

# Signal Transduction by Epidermal Growth Factor Occurs Through the Subclass of High Affinity Receptors

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**Abstract.** Many cell types display two classes of epidermal growth factor receptor (EGFR) as judged from EGF binding studies; i.e., a major class of low affinity EGFR and a minor class of high affinity EGFR. We have studied their respective contribution to the cascade of events elicited by EGF in human A431 carcinoma cells, using anti-EGFR mAb 2E9. This antibody specifically blocks EGF binding to low affinity EGFR, without activating receptors in intact cells, and thus enables us to study the effects of exclusive EGF binding to high affinity EGFR. We show that blocking of low affinity EGFR by mAb 2E9 has almost no effect on the activation of the receptor protein-tyrosine kinase by EGF, suggesting that EGFR kinase activation occurs exclusively through the subclass of high affinity EGFR (5–10%). In addition, we provide evidence that high affinity EGFR exists both in monomeric and dimeric forms, and that cross-phos-

phorylation of low affinity EGFR by high affinity EGFR may take place in dimers of both receptor types.

We demonstrate that the following early cellular responses to EGF are also unimpaired in the presence of mAb 2E9: (a) inositol phosphate production, (b) release of  $\text{Ca}^{2+}$  from intracellular stores, (c) rise in intracellular pH, (d) phosphorylation of EGF on threonine residue 654, (e) induction of c-fos gene expression, and (f) alteration in cell morphology. As possible nonspecific side effects, we observed that the EGF induced  $\text{Ca}^{2+}$  influx and fluid-phase pinocytosis were inhibited in A431 cells in the presence of mAb 2E9. We conclude, therefore, that the activation of the EGFR signal transduction cascade can occur completely through exclusive binding of EGF to the subclass of high affinity EGFR.

**E**PIDERMAL growth factor (EGF) is a common mitogen for a variety of animal cells. EGF exerts its action by binding to a specific plasma membrane-located receptor (EGFR),<sup>1</sup> a transmembrane glycoprotein of 170 kD with an extracellular EGF binding domain and an intracellular protein-tyrosine kinase domain (9, 36). EGF/EGFR interaction causes the activation of this kinase, resulting in (auto)phosphorylation of the receptor molecule, as well as phosphorylation of a number of cellular proteins on tyrosine residues (36). Importantly, this primary action of EGF appears to be a prerequisite for evoking further receptor-mediated responses, since these are absent in cells expressing a kinase-deficient, but otherwise intact, EGFR (11, 33, 35, 47).

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1. *Abbreviations used in this paper:* BCECF, bis(carboxyethyl)carboxyfluorescein;  $[\text{Ca}^{2+}]_i$ , intracellular free calcium concentration; DSS, disuccinimidylsuberate; EDC, 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide; EGFR, epidermal growth factor receptor; indo-1, indo-1 acetoxymethyl ester; InsPn, inositol phosphates; PKC, protein kinase C; TPA, 12-O-tetradecanoyl phorbol-13-acetate.

The elucidation of the mechanisms by which extracellular EGF binding activates the kinase on the intracellular part of the receptor is an intriguing and as yet unresolved question. In highly purified receptor preparations, EGF/EGFR interaction still results in EGFR-kinase activation. This implies that in vitro no molecules other than EGF and EGFR seem to be involved. Based upon this observation, two mechanisms for receptor activation have been proposed. In the intramolecular model (27), EGF binding leads to a conformational change in the receptor that is transmitted via the transmembrane stretch to the intracellular domain where it activates the kinase. In the intermolecular model (64), EGF binding shifts a hypothetical equilibrium of monomeric and dimeric receptors to the dimeric form. Dimerization of receptors in this model causes activation of the kinase.

The action of EGF has been studied most extensively in the human carcinoma cell line A431, as this cell type carries an unusually high number of EGFRs; i.e.,  $2 \times 10^6$  receptors per cell (23). In A431 cells, like in many other cell lines, two classes of EGFR can be detected as judged from EGF binding studies: a major class of low affinity EGFR and a mi-

nor class of high affinity EGFR, their respective binding affinity being different by more than an order of magnitude. In A431 cells, the high affinity EGFR constitute 5–10% of the total EGF binding capacity (19, 29).

The functional implications of the existence of high affinity and low affinity EGFRs are largely obscure, but a correlation between the relative number of high affinity EGFR and the activation of EGFR kinase has been established. Tumor promoters induce a concomitant decrease in high affinity EGF binding and EGF activation of the receptor kinase (26, 55), while the reverse is observed for cAMP treatment of PC12 cells (5). A comparison of A431 variants gave evidence for a similar correlation (19). In accordance with these observations, the high affinity EGFRs have been implicated as the subclass through which EGF predominantly exerts its mitogenic effect (39, 40, 54). So far, a lack of tools for the discrimination between high and low affinity EGFRs has hampered the analysis of their respective roles in the EGF-induced receptor activation. Monoclonal antibodies (mAb) against growth factor receptors could provide such tools (20, 21, 29, 53), provided that both receptor classes are immunologically distinct, or that the mAb interferes distinctly with the two classes. We have developed an mAb, 2E9-IgG1, that fulfills the latter requirement in that it blocks binding of EGF to low affinity EGFR without interfering with EGF binding to high affinity EGFR.

In this paper we characterize the binding properties of mAb 2E9, and study the respective contribution of high and low affinity EGFR to the cascade of events elicited by EGF in A431 cells, making use of the features of mAb 2E9. Our results indicate that (a) high affinity EGFRs are the subclass through which tyrosine kinase activation occurs predominantly, and (b) cross-phosphorylation of low affinity EGFRs by high affinity EGFRs may take place in dimers of both receptor types, (c) all further major early responses to EGF are unimpaired in the presence of mAb 2E9. We conclude that the activation of the EGFR signal transduction cascade can occur completely through exclusive binding of EGF to the subclass of high affinity EGFR.

## Materials and Methods

### Materials

EGF (receptor grade) was from Collaborative Research, Inc. (Waltham, USA).  $\gamma$ -[ $^{32}$ P]ATP, [ $^{32}$ P]orthophosphate, and Na- $^{125}$ I (carrier free) were from Amersham International (Houten, The Netherlands). For binding studies, EGF was labeled with Na- $^{125}$ I by the chloramine T method. Labeled EGF was separated from free label by ion-exchange chromatography on Dowex-1 (1  $\times$  8 200; Sigma Chemical Co., St. Louis, MO). The specific activity of the  $^{125}$ I-EGF thus obtained varied between 2 and 8  $\times$  10<sup>5</sup> cpm/ng EGF. 99.5% of the labeled material was precipitable by TCA. Antibody 528 was a kind gift of Dr. J. Mendelsohn (Sloan-Kettering Institute for Cancer Research, New York). Bis(carboxyethyl)carboxyfluorescein (BCECF) was obtained from HSC Research Development Corp. (Toronto, Canada). Indo-1 acetoxy-methyl ester (indo-1) was from Molecular Probes (Eugene, OR). [ $^3$ H]inositol was also from Amersham International. Fluorescein-complexon was from Eastman Kodak Co. (Tramedico, Weesp, The Netherlands).

### Cell Culture

3T3 cells transfected with human EGFR sequences were a gift of Dr. J. Schlessinger (Weizmann Institute, Rehovot, Israel). Human keratinocytes were obtained from Dr. M. Ponc (University Hospital, Leiden, The Netherlands). All cells were grown in DME (Gibco Europe, Breda, The Netherlands),

supplemented with 7.5% fetal calf serum (Hyclone Laboratories, Logan, UT) and buffered with 44 mM NaHCO<sub>3</sub> under a 7.5% CO<sub>2</sub> atmosphere. Throughout the paper, the term A431-2E9 cells refers to A431 cells that have been incubated with 300 nM mAb 2E9 for 30 min at 37°C before the addition of EGF. For each set of data, the effect of antibody treatment alone was compared with control cells. If not specifically stated otherwise, the antibody had no measurable effect on the parameter of interest.

### EGFR Antibodies

Antibody 2E9 was chosen from a panel of monoclonal antibodies. These were obtained after fusion of myeloma SP2/0 cells with spleen cells from Balb/c mice that had been immunized with plasma membranes from A431 cells. The antibodies were selected on the basis of various criteria, the most stringent being immunoprecipitation of a functional tyrosine kinase with a  $M_r$  of 170 kD capable of autophosphorylation (19). Polyclonal antiserum 281-7 was raised in New Zealand White rabbits against a synthetic peptide corresponding to residues 984–995 (43) of the human EGFR. All antibodies used in this study were purified by affinity chromatography on a protein A-Sepharose column (Pharmacia Fine Chemicals, Woerden, The Netherlands). For binding experiments, antibody was labeled as described for EGF. The specific activity of  $^{125}$ I-2E9 preparations varied between 1 and 5  $\times$  10<sup>4</sup> cpm/ng, while 99.1% of the material was TCA precipitable.

