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# Signal Transmission by Epidermal Growth Factor Receptor: Coincidence of Activation and Dimerization†

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*Received August 7, 1991; Revised Manuscript Received December 30, 1991*

ABSTRACT: Dimerization of epidermal growth factor receptor dissolved in a solution of nonionic detergent was followed with a resolution of 1 min by quantitative cross-linking with glutaraldehyde. Upon addition of epidermal growth factor to the solution, the initially monomeric protein dimerized in a reaction that was second-order in the concentration of receptor. A second-order rate constant, on the basis of enzymatic activity as a measure of the concentration of functional receptor, was calculated from time courses of dimerization at various initial concentrations of receptor. The activation of the protein tyrosine kinase of the receptor was monitored directly under the same conditions with an exogenous substrate. The increase in tyrosine kinase activity displayed kinetics that were also second-order in the concentration of receptor. A second-order rate constant for the activation of the tyrosine kinase could be calculated from the time courses. The second-order rate constant for the activation of the tyrosine kinase by epidermal growth factor was indistinguishable from the second-order rate constant for the dimerization induced by epidermal growth factor. Therefore, dimerization of epidermal growth factor receptor and activation of its tyrosine kinase are coincident events, both initiated by the binding of epidermal growth factor.

Epidermal growth factor receptor (EGFR)[1] is a transmembrane glycoprotein composed from a folded polypeptide 1190 amino acids in length (Ullrich et al., 1984). It is a member of a group of structurally and functionally related cell surface receptors characterized by possessing an intrinsic activity for protein tyrosine kinase (Yarden & Ullrich, 1988; Gill et al., 1987). It comprises an extracellular domain where the binding site for epidermal growth factor (EGF)[1] resides and a cytoplasmic domain where the active site for the tyrosine kinase is located. On the basis of the amino acid sequence of the protein, it has been proposed that a single membrane-

† This research was supported by Grant GM33962 from the National Institutes of Health that provides funds for the laboratory of Jack Kyte and by a postdoctoral fellowship from the Spanish Ministerio de Educacion y Ciencia.

[1] Abbreviations: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PVDF, poly(vinylidene difluoride); SDS-PAGE, electrophoresis on a polyacrylamide gel cast in a solution of sodium dodecyl sulfate.

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spanning segment links these two domains (Ullrich et al., 1984).

Binding of EGF to the receptor results in the transmission of a mitogenic signal through a cascade of intracellular responses that is not yet fully understood (Carpenter & Cohen, 1990). The transmission of this signal by EGFR is not only involved in the regulation of normal cellular growth but also different instances of abnormal signaling have been correlated with malignant growth (Yarden & Ullrich, 1988; Gill et al., 1987; Todaro et al., 1980; Yamamoto et al., 1983; Libermann et al., 1984; Xu et al., 1984). It is well established that the initial response caused by the binding of EGF to EGFR is the activation of its tyrosine kinase, leading to the phosphorylation of the receptor itself and several intracellular substrates (Hunter & Cooper, 1985; Glenny et al., 1988). It has been shown that the expression of this tyrosine kinase is required for all cellular responses to EGF, and therefore is essential for transmission of the signal (Prywes et al., 1986; Livneh et al., 1986; Chen et al., 1987; Honegger et al., 1987a,b; Moolenar et al., 1988). The mechanism by which the binding of EGF to the extracellular domain of EGFR produces the activation of the tyrosine kinase in its cytoplasmatic domain has been the subject of numerous studies (Northwood & Davis, 1988; Koland & Cerione, 1988; Biswas et al., 1985; Basu et al., 1986, 1989; Yarden & Schlessinger, 1987a,b; Cochet et al., 1988; Fanger et al., 1989; Kashles et al., 1988; Carpenter et al., 1991). Two different models are still in contention. According to the intramolecular model of activation (Koland & Cerione, 1988), a single folded polypeptide of EGFR would be sufficient to transmit the signal across the membrane. Binding of EGF to the extracellular domain would induce a conformational change that would be propagated through the membrane-spanning segment to produce the active conformation of the kinase. The alternative, intermolecular model of activation (Yarden & Schlessinger, 1987a; Schlessinger, 1988) proposes that binding of EGF would shift an equilibrium between inactive monomers of EGFR and active dimers of EGFR toward the dimers. The activation of the kinase would be the result of intersubunit interactions within the dimer.

The results reported here, describing the kinetics of the dimerization and the activation of the tyrosine kinase of EGFR, demonstrate that activation is a process second-order in monomers of EGFR and that activation is coincident with the dimerization of EGFR.

## Experimental Procedures

*Materials.* Cells of the A-431 human epithelioid carcinoma line (Haigler et al., 1978) were kindly provided by Dr. Gordon Gill, Department of Medicine, University of California at San Diego. *N*-(2-Hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid (HEPES),[1] Triton X-100, benzamidine hydrochloride, leucine aminopeptidase, aprotinin from bovine lung, bovine serum albumin, nitro blue tetrazolium, $Na_2ATP$, the *p*-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate, and Sephadex G-50 were purchased from Sigma; leupeptin, 9-fluorenylmethoxycarbonyl-blocked, protected amino acids and angiotensin II were purchased from Bachem; poly(vinylidene difluoride) (PVDF)[1] membranes were purchased from Millipore; a 25% solution of glutaraldehyde was purchased from ICN Pharmaceuticals; blotting-grade conjugate between goat antirabbit immunoglobulin G and alkaline phosphatase was purchased from Bio-Rad; carboxypeptidase Y was purchased from Worthington; the triethylammonium salt of adenosine 5'-[$\gamma$-$^{32}$P]triphosphate (3000 Ci mmol$^{-1}$) was purchased from Amersham; and phosphocellulose paper was purchased from Whatman. Epidermal growth factor, grade I from murine submaxillary glands, was purchased from Boehringer-Mannheim. Sodium dodecyl sulfate was recrystallized from 95% ethanol (Burgess, 1969).

The hexapeptide SEFIGA was synthesized by the 9-fluorenylmethoxycarbonyl method (Atherton & Sheppard, 1989). The peptide was purified by high-pressure liquid chromatography and identified by amino acid analysis. Its identity was established by quantitative amino acid analysis, digestion with leucine aminopeptidase, and digestion with carboxypeptidase Y.

