Implications of epidermal growth factor (EGF) induced egf receptor aggregation

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ABSTRACT To investigate the role of receptor aggregation in EGF binding, we construct a mathematical model describing receptor dimerization (and higher levels of aggregation) that permits an analysis of the influence of receptor aggregation on ligand binding. We answer two questions: (*a*) Can Scatchard plots of EGF binding data be analyzed productively in terms of two noninteracting receptor populations with different affinities if EGF induced receptor aggregation occurs? No. If two affinities characterize aggregated and monomeric EGF receptors, we show that the Scatchard plot should have curvature characteristic of positively cooperative binding, the opposite of that observed. Thus, the interpretation that the high affinity population represents aggregated receptors and the low affinity population nonaggregated receptors is wrong. If the two populations are interpreted without reference to receptor aggregation, an important determinant of Scatchard plot shape is ignored. (*b*) Can a model for EGF receptor aggregation and EGF binding be consistent with the "negative curvature" (i.e., curvature characteristic of negatively cooperative binding) observed in most Scatchard plots of EGF binding data? Yes. In addition, the restrictions on the model parameters required to obtain negatively curved Scatchard plots provide new information about binding and aggregation. In particular, EGF binding to aggregated receptors must be negatively cooperative, i.e., binding to a receptor in a dimer (or higher oligomer) having one receptor already bound occurs with lower affinity than the initial binding event. A third question we consider is whether the model we present can be used to detect the presence of mechanisms other than receptor aggregation that are contributing to Scatchard plot curvature. For the membrane and cell binding data we analyzed, the best least squares fits of the model to each of the four data sets deviate systematically from the data, indicating that additional factors are also important in shaping the binding curves. Because we have controlled experimentally for many sources of receptor heterogeneity, we have limited the potential explanations for residual Scatchard plot curvature.

INTRODUCTION

Ligand induced receptor clustering is a phenomenon common to many receptor systems. Ligands with valence greater than one, of the appropriate size and flexibility, can aggregate receptors by cross-linking them, i.e., bridging two or more receptors. Multivalent antigens aggregate cell surface immunoglobulin (Schreiner and Unanue, 1976), IgG-containing immune complexes aggregate FcRII receptors (Unkeless et al., 1981), and bivalent platelet-derived growth factor (PDGF) clusters PDGF receptors (Heldin et al., 1989). Although monovalent ligands are incapable of crosslinking receptors, some monovalent ligands, such as interleukin 2 (IL-2) (Hatakeyama et al., 1989) and epidermal growth factor (EGF) (Yarden and Schlessinger, 1987*b*), induce receptor clustering. Presumably, occupation of the receptor binding site by these ligands affects one or more extracellular domains responsible for receptor clustering, i.e., these ligands modulate receptor clustering by allosteric mechanisms.

Whenever ligands aggregate receptors the question arises as to what role receptor aggregation plays in transduction of signals across the plasma membrane. For some receptor systems it is clear that receptor aggregation is necessary for cell signaling. For example, monovalent ligands that bind to IgE on sensitized basophils and mast cells trigger no cellular responses (Siraganian et al., 1975), while multivalent ligands that aggregate IgE trigger a host of responses (reviewed in Metzger et al., 1986). For other receptor systems the necessity of receptor aggregation for the generation of transmembrane signals has not been demonstrated. Two IL-2 receptors have been isolated, a p55 and p75 chain that bind IL-2 with low and intermediate affinities respectively. These receptors cluster into p55-p75 heterodimers that bind IL-2 with high affinity (reviewed in Smith, 1989). The presence of p55-p75 heterodimers allows cells to bind IL-2 at pM rather than nM concentrations, suggesting that an important function of p55-p75 heterodimer formation is to increase the cell's sensitivity to IL-2. However, a unique signal function for the p55-p75 heterodimer has not been identified.

The binding of epidermal growth factor (EGF) to solubilized EGF receptors causes these receptors to dimerize rapidly (Yarden and Schlessinger, 1987*b*; Böni-Schnetzler and Pilch, 1987; Cochet et al., 1988; Fanger et al., 1989). The soluble, extracellular, EGF-binding domain is sufficient for receptor aggregation (Lax et al., 1991). Fluorescence resonance energy transfer studies of EGF receptors in membranes isolated from A431 cells indicate enhanced dimerization in the presence of EGF and divalent metal ion activators (Carraway III et al., 1989), but the method does not detect EGF induced dimerization on intact A431 cells (Carraway III and Cerione, 1991). EGF induced receptor dimerization has been detected in chemical cross-linking studies both on isolated plasma membranes (Cochet et al., 1988; Northwood and Davis, 1988*a*, *b*) and on intact cells (Fanger et al., 1986; Cochet et al., 1988; Northwood and Davis,

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1988*b*; Sorkin and Carpenter, 1991). Higher oligomers of EGF receptors have also been detected on isolated membranes (Northwood and Davis, 1988*a*) and in solution (Lax et al., 1991). On A431 cells dimerization is considerably reduced in the absence of EGF, but still detectable by chemical crosslinking (Cochet et al., 1988). However, fluorescence resonance energy transfer studies failed to observe EGF induced dimerization on A431 cells, even though the method detected significant dimerization on purified plasma membranes and membranes from disrupted cells (Carraway III and Cerione, 1991).

The mature EGF receptor is a single polypeptide chain of 1,186 amino acid residues possessing an extracellular EGF binding domain, a single membrane spanning region and an intracellular protein tyrosine kinase domain. Binding of EGF to the external portion of the EGF receptor activates the internal protein tyrosine kinase domain, leading to increased substrate phosphorylation and auto-phosphorylation of receptor tyrosine residues (reviewed in Carpenter, 1987).

Two classes of models have been proposed to explain how the binding of EGF to the extracellular binding domain of the receptor activates the cytoplasmic kinase domain. In one class of models, activation occurs through an intramolecular mechanism (Staros et al., 1985; Koland and Cerione, 1988). Binding of EGF is conjectured to induce a conformational change in the receptor which is transmitted through the transmembrane domain and activates intrinsic receptor protein tyrosine kinase activity (Bertics and Gill, 1985; Weber et al., 1984). In the other class of models, activation is controlled by receptor aggregation (Schlessinger, 1986; 1988; Yarden and Schlessinger, 1987*a*). In these models, the binding of EGF to monomeric receptors enhances receptor aggregation. The aggregated receptors have higher affinity for EGF than the unaggregated receptors and possess elevated protein tyrosine kinase activity. Both models are supported by experimental data. Because activation of tyrosine kinase by EGF has been observed for monomeric EGF receptors that are either detergent-solubilized (Koland and Cerione, 1988) or membrane bound (Northwood and Davis, 1988*a*), aggregation cannot be an absolute requirement for receptor activation. However, because cross-linking EGF receptors with bivalent antibodies stimulates receptor tyrosine kinase while exposure to Fab fragments does not (Spaargaren et al., 1991), aggregation is clearly sufficient to activate the receptor. Also, Honegger et al. (1989) showed that in a solution of two mutant receptors, one lacking and the other possessing kinase activity, the receptor lacking kinase activity was phosphorylated and phosphorylation was dependent on EGF. Thus, this "auto"-phosphorylation of solubilized mutant EGF receptors was mediated by intermolecular interactions. Honegger et al. (1989) proposed that phosphorylation was facilitated by EGF receptor oligomerization and they were able to isolate oligomers that contained both types of mutant EGF receptors.

A major component of the dimerization-activation hypothesis is that the affinity of EGF for aggregated receptors is higher than for monomeric receptors (Böni-Schnetzler and Pilch, 1987; Yarden and Schlessinger, 1987*a*; Northwood and Davis, 1988*a*). This was postulated to explain why a high affinity subclass of receptors appears dominant in mediating signal transduction and biological responses (Yarden and Schlessinger, 1987*a*; Defize et al., 1989; Bellot et al., 1990). The high affinity subclass has been proposed to represent the aggregated, active fraction of the receptor population (Schlessinger, 1988). Despite the evidence for EGF receptor dimer formation, its effect on EGF binding, and the related effect of EGF binding on receptor dimerization, published analyses of EGF binding data are based on models that do not include receptor aggregation. When EGF binds to EGF receptors, Scatchard plots usually show negative curvature, i.e., curvature associated with negatively cooperative binding. One way such curvature can arise other than by negative cooperativity is if there is heterogeneity in the binding affinities of the receptors. For this reason much of the published EGF binding data has been analyzed in terms of two populations of receptors, one with high and one with low affinity for EGF. Often, the implicit interpretation of the two populations is that one is a low affinity population of monomeric receptors and the other a high affinity population of receptors in dimers or higher oligomers. We will show that this interpretation is incorrect, because such a model predicts that a Scatchard plot will have curvature characteristic of positive cooperativity, the opposite of what is seen experimentally.

In this paper we develop a model for EGF receptor aggregation and binding, and use it to analyze equilibrium binding data. Through the model we investigate the implications of the three observations we have discussed concerning EGF receptor aggregation and EGF-EGF receptor binding: (*a*) EGF receptors self aggregate, i.e., in the absence of EGF there are some EGF receptor oligomers on the cell surface; (*b*) the addition of EGF increases the concentration of EGF receptors in oligomers; and (*c*) Scatchard plots for EGF binding to EGF receptors that are in solution, on closed vesicles or on cell surfaces do not exhibit positive cooperativity. We show that for these three results to be consistent with each other, either binding to oligomers must be negatively cooperative (i.e., the equilibrium constant for EGF binding to a single EGF receptor in a dimer must be higher when both sites are free than when only one site is free), or there must be significant contributions to the curvature in the Scatchard plot from sources other than genetic heterogeneity in the EGF receptor population, interactions with the cytoskeleton, or receptor-mediated endocytosis. These latter possibilities we eliminate by carrying out binding studies on closed membrane vesicles de-

rived from cells that normally express no EGF receptors and that have been transfected with either wild-type or mutant human EGF receptor cDNAs.

