Insufficiency of Self-Phosphorylation for the Activation of Epidermal Growth Factor Receptor†

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ABSTRACT: Polyclonal immunoglobulins were produced against the carboxy terminus, −SEFIGA, of the receptor for epidermal growth factor (EGF). The addition of these immunoglobulins to a solution containing EGF receptor resulted in the activation of its protein tyrosine kinase. The levels of activation were assessed by measuring the initial velocities of the phosphorylation of the tyrosine in angiotensin II. The enzymatic activity induced by the immunoglobulins was significant, usually 50−70% of the maximum activity induced by EGF, and the induction occurred over a narrow range of concentration of the immunoglobulins. In order to achieve the activation, the immunoglobulins had to be bivalent; the addition of monovalent Fab fragments to EGF receptor did not produce any activation of the protein tyrosine kinase. The activation produced by the immunoglobulins was found to be reversible upon the addition of the synthetic peptide SEFIGA against which the immunoglobulins had been produced. Self-phosphorylation of the EGF receptor also occurred as the enzyme was activated by the immunoglobulins. Tryptic peptide maps demonstrated that the self-phosphorylation caused by the immunoglobulins had the same signature as that produced by EGF. When the synthetic peptide that had been used as the hapten was added to EGF receptor that had been self-phosphorylated in the presence of the immunoglobulins, the stimulated enzymatic activity was lost even though the protein remained phosphorylated. It follows from the results of deletion mutation [Walton, G. M., Chen, W. S., Rosenfeld, M. G., & Gill, G. N. (1990) *J. Biol. Chem. 265*, 1750−1754] and the results reported here that self-phosphorylation is neither necessary nor sufficient for the activation of EGF receptor.

Epidermal growth factor receptor is an enzyme that is responsible for the first step in the regulation of the growth of many animal cells. Its responsibility is the transmission of a signal across the plasma membrane. Epidermal growth factor receptor is a membrane-spanning protein consisting of an extracellular domain for the binding of its ligand, epidermal growth factor (EGF[1]), and an intracellular domain for protein tyrosine kinase activity (Yarden & Ullrich, 1988; Gill et al., 1987). Each monomer of native EGF receptor contains only one membrane-spanning segment connecting these two domains (Cadena & Gill, 1993; Greenfield et al., 1989; Lax et al., 1991; Livneh et al., 1986; Ullrich et al., 1984; Wedegaertner & Gill, 1989). The biochemical signal transmitted is the phosphorylations catalyzed by the enzyme. The tyrosine kinase activity is generated inside the cell at the cytoplasmic surface of the protein only after EGF has bound to the EGF receptor at its extracytoplasmic surface and the enzyme has dimerized (Canals, 1992). The phosphorylation of intracellular substrates by EGF receptor initiates a plethora of events eventually leading to the cell growth and division that occurs in response to the exposure to EGF (Carpenter & Cohen, 1990; Egan et al., 1993; Koch et al., 1991; Margolis et al., 1989a; Meisenhelder et al., 1989).

Epidermal growth factor receptor is a member of a family of membrane-spanning protein tyrosine kinases whose enzymatic activities are expressed intracellularly upon the binding of the appropriate agonists on their extracellular surfaces (Yarden & Ullrich, 1988; Hunter & Cooper, 1985; Ullrich & Schlessinger, 1990). On the basis of a crystallographic molecular model of insulin receptor (Hubbard et al., 1994), another member of this family, it has been proposed that the phosphorylation of specific tyrosines within a molecule of a receptor of this family, catalyzed by the enzyme itself (Yarden & Ullrich, 1988; Hunter & Cooper, 1985; Ullrich & Schlessinger, 1990), is sufficient to produce the activation of its tyrosine kinase that is seen upon the binding of the agonist. To show that this hypothesis does not apply to a particular member of the family, it is necessary to show only that the self-phosphorylated form of the receptor is not necessarily activated.

It has been shown that the dimerization of EGF receptor and the activation of its tyrosine kinase occur coincidentally (Canals, 1992). Because dimerization is required for the activation of the tyrosine kinase, the addition of a bivalent immunoglobulin directed against EGF receptor, by causing its dimerization artificially, can activate its protein tyrosine kinase (Spaargaren et al., 1991, 1990; Defize et al., 1986; Das et al., 1984; Yarden & Schlessinger, 1987a). Moham-

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[1] Abbreviations: BSA, bovine serum albumin; C_{18}, octadecylsilyl silica gel; EDTA, disodium ethylenediaminetetraacetate; EGF, epidermal growth factor; Fmoc, 9-fluorenylmethyloxycarbonyl; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid; phosphate-buffered saline, 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.5; SDS, sodium dodecyl sulfate; t-Boc, *N*-*tert*-butoxycarbonyl; TFA, trifluoroacetic acid.

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madi et al. (1993) reported that agents which could aggregate the cytoplasmic domain of EGF receptor, such as bivalent immunoglobulins, ammonium sulfate, and polyethylene glycol, led to the activation of its tyrosine kinase. The level of activation of the cytoplasmic domain was found to correlate with the ability of these agents to aggregate or precipitate the protein. One of the reagents used in these earlier studies to induce dimerization of the cytoplasmic domain was anti-carboxy-terminal polyclonal immunoglobulins.

This paper describes the activation of intact EGF receptor produced by polyclonal immunoglobulins specific for its carboxy terminus. This activation proved to be rapidly reversible upon addition of the synthetic peptide against which the antibodies were raised. In this way the enzyme could be self-phosphorylated in the absence of EGF. When the immunoglobulins were removed, the enzyme returned to its resting activity even though it remained phosphorylated.

