Autophosphorylation of the Intracellular Domain of the Epidermal Growth Factor Receptor Results in Different Effects on Its Tyrosine Kinase Activity with Various Peptide Substrates

PHOSPHORYLATION OF PEPTIDES REPRESENTING Tyr(P) SITES OF PHOSPHOLIPASE C-γ*

(Received for publication, August 10, 1990)

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The effect of autophosphorylation on the tyrosine kinase activity of the epidermal growth factor receptor (EGFR) is not well understood. We previously demonstrated that phospholipase C-γ physically associates with the EGF-activated EGFR, but not with a kinase-negative mutant of the EGFR, and, moreover, that only the tyrosine-phosphorylated EGFR is able to associate with phospholipase C-γ. We have now investigated the effect of autophosphorylation on the tyrosine kinase activity of the EGFR by employing the purified kinase-active intracellular domain of the EGFR (EGFR-IC) produced by a baculovirus expression system. Synthetic peptides, including ones which contain the individual major tyrosine phosphorylation sites of phospholipase C-γ, were used as substrates. We found that the extensively prephosphorylated EGFR-IC exhibited similar reaction kinetics to the unphosphorylated EGFR-IC when angiotensin II was used as a nonspecific substrate. In contrast there was a clear stimulation of kinase activity due to autophosphorylation of the EGFR-IC when peptides representing either the major autophosphorylation site of the EGFR or the EGFR phosphorylation sites of phospholipase C-γ were used as substrates. However, the modes of stimulation for these peptides differed. The binding affinity (Km) for the unphosphorylated EGFR-IC for the peptide containing Tyr-771 of phospholipase C-γ was relatively poor compared with other peptides, but improved 5–6-fold when the EGFR-IC was prephosphorylated. On the other hand, autophosphorylation improved the reaction velocity (Vm) of the phosphorylation of other peptides by 2–3-fold, with little or no increase in affinity. These results suggest that autophosphorylation of the EGFR may induce a conformational change of its kinase domain which enhances its kinase activity with exogenous substrates and may induce association with phospholipase C-γ by increasing its affinity to a domain containing Tyr-771.

The epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein possessing a protein-tyrosine kinase domain within its cytoplasmic portion. The tyrosine kinase activity is essential for receptor-related signal transduction as well as intracellular routing of the receptor (1). Upon activation, the EGFR undergoes autophosphorylation of carboxyl-terminal tyrosine residues (2, 3) in an intermolecular reaction (4). In addition, it phosphorylates a number of exogenous substrates. Recently, it has been established that phospholipase C-γ is one such direct substrate of the EGFR kinase and that the EGFR and phospholipase C-γ are physically associated (5–7). Both these processes, phospholipase C-γ association with the EGFR and its tyrosine phosphorylation, are induced by EGF and are dependent on the tyrosine kinase activity of the EGFR. Moreover, prephosphorylation of the EGFR is required for its ability to associate with phospholipase C-γ in a cell-free reaction (8). These results suggest that autophosphorylation of the EGFR may enhance its affinity for the phospholipase C-γ substrate. As a way of examining this possibility, we determined the effects of prephosphorylation of the soluble intracellular kinase domain of the EGFR, purified from a baculovirus expression system (9), upon its kinetic activities with several different peptide substrates including those containing phospholipase C-γ phosphorylation sites.

The four major tyrosine phosphorylation sites of phospholipase C-γ have been recently identified (10, 11), and the phosphopeptide pattern of phospholipase C-γ phosphorylated by the cytoplasmic domain of the EGFR is similar to that obtained with the intact EGFR (12). We have synthesized three peptides, each containing an individual tyrosine phosphorylation site of phospholipase C-γ (Table I). The kinetics of phosphorylation of these substrates as well as of peptide K1, containing the major autophosphorylation site of the EGFR (Tyr-1173), and angiotensin II were studied. Our results demonstrate that receptor autophosphorylation differentially affected its kinase activity with different substrates. Prephosphorylation of the kinase did not significantly alter its activity with angiotensin II, whereas it had a prominent enhancing effect on its activity with the peptide containing Tyr-771 of phospholipase C-γ, primarily by enhancing the binding affinity between the kinase and the substrate. The phosphorylation rates of peptide K1 and peptides containing Tyr-783 and Tyr-1254 of phospholipase C-γ were also enhanced somewhat by prephosphorylation of the receptor kinase.

