Persistence of Dimerization and Enzymatic Activity of Epidermal Growth Factor Receptor in the Absence of Epidermal Growth Factor†

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Received March 24, 1997; Revised Manuscript Received August 26, 1997⊗

ABSTRACT: Dimerized and enzymatically active epidermal growth factor receptor, in the presence of saturating epidermal growth factor (EGF), was passed over a column containing an immunoadsorbent for EGF. The immunoadsorbent removed both EGF free in solution and EGF bound to EGF receptor, while EGF receptor passed through unimpeded. Both the dimerization of EGF receptor and the activation of its tyrosine kinase were arrested in samples containing EGF receptor that had been mixed with saturating EGF and then immediately passed over the immunoadsorbent for EGF. The EGF receptor in these samples, however, dimerized completely, and its tyrosine kinase became fully active upon readdition of EGF. Samples of EGF receptor that were fully dimerized and enzymatically active prior to being passed over the immunoadsorbent for EGF remained dimerized and enzymatically active even in the absence of bound EGF. The first-order rate constant for the inactivation of the tyrosine kinase of EGF receptor depleted of EGF was estimated by subtracting the rate constant of inactivation of the tyrosine kinase in samples replenished with EGF from the rate constant of inactivation of the tyrosine kinase in samples that had been depleted of EGF. The rate constant of inactivation was found to be 0.26 ± 0.06 h^{-1}.

The receptor on the cell surface for the mitogen epidermal growth factor (EGF)[1] is a member of a family of transmembrane peptide growth factor receptors that possess an inherent catalytic activity for phosphorylating tyrosine residues of particular cytosolic protein substrates (*1*, *2*). Each of these receptors consists of an extracellular, ligand-binding domain, a short membrane-spanning segment that likely traverses the bilayer only once, and an intracellular domain that contains the active site for the tyrosine kinase (*3*−*8*). The extracellular binding of EGF to EGF receptor results in the homodimerization of EGF receptor (*9*−*13*) and the coincident (*9*) activation of its intrinsic tyrosine kinase activity leading to the phosphorylation of tyrosines on EGF receptor itself along with tyrosines on other proteins in the cell. It is thought that the activation of this inherent tyrosine kinase activity of EGF receptor is the sole result of the transduction across the protein of the mitogenic signal, from the extracellular matrix through the cell membrane and into the cytoplasm (*14*, *15*). Ultimately, the message works its way into the nucleus, producing an activation of DNA synthesis and cell division.

To gain a better understanding of how the binding of EGF leads to the dimerization and activation of EGF receptor, experiments were conducted that examined the effects of rapidly removing EGF from a solution of fully dimeric, enzymatically active EGF receptor. The observations of Canals (*9*) suggested that, when EGF was saturating, the dimerization and activation of the tyrosine kinase displayed the kinetic properties of an irreversible bimolecular process. Further experiments (*16*), however, suggested that the properties of this process were more consistent with those of an approach to a reversible equilibrium for both activation and dimerization. There are other reports that favor reversible mechanisms (*17*−*22*). All of these proposals for the mechanism of activation assume that both liganded and unliganded forms of dimerized EGF receptor are active.

In the present studies, polyclonal immunoglobulins to EGF were raised in chickens, and an immunoadsorbent was made that could rapidly remove EGF from a solution of dimeric, enzymatically active EGF receptor. The immunoadsorbent for EGF was not only able to remove EGF from solution but also able to remove EGF that was bound to dimeric, fully active EGF receptor. It was observed that EGF receptor remained dimerized and its tyrosine kinase activity persisted over long intervals of time even in the absence of EGF.

EXPERIMENTAL PROCEDURES

Materials. Cells of the A431 human epithelioid carcinoma line (*23*) were kindly provided by G. Gill (Department of Medicine, University of California at San Diego). Human EGF was expressed in a strain of the yeast *Pichia pastoris* and purified from the cell-free broth. The broth was a generous gift from Sibia. Aprotinin, benzamidine hydrochloride, phenylmethanesulfonyl fluoride (PMSF),[1] bovine serum albumin (BSA),[1] Na_2ATP, Triton X-100 detergent, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES),[1] 2-mercaptoethanol, disodium ethylenediaminetetraacetate (EDTA),[1] Sephadex G-50, leupeptin, nitro blue tetrazolium, tris(hydroxymethyl)aminomethane (Tris),[1] and the *p*-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate

† This research was supported by Grant GM-33962 from the National Institutes of Health and Training Grant DK-07233 from the National Institutes of Health.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1997.

[1] Abbreviations: EGF, epidermal growth factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetate; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; Fmoc, 9-fluorenylmethyloxycarbonyl; SDS, sodium dodecyl sulfate; PVDF, poly(vinylidene difluoride); HPLC, high-pressure liquid chromatography; phosphate-buffered saline, 150 mM NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate (pH 7.2); elution buffer, 10% glycerol, 0.05% Triton X-100, 1 mM EDTA, 6 mM 2-mercaptoethanol, 10 μg mL^{-1} leupeptin, and 30 mM HEPES (pH 7.4).