EXPERIMENTAL PROCEDURES

Materials. Cells of the A431 human epithelioid carcinoma line (Haigler et al., 1978) were kindly provided by Dr. Gordon 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, phenylmethylsulfonyl fluoride, bovine serum albumin, Na_2ATP, Triton X-100, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 2-mercaptoethanol, disodium ethylenediaminetetraacetate (EDTA), papain, glucose, trypsin that had been treated with *N*-(*p*-tolylsulfonyl)-L-phenylalanyl chloromethyl ketone, Protein A Sepharose, and leupeptin were purchased from Sigma Chemical Co.; *N*-*tert*-butoxycarbonyl (t-Boc) and 9-fluorenylmethyloxycarbonyl (Fmoc) derivatives of the amino acids were purchased from Bachem, Inc; the triethylammonium salt of adenosine [γ-^{32}P]triphosphate (3000 mCi mmol^{-1}) was purchased from Amersham; hexokinase was purchased from Worthington Biochemical Corp.; trifluoroacetic acid (TFA) was purchased from Halocarbon Products Corp.; centrifugal concentrators (Centricons) with a cut off of apparent molecular mass 30 000 Da were purchased from Amicon; phosphocellulose paper was purchased from Whatman; Kodak X-OMAT film was purchased from Fotodyne Inc.; and Solvable was from DuPont-NEN Research Products. Sodium dodecyl sulfate (SDS) was purchased from Sigma Chemical Co. or Calbiochem Corp. and was recrystallized from 95% ethanol (Burgess, 1969).

Angiotensin II was synthesized from Fmoc amino acids (Stewart & Young, 1984; Thibault, 1993). The synthetic angiotensin II was purified on HPLC. It had the same chromatographic mobility as commercial material, and the amino acid composition after acid hydrolysis was $D_{1.2}R_{0.9}V_{1.2}Y_{0.9}I_{1.0}H_{1.0}P_{1.1}F_{0.8}$. The carboxy-terminal peptide of EGF receptor, SEFIGA, was synthesized by standard methods for solid phase t-Boc peptide chemistry (Stewart & Young, 1984; Bayer, 1990; Canals, 1992) and purified by high-pressure liquid chromatography. The purified peptide migrated on analytical HPLC as a single peak and upon acid hydrolysis gave the amino acid composition of $S_{1.0}E_{1.1}F_{1.0}I_{0.9}G_{0.9}A_{1.1}$.

Papain was coupled to Sepharose 4B-200 following cyanogen bromide activation of the agarose (March et al., 1974). The specific activity of the immobilized papain was determined by assaying for the activity of papain as described by the *Worthington Manual of Enzymes and Related Biochemicals*, Worthington Biochemical Corp.

Preparation of Extracts from A431 Cells. Cells of the A431 line were grown until they were confluent or just past confluence. The cells were then harvested, lysed, and homogenized at 0 °C in a buffer containing 10% glycerol, 1% Triton X-100, 1 mM EDTA, 5 mM ethylene glycol bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetate, 5 mM 2-mercaptoethanol, 2 mM benzamidine, 2.5 μg mL^{-1} aprotinin, 5 μg mL^{-1} leupeptin, and 30 mM NaHEPES, pH 7.4 (Canals, 1992). The homogenate was centrifuged at 200 000*g* for 30 min. The supernatant is referred to as an extract of A431 cells. The concentration of monomeric EGF receptor was estimated by the tyrosine kinase activity of the fully activated enzyme and its turnover number under these conditions (Sherrill & Kyte, 1996).

Preparation of Immunoglobulins and Monovalent Fab Fragments. Polyclonal immunoglobulins specific for the carboxy-terminal sequence −SEFIGA of EGF receptor (Canals, 1992) were produced by the immunization (Walter et al., 1980) of White New Zealand rabbits with the synthetic peptide SEFIGA cross-linked at 3 mol mol^{-1} as a hapten to bovine serum albumin (BSA) using glutaraldehyde (Kyte et al., 1987). Anti-SEFIGA antisera were purified over an affinity adsorbent constructed by coupling the peptide SEFIGA to a solid phase of Affi-Gel 10. The purified immunoglobulins were concentrated by pressure dialysis in a Centricon-30 centrifugal concentrator for further use.

To prepare monovalent Fab fragments of the immunoglobulins, anti-SEFIGA antisera were digested with immobilized papain (4.8 μmol min^{-1} mL^{-1} of resin) overnight (Harlow & Lane, 1988). After the anti-SEFIGA Fab fragments had been purified over the affinity adsorbent to ensure that they still recognized the antigen, they were separated from intact, undigested immunoglobulins by passing them over Sepharose to which Protein A had been attached (Surolia et al., 1982). The Fab fragments that passed through Protein A were collected and concentrated by pressure dialysis. The concentrations of the bivalent immunoglobulins and monovalent Fab fragments were estimated by their absorbance at 280 nm. All affinity adsorbents were equilibrated with 0.15 M NaCl, 0.1 mM EDTA, 20 mM sodium phosphate, pH 7.5 (phosphate-buffered saline) and eluted with 0.1 M sodium phosphate, pH 2.5.

Tyrosine Kinase Activity. The initial velocity of the protein tyrosine kinase of EGF receptor was assayed as previously described (Canals, 1992; Sherrill & Kyte, 1996). Samples (30 μL) of cell extracts (approximately 25 nM in EGF receptor) were mixed with EGF (final concentration 1.6 μM), phosphate-buffered saline (control), or the noted amount of bivalent or monovalent immunoglobulins in a final volume of 45 μL for 20 min at room temperature. The enzymatic assay was then initiated by the addition (to the noted final concentrations in a final volume of 60 μL) of a mixture of angiotensin II (1.25 mM), $MgCl_2$ (5 mM), $MnCl_2$ (2 mM), Na_3VO_4 (0.1 mM), ATP (10 μM), and [γ-^{32}P]ATP to give a final specific radioactivity of about 2 Ci (mmol of ATP)$^{-1}$. This mixture is referred to as the kinase mixture. The reactions were quenched after 1 min by adding tri-

chloroacetic acid to 5%, and the incorporation of [^{32}P]-phosphate into the peptide was assessed (Sherrill & Kyte, 1996).

