Self-Phosphorylation of Epidermal Growth Factor Receptor Is an Intermolecular Reaction†

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ABSTRACT: The binding of epidermal growth factor (EGF) to epidermal growth factor receptor (EGF receptor) results in the dimerization and self-phosphorylation of the receptor. Both of these responses were followed as a function of time and the concentration of EGF receptor. Dimerization of EGF receptor was monitored by immunoblotting the protein after it had been cross-linked with glutaraldehyde. The capacity for self-phosphorylation was followed by measuring the relative level of incorporation of [^{32}P]-phosphate into EGF receptor on autoradiograms of the same immunoblots used for the assay of its dimerization. When these two properties were followed as a function of time, it was found that dimerization preceded the appearance of the capacity for self-phosphorylation. Both dimeric and monomeric forms of EGF receptor were self-phosphorylated in the presence of EGF, but the dimeric form was phosphorylated preferentially to the monomeric form. When the dimerization and the capacity for self-phosphorylation were followed as a function of the concentration of dimeric EGF receptor, it was observed that the self-phosphorylation of dimeric EGF receptor increased as the concentration of dimeric EGF receptor increased. An equation including terms representing both intramolecular and intermolecular rates of self-phosphorylation was fit to the plots of self-phosphorylation as a function of concentration of EGF receptor. These fits demonstrate that intramolecular self-phosphorylation within dimers of EGF receptor is insignificant and that self-phosphorylation is an intermolecular process between dimers of EGF receptor.

Human epidermal growth factor receptor (EGF receptor),[1] a member of the family of receptor tyrosine kinases, is formed from a polypeptide 1186 amino acids in length that contains a single hydrophobic membrane-spanning region connecting an extracellular and an intracellular domain (*1*−*6*). Binding of epidermal growth factor (EGF)[1] at the extracellular domain of EGF receptor induces its dimerization and the activation of its intrinsic protein tyrosine kinase at the cytoplasmic side of the membrane (*7*−*10*). Signal transmission by EGF receptor triggers a mitogenic response of the cell to EGF (*11*).

Self-phosphorylation is the most obvious activity observed both *in vivo* and *in vitro* among all receptor tyrosine kinases (*12, 13*). It is defined operationally. Self-phosphorylation is the incorporation of phosphate into molecules of a protein kinase catalyzed by active sites of that same protein kinase. There is no requirement that the observed phosphorylation be either intramolecular or intermolecular to be designated as self-phosphorylation. The significance of self-phosphorylation varies among the subclasses of receptor tyrosine kinases. When the major sites of self-phosphorylation of insulin receptor and fibroblast growth factor receptor are mutated, the tyrosine kinase activity of these receptors cannot be significantly activated by their respective agonists (*14, 15*). In the case of EGF receptor, however, mutations of the self-phosphorylated tyrosines to phenylalanines have little effect on the activity of the enzyme (*16*−*18*), and a deletion mutant missing all of the self-phosphorylation sites proved to be efficiently activated by EGF (*19, 20*). It has also been demonstrated that self-phosphorylated EGF receptor can nevertheless be inactive (*21*). These results demonstrate that self-phosphorylation of EGF receptor is neither necessary nor sufficient for its activation and suggest that the sites of self-phosphorylation may only be important as locations for binding cytoplasmic signaling proteins downstream in the cascade from EGF receptor (*22*−*24*).

The sites for self-phosphorylation in EGF receptor are confined to the carboxy-terminal domain (*19, 25, 26*). Aspects of the mechanism by which self-phosphorylation occurs are still unclear. Intermolecular (*8*) and intramolecular (*27*−*29*) mechanisms have been proposed for the self-phosphorylation of EGF receptor. An intermolecular mechanism for the self-phosphorylation of EGF receptor is consistent with the observation that molecules of kinase-inactive EGF receptor could be phosphorylated by an enzymatically active deletion mutant of EGF receptor (*30, 31*). This observation, however, could not distinguish whether self-phosphorylation was occurring within a molecule of dimeric EGF receptor or was the result of one molecule of dimeric EGF receptor phosphorylating another molecule of dimeric EGF receptor. In this report, the former possibility will be referred to as "intramolecular" self-phosphorylation, and the latter as "intermolecular" self-phosphorylation in keeping with standard kinetic definitions of reaction order.

