Kinetics of Activation of the Tyrosine Kinase of a Deletion Mutant of Epidermal Growth Factor Receptor Missing the Sites of Self-Phosphorylation†

Jennifer M. Sherrill and Jack Kyte*

Department of Chemistry (0506), University of California at San Diego, 9500 Gilman Drive, La Jolla, California 92093-0506

Received September 22, 1998; Revised Manuscript Received November 23, 1998

ABSTRACT: The addition of epidermal growth factor (EGF) to epidermal growth factor receptor (EGF receptor) dissolved in a solution of the detergent Triton X-100 results in the activation of its protein tyrosine kinase. To investigate the importance of the sites for self-phosphorylation on the enzyme in this process, the kinetics of activation of a deletion mutant missing the last 195 amino acids of the protein, including all of the sites for self-phosphorylation, were followed by monitoring the initial velocity at which the enzyme catalyzes the phosphorylation of the exogenous substrate RRKGSTAENAEYLRV. The activation of the enzymatic activity of this deletion mutant of EGF receptor displays kinetics that are second-order with respect to the concentration of the enzyme as does wild-type EGF receptor. The second-order rate constant for its activation is $36 \pm 10\ \mu M^{-1}\ min^{-1}$, which is only 3-fold greater than the second-order rate constant for the activation of wild-type EGF receptor under the same conditions ($13 \pm 2\ \mu M^{-1}\ min^{-1}$). These results suggest that the mechanism by which the protein tyrosine kinase of the deletion mutant is activated is the same as that for the activation of the wild-type receptor and that the sites of self-phosphorylation in the wild-type EGF receptor do not participate in the mechanism of activation of the enzyme.

Human epidermal growth factor receptor (EGF receptor)[1] is a glycoprotein of 1186 amino acids consisting of an extracellular domain for binding its ligand, epidermal growth factor (EGF), a single membrane-spanning segment, and a cytoplasmic domain for expressing its protein tyrosine kinase activity (*1*−*6*). When EGF binds to EGF receptor at the outside surface of the cell, the receptor dimerizes, and its protein tyrosine kinase is activated (*7*−*10*). Both the dimerization and activation of the tyrosine kinase display kinetics that are second-order with respect to the concentration of EGF receptor (*10*). The dimerized, activated receptor then phosphorylates itself, in a process known as self-phosphorylation, as well as several cellular substrates (*11*−*13*).

Self-phosphorylation of EGF receptor occurs at five tyrosines near the carboxy terminus (*14*−*16*). Upon phosphorylation, these tyrosines have been shown to create binding sites that have a high affinity for the SH2 domains of intracellular signaling proteins (*17*−*19*). The interaction of SH2 domains with the phosphorylated tyrosines of EGF receptor is thought to initiate the transduction of the signal leading to growth and division. It has been suggested that self-phosphorylation and the subsequent recruitment of proteins to the plasma membrane are critical steps in signal transduction by EGF receptor (*13*), but a carboxy-terminal deletion mutant of EGF receptor missing all of its sites for self-phosphorylation is able to transform cells when expressed at levels similar to that of wild-type receptor (*20*, *21*). These results suggest that the associations between SH2 domains of cytoplasmic proteins and the sites of self-phosphorylation in the carboxy terminus of EGF receptor are not necessary for the cellular activation produced by EGF. In the presence of ligand, carboxy-terminal deletion mutants have also shown enhanced in vivo tyrosine phosphorylation of cellular substrates, suggesting that these mutants are at least as enzymatically active as the holoenzyme (*14*, *20*, *22*).

Although the participation of the sites of self-phosphorylation as positions of association important in the transduction of the signal would be sufficient explanation for their existence, there is at least one proposal in which they are viewed as essential for the normal activation of ligand-dependent protein tyrosine kinases (*23*). To address the question of the role of the sites of self-phosphorylation in the activation of the tyrosine kinase of EGF receptor, the rates of activation of a carboxy-terminal deletion mutant and wild-type EGF receptor have been measured and compared. Like the wild-type enzyme, EGF receptor truncated after glutamate 991 (Δ991 deletion mutant of EGF receptor), thereby lacking all of the sites of self-phosphorylation (*14*−*16*), displayed second-order kinetics of activation. The second-order rate constant for the activation of the Δ991 deletion mutant of EGF receptor is 3-fold greater than that for the wild-type enzyme, while the turnover number of the mutant is 5-fold smaller than that of the wild-type enzyme. These results suggest that deleting the sites of self-phosphorylation of EGF receptor has no significant effect on the mechanism of activation of its tyrosine kinase.

