Activation of Epidermal Growth Factor Receptor by Epidermal Growth Factor†

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ABSTRACT: The binding of epidermal growth factor (EGF) to epidermal growth factor receptor (EGF receptor) induces dimerization of the receptor and activation of its protein tyrosine kinase. Each of these three steps was followed as a function of the concentrations of EGF and of EGF receptor. Binding of EGF was followed by sedimentation of the complex between [^{3}H]EGF and EGF receptor, dimerization was measured by quantitative cross-linking with glutaraldehyde, and the activation of the protein tyrosine kinase was monitored under the same conditions by following the initial velocity of the phosphorylation of peptides containing tyrosine. The binding of epidermal growth factor to its receptor was measured as a function of the concentration of epidermal growth factor, and the relationship was sigmoid with an average value of 1.7 for the Hill coefficient. Both dimerization and the activation of the tyrosine kinase displayed saturation as a function of the concentration of EGF. The ranges of the concentrations of EGF where dimerization and activation of the tyrosine kinase activity were half-maximal were 15−30 and 50−200 nM, respectively, but the value for the concentration of EGF at the half-maximum for the activation of the tyrosine kinase was a complex function of the concentration of EGF receptor. The observed behavior of the binding of EGF, the dimerization of EGF receptor, and the activation of the tyrosine kinase were used as criteria against which to test mechanisms for the process of activation. Equations were derived for various reversible and irreversible mechanisms and used to calculate the theoretical behaviors of the three properties. In direct comparisons of the experimental and the theoretical data, several of the previously proposed reversible and irreversible mechanisms for the activation of EGF receptor were found to be inadequate, but a reasonable mechanism was formulated that was compatible with the experimental data. In this mechanism, dimeric EGF receptor must be occupied by two molecules of EGF for enzymatic activity to be expressed.

Epidermal growth factor receptor (EGF receptor)[1] plays a key role in regulation of the growth of many animal cells by mediating the transmission of a signal across the plasma membrane. This agonist-regulated enzyme is a member of the family of receptor tyrosine kinases, each of which contains an intrinsic protein tyrosine kinase activity necessary for transmission of the signal (Yarden & Ullrich, 1988; Gill et al., 1987). A membrane-spanning protein, EGF receptor, consists of an extracellular domain for binding its ligand, epidermal growth factor (EGF),[1] and a cytoplasmic domain for expressing its protein tyrosine kinase activity. These two domains are linked in the native protein by a single membrane-spanning segment (Cadena & Gill, 1993; Greenfield et al., 1989; Lax et al., 1991; Livneh et al., 1986; Ullrich et al., 1984; Wedegaertner & Gill, 1989). Transmission of the signal is initiated when EGF binds to EGF receptor at the outside surface of the cell and is completed upon generation of a biochemical message on the inside of the cell by phosphorylation of tyrosine residues on a set of target proteins which includes EGF receptor itself. This process is a required step in the cascade of events that eventually leads to growth and division (Carpenter & Cohen, 1990). In the absence of EGF, little or no tyrosine kinase activity is displayed by EGF receptor. After binding EGF, the receptor dimerizes, and its protein tyrosine kinase is simultaneously activated (Canals, 1992). Dimerization and activation have been shown to occur coincidentally in a reaction that displays second-order kinetics in the concentration of EGF receptor. The dimerized, activated enzyme phosphorylates itself as well as several intracellular substrates (Hunter & Cooper, 1985; Glenny et al., 1988).

The mechanism by which the binding of EGF leads to the dimerization and activation of EGF receptor is not known. Hypothetical schemes for this mechanism have been proposed. They include irreversible (Canals, 1992) and reversible mechanisms (Biswas et al., 1985; Basu, 1986; Basu et al., 1989; Yarden & Schlessinger, 1987a,b; Schlessinger, 1988). All of these proposals have as one of their assumptions that both liganded and unliganded forms of dimerized EGF receptor are active. An additional mechanism for activation of EGF receptor has been formulated on the basis of the behavior of growth hormone receptor. The extracellular domain of growth hormone receptor has been found to form a peculiar complex with growth hormone that contains one molecule of hormone bound between two monomers of the extracellular domain (de Vos, 1992). The yield of this complex is a biphasic function of the concentration of growth hormone with a distinct maximum (Cunningham et al., 1991), which should serve as a signature of this mechanism. Because EGF receptor dimerizes upon binding ligand and because the dimeric complex observed with the extracellular

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[1] Abbreviations: EGF receptor, epidermal growth factor receptor; EGF, epidermal growth factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, disodium ethylenediaminetetraacetate; t-Boc, *N*-*tert*-butoxycarbonyl; Fmoc, 9-fluorenylmethoxycarbonyl; PVDF, poly(vinylidene difluoride); SDS, sodium dodecyl sulfate.

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domain of growth hormone receptor might be involved in its activation, the mechanism for the activation of both of these receptors might have involved such an asymmetric complex.

To discriminate among these various mechanisms for EGF-dependent dimerization and activation of EGF receptor, the yields of dimerization of EGF receptor and activation of its tyrosine kinase were monitored as functions of both the concentration of EGF and the concentration of EGF receptor as well as the time of exposure to EGF. Results from these studies indicate that the activation of EGF receptor is an equilibrium process, that dimerization of monomeric EGF receptor does not occur in the absence of bound EGF, that dimeric EGF receptor is not active unless two molecules of EGF are bound to it, and that the active complex of EGF and EGF receptor is equimolar in its stoichiometry.

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. The placental tissues were received from the Department of Pathology at the Medical Center of the University of California at San Diego. The human EGF was expressed in a strain of the yeast *Pichia pastoris* and purified from the cell-free broth. The broth was a generous gift from Sibia. Aprotinin, benzamidine hydrochloride, phenylmethanesulfonyl fluoride, bovine serum albumin, Na_2ATP, Triton X-100 detergent, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES)[1], 2-mercaptoethanol, disodium ethylenediaminetetraacetate (EDTA)[1], papain, Protein A Sepharose, Sephadex G-50, Sephadex G-25, Sepharose 4B-200, leupeptin, nitro blue tetrazolium, and the *p*-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate were purchased from Sigma Chemical Co. Glutaraldehyde was purchased from Calbiochem-Novabiochem Corp, and *N*-*tert*-butoxycarbonyl (t-Boc)[1] and 9-fluorenylmethoxycarbonyl (Fmoc)[1] derivatives of the amino acids were purchased from Bachem, Inc. Angiotensin II was purchased from Bachem, Inc. and was also synthesized from Fmoc amino acids (Stewart & Young, 1984; Thibault, 1993). The synthetic angiotensin II was purified on HPLC. It had the same chromatographic mobility as the commercial material, and the amino acid composition after acid hydrolysis was $D_{1.2}R_{0.9}V_{1.2}Y_{0.9}I_{1.0}H_{1.0}P_{1.1}F_{0.8}$. Affi-gel 10 and goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase were purchased from Bio-Rad Laboratories Corp. Sodium [^{3}H]borohydride (100−1000 mCi mmol^{-1}) and [^{14}C]sucrose (1−5 mCi mmol^{-1}) were purchased from NEN Research Products, DuPont Co. The triethylammonium salt of adenosine [γ-^{32}P]triphosphate (3000 mCi mmol^{-1}) was purchased from Amersham. Membranes of poly(vinylidene difluoride) (PVDF)[1] were purchased from Millipore. Centrifugal concentrators (Centricons) with an apparent molecular mass cutoff of 30 000 Da were purchased from Amicon, and phosphocellulose paper was purchased from Whatman. Sodium dodecyl sulfate (SDS)[1] was purchased from Sigma Chemical Co. or Calbiochem Corp. and was recrystallized from 95% ethanol (Burgess, 1969).

The carboxy terminal peptide of EGF receptor, SEFIGA, was synthesized by standard methods for solid phase t-Boc peptide chemistry (Stewart & Young, 1984; Bayer, 1990; Canals, 1992) and purified by high-pressure liquid chromatography. The purified peptide migrated on analytical HPLC as a single peak and upon acid hydrolysis gave the amino acid composition of $S_{1.0}E_{1.1}F_{1.0}I_{0.9}G_{0.9}A_{1.1}$. The peptide RRKGSTAENAEYLRV, which is a reactant for the tyrosine kinase of EGF receptor, was synthesized from Fmoc amino acids (Stewart & Young, 1984; Thibault, 1993) and purified by HPLC. Acid hydrolysis of this purified peptide gave the following composition: $R_{3.1}K_{1.0}G_{1.1}S_{0.8}T_{0.9}A_{1.9}E_{2.2}N_{1.1}Y_{0.6}L_{1.1}V_{1.1}$.

Epidermal growth factor was reductively methylated as described by Rice and Means (1971) to a specific radioactivity of 3−20 cpm pmol^{-1}. After purification over a Sephadex G-25 gel filtration column, the specific radioactivity of each batch was established from the amino acid analysis of the radioactively labeled hormone and the counts per minute associated with the sample registered in the same channel of the scintillation counter used to count the samples from the binding assay.

Papain was coupled to Sepharose 4B-200 following cyanogen bromide activation of the agarose (March et al., 1974). The specific activity of the immobilized papain was determined by assaying for the activity of papain as described by the *Worthington Manual of Enzymes and Related Biochemicals*, Worthington Biochemical Corp.

Extracts of A-431 Cells. Cells of the A-431 human epidermal carcinoma line were grown until they were confluent or just past confluence. The cells were then harvested, lysed, and homogenized at 0 °C in a buffer containing 10% glycerol, 1% Triton X-100, 1 mM EDTA, 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate, 5 mM 2-mercaptoethanol, 2 mM benzamidine, 2.5 μg mL^{-1} aprotinin, 5 μg mL^{-1} leupeptin, and 30 mM HEPES adjusted to pH 7.4 with 1 M NaOH (Canals, 1992). The homogenate (1 mL in each tube) was centrifuged at 75 000 rpm in a TLA-100.2 rotor (Beckman Corp.) for 30 min. The supernatant fluid is referred to as an extract of A-431 cells.

Preparation of Placental Extracts. Human placental membranes were prepared as described by Koland and Cerione (1988). The membranes were dissolved by the addition of Triton X-100 to 2%, homogenization, and stirring on ice for 45 min. Phenylmethanesulfonyl fluoride was added to 0.2 mM and leupeptin to 0.1 μg mL^{-1}. The homogenate (1 mL in each tube) was centrifuged at 15 000 rpm in a microfuge (Beckman Corp.) for 30 min. The supernatant fluid from this centrifugation is referred to as a placental extract.

