Epidermal Growth Factor Induces Rapid, Reversible Aggregation of the Purified Epidermal Growth Factor Receptor†

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ABSTRACT: Epidermal growth factor (EGF) receptor from A-431 cells was purified by affinity chromatography with monoclonal anti-receptor antibodies. The purified radiolabeled receptor was incubated with EGF and then analyzed by gel electrophoresis under nondenaturing conditions. In these gels, the EGF receptor migrates in two forms: a fast-migrating (low) form and an EGF-induced slow-migrating (high) form. On the basis of the various control and calibration experiments described, it is concluded that the low form represents the monomeric 170-kilodalton EGF receptor and the high form represents an EGF receptor dimer. The binding of EGF causes a rapid, temperature-sensitive dimerization of the EGF receptor. Receptor dimerization is fully reversible and involves saturable, noncovalent interactions that are stable at neutral pH and in nonionic detergents. Both the monomeric and dimeric forms of the receptor bind EGF and undergo self-phosphorylation. The dimeric form of the receptor may possess higher ligand binding affinity, and it seems to be phosphorylated earlier than the monomeric form following the addition of EGF and [γ-^{32}P]ATP. On the basis of these results, it is concluded that receptor oligomerization is an intrinsic property of the occupied EGF receptor and that it may play a role in the activation of the kinase function and the subsequent transmembrane signaling process.

In the preceding paper we have addressed the question of signal transduction between the extracellular EGF[1]-binding domain and the cytoplasmic kinase region of the EGF receptor which allows ligand-induced regulation of the protein kinase function (Yarden & Schlessinger, 1987). On the basis of the primary structure of the EGF receptor (Ullrich et al., 1984) and on the basis of previous structural evidence for the existence of receptor oligomers (Zidovetzki et al., 1981) and their possible functional role (Schrieber et al., 1983), it has been proposed that the stimulation of the cytoplasmic kinase function by EGF may involve an intermolecular process. Moreover, an allosteric aggregation model was formulated as a framework for the various regulatory responses of the EGF receptor complex. The following lines of experimental evidence are compatible with an allosteric aggregation model. Ligand-induced self-phosphorylation of EGF receptor is dependent on the concentration of the receptor. Antibodies that cross-link the receptor molecules stimulate self-phosphorylation and increase the affinity of EGF toward the receptor. Finally, immobilization of EGF receptor on various solid matrices prevents EGF from activating the kinase function.

In the present study we further tested the following predictions made by the aggregation model described in the accompanying paper: (1) Does EGF receptor exist in a reversible equilibrium between monomeric and oligomeric states? (2) Is aggregation of receptors, in response to EGF, an intrinsic property of the receptor molecule?

On the basis of the higher ligand affinity of receptor aggregates as compared with dispersed receptors (see Figure 9 in the preceding paper), we estimated that the aggregated state would be stabilized by 1–5 kcal/mol as compared with the monomeric state. Hence, in order to detect the existence of receptor oligomers, it is necessary to employ approaches that will retard the dissociation of EGF receptor. One possible experimental approach is to use nondenaturing polyacrylamide gel electrophoresis (ND-PAGE). It is anticipated that within this gel the polymerized matrix will retard the dissociation reaction and will thus preserve the putative oligomeric state. By employing this approach here, we present evidence that EGF receptor induces rapid, reversible, oligomerization of the purified EGF receptor.

Materials and Methods

Materials. EGF was supplied by IDL (Jerusalem). Molecular weight marker proteins for ND-PAGE were from Pharmacia. Wheat germ lectin bound to agarose and aprotinin were from Sigma. Radioactive materials were purchased from Amersham. A-431 cells were grown as previously described (Yarden et al., 1985).

Receptor Purification. EGF receptor was purified from A-431 cells essentially as previously described (Yarden et al., 1985) except that all procedures were done in batches of 1.5 mL. Briefly, 2×10^7 A-431 cells were solubilized in 1 mL of solubilization buffer (50 nM Hepes buffer, pH 7.5, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 0.15 TIU/mL aprotinin). The cell extract was cleared by centrifugation for 10 min at 4 °C in an Eppendorf centrifuge. The clear extract was mixed with 0.07 mL of monoclonal anti-receptor antibody 29.1-IgG coupled to Sepharose beads (2 mg of antibody/mL of beads). After 1 h at 4 °C the beads were washed 3 times with 1 mL of HTG buffer (20 mM Hepes, pH 7.5, 0.1% Triton X-100, 10% glycerol), followed by two washes with 1 mL of borate buffer (50 mM borate buffer, pH 8.3, 0.7 M NaCl, 0.3 M $MgCl_2$, 10% glycerol, 0.1% Triton X-100) and two washes with 1 mL

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[1] Abbreviations: EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; ND-PAGE, nondenaturing polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; TIU, trypsin inhibitor unit(s); kDa, kilodalton(s).

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of HTG buffer. Radiolabeled receptor was obtained by incubating the receptor bound to the beads for 10 min at 4 °C with [γ-^{32}P]ATP (0.2 μM, 10–50 μCi) and $MnCl_2$ (5 mM) followed by washing with HTG buffer containing phosphatase inhibitors (1 mM ATP, 0.5 mM $ZnSO_4$, and 0.04 mM $NaVO_3$). Elution of the receptor from the immobilized antibody was carried out either by acidic buffer (50 mM glycine hydrochloride buffer, pH 2.5, 10% glycerol, 0.1% Triton X-100) or by 0.2 M *N*-acetylgalactosamine in HTG buffer. After the acid elution, the purified fraction was neutralized by the addition of concentrated Hepes buffer containing protease inhibitors (1 mM PMSF and aprotinin at 0.15 TIU/mL) and phosphatase inhibitors (0.5 mM $ZnSO_4$ and 0.04 mM $NaVO_3$). Receptor concentration in the purified fraction was determined by binding of radiolabeled EGF followed by poly(ethylene glycol) precipitation (Carpenter, 1979) and assuming monovalent interactions between the receptor and EGF. The purified fraction appears mostly as a single 170 000-dalton polypeptide in gels stained by Coomassie blue. Partial receptor purification was used for the experiment depicted in Figure 9. Wheat germ agglutinin (WGA) bound to agarose was used exactly as described above for 29.1-IgG–Sepharose except that 0.3 M *N*-acetylglucosamine was used for specific elution of the bound protein. Receptor concentration was determined by binding of ^{125}I-EGF (see below), assuming monovalent interactions.