In assays to which the synthetic peptide SEFIGA was added to block or reverse the binding of immunoglobulin, a solution of 1 mg mL^{-1} peptide and 5 mg mL^{-1} BSA was used. After exposing samples of cell extract to bivalent immunoglobulins for 20 min, the peptide solution (15 μL) was added for the noted time, and the enzymatic activity of the samples was then assayed for 1 min. Because the solution of peptide already contained BSA, the BSA normally added during the quenching of the reaction was not required for samples to which peptide had been added.

For samples that required EGF receptor to be fully self-phosphorylated before assaying the enzymatic activity, a kinase mixture from which angiotensin II had been omitted was used to effect the self-phosphorylation. After a 20 min interval with the immunoglobulins, EGF, or phosphate-buffered saline, a mixture of the following was added (to the noted final concentrations in 52.5 μL): $MgCl_2$ (5.5 mM), $MnCl_2$ (2.2 mM), Na_3VO_4 (0.11 mM), ATP (11 μM), NaHEPES (22 mM), pH 7.4, and [γ-^{32}P]ATP to a final specific radioactivity of about 2 Ci (mmol of ATP)$^{-1}$. The self-phosphorylation proceeded for 10 min before 15 μL of either a solution of 1 mg mL^{-1} of the peptide SEFIGA in 5 mg mL^{-1} BSA or 5 mg mL^{-1} BSA alone (control) was added for the noted time. The assay for the initial velocity of the phosphorylation of angiotensin II was then carried out by adding the full kinase mixture for 1 min and assessing the incorporation of [^{32}P] phosphate into the substrate.

Assay for the Self-Phosphorylation of EGF Receptor. Samples (30 μL) of cell extracts were mixed with EGF (final concentration of 1.6 μM), phosphate buffered saline (control), or the noted amount of bivalent immunoglobulins in a final volume of 45 μL for 20 min at room temperature. Self-phosphorylation was started by the addition (to the noted final concentrations in a volume of 52.5 μL) of $MgCl_2$ (5.5 mM), $MnCl_2$ (2.2 mM), Na_3VO_4 (0.11 mM), ATP (11 μM), NaHEPES (22 mM), pH 7.4, and [γ-^{32}P]ATP to give a final specific radioactivity of about 2 Ci (mmol of ATP)$^{-1}$. After 10 min, the excess ATP was removed by adding 5 μL of 1.26 units mL^{-1} hexokinase and 5 μL of 1.26 μmol mL^{-1} glucose. Either peptide solution (15 μL) or 5 mg mL^{-1} BSA (15 μL) was added for the noted time. The reaction was quenched by adding SDS to 1%. The samples were spun through a 1-mL column of Sephadex G-50 (Penefsky, 1977) equilibrated with 20 mM NaHEPES, pH 7.4, and submitted to electrophoresis on 5% polyacrylamide gels cast in a solution of 0.1% SDS (Laemmli, 1970). The gels were exposed to X-ray film at −70 °C. The bands of radioactivity corresponding to EGF receptor were sliced out of the gel, swollen in Solvable, and submitted to liquid scintillation.

The assay for the self-phosphorylation of EGF receptor was also performed by inactivating the protein with 25 μL of 5% phosphoric acid, after adding BSA or peptide. Aliquots of the samples were spotted on Whatman filter disks. The disks were washed with 4 L of 75 mM phosphoric acid and then submitted to liquid scintillation. A similar procedure was reported for assessing the self-phosphorylation of the intracellular domain of EGF receptor (Wedegaertner & Gill, 1989). The self-phosphorylation due only to the addition of EGF was assessed by subtracting the phosphorylation observed in its absence.

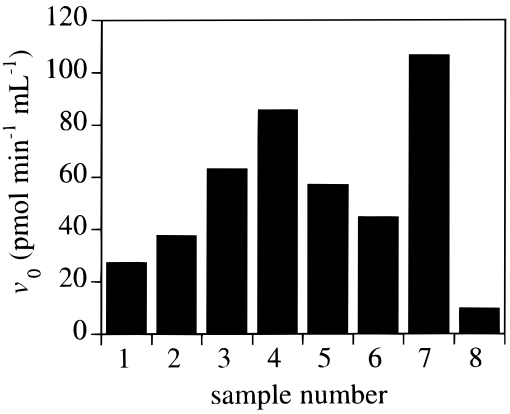

FIGURE 1: Activation of EGF receptor dependent on the immunoglobulins. The activation of the tyrosine kinase of EGF receptor by polyclonal immunoglobulins produced against the carboxy terminus, −SEFIGA, of EGF receptor was followed as a function of the amount of the immunoglobulins added to extracts of A431 cells. The immunoglobulins were affinity purified, and the concentration of the purified sample of immunoglobulins was determined by the absorbance at 280 nm. Samples of extracts of A431 cells (0.7 pmol of EGF receptor in a final volume of 45 μL) were exposed to the immunoglobulins, EGF, or phosphate-buffered saline for 20 min, and then the activity of the tyrosine kinase was followed over a period of 1 min by the incorporation of [^{32}P]phosphate (cpm) into angiotensin II, an exogenous substrate. Samples 1−6 were exposed to (1) 2.5, (2) 5, (3) 10, (4) 20, (5) 40, and (6) 80 pmol of anti-SEFIGA immunoglobulins. Samples 7 and 8 were exposed to a saturating amount (1.6 μM) of EGF and phosphate-buffered saline, respectively.

