A Carboxyl-terminal Mutation of the Epidermal Growth Factor Receptor Alters Tyrosine Kinase Activity and Substrate Specificity as Measured by a Fluorescence Polarization Assay*

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The expression of certain COOH-terminal truncation mutants of the epidermal growth factor receptor (EGFR) can lead to cell transformation, and with ligand stimulation, a broader spectrum of phosphorylated proteins appears compared with EGF-treated cells expressing wild-type EGFR. Accordingly, it has been proposed that elements within the COOH terminus may determine substrate specificity of the EGFR tyrosine kinase (Decker, S. J., Alexander, C., and Habib, T. (1992) J. Biol. Chem. 267, 1104–1108; Walton, G. M., Chen, W. S., Rosenfeld, M. G., and Gill, G. N. (1990) J. Biol. Chem. 265, 1750–1754). To address this hypothesis, we analyzed in vitro the steady-state kinetic parameters for phosphorylation of several substrates by both wild-type EGFR and an oncogenic EGFR mutant (the ct1022 mutant) truncated at residue 1022. The substrates included: (i) a phospholipase C-γ fragment (residues 530–850); (ii) the 46-kDa isoform of the Shc adapter protein; and (iii) the 13-residue peptide mimic for the region around the major autophosphorylation tyrosine and the Shc binding site (the Y1173 peptide); (iv) a poly(Glu,Tyr) 4:1 copolymer; and (v) the 8-residue peptide, angiotensin II. Our data demonstrate that the steady-state kinetic parameters for the ct1022 mutant differ from those of the wild-type enzyme, and the differences are substrate-dependent. These results support the concept that this oncogenic truncation/mutation alters EGFR substrate specificity, rather than causing a general alteration of activity. We performed the experiments using a non-radioactive fluorescence polarization assay that quantifies the degree of phosphorylation of peptide as well as natural substrates. The results are consistent with those from the traditional [γ-32P]ATP/filtration assay.

Binding of EGF1 and other ligands to EGFR and its relatives (ErbB2, ErbB3, and ErbB4) triggers a highly complicated signaling network (1, 2). Overexpression as well as oncogenic deletions and truncations of EGFR have been observed in many cancer types, including glioblastomas and non-small cell lung, pancreatic, breast, head and neck, colon, prostate, ovarian, and cervical tumors (3–5). The most commonly found mutation (EGFRvIII) involves a deletion in the NH2-terminal portion of EGFR, from residues 6 to 273 (5–7). Several other types of rearrangements have been found, however, including even a tandem duplication of the tyrosine kinase and calcium internalization domains (4, 5, 8). Half of all glioblastomas exhibit EGFR amplification, and of these, 15% possess COOH-terminal truncated EGFR (at residue 958) (5). Furthermore, expressing certain COOH-terminal truncation mutants in cell culture results in dramatic EGFR-induced increases in the variety of phosphorylated proteins present compared with those observed in cells expressing wild-type EGFR (9–12).

The cause of this increase in the assortment of phosphorylated proteins has not yet been completely explored; however, EGF-induced receptor internalization and degradation occurred at a slower rate with an oncogenic EGFR mutant possessing a COOH-terminal truncation at residue 973 rather than with wild-type EGFR (10, 13). Cells expressing this mutant develop a transformed phenotype when grown in the presence of low EGF concentrations (13). Moreover, data suggest that Δ973-EGFR and ErbB2 heterodimers form in cells expressing this mutant. The ErbB2 in these cells is tyrosyl phosphorylated in the absence of added ligand (12). Interestingly, the oncogene product of the avian erythroblastosis virus (v-erbB) is an NH2- and COOH-terminal truncated version of EGFR (ErbB1) (14, 15).

Cellular changes in phosphotyrosine levels may occur not only as a result of changes in the catalytic properties of the enzyme, but may also reflect alterations in the regulation of other kinases and/or phosphatases in addition to the possible changes in receptor internalization and dimerization already mentioned. For example, although cellular phosphorylation of caveolin-1 is more pronounced in cells expressing COOH-terminal truncated EGFR (9), whether the mutated EGFR directly phosphorylates this substrate remains unclear. Therefore, to precisely isolate the effects of an EGFR mutation on the tyrosine kinase activity, it was necessary to purify the mutant and compare its activity to that of wild-type enzyme in a biochemical assay.