Nondenaturing Polyacrylamide Gel Electrophoresis (ND-PAGE). Standard slab gels of 1.5-mm thickness (16 × 15 cm) were used. A linear gradient of 2–15% acrylamide was used with no stacking gel. Acrylamide and bis(acrylamide) dissolved in electrophoresis buffer [90 mM tris(hydroxymethyl)aminomethane, 80 mM boric acid, 2.5 mM EDTA, 0.3% Triton X-100, pH 8.4] were used at a ratio of 24:1. Gels were equilibrated for 20 min by electrophoresis at 70 V at 4 °C. Samples to be electrophoresed were mixed (1:1) with 2× concentrated sample buffer (2× concentrated electrophoresis buffer containing 27% sucrose and 0.002% bromophenol blue) and electrophoresed at 4 °C, first for 20 min at 70 V and then at a constant voltage of 150 V for 16 h. Gels were stained with Coomassie brilliant blue and destained according to published procedures. Then the gels were vacuum-dried and exposed to preflushed Agfa X-ray film with an intensifying screen. The molecular weight marker proteins (Pharmacia) used were thryoglobulin (monomer 670 kDa, dimer 1340 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (67 kDa). These marker proteins were labeled with rhodamine and used in the experiments in which nonfixed gels were used (Figures 7–9).

Biosynthetic Labeling. A-431 cells were grown in medium containing 10% fetal calf serum and combined antibiotics. Confluent monolayers (2×10^7 cells) were washed twice with methionine and cysteine-free Dulbecco's modified Eagle's medium and incubated overnight with e same medium containing 50 μCi of [^{35}S]methionine and 50 μCi of [^{35}S]cysteine per milliliter. The secreted EGF-binding domain (Weber et al., 1984a) was harvested from the medium of the labeled A-431 cells by using the 29.1-IgG–Sepharose beads as described above.

The gp74$^{v\text{-}erbB}$ protein was similarly extracted from ^{35}S-labeled GC-2 avian erythroblastosis virus (AEV) transformed erythroblasts (GC-2 cells) grown in RPMI medium containing 10% fetal calf serum and 1% chicken serum. Immunoaffinity purification of gp74$^{v\text{-}erbB}$ was accomplished by using RK2–Sepharose antibody (Kris et al., 1985) according to the protocol given above except that acid elution was used to release the protein from the beads. In some experiments gp74$^{v\text{-}erbB}$ was immunoprecipitated from nonlabeled cells and then internally labeled with [γ-^{32}P]ATP as described above for the EGF receptor.

Phosphorylation of EGF Receptor. Receptor self-phosphorylation was performed as described in the preceding paper (Yarden & Schlessinger, 1987), except that phosphorylation was rapidly followed by the addition of 0.025 mL of the concentrated gel sample buffer and then analyzed by ND-PAGE.

Radiolabeling of EGF. The lactoperoxidase method (Marchalonis, 1969) was used to label EGF to a specific radioactivity of 100 000 dpm/ng. For the experiment shown in Figure 3, the chloramine-T method (Hock & Hollenberg, 1980) was used for the preparation of ^{125}I-EGF. As previously reported, this method of radiolabeling enables direct linkage of EGF to its receptor (Linsley & Fox, 1980a).

Covalent Binding of ^{125}I-EGF to Its Receptor. EGF receptor was affinity purified by utilizing affinity chromatography with monoclonal antibody 29.1-IgG–Sepharose and then acid eluted and neutralized. Aliquots of the pure receptor [0.01 mL, 1.2 pmol as determined by binding experiments with ^{125}I-EGF labeled by the lactoperoxidase method and poly(ethylene glycol) precipitation] were incubated with ^{125}I-EGF labeled by the chloramine-T method at a final concentration of 1.8×10^{-7} M. The final reaction mixture (0.025 mL) contained, in addition to EGF and its receptor, 20 mM glycine, 50 mM Hepes, pH 7.5, 10% glycerol, 0.1% Triton X-100, 1 mM PMSF, and aprotinin at 0.15 TIU/mL. After the binding experiments, each sample was mixed with 0.025 mL of concentrated (2×) gel sample buffer and subjected to ND-PAGE.

Binding of ^{125}I-EGF to Its Receptor in Native Gel. Receptor containing samples were resolved by ND-PAGE. Following electrophoresis, the acrylamide gel was washed twice with binding buffer (25 mM Hepes buffer, pH 7.5, 10% glycerol, 0.1% Triton X-100, 0.1% albumin) and then incubated with 1.7×10^{-8} M ^{125}I-labeled EGF in 50 mL of the binding buffer. Binding was allowed for 1 h at 22 °C and followed by five washes of 3 min each with binding buffer containing 1 mM EGTA. The wet gel was directly exposed to X-ray film.

RESULTS

Ligand-Dependent Conversion between Two Molecular Forms of EGF Receptor. According to the allosteric aggregation model presented in the preceding paper, the binding of EGF to its receptor is thought to stabilize higher aggregation states of the receptor molecule. To test this prediction, the EGF receptor was purified from A-431 cells by utilizing monoclonal anti-receptor antibodies (Yarden et al., 1985), internally labeled with ^{32}P, incubated for 15 min at 0 °C with different concentrations of EGF, and then analyzed by gel electrophoresis under nondenaturing conditions. It is anticipated that higher aggregation states of EGF receptor, if existing and stable enough, will be resolved in the nondenaturing gel because of their differential electrophoretic mobility. Analysis of the gel autoradiogram (Figure 1A) reveals that the purified receptor exists in two molecular forms: a low form and a high form. In the presence of saturating concentrations of EGF, most of the low form converts to the high form. Unlike EGF, insulin did not have any effect on the relative amount of the two forms of the EGF receptor.

Figure 1B depicts the dose dependency of the EGF-induced molecular conversion of the EGF receptor. The radioactive contents of the gel regions corresponding to each molecular form were determined by monitoring their Cerenkov radiation. It appears that full occupancy of the receptor's binding sites

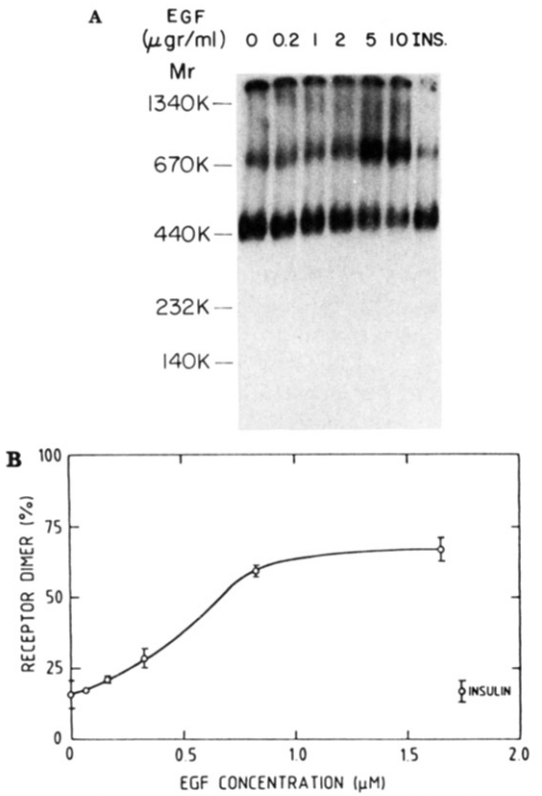

FIGURE 1: EGF induces appearance of a low-mobility form of EGF receptor in native gels. EGF receptor was purified from A-431 cells by immunoaffinity chromatography and internally labeled with ^{32}P as described under Materials and Methods. The radiolabeled receptor (0.8 pmol) was incubated for 15 min at 0 °C with different concentrations of EGF, insulin, or buffer alone and then subjected to gel electrophoresis under nondenaturing conditions as described under Materials and Methods. Panel A depicts the autoradiogram of the fixed and dried gel, which was exposed for 15 min. Panel B depicts the relative radioactive content of the two bands as a function of the concentration of EGF. The averages of duplicate determinations in separate gels and the corresponding ranges (bars) are indicated. Background radioactivity was determined by counting a piece of gel of equal dimensions from the 550-kDa region and subtracting its radioactivity from the total count.