*Detergent Extracts.* Cells of the A-431 human epidermal carcinoma line (0.1 mL of packed cells mL$^{-1}$) were lysed and homogenized at 0 °C in a buffer containing 30 mM HEPES, pH 7.4, 10% glycerol, 1% Triton X-100, 1 mM ethylenediaminetetraacetate, 5 mM ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetate, 5 mM 2-mercaptoethanol, 2 mM benzamidine, 2.5 $\mu$g mL$^{-1}$ aprotinin, and 5 $\mu$g mL$^{-1}$ leupeptin. These suspensions were clarified by centrifugation at 12000*g* for 10 min. The clarified supernatant is referred to as a detergent extract.

*Assay for Dimerization by Quantitative Cross-Linking.* Samples of a detergent extract were mixed at room temperature with a noted final concentration of EGF in a final volume of 45 $\mu$L. After a noted time, cross-linking was initiated by addition of 15 $\mu$L of a 4-fold-concentrated glutaraldehyde solution. After 1 min, the cross-linking was stopped by addition of 5 $\mu$L of 2 M glycine, pH 9, and the sample was immediately spun through a column (1 mL) of Sephadex G-50 (Penefsky, 1977). Samples were submitted to electrophoresis on gels of 5% polyacrylamide cast in solutions of 0.1% sodium dodecyl sulfate (SDS–PAGE),[1] electrotransferred to PVDF membranes, and immunostained by a standard procedure (Blake et al., 1984) using as the primary antibody anti-SEFIGA (an anti-peptide antibody against a peptide with the carboxy-terminal sequence of the human EGFR). These specific antibodies were produced using a glutaraldehyde conjugate of the synthetic peptide SEFIGA and bovine serum albumin to immunize White New Zealand rabbits (Walter et al., 1980), and the immunoglobulins were affinity-purified on a column of the peptide coupled to agarose (Kyte et al., 1987).

*Quantification of the Relative Amounts of Monomer and Dimer of EGFR from Immunoblots.* The fraction by mass of the dimer of EGFR in each sample was quantified by scanning the respective lane on an immunoblot with an LKB densitometer. Areas of absorbance of the peaks of stain corresponding to the monomer and covalent dimer of EGFR were calculated from the average of multiple scans of each lane. Standard curves of absorbance as a function of mass for the bands of monomer and covalent dimer, respectively, were prepared by scanning an immunoblot in which successive lanes had precise dilutions of the same sample of cross-linked EGFR. It was found that, because either of the procedure of immunoblotting or of limitations of the densitometer, the curves of measured area against relative mass of EGFR for both monomer and dimer showed deviation from linearity over the range of areas encountered in the cross-linking experiments themselves. Measured areas for the experiments were corrected for this deviation from linearity using these standard curves so that the corrected values were directly proportional to relative mass. Corrected areas will be referred to as $a_M$ and $a_D$, for the peaks of monomer and dimer, respectively.

It was also found that, during dimerization, the increase in absorbance of the dimer on the immunoblots was greater than the decrease in absorbance of the monomer. This indicated

that there was a difference in the extinction coefficients for monomer and dimer which may have resulted from differences in the efficiency of either the electrophoretic transfer or the staining procedure. For example, the covalent dimer, because it is a larger protein, might adsorb to the PVDF membranes more tightly or the covalent dimer might bind the immunoglobulins with a higher affinity because it is bivalent rather than monovalent. Correction for this difference was made in the following way. Extinction coefficients for monomer ($\epsilon_M$) and dimer ($\epsilon_D$) can be defined by

$$a_M = \epsilon_M[M] \quad (1)$$

$$a_D = \epsilon_D[D] \quad (2)$$

where [M] and [D] are the molar concentrations of monomer and dimer of EGFR, respectively, in the original sample loaded onto the polyacrylamide gel. These equations follow from the fact that the corrected areas, $a_M$ and $a_D$, are directly proportional to the corresponding concentrations. Since, in the cross-linking experiments, the amounts of protein and the volumes loaded in each lane of the polyacrylamide gel were identical, it follows that, for each set of samples, by conservation of mass:

$$[M] + 2[D] = [R]_{tot} \quad (3)$$

where $[R]_{tot}$, the total concentration of EGFR, is a constant over the series of lanes. From eq 1–3

$$a_D = \frac{[R]_{tot}\epsilon_D}{2} - a_M\frac{\epsilon_D}{2\epsilon_M} \quad (4)$$

The ratio between the extinction coefficients $\epsilon_D(2\epsilon_M)^{-1}$ was determined from the slope of plots of $a_D$ as a function of $a_M$ from the measurements from all of the lanes on a particular blot. Using this value, the fraction by mass of dimeric EGFR in each sample was

$$f_{dimer} \equiv \frac{2[D]}{[M] + 2[D]} = \frac{a_D}{a_M[\epsilon_D(2\epsilon_M)^{-1}] + a_D} \quad (5)$$

Observed values for $\epsilon_D(2\epsilon_M)^{-1}$ varied for different immunoblots between 1 and 4 depending on the conditions of electrophoretic transfer and staining. For blots corresponding to control samples (no EGF added), where a direct determination of this ratio was not possible, the value obtained for a second blot, which contained samples to which EGF had been added and which had been electrotransferred and stained simultaneously, was used.

*Tyrosine Kinase Activity.* The activity of the protein tyrosine kinase of EGFR was assayed as previously reported (Weber et al., 1984). Briefly, after samples of detergent extracts of EGFR (~1 pmol in 30 μL) had been mixed with EGF (final concentration 1 μM) for the noted times at room temperature, in a final volume of 45 μL, the assay for enzymatic activity was initiated by addition, to the noted final concentrations, of a mixture of angiotensin II to 1.25 mM, $MgCl_2$ to 5 mM, $MnCl_2$ to 2 mM, $Na_3VO_4$ to 0.1 mM, ATP to 10 μM, and [γ-$^{32}$P]ATP to give a final specific radioactivity of about 2 Ci (mmol of ATP)$^{-1}$ in a final volume of 60 μL. The reaction was stopped after 1 min by addition of trichloroacetic acid to a final concentration of 5%. After addition of bovine serum albumin to 0.5 mg mL$^{-1}$, the mixtures were kept on ice for 30 min and then centrifuged. Samples of the supernatants were spotted on disks of phosphocellulose paper, which were then washed with 75 mM $H_3PO_4$, dried, and submitted to scintillation counting. The enzymatic activity, expressed as nanomoles of phosphate incorporated into the tyrosine of angiotensin II per minute per liter, was calculated from the cpm of [$^{32}$P]ATP per microliter in each of the initial mixtures of ATP and EGFR and the molar concentration of ATP in the same mixture.

