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Anti-Epidermal Growth Factor Receptor Monoclonal Antibodies Affecting Signal Transduction

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Abstract Monoclonal antibodies prepared against tyrosine phosphorylated epidermal growth factor receptor (EGFR) were tested for their effects on transmembrane signal transduction in A431 tumor cells. Monoclonal antibodies (mab) defined by SDS-sensitive epitopes, i.e., epitopes with conformational specificity, were most effective. Mab 5-I25 reacting with a site of the extracellular EGFR domain blocked EGF-binding and cell proliferation in vitro, as well as tumor growth in vivo. However, this mab appeared not to be internalized upon binding to EGFR and did not trigger EGFR autophosphorylation. In contrast, mab 5-D43, also defined by an SDS-sensitive epitope and reacting with an extracellular EGFR site, did not block EGF binding but was readily internalized after binding to EGFR of untreated A431 cells. This mab induced EGFR tyrosine phosphorylation in cell lysates and tyrosine-specific autophosphorylation of insolubilized EGFR immune complexes. Cell growth in vitro was greatly stimulated in the presence of mab 5-D43. Since interaction of mab 5-D43 with EGFR induced most EGF-specific functions, although it did not bind to the EGF-specific site of EGFR, we have to assume that binding of mab 5-D43 to EGFR induced a conformational shift that activated the cytoplasmic EGFR kinase site. On the other hand, activation and/or accessibility of the EGFR kinase site could be blocked by mab 1-594, which is defined by an SDS-insensitive protein epitope of the cytoplasmic EGFR domain. Blocking of the EGFR kinase site by mab 1-594 also abolished EGF-induced tyrosine phosphorylation of endogenous cellular substrates with molecular masses of 145, 97, 85, 37, and 32 kDa, as well as of exogenous substrates such as GAT copolymer.

Key words: epidermal growth factor receptor, mab, tyrosine phosphorylation, conformational shift, endogenous cellular substrates

Tyrosine phosphorylation in receptor proteins appears to play a dominant role in the majority of regulatory systems, although the amount of phosphorylated tyrosine compared to the total of cellular acid-stable phosphorylated amino acid residues is only 0.02–0.06% [Cooper et al., 1983]. Tyrosine phosphorylation generally occurs as an essential step for signal transduction during effector/receptor interaction and is governed by the activation of a cytoplasmic receptor site exhibiting tyrosine-specific protein kinase activity [Yarden and Ullrich, 1988]. One of the most interesting regulatory systems, following this scheme of signal transduction, is the epidermal growth factor (EGF) and its receptor (EGFR). EGF is mitogenic for a variety of cell types, including fibroblasts and epithelial cells [Schlessinger et al., 1983]; together with an overexpression of EGFR, it might have an essential role in cellular transformation and oncogenesis as was initially suggested by Downward et al. [1984]. Overexpression of EGFR has been observed in various human cancers [Hendler and Ozanne, 1984; Fitzpatrick et al., 1984], especially in glioblastomas in which an amplification of the EGFR gene or of the protein products has been recorded for more than one-half of the tumors examined [Liberman et al., 1985; Wong et al., 1987].

The binding of EGF to EGFR initiates a series of rapid responses, most likely starting with phosphorylation of tyrosine residues within the EGFR itself, followed by phosphorylation of other cellular proteins, hydrolysis of phosphatidyl inositol, and release of Ca^{2+} from intracellular stores. In the case of the insulin receptor, which follows the same scheme of signal transduction, it was reported that, concurrent with receptor phosphorylation, the state of phosphorylation of a series of cellular proteins changes.

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However, this concerns for the most part proteins phosphorylated on serine and/or threonine residues [Rosen, 1987; Czech et al., 1988], which cannot be the direct substrates of the receptor tyrosine kinase. Therefore, it was suggested that this type of phosphorylation might require prior activation of the relevant kinases by tyrosine phosphorylation [Ullrich and Schlessinger, 1990; Chen et al., 1987]. One candidate substrate of activated EGFR is MAP kinase, a serine/threonine kinase that is transiently activated in many cell types by a variety of extracellular signals, which is tyrosine-phosphorylated and activated upon stimulation of cells with EGF [Rossomando et al., 1989; L'Allemain et al., 1991]. Another substrate shown to be directly phosphorylated by the EGFR tyrosine kinase domain upon EGF stimulation is phospholipase C II (PLC II or PLC-γ), which coprecipitates with EGFR in the presence of anti-PLC-γ [Margolis et al., 1989; Meisenhelder et al., 1989; Wahl et al., 1989]. Both substrates, MAP kinase and PLC-γ together with the GTPase-activating protein [Ellis et al., 1990; Margolis et al., 1990], appear to be important physiological mediators of the EGF-induced mitogenic signal but still some more substrates have to be identified to account for all different activities of the EGF receptor since EGFR internalization seems not to be required for tyrosine phosphorylation of PLC [Wahl et al., 1989]. The mechanism whereby EGF binding activates the EGFR tyrosine kinase site and elicits early and late responses remained unresolved up to now.