### Quantitative Binding and Binding Inhibition Studies

Cells were grown on 24-well tissue-culture clusters (Costar, Cambridge, MA) to a final density of 10<sup>5</sup> cells/cm<sup>2</sup> for A431 cells and to confluency for other cell types tested. After washing the cells with binding buffer (Hepes-buffered DME containing 0.1% BSA), mixtures of radiolabeled and unlabeled ligand were added to the same medium to give a final volume of 0.5 ml. Incubation was for 2 h at room temperature for antibody binding and at 0°C for EGF. After this, cells were washed five times with PBS, dissolved in 1 M NaOH, and cell-associated radioactivity was measured in a  $\gamma$ -counter. Nonspecific binding was determined using a 500–1,000-fold molar excess of unlabeled ligand. Data analysis, using the program LIGAND, was performed as described (6).

For binding inhibition assays, cells were preincubated with the first ligand for 60 min at 0°C in the case of EGF or for 2 h at room temperature in the case of antibody, after which period radiolabeled second ligand was added to the same medium to give a final volume of 0.5 ml.

### $^{125}$ -EGF/EGFR Cross-linking

A431 cells, grown on 19-cm<sup>2</sup> tissue culture dishes (Costar) were pretreated for 30 min at 37°C with binding buffer in the presence or absence of 300 nM mAb 2E9. The cells were subsequently chilled on ice,  $^{125}$ I-EGF (sp act, 180,000 cpm/ng) was added to a final concentration of 20 ng/ml with or without 1  $\mu$ g/ml of unlabeled EGF. After incubation for 60 min at 4°C, cells were washed three times with ice-cold PBS and incubated for a further 15 min at 4°C in a 1 mM solution of disuccinimidylsuberate (DSS; Pierce Chemical Co., Rockford, IL) in a 1:1 mixture of DMSO and PBS. The reaction was stopped by adding glycine (final concentration 100 mM, at pH 7.4) and incubated for 5 min at 4°C. After this, the cells were washed with PBS, scraped off the substratum with a rubber policeman, and pelleted for 2 min at 2,000 g. The cells were lysed in RIPA buffer (36) and insoluble material was removed by centrifugation at 15,000 g for 10 min at 4°C. The soluble fraction was boiled in Laemmli sample buffer and applied to a linear 7–9% gradient polyacrylamide gel. An autoradiograph of the gel was made using Kodak X-AR5 film.

### Immunoprecipitation of EGFR from Cell Lysates

After incubation with ligand, cells were washed and lysed by scraping in 500  $\mu$ l RIPA buffer. The lysates were clarified by centrifugation for 15 min at 15,000 g. 20  $\mu$ g 281-7-IgG bound to protein A-Sepharose (Pharmacia Fine Chemicals) was added to the lysates. The precipitate was washed once with buffer containing 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.5% Triton X-100, and twice with the same buffer containing 150 mM NaCl. For SDS-gel electrophoresis the precipitates were boiled in Laemmli sample buffer and applied directly to the gel.

### EGFR Protein-Tyrosine Kinase Activity Measurements in Intact Cells

Cells grown to subconfluency (100,000 cells/cm<sup>2</sup>) on 35-mm dishes (Cos-

tar) were incubated with 1 mCi/ml [<sup>32</sup>P]orthophosphate in phosphate-free DME for 16 h. Where indicated, antibody, EGF (50 ng/ml), or buffer were added to the same medium. After incubation, EGFR was immunoprecipitated as described above, and the precipitates were applied to a linear 5–15% SDS–polyacrylamide gel. The receptor band was localized in the dried gel by autoradiography on Kodak X-AR 5 films, cut out with a razor blade, and homogenized in 50 mM NH<sub>4</sub>HCO<sub>3</sub> containing 0.2% SDS and 2% β-mercaptoethanol. After incubation for 5 min at 100°C and 120 min at 20°C, the gel homogenate was spun at 10,000 g for 2 min and the supernatant was collected. After addition of 20 μg RNase (Sigma Chemical Co., St. Louis, MO) and incubation for 15 min on ice, TCA was added to a final concentration of 12.5%. The TCA precipitates were collected by centrifugation at 12,000 g for 5 min and washed once with pure ethanol. The pellets were dried under a stream of N<sub>2</sub>, resuspended in a small volume of formic acid, and an aliquot of each sample was hydrolyzed in 6 M HCl at 110°C for 60 min. The hydrolyzed samples (~1,000 cpm as determined by Cerenkov radiation) were applied to plastic foil thin-layer cellulose plates 5577; E. Merck, Darmstadt, FRG) together with 60 ng each of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine. Two-dimensional electrophoresis at pH 1.9 and 3.5 were as described by Cooper et al. (17). Phosphoamino acids were detected by spraying with 0.25% ninhydrin in acetone. After autoradiography, the phosphoamino spots were cut from the plate using a pair of scissors and radioactivity was measured using Dynagel (Baker Chemical Co., Deventer, The Netherlands) in a liquid scintillation counter.

### Surface Iodination and EGFR Cross-linking in Intact Cells

A431 cells, grown to subconfluency in 19-cm<sup>2</sup> dishes (Costar) were washed with PBS and surface iodinated by the lactoperoxidase method exactly as described (16), using 300 μCi of carrier-free Na-<sup>125</sup>I. The reaction was terminated by washing the cells extensively with DME containing 0.1% BSA, and a further incubation in this medium for 30 min at room temperature. Some of the dishes were subsequently incubated with 300 nM mAb 2E9 for 30 min at 37°C. The cells were cooled to 0°C, after which EGF was added to the same medium and the cells were incubated for an additional 30 min. After washing the cells, a 10 mM solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in PBS containing 1 mM glucose was added to the cells, and cross-linking was allowed to proceed for 30 min at 30°C. Cells were washed three times with PBS and lysed in 0.5% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 20 mM Tris-HCl, pH 7.4.

EGFR was immunoprecipitated from the lysates using antiserum 281-7, and the immunoprecipitates were subjected to electrophoresis on a 4–10% linear gradient SDS–polyacrylamide gel.

### Polyphosphoinositide Breakdown

Polyphosphoinositide breakdown was determined in A431 and A431-2E9 cells as inositol phosphates (InsP<sub>n</sub>) accumulation in the presence of LiCl as described by Tilly et al. (59).

### [Ca<sup>2+</sup>]<sub>i</sub> and pH<sub>i</sub> Measurements

Determination of intracellular free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) using indo-1, and pH<sub>i</sub> using BCECF were determined as described by Moolenaar et al. (47).

### Tryptic Phosphopeptide Mapping of the EGFR

Cells grown to subconfluency (150,000 cells/cm<sup>2</sup>) on 35-mm dishes (Costar) were labeled with 1 mCi/ml [<sup>32</sup>P]orthophosphate (Amersham International) in phosphate-free DME for 16 h. The cells were preincubated with mAb 2E9 in the same medium. EGF (50 ng/ml) or buffer were added to this medium for an additional 30 min. After this, cells were lysed in RIPA buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM EDTA, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% NP-40, 1% sodium desoxycholate, 0.1% SDS, 1 mM PMSF, 100 KIU/ml Aprotinin). The EGFR were precipitated using antiserum 281-7 and protein A-Sepharose beads (Pharmacia Fine Chemicals). The precipitate was applied to a linear 5–15% SDS–polyacrylamide gel. The receptor band was localized in the dried gel by autoradiography, cut out with a razor blade, and homogenized in 50 mM NH<sub>4</sub>HCO<sub>3</sub>, 0.2% SDS, 2% β-mercaptoethanol. After incubation for 5 min at 100°C and 120 min at 20°C, the homogenate was spun at 10,000 g for 2 min and the supernatant was collected. After addition of 20 μg RNase (Sigma Chemical Co.) and incubation for 15 min on ice, TCA was added to a final concentration of 12.5%. The TCA precipitates were collected by centrifugation at 12,000 g

for 5 min and washed once with pure ethanol. The pellets were dried under a stream of N<sub>2</sub>. By this procedure, ~70% of the counts present in the gel were recovered as measured by Cerenkov radiation. Samples were subsequently treated with performic acid and trypsin as described by Beemon and Hunter (2). Tryptic phosphopeptides were separated on thin-layer cellulose plates (E. Merck; 5716) by electrophoresis (pH 8.9 at 1,000 V) for 20 min in the first dimension, followed by ascending chromatography in *N*-butanol/pyridine/acetic acid/H<sub>2</sub>O (75:50:15:60).

### *c-fos* Expression

A431 cells, grown to 80% confluency, were washed and the medium was replaced by serum-free DME. 2 h later, agonists were added. 30 min later, the medium was aspirated and the cells were lysed in 5.7 M guanidinium isothiocyanate. RNA was isolated according to Chirgwin et al. (14), and quantitated by absorption at 260 nm. 20-μg aliquots were electrophoresed on a 0.7% agarose gel containing 2.2 M formaldehyde, after which the RNA was transferred to nitrocellulose. Hybridization was in 50% formamide with a nick-translated probe (sp act 5 × 10<sup>8</sup> cpm, 10<sup>6</sup> cpm/ml), for 16–20 h. Final washes were with 0.1 SSC and 0.1% SDS at 55°C. Autoradiography was on Kodak X-Omat XAR5 film.