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[1] Abbreviations: EGF, epidermal growth factor; EGF receptor, epidermal growth factor receptor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, 0.15 M NaCl, 1 mM EDTA, and 50 mM sodium phosphate, pH 7.2; SDS, sodium dodecyl sulfate; PVDF, poly(vinylidene difluoride); t-Boc, *N*-(*tert*-butoxycarbonyl).

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To examine steps in the sequence of events leading to fully self-phosphorylated, dimerized EGF receptor, the dimerization and capacity for self-phosphorylation of the enzyme were followed as a function of time and the concentration of EGF receptor. In the experiments following these properties as a function of time, the capacity for self-phosphorylation was found to appear later than dimerization when dimerization was rapid. When the extents of dimerization and self-phosphorylation of EGF receptor were assessed as a function of the concentration of active EGF receptor to elucidate the kinetic order in which self-phosphorylation occurs, it was found that the specific radioactivity of dimeric EGF receptor decreased to zero as the concentration of dimeric EGF receptor was decreased to zero. This observation indicates that self-phosphorylation is exclusively an intermolecular reaction between dimers of EGF receptor.

EXPERIMENTAL PROCEDURES

Materials. Cells of the A431 human epithelioid carcinoma line (*32*) were kindly provided by Dr. Gordon Gill, Department of Medicine, University of California at San Diego. Bovine serum albumin, Na_2ATP, Triton X-100 detergent, Sephadex G-50, leupeptin, nitro blue tetrazolium, and the *p*-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate were purchased from Sigma Chemical Co.; glutaraldehyde was purchased from Calbiochem−Novabiochem Corp; and *N*-(*tert*-butoxycarbonyl) (t-Boc)[1] derivatives of the amino acids were purchased from Bachem, Inc. Goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase were purchased from Bio-Rad Laboratories Corp.; the triethylammonium salt of adenosine [γ-^{32}P]triphosphate (3000 mCi mmol^{-1}) was purchased from Amersham; recombinant human EGF and *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES)[1] were purchased from Fisher Scientific; and membranes of poly(vinylidene difluoride) (PVDF)[1] were purchased from Millipore. Sodium dodecyl sulfate (SDS)[1] was purchased from Sigma Chemical Co. and Calbiochem Corp. and recrystallized from 95% ethanol (*33*). Kodak X-OMAT AR film, developer, and fixer were purchased from Fotodyne, Inc.

Detergent Extracts of A431 Cells, Immunoglobulins, and Quantification of the Concentration of EGF Receptor. Detergent extracts of A431 cells and immunoglobulins specific for the carboxy terminus of EGF receptor, −SEFIGA, were produced as described *(21, 34)*. The concentrations of EGF receptor in the extracts of A431 cells were determined as described *(34)*.

Assay for Dimerization and Self-Phosphorylation of EGF Receptor. Samples (30 μL) of extracts of A431 cells (3−50 nM in EGF receptor) were mixed with phosphate-buffered saline, 0.15 M NaCl, 1 mM EDTA, and 50 mM sodium phosphate, pH 7.2 (PBS),[1] or EGF to a final concentration of 1.6 μM in 45 μL for the noted amount of time at room temperature. A kinase mixture (15 μL) was added that had been prepared so that the final concentrations would be 5 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM Na_3VO_4, 10 μM ATP, 20 mM HEPES, pH 7.4, and [γ-^{32}P]ATP to a final specific radioactivity of 2 Ci (mmol of ATP)$^{-1}$. After 1 min, the reaction was quenched by cross-linking the enzyme with glutaraldehyde (15 μL) at a final concentration of 90 mM. After 1 min, the cross-linking reaction was quenched by the addition of 10 μL of 2 M glycine, pH 9. The polypeptides were unfolded by adding SDS, spun through 1 mL columns of Sephadex G-50 (*35*), submitted to electrophoresis on a 5% polyacrylamide gel, and electrotransferred to a membrane of PVDF (*36, 37*). The membrane of PVDF was exposed to X-ray film at −70 °C. Once the film had been developed, the membrane of PVDF was immunostained using the anti-SEFIGA antibody (*38*). The relative amounts of monomer and dimer were quantified from scans of the reflectance of the immunoblots as described previously (*10*).