Purification of Placental EGF Receptor. Epidermal growth factor receptor was purified from placental extract with the use of an immunoadsorbent that was constructed with purified monovalent Fab fragments of immunoglobulins that recognize the carboxy terminal sequence, SEFIGA, of EGF receptor (Canals, 1992). These specific immunoglobulins were produced by the immunization of White New Zealand rabbits with the synthetic peptide SEFIGA cross-linked as a hapten with glutaraldehyde to bovine serum albumin (Walter et al., 1980). Anti-SEFIGA antisera were digested with immobilized papain overnight (Harlow & Lane, 1988). Anti-SEFIGA Fab fragments were purified over an affinity adsorbent made from the synthetic peptide (Kyte et al., 1987) and separated from undigested immunoglobulins using Protein A Sepharose (Surolia et al., 1982). These

purified anti-SEFIGA Fab fragments were covalently attached by reaction with the *N*-hydroxysuccinimide esters of Affi-gel 10 (1 mL) to produce an immunoadsorbent (1 mL) with a measured capacity of 5 nmol for recognizing synthetic SEFIGA.

Samples of placental extract were recycled over the immunoadsorbent for 30 min, and the column was then washed with 10−20 volumes of a buffer containing 0.1% Triton X-100, 10% glycerol, 1 mM EDTA, 5 mM 2-mercaptoethanol, 2 mM benzamidine, 0.1 μg mL^{-1} leupeptin, and 20 mM HEPES (pH 7.4) (buffer A)[1]. Material bound to the column was eluted with a 1 mL solution of 1 mg mL^{-1} synthetic SEFIGA in buffer A. To ensure maximum recovery during the exchange of synthetic peptide for bound EGF receptor on the solid support, the elution buffer was left on the immunoadsorbent for 6−16 h. Another sample (1 mL) of the peptide solution was applied to the column, followed by five 1 mL samples of buffer A, to retrieve the rest of the released receptor. The eluted material was assayed for tyrosine kinase activity, and those fractions responsive to EGF were concentrated for further use in a Centricon-30 centrifugal concentrator at 6500 rpm in a SS34 rotor (Sorvall Division, DuPont Chemicals). The immunoadsorbent was washed with 10−20 column volumes of 0.1 M sodium phosphate buffer (pH 2.5) to elute the bound peptide, followed by 10 column volumes of 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate (pH 7.2) for storage at 4 °C.

Sedimentation of EGF Receptor. Samples (1 mL) of an extract of A-431 cells were centrifuged at 100 000 rpm in a TLA-100.2 rotor in a Beckman TL100 table top centrifuge for increasing amounts of time. Sodium dodecyl sulfate was added to the supernatant fluids which were then briefly brought to 100 °C, concentrated, and submitted to electrophoresis followed by immunoblotting. The immunoblot was submitted to densitometry. The area of absorbance from the immunostain of the band representing EGF receptor was followed as a function of time of ultracentrifugation. Because all of the EGF receptor in a sample could be sedimented in approximately 10 h, a time of 12 h was chosen for centrifugation during routine assays for the binding of EGF.

Binding Assay for EGF. Assays for the binding of EGF were performed using ultracentrifugation. When the concentration of specific binding sites for EGF was measured, several samples of up to 1000 μL of detergent extracts of EGF receptor were brought to 1.0 mL and mixed with [^{3}H]-EGF (3−20 cpm pmol^{-1}) at a saturating total concentration of 300 nM. To prevent nonspecific ion exchange, KCl was added to 100 mM. [^{14}C]Sucrose was used as an internal standard. The counts per minute of [^{14}C]sucrose added were adjusted so that the total number of counts per minute of [^{14}C]carbon were about equal to the total number of counts per minute of tritium. After 20 min, the samples (1.1 mL) were submitted to centrifugation at 100 000 rpm in a TLA-100.2 rotor for 12 h. The supernatant fluids were removed, and the pellets were dissolved in 88% formic acid. Both the supernatant fluids and the pellets were submitted to scintillation counting with windows set to discriminate between tritium and [^{14}C]carbon.

To assess what portion of the bound [^{3}H]EGF is specific for EGF receptor when the protein was sedimented from an extract, unlabeled EGF was added in increasing amounts to samples (1 mL) of a solution containing undiluted extract (80−85 nM in EGF receptor) and 75 nM [^{3}H]EGF. The bound [^{3}H]EGF that could not be chased by nonradioactive EGF could be estimated by extrapolation. The treatment of the data is justified by considering the following evaluation. If the binding of EGF to a binding site were defined by the equilibrium

$$\text{EGF} + \text{site} \overset{K_d}{\rightleftharpoons} \text{EGF}\cdot\text{site} \qquad (1)$$

where K_d is the dissociation constant, then by rearrangement of the equation for K_d

$$[\text{EGF}\cdot\text{site}] = \frac{[\text{EGF}]_{TOT}[\text{sites}]_{TOT}}{[\text{EGF}]_{free} + K_d + [\text{sites}]_{TOT}} \qquad (2)$$

where [EGF·site] is the concentration of EGF bound to the specific sites, $[\text{EGF}]_{TOT}$ is the total concentration of EGF, $[\text{site}]_{TOT}$ is the total concentration of specific binding sites for EGF, and $[\text{EGF}]_{free}$ is the concentration of free EGF in the assay, which can be determined by submitting the supernatant fluid to scintillation counting. The bound [^{3}H]-EGF in the pellet (cpm_{bound}) will include both [^{3}H]EGF that is bound nonspecifically (cpm_{nsp}) to the protein, lipid, and detergent in the pellet and [^{3}H]EGF that is bound specifically (cpm_{sp}) to EGF receptor in the pellet. If eq 2 defines the specific binding of EGF to EGF receptor, then

$$\text{cpm}_{bound} = \frac{\text{cpm}_{TOT}[\text{sites}]_{TOT}}{[\text{EGF}]_{free} + K_d + [\text{sites}]_{TOT}} + \text{cpm}_{nsp} \qquad (3)$$

From this equation, the counts per minute of nonspecifically bound [^{3}H]EGF could be determined by successive approximation. Values of $[\text{sites}]_{TOT}$ were estimated from the raw data before correction. Equation 3 was used to determine cpm_{nsp} from the intercept at the ordinate. This estimate of nonspecifically bound counts per minute was then subtracted from the counts per minute of bound [^{3}H]EGF to obtain a value referred to by the term "specifically bound [^{3}H]EGF." This value was used to obtain a corrected estimate of $[\text{sites}]_{TOT}$ and the process repeated. In practice, the values of cpm_{nsp} were so small that convergence was almost immediate.

Binding of EGF as a Function of Its Concentration. When the specific binding of EGF was measured as a function of the concentration of EGF, each sample contained the same amount of cell extract (0.6 or 1 mL) and [^{14}C]sucrose. Potassium chloride was added to a final concentration of 100 mM, and [^{3}H]EGF was added to the noted nanomolar concentration. It was found that the data obtained in these assays were fit best by a sigmoid function. The Hill equation used to fit a sigmoid curve to the data was

$$f([\text{EGF}]_{free}) = \frac{f([\text{EGF}]_{free})_{max}([\text{EGF}]_{free})^n}{(K_{1/2})^n + ([\text{EGF}]_{free})^n} \qquad (4)$$

where $f([\text{EGF}]_{free})$ is the bound [^{3}H]EGF, $[\text{EGF}]_{free}$ is the free concentration of EGF, and $K_{1/2}$, $f([\text{EGF}]_{free})_{max}$, and n are the three parameters of the equation being fit. In this formalism, the parameter $K_{1/2}$ is the value of the concentration of EGF at which the function displays half of its maximum value, $f([\text{EGF}]_{free})_{max}$.

Tyrosine Kinase Activity. The initial velocity of the protein tyrosine kinase of EGF receptor was assayed as previously reported (Canals, 1992). Samples (30 μL) of appropriately diluted detergent extracts of EGF receptor were mixed with EGF at noted final concentrations for noted times at room temperature. The enzymatic activity was then assayed by addition (to the following final concentrations in a final volume of 60 μL) 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 assays requiring the peptide RRKGSTAENAEYLRV as a substrate, the peptide was added to 0.25 mM in place of angiotensin II. The reactions were stopped after 1 or 5 min by adding trichloroacetic acid to 5%. The production of phosphorylated angiotensin II or RRKGSTAENAEYLRV, however, was linear with time for at least 10 min. Bovine serum albumin was added to 0.5 mg mL^{-1} 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 with 75 mM H_3PO_4, dried, and counted.

Quantitative Cross-Linking of Dimers of EGF Receptor. The assay for the dimerization of EGF receptor was performed as described previously (Canals, 1992). Relative amounts of dimers and monomers on immunoblots were quantified using a scanning densitometer. Each lane was scanned separately, and the areas of absorbance of the peaks of stain corresponding to dimer and monomer were calculated. Because of deviations from linearity of the measured areas as a function of the mass of protein for both monomeric and dimeric EGF receptor and differences in the extinction coefficients for monomer and dimer, measured areas were corrected as previously described (Canals, 1992).

All primary data, both the initial velocities of the tyrosine kinase activity and the measured fraction of dimer, were assumed to represent rectangularly hyperbolic functions of the total concentration of EGF. The equation used for the rectangular hyperbolas was

$$f([\mathrm{EGF}]_{\mathrm{TOT}}) = \frac{f([\mathrm{EGF}]_{\mathrm{TOT}})_{\mathrm{max}}[\mathrm{EGF}]_{\mathrm{TOT}}}{[\mathrm{EGF}]_{\mathrm{TOT},0.5} + [\mathrm{EGF}]_{\mathrm{TOT}}} \qquad (5)$$

where $f([\mathrm{EGF}]_{\mathrm{TOT}})$ is either the measured fraction dimerized or initial tyrosine kinase activity, $[\mathrm{EGF}]_{\mathrm{TOT}}$ is the total concentration of EGF, and $[\mathrm{EGF}]_{\mathrm{TOT},0.5}$ and $f([\mathrm{EGF}]_{\mathrm{TOT}})_{\mathrm{max}}$ are the two parameters of the fit.

