Self-Phosphorylation of Epidermal Growth Factor Receptor: Evidence for a Model of Intermolecular Allosteric Activation†

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ABSTRACT: The membrane receptor for epidermal growth factor (EGF) is a 170 000-dalton glycoprotein composed of an extracellular EGF-binding domain and a cytoplasmic kinase domain connected by a stretch of 23 amino acids traversing the plasma membrane. The binding of EGF to the extracellular domain activates the cytoplasmic kinase function even in highly purified preparations of EGF receptor, suggesting that the activation occurs exclusively within the EGF receptor moiety. Conceivably, kinase activation may require the transfer of a conformational change through the single transmembrane region from the ligand binding domain to the cytoplasmic kinase region. Alternatively, ligand-induced receptor–receptor interactions may activate the kinase and thus bypass this requirement. Both mechanisms were contrasted by employing independent experimental approaches. The following lines of evidence support an intermolecular mechanism for the activation of the detergent-solubilized receptor: (1) the EGF-induced receptor self-phosphorylation has a parabolic dependence on the concentration of EGF receptor, (2) cross-linking of EGF receptors by antibodies or lectins stimulates receptor self-phosphorylation, (3) immobilization of EGF receptor on various solid matrices prevents EGF from activating the kinase function, and (4) cross-linking of EGF receptors increases their affinity toward EGF. On the basis of these results, an allosteric aggregation model is formulated for the activation of the cytoplasmic kinase function of the receptor by EGF. This model may be relevant to the mechanism by which the mitogenic signal of EGF is transferred across the membrane.

Epidermal growth factor (EGF)[1] mediates its mitogenic response through interaction with a specific membrane receptor. The EGF receptor is a 170 000-dalton polypeptide with an intrinsic tyrosine protein kinase activity (Cohen et al., 1982a) similar to the kinase activity of the gene products of the src gene family of oncogenes (Hunter & Cooper, 1985). We have generated monoclonal anti-EGF receptor antibodies (Schreiber et al., 1983) and utilized them in immunoaffinity chromatography for the purification of EGF receptor (Yarden et al., 1985) for structural studies and for the determination of its primary structure (Downward et al., 1984b). From the complete primary structure of the EGF receptor, deduced from cDNA clones (Ullrich et al., 1984), it was concluded that the receptor molecule can be divided into three domains. The extracellular EGF-binding domain (621 amino acids) is connected by a single transmembrane region of 23 hydrophobic amino acids to the cytoplasmic tyrosine kinase domain (542 amino acids). Interestingly, the transforming protein of the avian erythroblastosis virus *v-erb-B* is a truncated EGF receptor that has lost the EGF-binding domain, retaining the cytoplasmic kinase portion, the transmembrane region, and a small extracellular domain (Downward et al., 1984b; Ullrich et al., 1984; Yamamoto et al., 1983). It has been proposed that autonomous growth can be achieved by an aberrant growth factor receptor that has lost its normal growth-control sequences, as apparently is the case for *v-erb-B*. In intact cells EGF receptor is phosphorylated in response to EGF on serine, threonine, and tyrosine residues (Hunter & Cooper, 1981). Tyrosine phosphorylation of EGF receptor in intact cells or in membranes appears to be due to self-phosphorylation of EGF receptor by its intrinsic tyrosine kinase activity (Cohen et al., 1982a; Ushiro & Cohen, 1980). However, phosphorylation at serine and threonine residues is probably mediated by protein kinase C (Cochet et al., 1984; Iwashita & Fox, 1984). The role of tyrosine phosphorylation in the mitogenic signal induced by EGF is not known (Yarden et al., 1982). However, the fact that various receptors for growth factors (Ek et al., 1982; Kasuga et al., 1982) and oncogene products possess this enzymatic activity raises the possibility that tyrosine phosphorylation may act as the first intracellular signal triggered by some mitogens. Hence, it is of interest to establish the mechanism by which EGF stimulates its receptor kinase activity.

Considering the single transmembrane domain of EGF receptor and the rapid activation by EGF of the kinase function even in highly purified receptor preparations (Cohen et al., 1982b), an important unanswered question is the mechanism underlying signal transfer between the EGF-binding domain and the cytoplasmic kinase domain. Essentially, two types of models can be proposed for signal transduction across the plasma membrane: an intramolecular model and an intermolecular model. In an intramolecular model it is assumed that the binding of EGF induces a conformational change in the extracellular domain which is transmitted through the transmembrane region to the kinase domain which is consequently activated. Alternatively, in an intermolecular model it is assumed that receptor–receptor interactions which are

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; PMSF, phenylmethanesulfonyl fluoride; PBS, Dulbecco's phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; TPA, 4β-phorbol 12β-myristate 13α-acetate; WGA, wheat germ agglutinin; Con A, concanavalin A.

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mediated by EGF lead to the activation of the receptor kinase. This type of mechanism will bypass the requirement for a conformational change to be transmitted through the single hydrophobic stretch connecting the two functional domains.

On the basis of these considerations and on the basis of the observation that in intact cells EGF induces receptor aggregation (Schlessinger et al., 1978; Haigler et al., 1978; Zidoveztki et al., 1981), we undertook a multiple-approach study to contrast these two models. Assuming that the activation of the tyrosine kinase by EGF involves an intermolecular process, then (1) it should be dependent on the concentration of EGF receptor; (2) cross-linking of EGF receptors by multivalent reagents may activate the kinase; (3) prevention of interactions between EGF receptors should abolish the ability of EGF to enhance kinase activity; and (4) if receptor aggregation is driven by ligand binding, then aggregated receptors should have higher affinity toward EGF as compared with dispersed receptors. These predictions were tested in an in vitro system composed of a detergent-solubilized EGF receptor. Our results appear to be compatible with an intermolecular mechanism rather than with an intramolecular mechanism for receptor activation. In the accompanying paper (Yarden & Schlessinger, 1987) we present complementary structural evidence that further supports this view of the EGF receptor.

Materials and Methods

Materials. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from the Radiochemical Centre, Amersham. EGF was supplied by IDL (Jerusalem) and radiolabeled with Na^{125}I by the lactoperoxidase method. Molecular weight standards for gel electrophoresis were from Bio-Rad. Wheat germ agglutinin (WGA) and *Lens culinaris* hemagglutinin were obtained from Bio-Yeda (Rehovot). Protein A coupled to Sepharose was obtained from Pharmacia. All other reagents were from Sigma.

Cells. Human epidermal carcinoma cells (A-431) were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated newborn calf serum (BioLab Laboratories, Jerusalem). Monolayers of A-431 cells were grown to confluency in 15-cm plastic dishes (Nunc).