Autophosphorylation of the insulin receptor (13–18) and of the pp60c-src kinase (19–21) has been shown to increase their kinase activities. In contrast, reports on the effects of autophosphorylation on the kinase activity of the EGFR have been inconsistent. In some studies (22, 23) autophosphorylation enhanced EGFR kinase activity whereas in others (24),
TABLE 1
Sequences of phospholipase C-γ peptides containing tyrosine phosphorylation sites

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC-771</td>
<td>Lys-Ile-Gly-Thr-Ala-Glu-Pro-Asp-Tyr-Gly-Ala-Leu-Tyr-Glu-Gly-Arg</td>
</tr>
<tr>
<td>PLC-783</td>
<td>Glu-Gly-Arg-Asn-Pro-Gly-Phe-Tyr-Val-Glu-Ala-Asn-Pro-Met-Pro-Thr-Phe-Lys</td>
</tr>
<tr>
<td>PLC-1254</td>
<td>Ala-Arg-Glu-Gly-Ser-Phe-Glu-Ala-Asp-Tyr-Gln-Glu-Pro-Phe-Glu-Asp-Phe-Arg-Ile</td>
</tr>
</tbody>
</table>

The phosphorylated tyrosine residue is underlined, and the numbering system is based on the deduced amino acid sequence of bovine brain phospholipase C-γ (28).

no effect was seen. It has been suggested (22, 25) that an important role of the autophosphorylation sites of the EGFR is to compete with exogenous substrates and thus serve an intrinsic negative regulatory function in the phosphorylation of these exogenous substrates. The results reported in this study show that, in addition, autophosphorylation has a positive regulatory effect. It increases either the binding affinity or the reaction velocity of the EGFR for different tyrosine phosphorylation sites of phospholipase C-γ, leading to enhanced phospholipase C-γ phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—Angiotensin II was purchased from Sigma. [γ-32P]ATP (6000 Ci/mmol) was from Du Pont-New England Nuclear. Peptide K1, containing the major autophosphorylation site of the EGFR (Tyr-1173), and three peptides, each containing an individual tyrosine phosphorylation site of phospholipase C-γ, as detailed in Table I, were synthesized using an Applied Biosystems 430 peptide synthesizer. After cleavage of the peptides from the resin, they were purified by chromatography on Bio-Gel P-4 (Bio-Rad) and analyzed by C18 reverse-phase high performance liquid chromatography.

Kinetic Analysis of Tyrosine Kinase Activity of EGFR Intracellular Domain—Experiments of time course analyses were performed either with 40 nM purified EGFR-IC and 300 μM peptide K1 or with 5 μM EGFR-IC and 10 μM peptide K1. The reaction with a low concentration of kinase was performed with 10 μM ATP, 50 μCi of [γ-32P]ATP, and 10 mM MnCl2 in HNTG (50 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol). The reaction with a high concentration of kinase was performed with 200 μM ATP, 100 μCi of [γ-32P]ATP, and 10 mM MnCl2 in HNTG. The prephosphorylated EGFR-IC (P-EGFR-IC) in each experiment was prepared by incubating the EGFR-IC with the appropriate concentrations of ATP and MnCl2 in HNTG on ice for 30–35 min. A premixed solution containing [γ-32P]ATP and peptide K1 was then added at zero time so that the initial total volume of the reaction was 100 μl. Samples of 15 μl were taken at the indicated time points and mixed immediately with an equal volume of 2× SDS sample buffer. Under these reaction conditions, a large excess of ATP was provided, such that the quantity of ATP used up by autophosphorylation during the preincubation was negligible (with either 40 nM or 5 μM kinase) and the specific radioactivities of ATP for peptide phosphorylation were nearly the same for the P-EGFR-IC and EGFR-IC reactions in each experiment. Experiments with varying concentrations of peptides were performed in 30-μl reaction mixtures, each containing 50 ng of the EGFR-IC (27 nM), 10 μM ATP, 10 μCi of [γ-32P]ATP, 10 mM MnCl2, and the appropriate concentration of peptide in HNTG. The P-EGFR-IC was prepared as described in the figure legends. Each reaction was allowed to proceed for 2 min on ice and then stopped by the addition of an equal volume of 2× SDS sample buffer. The samples were analyzed by electrophoresis on a 5–15% linear gradient (total of 16 ml) over a 20% (12 ml) polyacrylamide gel and by autoradiography. The bands of peptides and/or the EGFR-IC were then excised and quantitated by Cerenkov counting. Each experiment was repeated three times.

RESULTS

Effect of EGFR-IC Autophosphorylation on Its Kinase Activity with Peptide K1 and Angiotensin II—The purified baculovirus-expressed EGFR-IC (9) was used to study the effect of receptor autophosphorylation on its tyrosine kinase activity with various peptide substrates. We have shown previously (9) that the purified EGFR-IC is essentially not phosphorylated and that, upon cell-free autophosphorylation, it displays a phosphopeptide pattern which is identical to that of the intact EGFR.