RESULTS

Determination of the Concentration of EGF Receptor from Assays for EGF Binding. The binding of EGF to its receptor was measured by adding [^{3}H]EGF to samples of extracts and submitting the samples to centrifugation until all of the EGF receptor, but not the EGF, had pelleted. Saturating levels of [^{3}H]EGF (300 nM at 3−12 cpm pmol^{-1}) were added to increasing amounts of cell extract containing EGF receptor, and the individual samples were submitted to centrifugation. A control that did not contain any EGF receptor was also included. [^{14}C]Sucrose was used as an internal standard so that the cpm of [^{3}H]EGF in the supernatant fluid that had been collected with each pellet could be subtracted. After this first correction, it was found that 30−50 counts per minute of tritium had pelleted even in the absence of cell extract. This was assumed to represent free molecules of [^{3}H]EGF that had sedimented in the high centrifugal field, and these counts per minute were also subtracted from each of the samples containing EGF receptor. The final corrected values of counts per minute are referred to by the term "bound [^{3}H]EGF".

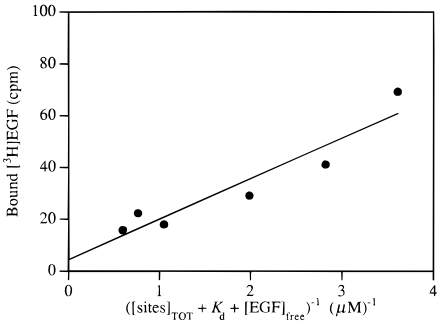


FIGURE 1: Competition of nonradioactive EGF with [^{3}H]EGF for the binding sites on EGF receptor in pellets from ultracentrifugation of an extract from A-431 cells. Each sample of an extract of A-431 cells (1 mL) was mixed with the same amount of [^{3}H]EGF (75 nM, 225 cpm) and increasing concentrations of nonradioactive EGF in a final volume of 1.1 mL so that the ratio of nonradioactive to radioactively labeled EGF increased in increments to a final value of 20:1. All samples were then centrifuged for 12 h. The supernatant fluids and the pellets dissolved in formic acid were submitted to scintillation counting. The counts per minute of bound [^{3}H]EGF in the pellets are plotted as a function of $([\mathrm{sites}]_{\mathrm{TOT}} + K_{\mathrm{d}} + [\mathrm{EGF}]_{\mathrm{free}})^{-1}$ in units of micromolar^{-1}, where the value of K_{d} for the binding of EGF was taken to be 100 nM and the value for $[\mathrm{sites}]_{\mathrm{TOT}}$ was estimated by successive approximation from the data.

To estimate the amount of bound [^{3}H]EGF that could be chased by the unlabeled ligand, a competitive binding assay was performed using increasing concentrations of nonradioactive EGF at a constant subsaturating concentration (75 nM) of [^{3}H]EGF (Figure 1). The measurements were plotted as counts per minute that had pelleted against the quantity $([\mathrm{EGF}]_{\mathrm{free}} + K_{\mathrm{d}} + [\mathrm{site}]_{\mathrm{TOT}})^{-1}$, where the value used for K_{d}, the dissociation constant for EGF, was taken to be 100 nM and the value of $[\mathrm{sites}]_{\mathrm{TOT}}$, the total molar concentration of specific sites for EGF in the solution before centrifugation, was determined by successive approximation. The choice of a dissociation constant was based on a binding curve for [^{3}H]EGF using the same assay (Figure 2). The extrapolated value of 10 counts per minute of bound [^{3}H]EGF at an infinite concentration of nonradioactive free EGF represented only a small fraction (3−5%) of the [^{3}H]EGF bound at saturation in the absence of nonradioactive EGF (Figure 2).

Before the concentration of EGF receptor was calculated from the specific radioactivity of the [^{3}H]EGF and the counts per minute bound to the pellet, the counts per minute of tritium from trapped supernatant fluid (usually 30−50 cpm), the counts per minute of tritium that had pelleted in the absence of receptor (usually <40 cpm), and the small amount of bound [^{3}H]EGF that could not be competed away (Figure 1) were subtracted from the counts per minute of tritium that were collected with the pellet when the receptor was present. These corrected values were used for the estimates of concentration of EGF receptor. The amount of bound [^{3}H]EGF was always directly proportional to the volume of the detergent extract added to the assay. Estimates of the concentration of EGF receptor were made from the slopes of the lines for bound [^{3}H]EGF at saturation as a function

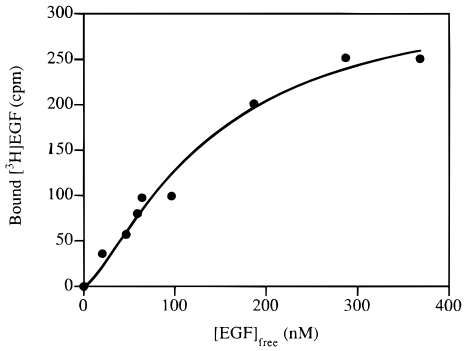


FIGURE 2: Binding of EGF to EGF receptor as a function of the free concentration of EGF. Samples (1 mL) of an extract from A-431 cells were mixed with increasing concentrations of [^{3}H]EGF (0−1600 nM) for 20 min and then centrifuged for 12 h. The values plotted for bound [^{3}H]EGF are the values of counts per minute that have been corrected for the counts per minute of unbound tritium trapped in the pellet and the counts per minute of free [^{3}H]-EGF that sedimented. The values of the nanomolar concentration of free EGF ($[EGF]_{free}$) were based on the counts per minute remaining in the supernatant fluid. The curve was produced with eq 4 using values for $K_{1/2}$ of 135 nM, $f([EGF]_{free})_{max}$ of 325 cpm, and n of 1.4. These parameters were obtained from a nonlinear least-squares fit of eq 4 to the data.

of the microliters of cell extract and the specific radioactivity of the [^{3}H]EGF. These assays were repeated three to five times for a given preparation of extract to determine the reproducibility of the binding assay, which was usually ±10%.

Concentrations of EGF receptor in the various extracts of A-431 cells were found to range from 30 to 80 nM. Using these concentrations of EGF receptor and the initial velocities (nanomoles of phosphate transferred per minute) of the tyrosine kinase assays with angiotensin II at saturating levels of EGF, the turnover numbers of the enzyme from cell extracts and placental extracts were calculated to be 5 ± 1 and 2 ± 0.5 min^{-1}, respectively. These values were used to determine the concentration of EGF receptor in the various assays of tyrosine kinase and dimerization. The value measured here for the turnover number of EGF receptor in extracts of A-431 cells is similar to the turnover numbers of 13 min^{-1} (Bertics & Gill, 1985) and 10 min^{-1} (Weber et al., 1984) previously reported for angiotensin II as a substrate.

Binding of EGF as a Function of Its Concentration. The binding of EGF to its receptor was also measured as a function of the concentration of EGF (Figure 2). When rectangular hyperbolas were fit to the data from several experiments by nonlinear least-squares analysis, the points at the lower concentrations of [^{3}H]EGF consistently fell below the smooth curves and those at the higher concentrations consistently fell above the curves. This behavior indicated that the binding of EGF to EGF receptor displayed sigmoid behavior, or a positive deviation from ideal hyperbolic behavior. For this reason, the data were fit with the Hill equation. In none of the experiments was the value of the Hill coefficient n less than 1 (the smallest value was 1.2), and its mean and standard deviation over all of the seven experiments were 1.7 ± 0.5. The mean and standard deviation of the value of $K_{1/2}$ were 70 ± 40 nM. The standard deviations are large because there was considerable variation from measurement to measurement, but for each run, the fits of the Hill equation were satisfactory (Figure 2). The important point, however, is that the mean value of the Hill coefficient n is significantly larger than 1.

Kinetics of Activation of Tyrosine Kinase as a Function of the Concentration of EGF. To analyze the activation of EGF receptor as a function of the concentration of EGF at various times of exposure, samples of either cell extracts or purified placental extracts were incubated with varying amounts of EGF for different intervals, and the initial velocity of the enzymatic reaction of the tyrosine kinase was subsequently measured. The extracts of A-431 cells were prepared as previously described (Canals, 1992). Placental extracts were partially purified over an immunoadsorbent recognizing the carboxy terminus of EGF receptor. When samples that had been eluted from the immunoadsorbent were submitted to electrophoresis, many bands other than EGF receptor appeared on the stained gel. For these experiments, however, it was not necessary to have homogeneous EGF receptor, only EGF receptor pure enough that its concentration could be conveniently increased. Extracts of A-431 cells were not so highly concentrated in protein as the initial placental extracts but could be concentrated up to 4-fold as needed. The activation of the protein tyrosine kinase after the noted exposure to EGF was followed by measuring the initial velocity (v_0) of the phosphorylation from [^{32}P]ATP of either the tyrosine in angiotensin II or the tyrosine in the synthetic peptide RRKGSTAENAEYLRV.

The activation of the tyrosine kinase displayed saturation as a function of the concentration of EGF for both long and short intervals of treatment. The analytical function for a rectangular hyperbola could be satisfactorily fit to these data with a nonlinear least-squares algorithm. The two parameters of the fit were V_{max}, the maximum initial velocity at saturation, and $[EGF]^{act}_{TOT,0.5}$ the total concentration of EGF at which the initial velocity was half the maximum value. The total concentration of EGF producing half-maximal activation of the tyrosine kinase ($[EGF]^{act}_{TOT,0.5}$) decreased as V_{max} increased during the activation of the tyrosine kinase (Table 1). This decrease in the value of the parameter $[EGF]^{act}_{TOT,0.5}$ from short to long intervals was consistently observed over a range of concentrations of EGF receptor and with EGF receptor in both placental and cell extracts. At the shorter intervals, the intermediates in the mechanism for the activation of the tyrosine kinase had not yet reached equilibrium. After 5−10 min, however, the value of the parameter $[EGF]^{act}_{TOT,0.5}$ did not change noticeably. This observation suggests that, by this time, the process of activation has reached equilibrium in this range of concentrations. For each experiment presented in Table 1, the concentration of EGF receptor was calculated from the V_{max} of the tyrosine kinase reaction at long intervals and the turnover number for the respective EGF receptor determined from the binding assays. The concentrations of EGF receptor noted in Table 1 are the final concentrations, after mixing with EGF, in the total volume of 45 μL before the assay for the tyrosine kinase was performed.