## Results

*Quantitative Cross-Linking of Dimers of EGFR.* Quantitative cross-linking with glutaraldehyde (Hermann et al., 1981, 1983, 1985; Craig, 1982a,b; Burns & Schachman, 1982) was used as an assay to quantify the type and relative amounts of the oligomers of EGFR dissolved in solutions of detergent. These solutions were made by suspending intact A-431 cells in 1% Triton X-100 and submitting the resulting suspensions to centrifugation at 12000*g* for 10 min. These clarified solutions of A-431 cells are referred to as detergent extracts. If it is assumed (Weber et al., 1984) that there are $4 \times 10^6$ molecules of EGFR per cell, the concentration of EGFR in these final detergent extracts was on the order of 30 nM. This value is in agreement with the receptor concentration that can be estimated from the intensities of bands of stain on the immunoblots used to analyze the samples.[2] Samples from detergent extracts of A-431 cells were allowed to stand at room temperature with or without EGF for different times, after which the cross-linking reaction was initiated by addition of a glutaraldehyde solution and then quenched with excess glycine. The samples were submitted to electrophoresis on polyacrylamide gels cast in solutions of sodium dodecyl sulfate (SDS–PAGE) and electroblotted onto PVDF membranes. These blots were then immunostained for EGFR using an affinity-purified anti-peptide antibody (Walter et al., 1980) raised against a peptide with the carboxy-terminal sequence of human EGFR (-SEFIGA). The relative amounts by mass of the monomer and covalent dimer of EGFR were then quantified by densitometry of the stained blots.

To determine the conditions necessary to achieve complete cross-linking of all the noncovalent dimer of EGFR present in a sample, with a time interval for the cross-linking reaction short enough to allow for studies of the kinetics of dimerization to be done (Hermann et al., 1981), the effect of the concentration of glutaraldehyde on the yield of the cross-linking reaction was studied (Figure 1). Samples of a detergent extract of A-431 cells were mixed with a saturating concentration of EGF for 10 min and then subjected to cross-linking with increasing concentrations of glutaraldehyde for 1 min. The results show that, at glutaraldehyde concentrations above about 80 mM, there is no significant increase in the fraction by mass of covalent dimer, and demonstrate that under these conditions quantitative cross-linking is achieved. From these results, it was concluded that cross-linking with 80 mM glutaraldehyde for 1 min under these conditions permits the accurate determination of the relative amounts of monomer and noncovalent dimer in a solution of EGFR immediately prior to the addition of the glutaraldehyde. Samples cross-linked in this way, even after exposure to EGF for up to 2 h, showed only two main bands, corresponding to the monomer and covalent dimer of EGFR.

*Kinetics of EGFR Dimerization.* Since the dimerization of EGFR induced by EGF must be preceded by the binding of EGF to the protein, experiments were carried out to determine the concentration of EGF necessary to attain immediate saturation of the sites. Samples of a detergent extract of A-431 cells were mixed for 1 min with increasing concentrations of EGF, and the amount of dimer formed over this short period was measured by cross-linking with glutaraldehyde

[2] G. Walton, personal communication.

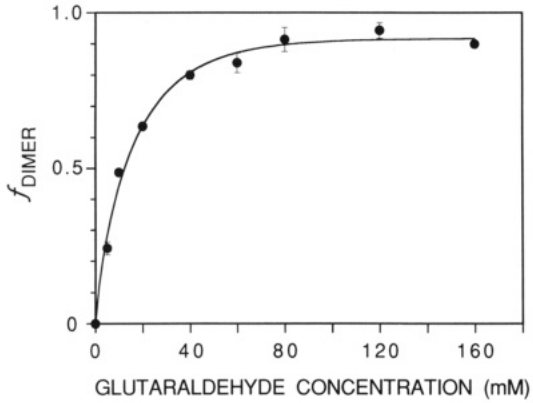


FIGURE 1: Effect of the concentration of glutaraldehyde on the cross-linking of the noncovalent dimer of EGFR. Equal samples (~1 pmol of EGFR) of a detergent extract of A-431 cells were mixed in a final volume of 45 $\mu$L with a final concentration of 1 $\mu$M EGF for 10 min. They were cross-linked for 1 min with glutaraldehyde at several final concentrations and then immediately quenched with glycine. The samples were subjected to SDS–PAGE, the separated proteins were transferred to PVDF membranes, and the blots were immunostained using an EGFR-specific anti-peptide antibody. The fraction by mass of the EGFR cross-linked to dimer, $f_{dimer}$, was quantified for each sample by densitometry. Error bars represent the spread of duplicate determinations. Similar results were obtained in three independent experiments.

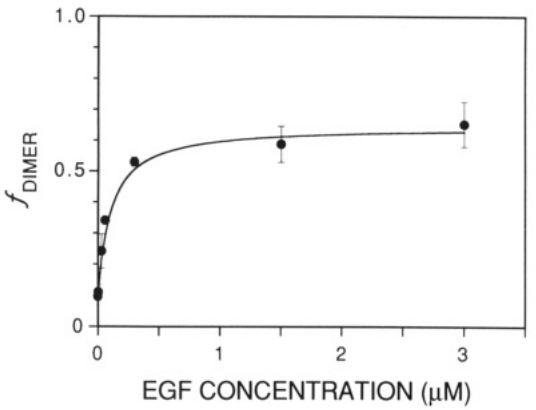


FIGURE 2: Influence of the concentration of EGF on the dimerization of EGFR at short time. Samples (~1 pmol of EGFR) of a detergent extract of A-431 cells were mixed for 1 min with varying final concentrations of EGF in duplicate in a final volume of 45 $\mu$L and then cross-linked with glutaraldehyde at a final concentration of 80 mM for 1 min. The fraction by mass of dimer, $f_{dimer}$, was determined as in Figure 1.