† This research was supported by Grant GM-47968 from the National Institutes of Health. Training Grants T32-GM08326 and T32-GM07313 from the National Institutes of Health also provided support to J.M.S.

[1] Abbreviations: EGF, epidermal growth factor; EGF receptor, epidermal growth factor receptor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Δ991 deletion mutant of EGF receptor, EGF receptor truncated after glutamate 991.

10.1021/bi982276d CCC: $18.00
Published on Web 02/13/1999

EXPERIMENTAL PROCEDURES

Materials. Cells of the A431 human epithelioid carcinoma line expressing wild-type EGF receptor (*24*) and the B82 murine epithelioid carcinoma line expressing the deletion mutants were provided by G. Gill of the Department of Medicine at the University of California at San Diego. Construction and expression of the Δ973 and Δ991 deletion mutants of EGF receptor have been previously described (*14*, *22*). Benzamidine hydrochloride, bovine serum albumin, phenylmethanesulfonyl fluoride, Na_2ATP, Triton X-100 detergent, aprotinin, and leupeptin were purchased from Sigma Chemical Co. Phosphocellulose paper was purchased from Whatman. 9-Fluorenylmethyloxycarbonyl derivatives of the amino acids were purchased from Bachem, Inc. The triethylammonium salt of adenosine [γ-^{32}P]triphosphate (3000 mCi mmol^{-1}) was purchased from Amersham. Recombinant human EGF, EDTA, and *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from Fisher Scientific. The peptide RRKGSTAENAEYLRV, which is a reactant for the tyrosine kinase of EGF receptor, was synthesized from 9-fluorenylmethyloxycarbonyl amino acids (*25*, *26*) and purified by HPLC. Acid hydrolysis of this purified peptide gave the following composition: $R_{3.1}K_{1.0}G_{1.1}S_{0.8}T_{0.9}A_{1.9}E_{2.2}N_{1.1}Y_{0.6}L_{1.1}V_{1.1}$.

Detergent Extracts of A431 Cells and Quantification of the Concentration of EGF Receptor. Detergent extracts of A431 cells were produced (*27*, *28*), and the concentrations of EGF receptors in the extracts of A431 and B82 cells were determined (*27*) as described previously.

Extracts of B82 Cells. Cells were grown to confluence, harvested, and lysed in 2 mM EDTA, 10 mM EGTA, 10 mM 2-mercaptoethanol, 4 mM benzamidine hydrochloride, 0.1 mM phenylmethanesulfonyl fluoride, 5 μg/mL aprotinin, 10 μg/mL leupeptin, and 30 mM HEPES (pH 7.4). The membranes were gathered by centrifugation at 15 000 rpm in a 50.2 Ti rotor (Beckman Corp.) for 30 min and dissolved at a final concentration of 1% Triton X-100 and 10% glycerol. The homogenate was centrifuged at 75 000 rpm in a TLA100.2 rotor (Beckman Corp.) for 30 min. The supernatant fluid is referred to as an extract of B82 cells.

Tyrosine Kinase Activity. The initial velocity of the protein tyrosine kinase of EGF receptor was assayed as previously reported (*10*, *27*, *28*). Samples (30 μL) of appropriately diluted extracts of the desired cells were mixed with 15 μL of 5 μM EGF in 0.15 M NaCl, 1 mM EDTA, and 50 mM sodium phosphate (pH 7.2) or 15 μL of 0.15 M NaCl, 1 mM EDTA, and 50 mM sodium phosphate (pH 7.2) as a control for the noted times at room temperature. The enzymatic activity was then assayed in a volume of 60 μL at the following final concentrations: 5 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM Na_3VO_4, 10 μM ATP, 0.375 mM RRKGSTAENAEYLRV, [γ-^{32}P]ATP to a final specific radioactivity of 2 Ci/mmol of ATP, and 20 mM HEPES (pH 7.4). The assay was quenched after 1 min by the addition of 25 μL of 20% trichloroacetic acid. Bovine serum albumin was added to 0.5 mg/mL before the samples were precipitated on ice for 30 min and centrifuged. Samples of the supernatant fluids were spotted on disks of phosphocellulose paper that were then washed four times with 1 L of 75 mM H_3PO_4, dried, and counted.