Buffered Solutions. HNEG buffer contained 20 mM Hepes buffer, pH 7.5, 150 mM NaCl, 1 mM EGTA, and 10% glycerol.

HNTG buffer contained 20 mM Hepes buffer, pH 7.5, 150 mM NaCl, 0.1% (w/v) Triton X-100, and 10% glycerol.

Solubilization buffer contained 50 mM Hepes buffer, pH 7.5, 150 mM NaCl, 1% (w/v) Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, aprotinin (0.15 trypsin inhibitor unit/mL), 1 mM PMSF, and 10 μg/mL leupeptin.

Binding buffer contained 50 mM Hepes buffer, pH 7.5, 0.1 mM NaCl, 0.1% (w/v) Triton X-100, and 0.1% (w/v) bovine serum albumin.

Dulbecco's phosphate-buffered saline (PBS) contained 137 mM NaCl, 2.7 mM KCl, 7.9 mM $NaHPO_4$, and 1 mM KH_2PO_4, pH 7.2.

Antibodies. Full details concerning the monoclonal antibodies TL5-IgG$_3$ and 29.1-IgG$_1$ were previously described (Schreiber et al., 1983). The generation and properties of the rabbit antibodies against a synthetic peptide from EGF receptor designated RK2-IgG were described elsewhere (Kris et al., 1985). Monovalent fragments of the RK2-IgG antibody were prepared by papain digestion (Harrison & Mage, 1967). Papain (1:100 w/w) was added to 30 mg of the antibody in 0.1 M sodium acetate buffer, pH 5.5, in the presence of ethylenediaminetetraacetic acid (2 mM) and cysteine (1 mM). The digestion was terminated after 8 h at 37 °C with iodoacetamide (1 mM), and the Fab fragment was purified by protein A affinity chromatography. SDS–PAGE analysis revealed the M_r 48 000 Fab-RK2 without traces of either the intact antibody molecule or the F_C fragment. The monovalent Fab fragment coupled to protein A–Sepharose beads conjugated to goat anti-rabbit antibodies was able to immunoprecipitate the EGF receptor, demonstrating that it retained its antigen binding capacity. Also, the radiolabeled Fab fragment specifically bound to plasma membrane vesicles of A-431 cells. Polyclonal antibodies to EGF receptor AgA-IgG were generated in rabbits by immunizing with shed membrane vesicles prepared from A-431 cells (Cohen et al., 1982a). All the antibodies used were ammonium sulfate precipitates of the corresponding antisera which were further purified by either ion-exchange chromatography (29.1-IgG$_1$; Yarden et al., 1985) or affinity chromatography on protein A–Sepharose (TL5-IgG$_3$, RK2-IgG).

Preparation of Plasma Membrane Fraction of A-431 Cells. Confluent dishes of A-431 cells were washed twice at 22 °C with 20 mL of phosphate-buffered saline at pH 7.2, scraped with a rubber policeman, and centrifuged (600*g*, 10 min at 4 °C). The cell pellet was suspended in 10 volumes of hypotonic buffer (10 mM Hepes buffer, pH 7.5, 1.5 mM $MgCl_2$, 1 mM PMSF, and 1 mM EGTA) and incubated for 10 min at 4 °C. The cellular lysate was briefly homogenized by three strokes with a glass Dounce homogenizer and further centrifuged. The pellet was discarded, and the supernatant was centrifuged for 30 min at 4 °C (24000*g*). The resulting pellet was homogenized in 20 volumes of HNEG buffer and loaded on a sucrose cushion (35% sucrose in phosphate-buffered saline). After a 30-min centrifugation (24000*g* at 4 °C) the interface fraction was collected, diluted (1:10) in 10 mM Hepes buffer, pH 7.5, and recentrifuged at 100000*g* for 20 min (4 °C). The resulting pellet was resuspended and stored in aliquots at −70 °C until used. the protein content of these preparations was quantitated by the procedure of Bradford (1976) with γ-globulin as a standard.

Extraction of EGF Receptor from A-431 Cells. Confluent monolayers of A-431 cells were washed twice with 20 mL of phosphate-buffered saline and with 20 mL of HNEG buffer. The cells were scraped into 20 mL of the HNEG buffer, centrifuged (600*g*, 10 min at 4 °C), and stored at −70 °C until used. Frozen cells were suspended in solubilization buffer (approximately 10^7 cells/mL of buffer) and then homogenized in a glass Dounce homogenizer (12 strokes). The insoluble material was removed by centrifugation at 40000*g* for 30 min at 4 °C. Aliquots of the supernatant were stored at −70 °C for 1 month without apparent loss of kinase activity.

Purification of EGF Receptor. A lectin-purified fraction of the EGF receptor was obtained by suspending a 1-mL fraction of plasma membranes of A-431 cells (3.3 mg of protein) in 1 mL of the solubilization buffer. The detergent-insoluble material was sedimented by centrifugation at 40000*g* for 30 min. The supernatant was mixed with 0.3 mL of wheat germ agglutinin–agarose beads (Sigma), and the mixture was shaken for 30 min at 4 °C. Unabsorbed material was removed by centrifugation, and the moist gel was washed 6 times with 10 mL of HNTG buffer and twice with 10 mL of the same buffer that also contained 1 M NaCl. The specifically absorbed material was eluted by suspending the beads in 2 volumes of HNTG buffer supplemented with 0.3 M *N*-acetylglucosamine. The eluted material was diluted (1:1) with binding buffer and stored in aliquots at −70 °C.

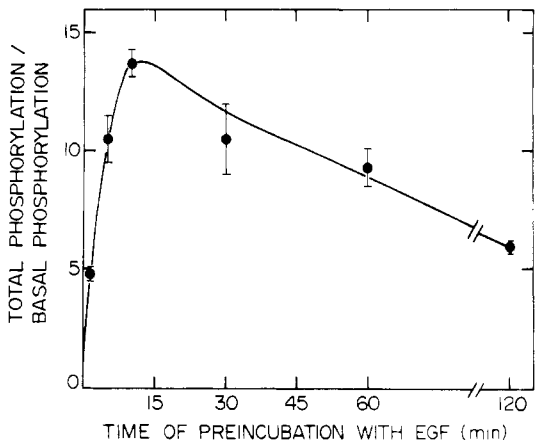


FIGURE 1: Receptor self-phosphorylation as a function of time of preincubation with EGF. Triton extracts from A-431 cells were incubated at 0 °C with EGF (1.7 μM) or with buffer alone for the indicated periods of time and then phosphorylated for 1 min at 0 °C. Duplicates of both EGF-treated (total phosphorylation) and nontreated (basal phosphorylation) receptor were taken. Receptor phosphorylation was quantitated by counting the radioactive content of the receptor band excised from the gels. The results are presented as the ratio of total phosphorylation to basal phosphorylation. Bars indicate the ranges of the corresponding averages.