MATERIALS AND METHODS

General

Mouse EGF was purified from submaxillary glands (Savage and Cohen, 1972). EGF was iodinated with ^{125}I (Amersham Corp., Arlington Heights, IL) using Iodo-Beads (Pierce Chemical Co., Rockford, IL) according to the manufacturer's recommendations and free iodine separated from the radiolabeled ligands by dialysis or by passing the mixture over a 0.8 × 20 cm column of Sephadex G-10 equilibrated with PBS. The specific activity of ^{125}I-labeled EGF was generally between 600 and 1,800 cpm/fmol. Protein concentration was determined using the BCA assay (Pierce Chemical Co.) and using bovine serum albumin as a standard.

Cell culture

B82 mouse L cells, which contain no endogenous EGF receptors, and B82 cells transfected with wild type or mutated (M^{721}, $A^{654}M^{721}c'1022$, $c'1022$, and $M^{721}c'1022$) human EGF receptors were generated as previously described (Chen et al., 1989). A modified dihydrofolate reductase gene was the selectable marker for all transfections. B82 cells were grown in Dulbecco's modified Eagle's medium (DME, Flow Laboratories, Inc., McClean, VA) containing dialyzed 10% calf serum (HyClone). 5 μM methotrexate was added to the medium for those cells transfected with human EGF receptor.

Isolation of plasma membrane vesicles

Cells were grown to confluence in roller bottles. After rinsing four times with phosphate-buffered saline lacking Ca^{2+} and Mg^{2+} at 0°C, the cells were removed using a rubber policeman and pelleted at 600 *g* for 1 min. The cell pellet was weighed, followed by the addition of a 20-fold volume of homogenization buffer consisting of 10 mM Tris-Cl (pH 8.0), 2 mM EDTA and 10 μg/ml each of the protease inhibitors leupeptin, chymostatin and pepstatin. The cells were allowed to swell 5 min on ice and then homogenized with 10 strokes of a tight-fitting dounce homogenizer. Cell breakage was monitored by phase-contrast microscopy and was >90%. The homogenate was brought to 1 mM $MgCl_2$, 1.5 mM $CaCl_2$ and the nuclei were removed by centrifugation for 2 min at 1,000 *g*. The postnuclear supernatant (12 ml) was loaded on a sucrose step gradient consisting of 7 ml 50% sucrose and 15 ml 30% sucrose and centrifuged for 20 min at 10,000 rpm in an SW28 rotor at 4°C. Membranes at the 30–50% interface were collected, resuspended in 4 volumes of homogenization buffer and pelleted by centrifugation at 7,000 *g* for 5 min. The vesicles were resuspended to a final concentration of 1–2 mg/ml using a dounce homogenizer.

Binding to intact cells

Cells grown to confluence in 35 mm dishes were switched from growth medium to serum free DME containing 20 mM HEPES (pH 7.4), 0.1% BSA and no bicarbonate (D/H/B) 18 h before experiments. Cells were rapidly chilled to 0°C by rinsing twice with ice-cold WHIPS saline (20 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml poly-vinylpyrrolidone) and cold D/H/B medium containing ^{125}I-EGF was added. Concentrations of ^{125}I-EGF ranged between 0.05–170 nM. The cells were allowed to reach equilibrium at 0°C (4–6 hrs), aliquots of the medium were taken for determination of free ligand concentration, and the cells were rinsed six times with cold WHIPS saline. Receptor-associated ligand was then removed by acid-stripping at 0°C using 50 mM glycine-HCl, 100 mM NaCl, 2 mg/ml PVP, 2 M Urea, pH 3.0, and then counted in a gamma spectrophotometer. Nonspecific binding was determined by parallel binding studies using B82 cells lacking EGF receptors and was always less than 5% of total binding. Cell number was determined with a Coulter Counter.

Binding to membranes

Membrane concentrations were adjusted to between 1–2 mg/ml protein in 100 mM Hepes buffer, pH. 7.0. A 40 μl aliquot of membranes was added to 100 μl of 100 mM Hepes, 1 mg/ml BSA containing ^{125}I-EGF at concentrations ranging between 0.15–40 nM in microfuge tubes. The tubes were capped, mixed and then brought to equilibrium for 4 h at 22°C on a shaker platform. To each individual tube was added 1 ml of ice-cold WHIPS saline containing 1 mg/ml of bovine serum albumin (WHIPS/BSA) immediately followed by collection of the membranes on millipore GVWP 025 filters using a filtration manifold. The tubes and filters were rinsed 3 times with cold WHIPS/BSA. After air drying, the filters were counted in a gamma spectrophotometer. Nonspecific binding was determined by parallel binding studies using membranes isolated from B82 cells lacking EGF receptors.

Data analysis

Parameter estimation was carried out using a nonlinear least squares fitting routine ZXSSQ, from the International Mathematics and Statistics Library (IMSL). The routine is based on a finite difference, Levenberg-Marquardt algorithm.

THE MODEL AND THEORETICAL RESULTS

We construct the simplest equilibrium model that includes EGF receptor aggregation and EGF binding. Then we determine restrictions on the parameters imposed by the following experimental observations: (*a*) The EGF receptor forms dimers in the absence of EGF; (*b*) EGF receptor aggregation is enhanced in the presence of EGF; and (*c*) Scatchard plots for the equilibrium binding of EGF to EGF receptors show no positive cooperativity. (Derivations of the conditions we obtain are included in the Appendix.)

Because we consider an equilibrium model where there is no internalization of EGF receptors, the theory we present is applicable only to EGF receptors in solution, on cell membrane fragments, on vesicles, and on cells where internalization is blocked. Further, we consider only the case where EGF receptors are genetically homogeneous and where protein kinase C does not act on the EGF receptor to alter its affinity, thereby introducing heterogeneity in receptor affinity. Finally, we assume that only EGF receptor dimers can form. The dimer model we arrive at is that first proposed by Levitzki and Schlessinger (1974). In the Appendix we show how the model can be generalized to include higher oligomers and discuss the questions that arise when this is attempted.

Fig. 1 illustrates the binding and aggregation reactions that can occur in the model. We define six equilibrium constants: three equilibrium binding constants, K, K_1 and K_2 that characterize respectively the binding of EGF to an isolated receptor, to a receptor in a dimer where both binding sites are free, and to a receptor in a dimer where one site is free and one bound; and three equilibrium aggregation constants, K_x, K_{x1}, and K_{x2} that charac-

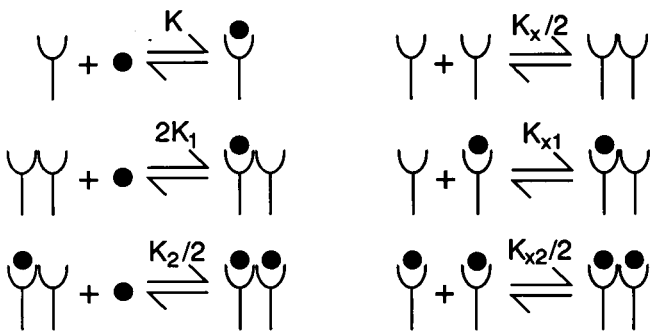

FIGURE 1 Shown are the binding and aggregation reactions that can occur when aggregation is restricted to dimer formation.

terize respectively the aggregation of two free receptors, one bound and one free receptor, and two bound receptors. (Note that the K's have units of volume whereas the K_x's have units of volume when the receptors are in solution and units of area when the receptors are on membranes.) Of the six equilibrium constants only four are independent. From detailed balance we have:

$$K_{x1} = K_x K_1 / K \tag{1}$$

$$K_{x2} = K_x K_1 K_2 / K^2. \tag{2}$$

In deriving Eqs. 1 and 2 we have assumed that the state of an aggregate is independent of the way in which it was formed. For example, Eq. 1 follows if a dimer with one site free and one bound is the same whether it is formed by an EGF molecule binding to a dimer with both sites free or by a free EGF receptor aggregating with a bound EGF receptor.

To be consistent with experimental observation 1, that EGF receptors aggregate in the absence of EGF, we simply require that

$$K_x \neq 0. \tag{3}$$

The second experimental observation is that the presence of EGF increases EGF receptor aggregation. For the model to be consistent with experiment, at a minimum it must have the property that the addition of EGF leads to an initial increase in receptor aggregation, i.e., in a plot of oligomerization versus EGF concentration the initial slope must be positive. For this to occur we must have $K_{x1} > K_x$ and therefore, from Eq. 1,

$$K_1 > K. \tag{4}$$

In Fig. 2 we show three different ways in which oligomerization could depend on the EGF concentration and increase initially with the addition of EGF. The concentration of EGF receptors in aggregates could be a monotonically increasing function of the EGF concentration, or it could increase initially to a maximum and then decline to a final value that is either greater than or less than its value in the absence of EGF. If the concentration of oligomers is greater in the presence of a large excess of EGF than in the absence of EGF, Fig. 2, *a* and *b*, then $K_{x2} > K_x$. This is because when there is a large amount of EGF present, at equilibrium almost all EGF receptors have bound EGF. Thus, at high EGF concentrations dimer formation takes place between EGF receptors that have bound EGF, and this is characterized by the aggregation constant K_{x2} (see Fig. 1). We note that from Eq. 2 the condition $K_{x2} > K_x$ implies that $K_1 K_2 > K^2$. In summary, both curves in Fig. 2, *a* and *b*, obey the conditions that $K_1 > K$ and $K_1 K_2 > K^2$. In addition to these conditions, in order for the curve to be monotonically increasing, Fig. 2 *a*, $K_2 > K$, whereas in Fig. 2 *b* we have that $K_2 < K$. In Fig. 2 *c*, where the initial slope is positive, but there are fewer dimers at very high EGF concentrations than in the absence of EGF, we have, as before, $K_1 > K$, but $K_1 K_2 < K^2$.