The capacity for self-phosphorylation of monomeric and dimeric EGF receptor over the 1 min reaction time was determined by scanning the transmittance of each lane on the autoradiograms and calculating the areas of the peaks of absorbance corresponding to monomeric and dimeric EGF receptor. The areas of absorbance of the bands represent the relative amounts of radioactivity in each sample. The films were exposed long enough to observe the bands of radioactivity but not so long that the intensity of the bands had reached saturation from overexposure of the film.

Extracts of A431 cells (15 μL) were diluted by adding the appropriate volume of a buffer containing 10% glycerol, 1% Triton X-100, and 30 mM NaHEPES, pH 7.4. In this way, the same amount of protein was in each sample. After making the appropriate dilutions, duplicate samples at the same concentration of EGF receptor were exposed to 1 μM EGF or PBS for 3 min, 5 min, and 10 min. The assays for self-phosphorylation and dimerization were then carried out as described above.

RESULTS

Self-Phosphorylation and Dimerization of EGF Receptor as a Function of Time. The time courses for both the dimerization of EGF receptor and the appearance of the capacity for self-phosphorylation induced by EGF were followed simultaneously by exposing samples of extracts of A431 cells to EGF or PBS for the noted time, adding [γ-^{32}P]-ATP for 1 min, and then cross-linking for 1 min with glutaraldehyde the protein that had dimerized. The proteins in the samples were then unfolded by adding SDS, and their polypeptides were separated by electrophoresis and electrotransferred to membranes of PVDF. The membranes of PVDF were exposed to X-ray film and then immunostained with polyclonal immunoglobulins produced against −SEFIGA, the carboxy terminus of EGF receptor (Figure 1). Over the shortest intervals, only a small amount of self-phosphorylated, dimeric EGF receptor was observed. As the interval of time with EGF increased, the capacity for self-phosphorylation and the amount of dimeric EGF receptor increased until nearly all of the EGF receptor had dimerized and the capacity for self-phosphorylation had reached a maximum. In the absence of EGF, only a small fraction of the EGF receptor appeared to be dimeric or become radioactively labeled with [^{32}P]phosphate.

The experiments described here were designed so that the dimerization and self-phosphorylation of EGF receptor could be followed as functions of the time with EGF and the concentration of EGF receptor. The concentration of EGF was chosen so that the hormone would be saturating at all concentrations of receptor. In this way, the concentration of hormone is not a variable in the dimerization and self-phosphorylation of EGF receptor. Detergent extracts were