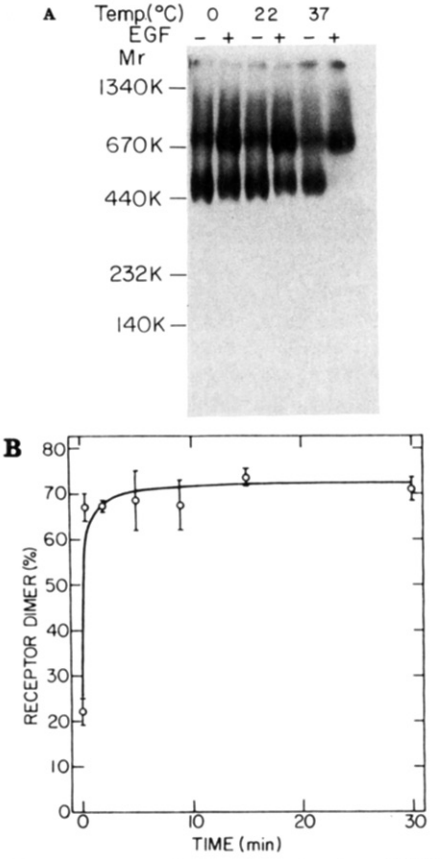

FIGURE 2: Temperature and time dependencies of EGF-induced appearance of the high molecular form. (A) Radiolabeled EGF receptor (0.8 pmol) was incubated for 10 min at various temperatures either with EGF (10 μg/μL) or with buffer alone and then subjected to gel electrophoresis under nondenaturing conditions. An autoradiogram of the gel exposed for 25 min on Agfa Gevearet X-ray film is shown. (B) Radiolabeled receptor was incubated with EGF for various times as indicated above, except that incubation was at 0 °C. The receptor–ligand mixtures were subjected to gel electrophoresis immediately after termination of the incubation period, and the radioactive contents of the receptor bands were determined by monitoring their Cerenkov radiation. The relative proportion (average and range of duplicates) of the higher form is given for each time point.

is required for the molecular conversion. The sharp effect observed with increasing concentrations of EGF may reflect the slow kinetics of binding of EGF at 0 °C or a cooperative step of unknown nature. The effect of EGF is saturable, and both molecular species are observed in the absence of EGF and in the presence of saturating concentrations of the growth factor.

Obviously, molecular weight determinations in native gels are not comparable with molecular weight determinations in SDS gels, especially for large hydrophobic glycoproteins such as the EGF receptor. However, in order to be able to compare and relate between experiments and in order to assess the reproducibility of this gel system, we have compared the mobility of the low and high forms of the EGF receptor to the mobility of standard water-soluble molecular weight markers (Pharmacia). It appears that the low form exhibits electrophoretic mobility similar to "440 kDa", and the mobility of the higher form corresponds to "750 kDa". These values reproducibly represent the two forms of the receptor. However, these numbers are not to be considered as molecular weight determinations in SDS gels; rather, they will be referred to as merely *numerical assignments* for the low and high forms of the receptor molecule.

EGF-Induced Conversion of Its Receptor Is Rapid and Temperature-Sensitive. The dependencies of the molecular conversion on the temperature and duration of preincubation with EGF are shown in Figure 2. In order to simplify the effect of binding kinetics at various temperatures, an oversaturating concentration of EGF (1.7 μM) was used. EGF clearly induced the formation of the higher receptor form at 0 °C (Figures 2A and 1). At 22 °C the conversion was more rapid, and at 37 °C an almost complete conversion took place. Residual receptor conversion and higher aggregates that do not penetrate the gel were observed at 37 °C even in the absence of EGF. Longer incubations with EGF at 0 or 22 °C did not significantly increase the proportion of the higher form, suggesting that rapid equilibrium is achieved even at low temperatures. The time course of the reaction shown in Figure 2B indicates that most of the conversion took place within a 1-min incubation with EGF at 0 °C. Shorter preincubations yield less reliable results as the reaction probably proceeds while the samples are loaded on the gel and even during the initial stage of electrophoresis.

Demonstration of the Molecular Conversion of EGF Receptor by Covalent Cross-Linking of Radiolabeled EGF. To demonstrate the molecular conversion of EGF receptor by an alternative method, we employed covalent cross-linking of the ligand to its receptor. According to this method (Linsley & Fox, 1980a), prolonged incubation of chloramine T radiolabeled ^{125}I-EGF at elevated temperatures results in covalent

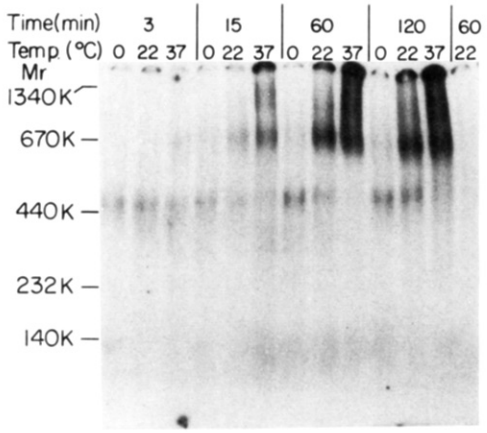

FIGURE 3: Covalent cross-linking of ^{125}I-labeled EGF to both molecular forms of the EGF receptor. Purified EGF receptor (1.2 pmol) was incubated with ^{125}I-EGF (labeled by the chloramine T method) for the indicated time periods and temperatures. The samples were analyzed by ND-PAGE, and an autoradiogram (2-h exposure) of the gel is shown. The right lane shows the result of incubation in the presence of 20-fold molar excess of nonlabeled EGF.

cross-linking of ^{125}I-EGF to its receptor. Although the exact nature of this reaction is unknown, it provides a useful method for labeling the extracellular domain of the EGF receptor. The addition of bifunctional cross-linking reagents was essentially ineffective because of the single amino group on the murine EGF and due to the interference caused by the detergent used to solubilize the receptor.