In the absence of EGF the fraction of receptors in dimers, d_0, is

$$d_0 = \frac{2K_x R_T + 1 - \sqrt{1 + 4K_x R_T}}{2K_x R_T}, \tag{5}$$

where R_T is the concentration of EGF receptors. Eq. 5 can be used to determine K_x, but this requires that both the concentration of EGF receptors and the fraction of these receptors in dimers in the absence of EGF be accurately determined.

It is useful to define the dimensionless lumped parameter,

$$k_x = K_x R_T, \tag{6}$$

because it is this parameter that determines what fraction of the EGF receptors are in dimers in the absence of EGF. For example, when $k_x = 1$, $d_0 = 0.38$, i.e., 38% of all EGF receptors are in dimers in the absence of EGF. Thus, in the absence of EGF, there is substantial dimerization when $k_x \geq 1$ and negligible dimerization when $k_x \ll 1$. (When $k_x \ll 1$ it follows from Eq. 5 that $d_0 \approx k_x$.)

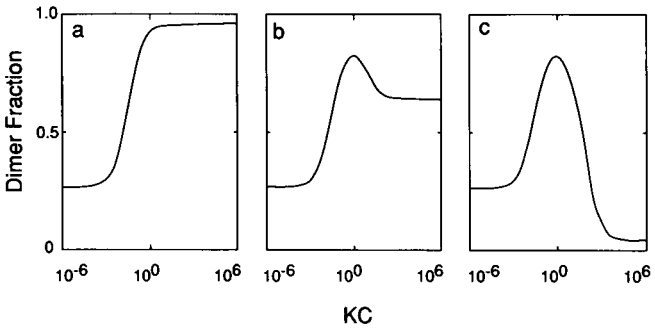

FIGURE 2 Shown are three possible ways in which dimer formation can depend on the free EGF concentration. The fraction of EGF receptors in dimers is plotted against the free EGF concentration. For each of the curves shown, $K_x R_T = 0.5$ and $K_1/K = 100$. The values of the parameter K_2/K were (*a*) 10, (*b*) 0.1 and (*c*) 0.001.

Finally, for our model to be consistent with experimental observation *c*, i.e., that the Scatchard plot shows no positive cooperativity, the following condition between the equilibrium binding and aggregation constants must be obeyed:

$$\frac{K_1(K_1 - K_2)}{(K_1 - K)^2} \geq \frac{\sqrt{1 + 4K_xR_T} - 1}{2K_xR_T\sqrt{1 + 4K_xR_T}}. \quad (7)$$

Because the right side of this inequality is positive the left side must also be. Therefore it is necessary, but not sufficient, that

$$K_1 > K_2. \quad (8)$$

This means that binding to dimers must exhibit negative cooperativity, i.e., the affinity for EGF binding to a single EGF receptor in a dimer when both binding sites are free must be greater than when only one site is free. This asymmetry in the binding properties of the dimer arises even though in the absence of EGF all EGF receptors are identical. One way that this could occur is through steric hindrance. The binding of EGF to one site on a dimer could partially block the second site. Another possibility is that the binding of EGF to one receptor in a dimer leads to alteration of the binding properties of the second receptor through an intermolecular mechanism. In Fig. 3 we illustrate that when $K_1 = K_2$ the model predicts a Scatchard plot characteristic of positive cooperativity. Such a Scatchard plot has been observed for the dimerization of human growth hormone by zinc (Cunningham et al., 1991), but not for the dimerization of EGF receptors by EGF.

For our model to be consistent with the three experimental observations we have considered, all the conditions on the parameters that we have obtained must be satisfied simultaneously. The restrictions this places on the parameters are illustrated in Fig. 4, *a*, *b*, and *c*, which correspond to the three possible ways in which dimer formation can depend on EGF concentration (see Fig. 2). In Fig. 4 the shaded areas correspond to the values of K_1 and K_2 that are allowable (consistent with experiment). If dimerization increases monotonically with EGF concentration, as shown for EGF receptors purified from A431 cells (Yarden and Schlessinger, 1987*b*) and EGF receptors on A431 cell membranes (Carraway III and Cerione, 1991), the parameters must fall within the shaded area of Fig. 4 *a*.

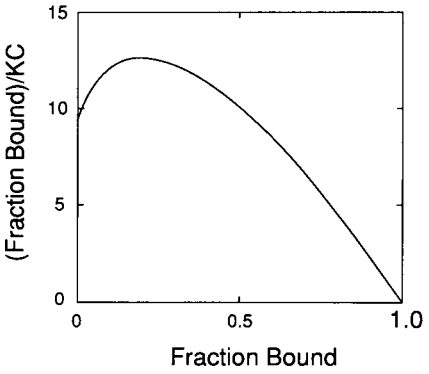

FIGURE 3 Our model predicts that if binding to a free EGF receptor site on a dimer is independent of the state of the adjacent site, i.e., whether it is free or occupied (this corresponds to $K_1 = K_2$), a Scatchard plot will show curvature characteristic of positive cooperativity. Shown is the predicted Scatchard plot when $K_1 = K_2 = 100$ K, $K_xR_T = 0.1$. *C* is the free EGF concentration and *K* is the equilibrium constant for EGF binding to a monomeric EGF receptor. The curve was calculated from Eq. A7 and the solution to Eq. A1.

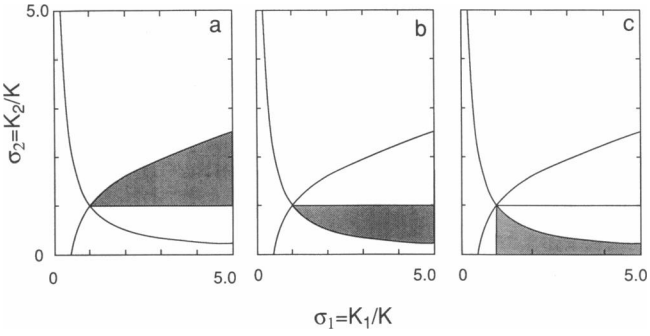

FIGURE 4 We require that our model predict the following: (1) that there be some EGF receptors in dimers in the absence of EGF; (2) at low EGF concentrations, the addition of EGF increases the concentration of EGF receptors in dimers; and (3) that Scatchard plots for EGF binding to EGF receptors do not show positive cooperativity. This places restrictions on the values of the parameters in the model. This is illustrated for the case when $K_xR_T = 0.1$, which corresponds to having 8% of the EGF receptors in dimers in the absence of EGF (see Eq. 5). The shaded areas in the plots show the three parameter sets leading to the three types of dimerization curve shown in Fig. 2. In the above figures, the monotonically increasing curve is Eq. 7 when equality holds. Then all of the shaded regions correspond to parameters satisfying the inequality given by Eq. 7, i.e., the condition under which a Scatchard plot does not indicate positive cooperativity. In addition, all of the shaded regions have $K_1 > K$, the condition for an initial increase in the fraction of EGF receptors in dimers, as the EGF concentration increases from 0. The monotonically decreasing curve is $K^2 = K_1K_2$. In Fig. 4 *a*, the shaded region has $K_2 \geq K$. In Fig. 4 *b*, the shaded region has $K_1K_2 \geq K^2$ and $K_2 \leq K$. In Fig. 4 *c*, the shaded region has $K_1K_2 \leq K^2$.

EXPERIMENTAL RESULTS

To focus on the effect of receptor aggregation on EGF binding data, we studied the equilibrium binding of EGF to receptors on closed membranes derived from B82 cells transfected with the human EGF receptor (Chen et al., 1987). The use of membranes eliminated the effects of internalization and cytoskeletal interactions on the EGF binding curves obtained. Because B82 cells lack endogenous EGF receptors and because we are transfecting with only a single receptor genotype for each set of binding studies, heterogeneity in primary receptor structure is eliminated. Membranes were isolated from cells expressing wild type and three mutant receptors. The