(Figure 2). No significant increase in the amount of dimer formed was observed at concentrations of EGF above about 1 $\mu$M. Similar behavior was observed when the same experiment was run with an exposure time to EGF of 0.5 min. In this case, the plateau value (encountered above 1 $\mu$M EGF) for the fraction of dimer was 0.45. These results are consistent with a kinetic mechanism involving a rapid equilibration of the binding of ligand within the first minute at all concentrations of EGF, so that the amount of dimer formed is proportional to the concentration of occupied receptor at equilibrium. The solid line (Figure 2) was fit to the data according to this model, by making the fraction of dimer observed at 1 min proportional to the concentration of EGFR occupied by EGF, as calculated using the reported value (100 nM) for the dissociation constant of EGF from EGFR dissolved in a solution of detergent (Yarden & Schlessinger, 1987a).

The time course of the dimerization of EGFR induced by EGF was followed by allowing samples of a detergent extract of A-431 cells to sit with or without added EGF for increasing

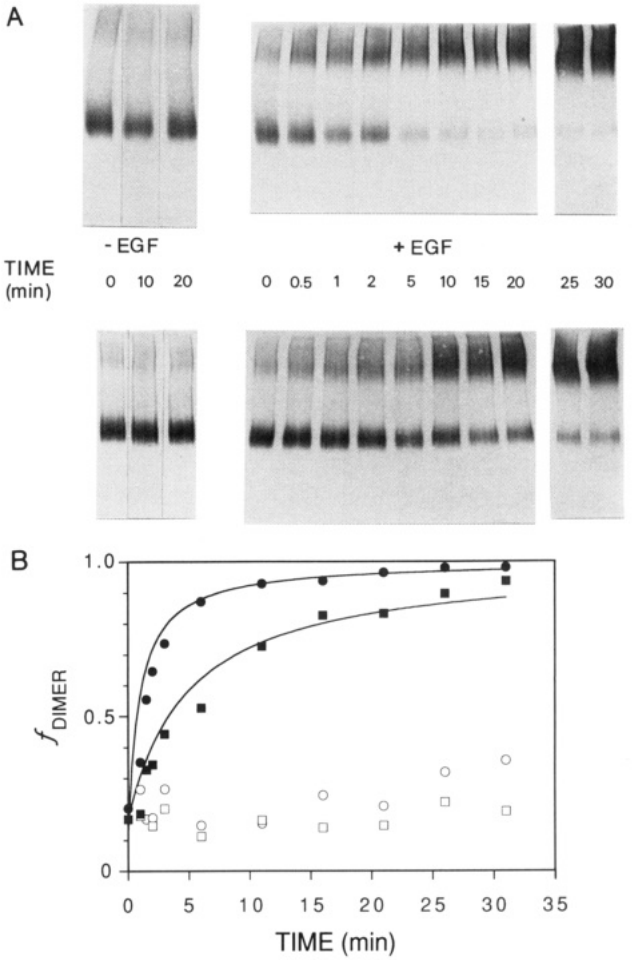


FIGURE 3: Kinetics of the dimerization of EGFR induced by EGF as a function of the concentration of EGFR. (A) Western blots of samples from 2 different detergent extracts of A-431 cells mixed with buffer alone (left 3 lanes) or EGF at the final concentration of 1 $\mu$M (right 10 lanes) for the times indicated (in minutes) and then cross-linked for 1 min with 80 mM glutaraldehyde. The bottom row of blots displays an experiment performed on a detergent extract with a 6-fold lower final concentration of EGFR than that used in the experiment shown in the top row of blots. This difference in concentration was established by densitometry of an immunoblot of samples prepared by serial dilutions of each of the two extracts used. For the experiment on the more dilute solutions, the samples were concentrated by lyophilization after being cross-linked, passed through Sephadex G-50, and dissolved with dodecyl sulfate. (B) Plot of the fraction by mass of dimer of EGFR as a function of the time of exposure to EGF plus the time (1 min) of the cross-linking reaction. Circles represent data from the experiment shown on the top row of (A). Squares represent data from the experiment shown in the bottom row of (A). Filled symbols are for samples incubated with EGF. Open symbols are for control samples without EGF. Solid lines were calculated using the equation for a second-order reaction (eq 7), taking as $f^{\circ}_{dimer}$ the average of the fraction of dimer in the corresponding samples from which EGF had been omitted, as $f^{\infty}_{dimer}$ unity, and as the rate constants those derived from linearizing the data by using eq 6.

time intervals and then submitting them to cross-linking by glutaraldehyde (Figure 3). Exposure to EGF was at a final concentration of 1 $\mu$M to ensure that binding of the EGF to EGFR was at saturation even at the shortest times (Figure 2) and the concentration of glutaraldehyde used (80 mM) was sufficient to produce quantitative intramolecular cross-linking (Figure 1). In these experiments (Figure 3), all of the samples in a given series had the same concentration of EGFR and were cross-linked with the same concentration of glutaraldehyde. Before EGF was added, only about 20% of the EGFR was cross-linked to covalent dimer; at long times and

high concentrations of protein, all of the EGFR was cross-linked to covalent dimer. Therefore, little, if any, intermolecular cross-linking of free monomers must have occurred, all dimers present in the solution immediately prior to adding the glutaraldehyde must have been cross-linked intramolecularly, and the cross-linking by glutaraldehyde must have monitored accurately the actual amounts of free monomer and noncovalent dimer in the solution as the dimerization progressed.

The observed time course for the dimerization of EGFR induced by EGF can be interpreted according to the kinetic mechanism shown in Scheme I where EGFR represents unoccupied, monomeric EGFR; M, monomeric EGFR occupied with EGF; D, dimeric EGFR; $K_{EGF}$, the dissociation constant for EGF from the monomer; and $k_{bi}$, the bimolecular rate constant for dimerization of receptor. Under the conditions where the time course of dimerization was determined, the step in which EGF binds is rapid and complete (Figure 2). Therefore, the observed time course can be interpreted as the result of a rate-limiting bimolecular dimerization. This process can be treated as an irreversible transformation rather than an approach to an equilibrium between the monomeric and dimeric forms because, in the range of receptor concentrations used, the dimerization proceeds to completion. Binding of EGF to the dimeric form is also irrelevant to the kinetics since glutaraldehyde will cross-link occupied or unoccupied dimers with the same efficiency.