In this study, an in vitro fluorescence polarization (FP) assay (described below) has been used quantitatively to compare the phosphorylation of peptide as well as natural substrates. The results are consistent with those from the traditional [γ-32P]ATP/filtration assay. The assay was used to assess the phosphorylation of a panel of peptides and to evaluate the effects of an oncogenic EGFR mutant (the ct1022 mutant) truncated at residue 1022. The substrates included: (i) a phospholipase C-γ fragment (residues 530–850); (ii) the 46-kDa isoform of the Shc adapter protein; (iii) the 13-residue peptide mimic for the region around the major autophosphorylation tyrosine and the Shc binding site (the Y1173 peptide); (iv) a poly(Glu,Tyr) 4:1 copolymer; and (v) the 8-residue peptide, angiotensin II. Our data demonstrate that the steady-state kinetic parameters for the ct1022 mutant differ from those of the wild-type enzyme, and the differences are substrate-dependent. These results support the concept that this oncogenic truncation/mutation alters EGFR substrate specificity, rather than causing a general alteration of activity. We performed the experiments using a non-radioactive fluorescence polarization assay that quantifies the degree of phosphorylation of peptide as well as natural substrates. The results are consistent with those from the traditional [γ-32P]ATP/filtration assay.

This paper is available on line at http://www.jbc.org

1 The abbreviations used are: EGF, epidermal growth factor; CR, cysteine-rich domain; ct1022 mutant, EGFR mutant possessing a truncation in the COOH terminus after residue 1022; EGFR, EGFR receptor; FP, fluorescence polarization; mP, millipolarization unit (1 polarization unit = 1000 mP units); Y1173 peptide, 13-residue peptide mimic for the region around the major autophosphorylation tyrosine (tyrosine 1173) and the Shc binding site; v-erbB, avian erythroblastosis virus; PLC-γ, phospholipase C-γ.
steady-state kinetic parameters of wild-type EGFR with those of the ct1022 mutant. FP measures molecular rotation occurring during the fluorescence lifetime, the period between excitation and emission of a fluorophore. Relatively small molecules tumble quickly in solution. When excited by plane-polarized light, these molecules emit light that is relatively depolarized. In contrast, larger molecules and complexes tumble more slowly in solution, and emit more highly polarized light (16). The FP competitive immunoassay involves the addition of a fluorescently labeled phosphopeptide tracer (a relatively small molecule) and an antibody (a relatively large molecule) specific for phosphorylated tyrosine to a kinase reaction. Initially, the phosphopeptide tracer binds to the antibody. As the kinase reaction proceeds, however, phosphorylated substrate binds to the antibody and the fluorescent tracer decreases the polarization value. Changes in polarization can readily be monitored with a FP instrument (Fig. 1) (17, 18). To determine whether the steady-state kinetic parameters for wild-type EGFR and for the ct1022 mutant would vary with the choice of substrate, five different substrates were employed: two synthetic peptide substrates, two natural substrates (the Shc adapter protein, and a 321-residue PLC-γ fragment containing the complete SH2-SH2-SH3 domains), and a poly(glu, tyr) 41 copolymer.

Fig. 2 depicts the basic structural features of EGFR (3). A recently solved crystal structure of EGFR encompassing residues 672–998 suggests that for this enzyme, interaction of the COOH-terminal substrate tyrosines with the active site, rather than phosphorylation of the kinase domain “activation loop,” is important for regulating cellular processes (19). Additionally, the structure reveals that Leu955 of the Leu955-Val956-Ile957 tripeptide is closely associated with the kinase domain, and we therefore hypothesize that truncations in the COOH terminus may cause conformational changes that alter substrate specificity.

In this paper, we used an FP competitive immunoassay to measure the amount of phosphorylated substrate produced in kinase reactions. We generated standard curves relating FP values to concentrations of phosphorylated forms of each substrate. In addition, we performed phosphorylation time course assays with EGFR and the ct1022 mutant for each of the five EGFR substrates described above at multiple substrate concentrations. We monitored the reaction progress using FP. We then determined the corresponding amount (pmol) of phosphorylated substrate generated at each time point using the standard curves for each substrate. We plotted the initial velocities resulting from these time course experiments as a function of substrate concentration and fitted the data to the Michaelis-Menten equation. We determined and compared the steady-state kinetic parameters for phosphorylation catalyzed by both wild-type EGFR and the ct1022 mutant for each substrate.
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ATP (23, 24). Aliquots of the reaction mixture (80 µL) were removed and quenched with 700 µL of 10% trichloroacetic acid. Three microliters (3 µL) of acetylated bovine serum albumin (10 mg/ml) was added, and the precipitated poly(Glu,Tyr) 4:1 copolymer or the Shc adapter protein was separated from the radiolabeled ATP by filtration. The radioactivity incorporated into the product was quantified by liquid scintillation counting.

Data Analysis—All data were fitted with Prism™ software, version 3.0 (GraphPad Software Inc.) using the equations below.

One-site competition, where mP = polarization expressed in units of milli-P (1 mP = 0.001 P),

\[
m_{P_{\text{observed}}} = m_{P_{\text{lowest}}} \cdot \frac{m_{P_{\text{observed}}}}{m_{P_{\text{observed}}} - m_{P_{\text{lowest}}}} + \log IC_{50} \tag{Eq. 1}\]

where IC_{50} refers to the polarization value observed when the amount of phosphate present in the substrate is at the midpoint between its lowest and highest values.