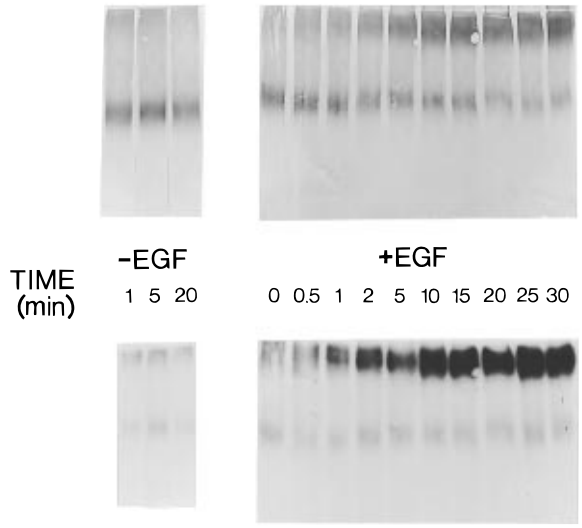

FIGURE 1: Immunoblots and autoradiograms following the dimerization and capacity for self-phosphorylation of EGF receptor as a function of time. Samples (30 μL) of an extract of A431 cells (5 nM in EGF receptor) were exposed to a final concentration of 1.6 μM EGF or PBS in a final volume of 45 μL for the noted time at room temperature. Self-phosphorylation and dimerization were assayed sequentially by first adding [^{32}P]ATP for 1 min and then cross-linking with 90 mM glutaraldehyde for 1 min. The cross-linking reaction was quenched by adding glycine to 0.9 M at pH 9. The proteins in the samples were unfolded by adding SDS, spun through a column (1 mL) of Sephadex G-50, submitted to electrophoresis, and electrotransferred to a membrane of PVDF. The membrane was exposed to X-ray film at −70 °C and then immunostained. The top row of images are immunostained blots on which dimerization can be followed as a function of time (minutes) between the addition of the EGF and the addition of the kinase mixture. The experiment was performed both in the absence (−EGF) and in the presence (+EGF) of EGF. The bottom row of images are autoradiograms of the same blots on which the appearance of the capacity for self-phosphorylation of EGF receptor can be followed as a function of time (minutes) in the absence or presence of EGF.

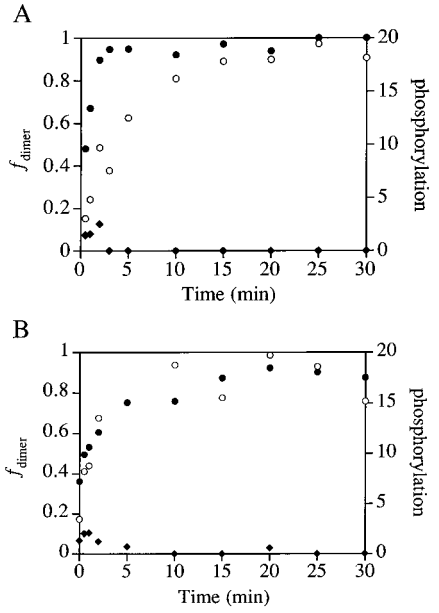

FIGURE 2: Dimerization and self-phosphorylation of EGF receptor as a function of time. Immunoblots and autoradiograms, similar to those shown in Figure 1, were scanned to determine the area of absorbance of each immunostained band of protein and the area of absorbance of the corresponding band on the film representing [^{32}P]-phosphorylated dimeric EGF receptor. The extent of dimerization (filled circles) in a given sample of EGF receptor is expressed as f_{dimer}, the fraction of the protein that had dimerized. The extent of self-phosphorylation (open circles) was assumed to be directly proportional to the area of each scan of the absorbance of the bands of phosphorylated, dimeric EGF receptor on autoradiograms, and the areas are expressed in arbitrary units. Both quantities are presented as a function of the time (minutes) between the addition of the EGF and the addition of the kinase mixture. The fraction of EGF receptor present as dimer was also determined in identical samples of extracts of A431 cells exposed only to PBS (filled diamonds). (A) Concentrations of EGF receptor in these samples were 30 nM. (B) Concentrations of EGF receptor in these samples were 8 nM, 4 times less than in (A).

used because it is not possible to quantify dimerization of EGF receptor when it is within cellular membranes because the frequency of collisions is so high that intermolecular cross-linking dominates the results.

The reflectance of the immunoblots and the transmittance of the autoradiograms were scanned in order to quantify the relative amounts of monomeric and dimeric protein and the levels of phosphorylated protein, respectively. The absorbances of the immunostained bands were corrected for the fact that the staining of dimeric EGF receptor and monomeric EGF receptor do not saturate at the same levels and the fact that dimeric EGF receptor stains more intensely than does monomeric EGF receptor (*10*). After these quantifications, the levels of dimerization and capacity for self-phosphorylation of the dimer could be compared on the same plot (Figure 2). In the presence of EGF, the amount of phosphorylated, dimeric EGF receptor was always much greater than the amount of phosphorylated, monomeric EGF receptor, and in many instances, the amount of self-phosphorylated, monomeric EGF receptor was difficult to detect. For these reasons, levels of dimerization of EGF receptor were plotted on the same graph with the self-phosphorylation of only dimeric EGF receptor rather than the self-phosphorylation of total enzyme.