Self-Phosphorylation of EGF Receptor. Detergent-solubilized receptor (20 μL) was preincubated for 15 min at 0 °C with 30 μL of binding buffer supplemented with either EGF (1.7 μM) or anti-receptor antibodies. Phosphorylation was initiated by the addition of 10 μL of ice-cold binding buffer containing 10 mM $MnCl_2$ and 50 μM ATP (5 μCi of [γ-^{32}P]ATP). After 20 s the phosphorylation was stopped by the addition of 15 μL of concentrated electrophoresis sample buffer followed by a 5-min incubation at 95 °C. The samples were then subjected to gel electrophoresis on 7.5% acrylamide gel. The region in the gel corresponding to an apparent molecular weight of 170 000 was excised after autoradiography of the dried gel and counted for Cerenkov radiation. The background radioactivity of the gel was determined by counting the radioactivity of a slice of the gel corresponding to an apparent molecular weight of 220 000.

EGF Binding to Solubilized EGF Receptor. The binding assay was carried out in a 50-μL final volume in binding buffer. Solubilized receptor prepared from plasma membrane fraction (10–30 μg of protein) was incubated for 4 h at 22 °C with different concentrations of ^{125}I-EGF (10^5 cpm/pmol). Separation of receptor-bound EGF from free ligand was carried out essentially as described (Carpenter, 1979), except that rabbit anti-receptor antibodies (125 μL of a 2 mg/mL solution) were added at the end of the binding reaction in order to augment precipitation by poly(ethylene glycol). Incubation with the antibodies was performed for 3 min at 0 °C, followed by the addition of 125 μL of 20% poly(ethylene glycol) in 20 mM Hepes buffer, pH 7.5. The precipitate was filtered on 0E-67 filters which were washed 4 times with 2 mL of 8% poly(ethylene glycol) in 20 mM Hepes buffer, pH 7.5. The nonspecific binding was determined in the presence of a 100-fold molar excess of unlabeled EGF. The binding data were analyzed according to the method of Scatchard (1949). The inclusion of the rabbit anti-receptor antibody in the assay increased the efficiency of receptor precipitation by 5-fold. Similar efficiency was achieved by replacing the antibody with a mixture of lectins (concanavalin A, wheat germ agglutinin, and *L. culinaris* lectin, 0.1 mg/mL each lectin). Under these conditions, preincubation of the receptor with TL5-IgG_3 antibody, before EGF binding, did not affect the efficiency of receptor precipitation, as was determined with ^{32}P-labeled receptor.

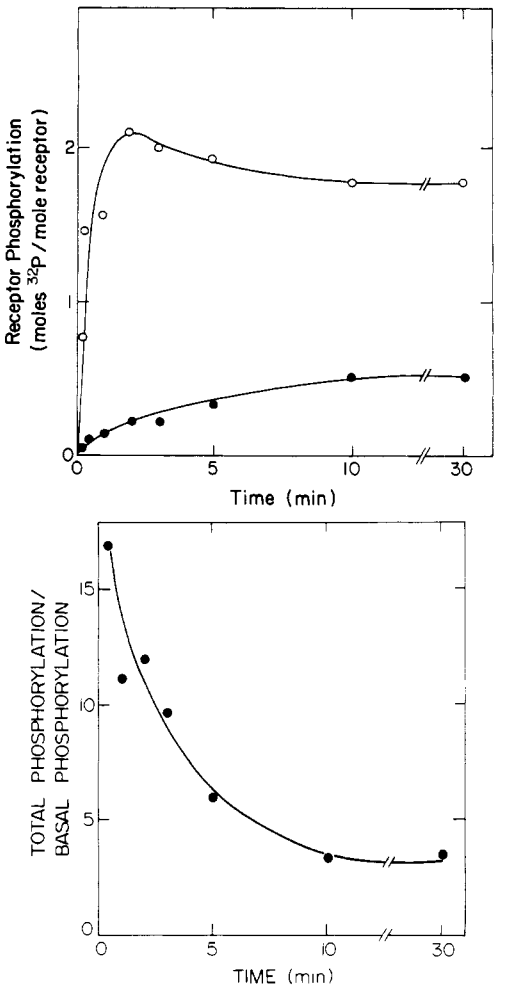


FIGURE 2: Receptor self-phosphorylation as a function of duration of phosphorylation reaction. (Top panel) Triton extracts of A-431 cells containing 40 nM EGF receptor were preincubated with EGF (1.7 μM; open circles) or buffer alone (closed circles) for 10 min at 0 °C and then subjected to phosphorylation at 0 °C for the indicated periods of time. (Bottom panel) Presentation of the data depicted in the top panel as the ratio between the amount of receptor phosphorylation in the presence vs. the absence of EGF.

RESULTS

The experimental system used in this study is a detergent-solubilized preparation of EGF receptor from A-431 cells of different degrees of purity. In establishing the experimental system, we have optimized various parameters that affect the self-phosphorylation of EGF receptor induced by EGF such as incubation times with EGF and with [γ-^{32}P]ATP and the effect of temperature. To assure full receptor occupancy, all the experiments were done with saturating concentrations of EGF (1.7 μM). Figure 1 shows that at 0 °C a 10-min incubation with EGF is required to obtain maximal receptor self-phosphorylation. Longer incubations with EGF result in a gradual reduction of EGF-induced receptor self-phosphorylation. Subsequent to preincubation with EGF, [γ-^{32}P]ATP and $MnCl_2$ were added to the reaction mixture for different periods, and the reaction was stopped by heating the samples. It appears that maximal receptor autophosphorylation was observed when the reaction was allowed to proceed for 20 s at 0 °C (Figure 2). The rate of the reaction was linear for 30 s, reaching a plateau after a 2-min incubation. On the basis of this analysis we conclude that