The activation of EGF receptor at saturating concentrations of EGF displays the properties of a bimolecular reaction that proceeds to completion (Canals, 1992). This observation suggested the possibility that, after sufficient time in the presence of any concentration of EGF, the final state achieved would not be a reversible equilibrium but the irreversible product of a complete chemical transformation. To provide evidence that the process of activation of EGF receptor has reached only a reversible equilibrium by 20 min,

Table 1: Kinetic Values for the Activation of EGF Receptor as a Function of Time of Exposure to EGF[a]

source of receptor	[EGF receptor][b] (nM)	time (min)	V_{max} (pmol mL^{-1} min^{-1})	$[EGF]^{act}_{TOT,0.5}$ (nM)
A-431 cells	30	1	60	290
		60	160	45
A-431 cells	20	1	40	85
		60	110	50
A-431 cells	25	1	25	70
		60	120	60
A-431 cells	10	1	20	190
		60	60	60
A-431 cells	25	1	75	270
		60	130	70
A-431 cells	5	1	20	210
		60	25	60
placenta[c]		5	0.65[d]	190
		10	0.7	190
		15	0.7	180
		15	1.2	75
		30	1.2	85
		60	1.0	65
membranes of A-431 cells[c]		1	0.8[d]	15
		2	0.9	50
		5	1.0	40
		10	1.0	30

[a] Samples (30 μL) of a placental extract or an extract of A-431 cells were exposed to several concentrations of EGF (in a final volume of 45 μL) for the noted times. The initial velocity (v_0) of the tyrosine kinase over a period of 1 min was then measured and was expressed in units of picomoles of phosphate incorporated into angiotensin II per minute. The data were fit with the equation for a rectangular hyperbola using a nonlinear least-squares program to obtain values for the parameters V_{max} and $[EGF]^{act}_{TOT,0.5}$. The parameter V_{max} was the value of the horizontal asymptote, and the parameter was the value of $[EGF]_{TOT}$ for the point on the hyperbola at which v_0 was equal to $V_{max}/2$. [b] Final concentration of EGF receptor in the volume of solution prior to the addition of reagents for the tyrosine kinase reaction. The value for this quantity was estimated from a turnover number of 5 min^{-1} and the value of the maximum velocity of the enzymatic reaction for a sample of the undiluted extract after 60 min of activation with saturating EGF. [c] The concentration of EGF receptor was not determined for these particular assays. [d] In these experiments, the activity at V_{max} reported for the shorter intervals is reported as the fraction of the V_{max} for the interval of 60 min.

samples of cell and placental extract were incubated at subsaturating concentrations of EGF (25 or 50 nM), and after 20 min, the samples were split so that one half of each sample remained at the subsaturating level of EGF while the second half was exposed to a saturating level of EGF (1 μM). The initial velocities of the tyrosine kinase activities were then assessed (Table 2). The samples incubated at 25 or 50 nM, respectively, because both of the intervals had low initial velocities after the two intervals; samples switched to saturating concentrations of EGF after the first interval, however, displayed the same high initial velocities as samples that had been activated only by 1 μM EGF for 20 min. Preincubation with subsaturating levels of EGF neither decreased nor augmented the effect of a subsequent exposure of the samples to 1 μM EGF. This result suggests that, after about 20 min in the presence of EGF, a reversible equilibrium has been established.

Effect of the Concentration of EGF Receptor on Its Activation. The next experiments were designed to determine the dependence of the activation of EGF receptor by EGF on the concentration of EGF receptor itself. Stock samples of cell extracts or concentrated, purified placental extracts were systematically diluted and mixed with various concentrations of EGF for 20 min. They were then

Table 2: Effect of Changing the Concentration of EGF after EGF Receptor Has Reached Equilibrium at Subsaturating Levels of EGF[a]

	$[EGF]_{TOT}$ (nM)		
experiment	first treatment	second treatment	v_0 (pmol min^{-1})
1	50	50	3
	50	1000	16
	25	25	1
	25	1000	16
	1000	—[b]	12
2	50	50	3
	50	1000	10
	25	25	2
	25	1000	14
	1000	—[b]	18
3	50	50	4
	50	1000	12
	25	25	1
	25	1000	12
	1000	—[b]	16

[a] Samples of placental extract were exposed to 25 or 50 nM EGF for 20 min. After 20 min, samples from the first treatment were split into two separate samples that were used for the second treatment. During the second treatment, the two samples were exposed to the same subsaturating level (25 and 50 nM) or a saturating level (1000 nM) of EGF for 20 min. At the end of the second treatment with EGF, the inital velocity for the tyrosine kinase activity was measured. Each result listed is from an experiment done in triplicate. [b] No second interval occurred.

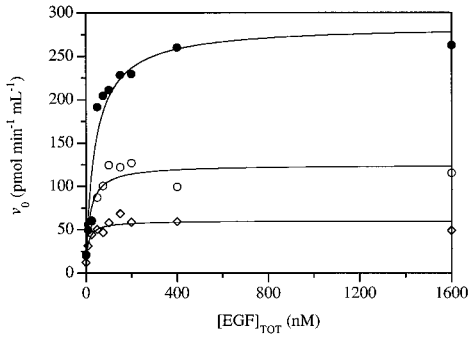


FIGURE 3: Activation of the tyrosine kinase of EGF receptor as a function of the total concentration of EGF (nanomolar) at various concentrations of EGF receptor. Samples (30 μL) of an extract from A-431 cells (30 nM in EGF receptor) were exposed to the noted total concentrations of EGF (in a final volume of 45 μL) for 20 min. Samples of the extract were undiluted (filled circles) or diluted 2-fold (open circles) and 4-fold (open diamonds). The activation of the tyrosine kinase achieved during this exposure to EGF was followed by measuring the initial velocity (v_0) over 1 min for the phosphorylation of angiotensin II (picomoles of phosphate incorporated into angiotensin II per minute per milliliter). The curves represent the nonlinear least-squares fits of the equation for a rectangular hyperbola.

monitored for the initial velocity of the tyrosine kinase (measured over an interval of 1 min) using angiotensin II as a substrate. The activation of the tyrosine kinase displayed saturation as a function of the concentration of EGF over a range of concentrations of EGF receptor (Figure 3). As before, the function for a rectangular hyperbola was fit to these data by a nonlinear least-squares algorithm.

To demonstrate that activation of the enzymatic activity by EGF remained at the saturating level and did not display a decrease at higher concentrations of EGF, assays were performed with the same extract (30 μL assay^{-1}) of A-431 cells at 1.6, 10, and 16 μM EGF. All of these concentrations of EGF produced the same activation of the tyrosine kinase activity (180 $\pm$ 20 pmol min^{-1} mL^{-1}). There was no

Table 3: Equilibrium Values for the Activation of EGF Receptor as a Function of Its Concentration[a]

source of receptor	[EGF receptor][b] (nM)	V_{max} (pmol mL^{-1} min^{-1})	$[EGF]^{act}_{TOT,0.5}$ (nM)
A-431 cells	25	130	65
	4	50	60
A-431 cells	30	150	80
	5	15	100
A-431 cells	30	150	50
	5	20	35
A-431 cells	40	200	85
	10	45	35
A-431 cells	25	130	70
	4	5	20
placenta	230	450	180
	120	290	130
	60	90	55
placenta	125	250	120
	25	25	80
placenta	75	145	150
	40	50	120
	15	25	100
A-431 cells	30	160	45
	15	110	20
	7	45	10
	4	20	40

[a] The values of V_{max} and $[EGF]^{act}_{TOT,0.5}$ were determined in experiments identical to those of Figure 3. Rectangular hyperbolas were fit to the data with a nonlinear least-squares program. The parameter V_{max} was the value of the horizontal asymptote, and the parameter $[EGF]^{act}_{TOT,0.5}$ was the value for $[EGF]_{TOT}$ for the point on the hyperbola at which v_0 was equal to $V_{max}/2$. [b] Final concentration of EGF receptor in the volume of the solution prior to the addition of reagents for the tyrosine kinase reaction. The values for this quantity were estimated from the respective turnover numbers and the maximum velocities of the enzymatic reactions for samples of each undiluted extract after 20 min of activation. The values tabulated are based on the dilutions of that extract performed in each experiment.

indication of any systematic decrease in the enzymatic activity at the higher levels of EGF.

The values for the parameter $[EGF]^{act}_{TOT,0.5}$ are listed for various concentrations of receptor (Table 3). These values are similar to those previously reported for this parameter at various fixed concentrations of EGF receptor (Bertics et al., 1988; Koland & Cerione, 1988; Pike et al., 1984). It was found in the present studies, however, in which the concentration of EGF receptor was varied systematically, that the values of $[EGF]^{act}_{TOT,0.5}$ for the activation of EGF receptor as a function of EGF increased as the concentration of EGF receptor was increased for both the purified, placental enzyme and the enzyme in the extracts of A-431 cells (Table 3). In the more concentrated samples of EGF receptor, a higher concentration of EGF was required to reach the point of half-maximal activation.

When values (Table 3) of the parameter $[EGF]^{act}_{TOT,0.5}$ were plotted as a function of the parameter V_{max}, they were found to increase monotonically as V_{max} increased (Figure 4). This increase could be due entirely to the inescapable decrease in the free concentration of EGF that results from the binding of EGF by EGF receptor if the concentrations of binding sites in the sample were in the same range as the concentrations of the EGF. If this were the case, these results would represent a kinetic titration of the binding sites (Jarrett & Kyte, 1979), and from the slope of the line, it would be possible to estimate the turnover number for the tyrosine kinase of EGF receptor. A linear fit was made through the data from each separate experiment listed in Table 3, where

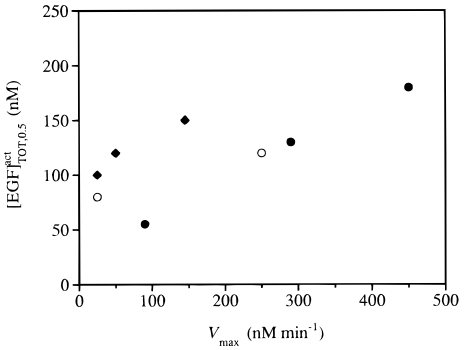


FIGURE 4: Relationship between the parameters V_{max} and $[EGF]^{act}_{TOT,0.5}$ for samples of partially purified placental extracts with different total concentrations of EGF receptor (Table 3). The total concentration of EGF (nanomolar) at which the activity of the tyrosine kinase was at the half-maximum ($[EGF]^{act}_{TOT,0.5}$) is shown as a function of the maximum velocity (V_{max}) of the tyrosine kinase (nanomolar minute^{-1}) following activation of EGF receptor in a given sample of diluted or undiluted detergent extract. The results from each experiment of Table 3 were given their own symbol.

each experiment is defined by a stock sample and its respective dilutions, and the slope for each experiment was determined. From the average slope (0.35 ± 0.15 nM EGF min mL pmol^{-1}) over all of these experiments, a turnover number for the enzymatic activity of EGF receptor (3 min^{-1}) could be calculated. This value agrees with the turnover number calculated independently from the binding and tyrosine kinase activity assays of 2−5 min^{-1}.