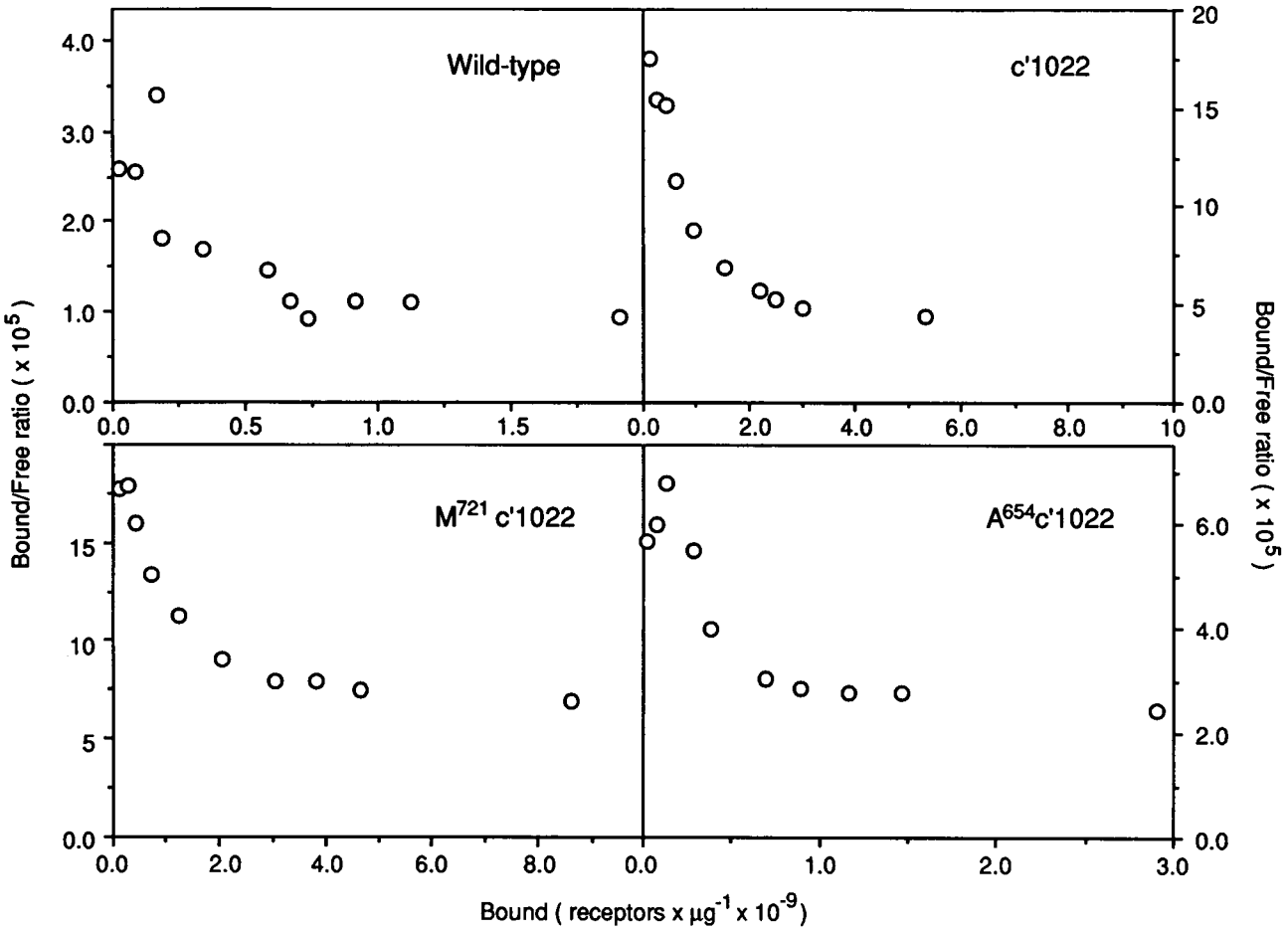

FIGURE 5 Scatchard plots for EGF binding to membranes from cells expressing wild type or mutant EGF receptors. Plasma membrane vesicles were isolated from mouse B82 cells expressing the indicated EGF receptor construction as described in Materials and Methods. They were incubated to equilibrium with ^{125}I-EGF at concentrations ranging from 0.15–40 nM, followed by separation of bound from free ligand by filtration. All data were corrected for nonspecific ligand binding.

cytoplasmic tail of all three receptor mutants was truncated from the C' end through amino acid residue 1023, leaving receptors that lack three identified tyrosine autophosphorylation sites (Downward et al., 1984) and a hydrophilic "hinge" region near residue 1037 (Gullick et al., 1985). The c'1022 receptor has the truncation only, whereas the M^{721}c'1022 receptor is both truncated and lacks tyrosine kinase activity due to replacement of Lys 721 in the ATP binding site of the receptor with Met (Glenney et al., 1988). The $A^{654}M^{721}$c'1022 is an M^{721}c'1022 receptor in which the protein kinase C phosphorylation site, Thr 654, was replaced by Ala. As can be seen in Fig. 5, Scatchard plots obtained for all of the receptors exhibit curvature characteristic of negatively cooperative binding. Our model predicts this qualitative behavior only if the binding of EGF to a dimer with one EGF already bound occurs with lower affinity than the initial binding event. If the affinities were equal, the curvature of the Scatchard plot would be characteristic of positively cooperative binding.

We also studied the equilibrium binding of EGF to B82 cells expressing either the wild-type or a mutant receptor M^{721} lacking tyrosine kinase activity. The experiments were done at 0°C to block internalization. EGF of either a constant specific activity or varying specific activity (by dilution with unlabeled ligand) was used to detect possible radiolabeling artifacts. As shown in Fig. 6, Scatchard plots were strongly nonlinear. Varying the specific activity of the EGF did not significantly affect the shape of these curves, indicating that heterogeneity in the ligand preparation did not contribute to curvature in the Scatchard plot.

To see if our model could account quantitatively for the observed curvature in the Scatchard plots we first carried out nonlinear least squares fits of the model to the membrane data. We did not fit the Scatchard plot data (Bound/Free vs Bound) but rather we fit the binding data (Bound vs Free) directly. The theoretical expression we used to predict the bound EGF concentration as a function of the free EGF concentration is given in the Appendix (Eq. A7 with X calculated from Eq. A1). The amount of EGF bound is a function of five parameters: R_T, the total number of receptors per vesicle; K_x, the equilibrium constant that characterizes the aggregation

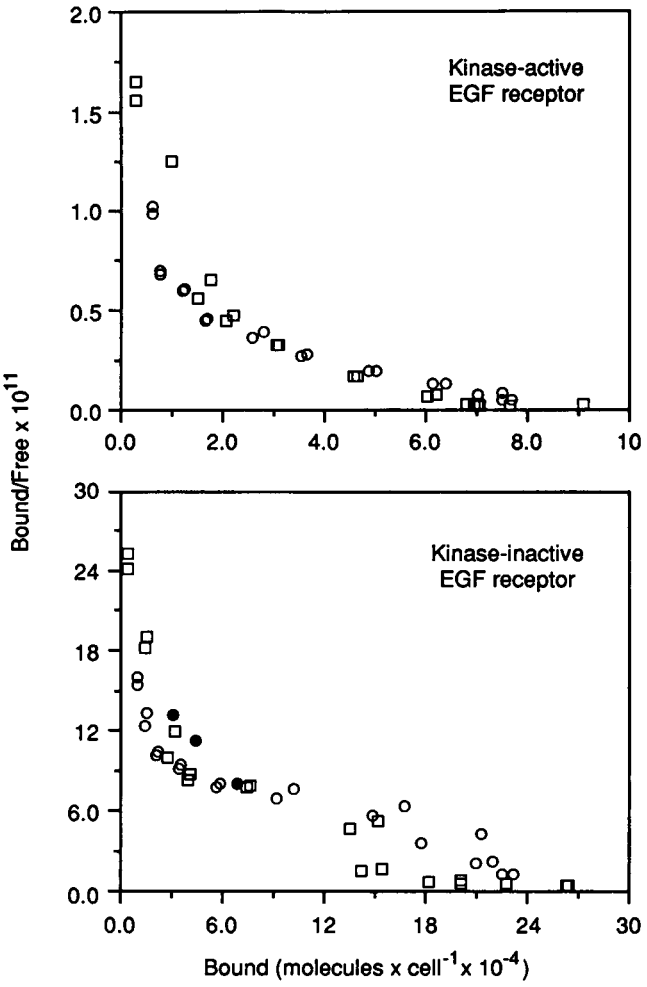

FIGURE 6 Scatchard plots for EGF binding to cells expressing wild type or mutant EGF receptors. Mouse B82 cells expressing either wild type (*top panel*) or M^{721} mutant receptors (*bottom panel*) were brought to equilibrium at 0°C with concentrations of ^{125}I-EGF ranging from 0.05–170 nM. The ^{125}I-EGF had either a constant specific activity of 1,600 cpm/fmol, or was systematically varied from 1.8 to 1800 cpm/fmol by the addition of unlabeled EGF.

of free EGF receptors on membranes; and K, K_1 and K_2, the three equilibrium constants that characterize respectively the binding of EGF to an unaggregated receptor, to a receptor in a dimer with both sites free, and to a receptor in a dimer with one site free and one bound. Not surprisingly, the data do not determine the five parameters uniquely. Additional experimental results on receptor aggregation as a function of EGF concentration would be needed to completely determine the parameter set. However, our first priority is not to determine parameter values, but to see if the model can fit the data and this can be done without completely determining the parameters. We found that if we fixed the value of K and the lumped parameter $k_x = K_x R_T$ and allowed the remaining three parameters to vary, the fitting program converged, i.e., we could determine the three free parameters R_T, K_1 and K_2 that minimized the sum of the squared deviations of the data from the model predictions. We carried out this procedure for many different values of K and k_x and found that the best fit was insensitive to the values of K and k_x, provided that these values were in a particular range. If K and k_x were outside this range we obtained poorer fits, i.e., larger sums of squared deviations. To illustrate, in the case of EGF binding to c'1022 EGF receptors on vesicles, the best fit was obtained when $k_x \leq 1 \times 10^{-1}$ and $K \leq 1 \times 10^8$ M^{-1}. For K and k_x in this range, the parameters K_2, R_T and $K_1 k_x$ were approximately constant with values $K_2 = 3.2 \times 10^9$ M^{-1}, $K_1 k_x = 2.5 \times 10^9$ M^{-1} and $R_T = 1.9 \times 10^{10}$ receptors/μg. (The total number of vesicles in the sample was unknown so we could not determine the number of receptors per vesicle. Here R_T is the total number of receptors on all vesicles per μg of protein in the sample.) Note that these parameters obey the following inequalities: $K_1 > K_2 > K$. When these inequalities hold the model predicts that the EGF receptor dimer concentration will be a monotonically increasing function of the EGF concentration (see Fig. 2*a*). Note also that for these parameters the model predicts from Eqs. 5 and A4 that less than 9% of EGF receptors are in dimers in the absence of EGF and greater than 96% are in dimers at very high EGF concentrations.