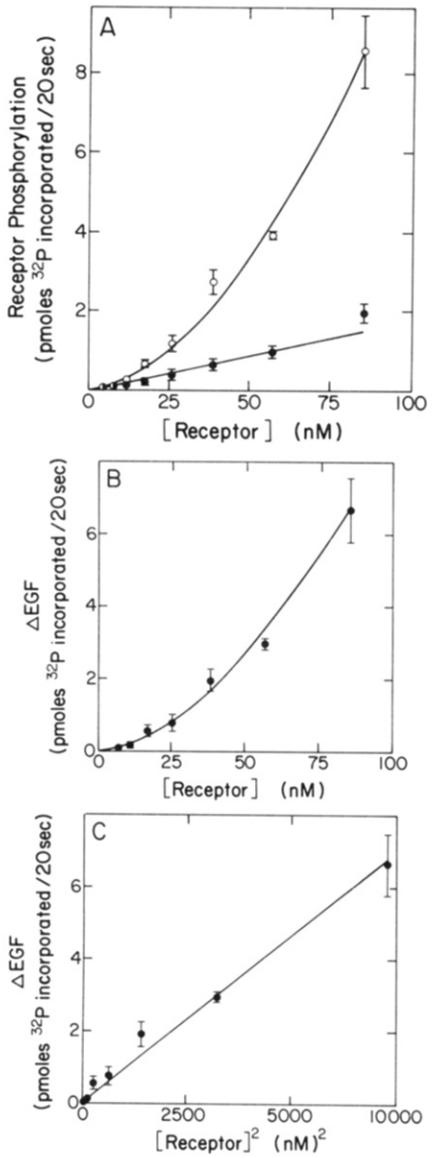


FIGURE 3: Effect of concentration of EGF receptor on EGF-induced receptor self-phosphorylation. EGF receptor was partially purified by wheat germ agglutinin affinity chromatography from the plasma membrane fraction of A-431 cells. This preparation was serially diluted and preincubated with either EGF (1.7 μM; open circles) or buffer alone (closed circles). Self-phosphorylation of EGF receptor was carried out and quantitated as described under Materials and Methods. Averages of duplicate samples and the corresponding ranges (bars) are given. Panel A depicts phosphorylation as a function of receptor concentration. Panels B and C depict the net EGF-induced phosphorylation (ΔEGF) as a function of either receptor concentration or the squared concentration of the receptor, respectively. Quantitation of EGF receptor was done by poly(ethylene glycol) precipitation of samples labeled with ^{125}I-EGF.

maximal response is obtained upon 10-min incubation with EGF followed by 20-s exposure to [γ-^{32}P]ATP at 0 °C. Under these conditions one to two phosphate groups are covalently attached to each receptor molecule.

EGF-Induced Self-Phosphorylation Is Dependent on Receptor Concentration. A partially purified preparation of EGF receptor, obtained by affinity chromatography with a lectin column, was serially diluted and subjected to phosphorylation reaction. Figure 3 depicts the effect of receptor concentration on the basal and EGF-induced phosphorylation of the receptor molecule. The basal activity increased linearly with receptor concentration, whereas the EGF-stimulated activity is concentration-dependent. When receptor phosphorylation is plotted as a function of the squared concentration of EGF

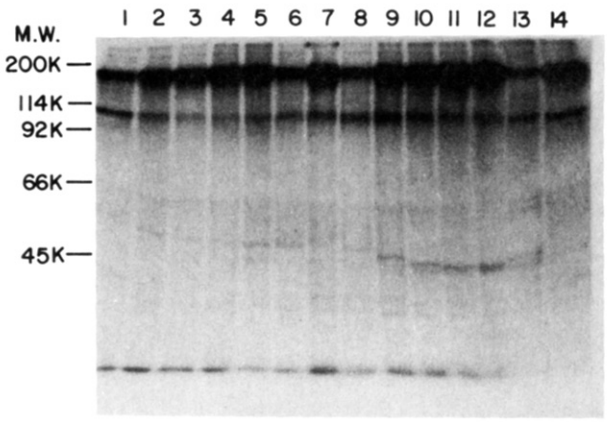


FIGURE 4: Effect of monoclonal anti-receptor antibodies and rabbit anti-receptor antibodies on EGF receptor autophosphorylation. Triton extracts of A-431 cells were preincubated for 20 min at 0 °C with EGF (1.7 μM; lanes 7 and 14), buffer alone (lanes 1, 6, and 8), monoclonal anti-receptor antibodies (TL5-IgG$_3$; lanes 2, 3, 4, and 5 at the following concentrations: 0.12, 0.25, 0.5 and 1 μM, respectively), rabbit polyclonal anti-receptor antibodies (AgA-IgG; lanes 9, 10, 11, and 12 at 0.06, 0.12, 2.5 and 5 μM, respectively) and nonimmune rabbit antibodies (lane 13; 2.5 μM). Receptor self-phosphorylation was quantitated as described undr Materials and Methods. The rabbit antibodies used are the γ-globulin fractions of the corresponding antisera, and monoclonal antibody is a pure IgG fraction of ascites fluid of mice.

receptor, a linear curve is obtained. Similar results were obtained when either detergent-solubilized plasma membranes or lysates of A-431 cells were used. Since this result is consistent with an intermolecular mechanism for stimulation of the kinase activation, additional experiments were performed to contrast between intramolecular and intermolecular mechanisms.

Cross-Linking of EGF Receptors Enhances Self-Phosphorylation. If EGF activates the kinase function by an intermolecular mechanism, then cross-linking of unoccupied receptors by multivalent agents may mimic the effect of EGF. To test this possibility, we employed various bivalent ligands (antibodies) or multivalent (lectins) ligands known to interact with EGF receptor. Two monoclonal antibodies, TL5-IgG$_3$ and 29.1-IgG$_1$, that recognize different carbohydrate residues associated with the EGF receptor of A-431 cells (Gooi et al., 1983) were able to enhance self-phosphorylation of the EGF receptor (Figures 4 and 7). Moreover, two rabbit anti-receptor antibodies directed to protein epitopes stimulated receptor phosphorylation (Figures 4 and 5). One of the polyclonal rabbit antibodies, AgA-IgG, reacts predominantly with the extracellular domain of the EGF receptor, whereas the second rabbit antibody, denoted RK2-IgG, is an anti-synthetic peptide antibody directed to the cytoplasmic domain of EGF receptor (Kris et al., 1985). In control experiments the corresponding preimmune or nonimmune antisera were found to have no effect on kinase activity. Finally, three plant lectins with different sugar specificities (concanavalin A, wheat germ agglutinin, and *L. culinaris* lectin) that bind to different carbohydrates of EGF receptor also mimicked the effect of EGF on receptor self-phosphorylation (Figure 6). As observed with EGF, all the various cross-linking agents specifically enhance the self-phosphorylation of the EGF receptor (170-kilodalton band). No other protein became signficantly phosphorylated.