Scheme I

$$2\text{EGFR} + 2\text{EGF} \overset{K_{EGF}}{\rightleftharpoons} 2\text{M} \xrightarrow{k_{bi}} \text{D}$$

The integrated rate equation for a second-order process is

$$1/[\text{M}] - 1/[\text{M}]_0 = k_{bi}t \qquad (6)$$

where [M] is the molar concentration of occupied monomer at time $t$ and $[\text{M}]_0$ is the molar concentration of occupied monomer in the solution immediately after the binding of EGF reaches equilibrium, which is well before significant dimerization has commenced. From eq 6 and the equation for the conservation of mass, the relationship for the measured fraction by mass of EGFR in the dimeric form, $f_{dimer}$ (eq 5), at any time, $t$, is

$$f_{dimer} = 1 - \frac{1 - f^{\circ}_{dimer}}{1 + (1 - f^{\circ}_{dimer})k_D^{app}t} \qquad (7)$$

where $k_D^{app} = k_{bi}([\text{M}]_0 + 2[\text{D}]_0)$, $f^{\circ}_{dimer}$ is the initial fraction of dimer (taken to be the fraction of dimer in the absence of EGF), and $[\text{D}]_0$ is the molar concentration of dimeric EGFR initially present in the solution. The observed data are in good agreement with this model (curves fit to data in Figure 3).

It is also possible to fit the data as satisfactorily with a curve defined by the integrated rate equation for a first-order process. The distinction between a first-order and a second-order process, however, is made by varying the initial concentration of reactant, in this case monomeric EGFR. Kinetic experiments were carried out with detergent extracts of A-431 cells at various concentrations of EGFR, achieved by dilution, to determine the effect of concentration on the time course of dimerization (two examples in Figure 3). In the two experiments presented in Figure 3, the total concentrations of EGFR ($[\text{M}]_0 + 2[\text{D}]_0$) differed by a factor of 6 between the two runs. This was established by densitometry of an immunoblot of samples prepared by serial dilutions of each of the two extracts used. The apparent rate constants, $k_D^{app}$, for the two curves differed by a factor of 5. This is the difference, within experimental error, expected from eq 7, the equation for a process second-order in concentration of EGFR. If a rate-limiting, first-order relaxation had governed the dimerization, the two apparent rate constants would have been identical.

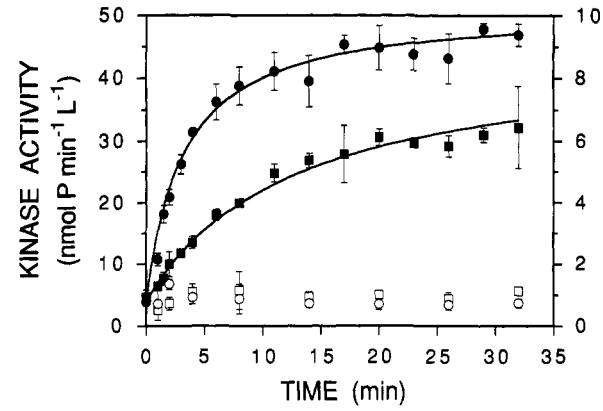


FIGURE 4: Kinetics of the activation of the tyrosine kinase of EGFR induced by EGF. The activity of tyrosine kinase in detergent extracts of A-431 cells was measured after incubation with 1 $\mu$M EGF (filled symbols) or buffer alone (open symbols) for different times as shown. The activity of the tyrosine kinase (KA) is expressed as nanomoles of phosphate incorporated into the tyrosine of angiotensin II per minute per liter. Data plotted correspond to two experiments with samples of an undiluted detergent extract (~15 nM EGFR; circles, left scale) and samples of the same extract diluted to a final concentration of one-fifth the former (squares, right scale). Solid lines are calculated according to eq 9 for a reaction second-order in monomers of EGFR taking as the value for $KA_0$ the average of the values of KA for the control samples (open symbols) to which EGF was not added. The line through the filled circles is the best fit of the data to eq 9 giving $KA_\infty$ = 51 nmol min$^{-1}$ L$^{-1}$. The line through the filled squares is the best fit of the data to eq 9 using a $KA_\infty$ = 9.3 nmol min$^{-1}$ L$^{-1}$ which is the average of the values obtained from the best fits to all of the data points ($KA_\infty$ = 8.3 nmol min$^{-1}$ L$^{-1}$) and to only the data up to 20 min ($KA_\infty$ = 10.3 nmol min$^{-1}$ L$^{-1}$). The data points shown are the average of quadruplicate determinations, and error bars correspond to standard deviations.

*Kinetics of Activation of Tyrosine Kinase of EGFR.* A study of the kinetics of the activation of the tyrosine kinase of EGFR under the same conditions as those in which the kinetics of the dimerization induced by EGF were determined should reveal whether the activation of the receptor precedes or follows its dimerization. This would provide a way to discriminate between the two proposed models for receptor activation (Koland & Cerione, 1988; Yarden & Schlessinger, 1987a). The time course for the activation of EGFR in detergent extracts of A-431 cells was studied by incubating samples with saturating EGF (1 $\mu$M final concentration) for different time intervals and then measuring their activity for tyrosine kinase (Figure 4). The enzymatic activity (Wong & Goldberg, 1983) was measured as the incorporation of radiolabeled phosphate into angiotensin II (Weber et al., 1984) over an interval of 1 min, chosen to parallel the interval used for the cross-linking by glutaraldehyde. The volumes of reagent added were also equivalent to those added in the cross-linking assay, to parallel any effects of dilution. Control samples, incubated without EGF, showed a steady level of tyrosine kinase activity about 10% of that induced by EGF which could have been due to active EGFR present initially in the extract, to other active tyrosine kinases, or to a combination of both of these possibilities.

The time course of activation was followed with two solutions that had different concentrations of EGFR to assess the dependence of the rate of activation on the concentration of EGFR (Figure 4). It is obvious that the rate for the activation of the tyrosine kinase is much slower when the concentration

of EGFR is lower. This suggests, as has been proposed (Yarden & Schlessinger, 1987a), that the dimerization of EGFR is involved in its activation.

The time courses for activation of the tyrosine kinase were determined at similar concentrations of EGFR and under identical conditions of temperature, pH, and ionic strength, as those for the time courses for dimerization, and often with the same extract. It was apparent from all of these experiments that, as the dimerization progressed (Figure 3), the tyrosine kinase activity increased (Figure 4) until all of the enzyme was a dimer, at which point the increase in tyrosine kinase activity ceased. These observations can only be explained if the specific activity of the tyrosine kinase is greater for the dimer than for the monomer of EGFR. In the development that follows, it is assumed, for mathematical simplicity, that the specific enzymatic activity of the monomer is negligible relative to that of the dimer. This is not a necessary assumption, however, to reach the conclusion that the actual time courses observed for the activation of the tyrosine kinase activity are consistent with the kinetic mechanism in Scheme I if, and only if, the increase in tyrosine kinase activity between monomer and dimer coincides with the dimerization.