At higher concentrations of EGF receptor where the rate of dimerization was rapid, a lag between dimerization and the appearance of the capacity for self-phosphorylation was observed (Figure 2A). At lower concentrations of EGF receptor, however, where the rate of dimerization is slower (*10*), the dimerization and appearance of the capacity for self-phosphorylation of EGF receptor were nearly coincident (Figure 2B). The lag at the higher concentration was observed in nine separate experiments.

The time intervals noted on the abscissas are for the time elapsed between the addition of the EGF and the addition of the kinase mixture. Dimerization, activation, and self-phosphorylation occur during the 1 min interval with the kinase mixture, which explains why significant dimerization and self-phosphorylation are observed at zero time. When samples of an extract of A431 cells were diluted 2-fold, 4-fold, or 6-fold, the respective initial rates of dimerization decreased by the same factors. This observation is consistent with the conclusion that the kinetics of dimerization are second order in the concentration of EGF receptor (*10*).

A rate constant for the appearance of the capacity for self-phosphorylation cannot be determined from these plots of dimerization and self-phosphorylation for several reasons. Each site of self-phosphorylation has been shown to be phosphorylated at a different rate (*25, 39*). It is also the case that the 1 min time period was too long to measure initial rates of self-phosphorylation, and as the concentration of active dimeric enzyme increased with time, this became

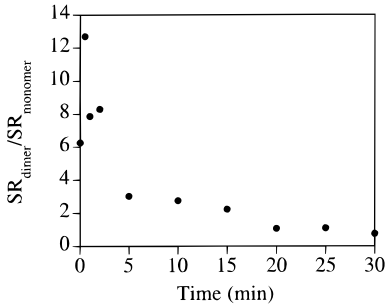

FIGURE 3: Ratios of specific radioactivities of dimeric and monomeric forms of EGF receptor as a function of time. To calculate the specific radioactivity of each species of EGF receptor, the area of absorbance of a band on the autoradiogram was divided by the corrected area of absorbance of the corresponding band of immunostain on the respective blot (Figure 1) for the experiment shown in Figure 2B. The ratio of the specific radioactivities (in arbitrary units) of dimeric EGF receptor to monomeric EGF receptor ($SR_{dimer}/SR_{monomer}$) is plotted as a function of time (minutes) with EGF.

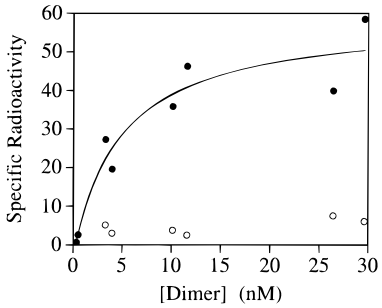

FIGURE 4: Specific radioactivities of self-phosphorylated dimeric and monomeric EGF receptor presented as a function of the concentration of dimeric EGF receptor. Equivalent samples (15 μL) of an extract of A431 cells were systematically diluted with 10% glycerol, 1% Triton X-100, and 30 mM NaHEPES, pH 7.4. The diluted samples were exposed to 1 μM EGF or PBS at room temperature. After 10 min, [^{32}P]ATP was added for 1 min to assay for the capacity for self-phosphorylation, and then glutaradehyde was added to a final concentration of 90 mM to assay for dimerization. The cross-linking reaction was quenched after 1 min with 0.9 M glycine, pH 9. The samples were mixed with SDS and analyzed for the specific radioactivities of the [^{32}P]phosphorylation by autoradiography and immunoblotting as described in Figures 1 and 3. To determine the specific radioactivities of dimeric and monomeric EGF receptor for only the phosphorylation resulting from the addition of EGF, the specific radioactivities of dimeric and monomeric EGF receptor resulting from phosphorylation in the absence of EGF were subtracted from the specific radioactivities of the same species in the presence of EGF. The fraction of dimeric EGF receptor (f_{dimer}) in each sample was determined from the immunoblots. The concentration of dimeric EGF receptor was calculated by multiplying the f_{dimer} by the concentration of EGF receptor in each sample which was established by dilution of an extract of known concentration. The values for the corrected specific radioactivities of dimeric (filled circles) and monomeric (open circles) EGF receptor were then plotted as a function of the concentration of dimeric EGF receptor (nM). The line in the figure was a least-squares fit of eq 1 to the data. The values for *a*, *b*, and *c* were -3, 13 nM^{-1}, and 0.2 nM^{-1}.

a more significant problem. At the highest concentrations, the protein became fully self-phosphorylated in less than 1 min.