### Fluid-phase Pinocytosis

A431 and A431-2E9 cells, at subconfluency on six-well tissue-culture clusters (Costar) were incubated with fluorescein–complexon (1 mg/ml) in Hepes-buffered DME containing 0.1% BSA. EGF (50 ng/ml) was added and at various time intervals the cells were washed five times in ice-cold PBS, followed by lysis in 2 ml of 0.05% Triton X-100 containing PBS. The lysis buffer was carefully aspirated and the amount of fluorescein–complexon measured by emission at a wavelength of 560 nm and an excitation wavelength of 500 nm in a Perkin-Elmer Corp. (Norwalk, CT) 3000 fluorimeter.

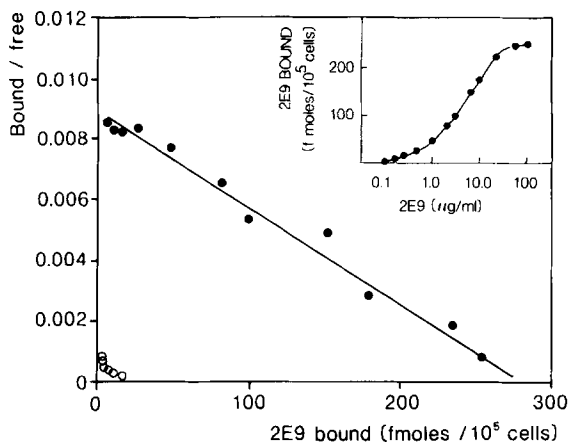
## Results

### 2E9–IgG1 Binds With a 1:1 Stoichiometry to the Protein Core of the EGFR

Several aspects of the production and biological properties of mAb 2E9–IgG1 have been described elsewhere (19–21). It is one of a panel of anti-EGFR mAbs obtained after fusion of SP2/0 myeloma cells with spleen cells of Balb/c mice immunized with plasma membrane vesicles of A431 cells. Immunoprecipitation experiments using A431 cells, metabolically labeled with [<sup>35</sup>S]methionine in the presence or absence of the glycosylation inhibitor tunicamycin, showed that mAb 2E9 recognizes the protein core of the receptor, since it precipitated the 130-kD nonglycosylated form (56) of this molecule (not shown).

To quantitate the binding properties of mAb 2E9, it was purified by affinity chromatography, and labeled with <sup>125</sup>I. At 20°C, <sup>125</sup>I-2E9 binding to A431 cells was analyzed according to Scatchard (52) as described in Materials and Methods. Binding equilibrium, as determined by measuring incubation time-dependent association of radiolabeled antibody, was reached after 60 min at room temperature (not shown). The Scatchard plot yielded a straight line (Fig. 1), indicating that the antibody binds with an apparent dissociation constant (*K<sub>d</sub>*) of 32 nM to a number of antigenic determinants that roughly equals the number of EGFR (2 × 10<sup>6</sup>/cell) (23). This indicates a 1:1 stoichiometry for 2E9–EGFR binding.

An important property of mAb 2E9 was revealed by measuring the effect of preincubating A431 cells with EGF or mAb 2E9 on the subsequent binding of either ligand. Preincubation with mAb 2E9 blocks EGF binding (Fig. 2 A) and, vice versa, preincubation with EGF blocks mAb 2E9 binding (Fig. 1) to intact A431 cells. Experiments in which the



**Figure 1.** Scatchard graph of mAb 2E9 binding to intact A431 cells. Cells were incubated at 20°C for 120 min with a constant amount of <sup>125</sup>I-labeled antibody together with increasing amounts of unlabeled antibody. Cell associated radioactivity was measured and Scatchard analysis was performed as described in Materials and Methods. Nonspecific binding was determined using a 1,000-fold excess of unlabeled antibody and did not exceed 5%. Inset shows saturation curve of the data. The open circles show Scatchard analysis of mAb 2E9 binding after preincubation with 50 ng/ml of EGF.

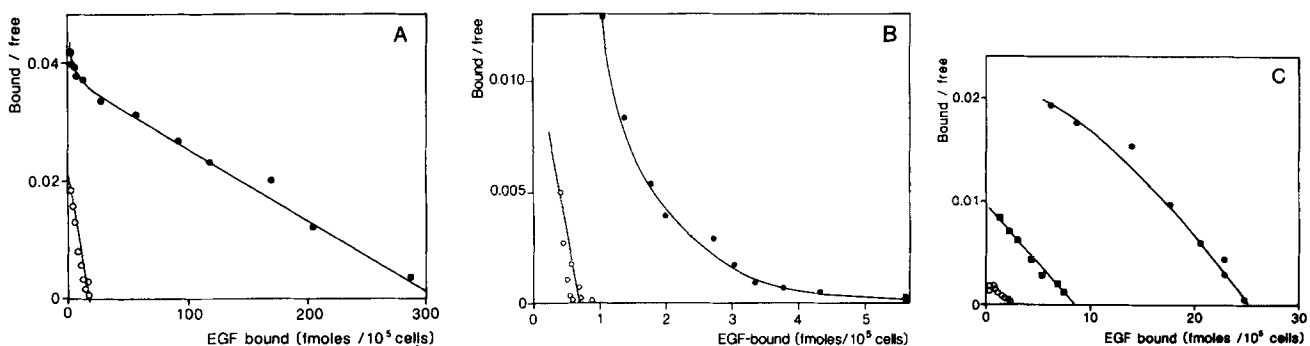
order of addition of the ligands was reversed, showed that neither EGF nor mAb 2E9 caused a significant change in the dissociation rate of either ligand (not shown). The former observation indicates that mAb 2E9 is directed against an antigenic determinant close to or at the binding domain for EGF. When similar experiments were carried out on *p*-formaldehyde-fixed A431 cells—a treatment that does not interfere with the specific binding of either ligand (7, 62)—the results were strikingly different. As before, preincubation with mAb 2E9 did inhibit EGF binding under these conditions, but preincubation with EGF now did not inhibit mAb 2E9 binding. This indicates that EGF and mAb 2E9 recognize different epitopes on the EGFR, and that EGF does not inhibit mAb 2E9 binding due to direct binding competition.

#### **mAb 2E9 Blocks EGF Binding to Low-affinity Receptors Only**

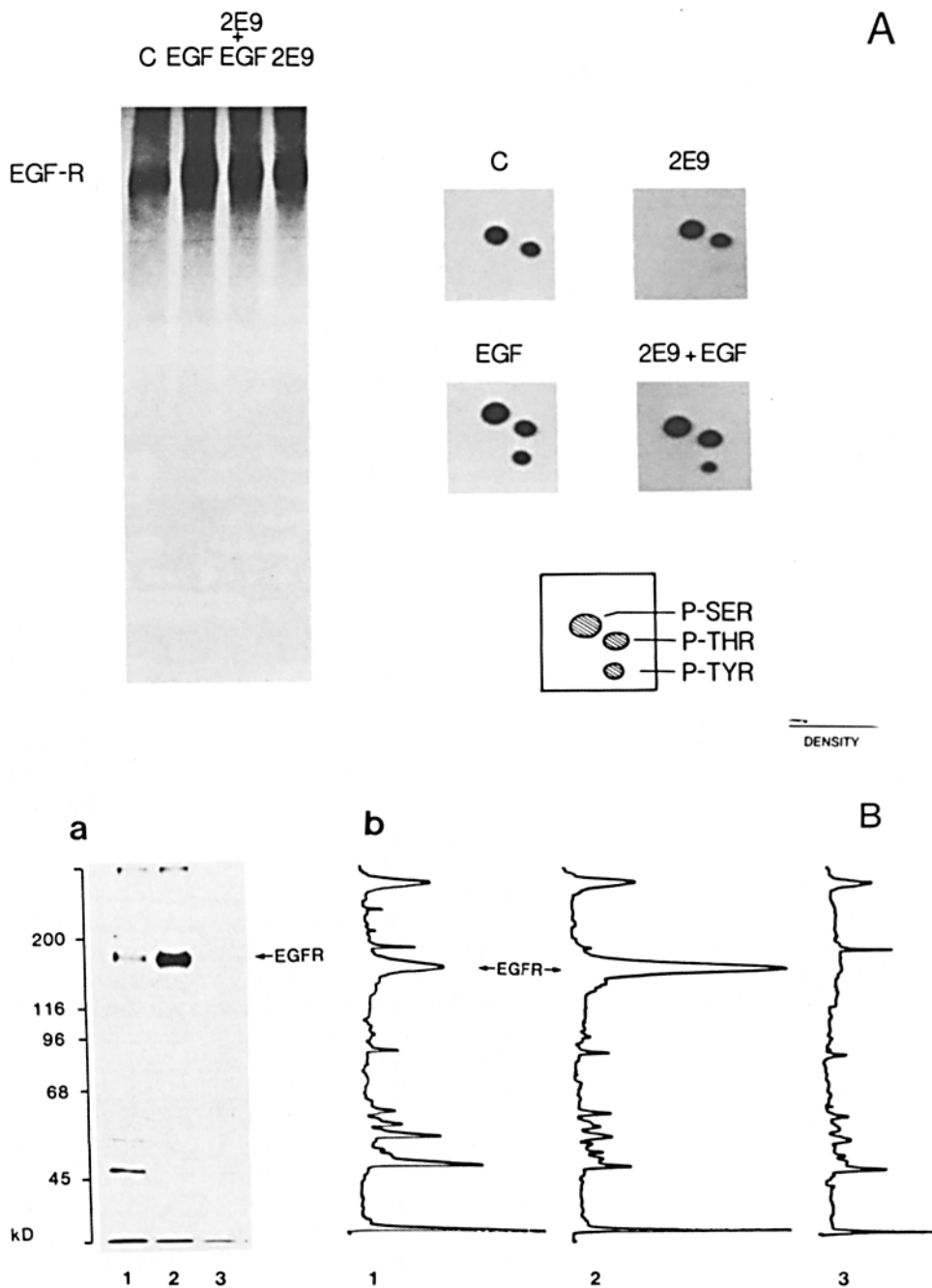
During the competition studies, the following intriguing phenomenon was observed: the extent of inhibition of EGF