When affinity-purified receptor is incubated with chloramine T labeled ^{125}I-EGF and then analyzed by ND-PAGE, a time- and temperature-dependent change in the electrophoretic mobility of EGF receptor is observed (Figure 3). Short preincubations with a less than saturating concentration of EGF (the method of radiolabeling limits the concentration of the radioactive ligand) result in an exclusive labeling of the lower form. However, upon longer incubations at higher temperatures the amount of radioactivity associated with the higher form gradually increased. The radiolabeling of both forms of the receptors was inhibited by unlabeled EGF, revealing the specificity of this approach. Thus the conversion of EGF receptor from a fast-migrating to slow-migrating form is independent of the labeling method. Both forms are observed when the radiolabeled ^{32}P tag is attached to the cytoplasmic domain of the EGF receptor or when the extracellular domain is directly conjugated to ^{125}I-EGF. Moreover, the experiment with ^{125}I-EGF (Figure 3) excludes the possibility that the high form represents occupied receptor and the low form is nonoccupied receptor as both forms covalently bind ^{125}I-EGF.

The Lower Form Is a Monomeric EGF Receptor, and the Higher Form Represents Receptor Dimers. Various experiments were performed to assess the nature of the low form (440 kDa) of the EGF receptor. EGF receptor was denatured by heating the samples for 5 min at 95 °C in the presence of 0.5% SDS or by reducing with dithiothreitol (20 mM, 30 min at 37 °C) in the presence of 4 M guanidine hydrochloride followed by alkylating with iodoacetamide (20 mM, 30 min at 37 °C). The denatured receptor, obtained by either procedure, still migrated in the nondenaturing gel solely as the 440-kDa form, and the higher form totally disappeared (not shown), indicating that the lower band seen in the native gel represents the monomeric receptor.

To investigate the difference between the electrophoretic mobility of the monomeric receptor in the nondenaturing gel (440 kDa) and its mobility in SDS gel (170 kDa), the electrophoretic mobilities of two molecules that correspond to different parts of the EGF receptor were examined. The secreted EGF-binding domain (Weber et al., 1984a; Ullrich et al., 1984) was purified from biosynthetically labeled A-431 cells by utilizing monoclonal anti-receptor antibodies. This protein, which has a molecular weight of 105 000 in SDS gel, migrates in the nondenaturing gel as a "250-kDa" band. The second variant of EGF receptor is the oncogene product of the avian erythroblastosis virus: gp74$^{v\text{-}erbB}$ (Graf & Beug, 1983; Beug & Hayman, 1984; Kris et al., 1985). This protein comprises most of the cytoplasmic portion of the avian EGF receptor, the transmembrane region, and a small extracellular region and has a molecular weight of 74 000 in SDS gel. The gp74$^{v\text{-}erbB}$ protein was purified from virally transformed chicken erythroblasts, internally labeled with ^{32}P, and subjected to gel electrophoresis under nondenaturing conditions. When calibrated with water-soluble molecular weight markers, the gp74$^{v\text{-}erbB}$ protein migrated in nondenaturing gels as a "180-kDa" band. Consistently, the sum of the mobilities of gp74$^{v\text{-}erbB}$ and the extracellular EGF receptor domain corresponds to the approximate mobility of the intact EGF receptor in the nondenaturing gel. We conclude that the 440-kDa form of EGF receptor is a monomeric receptor exhibiting slow molecular mobility in nondenaturing gel.

The higher molecular form (750 kDa) could represent an oligomeric receptor or a monomeric receptor that has experienced a large conformational change upon binding of EGF, resulting in marked retardation of its electrophoretic mobility. To contrast between these two possibilities, we determined the effect of receptor concentration on the appearance of both forms of the receptor. The effect of the concentration of EGF receptor on the EGF-induced high molecular form is depicted in Figure 4. This experiment demonstrates that the appearance of the high form of EGF receptor has a *parabolic* concentration dependence, thus indicating that it probably involves an intermolecular process mediated by receptor–receptor interactions rather than an intramolecular process associated exclusively with a conformational change. Moreover, the apparent second-order kinetics (Figure 4B) for the appearance of the high molecular form suggests that this form represents an EGF receptor dimer. This interpretation is also consistent with the relative mobilities of both forms and the lack of intermediate bands in the gel. Also supportive is the functional evidence for the necessity of receptor dimerization for kinase activation as detailed in the preceding paper (Yarden & Schlessinger, 1987). In conclusion, the low form represents monomeric EGF receptors, and the higher form of EGF receptor results from interreceptor interactions and probably represents a receptor dimer.

Receptor Dimerization Involves Reversible Noncovalent Interaction between EGF Receptors. According to the model described in the preceding paper, dimerization is thought to involve reversible molecular interactions. As was already mentioned, the free energy of this process was estimated to be in the range of 1–5 kcal/mol, much less than that expected for a covalent bond. Hence, the chemical nature of the molecular interactions stabilizing the EGF receptor dimer were studied by subjecting the dimer to various chemical conditions (Table I and Figure 5). The results of these experiments indicated that the EGF receptor dimer dissociates in the presence of ionic detergents or when exposed to alkaline or acidic buffers ($3 > pH > 11$). Both the dimers that are formed in response to EGF and also the residual dimerization observed in the absence of the growth factor dissociated under these conditions (Table I), suggesting that both the EGF-induced

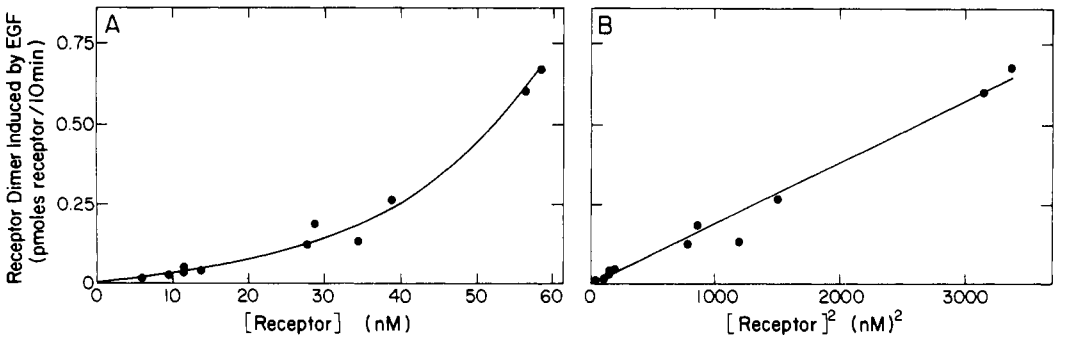

FIGURE 4: Effect of receptor concentration on EGF-induced appearance of the high form of EGF receptor. Purified EGF receptor, internally labeled with ^{32}P, was serially diluted (in 20 mM Hepes buffer, pH 7.5, 10% glycerol, 0.1% Triton X-100, 1 mM ATP, 0.2 mM $ZnSO_4$, and 0.04 mM $NaVO_3$) and incubated with EGF (1.7×10^{-6} M) or buffer alone. Incubation was done at 22 °C for 10 min and followed by ND-PAGE. The radioactivity of the two forms of the EGF receptor was determined by counting the appropriate gel slice. Background radioactivity was determined and subtracted as described in the legend to Figure 1. The amount of receptor dimer induced by EGF was determined by subtracting the corresponding level of receptor dimer in the absence of EGF. Panel A shows the EGF-induced appearance of the high molecular form of EGF receptor as a function of receptor concentration, and panel B shows the same parameter as a function of squared receptor concentration. The concentration of EGF receptor was determined by a radioreceptor assay with ^{125}I-EGF.