The role of antibody valency is depicted in Figure 5. As shown, the intact RK2-IgG antibody stimulated receptor autophosphorylation whereas its monovalent Fab fragment is inactive in spite of retaining binding capacity toward the EGF receptor (Figure 5). Consistently, the dose curves of all

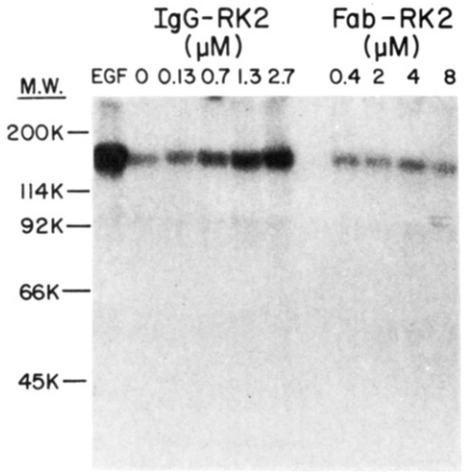


FIGURE 5: Effect of rabbit antibodies against synthetic peptide (RK2-IgG) and their monovalent fragment (RK2-Fab) on receptor self-phosphorylation. Detergent-solubilized plasma membranes from A-431 cells (30 μg of membrane protein) were incubated for 20 min at 0 °C with different concentrations of either IgG fractions of RK2 antibody, RK2-Fab, or EGF (1.7 μM). The samples were then phosphorylated with [γ-^{32}P]ATP and subjected to gel electrophoresis. The autoradiogram of the gel (1-h exposure) is shown. The experiment was repeated with Triton extract of A-431 cells, and the same results were obtained.

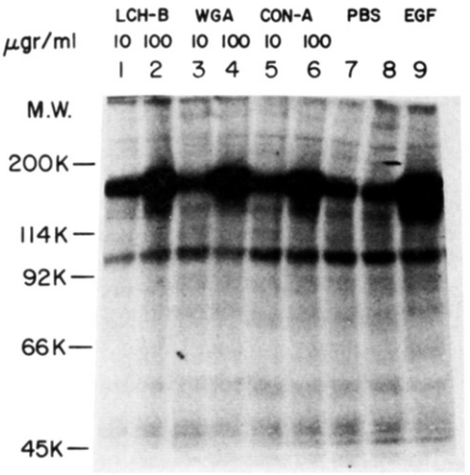


FIGURE 6: Effect of various lectins on receptor self-phosphorylation. Phosphorylation experiments with Triton X-100 extract of A-431 cells were carried out as described under Materials and Methods. Phosphorylation was preceded by a 10- or 30-min preincubation at 0 °C with either a 10 or 100 μg/mL concentration of the various lectins, respectively. The following lectins were used: *L. culinaris* lectin (LCH-B; lanes 1 and 2), wheat germ agglutinin (WGA; lanes 3 and 4), and concanavalin A (Con A; lanes 5 and 6). In control experiments (lanes 7 and 8) preincubations (10 and 30 min, respectively) were done with the buffer used in the stock lectin solution (PBS). The autophosphorylation of EGF receptor induced by 1.7 μM EGF for 10 min is shown in lane 9.

cross-linking agents employed in this study have bell-shaped profiles (Figure 7; data not shown for lectins). We interpret this result as an indication that maximal stimulation is achieved by multivalent binding of the ligands and their decreased effect is probably due to monovalent binding, which predominates at oversaturating ligand concentrations. Notably, all the cross-linking ligands employed achieved only 60–80% of the maximal stimulatory effect obtained with EGF. This may be due to the fact that the self-phosphorylation assay was optimized for EGF, conditions that are not necessarily optimal for the heterologous ligands. Alternatively, the failure to achieve maximal stimulatory effect may reflect mechanistic differences.

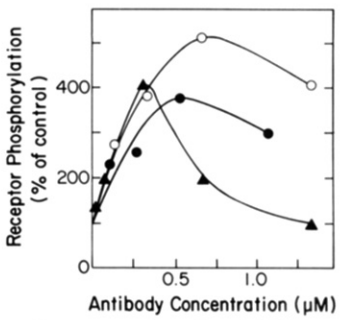


FIGURE 7: Effect of concentration of anti-EGF receptor antibodies on receptor self-phosphorylation. Triton extracts of A-431 cells were preincubated with increasing concentrations of anti-receptor antibodies and then phosphorylated as described under Materials and Methods. Antibodies used are 29.1-IgG$_1$ (triangles) and TL5-IgG$_3$ (closed circles) monoclonal anti-receptor antibodies and AgA-IgG rabbit anti-receptor antibodies (open circles). Quantitation of receptor phosphorylation was done by measuring the amount of ^{32}P label in the receptor band excised from the polyacrylamide gels. Receptor self-phosphorylation induced by EGF was 4–5-fold that of control values. These experiments were repeated 3 times with each antibody, with essentially the same results.

Table I: Phosphorylation of Immobilized Receptor[a]

immobilizing agarose matrix	EGF added before receptor immobilization	EGF added after receptor immobilization
29.1-IgG$_1$–Sepharose	12.2 ± 1.4	1.6 ± 0.4
TL5-IgG$_3$–protein A–Sepharose	6.0 ± 1.4	1.7 ± 0.3
RK2-IgG–protein A–Sepharose	4.6 ± 0.6	0.95 ● 0.1
Con A–agarose	4.7 ± 0.2	1.2 ± 0.2
WGA–Sepharose	6.4 ± 0.2	1.3 ● 0.4
Cibacrone blue–Sepharose	10.8 ± 2.5	1.1 ● 0.3

[a] Triton extracts of 1.5 × 10^5 A-431 cells were exposed to 1.7 μM EGF for 15 min at 0 °C or to buffer alone and then immobilized on the corresponding agarose beads. Three minutes later the receptor-bound beads were washed and immediately phosphorylated or first treated with EGF (1.7 μM; 15 min at 0 °C), washed, and then phosphorylated. Phosphorylation reaction was allowed for 20 s at 0 °C. Receptor phosphorylation was determined by gel electrophoresis as described under Materials and Methods. Results given represent receptor phosphorylation in the EGF-treated system relative to the corresponding buffer-treated experiments. Averages and corresponding ranges of duplicate experiments are given. The experiment was repeated 3 times with similar results. The extent of EGF-induced activation of soluble receptor kinase varied among different experiments in the range of 5–12-fold.

Receptor Immobilization Prevents EGF-Induced Kinase Activation. If kinase activation involves an intermolecular process, then receptor immobilization should prevent EGF from activating the kinase function. Receptor immobilization was accomplished by absorbing the receptor on agarose beads tagged with specific ligands that recognize the EGF receptor. A schematic description of the experiment is depicted in Figure 8.