Tryptic Peptide Mapping of EGF Receptor. Samples (100 μL) of extracts of A431 cells were exposed to immunoglobulins, EGF, or phosphate-buffered saline for 20 min in a final volume of 150 μL. Self-phosphorylation was initiated by adding (to the noted final concentrations in a volume of 175 μL) $MgCl_2$ (5.5 mM), $MnCl_2$ (2.2 mM), Na_3VO_4 (0.11 mM), ATP (11 μM), NaHEPES (22 mM), pH 7.4, and [γ-^{32}P]ATP to a final specific radioactivity of 2 Ci (mmol of ATP)$^{-1}$. After 10 min, the self-phosphorylation reaction was stopped by the addition of SDS at 100 °C. The samples were cooled, spun through 3-mL columns of Sephadex G-50, and submitted to electrophoresis on a 5% polyacrylamide gel. The gel was exposed to X-ray film so that the phosphorylated EGF receptor could be located and sliced out. The samples were in duplicate, so that two bands were isolated and digested with trypsin at the same time in the same tube. The slices were suspended in 2 mL of 0.2 mM $CaCl_2$ and 50 mM $NaHCO_3$, pH 7.8, and 1 mg of trypsin was added. After 12−14 h, more trypsin (1 mg) was added. The tryptic digestion lasted 24 h at 37 °C. The samples were dried under a vacuum, dissolved in 0.05% TFA in water, and injected onto an analytical C_{18} column (Vydac) with a mobile phase of 0.05% TFA in water. A linear gradient was developed from 0.05% TFA in water to 0.02% TFA in acetonitrile at 1% min^{-1} over 60 min. One-minute fractions were collected at a flow rate of 1 mL min^{-1} and submitted to liquid scintillation.

RESULTS

Activation of EGF Receptor Induced by Immunoglobulins. Polyclonal immunoglobulins raised against the haptenic peptide SEFIGA, which are specific for the carboxy terminus of EGF receptor, were able to activate EGF receptor over a range of concentrations (Figure 1). Samples of extracts from

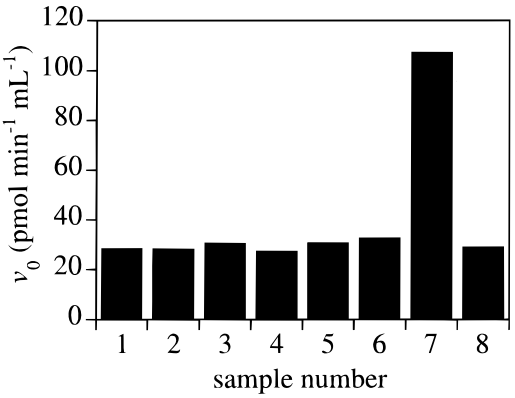

FIGURE 2: Activity of EGF receptor in the presence of Fab fragments. Samples of extracts of A431 cells (0.7 pmol of EGF receptor in a final volume of 45 μL) were exposed to Fab fragments of the immunoglobulins, EGF, or phosphate-buffered saline for 20 min, and the activity of the tyrosine kinase was then followed over a period of 1 min by the incorporation of [^{32}P]phosphate (cpm) into angiotensin II. Samples 1−6 were extracts of A431 cells exposed to (1) 200, (2) 100, (3) 50, (4) 25, (5) 12, and (6) 6 pmol of anti-SEFIGA Fab fragments. Samples 7 and 8 were exposed to a saturating amount (1.6 μM) of EGF and phosphate-buffered saline, respectively.

A431 cells were exposed to the affinity-purified immunoglobulins for 20 min, and the initial velocity of the tyrosine kinase was followed by the incorporation of radioactively labeled phosphate into angiotensin II. Significant activation (50−70% of the levels attained with EGF) of the tyrosine kinase was observed at optimal levels of the immunoglobulins.

Samples of extracts of A431 cells exposed to monovalent Fab fragments that had been prepared from the same preparations of immunoglobulins did not display any activation of the tyrosine kinase (Figure 2). These experiments were performed over a range of molar concentrations of Fab fragments encompassing those concentrations of intact immunoglobulins that were able to activate the tyrosine kinase activity. The dependence of the activation of EGF receptor on the bivalence of the immunoglobulin is consistent with the results of experiments with other antisera that are able to activate EGF receptor (Mohammadi et al., 1993; Schreiber et al., 1983; Spaargaren et al., 1991; Yarden & Schlessinger, 1987a).

The carboxy terminus of EGF receptor, -SEFIGA, was the antigen used to elicit the immunoglobulins that activate EGF receptor. When the synthetic peptide SEFIGA was mixed with the amount of purified immunoglobulins that produced the greatest activation of the tyrosine kinase activity and that mixture was then added to the extract of A431 cells, no activation of the tyrosine kinase was observed (sample 7, Figure 3). Because the synthetic peptide presumably inhibits the activation of EGF receptor by occupying all the combining sites for SEFIGA, the enzymatic activity observed after adding the immunoglobulins to EGF receptor presumably results from the binding of the immunoglobulins to the carboxy terminus, −SEFIGA, of EGF receptor and not from some other nonspecific effect.

Reversion of the Activation Caused by Immunoglobulins. The enzymatic activities of samples of extracts from A431 cells were measured after adding peptide to samples that had already been activated by mixing them with the immunoglobulins. After the samples had been activated by the optimal amount of purified immunoglobulins for 20 min, the synthetic peptide was added for intervals of 1−20 min (Figure 3). Even after the shortest interval, the activation produced by the immunoglobulins had been significantly reversed. When synthetic peptide was added to samples of extracts of A431 cells that had been exposed to EGF, the tyrosine kinase activity was not affected. This result demonstrated that the carboxy-terminal peptide was not inhibiting the tyrosine kinase of EGF receptor and was acting exclusively by affecting the ability of the immunoglobulins to activate the enzyme.