When we look at the fit of the model to the c'1022 data, Fig. 7 *a*, it is clear that there is a systematic error. When Scatchard plots of the experiment and theoretical prediction are compared, as in Fig. 7 *b*, the lack of agreement is even more striking. Fitting the Scatchard plot data directly improves the fit, as seen in Fig. 7 *c*, but a systematic error remains. Similar poor fits were obtained for binding experiments with the other two mutants and the wild type. Although the model as presented can give a negatively curved Scatchard plot, it cannot account fully for the binding data. Thus, even if binding to EGF receptors dimers is negatively cooperative, this cannot be the sole source of the observed curvature in the Scatchard plot.

There are other possible sources of curvature in the Scatchard plot that we have not been able to control for. For example, heterogeneity in receptor density can affect our model. (Such heterogeneity has no effect on binding models that ignore receptor aggregation.) If different vesicles have different surface densities of receptors, at the same EGF concentration they will have different fractions of receptors in monomers and dimers. Such heterogeneity can alter the shape of a Scatchard plot. To illustrate, we consider the binding of EGF to EGF receptors on two different populations of vesicles. The vesicles in both populations are the same size, but have different numbers of receptors on their surfaces. In particular, we assume a fraction f of the vesicles have R_1 receptors on their surface and a fraction $1 - f$ have R_2 receptors. In Fig. 8, *a* and *b*, we see that we can achieve excellent agreement with experiment by introducing such heterogeneity. Although modeling heterogeneity in cell surface

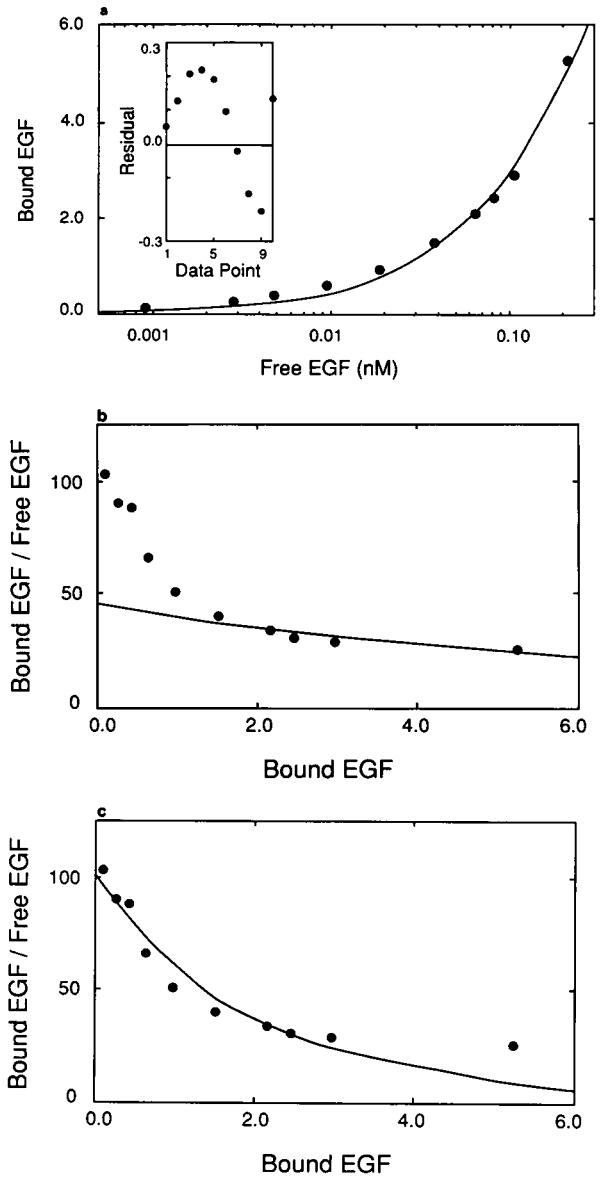

FIGURE 7 The equilibrium binding of EGF to mutant c'1022 receptors on vesicles. (*a*) The best fit (*solid line*) of the model to the binding data (Bound versus Free). The following two parameters were held fixed: $k_x = K_x R_T = 10^{-2}$ and $K = 10^7\ M^{-1}$. The best fit values for the three parameters that were varied were: $K_1 = 2.5 \times 10^{11}\ M^{-1}$, $K_2 = 3.2 \times 10^9\ M^{-1}$, and $R_T = 1.9 \times 10^{10}$ receptors/μg. A systematic difference between the theoretical fit and the data can be seen when the residuals, the differences between the experimental and theoretical values, are plotted against the number of the data point are plotted (*inset*). (*b*) Scatchard plots of the data and theoretical fit in (*a*). (*c*). The best fit of the model to the Scatchard data (Bound/Free versus Bound). As in (*a*), $k_x = 10^{-2}$ and $K = 10^7\ M^{-1}$ (held fixed). The best fit values for the three parameters that were varied were: $K_1 = 1.5 \times 10^{12}\ M^{-1}$, $K_2 = 7.1 \times 10^9\ M^{-1}$, and $R_T = 7.0 \times 10^9$ receptors/μg.

receptor density as simply two populations of identically sized vesicles with different receptor numbers is highly artificial, the results in Fig. 8 indicate that such heterogeneity can account for the discrepancies between the theory we developed in the previous section and the binding experiments presented here. However, flow cytometry studies on B82 cells expressing the wild type EGF receptor show a narrow distribution in receptor number. Because these cells are relatively uniform in size, the distribution in receptor density should also be narrow. Binding studies with these cells at 0°C for both the wild type and the receptor lacking kinase activity show curved Scatchard plots. When we fit our theoretical model to these binding data we obtain fits that show systematic errors, just as occurred when the binding studies were carried out on vesicles.

DISCUSSION

Scatchard plots of EGF binding usually show curvature associated with negatively cooperative binding. One way

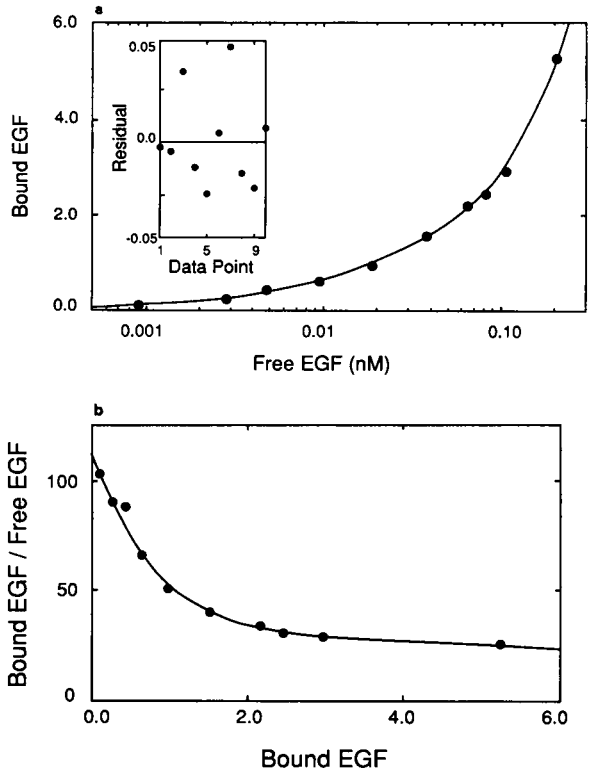

FIGURE 8 Fit of the equilibrium binding data for EGF binding to mutant c'1022 receptors on vesicles, assuming heterogeneity in the surface density of receptors. The data are the same as in Fig. 7. The model used to fit the data assumes all vesicles are the same size, with a fraction f having R_1 receptors per vesicle and $(1 - f)$ having R_2 receptors per vesicle. (*a*) The following three parameters were held fixed: $f = 0.999$, $K_x(R_1 + R_2) = 10^{-2}$ and $K = 10^7\ M^{-1}$. The best fit values for the four parameters that were varied were: $K_1 = 2.7 \times 10^{12}\ M^{-1}$, $K_2 = 7.5 \times 10^9\ M^{-1}$, $R_1 = 3.2 \times 10^{10}$ receptors/μg and $R_2 = 4.0 \times 10^{12}$ receptors/μg. No systematic difference between the theoretical fit and the data can be seen when the residuals, the difference between the experimental and theoretical values, versus the number of the data point are plotted (*inset*). Comparison of the fits obtained with and without allowance for heterogeneity in receptor density, based on the F statistic that would be appropriate if the regression were linear, indicates that the improvement evident in this figure is significant at $p < 0.001$. (*b*) Scatchard plots of the data and theoretical fit in (*a*).

such curvature can arise, other than by negative cooperativity, is by heterogeneity in receptor binding affinities. For this reason, much of the published EGF binding data has been analyzed in terms of two populations of receptors, one with high and one with low affinity for EGF. However, there is no evidence for two stable populations of EGF receptors. When genetically identical EGF receptors are expressed in cells that do not normally express EGF receptors, Scatchard plots show typical negative curvature (Glenney et al., 1988; Honegger et al., 1988; Heisermann et al., 1990). A single EGF receptor cannot have two binding sites with different affinities because it has a valence of one (Weber et al., 1984). The action of protein kinase C can alter the binding affinity of EGF for its receptor, and, thus, can produce heterogeneity in binding affinity in an initially homogeneous population of receptors (Cochet et al., 1984; McCaffrey et al., 1984; Downward et al., 1985; Lin et al., 1986). However, EGF receptors on vesicles in the absence of ATP or functioning protein kinase C show curved Scatchard plots similar to those seen when binding studies are carried out with cells (Downward et al., 1985; Davis, 1988). Thus, protein kinase C cannot fully explain the nonlinear Scatchard plots seen in binding studies with EGF receptors on cells and closed vesicles.