One-site competition, solving for log picomole of phosphate incorporated is as follows.

\[
\log(\text{pmol phosphate}) = \log\left(\frac{m_{P_{\text{highest}}} - m_{P_{\text{observed}}}}{m_{P_{\text{observed}}} - m_{P_{\text{lowest}}}}\right) + \log IC_{50} \tag{Eq. 2}\]

A single exponential decay,

\[
m_{P_{\text{observed}}} = (m_{P_{\text{highest}}} - m_{P_{\text{lowest}}})e^{-kt} + m_{P_{\text{lowest}}} \tag{Eq. 3}\]

where k = time or picomole of phosphate incorporated and k = the rate constant.

A single exponential decay, solving for picomole of phosphate incorporated is as follows.

\[
\text{Picomole of phosphate} = \left(\frac{1}{k}\right) \ln\left(\frac{m_{P_{\text{observed}}} - m_{P_{\text{lowest}}}}{m_{P_{\text{highest}}} - m_{P_{\text{lowest}}}}\right) \tag{Eq. 4}\]

Straight line is the following equation.

Michaelis-Menten equation is shown below.

\[
\frac{\text{Initial velocity}}{[\text{enzyme}]} = \frac{k_{\text{cat}}[\text{substrate}]}{K_{M} + [\text{substrate}]} \tag{Eq. 6}\]

where

\[
k_{\text{cat}} = \frac{V_{\text{max}}}{[\text{enzyme}]} \tag{Eq. 7}\]

Sigmoidal dose response (variable slope) is shown as,

\[
\text{Polarization} = \frac{m_{P_{\text{observed}}} - m_{P_{\text{lowest}}}}{1 + \frac{m_{P_{\text{lowest}}}}{m_{P_{\text{highest}}}} - \frac{m_{P_{\text{observed}}}}{m_{P_{\text{highest}}}}} \tag{Eq. 8}\]

where x = pmol of phosphate incorporated per 100-µL reaction.

RESULTS

Measuring Steady-state Kinetic Parameters for Wild-type EGFR and the ct1022 Mutant Using Competition Standard Curves—We created competition standard curves relating polarization values to the amounts of phosphorylated product formed using the phosphorylated forms of the substrates (Fig. 3A). We then used equations describing these relationships to calculate the amount of phosphorylated product that caused a given decrease in polarization (see representative data, Fig. 3, B and C). Two-fold serial dilutions of the phosphorylated substrates were prepared, then the antibody and tracer were added under the same conditions (buffer, temperature) used for (Equation 2), plotted versus time (A), and fitted to the equation for a straight line, with slope = initial velocity = 0.39 ± 0.01 pmol of phosphate incorporated per min and intercept = 0.52 ± 0.06 pmol of phosphate incorporated. Furthermore, 0.9 mM (0.3 units/100 µL) ct1022 mutant was preincubated as described above for wild-type EGFR, followed by the addition of the PLC-γ fragment to 0.25 µM. The decrease in polarization (C) could be described by the equation for a single exponential decay (Equation 3) with mP_{highest} − mP_{lowest} = 196 ± 2 mP, k = 0.0260 ± 0.0007 min⁻¹, and mP_{lowest} = 38 ± 0.4 mP. Phosphate incorporation was calculated