S0006-2960(97)00683-1 CCC: $14.00

were purchased from Sigma Chemical Co. Glutaraldehyde and appropriately protected 9-fluorenylmethyloxycarbonyl (Fmoc)[1] derivatives of the amino acids were purchased from Calbiochem-Novabiochem Corp. Affi-gel 10 and goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase were purchased from Bio-Rad Laboratories Corp. Sodium dodecyl sulfate (SDS)[1] was purchased from Sigma Chemical Co. or Calbiochem-Novabiochem Corp. and was recrystallized from 95% ethanol (*24*). The triethylammonium salt of adenosine [γ-^{32}P]triphosphate (3000 mCi mmol^{-1}) was purchased from Amersham. Membranes of poly(vinylidene difluoride) (PVDF)[1] were purchased from Millipore. The peptide RRKGSTAENAEYLRV, which contains the sequence surrounding the site of self-phosphorylation at tyrosine 1173 of EGF receptor and which is a substrate for its tyrosine kinase, was synthesized (*25*) from Fmoc amino acids and purified by high-pressure liquid chromatography (HPLC).[1]

Purification of EGF. Human EGF was purifed from the cell-free broth of the yeast *P. pastoris* expressing the protein by ion exchange on carboxymethylcellulose, reversed phase HPLC on a semipreparative C18 column (0.1 cm × 25.0 cm), reversed phase HPLC on an analytical C18 column (0.046 cm × 25.0 cm), and reversed phase HPLC on an analytical C4 column (0.046 cm × 25.0 cm). Its concentration was quantified by acid hydrolysis with 6 M HCl at 180 °C for 45 min followed by amino acid analysis.

Preparation of a Detergent Extract from A431 Cells. Cells of the A431 human epidermal carcinoma cell line were grown to confluency. The plates were scraped to release the cells, and the resulting suspension was spun at 3000 rpm for 30 min in a Sorvall SS-34 rotor at 4 °C. The pelleted cells were lysed by addition of 4 times the pelleted cell volume of 1 mM EDTA, 5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate, 5 mM 2-mercaptoethanol, 2 mM benzamidine, 0.1 mM PMSF, 2.5 μg mL^{-1} aprotinin, 5 μg mL^{-1} leupeptin, and 20 mM HEPES (pH 7.4). An equal volume of 2% Triton X-100, 20% glycerol, and 30 mM HEPES (pH 7.4) was added to the cell lysates, and the mixture was homogenized with 15−20 strokes in a Dounce homogenizer at 0 °C and then clarified by centrifugation at 100 000 rpm for 30 min at 4 °C in a TLA-100.2 rotor in a Beckman Tl-100 table top ultracentrifuge. The resulting extracts are referred to as detergent extracts of A431 cells. They were either used immediately or stored for future use at −70 °C.

Production of Polyclonal Anti-EGF Immunoglobulin. Epidermal growth factor (82 nmol) was conjugated to BSA (5 mg) with glutaraldehyde at a final concentration of 0.3%. The cross-linking proceeded at room temperature for 1 h prior to quenching with 0.2 M glycine at pH 9.0 for an additional 1 h at room temperature. The mixture was dialyzed against several changes of 150 mM NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate (pH 7.2) (phosphate-buffered saline)[1] overnight. Three 20-week-old White Leghorn laying hens were immunized (*26*) with the conjugate of EGF and BSA mixed with Freund's complete adjuvant. Four weeks after the primary immunization, the chickens received the first boosts.

Affinity Adsorbent for Anti-EGF Immunoglobulins. Epidermal growth factor (82 nmol) was immobilized on by mixing it (dissolved in 0.5 mL of phosphate-buffered saline) with 1 mL of Affi-gel 10 on a rotating wheel overnight at 4 °C. Any activated esters remaining on the Affi-gel 10 were capped off with 200 mM ethanolamine. The modified Affi-gel 10 was then placed into a small column (1.0 cm × 15 cm) and washed extensively with phosphate-buffered saline until the absorbance of the eluant at 280 nm was below 0.05.

Purification of Chicken Polyclonal Anti-EGF Immunoglobulins. Eggs were collected from the laying hens that had been immunized with the conjugate of EGF and BSA. Immunoglobulins were purified from the yolks of the eggs by the procedure of Dotan and Shechter (*26*). The pellets from the final precipitation with PEG were dissolved in 3−5 mL of phosphate-buffered saline and then dialyzed against phosphate-buffered saline overnight at 4 °C with several changes. The dialyzed solution of immunoglobulins was passed over the anti-EGF affinity adsorbent several times, and the affinity adsorbent was then washed with phosphate-buffered saline until the absorbance at 280 nm was below 0.1. The anti-EGF immunoglobulins were eluted from the affinity adsorbent with 0.1 M sodium phosphate (pH 2.5). The acid elutates containing the anti-EGF immunoglobulins were neutralized with a saturated solution of Tris-free base and then dialyzed at 4 °C extensively against phosphate-buffered saline.

Immunoadsorbent for EGF. The immunoadsorbent capable of binding EGF was made by incubating 1 mL of a concentrated solution of anti-EGF immunoglobulins (approximately 5 mg mL^{-1}) with 1 mL of Affi-gel 10. The resulting suspension was mixed overnight at 4 °C. Any activated esters remaining on the Affi-gel 10 were quenched with 200 mM ethanolamine. The resulting immunoadsorbent for EGF was then added to a small column (1.0 cm × 15 cm) and washed extensively with phosphate-buffered saline. The column was saturated with human EGF and rinsed extensively. Elution was performed with 0.1 M sodium phosphate (pH 2.5); the eluates were subjected to HPLC on a C18 reversed phase column, and the amount of EGF that had been retained by the immunoadsorbent was quantified by amino acid analysis. In experiments where detergent extracts of A431 cells were passed over this immunoadsorbent, the EGF receptor was eluted from the column with 10% glycerol, 0.05% Triton X-100, 1 mM EDTA, 6 mM 2-mercaptoethanol, 10 μg mL^{-1} leupeptin, and 30 mM HEPES (pH 7.4) (elution buffer).[1]