binding by mAb 2E9 depended upon the concentration of the growth factor, such that at low EGF concentrations (<0.1 nM) saturating doses of mAb 2E9 inhibited binding by only 60–70%, while at higher EGF concentrations (>1.0 nM) inhibition was as high as 90–95%. An explanation for this phenomenon was found in Scatchard analysis of EGF binding data to A431 cells with or without preincubation with a saturating amount of mAb 2E9 (300 nM for 120 min at 20°C). As shown in Fig. 2 *A* (solid circles), this analysis yielded a curvilinear graph, indicating the presence of two receptor classes in A431 control cells with different affinities for EGF. Obviously, it is difficult to determine accurately the number of high affinity binding sites from this graph, due to the very small initial curvature (see also Discussion). Using the program LIGAND (6), the estimated values for the number of receptors of both affinity classes and their affinity for EGF were 75,000 receptors, with an apparent  $K_d$  of 0.7 nM, and 1,900,000 receptors, with a  $K_d$  of 8.5 nM. Preincubation of A431 cells with mAb 2E9 followed by Scatchard analysis of <sup>125</sup>I-EGF binding yielded a linear Scatchard graph (Fig. 2 *A*, open circles) with a slope identical to the initial part of the graph in control cells, indicating a specific block of EGF binding to low affinity receptor sites only. The number of receptors remaining was 90,000, with an apparent  $K_d$  of 0.9 nM.

The specific block of EGF binding to the low affinity subclass by mAb 2E9 was not restricted to A431 cells. The antibody had the same effect on HeLa cells (Fig. 2 *B*), on human keratinocytes, and on 3T3 cells transfected with the wild-type human EGFR (line DHER 14, obtained from Dr. J. Schlessinger, Weizmann Institute) (data not shown), even after preincubation with supersaturating mAb 2E9 concentrations (1,200 nM; data not shown). Furthermore, a prolonged incubation with mAb 2E9 (24 h) did not alter the amount of high affinity sites as compared to a 1-h preincubation (data not shown), indicating that mAb 2E9 does not abolish a possible long-term recruitment of high affinity from low affinity receptors. When the same experiments were done with another anti-EGFR mAb, 528-IgG1, which blocks EGF binding to *p*-formaldehyde-fixed A431 cells by >95% (51), EGF binding to both receptor classes was blocked almost completely (not shown). This antibody, which is also directed against the protein core of the receptor (28), does not compete with mAb 2E9 for binding (not shown), indicating that the antibodies are directed against different epitopes.



**Figure 2.** Scatchard analysis of EGF binding to A431 cells (*A*), HeLa cells (*B*), and 3T3-IV cells (*C*, see text). Cells were either untreated (solid circles) or pretreated with 300 nM mAb 2E9 (open circles) for 120 min at room temperature. Subsequently, increasing concentrations of EGF were allowed to bind for 120 min at 0°C. Scatchard analysis of the amount of EGF bound to the cells was performed as described in Materials and Methods. The solid squares in *C* represent cells pretreated with antibody 2E9 at a concentration of 30 nM.



**Figure 3.** (A) EGFR (auto)phosphorylation in intact,  $^{32}\text{P}$ -labeled A431 cells. A431 cells, labeled overnight with [ $^{32}\text{P}$ ]-orthophosphate were treated (where indicated) with mAb 2E9 for 30 min at  $37^\circ\text{C}$ , followed by (where indicated) EGF for 5 min at  $37^\circ\text{C}$ . Subsequently, the cells were lysed in RIPA buffer and the EGFR was immunoprecipitated using antiserum 281-7. (Left) Autoradiograph of the immunoprecipitates analyzed by SDS gel electrophoresis. (Right) Phosphoamino acid analysis of the EGFR eluted from the gel and analyzed as described in Materials and Methods. Concentrations used were: EGF, 50 ng/ml; mAb 2E9, 50  $\mu\text{g}/\text{ml}$ . C, Control. (B) Analysis of the relative amount of receptors exposed to the cell surface. A431 cells, grown to subconfluency were incubated overnight with 20  $\mu\text{Ci}/\text{ml}$  of [ $^{35}\text{S}$ ]methionine in methionine-free DME supplemented with 1% FCS. Half of the cells were subsequently incubated with 100 nM of antibody 528 for 60 min at  $0^\circ\text{C}$ . The cells were washed, lysed in RIPA, and the 528-EGFR complex was immunoprecipitated with rabbit anti-mouse IgG coupled to protein A-Sepharose. In a parallel experiment, the cells were lysed before mAb 528 addition and immunoprecipitation. (a) An autoradiograph of the immunoprecipitates analyzed by SDS gel electrophoresis. (Lane 1) Surface-exposed receptors; (lane 2) receptors in total cell lysate. (b) Densitometric scan of the autoradiograph depicted in a.

That the blocking of EGF binding to low affinity receptors approached 100% was demonstrated in HeLa cells pretreated with the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), which in these cells abolishes high affinity EGF binding completely (55), as well as in 3T3 cells transfected with EGFR lacking almost the entire intracellular domain (3T3-IV cells) which display only one low affinity subclass (44). In both cases, preincubation with increasing amounts of mAb 2E9 led to a parallel shift of the Scatchard graph until, at saturating concentrations of antibody, EGF binding was no longer detectable (shown for 3T3-IV cells in Fig. 2 C).

#### Activation of EGFR Protein-Tyrosine Kinase Occurs through High Affinity EGFR

Several authors (39, 40, 54) have hypothesized that the high affinity receptor subclass is the major EGF signal transducing system. We could test this hypothesis by measuring receptor activation by EGF in intact cells pretreated with mAb 2E9. A431 cells were labeled to equilibrium with  $^{32}\text{P}$  and preincubated for 1 h with 300 nM mAb 2E9. Subsequently, EGF (50 ng/ml) was added to the same medium and the cells were incubated for various time intervals. After this the receptor was isolated and its phosphoamino acid content

Table I. Quantitation of Phosphorylation and Phosphoamino Acid Analysis of EGFR Isolated from <sup>32</sup>P-labeled A431 Cells

Incubation	Total <sup>32</sup> P incorporated in EGFR	Relative amount of cpm in phosphoamino acids		
		Phosphoserine	Phosphothreonine	Phosphotyrosine
		%	%	%
Control	12,827	61.3	36.3	2.3
EGF	27,728	58.3	25.5	16.1
mAb 2E9	17,745	64.4	33.8	1.8
2E9/EGF	22,748	58.8	31.8	9.4

EGFR protein immunoprecipitated from <sup>32</sup>P-labeled A431 cells was treated as described in Materials and Methods and the legend to Fig. 3. Total amount of counts per minute incorporated into receptor protein was measured in ethanol-washed TCA precipitates of EGFR material eluted from the SDS gel shown in Fig. 3. The relative amount of radioactivity in the three phosphoamino acids (phosphoserine, phosphothreonine, and phosphotyrosine) was determined by cutting out the phosphoamino acid spots from the thin-layer plate shown in Fig. 3 after scraping the surrounding areas. The isolated phosphoamino acid samples were counted with liquid scintillation fluid in a liquid scintillation counter. For background subtraction, several regions of the thin-layer plate were cut out and counted similarly. The data presented in the table are from a single experiment, which was repeated several times with essentially the same results.

was determined by two-dimensional analysis as described in Materials and Methods. It is clear from the autoradiograph of the SDS gel (Fig. 3 A, left panel) that a 1-h incubation with mAb 2E9 leads to enhanced incorporation of <sup>32</sup>P into the receptor. However, this incorporation is not on tyrosine residues (Fig. 3 A, right panel).

