes (Table 1). In general, the peptides with sequences based on the site of tyrosine phosphorylation in pp60$^{src}$ (peptides 1–5)

exhibited comparable $V_{max}$ values in both the presence and absence of EGF, the differences being within the range expected for variations in the specific activity of the kinase in different membrane preparations. The $K_m$ values for these same peptides showed slightly more variability, with changes being somewhat more pronounced in the presence of EGF. Modification of the sequence amino-terminal to the phosphorylated tyrosine caused 2- to 6-fold changes in the $K_m$ values for these peptides. However, none of the alterations appeared to have been sufficient to significantly decrease or prevent utilization of the peptide
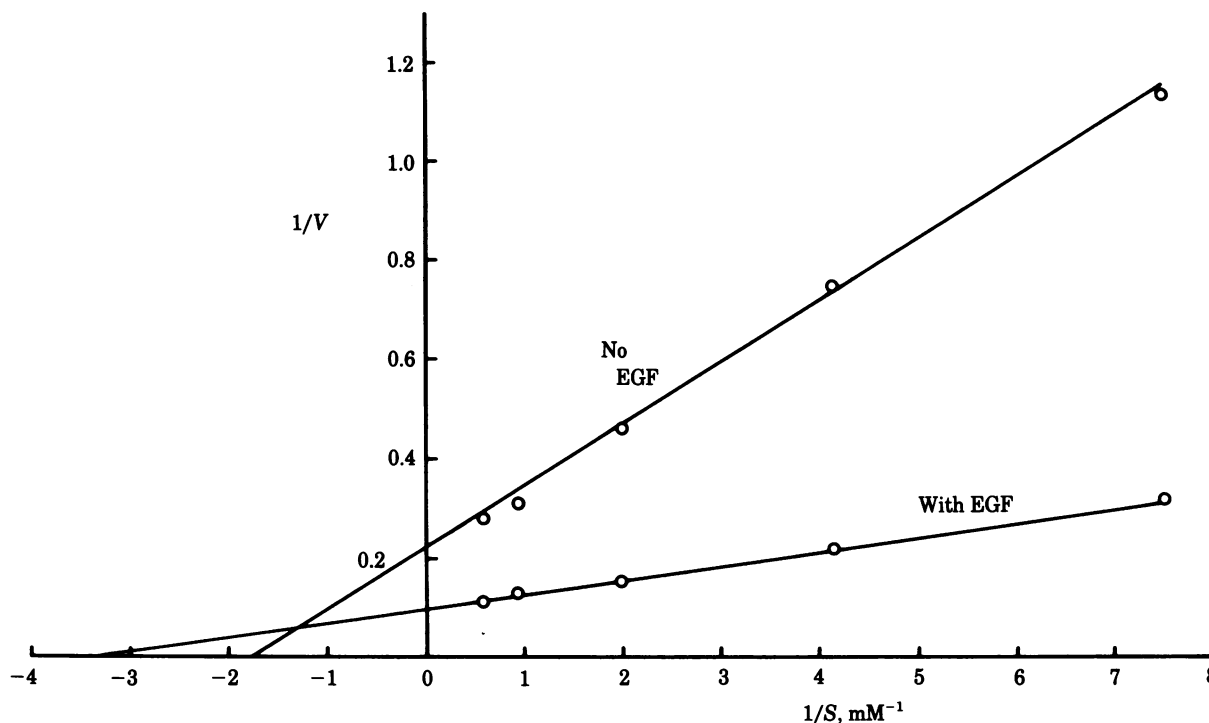


FIG. 5. Lineweaver–Burk plot for phosphorylation of peptide 2 by A431 membranes in the presence and absence of EGF. Phosphorylation assays were for 2 min.