FIG. 3. The elucidation of steady-state kinetic parameters for the phosphorylation of various substrates by wild-type EGFR and the ct1022 mutant. A, competition standard curves were generated to relate polarization to product formation. The phosphorylated forms of the synthetic peptide substrate or the phosphorylated reaction products were serially diluted as described, followed by the addition of antibody, tracer, and reaction buffer, and polarization was measured for the poly(Glu,Tyr) 4:1 copolymer (●), the pY1173 peptide (▲), the PLC-γ fragment (◇), and angiotensin II (♦) as described more fully under “Experimental Procedures” and “Results.” The inset illustrates a direct fit of the same data, where polarization is plotted as a function of picomole of phosphate present/100 µL, without the logarithmic scale. B, two representative time courses show the change in polarization upon phosphorylation of the Y1173 peptide and PLC-γ fragment, and the corresponding amounts of phosphate incorporated into each substrate. EGFR (0.8 nm, 0.2 units/100 µL) was preincubated with 2.5 mM ATP followed by the addition of the Y1173 peptide to 0.2 mM. The decrease in polarization was monitored (○), and data could be described by a single exponential decay (Equation 3) with mP_{highest} − mP_{lowest} = 183 ± 1 mP, k = 0.0135 ± 0.0005 min⁻¹, and mP_{lowest} = 38 ± 0.4 mP. The amount of phosphate incorporated for each data point was calculated from these FP data using Equation 2 and kinetic parameters from the competition standard curve, then plotted versus time (●), and data were fitted using the equation for a straight line, with slope = initial velocity = 0.136 ± 0.004 pmol of phosphate incorporated per min and intercept = 0.52 ± 0.06 pmol of phosphate incorporated. Furthermore, 0.9 mM (0.3 units/100 µL) ct1022 mutant was preincubated as described above for wild-type EGFR, followed by the addition of the PLC-γ fragment to 0.25 µM. The decrease in polarization (◇) could be described by the equation for a single exponential decay (Equation 3) with mP_{highest} − mP_{lowest} = 196 ± 2 mP, k = 0.0260 ± 0.0007 min⁻¹, and mP_{lowest} = 38 ± 0.4 mP. Phosphate incorporation was calculated
the assay (see “Experimental Procedures”). Competition standard curves for phosphorylated versions of angiotensin II and the Y1173 peptide were produced in this manner. Because phosphorylated forms of the PLC-γ fragment and poly(Glu,Tyr) 4:1 copolymer were not available, reactions were brought to completion by using a relatively high enzyme concentration. Each phosphorylation reaction contained 2.5 mM ATP and either 2.7 nM (0.3 units/100 µl) EGFR or 1.7 nM (0.6 units/100 µl) ct1022 mutant as well as either 0.12 µM PLC-γ fragment or 10 nM poly(Glu,Tyr) 4:1 copolymer, respectively, and was incubated at 30 °C for 2 h. Two-fold serial dilutions of these phosphorylated substrates were prepared and analyzed as described above for the peptide substrates. For each substrate, we measured and plotted polarization values as a function of the phosphorylated substrate concentration to create the competition standard curve, as shown in Fig. 3A. These data were described by a one-site competition equation (Equation 1), resulting in IC₅₀ values of 0.25 ± 0.06 pmol of phosphate for the poly(Glu,Tyr) 4:1 copolymer, 7.7 ± 0.8 pmol of phosphate for the pY1173 peptide, 9 ± 2 pmol of phosphate for the PLC-γ fragment, and 175 ± 13 pmol of phosphate for angiotensin II. The inset illustrates a direct fit of the same data using the equation for a single exponential decay (Equation 3) with β = 3.5 ± 0.5 pmol of phosphate −¹ for the poly(Glu,Tyr) 4:1 copolymer, 0.15 ± 0.02 pmol of phosphate −¹ for the pY1173 peptide, 0.098 ± 0.008 pmol of phosphate −¹ for the PLC-γ fragment, and 0.0053 ± 0.0006 pmol of phosphate −¹ for angiotensin II. The anomalously high IC₅₀ for angiotensin II may be because of its distinct structure (25). The results from control experiments involving higher concentrations of enzyme (3.6 and 2.6 nM, respectively, for the 0.12 µM PLC-γ fragment and 10 nM poly(Glu,Tyr) 4:1 copolymer substrates) indicated that our reaction conditions yielded completely phosphorylated substrates (data not shown).

After generating the competition standard curves, we performed FP assays and calculated the corresponding amounts of phosphorylated product formed from the transformed one-site competition equation for each substrate (Equation 2). Using FP, we measured substrate phosphorylation directly for each substrate at multiple substrate concentrations. Representative data for both the Y1173 substrate and the PLC-γ fragment are shown in Fig. 3B. In the FP assay, product formation is “coupled” to fluorescent phosphopeptide tracer dissociation from, and the concomitant binding of non-fluorescent phosphorylated substrate (product) to, the antibody, resulting in product de-

### Table I

| Enzyme                  | k₅₀, k₉⁰ | Kₐ | k₅₀K₉⁰ |
|-------------------------|----------|----|--------|
| Y1173 peptide           | 3.6 ± 0.3| 0.16 ± 0.04 µM | (4.0 ± 0.7) x 10² |
| ct1022 mutant           | 16 ± 2   | 0.23 ± 0.08 µM | (1.1 ± 0.2) x 10³ |
| Ratio                   | 2.8      | 1.4  | 2.8    |
| Poly(Glu,Tyr) 4:1 copolymer | 0.48 ± 0.03 | 18 ± 4 nM | (4.8 ± 0.8) x 10³ |
| ct1022 mutant           | 1.5 ± 0.1| 29 ± 8 nM  | (8 ± 2) x 10²    |
| Ratio                   | 3        | 1.6  | 1.7    |
| PLC-γ fragment          | 8.0 ± 2  | 0.23 ± 0.09 µM | (6 ± 2) x 10³    |
| ct1022 mutant           | 10 ± 4   | 0.3 ± 0.3 µM  | (5 ± 2) x 10⁴    |
| Ratio                   | 1.2      | 1.3  | 0.8    |
| Angiotensin II          | 13 ± 1   | 0.24 ± 0.06 µM | (9 ± 2) x 10²    |
| ct1022 mutant           | 5.7 ± 0.4| 0.17 ± 0.05 µM | (5.70 ± 0.03) x 10³ |
| Ratio                   | 0.4      | 0.7  | 0.6    |
| Shc adapter protein     | 0.17 ± 0.05 | 2 ± 1 µM | (1.4 ± 0.4) x 10³ |
| ct1022 mutant           | 0.035 ± 0.002 | 0.20 ± 0.05 µM | (3.0 ± 0.6) x 10³ |
| Ratio                   | 0.2      | 0.1  | 2      |