Cross-Linking of EGF Receptor, Electrophoresis, Electrotransfer, Immunostaining, Measurement of Tyrosine Kinase Activity, and Assay for Binding of EGF. Dimerization of EGF receptor was followed by quantitative cross-linking with glutaraldehyde (*9*). Electrophoresis was on 5% polyacrylamide gels cast in a solution of 0.1% SDS (*27*). Proteins were electrotransferred (*28*) to membranes of PVDF and immunostained (*29*) with a rabbit polyclonal immunoglobulin raised against the synthetic peptide SEFIGA (*9*), which corresponds to the carboxyl terminus of EGF receptor. The assay for tyrosine kinase (*16*) was initiated by the addition of a kinase mixture that produced final concentrations of 0.25 mM RRKGSTAENAEYLRV, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM Na_3VO_4, 10 μM ATP, and [γ-^{32}P]ATP to give a final specific radioactivity of about 2 Ci (mmol of ATP)$^{-1}$. The yield of phosphopeptide was then assessed (*9*). The assays for binding of EGF were performed as described by Sherrill and Kyte (*16*). When [^{125}I]EGF was used, however, the supernatant fluids were removed, and the pellets were submitted to γ counting.

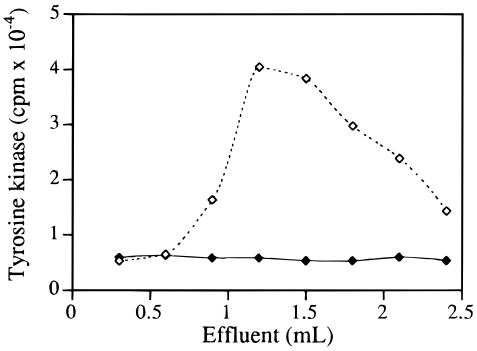

FIGURE 1: Assays for tyrosine kinase of EGF receptor activated by fractions of the eluate from the immunoadsorbent for EGF (closed symbols) or the control immunoadsorbent for KVLIE (open symbols). Solutions (0.45 mL) that were 830 nM in EGF were applied to columns (1.0 cm × 15.0 cm) containing immunoadsorbent (bed volume of 0.5 mL) for EGF or control immunoadsorbent for KVLIE and allowed to remain in the adsorbents for 2 min prior to eluting the columns with phosphate-buffered saline and collecting 0.3 mL fractions. A portion (15 μL) of each fraction was added to 30 μL of a detergent extract of A431 cells, and the mixture stood at room temperature for 20 min prior to the addition of 15 μL of a substrate mixture for the tyrosine kinase assay. The enzymatic reaction was allowed to proceed for 1 min at room temperature before it was quenched with trichloroacetic acid. The incorporation of ^{32}P into the substrate was then assessed. The tyrosine kinase activity (counts per minute) elicited by each fraction is presented as a function of elution volume (milliliters).

RESULTS

Polyclonal immunoglobulins were raised in laying hens by immunizing them with human EGF conjugated to BSA. Immunoglobulins were isolated from the yolks of the eggs by successive polyethylene glycol precipitations and further purified by passage over an affinity adsorbent made by coupling human EGF to agarose. These chicken polyclonal, anti-EGF immunoglobulins were then themselves coupled to agarose. The resulting immunoadsorbent was titered by passing human EGF over it and was found to have a capacity of 3 nmol.

Experiments were performed to make sure that the immunoadsorbent for EGF was able to remove EGF from a solution rapidly (2 min or less). Two identical solutions (0.45 mL) that were 830 nM in EGF were passed separately over the immunoadsorbent for EGF and a control immunoadsorbent specific for the unrelated peptide KVLIE (*30*). Fractions from these columns were assayed for their ability to activate the tyrosine kinase of EGF receptor in detergent extracts of A431 cells by monitoring the incorporation of [γ-^{32}P]phosphate into a synthetic peptide. The control immunoadsorbent was unable to remove EGF from solution because fractions of the eluate (open symbols) were still able to activate the tyrosine kinase of EGF receptor. In contrast, the fractions from the immunoadsorbent for EGF (closed symbols) were incapable of activating the tyrosine kinase of EGF receptor (Figure 1). In another experiment, identical (0.45 mL) solutions that were 830 nM in EGF were passed over both the immunoadsorbent for EGF and the control immunoadsorbent for KVLIE, and fractions of the eluate were assayed for their ability to induce dimerization of EGF receptor (*9*) in detergent extracts of A431 cells. As before, fractions of the eluate from the control immunoadsorbent were able to induce dimerization of EGF receptor, while fractions of the eluate from the immunoadsorbent for EGF were unable to induce any detectable dimerization of EGF receptor.

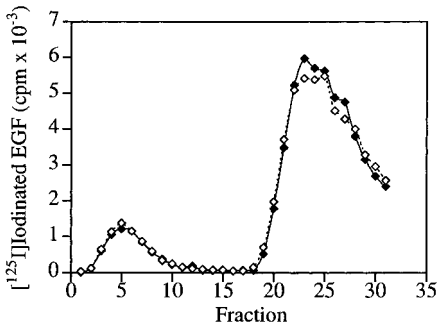

FIGURE 2: Removal of EGF bound to EGF receptor in a detergent extract of A431 cells by the immunoadsorbent for EGF. The sample of EGF receptor (open symbols) consisted of 270 μL of a detergent extract of A431 cells, 27 μL of 2.5 M KCl, and 150 μL of 2.5 μM [^{125}I]iodinated EGF. The control sample (closed symbols) consisted of 270 μL of a solution containing all of the components in the detergent extract except the A431 cells, 27 μL of 2.5 M KCl, and 150 μL of 2.5 μM [^{125}I]iodinated EGF (190 cpm pmol^{-1}). Each sample stood at room temperature for 10 min after adding the EGF prior to applying it to the immunoadsorbent for EGF. Each sample remained on the column of immunoadsorbent for 2 min before it was eluted with elution buffer. Fractions of 10 drops were separated directly into scintillation vials, and they were submitted to liquid scintillation. The immunoadsorbent was eluted until the counts per minute of ^{125}I decreased to background levels at which point the bound [^{125}I]iodinated EGF was eluted from the immunoadsorbent with 0.1 M sodium phosphate (pH 2.5). The amount of [^{125}I]-iodinated EGF in each fraction (counts per minute) is presented as a function of elution fraction.