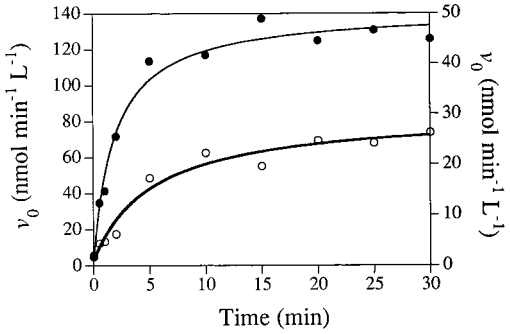

FIGURE 1: Activation of the tyrosine kinase of the Δ991 deletion mutant of EGF receptor as a function of time with EGF. Appropriately diluted samples (45 μL final volume) of an extract from B82 cells were exposed to a saturating concentration of EGF (1.6 μM) for the noted time and then assayed for tyrosine kinase activity. The enzymatic activity measured in the absence of EGF was subtracted from all of the data points from samples exposed to EGF. The concentrations of samples of extract were 16 (●) or 4 nM (○) in the Δ991 deletion mutant of EGF receptor. The solid lines through the data points represent the nonlinear least-squares fits of eq 1 for a reaction that is second order with respect to the concentrations of monomers of the Δ991 deletion mutant of EGF receptor.

RESULTS

The time course of activation of the tyrosine kinase of the Δ991 deletion mutant of EGF receptor was followed to determine whether this truncated enzyme displayed the same second-order kinetics of activation as those of the wild-type enzyme (*10*). The Δ991 deletion mutant of EGF receptor was expressed in murine B82 cells, which do not express endogenous EGF receptor (*29*). In this way, EGF-induced activation of the deletion mutant could be measured without the contamination of the wild-type enzyme.

The increase in enzymatic activity was followed by incubating the extracts of cells with saturating EGF (1.6 μM) for different time intervals and then monitoring the initial velocity of the incorporation of ^{32}P into the tyrosine-containing substrate, RRKGSTAENAEYLRV. Samples assayed in the absence of EGF showed a steady level of tyrosine kinase activity (5−10% of that induced by EGF) that was subtracted from all data points for samples exposed to EGF. The experiments were performed with the wild-type enzyme and the Δ991 deletion mutant to compare the rate constants for activation, k_A. When the time course of activation of the protein tyrosine kinase of the Δ991 deletion mutant of EGF receptor was followed (Figure 1), it was found that the data points could be fit with the equation

$$KA = KA_\infty - \frac{KA_\infty - KA_0}{1 + [\text{receptor}]k_A t} \qquad (1)$$

for a reaction that is second-order with respect to the concentration of receptor (*10*), where KA_∞ is the initial velocity of the tyrosine kinase at infinite time following the addition of EGF, KA is the initial velocity of the tyrosine kinase at any time (t), KA_0 is the initial velocity of the tyrosine kinase immediately before EGF was added, and [receptor] is the molar concentration of EGF receptor. This equation was derived from a series of equations that assume dimeric EGF receptor is the only active species and that at infinite time with saturating EGF, all of the EGF receptor in a sample will have dimerized (*10*, *27*).

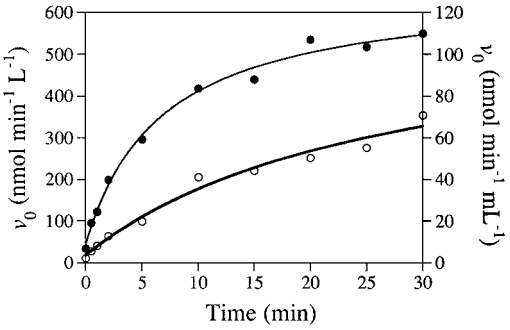

FIGURE 2: Activation of the tyrosine kinase of wild-type EGF receptor as a function of time with EGF. Appropriately diluted samples (45 μL final volume) of an extract from A431 cells were exposed to a saturating concentration of EGF (1.6 μM) for the noted time and then assayed for tyrosine kinase activity. The activity measured in the absence of EGF was subtracted from all of the data points from samples exposed to EGF. The concentrations of samples of extract were 10 (●) or 2.5 nM (○) in EGF receptor. The solid lines through the data points represent the nonlinear least-squares fits of eq 1 for a reaction that was second order with respect to the concentrations of monomers of EGF receptor.