Table I: Chemical Stability of the Dimeric Receptor[a]

treatment	receptor dimer (%)	
	buffer alone	EGF
buffer alone	19 ± 6	78 ± 5
pH 2.5	6	17
pH 11.5	12	24
EDTA (10 mM)	24	78
octyl β-glucoside (0.5%)	13	81
sodium deoxycholate (Na-DOC; 0.5%)	10	21
sodium dodecyl sulfate (SDS; 0.5%)	5	7
2-mercaptoethanol (25 mM)	24	82

[a] EGF receptor was treated with EGF (1.7 μM) or buffer alone as described in the legend to Figure 5. Aliquots were then incubated for 30 min at 22 °C with the indicated agents. Quantification of the resulting amounts of the monomeric and dimeric forms of the receptor was done by ND-PAGE. A duplicate determination of the control experiment was done, and the averages are given. Essentially the same results were obtained in two separate experiments.

and the basal dimerization involve interactions of similar chemical nature and that EGF acts by increasing the amount of the dimeric receptors. Nevertheless, various agents did not affect the integrity of the dimeric receptor. These include (Table I and Figure 5) nonionic detergents, cation chelator (10 mM EDTA), and exposure to reducing (0.5–50 mM dithiothreitol or β-mercaptoethanol) and alkylating agents (2–50 mM iodoacetamide or *N*-ethylmaleimide). Reduction and alkylation did not affect the EGF-induced aggregation when performed either before or after the addition of EGF to the pure receptor. The possible involvement of a thio ester bond was also excluded since alkylamines and various alkylating agents did not affect receptor aggregation. On the basis of these results, we propose that receptor dimerization induced by EGF involves noncovalent interactions between EGF receptors.

The noncovalent nature of receptor association within the dimer is further demonstrated by the reversibility of the aggregation process. Dilution of the already formed dimers resulted in their gradual disappearance and the concomitant appearance of receptor monomers (Figure 6). Moreover, the dissociated receptors were able to redimerize upon the addition of EGF and the readjustment of the concentration of the EGF receptor. Figure 6B depicts an experiment in which radiolabeled receptor dimers were allowed to dissociate by dilution and then EGF and nonlabeled EGF receptor were added until the original receptor concentration was reached. Quantification indicates that, in the presence of EGF and EGF receptor, almost a complete restoration of receptor dimers is achieved (not shown). Hence, we conclude that the EGF-induced dimerization of receptors involves reversible interactions between receptors molecules.

Functional Properties of Monomeric and Dimeric EGF Receptors. An important unanswered question relates to the functional properties of the monomeric and dimeric forms of the receptor molecule. We have attempted to analyze the binding properties and the kinase activity of both forms of the EGF receptor. In order to retain the enzymatic kinase activity of EGF receptor, the acid-release protocol used for the elution from the monoclonal antibody column was replaced by elution with specific sugars [Gooi et al. (1983) and our unpublished results]. Although this method resulted in lower recovery of receptor protein (about 20–40% of the recovery obtained upon elution with acidic pH), both ligand binding and kinase activity were full retained.

Preliminary experiments done with ^{125}I-EGF labeled by the lactoperoxidase method indicated that most of the bound ligand dissociates from its receptor upon ND-PAGE analysis. Residual radiolabeled EGF remained preferentially associated with the higher receptor band (data not shown). In the experiment depicted in Figure 7, affinity-purified active EGF receptor was initially incubated with various concentrations of nonlabeled EGF, insulin, or buffer alone and subsequently subjected to ND-PAGE. Following electrophoresis, the native gel was incubated with radiolabeled EGF (1.7×10^{-8} M; labeled by the lactoperoxidase method to avoid covalent cross-linking to the receptor). A less than saturating concentration of EGF was used to allow resolution of affinity differences. Evidently both the monomeric and dimeric forms of the receptor bind EGF (Figure 7). Moreover, as short as 1-min incubation with EGF prior to electrophoresis is sufficient to convert most of the monomeric form into the dimeric form. This effect is time- and concentration-dependent and EGF-specific in a manner similar to the effects observed with internally ^{32}P-labeled receptor (Figures 1 and 2). More importantly, the dimeric receptor, once formed, is more intensely labeled by ^{125}I-EGF, as compared with the monomeric form. (Compare, for example, the first two lanes in Figure 7.) This may suggest that the dimeric receptor has higher ligand capacity at the conditions used for labeling these gels. The most likely interpretation of this result is that the dimeric receptor has higher affinity to EGF as compared with the monomeric receptor. This conclusion is consistent with the experiment presented in the preceding paper, which showed that increased ligand affinity is associated with antibody-mediated oligomerization.