The experiments described above showed that the immunoadsorbent for EGF was able to remove EGF effectively from a solution. To determine if the immunoadsorbent could remove EGF that had been bound by EGF receptor, the following experiment was performed. A sample of a detergent extract of A431 cells containing 0.04 nmol of EGF receptor was mixed with 0.37 nmol of a nominally saturating solution (830 nM) of [^{125}I]iodinated EGF for 10 min and then passed over the immunoadsorbent for EGF. A control sample, containing identical additions except for the A431 cells, was also passed over the immunoadsorbent. In each case, after the unbound counts per minute had eluted, the counts per minute bound to the immunoadsorbent were eluted with an acidic solution. Each 10 drop fraction of the eluates for both the sample containing the detergent extract of A431 cells and the control sample was subjected to scintillation counting. This provided a complete analysis of the amount of [^{125}I]EGF that had passed through the immunoadsorbent and the amount of [^{125}I]EGF that had been specifically bound by it. When the profile of the counts per minute of ^{125}I on the chromatogram for the sample containing EGF receptor (open symbols) was compared to the chromatogram of the control (closed symbols), there was no significant difference (Figure 2). The small amount (less than 5% of the total counts per minute applied) of radioactivity that did not bind to the immunoadsorbent for EGF in both the chromatogram of the sample containing the detergent extract of A431 cells and the chromatogram of control samples to which no A431 cells were added could be due to ^{125}I that has become detached from the EGF or [^{125}I]iodinated EGF that had damaged epitopes.

One trivial explanation for this result would be that the minority of the molecules of EGF that are actually iodinated have a much greater dissociation constant from EGF receptor than the uniodinated majority of the molecules necessarily present in a preparation of labeled EGF. To examine this possibility, assays suitable for comparing dissociation con-

stants for binding of labeled forms of EGF by EGF receptor were conducted. Increasing amounts of a detergent extract of A431 cells (0−0.2 mL) containing EGF receptor were mixed with either saturating levels of [^{3}H]methylated EGF (600 nM at 80 cpm pmol^{-1}) or saturating levels of [^{125}I]-iodinated EGF (1 μM at 190 cpm pmol^{-1}). The assays for binding were performed as described by Sherrill and Kyte (*16*). The concentration of EGF receptor in the detergent extract of A431 cells was determined to be 160 nM when [^{125}I]iodinated EGF was used and 75 nM when [^{3}H]-methylated EGF was used. The fact that the concentration of EGF receptor was determined to be greater when [^{125}I]-iodinated EGF was used in the assay for binding than when [^{3}H]methylated EGF was used suggests that the dissociation constant of [^{125}I]iodinated EGF from EGF receptor has a value that is less than that for the dissociation constant of [^{3}H]methylated EGF from EGF receptor. Although the actual issue is the dissociation constant for [^{125}I]iodinated EGF relative to that for the unlabeled EGF inescapably present in the preparation of iodinated EGF, these results nevertheless demonstrate that [^{125}I]iodinated EGF binds well to EGF receptor and that it binds more tightly than [^{3}H]-methylated EGF.

On the basis of the assay just described for the binding of [^{125}I]iodinated EGF to EGF receptor, it could be calculated that an additional 8200 cpm (11% of the total counts per minute) from the sample containing the detergent extract of A431 cells beyond those from the control sample should have passed through the immunoadsorbent in the experiment presented in Figure 2 if the specifically bound [^{125}I]EGF had remained on the enzyme. The difference in the counts per minute that passed through the immunoadsorbent for the two samples, however, was less than 400 cpm. Additional experiments were performed in which, on the basis of the binding of [^{125}I]EGF, up to 30 000 cpm were specifically bound to EGF receptor prior to its passage over the immunoadsorbent for EGF. Again, there were no significant differences (<2000 cpm) in the profile of the counts per minute of ^{125}I between the chromatograms of the samples containing the detergent extracts of A431 cells or the control samples. In all of these experiments, the recovery of radioactivity in the acid eluates from the immunoadsorbent agreed well with the total amount of radioactivity added initially.

Experiments were performed to determine both the recovery of EGF receptor and the volume at which the EGF receptor in the extracts of A431 cells eluted from the immunoadsorbent. A total of 21 of the 22 pmol of phosphate per minute of EGF-dependent tyrosine kinase activity added to the immunoadsorbent for EGF was recovered in the second and third 0.3 mL fractions eluting from the column (bed volume of 0.5 mL). In addition, a sample from these two fractions was dissolved in a solution of SDS, submitted to electrophoresis on polyacrylamide gels, transferred to a membrane of PVDF, and immunostained. There was no apparent difference in the intensity of the immunostaining of this sample compared to that of an appropriately diluted sample of the initial detergent extract of A431 cells. Therefore, there was an almost complete recovery of EGF receptor following passage over the immunoadsorbent for EGF. Taken together, all of the experiments described so far demonstrate that the immunoadsorbent for EGF is able to remove not only EGF in solution but also EGF bound by EGF receptor.