This agreement provided an explanation for the linear increase observed when the values of the parameter $[EGF]^{act}_{TOT,0.5}$ were plotted as a function of the parameter V_{max}. This increase was due for the most part to the fact that the concentration of binding sites for EGF was on the same order of magnitude as the concentration of EGF. To avoid this complication, which seems common to many of the earlier experiments with EGF receptor, much smaller concentrations of EGF receptor had to be used in the enzymatic assay.

To determine the relationship between the parameter $[EGF]^{act}_{TOT,0.5}$ and the concentration of EGF receptor at lower concentrations of EGF receptor, the tyrosine kinase assays were run for 5 min and they were performed with the reactant RRKGSTAENAEYLRV. This reactant permits a much greater signal to be produced in the assays which in turn permits more dilute samples of an extract from A-431 cells to be assayed. To determine the molar concentration of EGF receptor, binding assays were performed with each of the extracts of A-431 cells . Unlike the behavior at the higher concentrations of EGF receptor (Figure 4), at low concentrations of EGF receptor, the value of the parameter $[EGF]^{act}_{TOT,0.5}$ decreased as the concentration of EGF receptor increased (Figure 5).

The experiments described to this point were performed with detergent extracts, and it was of interest to determine whether the response would be similar when EGF receptor was incorporated in a membrane. In membranes that had not seen detergent, the activation of EGF receptor reaches equilibrium rapidly (Table 1). The half-maximum point, $[EGF]^{act}_{TOT,0.5}$, for activation by EGF of the tyrosine kinase in the membranes, 30−50 nM, agreed with that observed at equilibrium for the activation of the tyrosine kinase in the

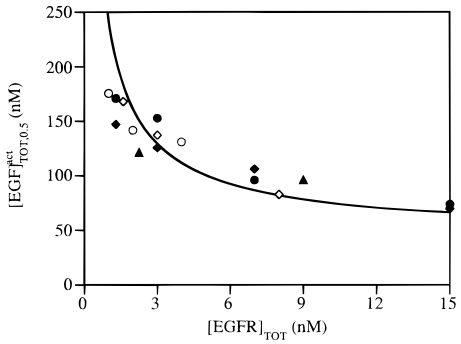


FIGURE 5: Behavior of the parameter $[EGF]^{act}_{TOT,0.5}$ as a function of the total concentration (nanomolar) of EGF receptor ($[EGFR]_{TOT}$) in dilute samples of an extract of A-431 cells. Each symbol represents an individual experiment where a sample of an extract from A-431 cells (15 and 20 nM) had been diluted 2-, 5-, 10- or 20-fold. The activation of each diluted sample was followed as a function of the total concentration of EGF by mixing the diluted samples with several concentrations of EGF and, after 60 min, measuring the initial velocity of the phosphorylation of the peptide RRKGSTAENAEYLRV. The total concentration of EGF (nanomolar) at which the activation of the tyrosine kinase was half-maximal $[EGF]^{act}_{TOT,0.5}$ was calculated from a nonlinear least-squares fit of the data from each individual experiment to the equation for a rectangular hyperbola. The curve represents theoretical results for scheme 11 with dissociation constants of $K_{dM} = 3.75$ μM, $K_{dD} = 7.5$ nM, $K_1 = 50$ μM, $K_2 = 1$ nM, and $K_3 = 2$ pM. Theoretical points were determined as a function of the total concentration of EGF for a particular concentration of EGF receptor using eqs 12, 14, and 15, and a rectangular hyperbola was fit to the points from which a value for the parameter $[EGF]_{TOT,0.5}$ was determined. A series of such theoretical values for the parameter $[EGF]_{TOT,0.5}$ were used to construct the theoretical curve.

detergent extracts of a higher concentration (Table 3). These results suggest that confinement in the membrane does not alter the effect of the concentration of EGF significantly. It is not possible, however, to vary systematically the concentration of EGF receptor when it is in a membrane.

Dimerization of EGF Receptor as a Function of the Concentrations of Both EGF and EGF Receptor. The samples assayed for dimerization of EGF receptor were exposed to EGF under the same conditions as those samples in which the activation of EGF receptor had been followed by the assays for the tyrosine kinase activity so that the kinetics of the two processes could be compared. Because the immunoblots for the dimerization assays performed with either purified placental extracts or unpurified placental extracts were too blurred to be interpreted quantitatively, all results for the dimerization assays came from extracts of A-431 cells. Samples (30 μL) of extracts of A-431 cells were mixed with EGF at the noted final concentrations for different intervals before the amount of dimer was measured by quantitative cross-linking with 220 mM glutaraldehyde for 1 min (Figure 6). As previously reported (Canals, 1992), a small amount (about 10%) of the EGF receptor in the various extracts was dimeric in the absence of EGF, and at the longer times and higher concentrations of EGF, almost all (>93%) of the EGF receptor has become dimeric.

The band of dimeric EGF receptor is always quite broad on gels of cross-linked protein (Figure 6; Canals, 1992). The reasons that we believe it to represent only dimeric EGF receptor are that its formation is second-order in the concentration of monomeric EGF receptor (Canals, 1992) and that there is no clear division of this band into dimer and trimer. One possible reason for its broadness is that,

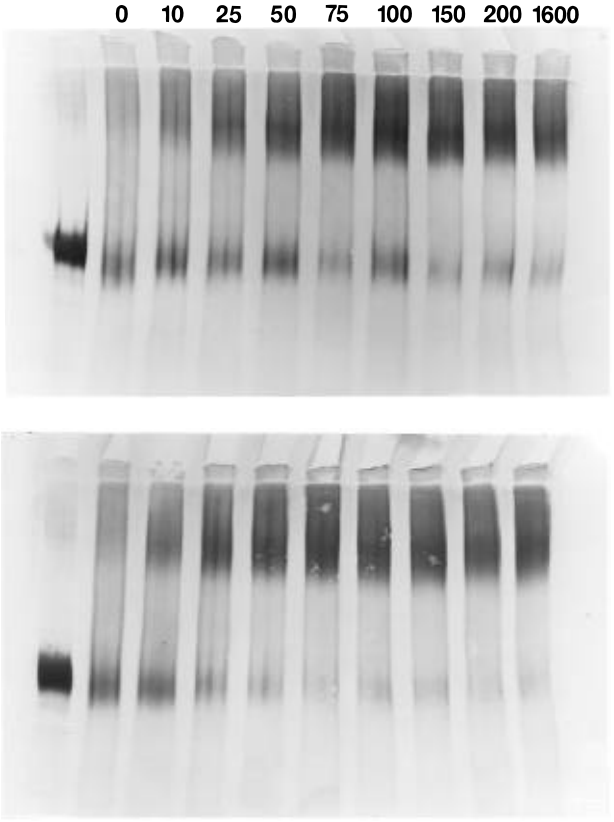


FIGURE 6: Immunoblots following the dimerization of EGF receptor as a function of the total concentration of EGF at different times of exposure. Samples of an extract of A-431 cells (30 nM in EGF receptor) were exposed to the noted total concentrations of EGF (nanomolar) for 5 min (upper panel) or 60 min (lower panel). After the specified time of exposure, glutaraldehyde was added to 220 mM for 1 min, and an excess of glycine was then added to quench the reaction. Samples were dissolved by adding SDS, spun through a G-50 spin column (1 mL), and submitted to electrophoresis and immunoblotting. The monomeric and dimeric forms of EGF receptor were stained using a primary antibody against the carboxy terminus of EGF receptor and a conjugate of alkaline phosphatase and goat anti-rabbit immunoglobulin.

following its dimerization, the dimer of EGF receptor could form heterocomplexes with other cellular proteins such as structural proteins or other proteins under its control. If such heterocomplexation were occurring, it would broaden the band. It would not, however, affect the quantification of the fraction of the protein that has dimerized, f_{dimer}, because this is based entirely on immunostaining with an immunoglobulin that recognized only the carboxy terminus of the polypeptide of EGF receptor. Therefore, as long as the corrections required are appropriate, as we believe them to be, the immunostaining measures only the relative numbers of polypeptides of EGF receptor in monomeric and dimeric complexes, respectively, regardless of how many other polypeptides are associated with either complex of EGF receptor.

The extent of dimerization of EGF receptor, f_{dimer}, displayed saturation as a function of the concentration of EGF, and the data could be satisfactorily fit by the equation for a rectangular hyperbola. The values for the parameter $[EGF]^{dimer}_{TOT,0.5}$, the total concentration of EGF producing half of the maximum dimerization, for assays performed at short and long times were determined (Table 4) from these fits. The values for the parameter $[EGF]^{dimer}_{TOT,0.5}$ for dimerization of EGF receptor as a function of EGF concentration change

Table 4: Kinetic Values for the Dimerization of EGF Receptor as a Function of Time of Exposure to EGF[a]

[EGF receptor][b] (nM)	time with EGF (min)	f_{dimer}^{MAX}	$[EGF]_{TOT,0.5}^{dimer}$ (nM)
35	1	0.75	10
	60	1.0	30
20	1	0.60	35
	60	1.0	20
20	1	0.50	8
	60	0.80	10
15	1	0.75	70
	60	0.80	15
5	1	0.45	60
	60	0.95	60
—[c]	5	0.90	45
	15	0.80	100
	30	1.0	50

[a] Samples (30 μL) of an extract of A-431 cells were exposed to several concentrations of EGF for the noted times. The dimerization of EGF receptor by EGF was followed in the various samples as described in Figure 6. The resulting immunoblots were then quantitatively analyzed using densitometry, and the areas of absorbance of the stain from bands representing monomeric and dimeric forms of EGF receptor were used, following several corrections, to determine the fraction of the EGF receptor that had dimerized (f_{dimer}) at a given total concentration of EGF ($[EGF]_{TOT}$). The data were then fit to the equation for a rectangular hyperbola using a nonlinear least-squares program to obtain values for the parameters f_{dimer}^{MAX} and $[EGF]_{TOT,0.5}^{dimer}$. The parameter f_{dimer}^{MAX} was the value of the horizontal asymptote, and the parameter $[EGF]_{TOT,0.5}^{dimer}$ was the value for $[EGF]_{TOT}$ for the point on the hyperbola at which f_{dimer} was equal to $f_{dimer}^{MAX}/2$. [b] The concentration of EGF receptor is given as the final concentration in the solution prior to adding the reagents for the assay of dimerization. The value for this quantity was estimated from a turnover number of 5 min^{-1} and the value of the maximum velocity of the enzymatic reaction for a sample of the undiluted extract after 60 min of activation. [c] The concentration of EGF receptor was not determined in this particular experiment.

only slightly with time at different concentrations of EGF receptor. A systematic change with time in the value of the parameter $[EGF]_{TOT,0.5}^{dimer}$ that was less than experimental variation or that proceeded at short times (<1 min) would have escaped detection.