Specific Radioactivities of Dimeric and Monomeric EGF Receptor as a Function of Time. To begin to understand the mechanism of the self-phosphorylation of dimeric and monomeric EGF receptor that is dependent upon the addition of EGF, the specific radioactivities of these species were followed as a function of the time during which the sample had been exposed to EGF. The specific radioactivities of dimeric and monomeric EGF receptor in each sample could be calculated from the respective areas of absorbance by quantifying the scans of the immunoblots and autoradiograms. To calculate the specific radioactivity of each species in a sample, the area of absorbance for the band from the autoradiogram was divided by the corrected area of absorbance for its corresponding band on the immunoblot.

When the ratio of the specific radioactivity of dimeric EGF receptor to the specific radioactivity of monomeric EGF receptor is plotted as a function of time with EGF, the ratio at early times is high and decreases as the length of time with EGF increases until the ratio is approximately unity (Figure 3). After short intervals of time with EGF, when the concentration of active enzyme is low, dimeric EGF receptor is phosphorylated preferentially to monomeric EGF receptor. After long times with EGF, when EGF receptor is fully activated, both dimeric EGF receptor and monomeric EGF receptor are phosphorylated to the same level. These results suggest that after the long exposures to EGF when the enzyme had become fully activated, most of the sites for tyrosine phosphorylation on both the monomers and dimers of EGF receptor were phosphorylated during the 1 min the sample was exposed to [^{32}P]ATP and that it is for this reason that the specific radioactivities of the monomeric and dimeric EGF receptor become the same. This explanation is consistent with the observation that at longer times (15−30 min), the specific radioactivities of both monomer (16) and dimer (17) after the 1 min interval for self-phosphorylation had become the same and were both greater than they were for monomer (1.4) and dimer (8) at the shortest times (1−2 min).

Specific Radioactivities of Dimeric and Monomeric EGF Receptor as a Function of the Concentration of Dimeric EGF Receptor. The specific radioactivities of dimeric and monomeric EGF receptor phosphorylated over the 1 min period after adding [γ-^{32}P]ATP were assessed as a function of the concentration of EGF receptor. Equal volumes of an extract of A431 cells (15 μL) were diluted appropriately and mixed with EGF or PBS for a short period of time (3−10 min). Adenosine [γ-^{32}P]triphosphate was then added for 1 min to monitor the capacity for self-phosphorylation, and cross-linking was performed to discriminate monomeric and dimeric forms of EGF receptor. The specific radioactivities for EGF-stimulated phosphorylation were calculated from the areas of absorbance both from the scans of the transmittance for the dimeric and monomeric EGF receptor on the autoradiograms and from the scans of the reflectance for dimeric and monomeric EGF receptor on the immunoblots. The corresponding specific radioactivities from a control lacking EGF receptor were subtracted from these experimental specific radioactivities. The corrected specific radioactivities were plotted as a function of the concentration of active dimeric EGF receptor (Figure 4). The concentration of dimeric EGF receptor in each sample was determined by multiplying the total concentration of EGF receptor in each of the diluted extracts of A431 cells by the value of f_{dimer}, the fraction of EGF receptor that had dimerized, for that dilution. It was found that the specific radioactivities for the self-phosphorylation of both monomeric and dimeric EGF receptor increased as a function of the concentration of dimeric EGF receptor and that the specific radioactivities of

the dimer were larger than those of the monomer at all concentrations. Similar increases in the specific radioactivities of both dimeric and monomeric EGF receptor as a function of the concentration of the protein were observed in five other experiments.