The state of dimerization of EGF receptor in detergent extracts of A431 cells activated to varying levels with EGF prior to removing the EGF by passing the samples over the immunoadsorbent for EGF was determined by subjecting the fractions of the eluate containing EGF receptor to quantitative cross-linking with glutaraldehyde. Samples of a detergent extract of A431 cells were activated with EGF and either immediately passed over the immunoadsorbent for EGF or passed over 10 min after the addition of EGF. The samples remained on the immunoadsorbent for EGF for 2 min before they were eluted. Fractions of the two eluates containing EGF receptor were pooled, and the two pools were each split into two identical samples. To one of each of these two samples was added phosphate-buffered saline; to the other was added EGF to 830 nM. These samples then stood at room temperature. At the indicated times, aliquots were removed, and glutaraldehyde was added for an additional 1 min before the cross-linking was quenched with glycine. The samples were then dissolved in a solution of SDS, run on polyacrylamide gels, transferred to a membrane of PVDF, and immunostained (Figure 3). The immunoadsorbent for EGF was able to arrest the dimerization of the EGF receptor that had been applied to the column immediately after the addition of EGF. This follows from the observation that the sample to which only phosphate-buffered saline was added following immunoadsorption had significant amounts of monomeric EGF receptor, while the sample to which EGF was added back had almost none (Figure 3A).

The immunoblots were quantified by scanning densitometry of the bands of monomer and dimer of EGF receptor on the immunostained membranes of PVDF (*9*). The percentages of monomeric EGF receptor in the fully dimerized sample from which the EGF had been removed by immunoadsorption (Figure 3B) were 3, 4, 6, 10, 8, and 5% at 15, 30, 50, 80, 100, and 140 min after the initial addition of EGF. Therefore, after the EGF had been removed by the immunoadsorbent, the amount of dimeric EGF receptor remained constant for up to 1.5 h. The percentages of monomeric EGF receptor in the fully dimerized sample from which the EGF had been removed and to which EGF was then readded (Figure 3C) were 3, 4, 6, 5, 6, and 5% at 18, 33, 55, 83, 113, and 143 min after the initial addition of EGF. It follows that activated, dimeric EGF receptor remained fully dimerized in the absence of EGF because there was no significant difference, as judged by densitometry, in the state of dimerization between the sample to which only phosphate-buffered saline had been added (Figure 3B) and the sample to which EGF had been readded (Figure 3C).

The tyrosine kinase activity of EGF receptor was followed in detergent extracts of A431 cells that were either immediately passed over the immunoadsorbent for EGF after the addition of EGF or allowed to dimerize and activate for 8 min before they were passed over the immunoadsorbent for EGF. Epidermal growth factor was added to samples of detergent extracts of A431 cells containing EGF receptor. Either immediately (Figure 4A) or after 8 min (Figure 4B), the extracts were applied to the immunoadsorbent for EGF. The pooled fractions containing EGF receptor from the two effluents were each split into two identical samples. To one of each of these two samples was added phosphate-buffered saline; to the other was added EGF back to 830 nM. The samples then stood at room temperature; aliquots were taken out at the noted times, and a mixture for assaying the tyrosine kinase was added. The immunoadsorbent for EGF was able

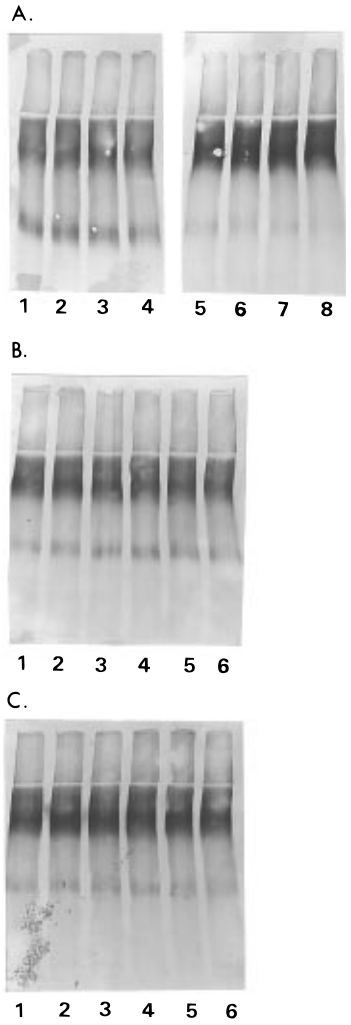

FIGURE 3: Cross-linking of EGF-activated EGF receptor with glutaraldehyde following removal of EGF by passage over the immunoadsorbent for EGF. Epidermal growth factor was added (0.15 mL at 2.5 μM) to samples of detergent extracts of A431 cells (0.3 mL) containing EGF receptor. Either immediately (A) or after 10 min (B and C), the samples were applied to the immunoadsorbent for EGF. The samples remained on the immunoadsorbents for 2 min prior to washing the column with elution buffer. Fractions of the eluate containing EGF receptor that had been depleted of EGF were pooled and split into two equal samples. To one of these samples (lanes 1−4 in panel A and every lane in panel B) was added phosphate-buffered saline; to the other (lanes 5−8 in panel A and every lane in panel C) was added EGF back to 830 nM. The samples then stood at room temperature, and aliquots were taken out at the noted times and cross-linked with glutaraldehyde (80 mM) for 1 min before quenching the cross-linking reaction with glycine (final concentration of 0.2 M). The proteins were unfolded in a solution of SDS [5 g of SDS (g of protein)$^{-1}$] for 1 min at 100 °C and submitted to electrophoresis on 5% polyacrylamide gels. The polypeptides were then electrotransferred to membranes of PVDF and immunostained with rabbit polyclonal immunoglobulins raised against the synthetic peptide SEFIGA corresponding to the carboxyl terminus of EGF receptor. (A) Lanes 1−4 are of the sample to which phosphate-buffered saline had been added after immunoadsorption and from which aliquots were cross-linked with glutaraldehyde 13, 15, 38, and 53 min after initial addition of EGF. Lanes 5−8 are of the sample to which EGF had been added back after immunoadsorption and from which aliquots were cross-linked 10, 20, 35, and 50 min after the initial addition of EGF. (B) Lanes 1−6 are of the sample to which phosphate-buffered saline had been added after immunoadsorption and from which aliquots were cross-linked 15, 30, 50, 80, 100, and 140 min after the initial addition of EGF. (C) Lanes 1−6 are of the sample to which EGF had been added after immunoadsorption and from which aliquots were cross-linked 18, 33, 55, 83, 113, and 143 min after initial addition of EGF.