In experiments following the dimerization of EGFR, some dimer was initially present in samples that were not mixed with EGF (Figure 3). Its percentage varied from extract to extract, but remained constant in the absence of EGF (Figure 3B). The amount of this preexisting dimer did not correlate with the EGF-independent activity of the tyrosine kinase in the same extracts, and it can be assumed that most, if not all, of this preexisting dimer is not enzymatically active. In any case, even if some of this preexisting dimer is enzymatically active, it does not participate in the dimerization, and its enzymatic activity is subtracted to obtain the measurement of tyrosine kinase induced by EGF. On the basis of this consideration, and the assumption that only dimeric EGFR shows significant activity, it follows that

$$KA - KA_0 = \alpha([D] - [D]_0) \quad (8)$$

where KA is the activity of tyrosine kinase at any time ($t$), $KA_0$ is the initial activity of tyrosine kinase, and $\alpha$ is a constant of proportionality converting the molar concentration of dimeric EGFR to enzymatic activity.

It will now be shown that the activation of the tyrosine kinase of EGFR is a second-order reaction in the concentration of EGFR. From eq 8, eq 6, the observation that at infinite time all of the EGFR is dimeric, and the conservation of mass, it follows, by the same development leading to eq 7, that

$$KA = KA_\infty - \frac{KA_\infty - KA_0}{1 + (KA_\infty - KA_0)k_A t} \quad (9)$$

where $k_A$ is equal to $2\alpha^{-1}k_{bi}$ and $KA_\infty$ is the activity of the tyrosine kinase at infinite time following the addition of EGF. The direct experimental determination of the values of $KA_\infty$ was not possible, because after long times of incubation with EGF the activity of tyrosine kinase began to decrease slowly. Values derived from fitting eq 9 to the data can be considered accurate for experiments done in concentrated extracts of EGFR (Figure 4, closed circles), where the reaction achieves more than 90% completion over a 30-min period. In experiments at lower concentrations of EGFR (Figure 4, squares), however, the inaccuracy in the extrapolated value of $KA_\infty$ introduces a significant source of error in the determination of the rate constant. Nevertheless, the values of $k_A$ for the two different concentrations of EGFR, obtained from the fits displayed in Figure 4, were $7 \times 10^{-3}$ and $9 \times 10^{-3}$ (nmol of P $min^{-1}$ $L^{-1})^{-1}$ $min^{-1}$. If eq 9 governs the process, these two rate constants should be identical, as they are, within experimental error.

From values of $KA_\infty - KA_0$ and the concentration of EGFR in the extracts used, estimated from staining on immunoblots,[2] the turnover number for the tyrosine kinase of the EGFR used in this experiment can be estimated to be 6 mol of phosphate (mol of EGFR)$^{-1}$ $min^{-1}$, in agreement with reported values for the phosphorylation of angiotensin II by the enzyme (Weber et al., 1984).

*Correlation of Dimerization and Activation of the Tyrosine Kinase of EGFR.* Before the time course of dimerization could be compared to that for activation of the tyrosine kinase, the fraction by mass of EGFR present as the dimer had to be corrected for the concentration of preexisting dimer. Because different detergent extracts showed different relative amounts of the dimer of EGFR present in the absence of EGF, the data were converted to represent the initial concentrations of monomeric EGFR actually responsive to EGF. Thus, the measured values of $f_{dimer}$ were converted to corrected values:

$$f_{dimer,corr} \equiv \frac{f_{dimer} - f^\circ_{dimer}}{1 - f^\circ_{dimer}} = \frac{[D] - [D]_0}{[D]_\infty - [D]_0} \quad (10)$$

which represents the molar concentration of dimer formed after time $t$ relative to the initial molar concentration of receptor present in monomeric form $[M]_0$.

In order to compare the observed second-order rate constants for dimerization induced by EGF with the observed second-order rate constants for the activation of the tyrosine kinase induced by EGF in extracts with different concentrations of EGFR, it was necessary to express the absolute concentrations of monomeric EGFR responsive to EGF in each particular extract in units that could be applied to the samples used in both of these types of experiments. I decided to use as units for the concentration of monomeric EGFR the total tyrosine kinase activity induced by EGF, $KA_\infty - KA_0$. This quantity is an unambiguous measurement, the value of which should be directly proportional to the molar concentration of EGFR responsive to EGF in a particular extract. The proportionality between $KA_\infty - KA_0$ and the molar concentration of competent EGFR was assessed in several experiments similar to the one described in Figure 4. Undiluted extracts and the same extracts diluted 5-fold showed values of final kinase activities induced by EGF that differed by factors of 5, within experimental error. On the basis of the fact that the monomer responds to EGF by dimerizing and the fact that the dimer has greater specific enzymatic activity than the monomer, I have assumed that it is the monomer of EGFR that is the form of the protein responsive to EGF. Furthermore, it can be concluded that all monomers in the solution are equally competent because all monomers dimerize in a homogeneous second-order process (Figure 3). All of these observations lead to the conclusion that the molar concentration of competent monomers of EGFR in any detergent extract is directly proportional to $KA_\infty - KA_0$ for that extract.