A large molar excess of receptor-specific groups on the inert support was used, and extensive washes were done to prevent interactions between neighboring receptors and to remove any nonimmobilized receptor from the experimental system. The results of this experiment are presented in Table I. It shows that receptor immobilization prevents EGF from stimulating the kinase activity. This treatment did not affect the affinity of EGF toward the receptor (data not shown), suggesting that the inhibition is due to immobilization rather than to a conformational change that may have been induced by the various immobilizing matrices. This interpretation is further supported by the fact that similar results were obtained by utilizing six different immobilizing ligands that bind to different sites on the receptor (see Table I). Since some of the ligands interact

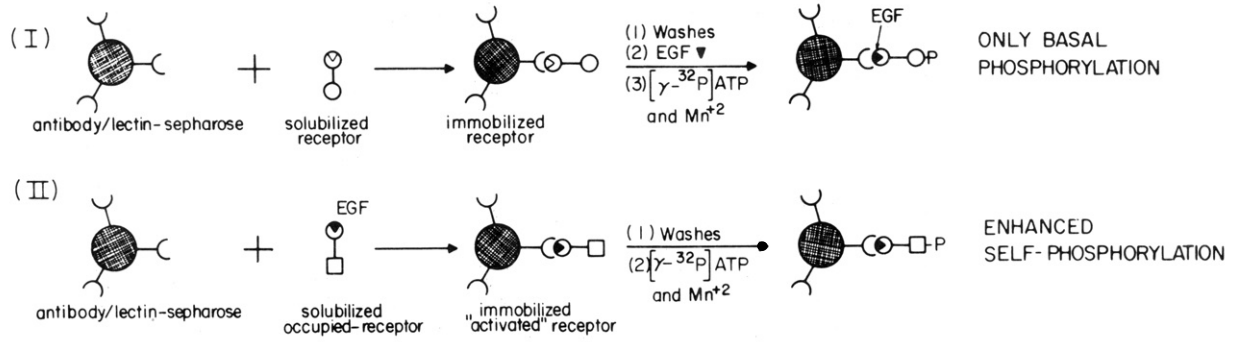


FIGURE 8: Schematic description of the protocol of the experiment used to determine effect of receptor immobilization on EGF-induced self-phosphorylation. (I) Solubilized receptor was immobilized on antibody or lectin–Sepharose. After several washes, EGF (or buffer alone), [γ-^{32}P]ATP, and Mn^{2+} were added. Only basal self-phosphorylation was detected in this experiment. (II) Solubilized receptor labeled with EGF was immobilized on antibody or lectin–Sepharose. The immobilized "activated" receptor was washed and then exposed to [γ-^{32}P]ATP and Mn^{2+}. Enhanced self-phosphorylation was detected in this experiment (see Table I).

with sugar moieties of the receptor molecule, it is unlikely that all of them will affect the conformation of the EGF receptor in a similar manner. In control experiments, first, EGF was added to the receptor and then the occupied receptors were immobilized prior to the phosphorylation reaction (Figure 8). Interestingly, preincubation of EGF receptor with EGF followed by immobilization resulted in an activated kinase, suggesting that once the kinase is activated, additional receptor–receptor interactions are not required for self-phosphorylation to occur. The simplest interpretation of the immobilization experiments is that EGF-induced receptor–receptor interactions are required for the stimulation of the kinase activity; however, once the kinase is activated, the autophosphorylation process is probably mediated by an intramolecular event.

Cross-Linking of EGF Receptors Increases Their Affinity toward EGF. The results presented so far appear to favor an intermolecular mechanism for the activation of the kinase function by EGF. Hence, one possible mechanism is that the EGF receptor exists in several aggregation states and that the oligomeric state is energetically stabilized by possessing higher ligand affinity as compared to the monomeric receptors. To test this possibility, we examined the effect of receptor cross-linking on the affinity of EGF toward the receptor. Short incubations of detergent-solubilized EGF receptor with monoclonal anti-receptor antibody TL5-IgG_3 resulted in higher ligand binding (Figure 9). Scatchard analysis of the binding curves in the absence of the antibody revealed a homogeneous population of binding sites for EGF with an apparent K_D of 100 nM. Upon cross-linking, a curvilinear Scatchard plot is obtained that indicates the appearance of high-affinity binding sites (K_D = 30 nM). Nevertheless, the total number of binding sites did not change, suggesting that the antibody-mediated cross-linking converted the low-affinity binding sites to high-affinity ones. Similar Scatchard plots were obtained when the cross-linking was induced by the monoclonal antibody 29.1-IgG_1 or by the RK2-IgG antiserum, which recognizes the cytoplasmic portion of EGF receptor, indicating that the increased affinity is due to receptor aggregation rather than to antibody binding to a specific epitope in the extracellular domain. No effect was observed when neither normal mouse monoclonal antibodies nor preimmune rabbit antibodies were used. Hence, we conclude that the cross-linking of EGF receptors transforms preexisting low-affinity binding sites to binding sites with higher affinity toward the ligand.

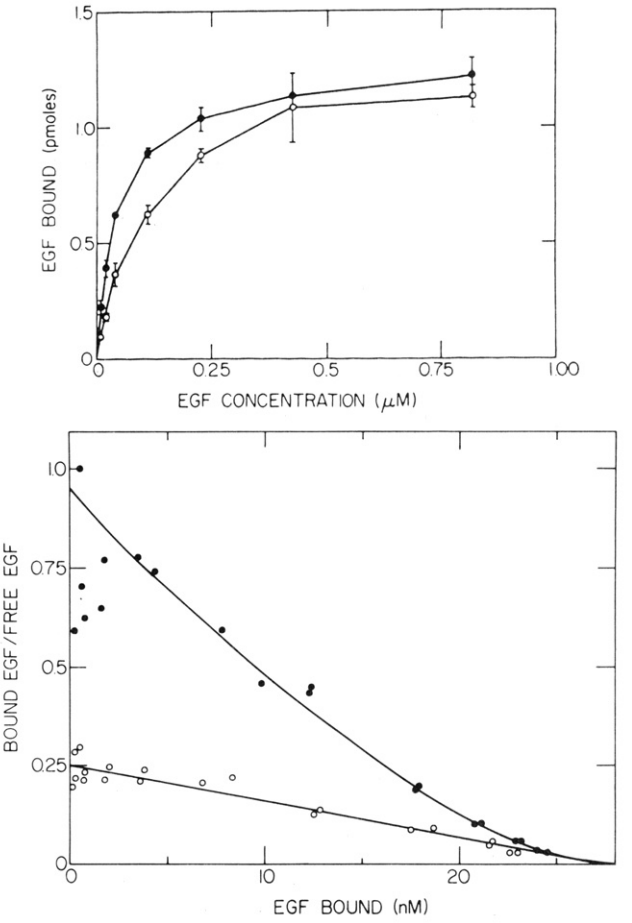


FIGURE 9: Effect of TL5-IgG_3 monoclonal anti-receptor antibody on the binding of EGF to detergent-solubilized receptor. Detergent-solubilized plasma membranes from A-431 cells (5 μg of membrane protein) were preincubated for 45 min at 0 °C with either TL5-IgG_3 (0.5 μM; closed circles) or buffer alone (open circles). Binding assay with radiolabeled EGF was carried out as described under Materials and Methods. Nonspecific binding was determined in the presence of a 100-fold molar excess of unlabeled EGF. Both the binding curves and the Scatchard analysis are given. Data points are the averages of triplicate results of two separate experiments. Bars represent standard deviation.