Table 1. Kinetic constants for tyrosine-containing peptides

| Pep-tide | Without EGF | | With EGF | |
|---|---|---|---|---|
| | $K_m$, mM | $V_{max}$, nmol/min/mg | $K_m$, mM | $V_{max}$, nmol/min/mg |
| 1 | 0.73 ± 0.3 | 2.5 ± 0.6 | 1.1 ± 0 | 11.5 ± 3 |
| 2 | 0.53 ± 0.2 | 3.8 ± 1.4 | 0.28 ± 0.01 | 7.5 ± 3 |
| 3 | 0.76 ± 0 | 4.2 ± 0.6 | 0.50 ± 0.03 | 9.8 ± 0.9 |
| 4 | 1.8 ± 0.2 | 1.9 ± 0.1 | 1.8 ± 0.1 | 9.2 ± 2.5 |
| 5 | 1.2 ± 0 | 2.6 ± 0.2 | 1.1 ± 0.1 | 8.5 ± 2 |
| 6 | 16.0 ± 4 | 0.84 ± 0.04 | 6.0 ± 0.7 | 1.2 ± 0.2 |

Assays were carried out for 2 min. Lines were established from six points determined in duplicate and represent a linear regression fit of the data. Correlation coefficients were 0.992 or better. Values shown are the mean ± half-range or standard deviation of two or three experiments performed with different membrane preparations.

as a substrate. Surprisingly, A431 cell membranes also phosphorylated peptide 6, which contains basic rather than acidic residues amino-terminal to the tyrosine and bears no relationship to the phosphorylation site sequence in pp60$^{src}$. In both the presence and absence of EGF the $K_m$ values for this peptide were significantly higher than those for peptides 1–5. In addition, the $V_{max}$ for this peptide in the presence of EGF was nearly an order of magnitude lower than the average value seen with the peptides containing acidic residues.

## DISCUSSION

The data presented here demonstrate that A431 cell membranes phosphorylate synthetic, tyrosine-containing peptides and that EGF stimulates this activity. Both peptides 1 and 2 had been shown previously to be phosphorylated by membranes from the lymphoid cell line LSTRA (18). As discussed by Casnellie *et al.* (18), the advantage of peptide 2 is the ability to use it in a simple phosphocellulose paper assay. Peptide 3 has the additional advantage of containing tyrosine as the only phosphorylatable amino acid in the sequence.

Addition of EGF to A431 cell membranes stimulated phosphorylation of peptide 2 2-fold over basal levels. Both basal and stimulated phosphorylation occurred on tyrosine residues and the dose–response curve for EGF was similar to that reported for the stimulation of endogenous protein phosphorylation by EGF in these membranes (5).

Kinetic analyses of the phosphorylation of peptide 2 yielded average $V_{max}$ values of 3.8 and 7.5 nmol/min per mg in the absence and presence of EGF, respectively. If it is tentatively assumed that the EGF receptor-associated protein kinase itself is the tyrosine kinase catalyzing the phosphorylation of peptide 2, then on the basis of published values for the number of EGF receptors per milligram of protein in this membrane preparation (22), a turnover number of 14–15/sec can be calculated for the enzyme. This value is nearly identical to the reported turnover number for cAMP-dependent protein kinase with a favorable hexapeptide substrate (20).

The A431 membrane enzyme exhibited $K_m$ values for peptide 2 of 0.53 mM in the absence and 0.28 mM in the presence of EGF. The $K_m$ values for other kinases with physiological protein substrates are significantly lower than the values reported here for peptide phosphorylation (23). Nonetheless, the $K_m$ values for the phosphorylation of peptide 2 by the EGF-stimulated kinase are slightly lower than those reported for the phosphorylation, by purified phosphorylase kinase, of a synthetic tetradecapeptide based on the phosphorylation site sequence in the enzyme's natural substrate, phosphorylase *b*. Thus, it is unclear whether the relatively high $K_m$ exhibited by A431 cell membranes for peptide 2 is a consequence of the fact that its se-

quence may be different from that of the phosphorylation site in the natural substrate for the enzyme or due to the small peptide nature of the substrate.