To examine the dimerization of EGF receptor as a function of the concentration of EGF at different concentrations of EGF receptor, dilutions of extracts of A-431 cells were made in the same manner as they were for the experiments monitoring activation of the tyrosine kinase activity. In some instances, the same cell extract was used for assays of both dimerization and tyrosine kinase to increase the reliability of comparisons. Samples (30 μL) of the variously diluted cell extracts were exposed to different concentrations of EGF (in a final volume of 45 μL) for 20 min, followed by quantitative cross-linking with 220 mM glutaraldehyde for 1 min. The results are presented as the fraction of dimer, f_{dimer}, as a function of the total concentration of EGF (Figure 7). The values of the parameter $[EGF]_{TOT,0.5}^{dimer}$ were determined in several such experiments (Table 5). As was the case with the values of the parameter $[EGF]_{TOT,0.5}^{act}$ for tyrosine kinase activation (Figure 4), the values of the parameter $[EGF]_{TOT,0.5}^{dimer}$ for the dimerization of EGF receptor increase monotonically with the concentration of EGF receptor (Figure 8), though the slope is considerably smaller. The value of the parameter $[EGF]_{TOT,0.5}$ for the dimerization of EGF receptor (Table 5) is always less than the value of the parameter $[EGF]_{TOT,0.5}$ for the activation of the tyrosine

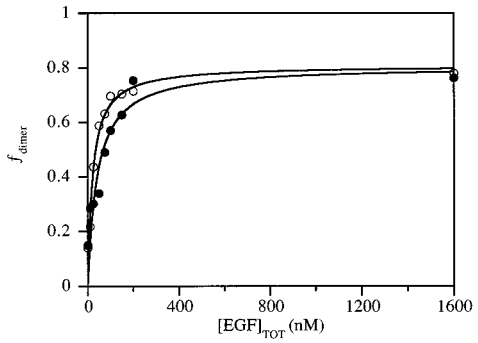


FIGURE 7: Dimerization of EGF receptor as a function of the total concentration of EGF at two concentrations of EGF receptor. A portion of an extract from A-431 cells (30 nM in EGF receptor) was diluted 6-fold, and samples from both this diluted extract and the undiluted extract were exposed to the noted total concentrations of EGF (nanomolar) for 20 min. Glutaraldehyde was then added to 220 nM to cross-link all dimers of EGF receptor, and the reaction was quenched with an excess of glycine after 1 min. The samples were then submitted to electrophoresis, and immunoblots were stained and then scanned using a densitometer. The fraction of dimeric EGF receptor (f_{dimer}) that had formed was calculated from the scans and is presented as a function of the total concentration of EGF ($[EGF]_{TOT}$). Rectangular hyperbolas were fit to the data by a nonlinear least-squares program.

Table 5: Equilibrium Values of the Parameter $[EGF]_{TOT,0.5}^{dimer}$ for the Dimerization of EGF Receptor as a Function of Its Concentration[a]

experiment	[EGF receptor][b] (nM)	$[EGF]_{TOT,0.5}^{dimer}$ (nM)
1	15	40
	2	20
2	35	45
	6	30
3	40	35
	6	2
4[c]	(1.0)	45
	(0.2)	15
5[c]	(1.0)	120
	(0.5)	150
	(0.25)	120

[a] The values of $[EGF]_{TOT,0.5}^{dimer}$ were determined in experiments identical to those of Figure 7. Rectangular hyperbolas were fit to the data with a nonlinear least-squares program. The parameter $[EGF]_{TOT,0.5}^{dimer}$ was the value of $[EGF]_{TOT}$ for the point on the hyperbola at which f_{dimer} was equal to $f_{dimer}^{MAX}/2$. [b] The concentration of EGF receptor is that of the final concentration in the volume of the mixture prior to the addition of the glutaraldehyde. The value for this quantity was estimated from a turnover number of 5 min^{-1} and the value of the maximum velocity of the enzymatic reaction for the sample of the extract of A-431 cells after 20 min of activation. [c] The concentration of EGF receptor was not determined in these assays so the dilution is noted in parenthesis, where the undiluted extract equals 1.0.

kinase at the same concentration of EGF receptor (Table 3) at both the longer and the shorter times (Tables 1 and 4). Consequently, the fraction of the EGF receptor that has dimerized (f_{dimer}) at a subsaturating level of EGF is always greater than the fraction of the EGF receptor that has been converted to active enzyme (v_0/V_{max}).

DISCUSSION

It has been shown that dimerization is the rate-limiting step for the activation of the tyrosine kinase of EGF receptor and that the activation of the tyrosine kinase is a second-order reaction in the concentration of EGF receptor (Canals, 1992). Both irreversible (Canals, 1992) and reversible

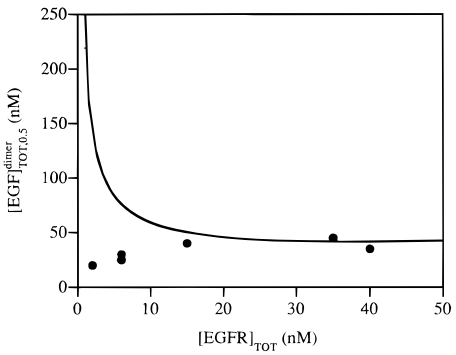


FIGURE 8: Relationship between the parameter $[EGF]^{dimer,}_{TOT,0.5}$ and the concentration of EGF receptor. The parameter $[EGF]^{dimer,}_{TOT,0.5}$ is the total concentration of EGF (nanomolar) at which the dimerization of EGF receptor was at half of the maximum value in a given experiment. Each value of the parameter $[EGF]^{dimer,}_{TOT,0.5}$ was obtained from a nonlinear least-squares fit of a rectangular hyperbola to the data as was done for the experiment displayed in Figure 7. The total concentrations of EGF receptor (nanomolar) were calculated from the values of V_{max} for each preparation of EGF receptor using the turnover number of 5 min^{-1} for angiotensin II observed for EGF receptor in extracts of A-431 cells. The points used in this plot are listed in Table 5. The curves drawn represent the behavior of theoretical values of the parameter $[EGF]_{TOT,0.5}$ for dimerization. For each concentration of EGF receptor, a set of theoretical points was generated with eqs 13−15 and fit with a hyperbola, and the resulting theoretical values of $[EGF]_{TOT,0.5}$ were then fit to smooth curves with cubic splines. The values for the dissociation constants used for the solid curve were the same as those used in Figure 5: $K_{dM} = 3.75$ μM, $K_{dD} = 7.5$ nM, $K_1 = 50$ μM, $K_2 = 1$ nM, and $K_3 = 2$ pM.

(Yarden & Schlessinger, 1987a,b; Schlessinger, 1988) mechanisms requiring dimerization during the activation of EGF receptor have been proposed as well as a mechanism similar to that recently proposed for the activation of growth hormone receptor by growth hormone (Cunningham et al., 1991; Lax et al., 1991). A common feature of all of these proposals is that all forms of dimeric receptor (H_iR_2, where $i = 0-2$) have been assumed to be active. These proposals can be discussed in turn, each in light of the results presented here. The peculiar features of the present results with which a given mechanism must be consistent are the sigmoid behavior of the binding of EGF (Figure 2), the lack of any inhibition of activation by high concentrations of EGF, the decrease in the value of the parameter $[EGF]_{TOT,0.5}$ for activation of the tyrosine kinase as the concentration of EGF receptor is increased (Figure 5), and the smaller value of the parameter $[EGF]_{TOT,0.5}$ for dimerization (Table 5) than for activation of the tyrosine kinase (Table 3).

In order to compare the simulations for the various mechanisms to be discussed with the experimental data presented in this report for the behavior of $[EGF]_{TOT,0.5}$ as a function of the concentration of EGF receptor (Figures 4, 5, and 8 and Tables 3 and 5), the following procedure was adopted. Schemes were drawn for the proposed mechanisms, and equations were derived for describing the activation of the tyrosine kinase or the fraction of dimer predicted by each of these schemes as a function of the total concentration of EGF and the total concentration of EGF receptor. Individual points of theoretical data were then calculated using these equations, and rectangular hyperbolas were fit to these theoretical data, just as they had been fit to the experimental data. From these rectangular hyperbolas, theoretical values for $[EGF]^{act}_{TOT,0.5}$ and $[EGF]^{dimer}_{TOT,0.5}$ were obtained over the range of the concentrations of both EGF and EGF receptor explored in the experiments. The various dissociation constants and rate constants in the equations were set so that the parameters $[EGF]^{act}_{TOT,0.5}$ or $[EGF]^{dimer}_{TOT,0.5}$ calculated from the theoretical mechanisms were as similar as possible to those of the experimental data in the range of the concentrations of EGF receptor used. It was possible to compare the behavior of each mechanism with the actual data by solving the equations and plotting curves of theoretical data for the various mechanisms.