Table 1: Second-Order Rate Constants for the Activation of the Δ991 Deletion Mutant and Wild-Type EGF Receptor[a]

source of receptor	[receptor] (nM)	k_A (μM^{-1} min^{-1})	source of receptor	[receptor] (nM)	k_A (μM^{-1} min^{-1})
A431 cells	10	12	B82 cells	16	26
	2.5	10		4	25
A431 cells	10	15	B82 cells	9.6	38
	2.5	15		2.4	26
A431 cells	7.5	13	B82 cells	20	20
	1.25	15		5	35
B82 cells	12	54	B82 cells	16	34
	3	60		4	40

[a] The values of the rate constant k_A were determined in experiments identical to those whose results are depicted in Figures 1 and 2. Equation 1 was fit to the data with a nonlinear least-squares program. The concentration of EGF receptor was determined from assays for the binding of EGF (*27*).

When eq 1 was fit to the kinetic data for the wild type (Figure 2) and the Δ991 deletion mutant of EGF receptor, it was found that the rate constants k_A for the diluted and undiluted samples of each were approximately the same. The values of the rate constant k_A for the two different concentrations of the Δ991 deletion mutant of EGF receptor (lines fit to the data in Figure 1) were 34 and 40 μM^{-1} min^{-1}. The values of the rate constants k_A for the two concentrations of wild-type receptor (lines fit to the data in Figure 2) were 15 and 15 μM^{-1} min^{-1}. Because the values of the rate constant k_A were nearly identical for different concentrations of either the wild type or the deletion mutant assayed in several different experiments (Table 1), the activation of the protein tyrosine kinases of both forms of EGF receptor proceeds according to eq 1 (*10*). These results demonstrate that EGF-induced activations of both the wild type and the Δ991 deletion mutant of EGF receptor are second-order with respect to receptor concentration. Therefore, dimerization is required for the activation of the Δ991 deletion mutant of EGF receptor, and the kinetic mechanism is the same as that of the wild-type enzyme.

The second-order rate constant k_A for the Δ991 deletion mutant of EGF receptor is 3-fold greater than that for the wild-type enzyme (Table 1). The turnover number for the deletion mutant (15 min^{-1}), however, is about 5-fold smaller than that for the holoenzyme (75 min^{-1}) when 0.375 mM reactant peptide was used. Under the same conditions, the Δ973 deletion mutant of EGF receptor has an even smaller turnover number (5 min^{-1}).

DISCUSSION

The carboxy-terminal portion of the amino acid sequence of EGF receptor that contains all of the sites for self-phosphorylation recruits other proteins to EGF receptor and regulates internalization, downregulation, and calcium responses (*22*, *30*, *31*). It has also been suggested that unphosphorylated sites of self-phosphorylation in this region are inhibitors of the protein tyrosine kinase and that this inhibition can be relieved when they become phosphorylated (*32*, *33*). According to this autoinhibition mechanism, deletion of this carboxy-terminal portion should produce a more active enzyme. The results reported here are inconsistent with this expectation because the Δ991 and Δ973 deletion mutants of EGF receptor, which are missing all of the sites for self-phosphorylation, are less active enzymes than the wild type under the conditions and with the concentrations of substrates used in the assay of enzymatic activity. The lower turnover numbers of these mutants also seem to disagree with the finding that the Δ991 and Δ973 deletion mutants of EGF receptor are more active in vivo than wild-type EGF receptor (*14*). Those earlier experiments, however, were examining the response of the whole signaling pathway to EGF and did not provide any kinetic evidence for a more active enzyme.

There are several reasons why the differences in turnover numbers observed in the experiments described here may be misleading. First, in the studies reported here we have evaluated intact and truncated receptor that were present in crude extracts prepared from different cell lines that are even from different species, human and mouse, respectively. It is possible that the modest differences in turnover number would disappear or even be reversed if these two proteins were compared in their pure state or in identical cellular backgrounds. Second, further carboxy-terminal truncation of EGF receptor to residue 944 produces an enzyme that is devoid of tyrosine kinase activity (*14*, *22*). This result and the fact that the Δ973 deletion mutant has a lower turnover number than that of the Δ991 deletion mutant suggest that the large truncations required to remove all of the carboxy-terminal sites of self-phosphorylation have also compromised the catalytic domain of EGF receptor somewhat and produced a less active enzyme. Third, only the peptide RRKGSTAENAEYLRV was used as a substrate. The differences observed between the mutant and the wild type might not have been the same if another peptide or a protein had been used as a substrate. Fourth, it is also possible that truncation could have increased dramatically the value of K_m for either the MgATP or the peptide and that the turnover number of the Δ991 deletion mutant at maximum velocity may be equal to or even greater than that of the wild-type enzyme. Because of all of these uncertainties, the differences in the turnover numbers observed in the studies described here neither support nor disprove the proposal for autoinhibition.