* Units of k₅₀ are picomoles of phosphate transferred per min/pmol of enzyme.
* Ratio is the steady-state kinetic parameter for the reaction catalyzed by the ct1022 mutant divided by the same kinetic parameter for wild-type EGFR.
* k₅₀ has been normalized for variations in wild-type EGFR specific activity.

Similar experiments were performed at multiple substrate concentrations, and Fig. 3C depicts representative data for the Y1173 peptide substrate. Time courses were prepared involving the preincubation of 0.4–1.7 nM (0.1–0.4 units/100 µl) wild-type EGFR or 0.5 nM (0.2 units/100 µl) ct1022 mutant with 2.5 mM ATP, followed by the addition of the Y1173 peptide. Polarization values were transformed into picomole of phosphate transferred/min/pmol of EGFR (Equation 2) as described, and plotted as a function of substrate concentration. Data were fitted to the Michaelis-Menten equation (Equation 6) (26), and the steady-state kinetic parameters were thus determined. The mutant enzyme yielded a 4-fold higher turnover number for the phosphorylation of the Y1173 peptide substrate than did EGFR. The steady-state kinetic parameters determined in this manner for each substrate are described in Table I.

Because the poly(Glu,Tyr) 4:1 copolymer and the PLC-γ fragment contain more than one potential phosphorylation site, the kinetic parameters for these substrates are apparent values (27). Full-length PLC-γ has four tyrosines that are phosphorylated by EGFR (Tyr-472, Tyr-771, Tyr-783, and Tyr-1254) (28). Therefore, the fragment of PLC-γ we used as a substrate (residues 538–850) possesses two tyrosines that EGFR can phosphorylate (Tyr-771 and Tyr-783). Steady-state kinetic parameters for angiotensin II and the PLC-γ fragment were within experimental error of those previously measured using the [γ-³²P]ATP phosphate incorporation assay (24).²

² P. J. Berties, unpublished data.
Verification of Competition Standard Curve Results for the Poly(Glu,Tyr) 4:1 Copolymer Substrate—Although previous control experiments indicated that the poly(Glu,Tyr) 4:1 copolymer and PLC-γ fragment used for generating the competition standard curves had been completely phosphorylated, we further verified these results by employing an additional method for generating a standard curve for the poly(Glu,Tyr) 4:1 copolymer. This second approach calls for performing identical reactions and evaluating them by different methods. We analyzed one reaction with the traditional kinase assay using [γ-32P]ATP, and two other reactions with the FP technique. Given that conditions (enzyme and substrate concentrations, temperature, buffer, etc.) were the same for all reactions, the extent of substrate phosphorylation required to produce a given decrease in polarization could be calculated. We measured the phosphorylation of the poly(Glu,Tyr) 4:1 copolymer (150 nm) by the ct1022 mutant (0.5 nm, 0.13 units/100 μl) in three reactions: the FP assay in both real-time and end-point modes and by the [γ-32P]ATP assay (Fig. 4A) as described under “Experimental Procedures.” Each time course was set up under identical conditions (10 μM ATP and the buffer and temperature described), except that the [γ-32P]ATP assay reaction included 7.5 μCi of [γ-32P]ATP/100 μl (inset, left side). Data for the [γ-32P]ATP time course were fitted to the equation for a straight line, with slope = initial velocity = 0.070 ± 0.008 pmol of phosphate transferred per min, and intercept = −0.1 ± 0.1 pmol of phosphate transferred. For each time point of the FP assays, the amount of phosphate transferred to the substrate was calculated using the equation that describes the [γ-32P]ATP time course: picomole of phosphate incorporated = 0.070 pmol of phosphate incorporated per min × time − 0.1 pmol of phosphate incorporated. Polarization was plotted as a function of picomole of phosphate incorporated, and data were fitted to Equation 7, with IC50 real-time assay = 0.27 ± 0.01 pmol of phosphate incorporated, Hill slope = −1.36 ± 0.03, and IC50 end-point assay = 0.26 ± 0.01 pmol of phosphate incorporated, Hill slope = −2.5 ± 0.2. These resulting IC50 values were very similar to each other and to that determined previously using a competition standard curve (Fig. 3A) (0.25 pmol of phosphate incorporated). Therefore, the kinetic parameters determined by converting polarization to phosphorylated product with either method of generating a standard curve should be essentially identical. Furthermore, the inset (right side) presents the same data without a log scale, described by Equation 3. For the real-time assay, mPhigh − mLowe st = 149.3 ± 0.8 mP, k = 2.77 ± 0.03 pmol of phosphate; k, and mLowe st = 43.9 ± 0.2 mP. For the end point assay, mLowe st = 220 ± 9 mP, k = 3.0 ± 0.3 pmol of phosphate; k, and mLowe st = 34 ± 8 mP. A control reaction was performed that also contained 7.5 μCi of [γ-32P]ATP/100 μl, but was analyzed for generating a standard curve for the poly(Glu,Tyr) 4:1 copolymer to 15 nM. The decrease in polarization was monitored, and data could be described by a single exponential decay (Equation 3), with mLowe st − mLowe st = 174 ± 3 mP, k = 0.042 ± 0.002 min−1, mLowe st = 63 ± 4 mP, and R2 = 0.9996. The increase in the amount of phosphate incorporated was calculated from each data point (Equation 4) using kinetic parameters from the standard curve. The amount of phosphate transferred to substrate was plotted with respect to time. These data were then fitted using the equation for a straight line, with slope = initial velocity = 0.0117 ± 0.0001 pmol of phosphate incorporated per min and intercept = 0.028 ± 0.001 pmol of phosphate incorporated. C, representative steady-state kinetic data for the catalysis of phosphorylation of the poly(Glu,Tyr) 4:1 copolymer by wild-type EGFR (●) and the ct1022 mutant (○) determined by FP (as described more fully under “Experimental Procedures” and “Results”).