Recent studies of growth hormone receptor have shown that two molecules of the artificially produced, monomeric extracellular domain of the receptor can cooperate to bind one molecule of growth hormone and form an asymmetric complex (de Vos et al., 1992; Cunningham et al., 1991). The relevance of such a complex to the mechanism of activation of growth hormone receptor is unknown. In the proposal for the activation of EGF receptor that is based on the behavior of the extracytoplasmic domain of growth hormone receptor, the only accessible dimer would be a complex containing two monomers of EGF receptor and one molecule of EGF, and this would also be the only enzymatically active form. Application of the proposed mechanism for the activation of growth hormone receptor to the activation of EGF receptor would produce the scheme

$$\begin{array}{ccccc} 2R + 2H & \underset{K_{dM}/2}{\rightleftharpoons} & R + H + HR & \underset{2K_{dM}}{\rightleftharpoons} & 2RH \\ & & \Big\downarrow\Big\uparrow K_2' & & \\ & & RHR + H & & \end{array} \qquad (6)$$

where R is the monomer of EGF receptor, H is epidermal growth factor, K_{dM} is the dissociation constant for EGF from its binding site on monomeric EGF receptor, and K_2' is the dissociation constant for the dimer. An equation was derived for the fraction of activated receptor, RHR, as a function of the concentration of free epidermal growth factor, containing as parameters the dissociation constant K_{dM}, the dissociation constant K_2', and the total concentration of receptor $[R]_{TOT}$. From this expression, theoretical values for v_0/V_{max} could be calculated

$$\frac{v_0}{V_{max}} = \frac{2[RHR]}{[R]_{TOT}} = \frac{K_{dM}K_2'}{8[R]_{TOT}[H]}\left\{\left[\left(1 + \frac{[H]}{K_{dM}}\right)^2 + \frac{8[R]_{TOT}[H]}{K_{dM}K_2'}\right]^{1/2} - 1 + \frac{[H]}{K_{dM}}\right\}^2 \qquad (7)$$

where v_0 is the initial velocity of the enzymatic reaction of EGF receptor activated at any concentration of EGF, V_{max} is the initial velocity that EGF receptor would display if all of it were in the form RHR, [RHR] is the concentration of active dimeric receptor, [H] is the concentration of free EGF in solution, and $[R]_{TOT}$ is the concentration of EGF receptor in all of its forms. The factor of 2 before [RHR] is required to adjust for the fact that there are two monomers, R, in each molecule of RHR. The amount of receptor−hormone complex that would be formed as a function of the total concentration of EGF in all of its forms, $[H]_{TOT}$, and hence

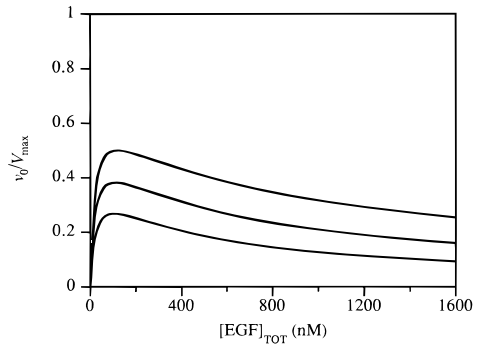


FIGURE 9: Theoretical activation of EGF receptor predicted by the mechanism that has been proposed for growth hormone receptor. Equations 7−9 were used to calculate the theoretical tyrosine kinase activity (v_0/V_{max}) of activated EGF receptor as a function of the total concentration of EGF (nanomolar) according to the proposed mechanism of activation for growth hormone receptor (scheme 6). The parameter V_{max} is the value of the initial velocity that would have been observed if all of the EGF receptor had dimerized and were active. Theoretical data were fit by cubic splines to produce the smooth curves. Equilibrium constants were chosen ($K_{dM} = 100$ nM, $K_2' = 10$ nM) to give half-maximum points similar to the experimental half-maximum values for the activation of EGF receptor. Each curve was for a different value of the total concentration of EGF receptor (10, 20, and 40 nM in ascending order).

the value for v_0/V_{max} (Figure 9), can then be calculated by noting that

$$[H]_{TOT} = [H]\left(1 + \frac{[R]}{K_{dM}} + \frac{[R]^2}{K_{dM}K_2'}\right) \quad (8)$$

where [R] is the concentration of unliganded, monomeric EGF receptor

$$[R] = \frac{\left[\left(1 + \frac{[H]}{K_{dM}}\right)^2 + \frac{8[H][R]_{TOT}}{K_{dM}K_2'}\right]^{1/2} - 1 + \frac{[H]}{K_{dM}}}{\frac{4[H]}{K_{dM}K_2'}} \quad (9)$$

As the concentration of hormone increases, the amount of the complex between growth hormone and the two domains of growth hormone receptor reaches a maximum and then decreases at higher concentrations of hormone (Cunningham et al., 1991). The same effect is observed in the theoretical behavior predicted by scheme 6 (Figure 9). The experimentally observed activation of EGF receptor, however, does not display this behavior because the concentration of active tyrosine kinase increases as a function of the concentration of EGF until it reaches saturation (Figure 3) and does not display any inhibition by EGF. There was no indication of any decrease in enzymatic activity at the higher levels of EGF. Therefore, the EGF-dependent activation of EGF receptor does not resemble the formation of the crystallographically observed complex between the extracytoplasmic domain of growth hormone receptor and growth hormone.

The behavior observed experimentally at equilibrium, however, is not inconsistent with scheme 6. If the value of K_2' is made several orders of magnitude smaller than K_{dM}, the inhibition by high concentrations of EGF is not significant even within the large range of concentrations that was sampled in the experiments described here. For example, if the value of K_{dM} is made 10 000 nM and the value of K_2' is made 0.1 nM, the data displayed in Figure 3 can be fit by eqs 7−9, and there is no significant inhibition by EGF even at concentrations of 16 μM. When equations for rectangular hyperbolas are fit to sets of such theoretical data, the behavior of the parameter $[EGF]_{TOT,0.5}$ as a function of the concentration of EGF receptor also was similar to the experimental data (Figures 4 and 5). It should be noted, however, that, if the value of K_2' is so much smaller than the value of K_{dM}, the association of the two monomers cannot be the result of forming only the two interfaces between the two respective monomers of EGF receptor and the one molecule of EGF. It is also necessary that either a global conformational change occur in the EGF upon the formation of the first interface to increase its affinity for the second monomer dramatically or there must be extensive energetically favorable contacts, which do not involve the molecule of EGF, between the two protomers of EGF receptor themselves.

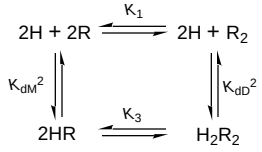
The scheme based on the behavior of the extracellular domain of growth hormone receptor (scheme 6), however, is inconsistent with the behavior of the enzymatic activity of EGF receptor as a function of the concentration of EGF at short times of exposure, with the differences seen between activation and dimerization, and with the sigmoid behavior of the binding of EGF. Theoretical curves generated by kinetic equations describing the behavior of scheme 6 immediately after the addition of EGF to the solution do not display saturation in the range of concentrations sampled in the experiments but pass through maxima at much higher levels of EGF. The values of the parameter $[EGF]^{act}_{TOT,0.5}$ calculated from these curves are much greater than any of the values measured experimentally at the shorter times (Table 1). In addition, if scheme 6 were operating, the values of the parameter $[EGF]_{TOT,0.5}$ would have to be the same for both activation and dimerization because the only active enzyme would be the one that has dimerized, RHR. This aspect of scheme 6 disagrees with the experimental results, because the value of the parameter $[EGF]^{dimer}_{TOT,0.5}$ (Tables 4 and 5) is always less than the value of the parameter $[EGF]^{act}_{TOT,0.5}$ (Tables 1 and 3). In addition, if scheme 6 were the correct description of the mechanism of activation, the maximum velocity of the activated enzyme should not necessarily be proportional to the concentration of EGF receptor (Figure 9), as it is (Table 3). For the observed maximum velocity to be proportional to the concentration of EGF receptor, the maxima of the theoretical curves of v_0/V_{max} would all have to have the same value for every concentration of EGF receptor tried, which they usually do not (Figure 9).

The final inconsistency between the experimental results and the theoretical behavior predicted by scheme 6 is the sigmoid behavior observed in the binding of EGF. When binding curves for EGF were calculated for scheme 6, the parameter n was always less than 1 when dissociation constants were used that gave theoretical data similar to the data observed experimentally (Figure 3). According to the nonlinear, least-squares fits, however, every binding curve for EGF displayed sigmoid behavior with a value of n greater than 1. Even though the experimental fluctuation was high and even though, of necessity, the values of the Hill coefficient n depend on the deviation from ideality of the data at low concentrations of [^{3}H]EGF where the bound counts per minute were usually less than 100 cpm, the results

were consistently sigmoid, and over seven experiments, the mean of the values for the Hill coefficient was statistically greater than 1. Because of the sigmoid behavior in the binding of hormone, the proportionality between V_{max} and the concentration of EGF receptor, the difference between the values of the parameter $[EGF]_{TOT,0.5}$ for dimerization and activation, and the saturation observed at short times of exposure to hormone, scheme 6 is inconsistent with the experimental observations.

In a previously described irreversible mechanism for the activation of EGF receptor (Canals, 1992), it was proposed that the dimerization of the protein would involve an irreversible step preventing the dimers of EGF receptor from dissociating to monomers. If EGF receptor, however, were activated by an irreversible mechanism, its level of activation would continuously increase with time until completion of the reaction, and the values of the parameter $[EGF]^{act}_{TOT,0.5}$ would decrease continuously with time. This would mean that any level of EGF could activate all of the EGF receptor in a given sample given enough time. In fact, however, after EGF receptor has been exposed to EGF for 10−15 min, the value of the parameter $[EGF]^{act}_{TOT,0.5}$ does not change significantly, even at exposure times of 60 min (Table 1). These results are inconsistent with an irreversible mechanism of activation.

Another proposal for the mechanism of activation of EGF receptor is that the monomeric and dimeric forms of the enzyme are in equilibrium and the binding of EGF shifts this equilibrium to favor the enzymatically active, dimeric form of EGF receptor (Yarden & Schlessinger, 1987b). This reversible mechanism for the activation of EGF receptor by EGF is



(10)

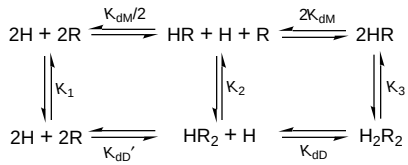
where K_1 and K_3 are the dissociation constants for dimeric forms of unoccupied EGF receptor and fully occupied EGF receptor, respectively. The active species in this mechanism are those receptors that are dimeric, and they include R_2 and H_2R_2. The steps required for dimerization and activation in this mechanism are the bimolecular steps governed by K_1 and K_3. To determine the fraction of active EGF receptor as a function of the concentration of EGF, equations were derived for the behavior of the levels of tyrosine kinase activity as a function of the total concentrations of EGF and EGF receptor.