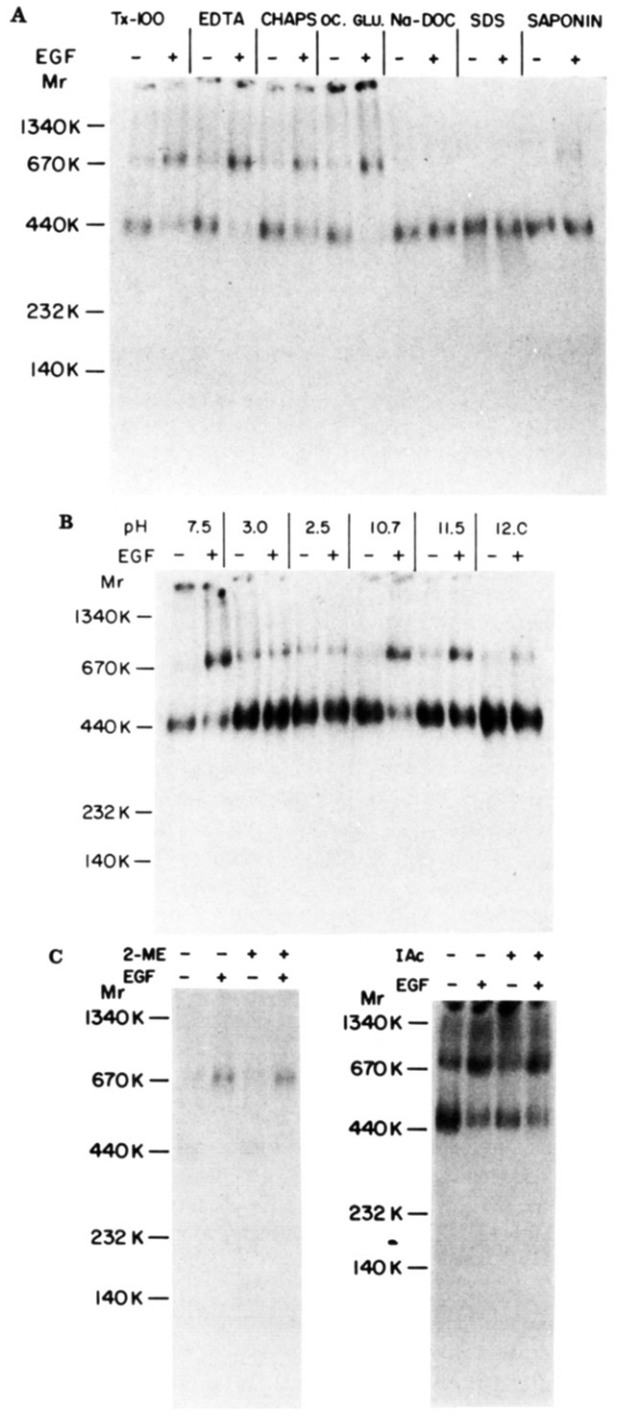

FIGURE 5: Chemical stability of EGF receptor dimers. Affinity-purified EGF receptor (0.2–1.1 pmol) internally labeled with ^{32}P was incubated for 10 min at 22 °C with either 1.7×10^{-6} M EGF or buffer alone. Aliquots (0.01 mL) of each sample were then incubated for 30 min at 22 °C with 0.03 mL of dilution buffer (see legend to Figure 4) and the various agents. At the end of the incubation, aliquots containing 0.01 mL of each reaction mixture were diluted (1:5) in gel sample buffer and subjected to ND-PAGE. Since dilution affects the relative amount of the dimer, an appropriately diluted control experiment is also shown in each autoradiogram. (A) The following detergents were used at concentrations of 0.5% (w/v): 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), octyl β-glucoside, Triton X-100, sodium deoxycholate (Na-DOC), SDS, and saponin. The effect of 10 mM EDTA is also shown. (B) The following buffers were used to maintain the corresponding pH values: 0.1 M citrate buffer (pH 3.0), 50 mM glycine hydrochloride buffer (pH 2.5), 50 mM triethylammonium chloride buffer (pH 10.7), 0.1 M lysine hydrochloride buffer (pH 11.5), and 0.1 N NaOH (pH 12.0). (C) For reduction and alkylation, 50 mM 2-mercaptoethanol (2-ME) and 50 mM iodoacetamide (IAc) were used where indicated.

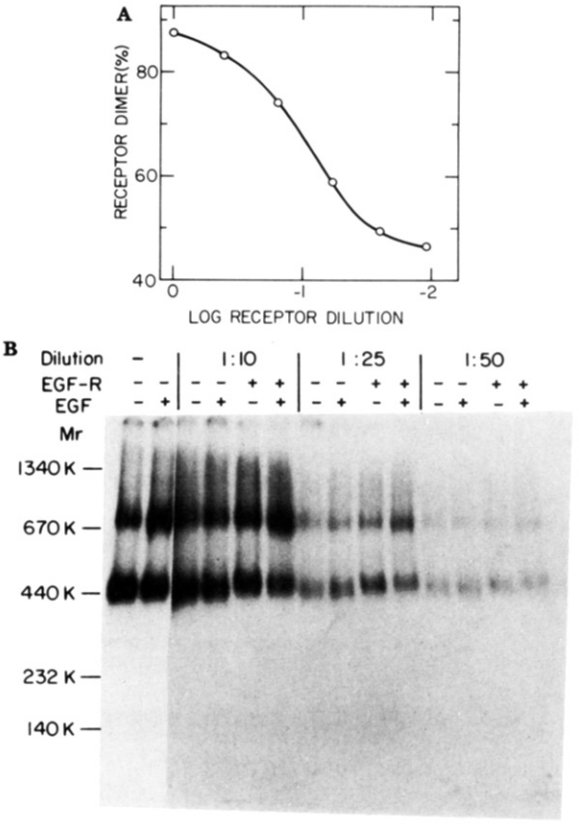

FIGURE 6: Reversibility of dimerization of EGF receptor. Receptor dimers were formed by incubation of ^{32}P-labeled receptor (at 48 nM final concentration) with EGF (1.7×10^{-7} M, 10 min at 0 °C) and in control experiments with buffer alone. Aliquots were serially diluted as indicated in solution containing 20 mM Hepes buffer, pH 7.5, 10% glycerol, 0.1% Triton X-100, 1% aprotinin, 1 mM ATP, 0.2 mM $ZnSO_4$, and 0.04 mM $NaVO_3$ and then further incubated for 30 min at 22 °C. The relative amounts of the dimer form of the receptor (panel A) were quantified as described in the legend to Figure 1. To follow the reappearance of the higher form, the diluted samples were further incubated for 10 min at 0 °C with either one or a combination of the following, as indicated by the + symbols: EGF (1.7 μM), nonlabeled EGF receptor (EGF-R) added to adjust for the original receptor concentration, or buffer alone. Samples were then resolved by ND-PAGE, and the resulting autoradiogram is given in panel B. Exposure to the X-ray film was for 1 h, except for the left two slots, which were separately exposed for 10 min.

To test the kinase function of the monomeric and dimeric forms of the receptor, purified receptor was incubated at 0 °C with EGF and then subjected to brief self-phosphorylation by adding [γ-^{32}P]ATP and Mn^{2+}. The results, shown in Figure 8, indicate that in the absence of EGF the monomeric form is almost exclusively phosphorylated. Upon incubation with EGF, a labeled dimeric form gradually appeared. However, EGF increased the self-phosphorylation of both the monomeric and dimeric forms. The increase in the phosphorylation of the dimeric receptor was time- and dose-dependent with a profile similar to those of the formation of the dimeric form (Figure 1A) and the binding of radiolabeled EGF (Figure 7). Taken together, it appears that the amount of phosphate labeling found in the higher form may simply represent the relative amount of the dimeric form with no indication for preferential self-phosphorylation of the dimeric receptor.