Pretreatment with mAb 2E9 leads to a reduction in the activation of EGFR by EGF as measured by receptor tyrosine phosphorylation. However, this reduction is relatively small as compared to the reduction in the number of receptors accessible to EGF after mAb 2E9 pretreatment. Quantitative data, obtained by counting the individual phosphoamino acid spots in a  $\beta$ -counter are presented in Table I. While, after mAb 2E9 pretreatment, binding of EGF to its receptor is blocked by  $\sim 95\%$ , receptor tyrosine (auto)phosphorylation induced by the growth factor is only reduced by  $\sim 30\%$ . Time-course analysis showed no effect of mAb 2E9 on the rapidity of the EGF-induced EGFR tyrosine (auto)phosphorylation (not shown). In antibody-pretreated as well as in control cells, this was maximally induced 1 min after the addition of EGF. From these data, we conclude that EGFR activation, as measured by receptor autophosphorylation, occurs predominantly if not exclusively through high affinity binding sites.

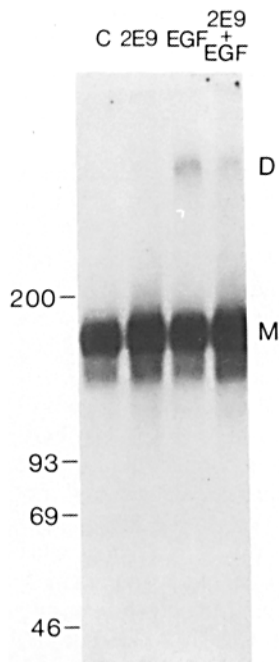
To establish the amount of EGFR exposed at the cell surface as a fraction of the total receptor population, we incubated [<sup>35</sup>S]methionine-labeled A431 cells at 0°C (to prevent internalization) with 100 nM of antibody 528 (39) in order to occupy all membrane-exposed receptors. Subsequently, the cells were washed, lysed in RIPA buffer, and the 528-EGFR complexes immunoprecipitated from the lysates. In a parallel experiment, cells were lysed, followed by incubation with antibody 528, and immunoprecipitation. The precipitates were analyzed by SDS gel electrophoresis and autoradiography. An autoradiograph of this experiment is shown in Fig. 3 B, together with a densitometric scan. From this, we calculate that 30% of the total receptor population is localized at the cell surface, in accordance with earlier estimates (10, 42). Thus, high affinity EGFRs, which represent  $\sim 5$ – $10\%$  of membrane-exposed receptors, constitute only  $\sim 2$ – $3\%$  of the total receptor population. However, estimates about the fraction of EGFRs that becomes phosphorylated on tyrosine residues after EGF treatment vary from 20 (reference 15) to 35% or more (58) of the total receptor population, and thus receptor (auto)phosphorylation does not occur exclusively on high affinity receptors. Activation of EGFR

protein-tyrosine kinase however, occurs predominantly through the high affinity subclass (see above). Since our data indicate that low affinity receptors have to be (auto)phosphorylated in response to EGF, this indicates that phosphorylation of low affinity receptors occurs through activation of high affinity receptors, possibly via cross-phosphorylation.

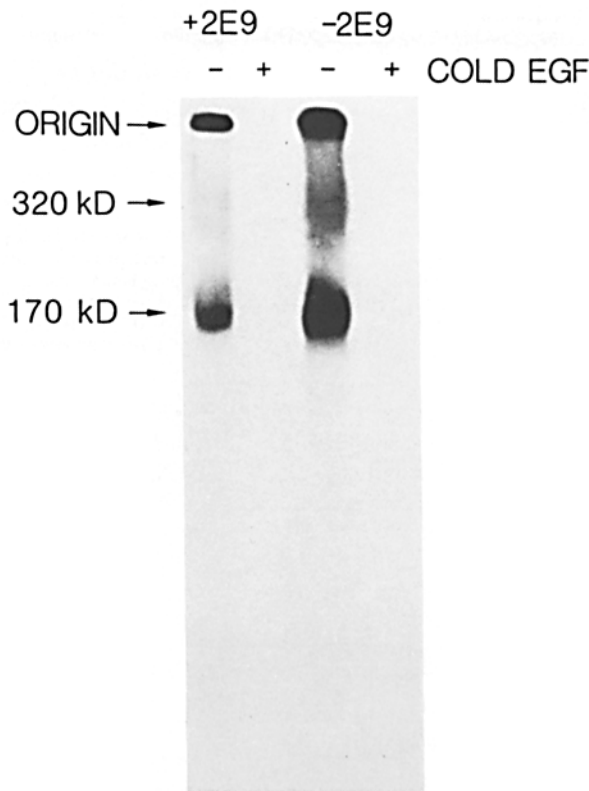
### EGFR Dimerization Is Efficiently Driven Through High Affinity EGF Binding

Cross-phosphorylation of low affinity by high affinity receptors implies a physicochemical interaction between the two molecules. The EGF driven formation of receptor dimers as described by Cochet et al. (16) could well be a reflection of this process. To test whether dimerization between high and low affinity receptors indeed occurs, A431 cells were surface labeled with <sup>125</sup>I using lactoperoxidase as described by Cochet et al. (16). The labeled cells were treated with mAb 2E9, followed by EGF at 0°C, and possible receptor-receptor complexes were cross-linked with EDC as described (16). Cells were lysed in RIPA buffer and EGFR was immunoprecipitated as described in Materials and Methods. The immunoprecipitates were analyzed by SDS gel electrophoresis and autoradiography.

As shown in Fig. 4 A, the antibody itself does not cause receptor dimerization. Furthermore, receptor dimers are still formed in response to EGF in mAb 2E9-pretreated cells albeit at a reduced rate, implying that receptor dimerization is driven by EGF even when it binds to high affinity receptors only. This result however, could also imply that dimers are formed exclusively in the high affinity subpopulation. To test this possibility, we measured the amount of receptor oligomers cross-linked to <sup>125</sup>I-EGF before and after mAb 2E9 treatment. A431 cells were preincubated with 300 nM mAb 2E9, followed by an incubation with <sup>125</sup>I-EGF at a concentration of 20 ng/ml to prevent EGF-induced receptor internalization. After extensive washing, receptor/<sup>125</sup>I-EGF complexes were cross-linked with the bivalent reagent DSS as described by Fanger et al. (24). The cells were taken up in SDS sample buffer and applied to a 7–9% linear gradient polyacrylamide gel. Fig. 4 B shows an autoradiograph of such a gel. In cells not pretreated with antibody, two labeled complexes were found, which had an *M<sub>r</sub>* of 170 (monomeric form) and 325 kD (dimeric form), respectively, in accordance with the data of Fanger et al. (24) and Cochet et al. (16). In parallel dishes, where a high amount of unlabeled EGF was



A



B

**Figure 4.** (A) EGF-induced EGFR dimerization in intact, surface-iodinated A431 cells. A431 cells were surface iodinated exactly as described by Cochet et al. (16). Subsequently, the cells were treated (where indicated) with mAb 2E9 for 30 min at 37°C. After this, EGF was added (where indicated) and cells were incubated for a further 30 min at 0°C. Cells were washed and treated with EDC (see text) for 30 min at 30°C. Subsequently, the cells were lysed in RIPA buffer, the receptor complexes were immunoprecipitated using antiserum 281-7 and subjected to SDS gel electrophoresis on a 7% polyacrylamide gel with a 5% stacking gel. The gel was dried and autoradiographed on Kodak X-AR 5 film. *M*, EGFR monomer ( $M_r$  170 kD), *D*, EGFR dimer ( $M_r$  325 kD). (B) Cross-linking of  $^{125}\text{I}$ -labeled EGF to untreated (-2E9) or mAb 2E9-pretreated (+2E9) A431 cells. After pretreatment for 30 min at 37°C with 300 nM mAb 2E9 (where indicated), cells were incubated with  $^{125}\text{I}$ -EGF (20 ng/

ml; sp act,  $3 \times 10^5$  cpm/ng) for 60 min at 4°C. To some dishes a 50-fold excess of unlabeled EGF was added (+ lanes). After a 60-min incubation at 4°C, EGF/EGFR complexes were cross-linked with DSS as described in Materials and Methods. The cross-linked complexes were separated on a 7-9% linear polyacrylamide gel. Autoradiography was with Kodak XAR-5 film. Exposure time was 50 h at -70°C.

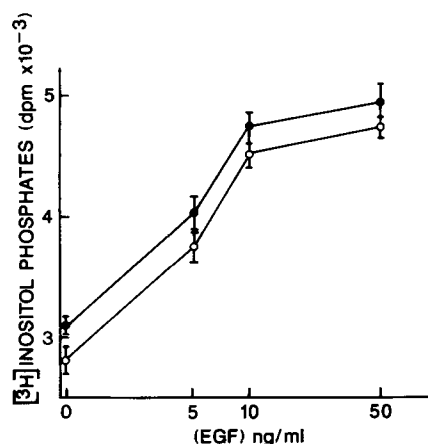
added, essentially no radioactivity was found in the gel, indicating a high specificity of labeling. After preincubation with mAb 2E9 both monomeric and dimeric receptor forms are still observed. These results demonstrate clearly that the high affinity receptor fraction does not represent exclusively dimers, but contains also monomers.