In the studies reported here, the initial velocity of the tyrosine kinase activity of EGF receptor was used to measure

the concentration of the active, dimeric form of EGF receptor and the Δ991 deletion mutant. Because previous studies (*32*, *33*) have presented evidence suggesting that self-phosphorylation of EGF receptor affects the interaction between the receptor and its peptide substrates, it is important to consider the possibility that changes in V_{max} or K_m produced by self-phosphorylation could compromise the relationship between the initial velocity and the concentration of active dimeric enzyme. This would only be a problem with the wild-type enzyme, because the Δ991 deletion mutant cannot be self-phosphorylated, and consequently, there should be only the unactivated, monomeric form and the activated, dimeric form of this mutant in solution during the course of the activation. As far as the wild-type form of the enzyme is concerned, it has already been demonstrated (*10*) that the use of the increase in enzymatic activity observed upon the addition of EGF does follow the concentration of activated, dimeric EGF receptor accurately and reliably. The fact that the kinetics of activation as monitored by the enzymatic activity do follow a rate law that is second-order with respect to the concentration of EGF receptor (Table 1) also suggests that this procedure is valid in the studies described here. Therefore, if changes in the K_m or V_{max} of the enzymatic activity do occur on self-phosphorylation, they do not appear to affect significantly the ability of the initial velocity of the enzymatic reaction to register the concentration of activated, dimeric enzyme.

The observations reported here can be integrated into a comprehensive synthesis of the results obtained from experiments performed in our laboratory (*10*, *27*, *28*, *34*−*36*) that clarify the steps in the mechanism of the activation of EGF receptor by EGF. Each of these experiments was performed on intact human EGF receptor or one of its fragments dissolved at concentrations between 1 and 250 nM in similar if not identical solutions of the detergent Triton X-100 at room temperature, but the various deletion mutants compared in these studies were expressed in different cell lines. The wild-type EGF receptor was in crude extracts of human epithelioid A431 carcinoma cells, and the Δ991 deletion mutant and the Δ647 deletion mutant were in crude extracts of murine epithelioid B82 carcinoma cells. It is possible that the magnitudes of the various differences we have observed between the two mutants and the wild-type enzyme would not have been so large if the wild-type enzyme had also been expressed in and extracted from B82 cells, but during all of the experiments, the three proteins were in free solution occupying their own individual micelles of Triton X-100.

Although the enzyme was not incorporated into a phospholipid bilayer as it is under physiological conditions, similar steps must take place in both the plasma membrane and the solution of detergent. In the plasma membrane, a molecule of EGF receptor diffuses in two dimensions, colliding with other molecules of EGF receptor and other proteins, each of which is aligned by the bilayer. In the solution of Triton X-100, each molecule of EGF receptor or one of its fragments finds itself most of the time within its own micelle, but that occupied micelle is diffusing in three dimensions and continuously and rapidly colliding, fusing, and unfusing with other micelles, some empty and some occupied with a molecule of EGF receptor or with another molecule of protein. The first step in the dimerization of EGF receptor is probably the fusion of the two micelles each containing a separate monomer of EGF receptor. Although the larger micelle that results from this fusion can exert some orientational alignment of the two molecules of protein, they are probably not aligned so precisely as they are by the planarity of the bilayer of the plasma membrane. Nevertheless, the differences between the collision of two molecules of EGF receptor in a plasma membrane and in a micelle of Triton X-100 are not fundamental; rather, they are differences in degree.

In the absence of EGF, EGF receptor is monomeric; each folded polypeptide occupies its own micelle (*10*). Even at concentrations of EGF receptor as high as 40 nM, there is no evidence for the presence of dimeric enzyme (*10*, *27*). Nor are there any observations suggesting that the background enzymatic activity in the absence of EGF varies with the concentration of EGF receptor as it would if the unliganded enzyme were capable of dimerizing.