Discussion

An interesting conclusion drawn from the primary structure of EGF receptor (Ullrich et al., 1984) is that the extracellular ligand binding domain is connected to the cytoplasmic kinase region by a single transmembrane region. This is in contrast to other transmembrane proteins that are involved in signal transduction across the plasma membrane and that usually traverse the lipid bilayer more than once. Thus in rhodopsin and bacteriorhodopsin, seven α-helices span the membrane (Stoeckenius, 1985). Moreover, the β-adrenergic receptor

spans the plasma membrane seven times (Dixon et al., 1986; Yarden et al., 1986), the α subunit of the nicotinic acetylcholine receptor has five transmembranal regions (Young et al., 1985), and the bacterial chemosensory transducers have two membrane-spanning regions (Bollinger et al., 1984). Hence, multiple crosses of the plasma membrane seem to be often associated with proteins that mediate ligand-induced signaling across the membranes. Two distinct mechanisms can be reasoned for the transmembrane signaling of membrane proteins with a single transmembrane region such as the EGF receptor. In an intramolecular model a vertical dislocation of the membranal hydrophobic stretch of EGF receptor is required for the transfer of a conformational change from the ligand binding domain to the cytoplasmic kinase region. However, the juxtamembranal sequence of EGF receptor contains charged amino acids at both faces of the plasma membrane. Moreover, several proline residues reside at the cytoplasmic face of the plasma membrane of the EGF receptor (Ullrich et al., 1984; Livneh et al., 1985). This configuration may impose a high-energy barrier for a putative vertical conformational change. Moreover, comparison of the transmembrane amino acid sequence of the human EGF receptor with its *Drosophila* homologue (Livneh et al., 1985) indicates that, besides its hydrophobic nature, this is one of the most variable regions of the EGF receptor.

An alternative mechanism involves an intermolecular allosteric process in which ligand-induced receptor–receptor interactions lead to the activation of the kinase function by subunit interaction between neighboring cytoplasmic domains.

In this paper we employ an in vitro system to contrast between these two mechanisms. The following three separate lines of experimental evidence seem to be consistent with an intermolecular mechanism rather than with an intramolecular activation process: (1) EGF-induced self-phosphorylation of its solubilized receptor exhibits a parabolic concentration profile, suggesting that a bimolecular step is involved in receptor activation. (2) Cross-linking of detergent-solubilized receptors by different bivalent and multivalent ligands (antibodies and lectins) activates the kinase function without the involvement of EGF. Bivalency appears to be essential, as if receptor aggregation is sufficient for at least partial activation of the kinase function. (3) Prevention of receptor interactions by means of immobilization on an inert support abolishes EGF-induced activation of the immobile receptor.

It seems, therefore, that receptor–receptor interactions are essential for the ligand-induced activation. Moreover, these results are inconsistent with an intramolecular activation in which the conformational change propagates from the extracellular to the cytoplasmic domain of an individual receptor molecule. This conclusion is consistent with Rubin and Earp (1983a,b), who studied the dependencies of kinase activation upon receptor and EGF concentrations and concluded that a bimolecular step involving two monomeric receptors may take place in the activation process. Gill et al. (1984) reported that a monoclonal anti-receptor antibody is a partial agonist for the self-phosphorylation reaction. On the other hand, Weber et al. (1984) reported zero-order kinetics for receptor phosphorylation and concluded that an intramolecular autophosphorylation follows EGF binding.

It appears that minimal oligomerization, namely, EGF receptor dimerization, is sufficient for the activation of the kinase function of the solubilized receptor. This conclusion is based on the fact that receptor self-phosphorylation exhibits bimolecular kinetics (Figure 3C). Moreover, a bivalent antibody, which recognizes a single epitope on the receptor molecule (RK2-IgG), is an efficient agonist of the receptor kinase (Figure 6). However, all the multivalent ligands tested appear to be less active than EGF in activating the autophosphorylation of EGF receptors. This may suggest that the mechanism by which EGF activates the receptor is more efficient than simple dimerization. In contrast to previously described agonistic antibodies 2G2-IgM (Schreiber et al., 1981) and 528-IgG (Gill et al., 1984), the cross-linking ligands tested in the present study do not compete with EGF for the receptor binding site. Hence, it seems that kinase activation may be accomplished in either one of two ways: (1) monovalent interaction of EGF with the EGF binding site on the receptor, probably followed by a conformational change that facilitates receptor–receptor interactions, and (2) bivalent or multivalent interactions of heterologous ligands with sites other than the binding site, thereby directly cross-linking the unoccupied receptors. As indicated, the latter route of receptor activation seems to be less efficient than the EGF-mediated process.

Although EGF-induced activation of its receptor kinase seems to involve an intermolecular step, the autophosphorylation of the receptor molecule seems to be an intramolecular event. Two lines of experimental evidence support this conclusion. First, the basal self-phosphorylation of the receptor in the absence of EGF exhibits first-order kinetics (Figure 3). Second, and more important, the EGF-stimulated receptor exhibits elevated self-phosphorylation even under conditions where intermolecular interactions are not allowed (Table I). Thus, both the basal and the EGF-induced self-phosphorylation of EGF receptor seem to be mediated by an intramolecular reaction. This conclusion is consistent with the results of Weber et al., who employed an immunoaffinity-purified receptor and concluded that the kinetically preferred incorporation of the first phosphate is an intramolecular reaction (Weber et al., 1984). However, as EGF was used to elute the purified receptor from the affinity column, these results are relevant to the EGF-stimulated state of the receptor.