#### Stimulation of Polyphosphoinositide Breakdown

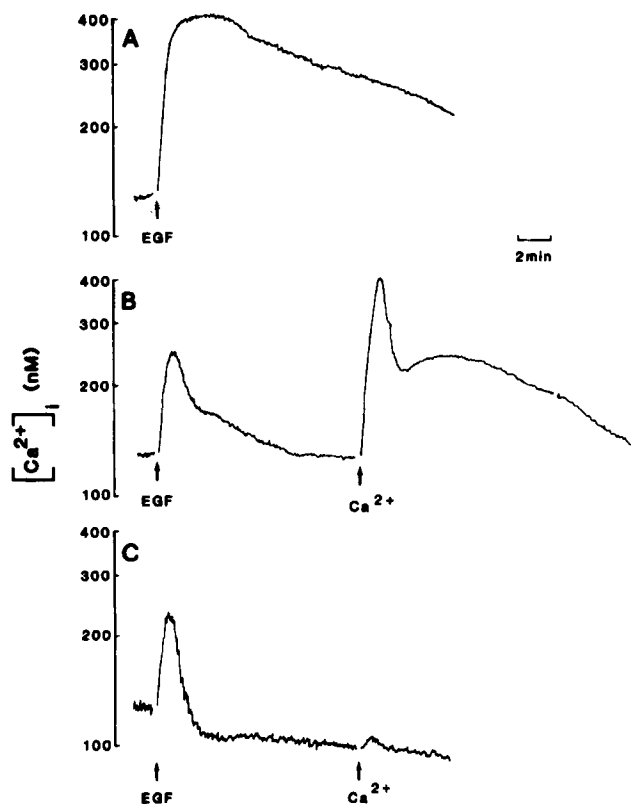
Among the earliest detectable secondary responses to the activation of a wide variety of membrane receptor systems is the activation of phospholipase C, resulting in the hydrolysis of polyphosphoinositides. As a consequence at least two important second messengers are produced; i.e., inositol trisphosphate and 1,2-diacylglycerol. Although quantitative differences are found for different hormones and different cell types, EGF is no exception to this rule (4, 8). Here we determined the activation of phospholipase C by determining the accumulation of InsPn in the presence of LiCl (8). A dose-response curve of the EGF-induced InsPn accumulation is shown in Fig. 5. Half-maximal stimulation occurred at 5 ng/ml of EGF. In A431-2E9 cells (see Materials and Methods), InsPn formation was slightly decreased without a significant change in the shape of the dose-response curve, showing that EGF occupation of high affinity sites alone is sufficient for the activation of phospholipase C.

#### Intracellular Free $\text{Ca}^{2+}$

Many mitogens, including EGF, evoke a rapid (within seconds after addition) rise in the  $[\text{Ca}^{2+}]_i$ . It has been estab-



**Figure 5.** InsPn formation in A431 and A431-2E9 cells. Myo- $^3\text{H}$ inositol-labeled A431 and A431-2E9 cells were treated with the indicated concentrations of EGF for 30 min in the presence of 10 mM LiCl. Formation of InsPn was determined as described (59). Solid circles represent A431 cells; open circles, A431-2E9 cells. Data are expressed as mean  $\pm$  SEM ( $n = 3$ ).



**Figure 6.**  $[Ca^{2+}]_i$  measurements. A431 (A and B) and A431-2E9 (C) cells, loaded with indo-1 were treated with EGF (50 ng/ml) and  $Ca^{2+}$  (4 mM) as indicated, in the presence (A and C) or absence (B) of  $Ca^{2+}$ . Indo-1 fluorescence was continuously monitored using a fluorimeter. Cells in B were treated with 4 mM EGTA before EGF addition.

lished that in the majority of cases this is primarily due to the release of  $Ca^{2+}$  from intracellular stores (4) evoked by the production of inositol trisphosphate. In a variety of cell lines it has been found that the EGF effect is largely dependent on the presence of  $Ca^{2+}$  in the extracellular medium (46). Hepler et al. (32) and Wheeler et al. (60) showed that in A431 cells the EGF-induced  $Ca^{2+}$  signal is biphasic, consisting of a small, transient release of  $Ca^{2+}$  from intracellular stores accompanied by a large, prolonged influx from the extracellular medium.

A431 cells were labeled with indo-1, a fluorescent dye which allows detection of minor changes in  $[Ca^{2+}]_i$ . In control cells, EGF addition resulted in a broad and prolonged response (Fig. 6 A) in accordance with previous results (32, 46, 60). Addition of EGF in  $Ca^{2+}$ -free medium resulted in a single, transient peak (Fig. 6 B). When extra  $Ca^{2+}$  was added to these cells, a second peak of  $[Ca^{2+}]_i$  increase due to influx was observed (Fig. 6 B), in accordance with the results of Wheeler et al. (60). EGF addition to A431-2E9 cells (Fig. 6 C) resulted in a  $Ca^{2+}$  signal much like that measured in the presence of EGTA (Fig. 6 B). The rise in  $[Ca^{2+}]_i$  is rapid and transient, suggesting that mAb 2E9 does not impair  $Ca^{2+}$  mobilization, but causes a block of  $Ca^{2+}$  influx. In line with this assumption, addition of  $Ca^{2+}$  did not have any effect on  $[Ca^{2+}]_i$  (Fig. 6 C). Our results thus indicate that  $Ca^{2+}$  mobilization is unaffected when EGF binding to low affinity EGFR is blocked by mAb 2E9. Whether the observed inhibition of

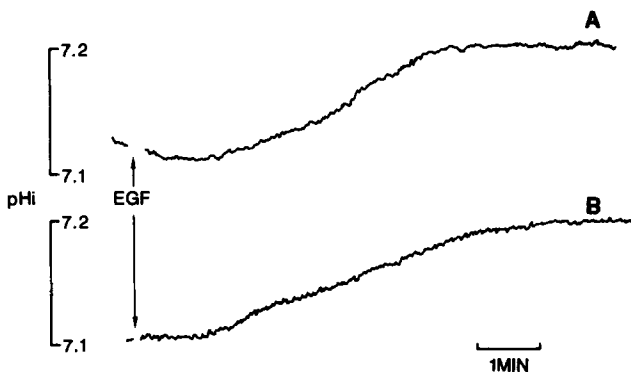
EGF induced  $Ca^{2+}$  influx by mAb 2E9 is a nonspecific side effect of the antibody, or results from a specific 2E9-EGFR interference, remains to be determined.

### Intracellular pH ( $pH_i$ )

Mitogenic stimulation of quiescent cells under bicarbonate-free conditions leads to a rapid and sustained increase in  $pH_i$ , mediated by activation of the amiloride sensitive  $Na^+/H^+$  exchanger (45). A change in  $pH_i$  evoked by EGF can be optimally measured in A431 cells when the cells have been incubated under serum-free conditions at a relatively low external pH ( $pH_o$ ) (6.8) for 24 h (reference 20, unpublished observations). Incubation at pH 6.8 does not influence binding of mAb 2E9 (not shown). Addition of EGF to control cells leads to an increase in  $pH_i$  of  $\sim 0.1$  U (Fig. 7 A). EGF treatment of A431-2E9 cells leads to a comparable increase in  $pH_i$  as shown in Fig. 7 B. This contrasts an earlier observation (20), in which 2E9 was reported to block the EGF-induced  $pH_i$  response at  $pH_o$  7.2. We do not know what caused the inhibitory effect at that time, but despite numerous efforts, we are not able even in control cells to measure any  $pH_i$  change at this  $pH_o$  anymore. Clearly, at least at  $pH_o$  6.8, occupation of high affinity receptors by EGF is sufficient to activate the  $Na^+/H^+$  exchange system in A431 cells with similar kinetics as when the total receptor population is available to EGF.