When EGF is added at saturating concentrations to a solution of unliganded, monomeric EGF receptor, the enzyme rapidly begins to dimerize (*10*). At all concentrations of EGF receptor that have been examined directly for oligomeric distribution (down to 5 nM), the rate of the dimerization at saturating concentrations of EGF is second-order with respect to the concentration of EGF receptor, and the reaction proceeds to completion (*10*, *27*). The second-order rate constant for the dimerization of full-length wild-type EGF receptor at room temperature is $13 \pm 2\ \mu M^{-1}\ min^{-1}$ (Table 1); the second-order rate constant for the dimerization of the Δ991 deletion mutant of EGF receptor is $36 \pm 10\ \mu M^{-1}\ min^{-1}$ (Table 1), and the rate constant for the dimerization of the carboxy-terminal deletion mutant Δ647, which is truncated just beyond the membrane-spanning segment and is missing the entire cytoplasmic domain of the protein, is $>300\ \mu M^{-1}\ min^{-1}$ (*36*). These rate constants suggest that both the domain of self-phosphorylation and the catalytic domain of EGF receptor sterically interfere with its dimerization.

In contrast to these observations, the rate of dissociation to the monomer of dimeric EGF receptor from which the EGF has been removed is $0.3\ h^{-1}$ (*35*) while that of the dimeric deletion mutant Δ647 is $>10\ h^{-1}$ (*36*). The large difference between these two rate constants indicates that even though the two respective cytoplasmic domains slow the association of two liganded monomers of EGF receptor, once the dimeric complex is formed, one or more interfaces of significant stability have been established between the two cytoplasmic domains. When two full-length wild-type monomers of EGF receptor are brought together, however, by a bivalent immunoglobulin bound at their respective carboxy termini, this adventitious but enzymatically active complex dissociates with a rate constant of $10\ h^{-1}$ (*28*) upon removal of the immunoglobulin. This observation suggests that the interfaces, if any, that form between the cytoplasmic domains when two monomers are held together at their carboxy termini by an immunoglobulin are different from the interface or interfaces formed between them when EFG is bound even though the complex formed by the immunoglobulin does display enzymatic activity. Therefore, the binding of EGF to the extracytoplasmic domain of EGF receptor permits an interface or interfaces to form within one or more of the other domains that cannot form in the absence of EGF. This is consistent with the proposal (*36*) that the extracytoplasmic

domain sterically prevents strong associations between other domains of the enzyme from forming in the absence of EGF.

Three of our observations demonstrate that each monomer of EGF receptor in a dimeric complex associates with its own molecule of EGF (*37*) and that neither of the two molecules of EGF in the complex bridges the two monomers of EGF receptor. First, the binding of EGF to EGF receptor displays sigmoid behavior (*27*) with a Hill coefficient of 1.7 ± 0.5. Second, the activation of EGF receptor does not display inhibition at high concentrations of EGF (*27*). Third, when EGF is removed from a dimeric complex of full-length wild-type EGF receptor, the resulting unliganded, enzymatically active dimer dissociates slowly over a period of hours (*35*), an observation inconsistent with the obligatory enclosure of a molecule of EGF within that dimer.

The dimerization of EGF receptor is a necessary step in the activation of its tyrosine kinase. At concentrations of EGF receptor of <30 nM, the appearance of its enzymatic activity, which is also second-order with respect to the concentration of enzyme (Table 1), is coincident with its dimerization (*10*). The free concentration of EGF needed to produce half-maximum activation increases as the concentration of EGF receptor is decreased (*27*), as required by an enzymatic activation linked to a dimerization of the enzyme. The activation of the protein tyrosine kinase by an immunoglobulin directed against the carboxy terminus of EGF receptor displayed all of the properties expected if that activation had resulted only from the adventitious dimerization of the enzyme (*28*).

Following the activation of the protein tyrosine kinase of EGF receptor, the enzyme itself becomes phosphorylated on five tyrosines located beyond glutamate 991 in its amino acid sequence (*14*−*16*). This self-phosphorylation, however, is neither necessary (*14*) nor sufficient (*28*) for the activation of the protein tyrosine kinase. In fact, the Δ991 deletion mutant of EGF receptor that lacks all of the sites of self-phosphorylation displays little if any enzymatic activity in the absence of EGF and is activated upon addition of EGF (Figure 1). The rate of activation of the protein tyrosine kinase is second-order with respect to the concentration of protein (Table 1) as is that of the full-length wild-type enzyme (*10*). All of these results demonstrate the irrelevance of self-phosphorylation to the mechanism of activation of EGF receptor. Nor does it appear that this self-phosphorylation is an intramolecular transphosphorylation, as was once proposed, because the rate of self-phosphorylation decreases as the concentration of EGF receptor decreases, as expected for an intermolecular reaction in which a dimer of EGF receptor cannot phosphorylate itself but rapidly phosphorylates other dimers (*34*).