Because there is evidence for differential affinities of EGF for monomeric and dimeric EGF receptors, it is tempting to interpret results of the two-affinity Scatchard plot analysis as reflecting affinities of receptors in monomers and dimers (or higher oligomers). As we have shown, this is incorrect. To explore the relation between receptor oligomerization and EGF receptor affinity, we presented the simplest model that was consistent with the known binding and aggregation properties of the EGF receptor. This model is a generalization of the model of Yarden and Schlessinger (1987*a*), modified to allow the EGF receptor dimer to bind to a first and second EGF with different affinities (see Fig. 1). It was first introduced by Levitzki and Schlessinger (1974) to study general features of ligand induced protein aggregation. To test the model, we did EGF binding studies on membrane vesicles. These experiments eliminated several sources of heterogeneity that might lead to negatively curved Scatchard plots. The population of EGF receptors on vesicles was homogeneous with respect to primary structure because the vesicles were derived from transfected cells expressing only one receptor genotype. The use of membrane vesicles eliminated the possibility that cytoskeletal interactions could induce a higher affinity state of the EGF receptor (Wiegant et al., 1986). Using vesicles also eliminated receptor internalization and the resulting ambiguities in interpreting binding data (Gex-Fabry and DeLisi, 1984). Receptor mutants were used that lacked kinase activity (Chen et al., 1989), the major receptor autophosphorylation sites (Downward et al., 1984), and the ability to be phosphorylated by protein kinase C (Lund et al., 1990). Heterogeneity in the phosphorylation state of the EGF receptor was further reduced by doing all binding studies in the absence of ATP and protein kinase C. Heterogeneity in the EGF preparation was shown not to contribute to curvature in the Scatchard plot (see Fig. 6). However, we have probably not eliminated all sources of heterogeneity that affect the affinity of EGF for its receptor. For example, some receptors may be only partially glycosylated. Because the state of glycosylation influences the affinity of EGF for its receptor this could contribute to curvature in the Scatchard plot (Slieker and Lane, 1985). EGF receptors might interact with other proteins in the membrane forming EGF receptor-protein complexes that have altered affinities (Mayo et al., 1989; Gex-Fabry and DeLisi, 1986). During vesicle preparation some EGF receptors might be partially denatured and bind EGF with a reduced affinity. With the model we developed we looked at whether the dominant source of curvature in the Scatchard plots we obtained from membrane binding studies can be EGF induced EGF receptor aggregation.

If EGF has one affinity for its receptor when the receptor is monomeric and another when it is in an oligomer, our model predicts that a Scatchard plot should show positive cooperativity, something not seen for EGF binding to its receptor, but that has been seen for the dimerization of human growth hormone by zinc (Cunningham et al., 1991). The prediction of positive cooperativity is independent of whether the high affinity state is the dimer (Yarden and Schlessinger, 1987*a*) or the monomer (Biswas et al., 1985). We found that models postulating aggregation as a source of negatively curved Scatchard plots only work if there is negative cooperativity of the distinct receptors in a dimer (or higher oligomer). That is, EGF binds with higher affinity to a receptor in a dimer with both sites free than to the second receptor in a dimer with one site already occupied. If the affinities are the same for the first and second binding event, the Scatchard plot will have positive curvature. This result is not altered when the model is extended to include the formation of higher oligomers. We also showed that there are further restrictions on the set of parameters consistent with a negatively curved Scatchard plot (Eq. 7 gives that exact condition.)

When we fit the aggregation model to the binding data (i.e., determined parameters that gave the best least squares fit of the predicted amounts of bound EGF to those measured experimentally), the parameters were consistent with negatively cooperative binding and the predicted Scatchard plots showed characteristic curvature. However, even with the parameters that gave the best fit to the data, there was a systematic error. The curvature in the experimental Scatchard plots was more pronounced than could be explained by the model. Introducing heterogeneity in membrane receptor density improved our fits to the data, but there is no evidence for

such heterogeneity. Rather, flow cytometry studies on whole B82 cells show no pronounced heterogeneity in receptor number or cell size (data not shown), suggesting that it is unlikely that vesicles derived from these cells have sufficient heterogeneity in receptor density to explain the data. As we have discussed, there are other possible sources of heterogeneity that could contribute to the observed curvature of the Scatchard plots. Heterogeneity is usually modeled in terms of two populations of EGF receptors with different affinities. Note however, that if one were to fit the binding data with just a two population model, i.e., a model where R_1 receptors have a low affinity and R_2 have a high affinity, the parameter values one obtained from the fit would not be meaningful unless there were no EGF-induced receptor aggregation. If EGF induced receptor aggregation occurs, then it will contribute to the curvature in the Scatchard plot and must be accounted for.

APPENDIX

Here we develop the model more fully. Beginning with the case where dimers are the only aggregates that form, we derive an equation for the fraction of EGF receptors in dimers, $d(C)$, as a function of the EGF concentration C. We use this equation to obtain an expression for the concentration of EGF receptors in dimers in the absence of EGF, Eq. 5, and to determine parameter ranges where EGF enhances dimerization. There are three criteria we consider for ligand-enhanced receptor aggregation: $d(C)$ is larger at very high EGF concentrations than at very low concentrations [$d(\infty) > d(0)$]; $d(C)$ increases initially ($d'(0) > 0$); and $d(C)$ increases monotonically ($d'(C) > 0$ for all $C > 0$). The three criteria correspond to different restrictions on the parameters. Next we derive conditions for positive (negative) cooperativity of the Scatchard plot. Finally, we show how the model can be extended if larger oligomers form.

Ligand-enhanced receptor dimerization

Fig. 1 summarizes our model for EGF receptor dimer formation and EGF binding when dimers are the only aggregates that form. The six equilibrium association constants are related by the detailed balance Eqs. 1 and 2. Then the following conservation law holds:

$$R_T = X(1 + KC) + K_x X^2(1 + 2K_1C + K_1K_2C^2), \quad \text{(A1)}$$

where R_T is the total concentration of EGF receptors on the cell surface, X is the concentration of free (unbound, monomeric) EGF receptors and C is the concentration of EGF in solution.

The fraction of EGF receptors in dimers, as a function of the EGF concentration C, is given by:

$$d(C) = 1 - X(1 + KC)/R_T. \quad \text{(A2)}$$

Eq. 5 for the concentration of EGF receptors in dimers in the absence of EGF ($C = 0$) follows from substitution of the unique non-negative root X of the quadratic Eq. A1 into Eq. A2.

The first criterion we consider for EGF-enhanced receptor aggregation is $d(\infty) > d(0)$. We have given an intuitive argument that this occurs when $K_{x2} > K_x$ or equivalently, $K_1K_2 > K^2$. To derive this condition from the equations, we rewrite Eq. A1 in terms of the monomeric receptor fraction $1 - d(C)$ (from Eq. A2) in the two limits of interest:

$$1 = [1 - d(0)] + K_xR_T[1 - d(0)]^2 \quad \text{(A3)}$$

$$1 = [1 - d(\infty)] + K_xR_T(K_1K_2/K^2)[1 - d(\infty)]^2. \quad \text{(A4)}$$

(Eq. A4 depends on the observation that for the conservation law, Eq. A1, to hold as $C \to \infty$, XC must approach a finite limit.) Then from Eqs. A3 and A4,

$$d(\infty) > d(0) \Leftrightarrow K_1K_2/K^2 > 1.$$

The other criteria we consider for ligand-enhanced dimerization involve the derivative of $d(C)$. From Eq. A2,

$$d'(C) = -\frac{dX}{dC}\left(\frac{1 + KC}{R_T}\right) - \frac{KX}{R_T}. \quad \text{(A5)}$$

Differentiating Eq. A1, solving for dX/dC, substituting into Eq. A5 and simplifying, we find that

$$d'(C) > 0 \Leftrightarrow K_1 + K_1K_2C > K + KK_1C$$

$$d'(0) > 0 \Leftrightarrow K_1 > K$$

$$d'(C) > 0 \quad \text{for all} \quad C > 0 \Leftrightarrow K_1 > K \quad \text{and} \quad K_2 > K.$$

Scatchard plot curvature

We turn now to the Scatchard plot analysis, i.e., we consider the graph of B/C plotted as a function of B, where B is the number of ligands bound per cell. The plot is considered to have "negative curvature," characteristic of negative cooperativity of ligand binding to distinct receptors, if the curve lies below the straight line joining the two intercepts (B/C at $B = C = 0$ and $B = R_T$ as $C \to \infty$ and $B/C \to 0$), and positive curvature if the graph lies above the line. The criterion we will use compares the initial slope of the Scatchard plot with the slope of the straight line ($-\lim_{C\to 0}(B/C)/R_T$). For the Scatchard plot to lie on or below the line we must have:

$$\lim_{C\to 0}\left[\frac{d(B/C)}{dB} + \frac{B/C}{R_T}\right] \le 0. \quad \text{(A6)}$$