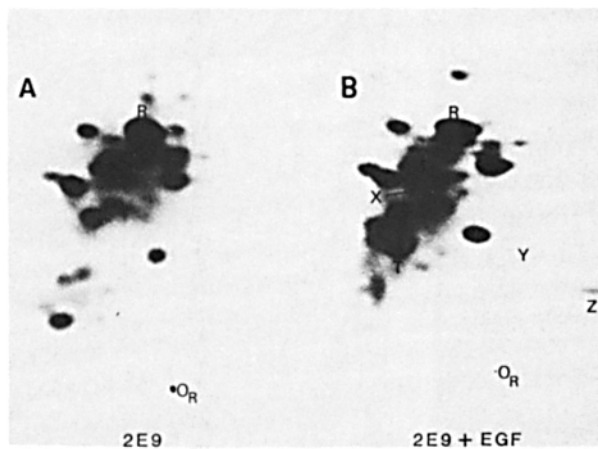
### Phosphorylation of EGFR on Threonine 654

The hydrolysis of polyphosphoinositides, as a result of phospholipase C activation, leads to protein kinase C (PKC) activation by 1,2-diaclyglycerol, one of the hydrolytic products. One of the best characterized substrates for PKC is the EGFR. Phosphorylation of the EGFR by PKC occurs predominantly on a threonine residue (threonine 654) located close to the plasma membrane (37). This results in a specific loss of high affinity EGF binding and a decreased ability for EGF-mediated protein-tyrosine kinase activation. Also EGF induces threonine 654 phosphorylation (61), thus constituting a negative feedback mechanism in the action of the growth factor. In tryptic phosphopeptide maps of EGFR isolated from A431 cells treated with the phorbol ester TPA, the



**Figure 7.**  $pH_i$  measurements. A431 (A) and A431-2E9 (B) cells, grown on glass coverslips and loaded with BCECF were treated with EGF (50 ng/ml) as indicated. BCECF fluorescence was continuously monitored using a fluorimeter.

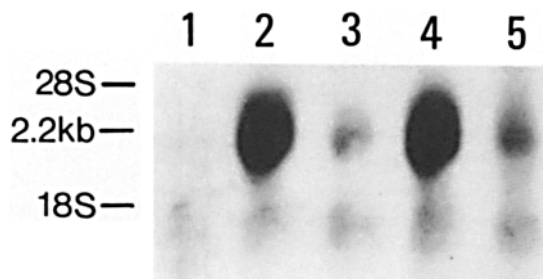




**Figure 8.** Tryptic phosphopeptide maps of EGFR isolated from  $^{32}\text{P}$ -labeled A431 cells. A431-2E9 cells, labeled to equilibrium with [ $^{32}\text{P}$ ]orthophosphate were treated with EGF (**B**) or not (**A**). The cells were lysed and the EGFR was immunoprecipitated. Subsequently, EGFR was isolated from SDS gels and treated with trypsin as described in Materials and Methods. Shown are autoradiographs of two-dimensional, high performance, thin-layer chromatograms of the tryptic peptides. X, Y, and Z are peptides containing threonine 654. T, the peptide containing tyrosine 1173. O<sub>R</sub>, the site of application. First dimension, high voltage electrophoresis at pH 8.9 (anode left, cathode right); second dimension, ascending chromatography.

phosphopeptides containing threonine 654 are visible as three distinct spots that have been labeled X, Y, and Z, respectively, by Hunter et al. (37).

Incubation of  $^{32}\text{P}$ -labeled A431 cells with mAb 2E9 yielded the same pattern of labeled EGFR tryptic phosphopeptides as in control cells (map shown for A431-2E9 cells in Fig. 8 **A**). Addition of EGF to A431-2E9 cells resulted in the appearance of several new spots (Fig. 8 **B**). On the basis of parallel phosphopeptide maps made from EGFR isolated from cells treated with the PKC-activating tumor promoter TPA (not shown), we have identified three of these



**Figure 9.** Northern blot analysis of *c-fos* expression. Subconfluent A431 cells were treated with the indicated ligands for 30 min followed by cell lysis, extraction of RNA, and Northern blotting. Indicated are the positions of the 2.2-kb *fos* mRNA, and 18-S and 28-S ribosomal RNAs. Lanes 1-3, A431 cells; lanes 4 and 5, A431-2E9 cells. Ligands added: (lane 1) mAb 2E9; (lanes 2 and 4) EGF; (lanes 3 and 5) TPA.

as peptides X, Y, and Z. The extent and rapidity of phosphorylation on residues X, Y, and Z equaled that in EGF-treated control cells (not shown), indicating that in A431-2E9 cells the same amount of receptors is subject to this feedback regulation mechanism. Thus, EGF is able to induce phosphorylation of the EGFR residue threonine 654, most probably via activation of PKC, through binding to high affinity receptors only. A fourth spot (labeled T in Fig. 8 **B**) was identified as tyrosine 1173, the major EGFR (auto)phosphorylation site (22, 42).

### Induction of the *c-Fos* Protooncogene

Bravo et al. (8) have shown a rapid induction of *c-fos* by EGF in A431 cells as well as in subclones of this cell line that are not growth inhibited by EGF. Accordingly, measurements of *c-fos* expression by RNA blot analysis (Fig. 9) showed a strong induction of the protooncogene by EGF. In A431-2E9 cells, EGF-induced *c-fos* expression was as strong as in control cells, and occurred with a similar time course (not shown), demonstrating that also in this case, EGF acts predominantly via high affinity receptors.

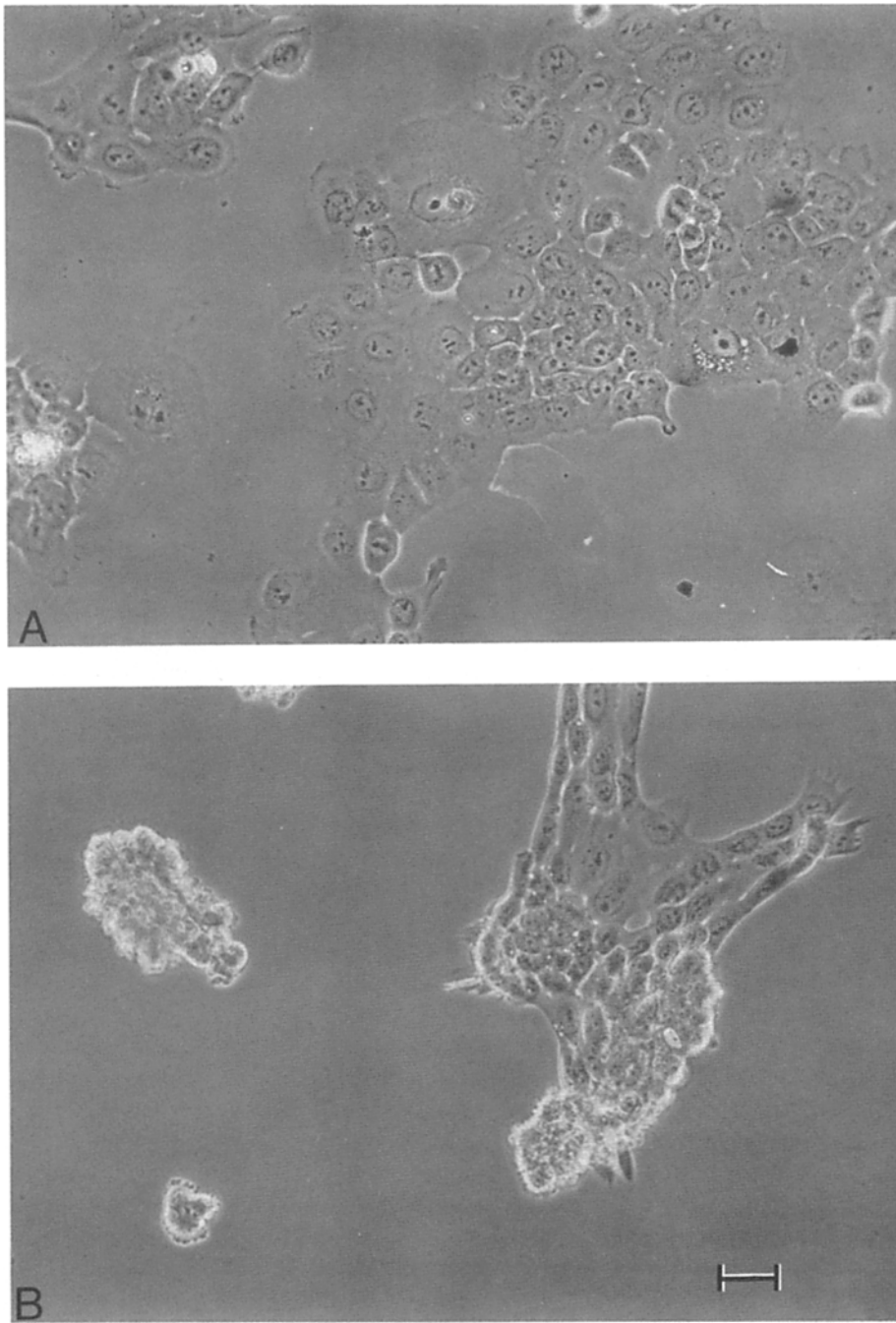
### Fluid-phase Pinocytosis and Morphological Changes

Fluid-phase pinocytosis (the uptake of small droplets of extracellular medium via plasma membrane invaginations resulting in easily visible, vacuole-like structures) is elicited rapidly in A431 cells by very low concentrations of EGF (30), suggesting that it might occur via high affinity EGFR. Here, we measured pinocytosis by adding EGF in combination with the fluorescent dye fluorescein-complexon to A431 and A431-2E9 cells. After incubation at 37°C, cells were washed and lysed. Fluorescence in the lysates was measured at the appropriate wavelength in a fluorimeter. In A431-2E9 cells, the pinocytotic activity is drastically reduced (not shown). This is not due to a previous induction of pinocytosis by the antibody. As for the effects of mAb 2E9 on EGF-induced  $\text{Ca}^{2+}$  influx, it is unclear at present whether we are dealing here with a nonspecific side effect of the antibody or not.