Comparison of the kinetic parameters for the phosphorylation of the several different peptides studied (Table 1) suggests that those with sequences similar to the site of tyrosine phosphorylation in pp60$^{src}$ (peptides 1–5) are better substrates than a tyrosine-containing peptide of totally unrelated sequence (peptide 6). Because one difference between peptides 1–5 and peptide 6 is the presence of acidic residues amino-terminal to the phosphorylated tyrosine in the former, the data suggest that the tyrosine-directed protein kinase in A431 cells may prefer to phosphorylate sequences with acidic residues in this region. However, changes in peptide length and substitutions carboxy-terminal to the tyrosine also may have been a factor.

The ability of the EGF-stimulated kinase to phosphorylate peptide 6 is of considerable interest. An apparent lack of specificity of a protein kinase with simple peptide substrates, as opposed to protein substrates, is not unprecedented. cAMP-dependent protein kinase does not phosphorylate phosphorylase *b*. However, the cyclic nucleotide-dependent kinase will phosphorylate a synthetic peptide based on the sequence of phosphorylase *B* (23). Thus, phosphorylation of peptide 6 (which has a vastly different sequence from peptides 1–5) by the EGF-stimulated kinase suggests that secondary or tertiary structure of the substrate may be important for determining kinase specificity *in vivo*.

Under the assay conditions we used, basal and EGF-stimulated phosphorylation of peptide 2 is rapid and linear for approximately 3 min. The essentially immediate stimulation of peptide phosphorylation upon addition of EGF suggests that activation of a tyrosine-directed protein kinase occurs very rapidly after binding of the hormone to its receptor. Thus, the initial response to EGF may occur at the level of the plasma membrane and may not require hormone–receptor complex internalization.

The exact mechanism whereby EGF stimulates peptide 2 phosphorylation remains to be elucidated. In view of the reports of an EGF receptor-associated tyrosine-directed protein kinase activity (3–5), the simplest interpretation of the present data is that binding of EGF to its receptor stimulates the intrinsic activity of the receptor itself or of the receptor-associated protein kinase. However, it is also possible that EGF-stimulated phosphorylation of tyrosine-containing peptides is carried out by a protein kinase that is not directly associated with the EGF receptor but is activated subsequent to the initial response to the hormone.

The fact that a growth hormone increases the phosphorylation of a synthetic peptide derived from pp60$^{src}$ suggests a possible relationship between the EGF-stimulated protein kinase and the virus-encoded transforming protein. pp60$^{src}$ has been reported to autophosphorylate on a tyrosine residue (10). However, not all purified preparations of this viral kinase exhibit autophosphorylation activity (9). The ability of the EGF-stimulated tyrosine-directed protein kinase in A431 cells to phosphorylate a peptide derived from pp60$^{src}$ suggests that the growth factor-responsive kinase has the potential for carrying out the phosphorylation of the viral kinase or possibly its closely related cellular homolog, pp60$^{sarc}$. However, in view of the broad specificity for peptide substrates demonstrated by the A431 membrane enzyme, it is unclear which peptide sequences, if any, represent *in vivo* sites of protein phosphorylation by the EGF-stimulated kinase.

A431 cell membranes contain significant levels of basal peptide 2 phosphorylating activity. Whether this represents the activity of an enzyme different from the EGF-stimulated kinase

is not known. Membrane preparations from various other cell types also exhibit basal peptide 2 phosphorylating activity including LSTRA cells (18), Rous sarcoma virus-transformed rat fibroblasts, U251 MG glioma cells, and 3T3 cells (unpublished results). Kinetic analyses of peptide 2 phosphorylation in LSTRA cell membranes yield a $K_m$ of 3–5 mM for the peptide (18). This indicates that the LSTRA cells contain a tyrosine-directed protein kinase distinctly different from that found in A431 cells. Thus, various tyrosyl protein kinases with different kinetic properties and possibly different specificities may exist *in vivo.*

Assays based on the phosphorylation of pp60$^{src}$-derived synthetic peptides provide a useful tool for the study of tyrosine-directed protein kinases in general and of EGF-stimulated phosphorylation in particular. The ability to correlate EGF binding with a biological response—peptide phosphorylation—should permit a more detailed investigation of the mechanism of EGF action. In addition, the availability of a simple assay for tyrosine-directed protein kinases should facilitate the purification and characterization of these enzymes.

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