These observations suggest that self-phosphorylation is an intermolecular process. The data in Figure 4 were fit to the arbitrary equation:

$$\text{specific radioactivity} = a + \frac{b[\text{dimer}]}{1 + c[\text{dimer}]} \qquad (1)$$

where a represents the rate of intramolecular self-phosphorylation; b[dimer], the rate of intermolecular self-phosphorylation; and bc^{-1}, the maximum level of self-phosphorylation. The value of a from the data in Figure 4 is slightly less than zero (−3). Because the specific radioactivities are expressed in arbitrary units, the maximum level of self-phosphorylation of the experiments ranges from 20 to 60. In order to compare the values of a from all of these experiments, each value of a was calculated as a percentage of the maximum level of phosphorylation. The percentage obtained for the value of the parameter a by averaging the data from the six different experiments was $-13 \pm 9\%$. Because the value of this parameter representing the rate of intracellular self-phosphorylation is indistinguishable from zero, no significant intramolecular self-phosphorylation could have been occurring. It has already been noted that the values for self-phosphorylation probably reach a maximum (bc^{-1} in eq 1 and the plateau in Figure 4) because at high concentrations of active dimeric EGF receptor, all of the sites for self-phosphorylation have become filled during the 1 min interval. It follows that this is probably a false maximum and that the actual initial rate of self-phosphorylation continuously increases as the concentration of active dimeric EGF receptor is increased. This consideration only magnifies the insignificance of intramolecular self-phosphorylation.

In most of the experiments, the specific radioactivity of dimeric EGF receptor seemed to reach a plateau at the higher concentrations. The specific radioactivity of monomeric EGF receptor, however, was still increasing at the higher concentrations (the slope of a line fit through the data is 0.2 nM^{-1}). As the levels of phosphorylation of the monomer were much lower, the incorporation of [^{32}P]phosphate presumably has not yet even approached saturation of the sites for phosphorylation on the monomer.

The results just reported were for EGF-stimulated self-phosphorylation. When phosphorylation and dimerization were followed over a range of concentrations of EGF receptor in the absence of EGF, it was found that no dimeric EGF receptor could be detected and that the specific radioactivity of monomeric EGF receptor, which was much lower than it was in the presence of EGF, remained constant. This small amount of phosphorylated EGF receptor observed in the absence of EGF can probably be attributed to serine and threonine kinases in the extracts of A431 cells *(40−42)*. If the phosphorylation of EGF receptor observed in the absence of hormone is a true measure of the full activity of the serine and threonine kinases present in these extracts, then the amount of phosphorylation resulting from these other kinases is insignificant (1%) compared to EGF-induced self-phosphorylation (Figure 1) and should not affect the results presented here.

DISCUSSION

Self-phosphorylation was one of the first characteristics of EGF receptor observed in the presence of EGF *(43)*. Early studies had demonstrated ligand-induced aggregation of EGF receptor, but dimerization of EGF receptor was only detected when reagents that could covalently cross-link the dimers of EGF receptor were applied *(44−47)*. It was then shown that dimerization of EGF receptor either must precede or coincide with the activation of its protein tyrosine kinase *(10)*.

This report demonstrates that the capacity for self-phosphorylation succeeds but does not coincide with the dimerization of EGF receptor. When the rate of dimerization was increased by increasing the concentration of EGF receptor, a lag between dimerization and self-phosphorylation could be observed (Figure 2). It follows that there must be a step in the mechanism of EGF receptor between its dimerization and the appearance of its capacity for self-phosphorylation. This conclusion agrees with the finding that the appearance of the capacity for self-phosphorylation lags appreciably behind the binding of EGF (*29*) while dimerization occurs rapidly after its addition *(9, 10)*.

The fact that when dimerization is slowed, dimerization and self-phosphorylation are coincident (Figure 2B) suggests that the dimers that are quantified are active dimers and are able to self-phosphorylate. It agrees with a previous report from our laboratory which proved that the dimerization of EGF receptor, as measured quantitatively with the same cross-linking reagent used in the present experiments, was coincident with activation of the tyrosine kinase (Canals, 1992). Although none of these results rules out the possibility that some of the dimers formed after adding EGF are inactive, it would have to be the case that the rate constant for the formation of an inactive dimer was about the same as that for the formation of an active dimer, which is unlikely. As has been documented previously *(10)*, glutaraldehyde does not produce artifactual intermolecular cross-links but only cross-links oligomers intramolecularly. Thereby, it registers quantitatively the EGF-dependent dimerization that has occurred before it is added.