On the basis of the assumption that the dimer of EGFR is the only significantly active species, the observation that the monomer was absent at infinite time, the conservation of mass, and eq 8, the proportionality between $KA_\infty - KA_0$ and the initial concentration of monomer, $[M]_0$, can be written as

$$[M]_0 = 2([D]_\infty - [D]_0) = 2\alpha^{-1}(KA_\infty - KA_0) \quad (11)$$

From eq 10, eq 11, and the conservation of mass, it follows that

$$[M] = [M]_0 - 2([D] - [D]_0) = 2\alpha^{-1}(KA_\infty - KA_0)(1 - f_{dimer,corr}) \quad (12)$$

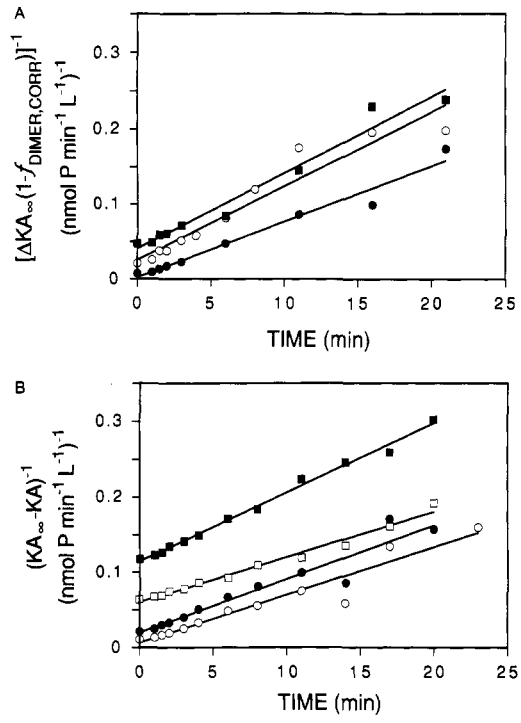


FIGURE 5: Determination of the second-order rate constants for dimerization (A) and activation of the tyrosine kinase (B) by linearization of the data from the experiments presented in Figures 3 or 4, respectively, and other similar, independent experiments. (A) For the dimerizations, the variable $f_{dimer,corr}$ was calculated by eq 10 for each point, and $\Delta KA_\infty$ was measured for the corresponding extract and corrected for the dilutions used. The quantity $[\Delta KA_\infty(1 - f_{dimer,corr})]^{-1}$ was plotted as a function of time as required by eq 13. (B) For the measurements of the activation of tyrosine kinase by EGF, values of enzymatic activity were used directly to plot $(KA_\infty - KA)^{-1}$ as a function of time as required by eq 14. The various symbols in the two graphs (A and B) represent data from separate experiments (Table I), each carried out at different initial concentrations of EGFR. Because $f_{dimer,corr}$ is zero at zero time and KA is equal to $KA_0$ at zero time, the initial point in each data set is the reciprocal of the enzymatic activity of the tyrosine kinase activated by EGF ($\Delta KA_\infty$, Table I). This enzymatic activity is directly proportional to the initial molar concentration of EGFR capable of being activated by EGF.

With these substitutions, the integrated rate equation for a second-order reaction (eq 6) can be rewritten as

$$\frac{1}{\Delta KA_\infty(1 - f_{dimer,corr})} - \frac{1}{\Delta KA_\infty} = k_D t \qquad (13)$$

where $\Delta KA_\infty$ is $KA_\infty - KA_0$ and $k_D$ is $2\alpha^{-1}k_{bi}$. Equation 9 can be rearranged accordingly to an analogous linear form:

$$\frac{1}{KA_\infty - KA} - \frac{1}{\Delta KA_\infty} = k_A t \qquad (14)$$

where $k_A$ is also equal to $2\alpha^{-1}k_{bi}$. Equations 13 and 14 provide a way of comparing the second-order rate constants of dimerization and activation of the kinase in the same units of concentration. Furthermore, both of these processes are expressed in terms of the concentration of EGFR that is actually participating in the observed response to EGF. The data for either dimerization or activation of the tyrosine kinase were plotted in the linear forms defined by eq 13 and 14, respectively (Figure 5). In each case, the slopes of the lines are values for $k_D$ or $k_A$, respectively. If each of these processes displays second-order kinetics, the value for the respective rate constants should be invariant with initial concentration, as is the case (Figure 5 and Table I). The values obtained for $k_D$ are, within experimental error, equal to the values for $k_A$ (Table I), in agreement with the proposed kinetic mechanism (Scheme I) and the assumption that activation of the receptor is coincident with its dimerization.

If the value of the turnover number of EGFR for angiotensin II under these circumstances is about 10 min$^{-1}$ (Weber et al., 1984), then the average second-order rate constant for dimerization [8 ($\mu$mol of P min$^{-1}$ L$^{-1}$)$^{-1}$ min$^{-1}$] is equivalent to a second-order rate constant of $10^6$ M$^{-1}$ s$^{-1}$, which is well below the rate constant expected of a reaction under diffusion control.

## Discussion

The ability of EGFR to form dimers in response to the binding of EGF has been previously demonstrated through covalent cross-linking with several reagents (Northwood & Davis, 1988; Cochet et al., 1988; Fanger et al., 1989). This fact has been presented as one of the main lines of evidence for a mechanism of signal transmission in which dimerization of EGFR would play a central role and would be a prerequisite for the activation of the tyrosine kinase of the receptor. Nevertheless, in all the results of such experiments reported so far, the low yield of the covalent dimers of the receptor and the long reaction time required for the cross-linking reaction prevent drawing any conclusion as to whether the dimerization of the receptor precedes the activation of its tyrosine kinase or is an unrelated effect of the binding of the ligand. In addition, because quantitative cross-linking was never achieved in these experiments, the possibility that EGFR was a dimer even in the absence of EGF and that the observed increase in cross-linking was only the result of a change in its conformation could not be ruled out.

Other reported results favoring this dimerization model for the activation of EGFR also fail to provide conclusive evidence. Thus, as it has been pointed out (Honegger et al., 1989), the ability of dimers of EGFR to undergo cross-phosphorylation (Ballotti et al., 1989; Honegger et al., 1989) does not exclude the possibility of an intramolecular mechanism of activation. The same is true for negative evidence derived from the study of mutants of EGFR with altered transmembrane regions (Kashles et al., 1988; Carpenter et al., 1991). Results from studies using antibodies that can mimic the response of the receptor to EGF (Yarden & Schlessinger, 1987a; Spaargaren et al., 1991), or studies concerning the ability of a mutant form of EGFR to suppress the activation of the wild-type protein by heterodimerization (Basu et al., 1989; Kashles et al., 1991), can be interpreted according to the dimerization model and provide stronger support. They are open, however, to other interpretations.

Cross-linking with glutaraldehyde has been previously used to determine quantitatively the concentration of oligomers present in preparations of an integral membrane-bound protein dissolved in solutions of detergent (Craig, 1982a,b). This technique has also been shown to be a suitable approach to the study of the kinetics of assembly of oligomers in studies of the renaturation of soluble enzymes (Hermann et al., 1981, 1983, 1985). The results reported here show that cross-linking with glutaraldehyde permits the accurate quantification of the relative amounts of monomeric and dimeric EGFR dissolved in a solution of detergent. Moreover, the quantitative cross-linking described here can be accomplished in a short enough interval to study the kinetics of the dimerization induced by EGF.