The inability to detect a dimeric receptor possessing elevated enzymatic activity is probably due to the reversibility of the dimerization process (Figure 6), to the short lifetime of the putative activated dimers, and to the poor time resolution of the gel system. To test this possibility, we have shortened the incubation time with EGF and used higher concentrations of EGF receptor. As shown in Figure 9, after 30 s of incubation

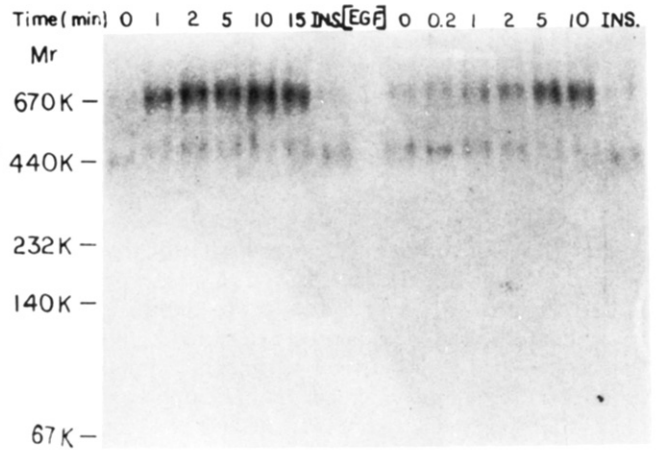

FIGURE 7: Binding of ^{125}I-EGF to EGF receptor in nondenatured polyacrylamide gel. EGF receptor was purified as described under Materials and Methods except that it was eluted from the immobilized antibody with 0.2 M *N*-acetylgalactosamine. The receptor (0.1 pmol in 0.02 mL) was then incubated with either EGF at various concentrations, insulin (INS), or buffer alone, and the samples were subjected to ND-PAGE. Following electrophoresis, the gel was incubated for 1 h at 22 °C with radiolabeled EGF (1.7×10^{-8} M). An autoradiogram of the washed gel (see Materials and Methods) is given. Molecular weights were determined with fluorescently labeled proteins. (Left) Purified receptor was incubated for the indicated periods of time at 22 °C with 1.7 μM EGF, 1.8 μM insulin, or buffer alone. Incubation with insulin was for 15 min. (Right) Purified receptor was incubated for 15 min at 22 °C with different concentrations of EGF as indicated in the figure. Numbers refer to concentration in micrograms per milliliter. Insulin concentration used was 1.8 μM.

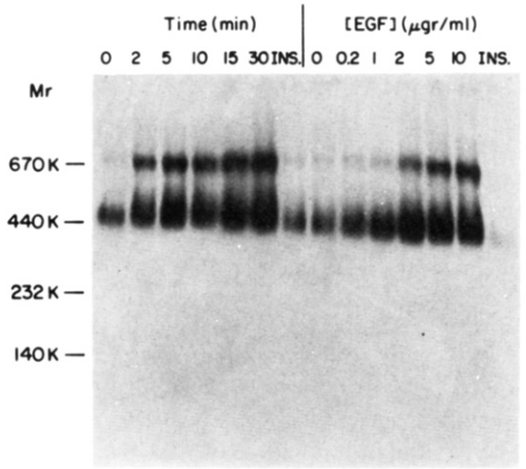

FIGURE 8: EGF-induced self-phosphorylation of monomeric and dimeric forms of EGF receptor. EGF receptor was purified and treated as described in the legend to Figure 7, except that at the end of the incubation with EGF the receptor was allowed to undergo self-phosphorylation. The reaction was started by the addition of [γ-^{32}P]ATP (0.2×10^{-6} M, 3 μCi) and $MnCl_2$ (5 mM) and terminated after 90 s at 0 °C by the addition of gel sample buffer containing 2 mM ATP. The sample was immediately subjected to nondenaturing gel electrophoresis. An autoradiogram (20-min exposure) of the dried and fixed gel is given. Incubation with EGF (1.7×10^{-6} M, unless otherwise indicated) or insulin (INS; 1.8×10^{-6} M) was for 15 min at 0 °C unless other incubation periods are indicated.

with EGF the dimeric form is preferentially labeled with ^{32}P. This was less apparent after 2 min and after 15 min of incubation with EGF, suggesting that rapid equilibrium between the monomeric and dimeric species indeed took place. Hence, our results are consistent with the existence of an enzymatically active dimer. However, further experiments utilizing measurements that allow rapid determination of the phosphorylation of exogenous substrates are required to unequivocally determine the mechanism of this process.

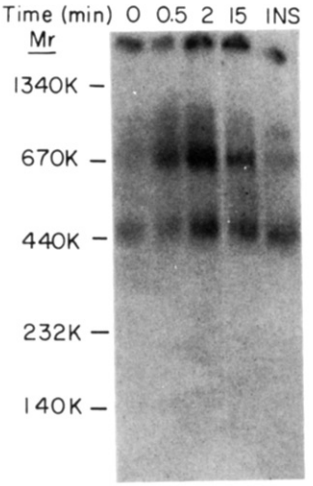

FIGURE 9: Self-phosphorylation of receptor forms after short incubation with EGF. EGF receptor was purified from A-431 cells by affinity chromatography with wheat germ lectin bound to agarose and specifically eluted with *N*-acetylglucosamine. The receptor (0.6 pmol in 0.02 mL) was subjected to self-phosphorylation and gel electrophoresis exactly as described in the legend to Figure 8, except that shorter incubation periods with EGF (1.7×10^{-6} M) were used. Incubation with insulin (INS; 1.8×10^{-6} M) was for 15 min.

Discussion

The experimental basis for the present work was our study of the mechanism by which soluble EGF receptor kinase is activated upon ligand binding (Yarden & Schlessinger, 1987). By employing several independent experimental approaches, we conclude that EGF activation of its own receptor kinase involves the formation of an oligomeric active state, possibly a dimeric receptor. In this work we tried to further explore this mechanism by providing structural evidence for receptor oligomerization and its functional consequences.

Experimental evidence for the aggregation of the soluble EGF receptor was previously obtained by employing gel filtration chromatography and velocity sedimentation (Linsley & Fox, 1980b). More recently, two forms of soluble EGF receptor were separated by sedimentation in sucrose gradients: a 7.7S monomeric receptor and a 12S form, which is probably a dimeric receptor (Biswas et al., 1985). Similarly, polyacrylamide gel electrophoresis under nondenaturing conditions revealed the existence of two receptor forms (Figure 1). On the basis of determination of the electrophoretic mobilities of denatured receptor and two receptor-related molecules, we concluded that the lower, fast-migrating form is a monomeric receptor representing the single M_r 170 000 receptor molecule. The higher, slow-migrating form represents an oligomeric receptor and is probably a receptor dimer. The fast-migrating form in the native gel is likely to be identical with the 7.7S form observed in sucrose gradients, and the slow-migrating band probably corresponds to the 12S form (Biswas et al., 1985). By employing covalent cross-linking reagents, we have recently demonstrated that EGF induces the dimerization of the purified EGF receptor. Analysis of the cross-linked EGF receptor by SDS–PAGE revealed a molecular weight of 340 000, which corresponds to an EGF receptor dimer (Cochet et al., unpublished results).

The nondenaturing gel system, by enabling simultaneous processing of multiple samples, allowed close examination of the properties of the oligomerization process of EGF receptors. The following characteristics were observed:

(*a*) *EGF Dependency*. EGF binding dramatically shifts the distribution of its receptor in favor of the oligomeric state. In

the absence of EGF most of the receptor (70–80%) is found in a monomeric form, whereas upon EGF binding most of it (70–85%) appears as dimeric receptors (Figure 1). The effect is specific for EGF, as other hormones or proteins do not induce this process. Although receptor occupation is required for dimerization, it is not clear whether the occupation of both binding sites is required for dimerization. It is noteworthy that both receptor forms exist, albeit in different proportions, under all the conditions tested in this study. This result is consistent with an equilibrium between two aggregation states which can be modulated by EGF binding.