When the concentration of EGF receptor is low, the dimerization reaction is slowed, and even after long periods of time (20−30 min), EGF receptor in diluted samples has not fully dimerized (Figures 1 and 2B; *10*). Under these conditions, it was possible to measure the specific radioactivities of both self-phosphorylated monomeric and self-phosphorylated dimeric forms of EGF receptor as the reaction progressed. Because the ratio of the specific radioactivities of dimer to monomer is high at short times and decreases over time until it is approximately 1 (Figure 3), the dimer must be preferentially phosphorylated by activated EGF receptor. This agrees with an earlier report that dimeric EGF receptor is self-phosphorylated preferentially to the monomeric form (*9*). After long exposure times to EGF (20−30 min), most of the receptor in the sample has been activated and is able to phosphorylate rapidly both dimeric and monomeric forms of EGF receptor, resulting in specific radioactivities that are the same (Figure 3).

The capacity of EGF receptor for self-phosphorylation is elevated upon the addition of bivalent immunoglobulins and other reagents that produce the aggregation of EGF receptor (*8, 21, 48−50*). From these results, as well as the depen-

dence of the appearance of the capacity for self-phosphorylation on the concentration of EGF receptor, an interprotomeric mechanism was proposed for the activation of its protein tyrosine kinase (*8*). This earlier study, however, could not distinguish whether self-phosphorylation had occurred as an intermolecular process between molecules of dimeric EGF receptor or as an intramolecular process within a molecule of dimeric EGF receptor. Active sites on protomers of EGF receptor that were enzymatically active but made shorter by a carboxy-terminal deletion of 63 amino acids were able to phosphorylate full-length protomers of EGF receptor that had been made enzymatically inactive by a point mutation in the tyrosine kinase domain (*30*, *31*). These results and others like them (*51*−*53*) demonstrated that self-phosphorylation is an interprotomeric process but did not determine whether self-phosphorylation was occurring intramolecularly within the dimers of EGF receptor or intermolecularly between the dimers of EGF receptor.

Evidence for an exclusively intermolecular mechanism in which self-phosphorylation occurs between molecules of dimeric EGF receptor rather than within them is provided by the data presented in this report (Figure 4). When the specific radioactivities of EGF receptor were followed as a function of the concentration of dimeric EGF receptor after short times with EGF, the specific radioactivities of both monomer and dimer were found to increase as the concentration of active, dimeric EGF receptor increased. If self-phosphorylation were only an intramolecular reaction occurring within the dimer, then the specific radioactivity of the dimer would not have increased as a function of the concentration of dimeric EGF receptor. This observation rules out an obligatorily intramolecular mechanism for the self-phosphorylation of EGF receptor and suggests that most of the self-phosphorylation observed is intermolecular. The fact that in six experiments, the values for the specific radioactivities for self-phosphorylated dimeric EGF receptor extrapolated to zero as the concentration of the active enzyme was decreased toward zero suggests that interprotomeric, intramolecular self-phosphorylation within the dimer (*30*, *31*, *51*−*53*) cannot occur and that all self-phosphorylation is intermolecular, between protomers in different dimers.

That EGF induced the self-phosphorylation of monomeric receptor (Figure 1) was an unexpected observation. After EGF has been added, there are at least three scenarios that might explain the observed phosphorylation of the monomeric form. The first would be that monomeric EGF receptor phosphorylates itself intramolecularly, but this possibility is inconsistent with the apparent increase in the specific radioactivity of monomeric EGF receptor as the concentration of EGF receptor increases, even though this increase is not dramatic (Figure 4) or perhaps even statistically significant. The second would be that EGF receptor is dimerizing, being self-phosphorylated only within the dimer, and then dissociating to monomer which is thus phosphorylated. The third would be that active, dimeric EGF receptor is intermolecularly phosphorylating other molecules of both dimeric and monomeric EGF receptor. The results described in this report seem to be most consistent with this third scenario. By this mechanism, monomeric EGF receptor could also have been self-phosphorylated while in a dimer by another molecule of dimer and then dissociate to monomer which is phosphorylated.

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