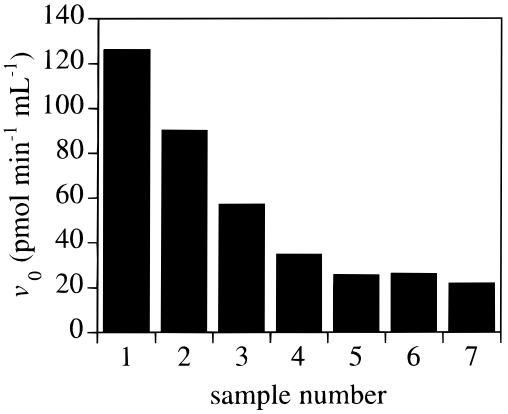

FIGURE 3: Reversibility of immunoglobulin-dependent activation of EGF receptor upon addition of the peptide, SEFIGA. Samples of extracts of A431 cells (0.5 pmol of EGF receptor in a final volume of 45 μL) were exposed to optimal levels of the immunoglobulins (10 pmol) for 20 min to achieve activation of the protein tyrosine kinase (sample 2). Identical samples were then exposed to the peptide SEFIGA (24 nmol), for increasing intervals of time (samples 3−5): (3) 1, (4) 10, and (5) 20 min. Samples 1 and 6 were samples of extracts of A431 cells that had been exposed to a saturating amount (1.6 μM) of EGF and phosphate-buffered saline, respectively. Sample 7 was an extract of A431 cells that had been mixed with the immunoglobulins, after they had been exposed to the peptide SEFIGA for 20 min. Following the addition of peptide, the phosphorylation of angiotensin II with [^{32}P]ATP (cpm) was measured over 1 min.

Dissociation of Self-Phosphorylation from Activation of the Tyrosine Kinase. In samples of extracts of A431 cells that had been activated by the immunoglobulins, EGF receptor displayed self-phosphorylation (Figure 4B). This observation agrees with those made in previous studies. In fact, self-phosphorylation of EGF receptor is the usual way in which the stimulation produced by the addition of bivalent immunoglobulins against EGF receptor is assayed (Spaargaren et al., 1991; Mohammadi et al., 1993; Yarden & Schlessinger, 1987a; Das et al., 1984). The incorporation of [^{32}P]phosphate into EGF receptor in the present experiments reached saturation between 5 and 10 min after the kinase mixture was added. Routinely, the reaction was run for 10 min to ensure that EGF receptor was fully self-phosphorylated.

Identical samples of cell extract were used to follow both the enzymatic activity and the self-phosphorylation of EGF receptor. The samples were exposed to EGF, the immunoglobulins, or phosphate-buffered saline for 20 min; the kinase mixture was added without substrate to elicit self-phosphorylation of the protein; and, after 10 min, peptide was added for increasing intervals of time to remove the immunoglobulins. It was found that the sample had to be exposed to peptide for at least 20 min to reverse completely the activation induced by the immunoglobulins (Figure 3).

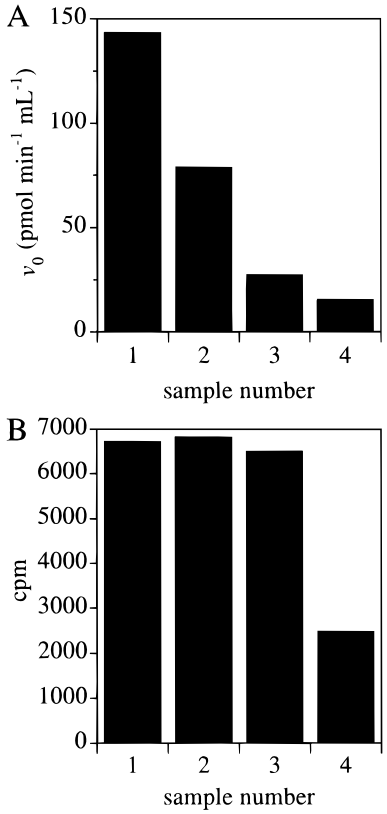

FIGURE 4: Reversibility of the activation and the irreversibility of the self-phosphorylation of EGF receptor activated by the immunoglobulins following the addition of the peptide SEFIGA. (A) Samples of extracts of A431 cells (0.8 pmol of EGF receptor in a final volume of 45 μL) were exposed to optimal levels of immunoglobulins for 20 min to achieve activation of the protein tyrosine kinase. [γ-^{32}P]Adenosine triphosphate was added to samples for 10 min in a final volume of 52.5 μL, followed by the addition of the peptide SEFIGA for 20 min (sample 3). Samples of extracts of A431 cells were also exposed to a saturating amount (1.6 μM) of EGF (sample 1), phosphate-buffered saline (sample 4), or the immunoglobulins without peptide (sample 2). After all additions had been made, the incorporation of [^{32}P]phosphate into angiotensin II was measured in a 1-min assay. (B) Samples of A431 cells (0.8 pmol of EGF receptor in a final volume of 45 μL) were exposed to 1.6 μM EGF (sample 1), optimal levels of immunoglobulins (samples 2 and 3), or phosphate-buffered saline (sample 4). After 20 min, [γ-^{32}P]adenosine triphosphate was added to samples for 10 min in a final volume of 52.5 μL, followed by the addition of hexokinase and glucose for 1 min to consume the remaining ATP. The peptide SEFIGA was then added to sample 3. After 20 min, SDS was added to 0.1%, and the samples were spun through columns of Sephadex G-50 to remove the free [γ-^{32}P]-ATP. Phosphorylated protein was isolated by running the samples on 5% polyacrylamide gels and located by exposing the gel to Kodak OMAT X-ray film at −70 °C. The resulting films displayed only one band of radioactivity, corresponding to EGF receptor, and this band of radioactivity was cut out, the polyacrylamide was swollen with Solvable, and the solution was submitted to liquid scintillation.