EGF induces membrane ruffling and rounding up of A431 cells within minutes (12, 13). Under  $\text{Ca}^{2+}$ -free conditions these effects are particularly dramatic, but also in normal medium they can easily be observed. Chinkers et al. (12) have suggested that fluid-phase pinocytosis and membrane ruffling are closely associated. It was therefore of interest to determine this effect in A431-2E9 cells. Fig. 10 **A** shows a phase-contrast image of A431-2E9 cells, and Fig. 10 **B** shows the same cells after addition of 5 ng/ml of EGF for 10 min at 37°C. Clearly, there is extensive cell rounding particularly at the edges of colonies, in agreement with the observation of Chinkers et al. (12). Thus, EGF-induced cell rounding occurs via high affinity receptors and in the absence of pinocytosis.

### Discussion

EGF receptors exist in two forms on the cell surface of a variety of cells. These subclasses differ prominently in their binding affinity for EGF, the high affinity EGFR being the minor fraction. At present, a direct biochemical identifica-



**Figure 10.** EGF-induced morphological changes. Half-confluent A431 and A431-2E9 cells were treated with EGF (50 ng/ml) for 10 min at 37°C. *A* shows a phase-contrast image of untreated A431-2E9 cells. *B* shows the same cells after EGF treatment. Bar, 50  $\mu\text{m}$ .

tion of high affinity EGFR is lacking, but a number of data apart from Scatchard analysis can be considered as evidence for their existence: (a) treatment of cells with agents that activate PKC results in a specific inhibition of EGF binding to high affinity receptors, without changing the total receptor number (55); (b) treatment of HeLa cells with glucocorticoids leads to a specific increase in high affinity receptor number (25); (c) 3T3 and Chinese hamster ovary cells transfected with single, EGFR-encoding cDNA sequences display both receptor classes (48), but several mutations in the intracellular domain of such receptors specifically lead to abolishment of high affinity sites (44, 48); and (d) the present study, along with a number of other reports on EGFR mAbs shows

that high and low affinity receptors can be recognized on the basis of their immunoreactivity.

The availability of an agent like EGFR-reactive mAb 2E9, that specifically interferes with EGF binding to the low affinity subclass, enables investigation of both structural and functional parameters of high affinity EGFR. mAb 2E9 is not unique in this respect. Antibodies B4G7-IgG, described by Behzadian and Shimizu (3), and EGR/G49, described by Gregoriou and Rees (29), also specifically block EGF binding to low affinity receptors, but only partially. Kawamoto et al. (39) reported the presence of a very small (<0.2%) population of very high affinity ( $K_d$   $7 \times 10^{-11}$  M) EGFR on formaldehyde-fixed A431 cells, based upon saturation curves

of EGF binding when added together with antibody 528. In our hands, however, on unfixed cells preincubation with antibody 528 efficiently blocks binding to both low and high affinity receptors.

In a previous paper (20), we reported that antibody 2E9 and two other EGFR-reactive mAbs stimulated EGFR protein-tyrosine kinase activity *in vitro* as well as in intact cells. However, as shown here for mAb 2E9, when these results were reevaluated using the method of phosphoamino acid analysis described in Materials and Methods, receptor activation by the antibodies was not detected in intact cells. Additional experimental evidence (not shown) indicated that the spot originally identified by us as phosphotyrosine in a one-dimensional high voltage thin-layer chromatograph was most probably uridine monophosphate, present due to hydrolysis of RNA contaminating the EGFR immunoprecipitates. Therefore, one-dimensional phosphoamino acid analysis at pH 3.5 may yield unreliable results, even when it is performed on immunoprecipitated material from  $^{32}\text{P}$ -labeled cells which has been subjected to SDS gel electrophoresis.

The usefulness of antibody 2E9 in the analysis of high affinity EGFR receptors becomes even more apparent when it is realized that such studies cannot be made by using low concentrations of EGF with the idea of specifically occupying high affinity sites. One can calculate that even at extremely low concentrations of EGF ( $<0.01$  nM), a substantial amount ( $\sim 35\%$ ) will bind to low affinity sites. An additional problem is that under such circumstances only a very small part of the total high affinity receptor population will be occupied ( $\sim 5\%$  at equilibrium). Studies in which low amounts of EGF are used to measure high affinity receptor-mediated effects (49) should therefore be interpreted with caution. Blocking low affinity sites with mAb 2E9 makes it possible not only to specifically saturate the remaining high affinity receptors, but also to achieve this rapidly with a high dose of EGF. We realize that this approach is also subject to criticism. At the standard concentration of mAb 2E9 used in our experiments (300 nM), only 90% of the maximal amount of antibody binding sites are occupied, given its  $K_d$  of 32 nM. Furthermore, our data do not rule out the possibility that mAb 2E9 itself might influence the behavior of high affinity receptors. However, in comparison with EGF binding data obtained in the absence of mAb 2E9, the antibody does not disturb EGF binding to the high affinity subclass, even after a prolonged incubation with supersaturating amounts of antibody (1,200 nM for 3 h, not shown).

Our data indicate that in A431 cells, high affinity receptors are the major subpopulation through which receptor activation occurs: while mAb 2E9 effectively blocks EGF binding to 95% of the surface-exposed receptor population, EGF is still capable of causing the major part of surface-exposed receptors to become phosphorylated on tyrosine residues (70% of the amount phosphorylated in control cells). Since the high affinity receptor subpopulation alone is far too small to account for at least 70% of the autophosphorylation signal (see Results), phosphorylation of low affinity receptors has to occur, even when they are inaccessible to EGF. We have considered the possibility that high affinity receptors are very rapidly internalized and replaced by new receptors, thus accounting for more than the apparent 5% of membrane-exposed receptors. However, the same results were obtained

when mAb 2E9-treated cells were incubated with EGF at  $0^\circ\text{C}$  (data not shown), a temperature at which internalization does not occur (63), in contrast to EGF-induced dimerization (16).

The results obtained in surface-labeled cells show that EGFR dimer formation can be driven through high affinity receptors only. Furthermore, the data obtained using radiolabeled EGF and DSS as cross-linking agent, indicate that high affinity receptors contain dimers as well as monomers.

In the present study, receptor kinase activation was measured as an increase in tyrosine phosphorylation of the receptor itself, which is commonly referred to as autophosphorylation (i.e., intramolecular). However, recent observations suggest that (auto)phosphorylation might occur through intermolecular cross-phosphorylation: Stern and Kamps (57) and King et al. (41) reported that the *c-erbB2/neu* proto-oncogene product p185<sup>neu</sup> becomes cross-phosphorylated on tyrosine residues when EGF is added to cells in which both this protein and the EGFR are expressed. P185<sup>neu</sup> and EGFR share an extensive overall structural homology (18). Furthermore, the cross-phosphorylation of p185<sup>neu</sup> by EGFR occurs with similar kinetics as receptor (auto)phosphorylation (57). Direct evidence for intermolecular EGFR cross-phosphorylation was recently obtained by J. Schlessinger and co-workers using 3T3 cells that were double transfected with two mutated EGFR sequences (34). In combination, the results lead us to propose that, at least in intact A431 cells, EGF induces intermolecular activation of low affinity receptors by high affinity receptors through the formation of oligomers between the two receptor types; i.e., cross-phosphorylation.

In addition, we have analyzed the effects of blocking low affinity EGFR by mAb 2E9 on the early EGF-induced signal transduction cascade. The results are quite straightforward. Exclusive EGF binding to the high affinity EGFRs leads to responses qualitatively and quantitatively similar to those normally observed. These responses include all secondary effects tested that have been linked before to second messenger production resulting from the breakdown of phosphoinositides by phospholipase C activation. These are (a) InsPn production, (b)  $\text{Ca}^{2+}$  mobilization, (c)  $\text{Na}^+/\text{H}^+$  exchange activation, (d) phosphorylation of threonine residue 654 of the EGFR, and (e) induction of *c-fos* gene expression. Other cellular responses that occur normally in the presence of mAb 2E9 are EGF internalization and cell rounding. The only exceptions found are the inhibition of  $\text{Ca}^{2+}$  influx and fluid-phase pinocytosis. Pinocytosis is a rapid and transient event in A431 cells (30). It involves uptake of relatively large amounts of extracellular fluid and therefore the EGF-induced  $\text{Ca}^{2+}$  influx may be a direct consequence of this event. However, we render it likely that these are nonspecific side effects of the antibody in combination with EGF, but direct evidence hereto is lacking.

In conclusion, our present results for the first time provide direct evidence for a major role of high affinity EGFR in the complex process of early intracellular signal transduction.

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