From the observation that the tyrosine kinase activity of EGFR increases in concert as dimerization progresses and the observation that the increase in enzymatic activity ceases when all of the EGFR is dimeric, it follows that the specific activity of the dimer of EGFR must be greater than that of the monomer. All of the mathematical development presented so far

Table I: Second-Order Rate Constants for the Dimerization and Activation of EGFR

| dimerization | | | activation of tyrosine kinase | | |
|---|---|---|---|---|---|
| $\Delta KA_\infty$[a] (nmol of P $min^{-1}$ $L^{-1}$) | relative $[M]_0$[b] | $k_D$[c] [($\mu$mol of P $min^{-1}$ $L^{-1})^{-1}$ $min^{-1}$] | $\Delta KA_\infty$[a] (nmol of P $min^{-1}$ $L^{-1}$) | relative $[M]_0$[b] | $k_A$[c] [($\mu$mol of P $min^{-1}$ $L^{-1})^{-1}$ $min^{-1}$] |
| 126 | 1 | 7 | 92 | 0.71 | 6 |
| 108 | 0.86 | 8 | 66 | 0.53 | 6 |
| 47 | 0.38 | 10 | 49 | 0.39 | 9 |
| 20 | 0.16 | 6 | 47 | 0.38 | 7 |
| 20 | 0.16 | 10 | 16 | 0.12 | 6 |
| | | | 9.0 | 0.07 | 9 |
| | | | 8.5 | 0.07 | 9 |

[a] Values for $\Delta KA_\infty$ were obtained by extrapolation of curves as in Figure 4. [b] The numbers tabulated for relative $[M]_0$ are based on the values for $\Delta KA_\infty$ with the assumption that tyrosine kinase induced by EGF is directly proportional to the initial molar concentration of those monomers capable of being converted to dimers. [c] The rate constants $k_D$ and $k_A$ were determined from the slopes of lines plotted as in Figure 5.

can be modified, for the possibility that the monomer of EGFR does have significant enzymatic activity, and all of the same conclusions can be drawn, although the kinetic derivations are somewhat more complicated. As it would be difficult if not impossible to define the contribution of the monomeric form of EGFR to the small amount of tyrosine kinase activity ($\sim$ 10%) seen in the absence of EGF, this was not attempted. Therefore, even though this was assumed in the derivations, it cannot be concluded that the monomer of EGFR has no tyrosine kinase activity, only that it has very little relative to that of the dimer.

The second-order dependence on the concentration of EGFR observed for the kinetics of the activation of the tyrosine kinase (Figure 4) rules out an entirely intramolecular mechanism of activation, for which the apparent rate of activation would be independent of the concentration of EGFR. Because the activation of the tyrosine kinase of EGFR is a second-order reaction, the transition state of the rate-limiting step in the process of activation must be a dimer of EGFR. Therefore, dimerization of EGFR must precede or coincide with its activation. Within the resolution of the observed kinetics (<2–3 min), this activation occurs coincident with or immediately after the dimerization itself. The simplest explanation for this coincidence between the second-order rate constants for dimerization and activation of the tyrosine kinase activity (Table I) is that the dimerization itself is the rate-limiting step in the activation of the tyrosine kinase. It should be noted, however, that any number of rapid steps could occur after the rate-limiting step and before the actual activation of the tyrosine kinase.

Although these results strictly apply only to EGFR dissolved in detergent, it is likely that the same mechanism of activation operates while the enzyme is in the plasma membrane. Formation of dimers of EGFR induced by EGF has been shown, by chemical cross-linking, to take place both in isolated membranes (Northwood & Davis, 1988) and in intact cells (Fanger et al., 1986; Cochet et al., 1988; Northwood & Davis, 1989). Microaggregation of EGFR in isolated plasma membranes has also been detected by fluorescence resonance energy transfer (Carraway et al., 1989), and aggregation increased under conditions where the receptor was active as a tyrosine kinase. This technique, however, failed to detect microaggregation of EGFR in intact cells under the same conditions (Carraway & Cerione, 1991). The conclusion drawn by these authors was that "EGF does not cause the microaggregation of the majority of its receptors within the time period of the early events associated with growth factor action". It is clear from this statement that the authors are aware that the dimerization of a minority of the EGF receptors over the early time period, sufficient to produce enough tyrosine kinase to initiate the early events, could not have been detected by their procedures. It is common for cells to have total surface concentrations of receptors in large excess over the concentration of activated receptors necessary to initiate a response. This is probably the case with A431 cells, which have an abnormally high concentration of EGF receptors.

In the model proposed earlier for signal transmission through dimerization (Yarden & Schlessinger, 1987a), it was assumed that the monomeric and dimeric forms of EGFR are in an equilibrium, which, upon binding of EGF, is shifted to favor the enzymatically active, dimeric form by the higher affinity of the ligand for the dimer of EGFR. The results presented here are consistent with this model, but no evidence for reversibility of the dimerization process was found in the range of the concentrations of EGFR used. In addition, for a model of activation of the tyrosine kinase based on an equilibrium between monomer and dimer, the binding of EGF to EGFR should display cooperativity with respect to the concentration of EGF, which has not been observed (Yarden & Schlessinger, 1987a; Hock & Hollenberg, 1980; Cohen et al., 1982; Yarden et al., 1985; Payrastre et al., 1988). Taking this into account, an alternative model for signal transmission can be proposed which is consistent with all the present evidence. Binding of EGF to its receptor would produce a reversible conformational change in the monomer of EGFR that would case the dimeric form to become the more stable and lead to the formation of dimers of EGFR. Dimerization would trigger an essentially irreversible change, locking the protein in the dimeric form. Interaction between the apposed intracellular kinase domains of the two molecules of EGFR in the dimer would lead to the activation of the tyrosine kinase, and this would complete the transmission of the signal across the cell membrane.

## ACKNOWLEDGMENTS

I thank Gordon Gill and Gordon Walton for providing the A-431 cells for these experiments, for teaching me the necessary assays and immunochemical methods, and for providing advice and encouragement. I also thank Jack Kyte, in whose laboratory these experiments were conducted, for advice and support.

**Registry No.** EGF, 62229-50-9; EGF receptor tyrosine kinase, 79079-06-4.

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