Our aim is to derive Eq. 7, which gives conditions for Eq. A6 to hold, guaranteeing that the Scatchard plot is not positively curved.
The number of ligands bound per cell is:

$$B = KCX + K_xX^2(K_1C + K_1K_2C^2). \quad \text{(A7)}$$

The easiest way to obtain $d(B/C)/dB$ as $C \to 0$ is to differentiate the expressions for B and B/C (both obtained from Eq. A7) with respect to C, substitute the expression for dX/dC obtained from differentiation of Eq. (A1), take the limits as $C \to 0$, and finally use the chain rule relation: $d(B/C)/dB = d(B/C)/dC \div dB/dC$. Then:

$$\lim_{C\to 0}\frac{d(B/C)}{dB} = -\frac{(KX_0 + 2K_1K_xX_0^2)^2}{(KX_0 + K_1K_xX_0^2)(X_0 + 2K_xX_0^2)} + \frac{K_1K_2K_xX_0^2}{(KX_0 + K_1K_xX_0^2)}, \quad \text{(A8)}$$

where X_0 is the solution to the conservation law, Eq. A1, evaluated at $C = 0$. Also from Eq. A7 we have:

$$\lim_{C\to 0}\frac{B/C}{R_T} = \frac{KX_0 + K_1K_xX_0^2}{R_T}. \quad \text{(A9)}$$

Summing the expressions in Eqs. A8 and A9, substituting $R_T - X_0$ for $K_xX_0^2$ (from Eq. A1 at $C = 0$), and simplifying, we find that the inequality given by Eq. A6 holds if and only if:

$$(K_1 - K)^2X_0^2 - K_1(K_1 - K_2)(2R_T^2 - R_TX_0) \le 0. \quad \text{(A10)}$$

By Eq. A1, $2R_T - X_0 = 2(X_0 + K_xX_0^2) - X_0 = X_0 + 2K_xX_0^2$, and hence, Eq. A10 can be written in the form:

$$\frac{K_1(K_1 - K_2)}{(K_1 - K)^2} \geq \frac{X}{(1 + 2K_xX_0)}.$$

Substituting for X_0 the solution to Eq. A1 at $C = 0$ yields Eq. (7), completing the proof that the condition for the Scatchard plot not to exhibit positive cooperativity is that the parameters satisfy Eq. (7).

Theory for higher oligomers

So far we have viewed EGF receptor aggregation as a monovalent interaction, i.e., if dimers are the only aggregates that form, this means that an EGF receptor binds to at most one other EGF receptor. The simplest model where higher oligomers form treats the EGF receptor as bivalent for other EGF receptors. Then each receptor binds at most two others and only linear chains and rings of receptors can form. This situation is considerably more tractable than if receptors have valence of three or more, because then branched networks of receptors can form. We will present the bivalent aggregation model and show that, as in the monovalent aggregation (dimer) model, if the affinity of EGF for its receptor depends only on whether the receptor is isolated or aggregated, the Scatchard plot has curvature characteristic of positive cooperativity.

Fig. 9 shows the binding and aggregation reactions and equilibrium constants in the model. We are considering the case where EGF has one equilibrium association constant K for isolated EGF receptors and another, K_1, for all receptors in chains of two or more receptors, independent of chain length, receptor position in a chain, and the binding state of neighboring receptors. We assume that the formation of closed rings of EGF receptors is negligible. We allow the initial aggregation step (the "seeding" of an aggregate) to occur with a different (perhaps lower) equilibrium constant than the subsequent additions to the chain.

For this model, the conservation law for the concentration of EGF receptors in chains of all lengths is:

$$R_T = (1 + KC)X + 2K_xX^2 \sum_{n=2}^{\infty} n(1 + K_1C)^n(2K_x^*X)^{n-2}. \quad (A11)$$

The most convenient form of Eq. A11 for the Scatchard plot calculation is:

$$R_T = (1 + KC)X + 2K_xX^2(1 + K_1C)^2 \times \left[\frac{1}{1 - 2K_x^*(1 + K_1C)} + \frac{1}{[1 - 2K_x^*(1 + K_1C)]^2}\right]. \quad (A12)$$

A similar summation gives the equilibrium concentration of bound ligand:

$$B = KCX + 2K_xX^2K_1C(1 + K_1C) \times \left[\frac{1}{1 - 2K_x^*(1 + K_1C)} + \frac{1}{[1 - 2K_x^*(1 + K_1C)]^2}\right]. \quad (A13)$$

Note that when $K_x^* = 0$, and with the appropriate identification of the dimer formation constants (i.e., K_x for monovalent aggregation is identified with $4K_x$ in the bivalent aggregation model), Eq. A13 reduces to the dimer model's Eq. A7, in the special case when there is only a single affinity for binding to a receptor in a dimer, i.e., when $K_1 = K_2$.

As before, we find the difference between the initial slope of the Scatchard plot and the slope of the straight line joining the intercepts. In this case we find that the difference is always positive, showing that the Scatchard plot has the type of curvature associated with positively cooperative binding.

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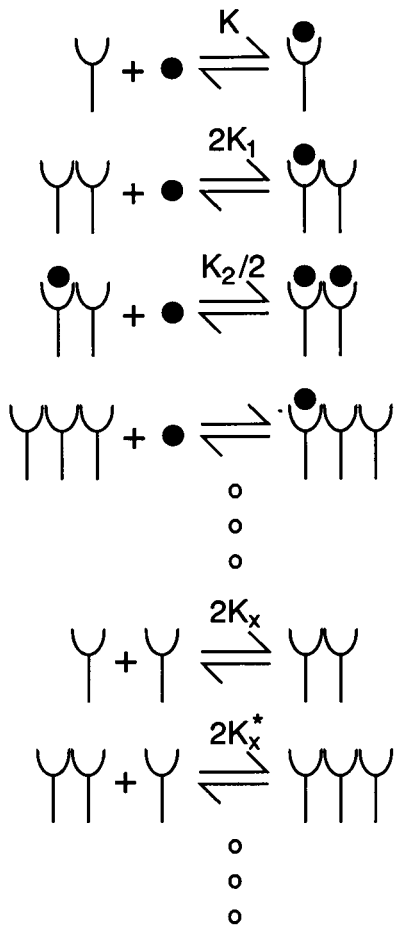

FIGURE 9 Shown are binding and aggregation reactions for one possible model where EGF receptors can form oligomers larger than dimers. Here we assume that an EGF receptor can aggregate with at most two other EGF receptors so that only chains or rings of EGF receptors can form. We neglect rings, but they can be added to the theory (e.g., Dembo and Goldstein, 1978). We take the equilibrium cross-linking constant for dimer formation to be K_x and the equilibrium cross-linking constant for further growth of a chain to be K_x^*, which may or may not equal K_x. As before, we take K to be the equilibrium constant for EGF to bind to a single monomeric EGF receptor. K_1 is the equilibrium constant for EGF to bind to a single EGF receptor in a chain. We assume that the binding to a free EGF receptor site in a chain is independent of the state of the other EGF receptor sites in the chain, i.e., there is no cooperative binding within a chain.

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REFERENCES

Bellot, F., W. Moolenaar, R. Kris, B. Mirakhur, I. Verlaan, A. Ullrich, J. Schlessinger, and S. Felder. 1990. High-affinity epidermal growth factor binding is specifically reduced by a monoclonal antibody, and appears necessary for early responses. *J. Cell Biol.* 110:491–502.

Bertics, P. J., and G. N. Gill. 1985. Self-phosphorylation enhances the protein-tyrosine kinase activity of the epidermal growth factor receptor. *J. Biol. Chem.* 260:14642–14647.

Biswas, R., M. Basu, A. Sen-Majumdar, and M. Das. 1985. Intrapep-

tide autophosphorylation of the epidermal growth factor receptor: regulation of kinase catalytic function by receptor dimerization. *Biochemistry.* 24:3795–3802.

Böni-Schnetzler, M., and P. F. Pilch. 1987. Mechanism of epidermal growth factor receptor autophosphorylation and high-affinity binding. *Proc. Natl. Acad. Sci. USA.* 84:7832–7836.

Carpenter, G. 1987. Receptors for epidermal growth factor and other polypeptide mitogens. *Annu. Rev. Biochem.* 56:881–914.

Carraway III, K. L., and R. A. Cerione. 1991. Comparison of epidermal growth factor (EGF) receptor–receptor interactions in intact A431 cells and isolated plasma membranes. *J. Biol. Chem.* 266:8899–8906.

Carraway III, K. L., J. G. Koland, and R. A. Cerione. 1989. Visualization of epidermal growth factor (EGF) receptor aggregation in plasma membranes by fluorescence resonance energy transfer. *J. Biol. Chem.* 264:8699–8707.

Chen, W. S., C. S. Lazar, K. A. Lund, J. B. Welsh, C.-P. Chang, G. M. Walton, C. J. Der, H. S. Wiley, G. N. Gill, and M. G. Rosenfeld. 1989. Functional independence of the epidermal growth factor receptor from a domain required for ligand induced internalization and calcium regulation. *Cell.* 59:33–43.

Cochet, C., G. N. Gill, J. Meisenhelder, J. A. Cooper, and T. Hunter. 1984. C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity. *J. Biol. Chem.* 259:2553–2558.

Cochet, C., O. Kashles, E. M. Chambaz, I. Borrello, C. R. King, and J. Schlessinger. 1988. Demonstration of epidermal growth factor-induced receptor dimerization in living cells using a chemical covalent cross-linking agent. *J. Biol. Chem.* 263:3290–3295.

Cunningham, B. C., M. G. Mulkerrin, and J. A. Wells. 1991. Dimerization of human growth hormone by zinc. *Science (Wash. DC).* 253:545–548.

Davis, R. J. 1988. Independent mechanisms account for the regulation by protein kinase C of the epidermal growth factor receptor affinity and tyrosine-protein kinase activity. *J. Biol. Chem.* 263:9462–9469.