When the synthetic peptide SEFIGA was added to samples of cell extract that had been activated by immunoglobulins and self-phosphorylated with [^{32}P]ATP, the enzymatic activity of the tyrosine kinase was lost, but the EGF receptor remained phosphorylated (Figure 4). The levels of the radioactivity of [^{32}P]phosphorylated EGF receptor were always similar (97% ± 16%, $n = 4$) before (sample 2, Figure 4B) and after (sample 3, Figure 4B) the addition of the synthetic peptide SEFIGA. The calculation of these levels has been based on the difference between incorporation of [^{32}P]phosphate in the presence of the immunoglobulins (samples 2 and 3, Figure 4B) and incorporation in their absence (sample 4, Figure 4B). Even though the levels of self-phosphorylation were unchanged, the activation of the protein tyrosine kinase was reversed (17 ± 7%, $n = 4$) upon addition of the peptide (Figure 4A). These results show that phosphorylation of EGF receptor alone is not sufficient for the activation of the tyrosine kinase.

In assays following the self-phosphorylation of EGF receptor by spotting samples on filter paper rather than purifying the product by gel electrophoresis, identical samples of extracts of cells also were used to follow the self-phosphorylation and tyrosine kinase activity of EGF receptor. The percentage of self-phosphorylation and tyrosine kinase activity remaining after 20 min with the peptide SEFIGA compared to the levels before peptide was added were 100 ± 20% and 16 ± 8% ($n = 9$), respectively. The problem with these experiments was that the unspecific background of phosphorylated protein (for example, see sample 4, Figure 4) was much higher because other phosphorylated proteins were included. Nonetheless, these results agree with those obtained when electrophoresis was used to purify phosphorylated EGF receptor (Figure 4).

Phosphopeptide Maps of EGF Receptor. To demonstrate that the same amino acid residues of EGF receptor were self-phosphorylated in the presence of immunoglobulin as in the presence of EGF, phosphopeptide mapping of self-phosphorylated EGF receptor was performed (Figure 5). Activation of EGF receptor was induced by the addition of the immunoglobulins or EGF for 20 min. A control to which only phosphate-buffered saline had been added was also included. The self-phosphorylation reaction was then initiated by adding adenosine [γ-^{32}P]triphosphate, and it lasted for 10 min before a solution of SDS at 100 °C was added to stop the reaction. After purifying the polypeptide of EGF receptor by electrophoresis on a polyacrylamide gel, the protein was digested with trypsin and the digest was submitted to high-pressure liquid chromatography.

The tryptic digests of self-phosphorylated EGF receptor gave rise to six major phosphopeptides above a background ranging from 25 to 50% of the counts per minute associated with the major peaks. Many of the background counts per minute (cpm) are probably due to peptides containing phosphoserine and phosphothreonine residues. Most of the phosphorylation sites on serines and threonines of EGF receptor are modified to a lesser extent than the self-phosphorylation sites on tyrosines (Downward et al., 1984). The threonine and serine residues of EGF receptor are phosphorylated by cytoplasmic proteins such as protein kinase C (Hunter et al., 1984; Cochet et al., 1984; Heiserman & Gill, 1988). The EGF receptor used in these experiments had not been purified by immunoadsorption from the extracts of A431 cells because immunopurification would have activated EGF receptor, and I did not want to start with activated EGF receptor in any of my experiments. As a result, these phosphopeptide maps have a more complex pattern of peaks (Margolis et al., 1989b; Downward et al., 1984; Walton et al., 1990).

The counts per minute displayed in Figure 5 are the result of only phosphorylations induced by immunoglobulins and EGF, respectively. The incorporation of [^{32}P]phosphate that occurred in the control in the presence of phosphate-buffered saline was subtracted from both sets of data. The mean

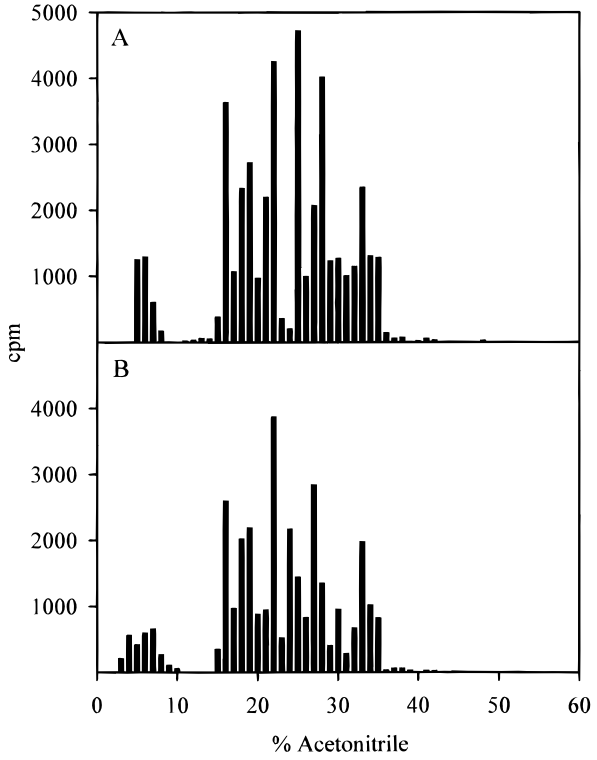

FIGURE 5: Maps of phosphopeptides in tryptic digests of EGF receptor self-phosphorylated in the presence of immunoglobulins (A) or EGF (B). Samples of extracts of A431 cells (100 μL) were exposed to immunoglobulins, EGF, or phosphate-buffered saline in a final volume of 150 μL for 20 min. [γ-^{32}P]Adenosine triphosphate (11 μM, 2 Ci mmol^{-1}) and other required reagents were added to the samples for 10 min in a final volume of 175 μL, followed by the addition of SDS to 0.1% at 100 °C. Samples were spun through columns of Sephadex G-50 (3 mL) and submitted to electrophoresis. An autoradiogram of the resulting gel was developed, and the bands of radioactivity corresponding to EGF receptor were cut out of the polyacrylamide gel. The slices were suspended in solutions containing trypsin (0.5 mg mL^{-1}). After 24 h, the samples were dried down and injected onto an analytical C_{18} column. The gradient was developed from 0% to 60% acetonitrile over 60 min at a flow rate of 1 mL min^{-1}. One-minute fractions were collected and submitted to liquid scintillation. Counts per minute (cpm) are presented as a function of fraction number (designated by % acetonitrile).

number of cpm in the fractions from the digested EGF receptor that had been exposed only to phosphate-buffered saline was 400 cpm, about 10−20% of the cpm in the major peaks.