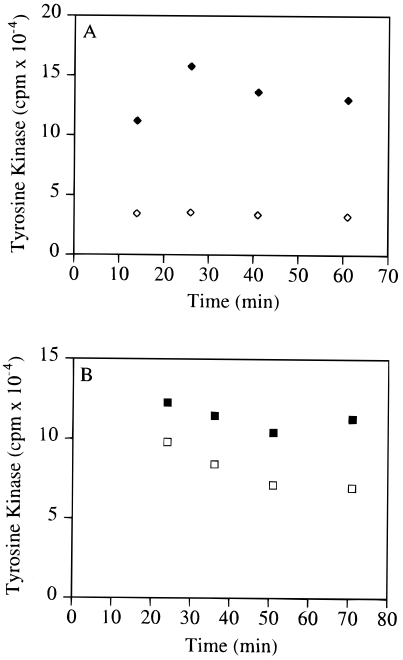

FIGURE 4: Tyrosine kinase activity of EGF receptor following removal of EGF by the immunoadsorbent for EGF. Epidermal growth factor (0.15 mL at 2.5 μM) was added to two 0.3 mL samples of a detergent extract of A431 cells. One sample was immediately applied (A), and the other was applied after 8 min (B), to the immunoadsorbent for EGF. The extracts remained on the column for 2 min prior to eluting them with elution buffer. The fractions containing EGF receptor were pooled, and the pools were split into two identical samples. Phosphate-buffered saline was added to one sample (open symbols), and EGF was added back to 830 nM to the other (closed symbols). Aliquots (30 μL) were removed at the specified times and assayed for tyrosine kinase. Tyrosine kinase activity (counts per minute) is presented as a function of the time (minutes) that had elapsed since mixing the EGF and the EGF receptor before immunoadsorption.

to arrest the activation of the tyrosine kinase of EGF receptor that had been applied to the column immediately after the addition of EGF. This conclusion follows from the low tyrosine kinase activity of the portion to which only phosphate-buffered saline had been added after the EGF had been removed (Figure 4A, open symbols). The EGF receptor in the corresponding portion that had been replenished with EGF, however, was able to become rapidly and fully activated (Figure 4A, closed symbols). This further demonstrates that the immunoadsorbent does not remove the EGF receptor from solution, nor does it inactivate EGF receptor. The tyrosine kinase of EGF receptor in the sample that had been activated with EGF for 8 min prior to passing it over the immunoadsorbent for EGF remained almost fully activated in the absence of EGF. This conclusion follows from the fact that the tyrosine kinase activity was high and could only be activated slightly (15%) upon readdition of EGF (Figure 4B). It was also noted that the tyrosine kinase of EGF receptor in the sample that had been activated with EGF for 8 min prior to passing it over the immunoadsorbent for EGF retained an elevated level of tyrosine kinase for up to 1 h after removal of EGF by the immunoadsorbent for EGF. This result and the results of quantitative cross-linking with glutaraldehyde (Figure 3) demonstrate that EGF receptor remains enzymatically active and dimerized for greater than 1 h in the absence of EGF.

Experiments were then performed in order to assess more quantitatively the decay of the tyrosine kinase of EGF receptor that had been activated with EGF and then stripped

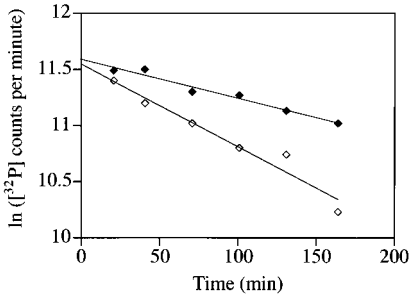

FIGURE 5: Rate of inactivation of the tyrosine kinase of EGF receptor in the presence or absence of EGF. Epidermal growth factor (0.15 mL at 2.5 μM) was added to 0.3 mL of a detergent extract of A431 cells. After 10 min, the activated extract was applied to the immunoadsorbent for EGF. The extract remained on the column for 2 min prior to eluting with an elution buffer. The fractions containing EGF receptor were pooled, and the pool was split into two identical samples. Phosphate-buffered saline was added to one sample (open symbols), and EGF was added back to 830 nM to the other (closed symbols). Aliquots (30 μL) were removed at the specified times and assayed for tyrosine kinase. The natural logarithm of the enzymatic activity (^{32}P counts per minute) is presented as a function of the time (minutes) since mixing the EGF and the EGF receptor before the immunoadsorption. Lines were fit to both sets of data by least-squares numerical analysis.

of its EGF by passage over the immunoadsorbent. A detergent extract of A431 cells was activated for 10 min prior to passage over the immunoadsorbent. Fractions from the eluate containing EGF receptor were assayed for tyrosine kinase over 2.5 h. At the earliest times, the tyrosine kinase of EGF receptor in samples that had not been replenished with EGF after passage over the immunoadsorbent for EGF was comparable to that in samples to which EGF had been added back following immunoadsorption. The tyrosine kinase activity of EGF receptor in samples that had been depleted of EGF, however, appeared to be lost at a somewhat faster rate than that in samples to which EGF had been added back. The natural logarithm of the tyrosine kinase activity was plotted against the time after which the sample of detergent extract of A431 cells was originally activated with EGF (Figure 5), and the rate of inactivation for the tyrosine kinase of EGF receptor in the presence of saturating EGF (closed symbols) was compared to that of EGF receptor from which EGF had been removed (open symbols). The first-order rate constant for the inactivation of the tyrosine kinase of EGF receptor in the presence of saturating EGF was subtracted from the first-order rate constant for the inactivation of EGF receptor in the absence of EGF to give a rate constant for the inactivation of EGF receptor due only to the removal of the EGF. The values for this corrected rate constant from three separate experiments in which EGF receptor was activated for 10−20 min before the immunoadsorption were 0.22, 0.32, and 0.23 h^{-1}.