Fig. 4. The measurement of steady-state kinetic parameters for the poly(Glu,Tyr) 4:1 copolymer by FP using a radioactive phosphate incorporation assay to generate the standard curves. A, three reactions involving the catalysis of phosphorylation of the poly(Glu,Tyr) 4:1 copolymer by the ct1022 mutant were measured by the traditional [γ-32P]ATP assay (lower left inset (●)), the FP assay in real time (○) using the Beacon2000 fluorescence polarization instrument, and the end point assay (●), in which time points were quenched with 6 mM EDTA (Tecan Ultra). Furthermore, the inset (lower right) presents the same data shown in the main figure, but as a direct fit (not using a log scale). B, representative data illustrating the drop in polarization for the phosphorylation of the poly(Glu,Tyr) 4:1 copolymer (●), and the corresponding amounts of phosphate incorporated into substrate (●) are shown. EGFR (0.5 nm, 0.13 units/100 μl) was preincubated in reaction buffer and 2.5 mM ATP followed by the addition of the poly(Glu,Tyr) 4:1 copolymer to 15 nm. The decrease in polarization was monitored, and data could be described by a single exponential decay (Equation 3), with mLowe st − mLowe st = 174 ± 3 mP, k = 0.042 ± 0.002 min−1, mLowe st = 63 ± 4 mP, and R2 = 0.9996. The increase in the amount of phosphate incorporated was calculated from each data point (Equation 4) using kinetic parameters from the standard curve. The amount of phosphate transferred to substrate was plotted with respect to time. These data were then fitted using the equation for a straight line, with slope = initial velocity = 0.0117 ± 0.0001 pmol of phosphate incorporated per min and intercept = 0.028 ± 0.001 pmol of phosphate incorporated. C, representative steady-state kinetic data for the catalysis of phosphorylation of the poly(Glu,Tyr) 4:1 copolymer by wild-type EGFR (●) and the ct1022 mutant (○) determined by FP (as described more fully under “Experimental Procedures” and “Results”).
in real time by FP in the Beacon™ 2000 fluorescence polarization instrument. Results were within experimental error of those obtained in the absence of [γ-32P]ATP (data not shown).

Fig. 4B illustrates a representative time course in which the reaction was monitored by FP, and the amount of phosphate incorporated at each time point was calculated from the standard curve. Similar data were collected at multiple substrate concentrations, and steady-state kinetic parameters were measured (Fig. 4C). EGFR (0.5–0.8 nM, 0.1–0.2 units/100 μl) or the ct1022 mutant (0.3–0.5 nM, 0.1–0.2 units/100 μl) were preincubated with 2.5 mM ATP for 15 min, then substrate was added and polarization was monitored. Polarization values were transformed into picomole of phosphate transferred per min/pmol of EGFR as described, plotted versus time, and initial velocities were described by the slopes of these lines. Initial velocities were then graphed as a function of substrate concentration, and these data were fitted to the Michaelis-Menten equation. The steady-state kinetic parameters thus determined for the poly(Glu,Tyr) 4:1 copolymer as well as the other substrates are shown in Table I, and the turnover number for the phosphorylation of this substrate is 3-fold higher with the ct1022 mutant.