The initial experiments were carried out with two previously studied peptide substrates, peptide K1, containing the major autophosphorylation site (Tyr-1173) of the EGFR (25), and angiotensin II. A time course analysis of peptide K1 phosphorylation by either the P-EGFR-IC (I) or the unphosphorylated (II) EGFR-IC is shown in Fig. 1 (upper). In this experiment, we used low levels of kinase (40 nM) and excess substrate (300 μM). The prephosphorylation reaction (carried out as described under “Experimental Procedures” and in the legend to Fig. 1) was very efficient, as evidenced by the complete shift to the slower migrating form upon SDS-PAGE and the low level of 32P labeling of this band when [γ-32P]ATP was added following the completion of the reaction in the presence of unlabeled ATP. From other experiments, when the EGFR-IC was preincubated in the presence of [γ-32P]ATP, we have calculated that the extent of prephosphorylation during this incubation was ~2–2.5 mCi of phosphate/mmol of the P-EGFR-IC (Fig. 2C, inset). The extent of phosphorylation of peptide K1 by the P-EGFR-IC was ~70% higher than that by the (nonprephosphorylated) EGFR-IC at

![FIG. 1. Time course analysis of effect of autophosphorylation on kinase activity with peptide K1 as substrate. Reaction mixtures contained 40 nM purified EGFR-IC (22), 10 μM ATP, 50 μCi of [γ-32P]ATP, 10 mM MnCl2, and 300 μM peptide K1 in 100 μl of HNTG. The P-EGFR-IC was prepared by incubating the EGFR-IC with MnCl2 and unlabeled ATP in HNTG for 30–45 min at 4 °C before the addition of peptide K1 and [γ-32P]ATP. For the unphosphorylated EGFR-IC reaction, the EGFR-IC was preincubated with MnCl2 only for 30–45 min at 4 °C and then added to a mixture containing ATP, [γ-32P]ATP, and peptide K1. Aliquots of 15 μl were removed at the indicated times and analyzed by SDS-PAGE analysis on a 5–15% linear polyacrylamide gradient over a 20% gel cushion. After autoradiography, peptide K1 bands were cut and counted by Cerenkov counting. Upper, autoradiograph of P-EGFR-IC (I) and EGFR-IC (II) reactions; lower, graphic plots of counts in peptide K1 at each time point.](image-url)
Menten analysis, using EGFR-IC or EGFR-IC and the indicated concentrations of peptide K1, P-EGFR-IC was prepared by incubating the EGFR-IC with MnCl₂, unlabeled ATP, and [γ-³²P]ATP for 30 min at 4°C. The reaction, initiated by the addition of peptide, was allowed to proceed for 2 min at 4°C. A and B, autoradiographs of the SDS-PAGE analysis of the reactions using the EGFR-IC and P-EGFR-IC, respectively; C, plots of the rate of phosphate incorporation into peptide K1 as a function of peptide K1 concentration (inset, stoichiometries of phosphate incorporation into the EGFR-IC molecule at the end of each reaction); D, double-reciprocal plots of the same data for determination of the kinetic parameters (Table II).

**Table II**

Summary of the effects of EGFR-IC autophosphorylation on its kinase activity with various peptide substrates

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Kₐ (μM)</th>
<th>Vₐ (pmol P/min/µg EGFR-IC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-EGFR-IC</td>
<td>K1</td>
<td>41 ± 3</td>
</tr>
<tr>
<td></td>
<td>K1</td>
<td>72 ± 25</td>
</tr>
<tr>
<td>P-EGFR-IC</td>
<td>Angiotensin II</td>
<td>6400 ± 800</td>
</tr>
<tr>
<td></td>
<td>Angiotensin II</td>
<td>7200 ± 900</td>
</tr>
<tr>
<td>P-EGFR-IC</td>
<td>PLC-771</td>
<td>385 ± 100</td>
</tr>
<tr>
<td></td>
<td>PLC-771</td>
<td>2170 ± 700</td>
</tr>
<tr>
<td>P-EGFR-IC</td>
<td>PLC-783</td>
<td>460 ± 60</td>
</tr>
<tr>
<td></td>
<td>PLC-783</td>
<td>678 ± 57</td>
</tr>
<tr>
<td>P-EGFR-IC</td>
<td>PLC-1254</td>
<td>369 ± 100</td>
</tr>
<tr>
<td></td>
<td>PLC-1254</td>
<td>350 ± 25</td>
</tr>
</tbody>
</table>