Previously published phosphopeptide maps of self-phosphorylated EGF receptor (Margolis et al., 1989b; Downward et al., 1984; Walton et al., 1990) are similar to those shown here (Figure 5). The biggest difference between the two peptide maps displayed in Figure 5 is that the peptides in Figure 5B are running slightly faster than the peptides in Figure 5A. For example, the fraction at 25% acetonitrile in the sample exposed to the immunoglobulins (Figure 5A) is bigger than the fraction at 25% in the sample exposed to EGF (Figure 5B), but this peak in the latter chromatogram is actually coming out in two fractions, those at 24% and those 25% acetonitrile. The fraction at 25% acetonitrile in Figure 5A has approximately 4700 cpm, and the two fractions at 24−25% acetonitrile in Figure 5B have 4200 cpm. The fraction at 28% acetonitrile in Figure 5A has 4000 cpm, and the two fractions at 27−28% acetonitrile in Figure 5B together have 4000 cpm. For both maps, the cpm in fractions 18−19 are probably part of the same peak and should be added to give 5000 cpm for the sample exposed to the immunoglobulins and 4200 cpm for the sample exposed to EGF. Peaks eluting at 16%, 22%, and 33% acetonitrile are not split into two fractions. Four experiments were performed, and in each, the pattern of peaks was the same.

DISCUSSION

Previous studies have reported activation of EGF receptor induced by antisera and protein A purified monoclonal and polyclonal immunoglobulins against known and unknown epitopes of EGF receptor (Spaargaren et al., 1991, 1990; Yarden & Schlessinger, 1987a; Das et al., 1984; Gill et al., 1984; Schreiber et al., 1983, 1981). Some of the earliest studies reported an increase in DNA synthesis (Schreiber et al., 1981) and clustering of EGF receptor on the surface of cells when monoclonal anti-EGF receptor antibodies were added to A431 cells (Defize et al., 1986; Schreiber et al., 1983). In other studies, the self-phosphorylation of EGF receptor (Yarden & Schlessinger, 1987a; Das et al., 1984; Gill et al., 1984) and the phosphorylation of endogenous protein substrates in membrane preparations were followed (Spaargaren et al., 1991; Schreiber et al., 1981). Because the assay for phosphorylation of either endogenous substrates or EGF receptor itself is far more sensitive and significantly less quantitative than the assay for phosphorylation of an exogenous peptide, it is possible that only low levels of activation were attained in these earlier experiments. The phosphorylation of an exogenous peptide by both dissolved (Gill et al., 1984) and undissolved (Spaargaren et al., 1991; Spaargaren et al., 1990) membranes of A431 cells was also measured, but in these experiments the activity was induced by monoclonal immunoglobulins that were produced against unknown epitopes of EGF receptor at its extracellular domain. In fact, these immunoglobulins had been selected for their ability to inhibit the binding of EGF. The activation produced by immunoglobulins raised against regions in the EGF binding domain (Spaargaren et al., 1991; Yarden and Schlessinger, 1987a; Gill et al., 1984; Schreiber et al., 1983, 1981) is difficult to analyze because of possible interactions between the immunoglobulins and the binding site for EGF. The activation observed in the present experiments was not complicated by any of these shortcomings. The epitope is known, it is on the cytoplasmic surface of the enzyme well removed from the binding site for EGF, and a quantitative enzymatic assay was used to assess the activation.

If a population of EGF receptors is forced to dimerize by the addition of immunoglobulins to a sample of cell extract in the same way that it does when EGF is added, then tyrosine kinase activity should be observed. The data shown here demonstrate that the binding of polyclonal immunoglobulins directed against the carboxy terminus of the EGF receptor, which is located in its cytoplasmic domain, is sufficient to activate the tyrosine kinase. The activation of EGF receptor was dependent on the concentration of the immunoglobulins and displayed a maximum (Figure 1). At low relative concentrations of a bivalent immunoglobulin, each of its combining sites is occupied by a monomer of EGF receptor, and these bivalent complexes containing two monomers of EGF receptor bound to one immunoglobulin should be enzymatically active. Therefore, in this low range of concentrations, the level of activation of the tyrosine

kinase should be a linear function of the concentration of the immunoglobulins. High concentrations of immunoglobulins deplete the free concentration of EGF receptor in the solution so that each immunoglobulin binds to only one molecule of EGF receptor rather than two, and these monovalent complexes should be inactive. As the concentration of the immunoglobulins increases beyond the point at which bivalent complexes predominate, the activity of the tyrosine kinase should decrease (Figure 1). At the optimal concentration of the immunoglobulins, the prevailing species should be one in which two monomers of EGF receptor are bound to one molecule of immunoglobulin, and the consequent phosphorylation of exogenous substrate should be significant, and it was (50−70% of the activity induced by EGF). The activation observed was reversed by adding the synthetic peptide that was the original antigen (Figure 3), and no activation was elicited by Fab fragments (Figure 2). All of these observations are consistent with the conclusion that it is the dimerization produced by the bivalent immunoglobulins binding to the carboxy termini of EGF receptors that is responsible for the activation of the protein tyrosine kinase. The activation displayed by the bivalent complexes may even have been as great as that seen when EGF is added, because some univalent complexes must have been present at the optimal ratios of the immunoglobulin to EGF receptor.