Defize, L. H. K., J. Boonstra, J. Meisenhelder, W. Kruijer, L. G. J. Tertoolen, B. C. Tilly, T. Hunter, E. N. van Bergen, P. M. P. Henegouwen, W. H. Moolenaar, and S. W. de Laat. 1989. Signal transduction by epidermal growth factor occurs through the subclass of high affinity receptors. *J. Cell Biol.* 109:2495–2507.

Dembo, M., and B. Goldstein. 1978. Theory of equilibrium binding of symmetric bivalent haptens to cell surface antibody: application to histamine release from basophils. *J. Immunol.* 121:345–353.

Downward, J. M., J. Parker, and M. D. Waterfield. 1984. Autophosphorylation sites on the epidermal growth factor receptor. *Nature (Lond.).* 311:483–485.

Downward, J. M., D. Waterfield, and P. J. Parker. 1985. Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 260:14538–14546.

Fanger, B., J. E. Stephens, and J. V. Staros. 1989. High yield trapping of EGF-induced receptor dimers by cross-linking. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:71–75.

Fanger, B. O., K. S. Austin, H. S. Earp, and J. A. Cidlowski. 1986. Cross-linking of epidermal growth factor receptors in intact cells: detection of initial stages of receptor clustering and determination of molecular weight of high affinity receptor. *Biochemistry.* 25:6414–6420.

Gex-Fabry, M., and C. DeLisi. 1984. Receptor-mediated endocytosis: a model and its implication for experimental analysis. *Am. J. Physiol.* 247:R768–R779.

Gex-Fabry, M., and C. DeLisi. 1986. Regulation of interacting populations during endocytosis: models of growth factor-tumor promoter dynamics. *Am. J. Physiol.* 250:R1123–R1132.

Glenney, Jr., J. R., W. S. Chen, C. S. Lazar, G. M. Walton, L. M. Zokas, M. G. Rosenfeld, and G. N. Gill. 1988. Ligand-induced endocytosis of the EGF-receptor is blocked by mutational inactivation and by microinjection of anti-phosphotyrosine antibodies. *Cell.* 52:675–684.

Gullick, W. J., J. Downward, and M. D. Waterfield. 1985. Antibodies to the autophosphorylation sites of the epidermal growth factor receptor protein-tyrosine kinase as probes of structure and function. *EMBO (Eur. Mol. Biol. Org.) J.* 4:2869–2877.

Hatakeyama, M., M. Tsudo, S. Minamoto, T. Kono, T. Doi, T. Miyata, M. Miyasaka, and T. Taniguchi. 1989. Interleukin-2 receptor β chain gene: generation of three receptor forms by cloned human α and β chain cDNA's. *Science (Wash. DC).* 244:551–556.

Heisermann, G. J., H. S. Wiley, B. J. Walsh, H. A. Ingraham, C. J. Fiol, and G. N. Gill. 1990. Mutational removal of the Thr669 and Ser671 phosphorylation sites alters substrate specificity and ligand-induced internalization of the epidermal growth factor receptor. *J. Biol. Chem.* 265:12820–12827.

Heldin, C-H., A. Ernlund, C. Rorsman, and L. Rönnstrand. 1989. Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation. *J. Biol. Chem.* 264:8905–8912.

Honegger, A., T. J. Dull, D. Szapary, A. Komoriya, R. Kris, A. Ullrich, and J. Schlessinger. 1988. Kinetic parameters of the protein tyrosine kinase activity of EGF-receptor mutants with individually altered autophosphorylation sites. *EMBO J.* 7:3053–3060.

Honegger, A. M., R. M. Kris, A. Ullrich, and J. Schlessinger. 1989. Evidence that autophosphorylation of solubilized receptors for epidermal growth factor is mediated by intermolecular cross-phosphorylation. *Proc. Natl. Acad. Sci. USA.* 86:925–929.

Koland, J. G., and R. A. Cerione. 1988. Growth factor control of epidermal growth factor receptor kinase activity via an intramolecular mechanism. *J. Biol. Chem.* 263:2230–2237.

Lax, I., A. K. Mitra, C. Ravera, D. R. Hurwitz, M. Rubinstein, A. Ullrich, R. M. Stroud, and J. Schlessinger. 1991. Epidermal growth factor (EGF) induces oligomerization of soluble, extracellular, ligand-binding domain of EGF receptor. *J. Biol. Chem.* 266:13828–13833.

Levitzki, A., and J. Schlessinger. 1974. Cooperativity in associating proteins. Monomer-dimer equilibrium coupled to ligand binding. *Biochemistry.* 13:5214–5219.

Lin, C. R., W. S. Chen, C. S. Lazar, C. D. Carpenter, G. N. Gill, R. M. Evans, and M. G. Rosenfeld. 1986. Protein kinase C phosphorylation at Thr 654 of the unoccupied EGF receptor and EGF binding regulate functional receptor loss by independent mechanisms. *Cell.* 44:839–848.

Lund, K. A., C. S. Lazar, W. S. Chen, B. J. Walsh, J. B. Welsh, J. J., Herbst, G. M. Walton, M. G. Rosenfeld, G. N. Gill, and H. S. Wiley. 1990. Phosphorylation of the epidermal growth factor receptor at threonine 654 inhibits ligand-induced internalization and downregulation. *J. Biol. Chem.* 265:20517–20523.

Mayo, K. H., M. Nunez, C. Burke, C. Starbuck, D. Lauffenburger, and C. R. Savage, Jr. 1989. Epidermal growth factor receptor binding is not a simple one-step process. *J. Biol. Chem.* 264:17838–17844.

McCaffrey, P. G., B. A. Friedman, and M. R. Rosner. 1984. Diacylglycerol modulates binding and phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 259:12502–12507.

Metzger, H., G. Alcaraz, R. Hohman, J.-P. Kinet, V. Pribluda, and R. Quarto. 1986. The receptor with high affinity for immunoglobulin E. *Annu. Rev. Immunol.* 4:419–470.

Northwood, I. C., and R. J. Davis. 1988*a*. Activation of the epidermal growth factor receptor tyrosine protein kinase in the absence of receptor oligomerization. *J. Biol. Chem.* 263:7450–7453.

Northwood, I. C., and R. J. Davis. 1988*b*. Protein kinase C inhibition of the epidermal growth factor receptor tyrosine protein kinase activ-

ity is independent of the oligomeric state of the receptor. *J. Biol. Chem.* 264*b*:7450–7453.

Savage, C. R., Jr., and S. Cohen. 1972. Epidermal growth factor and a new derivative: rapid isolation procedures and biological and chemical characterization. *J. Biol. Chem.* 247:7609–7611.

Schlessinger, J. 1986. Allosteric regulation of the epidermal growth factor receptor kinase. *J. Cell Biol.* 103:2067–2072.

Schlessinger, J. 1988. Signal transduction by allosteric receptor oligomerization. *TIBS (Trends Biochem. Sci.)* 13:443–447.

Schreiner, G. G., and E. R. Unanue. 1976. Membrane and cytoplasmic changes in B-lymphocytes induced by ligand-surface immunoglobulin interaction. *Adv. Immunol.* 24:37–165.

Siraganian, R. P., W. A. Hook, and B. B. Levine. 1975. Specific in vitro histamine release from basophils by bivalent haptens: evidence for activation by simple bridging of membrane bound IgE. *Immunochem.* 12:149–157.

Slieker, L. J., and M. D. Lane. 1985. Post-translational processing of the epidermal growth factor receptor. *J. Biol. Chem.* 260:687–690.

Smith, K. A. 1989. The interleukin 2 receptor. *Annu. Rev. Cell Biol.* 5:397–425.

Sorkin, A., and G. Carpenter. 1991. Dimerization of internalized epidermal growth factor receptors. *J. Biol. Chem.* 266:23453–23460.

Spaargaren, M., L. H. K. Defize, J. Boonstra, and S. W. de Laat. 1991. Antibody-induced dimerization activates the epidermal growth factor receptor tyrosine kinase. *J. Biol. Chem.* 266:1733–1739.

Staros, J. V., S. Cohen, and M. W. Russo. 1985. Epidermal growth factor receptor: characterization of its protein kinase activity. *In* Molecular Mechanisms of Transmembrane Signalling. P. Cohen and M. D. Houslay, editors. Elsevier, Amsterdam/New York. 253–278.

Unkeless, J. C., H. Fleit, and I. Mellman. 1981. Structual aspects and heterogeneity of immunoglobulin Fc receptors. *Adv. Immunol.* 31:247–270.

van Belzen, N., P. J. Rijken, W. J. Hage, S. W. de Laat, R. J. Verkleij, and J. Boonstra. 1988. Direct visualization and quantitative analysis of epidermal growth factor-induced receptor clustering. *J. Cell. Phys.* 134:5373–5393.

Weber, W., P. J. Bertics, and G. N. Gill. 1984. Immunoaffinity purification of the epidermal growth factor receptor: stoichiometry of binding and kinetics of self-phosphorylation. *J. Biol. Chem.* 259:14631–14636.

Wiegant, F. A. C., F. J. Blik, L. H. K. Defize, W. A. M. Linnemans, A. J. Verkleij, and J. Boonstra. 1986. Epidermal growth factor receptors associated to cytoskeletal elements of epidermoid carcinoma (A431) cells. *J. Cell Biol.* 103:87–94.

Yarden, Y., and J. Schlessinger. 1987*a*. Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation. *Biochemistry.* 26:1434–1442.

Yarden, Y., and J. Schlessinger. 1987*b*. Epidermal growth factor induces rapid, reversible aggregation of the purified growth factor receptor. *Biochemistry.* 26:1443–1451.