Similarly, a standard curve for the 46-kDa isoform of the Shc adapter protein was generated by running two identical reactions (0.5 μM Shc, 10 μM ATP, and 5.1 nM ct1022 mutant at 30 °C); one analyzed by FP in real time using the Beacon™ 2000 fluorescence polarization instrument, and the other by the radioactive assay as described. An initial velocity of 0.025 ± 0.004 pmol/min was measured (data not shown) and standard curves similar to those for the poly(Glu,Tyr) 4:1 copolymer were generated, with k = 1.88 ± 0.03 pmol−1 from the direct fit using a single exponential decay, and IC50 = 0.46 ± 0.03 pmol of phosphate incorporated (data not shown). Steady-state kinetic parameters for catalysis of phosphorylation by wild-type and mutant enzyme were determined by fitting plots of initial velocity versus substrate concentration to the Michaelis-Menten equation (Equation 6), and Table I summarizes the kinetic parameters so determined for the Shc adapter protein as well as all of the other substrates.

### DISCUSSION

The Quantitative Use of the FP Assay to Measure Kinetic Parameters—We have used two different methods to demonstrate that a tyrosine kinase FP assay may be used quantitatively. Competition standard curves may be employed (Fig. 3A), or one may conduct a traditional [γ-32P]ATP/filtration time course under identical conditions as an FP time course to relate the amount of phosphorylated product formed to the observed polarization value (Fig. 4A). For each substrate, substrate phosphorylation was measured by FP as a function of time at multiple substrate concentrations. We have provided representative data (Figs. 3B and 4B) to illustrate the use of each method. The initial velocities determined in this manner were plotted as a function of substrate concentration for each substrate, and data were described by the Michaelis-Menten equation resulting in the determination of steady-state kinetic parameters. Representative data are shown (Figs. 3C and 4C). These steady-state kinetic parameters determined by FP are described in Table I.

The FP assay has the advantages of being nonradioactive and amenable to high throughput screening. Furthermore, we have shown that this particular FP assay can be performed at relatively high ATP concentrations (2.5 mM), which would be more difficult with the traditional radioactive assay. In the [γ-32P]ATP assay, sensitivity is directly proportional to the concentration of radioactive ATP and inversely proportional to the concentration of unlabeled ATP. Therefore, the amount of radioactive ATP needed to conduct assays at physiological ATP concentrations, which are often in the millimolar range, would be quite high. Some examples of intracellular ATP concentrations include 3 mM for yeast (29), 1 mM in smooth muscle cells (30), and 2.6 mM in human astrocytoma cells (31). We found that the Km values for ATP were in the micromolar range for certain substrates. For example, for the poly(Glu,Tyr) 4:1 copolymer (substrate concentration = 2 ng/μl (60 nM), using the FP assay at room temperature, as described under “Experimental Procedures”), the Km(ATP,EGFR (wild-type)) = 6 ± 2 μM and the Km(ATP,ct1022 mutant) = 1.9 ± 0.8 μM, data not shown. However, we observed that the Km values for ATP with the PLC-γ fragment (wild-type EGFR, substrate concentration = 0.2 μM, using the FP assay at 30 °C, as described under “Experimental Procedures”) is much greater than 50 μM (data not shown). Therefore, the amount of [γ-32P]ATP required in the [γ-32P]ATP filtration assay (if it were conducted near or above the Km for ATP) would increase dramatically. Furthermore, for the Shc adapter protein, while we found that Km(ATP,ct1022) was less than 10 μM (substrate concentration = 0.5 μM, using the FP assay at 30 °C), the Km(ATP,EGFR (wild-type)) was greater than 100 μM (substrate concentration = 1.5 μM, using the FP assay at 30 °C). Consequently, FP may be an easier method for analyzing the kinetics of the wild-type enzyme for this natural substrate.

Moreover, we have demonstrated that this assay is accurate in real time (Fig. 4A). The comparison of the real time versus the end point assay for the poly(Glu,Tyr) 4:1 copolymer suggests that, for this particular FP assay, the time required for phosphorylated product to come to equilibrium with the antibody and tracer is so brief that the results are unaffected. Thus, the assay may be performed directly in an instrument measuring FP, rather than by quenching individual time points with EDTA and waiting for antibody and tracer to come to equilibrium. Therefore, during screening experiments, control reactions run in the absence of inhibitory could be monitored to determine the appropriate time to quench the reaction with EDTA.

It is important to keep in mind that the relationship of polarization to phosphorylation of substrate is not linear (Figs. 3, A and B, and 4, A and B), and that the assay is more sensitive to phosphorylation before all of the tracer is displaced. In measuring initial rates to determine kinetic parameters, continuous assays are always preferable to single time point assays (32), and this is also true for the FP technique. A standard curve, however, takes into account the “leveling off” of polarization as tracer is displaced.