(*b*) *Kinetics.* Although difficult to determine precisely, dimer formation induced by EGF is probably complete within a few minutes (Figure 2B). The process exhibited temperature dependency (Figure 2A), which may be limited in part by the rate of EGF binding to the receptor at various temperatures. However, even at 0 °C, close to maximal dimerization occurs after 1 min. The kinetics of the dimerization process is not affected by the integrity of the kinase function, as receptors devoid of kinase activity still undergo efficient EGF-induced dimerization.

(*c*) *Reversibility.* The oligomerization of EGF receptors is a reversible process (Figure 6A). The re-formation of receptor dimers requires EGF and adjustment of the concentration of EGF receptor as expected for a reaction in equilibrium. A striking similarity exists between the above-mentioned characteristics of dimer formation and the activation of the EGF receptor kinase function (Carpenter et al., 1979; King et al., 1980). Both are specifically stimulated by EGF binding and show basal activity in the absence of the ligand. Moreover, both processes are very rapid and occur at low temperatures. However, maximal self-phosphorylation of soluble receptor requires a 10-min incubation with EGF, whereas dimer formation seems to be more rapidly coupled to EGF binding.

The interactions that stabilize the oligomeric receptor are noncovalent; the slow-migrating form disappears upon receptor dilution (Figure 6) or mild treatments (Table I). The pH sensitivity of the dimeric form and its breakdown by ionic detergents, but not by uncharged detergents, may suggest the involvement of hydrophilic noncovalent interactions in the process of dimer formation.

It appears that receptor oligomerization is an intrinsic property of the receptor molecule. However, the inability to detect oligomers of both receptor-related molecules, $gp74^{v\text{-}erbB}$ and the secreted EGF-binding domain (see Results), may imply that the integrity of the receptor molecule is essential for receptor oligomerization. An interesting question is the identification of the receptor domain that mediates the protein–protein interactions required for oligomerization. The close correlation between EGF binding and receptor oligomerization may imply a direct role for the extracellular ligand-binding domain in mediating the oligomerization process. This 621 amino acid long extracellular domain contains two cysteine-rich regions (Ullrich et al., 1984) of as yet unknown function. Similarity matrix comparisons of the EGF receptor (Toh et al., 1985) revealed the presence of extensive homology between two regions of ~300 residues, covering almost the entire extracellular portion. This structural organization may be of functional importance, as suggested by high conservation in the *Drosophila* homologue of EGF receptor (Livneh et al., 1985). However, it probably does not serve as a ligand-binding region since a similar cysteine-rich region exists in the extracellular binding domain of the insulin receptor (Ullrich et al., 1986) and other growth factor receptors with different ligand specificities. Whether these structural elements are involved in the stabilization of a receptor dimer is not known. However, it is noteworthy that the immunoglobulin molecule exhibits strong noncovalent interactions between highly homologous and cysteine-stabilized structures [so-called "homology subunits"; see Amzel and Poljak (1979) for a review].

In this study we attempted to link the activation of EGF receptor kinase to the formation of an oligomeric receptor. The oligomerization of EGF receptor is a reversible process, and therefore, the analysis of the functional properties of each receptor form may be hindered by the reversibility of this process. Practically, two experimental approaches are available: (1) a direct approach that attempts to analyze the ligand binding and kinase activity of each receptor form; (2) an indirect approach that tests predictions made on the basis of an oligomerization-activation model. The latter approach, presented in the preceding paper, suggested the existence of a kinase activation step that involves receptor oligomerization. The dimerization of EGF receptor detected by the native gel system is consistent with the theoretical and experimental framework established in the preceding paper. Moreover, it was proposed that the driving force for receptor oligomerization is mediated by the higher binding affinity of the oligomeric receptor toward EGF as compared with the binding affinity of the monomeric receptor. Indeed, receptor aggregation increases the apparent binding affinity of ^{125}I-EGF to the receptor (Yarden & Schlessinger, 1987).

In situ examination of protein kinase activity of both receptor forms in a nondenaturing gel was not successful, possibly due to the inactivation of the kinase function under these conditions. Hence, we have analyzed the autophosphorylation of the two receptor forms by nondenaturing gel immediately after self-phosphorylation. It appears that both receptor forms are self-phosphorylated and the dimeric receptor is phosphorylated earlier than the monomeric receptor. Since receptor oligomerization is a reversible process, we were not able to distinguish between the following two possibilities: (1) increased self-phosphorylation is exclusively associated with the oligomeric form, which then rapidly exchanges with the monomeric form, or (2) the activation process is of a collision-type mechanism whereby a short-lived dimer yields to active monomeric receptors. Certainly more experiments are required to distinguish between these two and other possibilities.

In summary, the following cascade of events seems to follow the binding of EGF leading to the activation of the receptor kinase. It appears that both receptor monomers and oligomers (probably dimers) exist in solution but EGF binding favors the oligomeric state due to its higher binding affinity. Once oligomerized, the receptor kinase is activated, and since the oligomers are in equilibrium with monomeric receptors, both active monomers and dimers probably exist. It appears that these two forms undergo self-phosphorylation by an intramolecular reaction (Weber et al., 1984b; Yarden & Schlessinger, 1987) and are gradually deactivated by an as yet unknown mechanism. A different model was recently proposed by Das and her collaborators (Biswas et al., 1985), who studied receptor oligomerization by means of sucrose gradients and aging of receptor preparations. They concluded that kinase activation requires the formation of receptor monomers and receptor inactivation is due to dimerization. The reason for the discrepancy between these two models is unknown. However, it may be, at least in part, due to differing procedures for receptor purification, storage conditions, and methods used to detect oligomerization.

The studies presented in this paper and the preceding paper demonstrate a linkage between ligand binding, receptor oligomerization, and kinase activation for the soluble receptor in solution. Whether these relationships are relevant to the living cell is an important, though yet unanswered, question. Numerous studies utilizing various approaches have shown that in intact cells EGF induces aggregation of its randomly dispersed membrane receptors (Schlessinger et al., 1978; Haigler et al., 1979; Hopkins et al., 1981; Zidovetzki et al., 1981; Hillman & Schlessinger, 1982). Moreover, the initial step of ligand-induced receptor aggregation seems to play a role in the cascade of events leading to the mitogenic signal: it was shown that various anti-receptor antibodies capable of cross-linking the receptor can mimic the growth factor and stimulate DNA synthesis (Schreiber et al., 1981; Chandler et al., 1985; Fernandez-Pol, 1985). However, other antibodies that can also cause receptor aggregation fail to induce DNA synthesis (Schreiber et al., 1983; Gill et al., 1984; Gregoriou & Rees, 1984; Chandler et al., 1985), suggesting that receptor aggregation per se is an insufficient stimulus for DNA synthesis. Certainly more studies will be required to correlate the observed ligand-induced aggregation of EGF receptors in solution and the mitogenicity of EGF.

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