DISCUSSION

The main question that has been addressed in the present study is what happens when EGF is rapidly removed from a solution of a detergent extract of A431 cells containing EGF receptor. The effect of removing the EGF was assessed by assaying the state of dimerization and the tyrosine kinase activity of the protein. Possible outcomes of removing the EGF included reversion of EGF receptor to a monomer with retention of the tyrosine kinase activity, reversion of EGF receptor to a monomer with subsequent loss of the tyrosine kinase activity, retention of the dimer but loss of the tyrosine kinase activity, or retention of the dimer and retention of tyrosine kinase activity.

Initial experiments designed to separate EGF from EGF receptor by rapid gel filtration on spin columns (*31*) proved unsuccessful. Significant quantities of EGF passed through the spin columns as assayed by the ability of the eluate to induce the dimerization and activation of the tyrosine kinase of EGF receptor in detergent extracts of A431 cells. It was found, however, that making an immunoadsorbent for EGF by coupling anti-EGF polyclonal immunoglobulins to agarose and placing the resulting solid phase in a small column was an effective method of removing EGF from solution. Chickens were chosen to produce the anti-human EGF immunoglobulins since commercially available anti-human EGF immunoglobulins that had been raised in rabbits or mice proved to be of low titer. This latter observation is not surprising since EGF is highly conserved in mammals. It was hoped that using a species more distantly related to humans would result in an increased immune response. The immune response in the chickens proved satisfactory, and sufficient yields of specific anti-EGF immunoglobulins were obtained to make an immunoadsorbent to EGF of high capacity. An anti-EGF immunoadsorbent was made by coupling these immunoglobulins to agarose after they had been affinity purified. The capacity of the anti-EGF immunoadsorbent was high, and the EGF could be removed rapidly from a solution of enzymatically active, dimerized EGF receptor that had been saturated with the hormone. The total amount of EGF present in the experiments reported here was always less than 400 pmol, well below the capacity of the immunoadsorbent for EGF.

To investigate fully the effects of removing EGF from a solution of dimerized and enzymatically active EGF receptor that was saturated with EGF, it was necessary to determine if the immunoadsorbent for EGF was capable of removing not only EGF in solution but also EGF bound by EGF receptor. Most of the proposed mechanisms for activation of EGF receptor assume that both liganded and unliganded forms of dimerized EGF receptor are active (*9*, *17*−*22*). Thus, not knowing if EGF receptor retained any tightly bound EGF after passing over the immunoadsorbent for EGF would complicate the analysis of the fate of the activated EGF receptor. If it proved to be the case that EGF receptor retained a significant amount of EGF following immunoadsorption, then that amount of EGF would have to be accounted for in analyzing the tyrosine kinase activity and state of dimerization of EGF receptor. Off rates for EGF would have to be estimated and equilibrium concentrations of EGF determined. As it turned out, however, the immunoadsorbent for EGF removed all the EGF, both free and bound. This was determined by mixing equivalent samples of [^{125}I]EGF with an A431 cell detergent extract or a control of identical composition but without the A431 cells, and then passing these samples over the immunoadsorbent for EGF and examining the chromatograms of ^{125}I counts per minute for each of the samples (Figure 2). There was no significant difference when the profiles of the counts per minute of ^{125}I in the two chromatograms were compared. If all of the specifically bound [^{125}I]EGF (8000 cpm) had remained bound, it would have been easily detected. Because the yield of EGF receptor itself from the immunoadsorbent for EGF was almost complete, the immunoadsorbent must be able to remove both free EGF and EGF that has been bound by EGF receptor.

The immunoadsorbent for EGF was also able to arrest both the dimerization of EGF receptor and the activation of its tyrosine kinase in samples that were passed over it immediately after the addition of EGF to the protein (Figures 3A and 4A). The fact that EGF receptor dimerizes completely and its tyrosine kinase becomes fully active upon adding back EGF demonstrates that the immunoadsorbent arrests dimerization and the activation of the tyrosine kinase not by altering the normal responsiveness to EGF of the enzyme that has passed through the immunoadsorbent but simply by removing the agonist. The most striking observation, however, is the fact that EGF receptor that had been incubated with EGF for a length of time sufficient to ensure complete dimerization and activation of its tyrosine kinase remained fully dimerized (Figure 3B) and enzymatically active (Figure 4B) after the EGF had been removed. This persistent dimerization and enzymatic activity of EGF receptor in the absence of EGF is an unexpected result.