FIG. 2. Kinetic analyses of effect of autophosphorylation of EGFR-IC on its kinase activity with peptide K1. Michaelis-Menten analysis, using a 27 nM concentration of either the P-EGFR-IC or EGFR-IC and the indicated concentrations of peptide K1, was carried out as detailed under “Experimental Procedures.” The P-EGFR-IC was prepared by incubating the EGFR-IC with MnCl₂, unlabeled ATP, and [γ-³²P]ATP for 30 min at 4°C. The reaction, initiated by the addition of peptide, was allowed to proceed for 2 min at 4°C. A and B, autoradiographs of the SDS-PAGE analysis of the reactions using the EGFR-IC and P-EGFR-IC, respectively; C, plots of the rate of phosphate incorporation into peptide K1 as a function of peptide K1 concentration (inset, stoichiometries of phosphate incorporation into the EGFR-IC molecule at the end of each reaction); D, double-reciprocal plots of the same data for determination of the kinetic parameters (Table II).
the EGFR-IC when excess substrate over enzyme was used in the reaction (Fig. 2C, inset).

A similar study was performed with angiotensin II as a substrate (Fig. 3). The affinity of the EGFR-IC kinase for angiotensin II is ~100-fold lower than that for peptide K1 (Table II), and neither the affinity nor the reaction velocity is substantially increased upon kinase autophosphorylation (Fig. 3, and Table II), as distinct from the reaction with peptide K1.

**Effect of EGFR-IC Autophosphorylation on Its Tyrosine Kinase Activity with Phospholipase C-γ Peptides**—Recently, the four major tyrosine residues in bovine brain phospholipase C-γ that are phosphorylated in vitro by the EGFR have been identified. These are tyrosines 472, 771, 783, and 1254. The rate of phosphorylation was greatest at positions 771 and 783, less at position 1254, and least at position 472 (10, 11). Phospholipase C-γ phosphorylated in cells, in response to EGF, contains at least four tyrosine phosphate-containing peptides. Two of these phosphorylated tyrosines have been identified as residues 771 and 1254 (10, 11). We have synthesized three peptides, each containing one of the most efficient sites in the cell-free phosphorylation reaction. The sequences of these peptides are shown in Table I. Three of these peptides were used in kinetic analyses with the soluble kinase as a model system for “exogenous substrate” phosphorylation by the EGFR. The phosphopeptide pattern of phospholipase C-γ phosphorylated by the baculovirus-expressed kinase domain of the EGFR was similar to that obtained with the intact EGFR (12). We examined the effects of EGFR-IC autophosphorylation on its tyrosine kinase activity with the three phospholipase C-γ peptides in an attempt to establish whether the observation, made previously on intact cells demonstrating that autophosphorylation of the intact EGFR enhances its affinity for the phospholipase C-γ protein (8), could also be manifested at the level of one (or more) of the individual tyrosine phosphorylation sites.

Results of a representative kinetic analysis of peptide PLC-771 phosphorylation by the EGFR-IC and P-EGFR-IC are shown in Fig. 4. The $K_m$ and $V_{max}$ values derived from the double-reciprocal plots are listed in Table II. Clearly, autophosphorylation of the soluble kinase results in a marked (5–6-fold) increase in its affinity for the Tyr-771-containing site of phospholipase C-γ.

The same analysis, performed with the other two phospholipase C-γ peptides, PLC-783 (Fig. 5, upper) and PLC-1254 (Fig. 5, lower), revealed that, with these two peptides, autophosphorylation of the EGFR-IC did not substantially increase its affinity for the substrates. However, reaction velocity was enhanced 2–3-fold by kinase autophosphorylation.

Therefore, these results demonstrate that autophosphorylation of the soluble kinase stimulates its activity with all three phospholipase C-γ peptides tested. The mode of stimulation, however, differed with the particular peptides.

**Competition between Peptide Substrates for Phosphorylation by EGFR-IC**—To test whether the differential effect of EGFR-IC autophosphorylation on its kinase activity with various peptide substrates is, in fact, manifested by a single domain of the substrate-binding site, we carried out competition experiments with two different peptides. Since for most substrates the prephosphorylated enzyme was the more active kinase, we used it in these experiments. The experiment described in Fig. 6 suggests that, under conditions of substrate...
performed exactly as detailed for PLC-771 in the legend to Fig. 4.

**EGFR-IC**

Peptide K1 concentrations of $-50 \text{ pM}$ ATP, $10 \text{ pCi of } [\gamma^{-32}\text{P}]\text{ATP, } 10 \text{ mM MnCl}_2$, $600 \text{ pM peptide PLC-783; lower, peptide PLC-1254.**

Only the double-reciprocal plots are presented. These results suggest that the receptor kinase can phosphorylate only one substrate at a time in a single catalytic domain.