The theoretical curves for ν_0/V_{max} as a function of the total concentration of EGF generated in these simulations describe behavior displaying saturation and resembling that observed in Figure 3. The value of the parameter $[EGF]_{TOT,0.5}$ as a function of the concentration of EGF receptor was also similar to that of the values for the parameter $[EGF]_{TOT,0.5}$ for the activation of the tyrosine kinase that were determined experimentally (Figures 4 and 5).

One possible inconsistency between scheme 10 and the experimental observations lies in the fact that unliganded, dimeric EGF receptor has yet to be observed. As mentioned earlier, about 10% of the EGF receptor isolated from A-431 cells is dimeric as determined by cross-linking with glutaraldehyde in the absence of EGF. This population probably arises from nonspecific or artifactual dimerization or aggregation. When the concentration of EGF receptor is increased in the absence of EGF, the fraction of this dimeric receptor does not increase (Canals, 1992) as it must if this unliganded dimer were involved in the equilibria described in scheme 10. Therefore, there is no evidence for the unliganded, dimeric form of EGF receptor described in scheme 10 that is in free equilibrium with the monomeric form. For scheme 10 to fit the experimental results, the parameter K_1 must be large enough to prevent dimerization from becoming significant in the absence of EGF. Even at the highest concentrations of EGF receptor examined in these studies (80−100 nM), there was no indication that the dimer could form in the absence of EGF, so it must be the case that K_1 is greater than 500 nM.

The experimental observations presented in this report are inconsistent with the mechanisms for the activation of EGF receptor shown in scheme 10 in another aspect. Because all forms of dimeric EGF receptor are active in scheme 10, there should not be any difference between the parameters $[EGF]_{TOT,0.5}$ for the dimerization of EGF receptor and the activation of the tyrosine kinase. The results reported here show that there is (Tables 3 and 5).

As the equations for the previously proposed mechanisms and other possible mechanisms were being derived, it became clearer which features were necessary in a mechanism that could explain the experimental observations for the binding of EGF, the dimerization of EGF receptor, and the activation of the tyrosine kinase. A mechanism that is consistent with the data gathered in these experiments is



(11)

where K_2 is the dissociation constant for partially occupied dimeric EGF receptor and K_{dD}' is the dissociation constant for hormone from HR_2. The only active species is H_2R_2, and in the range of concentrations sampled in these experiments, dimerization occurs only when at least one of the two monomers of EGF receptor has EGF bound to it. The three dimeric forms of EGF receptor are R_2, HR_2, and H_2R_2, but R_2 is never present in significant concentrations and HR_2 does not display tyrosine kinase activity; only the complex H_2R_2 is enzymatically active. The fraction of enzymatically active EGF receptor would be

$$\frac{\nu_0}{V_{max}} = \frac{2[H_2R_2]}{[R]_{TOT}} = \frac{2[H]^2[R]^2}{K_{dM}{}^2K_3[R]_{TOT}} \qquad (12)$$

and the fraction of dimeric EGF receptor would be

$$f_{dimer} = \frac{2[R_2]_{all}}{[R]_{TOT}} = \frac{2[R]^2}{[R]_{TOT}}\left(\frac{1}{K_1} + \frac{[H]}{K_2K_{dM}} + \frac{[H]^2}{K_3K_{dM}{}^2}\right) \qquad (13)$$

where $[R_2]_{all}$ is again all forms of dimeric EGF receptor, R_2,

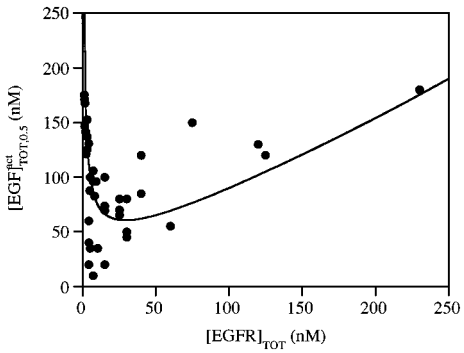


FIGURE 10: Relationship between the experimental measurements of the parameter $[EGF]^{act}_{TOT,0.5}$ for activation of EGF receptor (points) and theoretical estimates of its behavior (curves) as a function of the total concentration of EGF receptor. The points are the data from all of the experiments (Table 3 and Figures 4 and 5). The total concentration of EGF receptor (nanomolar) was either determined directly by the assay of EGF binding or calculated from the value of the parameter V_{max} for the preparation of EGF receptor and the appropriate turnover numbers for phosphorylation of angiotensin II. The solid curve was produced with eqs 12, 14, and 15 using values for the dissociation constants of $K_{dM} = 3.75$ μM, $K_{dD} = 7.5$ nM, $K_1 = 50$ μM, $K_2 = 1$ nM, and $K_3 = 2$ pM. For each concentration of EGF receptor, a set of theoretical points was generated and fit with a hyperbola. The resulting theoretical values of $[EGF]^{act}_{TOT,0.5}$ were used to construct the smooth curve with cubic splines.

HR_2, and H_2R_2, [R] is the concentration of free EGF receptor

$$[R] = \left[\left[\left(1 + \frac{[H]}{K_{dM}}\right)^2 + 8[R]_{TOT}\left(\frac{1}{K_1} + \frac{[H]}{K_2K_{dM}} + \frac{[H]^2}{K_3K_{dM}^2}\right)\right]^{1/2} - 1 + \frac{[H]}{K_{dM}}\right]/4\left(\frac{1}{K_1} + \frac{[H]}{K_2K_{dM}} + \frac{[H]^2}{K_3K_{dM}^2}\right) \quad (14)$$

and [H] is the concentration of free EGF

$$[H]_{TOT} = [H]\left(1 + \frac{[R]}{K_{dM}} + \frac{[R]^2}{K_2K_{dM}} + \frac{2[H][R]^2}{K_3K_{dM}^2}\right) \quad (15)$$

The main difference between scheme 10 and scheme 11 is that the only active dimeric form of EGF receptor in scheme 11 is H_2R_2. Although we have included the unliganded, dimeric form of receptor, R_2, there is no reason to believe that unliganded, dimeric EGF receptor would ever form in the cell. Because its concentration has never been significant under the explored experimental conditions, its properties and even its existence are in doubt.

The values for the various dissociation constants in eqs 12−15 were varied by trial and error until the calculated values resembled the experimental values for the parameter $[EGF]_{TOT,0.5}$ for both tyrosine kinase activity and dimerization. The equations derived from the mechanism shown in scheme 11 could generate theoretical curves for the activation of the tyrosine kinase activity (Figure 10) that closely resemble the behavior displayed by the experimental data (Table 3 and Figures 4 and 5) both at high concentrations of EGF receptor and at low concentrations (see the theoretical curve in Figure 5). The theoretical curves for the levels of dimerization (Figure 8) display the differences observed between the parameters $[EGF]_{TOT,0.5}$ for activation of the tyrosine kinase and the dimerization. The data for dimerization, however, do not display the increase in the parameter $[EGF]^{dimer}_{TOT,0.5}$ predicted at low concentrations of EGF receptor. It may be that the concentrations of EGF receptor examined experimentally could not be made low enough. The quantification of the dimerization of EGF receptor is limited by the sensitivity of the immunoblots that are probed with polyclonal antibodies recognizing the carboxy terminus of the receptor. At very low concentrations of EGF receptor, it would be difficult to quantify the fraction of dimeric and monomeric forms of EGF receptor present at a particular concentration of EGF. Nevertheless, using the same set of parameters (curves in Figures 8 and 10), it was possible to produce values for the parameter $[EGF]_{TOT,0.5}$ that mimicked the increase as a function of the concentration of EGF receptor seen for both dimerization and tyrosine kinase activity at the higher concentrations.

Equation 14 was also used to calculate curves for the binding of EGF to EGF receptor together with the equation

$$[H\cdot site] = \frac{[H][R]}{K_{dM}}\left(1 + \frac{[R]}{K_2} + \frac{2[H][R]}{K_2K_{dD}}\right) \quad (16)$$

where [H·site] is the concentration of all forms of bound EGF. In several simulations in which the dissociation constants for scheme 11 were varied, values for n as large as 1.6 were readily observed; none was less than 1. This observation establishes that sigmoid behavior for the binding of hormone is required by this mechanism. This requirement agrees with the experimental observations (Figure 2).

Because the equations derived from the mechanism of scheme 11 were complex and each contained several adjustable parameters, it was not possible to fit any of the equations to the data by any program at our disposal for nonlinear least-squares numerical analysis. The theoretical curves that have been presented were all fit to the data by trial and error and do not necessarily represent the most ideal choice of the fitting parameters. Therefore, the values of the parameters used are not definitive or probably even close to their actual values. Ideally, there should be a set of dissociation constants that can duplicate the experimental data for the binding of EGF, the dimerization of the EGF receptor, and the activation of the tyrosine kinase. It would probably be possible to find dissociation constants for the theoretical mechanism shown in scheme 11 that do fit all of the experimental results more closely if a least-squares computing program were available that could handle equations as complex as these.

The mechanism proposed in scheme 11 has several unique features. Dimeric EGF receptor may form in the absence of EGF but is not present at significant concentrations in the range examined in the experiments. The complexes R_2 and HR_2 are dimeric but inactive. Dimeric EGF receptor must be occupied by two molecules of EGF for enzymatic activity to be expressed. The most important conclusion, however, that follows from these simulations is that the behavior of the parameter $[EGF]_{TOT,0.5}$ as a function of the concentration of EGF receptor is consistent with an absolute requirement for dimerization of the protein in its activation. The apparent invariance of this parameter at the higher concentrations of EGF receptor (Figures 4 and 8) was not due to the absence of such a requirement, as had been previously proposed (Koland & Cerione, 1988), but to the fact that the concentrations of EGF and EGF receptor were in the same range so that the binding of the EGF was

significantly decreasing the free concentration of the EGF. When this is accounted for, the data are readily reconciled with a mechanism requiring dimerization for activation. This reconciliation is reinforced by the increase in the value of the parameter $[EGF]^{act}_{TOT,0.5}$ at the lower concentrations of EGF receptor (Figure 5).

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