The molar ratio of bivalent immunoglobulins to EGF receptor in the sample that yielded the highest levels of enzymatic activity was 30:1 (in Figure 1, for example, 15 nM EGF receptor and 450 nM immunoglobulins). This ratio appears high if it is the case that the immunoglobulin activates EGF receptor by binding in a 1:2 ratio. These immunoglobulins, however, are polyclonal; they were produced against a synthetic peptide not the actual receptor; and they were purified on an affinity adsorbent to which synthetic peptide had been coupled. One possible reason for the high ratio of the immunoglobulins to receptor required for maximum activation would be that the carboxy terminus of EGF receptor is folded in such a way that it is not recognized by the majority of the immunoglobulins. In this case, only a minority (3−4%) would be responsible for binding and activating the native protein. It is also possible that, having been eluted under acidic conditions from the affinity adsorbent, some of the immunoglobulins had been partially unfolded by the low pH and were unable to refold properly as the pH was raised. Because the concentration of the immunoglobulins was determined by their absorbance, this measurement would not distinguish between the immunoglobulins unable to bind to the native enzyme, improperly folded immunoglobulins, and active immunoglobulins. Therefore, the concentration of the immunoglobulins determined spectrophotometrically could be significantly greater than the concentration of the immunoglobulins actually responsible for the effects observed.

The activation of EGF receptor by these immunoglobulins is interesting because they are binding to the carboxy terminus of EGF receptor, which is at the opposite end of the enzyme from the extracellular binding site for EGF. Mohammadi et al. (1993) have demonstrated that immunoglobulins raised against the carboxy-terminal sequence of EGF receptor are able to activate its cytoplasmic domain, but because the levels of full activation for this domain could not be assessed and the assay was only for the self-phosphorylation of the enzyme, the degree of the activation produced by the immunoglobulins was not quantitatively established. In the present experiments, however, the assays employed were quantitative and demonstrated that levels of tyrosine kinase 50−70% of those attained with EGF could be elicited. The activation produced by the binding of immunoglobulins specific for the carboxy terminus demonstrates that simply having two monomers of EGF receptor in close proximity is sufficient for satisfactory activation of the tyrosine kinase of EGF receptor.

When the active complex of dimeric EGF receptor and immunoglobulin was exposed to the synthetic peptide SEFIGA against which the polyclonal immunoglobulins were raised, the enzymatic activity rapidly declined (Figure 3). Therefore, the activation of EGF receptor produced by the immunoglobulins is rapidly reversible in solution. Prior to this paper, the reversibility of immunoglobulin-activated EGF receptor has not been demonstrated. This result is consistent with proposed reversible models for the activation of EGF receptor in which the active, dimeric EGF receptor is in equilibrium with the inactive forms of the EGF receptor (Sherrill & Kyte, 1996; Schlessinger, 1988; Yarden & Schlessinger, 1987a,b; Basu et al., 1989).

The role of self-phosphorylation in the activation of EGF receptor has been the source of great confusion because of the number of contradictory reports. It was recently proposed that the self-phosphorylation of tyrosine 1162 is an inescapable step in the activation of the tyrosine kinase of insulin receptor (Hubbard et al., 1994). In the crystallographic structure of unphosphorylated insulin receptor, tyrosine 1162 is bound at the active site apparently preventing the enzyme from binding ATP. Upon activation of insulin receptor, this tyrosine becomes self-phosphorylated (White et al., 1988). It has been proposed (Hubbard et al., 1994) that, upon its phosphorylation, the segment of polypeptide containing tyrosine 1162 moves away from the active site allowing access for ATP and cellular substrates. The authors suggest that this "activation mechanism will apply to many protein tyrosine kinase family members". Unfortunately, the region of EGF receptor homologous to this proposed self-inhibitory site in insulin receptor does not contain any sites of self-phosphorylation. In addition, the tyrosines known to be self-phosphorylated in EGF receptor are not homologous to any of the self-phosphorylated tyrosines in insulin receptor (White et al., 1988). Therefore, all that can be concluded at the present time is that self-phosphorylation is not sufficient to activate EGF receptor.

While self-phosphorylation may not lead to the activation of EGF receptor, studies have demonstrated that self-phosphorylation enhances the protein tyrosine kinase activity of EGF receptor once it has been switched on (Bertics & Gill, 1985; Bertics et al., 1988). Bertics and Gill have reported results which suggest that at least one of the tyrosines that is self-phosphorylated can act as a partial auto-inhibitor at the active site. According to this proposal, once these auto-inhibitory tyrosines are self-phosphorylated, they no longer interact so strongly with the active site and the phosphorylated receptor displays enhanced activity.

Other studies have demonstrated that EGF receptor truncated at its carboxy terminus, so that it is missing all of the sites of self-phosphorylation and has if anything a greater tyrosine kinase activity than the wild-type receptor in the presence of EGF (Walton et al., 1990; Li et al., 1994). This deletion mutant of EGF receptor, however, does not display

elevated enzymatic activity in the absence of EGF. This result demonstrates conclusively that the phosphorylation of an internal, inhibitory tyrosine-containing peptide is not a necessary step in the activation of EGF receptor.

When the EGF receptor is activated by the immunoglobulins raised against its carboxy terminus, as when it is activated by EGF, it is capable of self-phosphorylation (Figure 4B) at the same tyrosines (Figure 5). The data presented here show that EGF receptor activated by these immunoglobulins remained fully self-phosphorylated after the synthetic peptide SEFIGA had been added but that the enzymatic activity was rapidly lost (Figure 4). This result demonstrates that self-phosphorylation is not sufficient for the activation of the protein tyrosine kinase of EGF receptor and together with the results obtained with the carboxy-terminal deletion mutants suggest that self-phosphorylation is inconsequential to the process of activation of EGF receptor. The role of the self-phosphorylation sites in EGF receptor seems to be restricted to their association with cellular proteins downstream of EGF receptor in the cascade of events leading to cell growth and mitogenesis (Coffer & Kruijer, 1995; Egan et al., 1993; Koch et al., 1991; Margolis et al., 1990).

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