There remain several puzzling features of the activation of EGF receptor by EGF. Because, over the range of protein concentrations examined, no dimeric, active enzyme can be observed in the absence of EGF (*10*, *27*) and little or no monomeric enzyme persists following activation of the enzyme with saturating concentrations of EGF (*10*, *27*), the difference in the numerical values for the equilibrium constants for dissociation of unliganded dimeric EGF receptor and fully liganded dimeric EGF receptor must be greater than a factor of 100. This factor is probably far greater than 100 unless by chance the concentrations of EGF receptor used in these experiments fortuitously split the difference. If dimerization is linked to the binding of EGF, then the dissociation constants of EGF for dissociation from monomeric EGF receptor and from dimeric EGF receptor must differ by a great deal more than a factor of 10. Yet values for the dissociation constants and apparent dissociation constants for dissociation of EGF from intact EGF receptor and its various fragments over a range of protein concentrations usually fall between 20 and 500 nM (*8*, *27*, *37*−*41*). The most unexpected observation is that the dissociation constant for EGF from monomeric forms of EGF receptor appears to be less than 500 nM (*10*, *37*, *38*, *41*), which seems much too small (*27*).

Another peculiar feature of the dimerization and activation of EGF receptor is that dimeric EGF receptor formed upon addition of EGF only slowly (0.3 h^{-1}) loses its enzymatic activity and its dimeric state after the EGF is rapidly removed (*35*) but the equilibrium between the monomer and dimer of EGF receptor adjusts rapidly to changes in the levels of EGF (*27*). These apparently contradictory results suggest that rapid equilibration among the various liganded and unliganded forms of monomeric and dimeric EGF receptor requires EGF to be present. One possibility is that it is through the asymmetric dimer to which only one molecule of EGF is bound that the dimer and monomers rapidly equilibrate. There is some evidence suggesting that this singly occupied form of the dimeric protein has a significantly lower enzymatic activity than either the unoccupied dimer or the fully occupied dimer (*27*). The singly occupied dimer, because of its asymmetry, may be quite different from either of its symmetric siblings.

REFERENCES

1. Yarden, Y., and Ullrich, A. (1988) *Annu. Rev. Biochem. 57*, 443−478.
2. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. (1984) *Nature 309*, 418−425.
3. Cadena, D. L., and Gill, G. N. (1993) *Protein Expression Purif. 4*, 177−186.
4. Wedegaertner, P. B., and Gill, G. N. (1989) *J. Biol. Chem. 264*, 11346−11353.
5. Greenfield, C., Hiles, I., Waterfield, M. D., Federwisch, M., Wollmer, A., Blundell, T. L., and McDonald, N. (1989) *EMBO J. 8*, 4115−4123.
6. Hsu, C. Y., Mohammadi, M., Nathan, M., Honegger, A., Ullrich, A., Schlessinger, J., and Hurwitz, D. R. (1990) *Cell Growth Differ. 1*, 191−200.
7. Cohen, S., Fava, R. A., and Sawyer, S. T. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6237−6241.
8. Yarden, Y., and Schlessinger, J. (1987) *Biochemistry 26*, 1434−1442.
9. Yarden, Y., and Schlessinger, J. (1987) *Biochemistry 26*, 1443−1451.
10. Canals, F. (1992) *Biochemistry 31*, 4493−4501.
11. Carpenter, G., and Cohen, S. (1990) *J. Biol. Chem. 265*, 7709−7712.
12. Ullrich, A., and Schlessinger, J. (1990) *Cell 61*, 203−212.
13. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) *Cell 64*, 281−302.
14. Walton, G. M., Chen, W. S., Rosenfeld, M. G., and Gill, G. N. (1990) *J. Biol. Chem. 265*, 1750−1754.