The ability of EGF to increase the interaction between EGF receptors is a central feature of the proposed intermolecular activation mechanism. As the sole source of energy is the formation of hormone–receptor complexes, we reasoned that a higher affinity for the oligomeric receptor may provide the driving force for increased receptor–receptor associations. The results presented in this paper indicate that soluble aggregates of EGF receptors possess elevated ligand affinity. The increased affinity is observed when the antibodies used to cross-link the receptor were directed against either extracellular or cytoplasmic epitopes, thus suggesting that receptor clustering per se may create a high-affinity state. The curvilinear Scatchard plot obtained for the EGF-binding data in the presence of the antibody implies that only part of the binding sites converted their affinity, perhaps due to incomplete clustering mediated by the antibody. Curvilinear Scatchard plots indicative of high- and low-affinity EGF receptors were observed in binding experiments with ^{125}I-EGF to numerous cultured cells (King & Cuatrecasas, 1982; Adamson & Rees, 1981; Fearn & King, 1985; Kawamoto et al., 1983). However, detergent-solubilized receptors usually (Cohen et al., 1982a; Yarden et al., 1985) exhibit a single low-affinity class of binding sites. This may reflect the adverse effects of the detergent, which may disrupt the high-affinity state of the putative oligomeric receptors by solubilization.

On the basis of the results presented in this paper we propose an allosteric aggregation model for the activation of the EGF receptor kinase function by the growth factor (Figure 10).

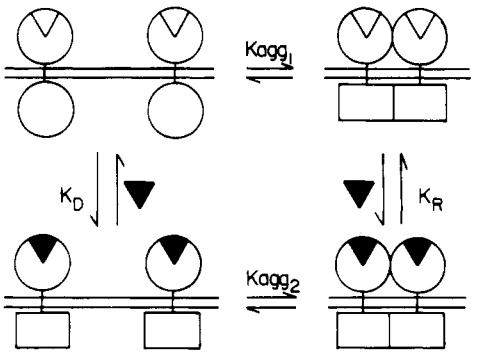
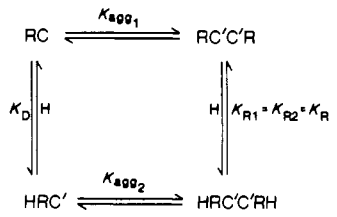


FIGURE 10: Allosteric oligomerization model for activation of EGF receptor kinase by EGF. EGF receptor is schematically depicted as a biglobular transmembrane structure (receptor domains are not drawn to scale). It is proposed that monomeric EGF receptor is in equilibrium with receptor oligomers. To simplify the picture, we describe aggregation as dimer formation. RC stands for monomeric EGF receptor, R stands for the binding domain of EGF receptor, C stands for the kinase domain, and H stands for the ligand. It is assumed that the monomeric receptor has low kinase activity (RC) and low binding affinity toward EGF (K_D) and that the dimeric receptor has stimulated kinase activity (RC'C'R) and high binding affinity toward EGF (K_{R1}, K_{R2}). To further simplify the picture, we assume that the sites on each dimer are identical and that they do not interact, namely, $K_{R1} = K_{R2} = K_R$. $K_{agg_1} = [RC]^2/[RC'C'R]$ is the aggregation



constant in the absence of the ligand, and $K_{agg_2} = [HRC']^2/[HRC'C'RH]$ is the aggregation constant in the presence of the ligand. $K_D = [RC][H]/[RC'H]$, $K_{R1} = [H][RC'C'R]/[RC'C'RH]$, and $K_{R2} = [H][RC'C'RH]/[HRC'C'RH]$. On the basis of microscopic reversibility, $K_{agg_2}K_D^2 = K_{agg_1}K_{R1}K_{R2}$ or $K_{agg_2}/K_{agg_1} = K_R^2/K_D^2$. If $K_R > K_D$ by a factor of 10–100, then $K_{agg_2} > K_{agg_1}$ by a factor of 100–10000. Hence, the binding energy $G = -RT \ln (K_R/K_D)$ confers the aggregated state upon the EGF receptor. This in turn leads to elevated tyrosine kinase activity.

According to this model EGF receptors exist in equilibrium between a monomeric state and an oligomeric state. It is assumed that the oligomeric state has higher affinity toward the ligand, and therefore the binding of EGF shifts the equilibrium toward receptor oligomers. It is proposed that simple juxtaposition of occupied receptors is sufficient to activate their intrinsic kinase function by allosteric subunit interaction.

Obviously, further experiments are required to clarify the validity of the allosteric model for the activation of EGF receptor in living cells. However, it is well established that in intact cells EGF induces the aggregation of EGF receptors (Schlessinger et al., 1978; Haigler et al., 1978; Zidovetzki et al., 1981). Moreover, analysis of the rotational diffusion of EGF receptor in membrane vesicles indicated that under these conditions receptor microaggregation is a partially reversible process independent of metabolic energy or external interactions (Zidovetzki et al., 1986). Receptor clustering, induced by multivalent antibodies, is sufficient to induce DNA synthesis in human and mouse fibroblasts (Schechter et al., 1979; Schreiber et al., 1981, 1983; Chandler et al., 1985; Fernandez-Pol, 1985). As already discussed (Schreiber et al., 1983; Dower et al., 1981), ligand-induced aggregation of binding sites and their apparent ligand affinity are mutually dependent parameters. Indeed, mitogenicity was attributed to a small class of high-affinity receptors for EGF (Schechter et al., 1978; Kawamoto et al., 1983; King & Cuatrecasas, 1982). Whether these sites represent oligomeric EGF receptors is still an open question (Rees et al., 1984).

The role of tyrosine phosphorylation of EGF receptor in the generation of the mitogenic signal is not clear (Schreiber et al., 1981; Yarden et al., 1982). It was suggested that self-phosphorylation enhances the kinase activity toward endogenous substrates (Betrics & Gill, 1985). The picture is probably more complicated, as in living cells it was shown that EGF receptor is also phosphorylated on serine and threonine residues (Hunter & Cooper, 1981). It was further shown that kinase C phosphorylates EGF receptor on Thr-654, a residue located 10 amino acids beneath the transmembrane region of EGF receptor (Hunter et al., 1984; Davis & Czech, 1985). TPA, which activates the kinase, was shown to abolish the high-affinity state of EGF receptor and to reduce its kinase activity (Friedman et al., 1984; Fearn & King, 1985). Therefore, it was proposed that the phosphorylation of Thr-654 may induce an allosteric transition in EGF receptor which in turn will modulate receptor ligand affinity and tyrosine kinase activity. Alternatively, it is possible that the phosphorylation of Thr-654 may prevent the aggregation of EGF receptor, thus favoring a monometric receptor with decreased affinity toward EGF and diminished tyrosine kinase activity.

In summary, the data presented here provide evidence that EGF-induced oligomerization of its soluble receptors may mediate the activation of the cytoplasmic tyrosine kinase function. In the accompanying paper (Yarden & Schlessinger, 1987) we provide structural evidence that further supports this mechanism. The relevance of the suggested molecular mechanism to the mitogenic signaling by EGF is a matter for future research.

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