**DISCUSSION**

The effect of autophosphorylation of the EGFR on its intrinsic tyrosine kinase activity has been a subject of controversy in the literature. Researchers using different peptide substrates, including angiotensin II (22, 23), an src-derived peptide (22), and peptides containing the major autophosphorylation sites of the EGFR (24), reported either enhancement (22, 23) or no effect (24) of autophosphorylation on its kinase activity. We (6) and others (5, 7) have demonstrated that phospholipase C-$\gamma$ is physically associated with and directly tyrosine phosphorylated by the EGFR. Furthermore, a kinase-negative mutant of the EGFR does not associate with phospholipase C-$\gamma$, and the wild-type EGFR associates with phospholipase C-$\gamma$ only when it is in its phosphorylated form (8).

In this study, we further investigated the effect of autophosphorylation on the tyrosine kinase activity of the soluble EGFR-IC (9) with several peptide substrates, including three containing individual phosphorylation sites of phospholipase C-$\gamma$ (10, 11). Interestingly, autophosphorylation of the EGFR-IC has different effects on its kinase activity with different substrates. The most dramatic effect was observed with the synthetic peptide containing Tyr-771 of phospholipase C-$\gamma$. Phosphorylation of the EGFR-IC enhances the affinity for this peptide by a factor of 5-6. Therefore, it is tempting to speculate that Tyr-771 is contained within a domain of phospholipase C-$\gamma$ which is involved in the association of this enzyme with the intact EGFR. Recent evidence from site-directed mutagenesis is consistent with Tyr-771 being a site of subtle regulation of phospholipase C-$\gamma$ activation, whereas phosphorylation of Tyr-783 is essential for activation of enzymatic activity. The reaction velocity with peptide PLC-771, however, is not substantially increased by EGFR-IC phosphorylation. On the other hand, EGFR-IC prephosphorylation enhances the reaction velocities 2-3-fold for peptides K1, PLC-783, and PLC-1254, but affects the affinities for these three peptides to a much less extent than that for PLC-771.

In contrast to the observations with peptide K1 and the three phospholipase C peptides, autophosphorylation of the receptor kinase has little effect on the phosphorylation of the small, nonspecific substrate angiotensin II. Previous reports using angiotensin II as a substrate did show a small effect caused by EGFR autophosphorylation, either as a 30-40% increase of the kinase activity (23) or as a slight decrease in $K_m$ value (22). These results are actually similar to our observations with angiotensin II. However, the possible significance of the slight increase in affinity for this very inefficient substrate is questionable, particularly when compared with the dramatic (5-6-fold) enhancement that EGFR autophosphorylation has on its affinity for peptide PLC-771, which is derived from a physiologically important exogenous substrate. Among the five peptides tested, the kinase has the highest affinity for the substrate representing the major autophosphorylation site of the EGFR itself, peptide K1. This may be related to the important regulatory function of the autophosphorylation sites in the carboxyl-terminal tail of the receptor in receptor signaling. It thus appears likely that the EGFR kinase is first stimulated in vivo by EGF binding, which results in receptor autophosphorylation. This process, in turn, enables the receptor to associate with phospholipase C-$\gamma$, possibly at a domain containing Tyr-771 of phospholipase C-$\gamma$. In fact, Tyr-771 phosphorylation is an early event in phospholipase C-$\gamma$ phosphorylation catalyzed by the EGF-stimulated EGFR kinase (10).

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2 S. G. Rhee, personal communication.
Phosphorylation of Peptides Representing Tyr(P) Sites

The mechanism of the enhancement of the EGFR kinase activity by autophosphorylation is not known. Removal of the intrinsic competitive sites is not likely the sole explanation for its enhancement as judged by our kinetic experiments. It is likely that receptor autophosphorylation at the carboxyl terminus results in a conformational change in the kinase domain and consequently enhances its kinase activity. The mode of stimulation (enhancement of substrate binding affinity or reaction velocity) depends on the particular properties (sequence and secondary structure) of the substrates. Actual conformational change elicited by growth factor-induced autophosphorylation has been observed for the platelet-derived growth factor receptor (26, 27). This conformational change may be important for the mitogenic effect of the platelet-derived growth factor (26). The conformational change of the kinase domain of the EGFR may be probed by biophysical and immunological analyses.

Acknowledgments—We would like to thank Sue Goo Rhee for communicating the sequences of the phosphorylation sites of phospholipase C-γ and the results of the site-directed mutagenesis of phospholipase C-γ before publication. We would also like to thank Rosalie Ratkiewicz for typing this manuscript.

REFERENCES