15. Downward, J., Parker, P., and Waterfield, M. D. (1984) *Nature 311*, 483−485.
16. Margolis, B. L., Lax, I., Kris, R., Dombalagian, M., Honegger, A. M., Howk, R., Givol, D., Ullrich, A., and Schlessinger, J. (1989) *J. Biol. Chem. 264*, 10667−10671.
17. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991) *Science 252*, 668−674.
18. Coffer, P. J., and Kruijer, W. (1995) *Biochem. Biophys. Res. Commun. 210*, 74−81.
19. Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. R., Zilberstein, A., Ullrich, A., Pawson, T., and Schlessinger, J. (1990) *EMBO J. 9*, 4375−4380.
20. Li, N., Schlessinger, J., and Margolis, B. (1994) *Oncogene 9*, 3457−3465.
21. Wells, A., Welsh, J. B., Lazar, C. S., Wiley, H. S., Gill, G. N., and Rosenfeld, M. G. (1990) *Science 247*, 962−964.
22. Chen, W. S., Lazar, C. S., Lund, K. A., Welsh, J. B., Chang, C., Walton, G. M., Der, C. J., Wiley, H. S., Gill, G. N., and Rosenfeld, M. G. (1989) *Cell 59*, 33−43.
23. Hubbard, S. R., Wei, L., Ellis, L., and Hendrickson, W. A. (1994) *Nature 372*, 746−754.
24. Haigler, H., Ash, J. F., Singer, S. J., and Cohen, S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3317−3321.
25. Stewart, J. M., and Young, J. D. (1984) *Solid-Phase Peptide Synthesis*, 2nd ed., Pierce Chemical Co., Rockford, IL.
26. Thibault, D. (1993) *Biochemistry 32*, 2813−2821.
27. Sherrill, J. M., and Kyte, J. (1996) *Biochemistry 35*, 5705−5718.
28. Sherrill, J. M. (1997) *Biochemistry 36*, 5677−5684.
29. Lin, C. R., Chen, W. S., Lazar, C. S., Carpenter, C. D., Gill, G. N., Evans, R. M., and Rosenfeld, M. G. (1986) *Cell 44*, 839−848.
30. Chen, W. S., Lazar, C. S., Poenie, M., Tsien, R. Y., Gill, G. N., and Rosenfeld, M. G. (1987) *Nature 328*, 820−823.
31. Chang, C., Kao, J. P. Y., Lazar, C. S., Walsh, B. J., Wells, A., Wiley, H. S., Gill, G. N., and Rosenfeld, M. G. (1991) *J. Biol. Chem. 266*, 23467−23470.
32. Bertics, P. J., Weber, W., Cochet, C., and Gill, G. N. (1985) *J. Cell. Biochem. 29*, 195−208.
33. Bertics, P. J., Chen, W. S., Hubler, L., Lazar, C. S., Rosenfeld, M. G., and Gill, G. N. (1988) *J. Biol. Chem. 263*, 3610−3617.
34. Sherrill, J. M. (1997) *Biochemistry 36*, 12890−12896.
35. Tanner, K. G. (1997) *Biochemistry 36*, 14889−14896.
36. Tanner, K. G. (1997) Persistence of Dimerization and Enzymatic Activity of Epidermal Growth Factor Receptor in the Absence of Epideremal Growth Factor and Dimerization of the Extracellular Domain of Epidermal Growth Factor Receptor Containing the Membrane-Spanning Segment in Response to Treatment with Epidermal Growth Factor: A Model for the Dimerization of Epidermal Growth Factor Receptor, Ph.D. Dissertation, University of California at San Diego, La Jolla, CA.
37. Lemmon, M. A., Bu, Z., Ladbury, J. E., Zhou, M., Pinchasi, D., Lax, I., Engelman, D. M., and Schlessinger, J. (1997) *EMBO J. 16*, 281−294.
38. Günther, N., Betzel, C., and Weber, W. (1990) *J. Biol. Chem. 265*, 22082−22085.
39. Hurwitz, D. R., Emanuel, S. L., Nathan, M. H., Sarver, N., Ullrich, A., Felder, S., Lax, I., and Schlessinger, J. (1991) *J. Biol. Chem. 266*, 22035−22043.
40. Lax, I., Mitra, A. K., Ravera, C., Hurwitz, D. R., Rubinstein, M., Ullrich, A., Stroud, R. M., and Schlessinger, J. (1991) *J. Biol. Chem. 266*, 13828−13833.
41. Zhou, M., Felder, S., Rubinstein, M., Hurwitz, D. R., Ullrich, A., Lax, I., and Schlessinger, J. (1993) *Biochemistry 32*, 8193−8198.

BI982276D