While the tyrosine kinase of EGF receptor remained persistently active in the absence of any free or bound EGF, it appeared as though the tyrosine kinase in the samples that had been depleted of EGF did decay at a significantly faster rate than that in samples in which EGF had been replenished. The rates of inactivation of the tyrosine kinase activity in samples that had been depleted of EGF by passing them over the immunoadsorbent for EGF were compared to those in identical samples that had been replenished with EGF (Figure 5). The rate constant for inactivation of the tyrosine kinase of EGF receptor in the absence of EGF was corrected by subtracting the rate constant for inactivation of the tyrosine kinase of EGF receptor in samples containing EGF. The corrected rate constant of inactivation of unoccupied EGF receptor was found to be 0.26 ± 0.06 h^{-1}. As the dissociation constant for the unliganded dimer of EGF receptor seems to be greater than 500 nM (*16*), the rate constant for the association of two unliganded monomers should be less than 0.5 μM^{-1} h^{-1}. This very slow rate constant may in part explain why unliganded dimer is not observed even at high concentrations of EGF receptor in the absence of EGF.

It has been shown (*20, 32*−*36*) that the tyrosine kinase of EGF receptor can be activated by various immunoglobulins. The concentration of bivalent immunoglobulins at which the tyrosine kinase of EGF receptor is maximally activated likely corresponds to a condition in which a complex between two monomers of EGF receptor and one immunoglobulin predominates. At the optimal concentration of immunoglobulins, the tyrosine kinase activity of the enzyme can be as high as 50−70% of that induced by EGF (*32*). Thus, it appears that immunoglobulins are able to bring two monomers of EGF receptor together and that this is sufficient to activate its tyrosine kinase. In at least one case, however, the complex of immunoglobulin and two monomers of EGF receptor could be rapidly dissociated by adding the peptide against which the antibodies had been raised. When the antigen−antibody complex was dissociated, the activation of the tyrosine kinase was rapidly reversed (*32*). This result is in contrast to the behavior of dimeric, active EGF receptor from which the EGF has been removed by passage over the immunoadsorbent for EGF. The form of unliganded dimer produced in this sequence of steps remains dimerized and loses its tyrosine kinase slowly over several hours. This observation suggests that the contacts formed in the dimer of EGF receptor within the complex with an immunglobulin are somehow different from the contacts that are present in the dimer of EGF receptor produced by the binding of EGF. One possibility is that the conformation of the extracellular ligand binding domain of EGF receptor that has never bound EGF sterically inhibits its dimerization. This steric inhibition would dominate the dissociation of the complex once the physical constraint of the immunoglobulin holding two monomers of EGF receptor together in close proximity has been removed by its dissociation. This possibility would provide a reason the protein that is expressed from the v-erb B gene is associated with cell transformation. The sequence of this protein is highly homologous to EGF receptor (*37*), but nearly all of the extracellular portion of the protein is deleted along with the carboxyl-terminal amino acids. It is possible that in part it is the absence of an inhibitory extracellular EGF binding domain that leads to constitutive dimerization and activation of its tyrosine kinase.

In addition to the differences in the behavior of the unliganded dimer formed in the presence of a bivalent immunoglobulin and that of the unliganded dimer produced by removing EGF from the protein rapidly, there are differences in the kinetic behavior of the latter unliganded dimer and dimeric EGF receptor in the presence of EGF. When EGF is added to monomeric EGF receptor, it dimerizes and activates in a reaction that has all of the characteristics of a rapidly equilibrating system (*16*). Yet the unliganded dimer produced by rapidly removing its bound EGF only slowly reverts to its inactive form. One possible explanation for these seemingly contradictory observations is the fact that a symmetric dimer formed from two unliganded monomers displays slow rates of dissociation while the unsymmetric dimer formed from one liganded and one unliganded monomer displays rapid rates of dissociation and association. In this view, it is the asymmetric form of the dimer that displays the rapid kinetics.

Numerous studies have shown that EGF receptor, following the binding of EGF, is rapidly internalized through clathrin-coated pits into acidified endosomes in which both EGF receptor and EGF alike are somehow sorted and then subjected to lysosomal degradation (*38*−*41*). The results presented in this study show that the removal of EGF from dimerized and enzymatically active EGF receptor does not turn it off. Perhaps this is why clathrin-mediated endocytosis for EGF receptor and other ligand-activated receptor tyrosine kinases is necessary. It provides a mechanism by which activated receptors are rapidly cleared from the surface of the cell and their signals downregulated. This would be important if simple removal of the agonist does not turn off the enzyme.

The behavior of the extracellular domain of the growth hormone receptor with growth hormone provides one model for the mechanism of activation of EGF receptor by EGF. The extracellular domain of growth hormone receptor has been shown to form a complex with growth hormone in which one growth hormone protomer resides between two extracellular domains of the receptor (*42*). Human growth hormone acts as a bivalent dimerizing agent. On the surface of the hormone, there is a high-affinity binding site for one extracellular domain and a low-affinity site for another. Thus, the yield of the 2:1 complex is a biphasic function of the concentration of growth hormone with a distinct maximum (*43*). A recent study (*16*) has demonstrated that the behavior of activation of EGF receptor by EGF is inconsistent with the behavior that would result if EGF receptor followed the

mechanism that has been proposed for growth hormone receptor. This was determined by monitoring the yields of dimerization of EGF receptor and activation of its tyrosine kinase as a function of both the concentration of EGF and of EGF receptor as well as the time of exposure to EGF. The present study shows that EGF receptor remains dimerized and enzymatically active in the absence of EGF. This observation eliminates the growth hormone model for the mechanism of dimerization and activation of EGF receptor. The present results are direct and unambiguous and do not rely on comparing experimentally derived parameters with theoretical models.

ACKNOWLEDGMENT

I thank Dr. Gordon Gill for providing me with the A-431 cells used in these experiments and for his consistent interest and encouragement. The broth from the strain of *P. pastoris* expressing EGF was the generous gift of Sibia. I am grateful to Dr. Jack Kyte, in whose laboratory this research was performed, for his insight, support, and guidance.

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BI9706834