Comparison of Kinetic Parameters for Reactions Catalyzed by the ct1022 Mutant to Those for Wild-type EGFR—Our data demonstrate that the steady-state kinetic parameters for the ct1022 mutant differ from those of the wild-type enzyme, and these differences depend on the identity of the substrate (Table I). The increases in turnover number for certain substrates along with the studies of internalization and possible heterodimer formation with ErbB2 may explain the broader specificity of EGFR. The Y1173 peptide and the poly(Glu,Tyr) 4:1 copolymer is 3-fold higher with the ct1022 mutant. The phosphorylation of this substrate is 3-fold higher with the ct1022 mutant. Furthermore, we have shown that this particular FP assay can be performed at relatively high ATP concentrations (2.5 mM), which would be more difficult with the traditional radioactive assay. In the [γ-32P]ATP assay, sensitivity is directly proportional to the concentration of radioactive ATP and inversely proportional to the concentration of unlabeled ATP. Therefore, the amount of radioactive ATP needed to conduct assays at physiological ATP concentrations, which are often in the millimolar range, would be quite high. Some examples of intracellular ATP concentrations include 3 mM for yeast (29), 1 mM in smooth muscle cells (30), and 2.6 mM in human astrocytoma cells (31). We found that the Km values for ATP were in the micromolar range for certain substrates. For example, for the poly(Glu,Tyr) 4:1 copolymer (substrate concentration = 2 ng/μl (60 nM), using the FP assay at room temperature, as described under “Experimental Procedures”), the Km(ATP,EGFR (wild-type)) = 6 ± 2 μM and the Km(ATP,ct1022 mutant) = 1.9 ± 0.8 μM, data not shown. However, we observed that the Km values for ATP with the PLC-γ fragment (wild-type EGFR, substrate concentration = 0.2 μM, using the FP assay at 30 °C, as described under “Experimental Procedures”) is much greater than 50 μM (data not shown). Therefore, the amount of [γ-32P]ATP required in the [γ-32P]ATP filtration assay (if it were conducted near or above the Km for ATP) would increase dramatically. Furthermore, for the Shc adapter protein, while we found that Km(ATP,ct1022) was less than 10 μM (substrate concentration = 0.5 μM, using the FP assay at 30 °C), the Km(ATP,EGFR (wild-type)) was greater than 100 μM (substrate concentration = 1.5 μM, using the FP assay at 30 °C). Consequently, FP may be an easier method for analyzing the kinetics of the wild-type enzyme for this natural substrate.

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In addition to the investigations performed with purified proteins, differences in the amounts of specific phosphorylated proteins formed in the presence of COOH-terminal truncated versions of EGF compared with those formed in the presence of wild-type EGF have been explored in transfected cells. The results suggest that phosphorylation of both PLC-γ and Ras-GTPase activating protein-associated protein p62 was dramatically reduced in EGF mutants truncated at residues 1011 or 973; however, the truncated EGF receptors could still induce phosphorylation of the Shc adapter protein (10, 36–38). Furthermore, caveolin-1 phosphorylation occurred in cells expressing truncated EGF receptors, but not in cells expressing wild-type or kinase-inactive EGF (9). As mentioned earlier, changes in phosphotyrosine levels observed in vitro may be because of changes in the regulation of other kinases and/or phosphatases, and not due simply to an alteration in the catalytic properties of the enzyme in question. However, EGF-dependent cell growth did not occur in cells expressing a ct1022 kinase negative double mutation, but did occur in cells expressing an EGF mutant truncated at residue 973, suggesting the phenotypic importance of kinase activity for COOH-terminal truncation mutants (13).

Interestingly, we observed that the ct1022 mutant phosphorylated the PLC-γ fragment in vitro as efficiently as did purified wild-type EGF. Only one of the five tyrosine autophosphorylation sites remains in the ct1022 mutant (Tyr-992), and phosphorylation sites remain in the ct1022 mutant (compared with the wild-type enzyme) for phosphorylating the Shc adapter protein may be because of slower product dissociation, because, as noted earlier, product dissociation is rate-limiting for ErbB2 (33). Furthermore, the lower $K_m$ might suggest a tighter association between the ct1022 mutant and Shc than between wild-type EGF and Shc. In the absence of data describing the individual rate constants, we cannot determine whether the $K_m$ equals the dissociation constant for enzyme and substrate. However, we expect that the Shc adapter protein as well as GrbB2 associates with EGF in vitro and also participates in the initiation of the mitogen-activated protein kinase cascade (1).

In sum, when compared with wild-type EGF, several substrates are more efficiently phosphorylated by the ct1022 mutant, whereas other substrates are equally well phosphorylated or even less efficiently phosphorylated by the mutant receptor. These data reveal that COOH-terminal sequences may participate in determining the substrate specificity of EGF, in addition to serving as docking sites for various effector molecules.

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