In order to obtain more detailed information on the structural criteria of EGFR action, we prepared a series of anti EGFR monoclonal antibodies that differ in their blocking effects on various EGFR activities. Our approach was based on the use of highly purified EGFR [Steinhilber et al., 1990] as immunizing antigen yielding monoclonal antibodies (IgG) that show distinct effects on cell growth, signal transduction, EGF binding, autophosphorylation, and kinase activity.

MATERIALS AND METHODS

Cells

The following cell lines were used: human epidermoid vulva carcinoma cell line A431 [Fabricant et al., 1977], human glioblastoma cell line N28, and human astrocytoma cell lines N31 and N63 [Stavrou et al., 1987]. The cells were grown in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 μg/ml ciprofloxacin (Bayer, Leverkusen, FRG) to avoid growth of mycoplasmas [Schmitt et al., 1988]. Mouse myeloma cells Sp2/O-Ag.14 [Shulman et al., 1978] were used in fusion experiments.

Preparation of Monoclonal Antibodies

Highly purified human EGFR was prepared from A431 tumor cells by affinity chromatography on antiphosphotyrosine-coated columns [Steinhilber et al., 1990]. Female BALB-c mice, 8 weeks old, were immunized i.p. with 50 μg purified EGFR in complete Freund's adjuvant, followed by booster injections with 25 μg EGFR in incomplete Freund's adjuvant at day 21 and 42 after the first injection. Two further booster injections, the last dissolved in PBS, were given after intervals of 10 weeks. Increase of anti-EGFR titers was followed by enzyme-linked immunosorbent assay (ELISA) (30 ng EGFR/well), and mice used for fusion experiments, had titers exceeding $1:10^6$. Three days after the last injection, the animals were sacrificed and the splenocytes of the immunized animals were fused with Sp2/O-Ag.14 mouse myeloma cells as described by Galfré and Milstein [1981]. Thereafter, the cells were plated with feeder peritoneal cells using RPMI-HAT medium. Supernatants of growing colonies were screened for specific antibody production using dot-immunobinding to select for IgG production [Hawkes et al., 1982] and an ELISA (30 ng EGFR per well) for EGFR specificity using peroxidase-conjugated goat antimouse IgG (Dianova, Hamburg, FRG) as second antibody [Engvall, 1980]. All hybridoma clones with positive reactions were further screened for extracellular and intracellular binding of antibodies to EGFR domains in A431 tumor cells using indirect immunofluorescence microscopy. The cells grown on 12-mm glass coverslips were stimulated with 75 ng/ml EGF (Sigma, Deisenhofen, FRG) at 37°C for 10 min and then fixed with 3.7% formaldehyde in PBS at room temperature for 15 min. For the preparation of permeabilized cells, part of the fixed cells were treated with a mixture of methanol/acetone (2:1) at −20°C for 5 min. Thereafter, the coverslips were incubated with the hybridoma supernatants for 30–45 min at 37°C, washed 3 times with PBS followed by incubation with goat antimouse IgG-F(ab')$_2$ fluorescein isothiocyanate conjugates (Dianova, Hamburg,

FRG), and then again washed 3 times with PBS prior to microscopy.

Hybridomas with supernatants yielding positive immunofluorescence were cloned at least twice by serial dilution, and subclasses of monoclonal antibodies were determined by Ouchterlony immunodiffusion analysis using subclass-specific rabbit antibodies (Nordic Immunological Laboratories, Bochum, FRG). Monoclonal antibodies in hybridoma supernatants were purified by chromatography on protein A–Sepharose CL-4B columns (Pharmacia, Uppsala, Sweden). The protein content was determined with the method of Bradford modified by Rylatt and Parish [1982] or with the method of Peterson [1977].

Cell Lysis and Immunoprecipitation

Cell lysates were prepared from about 10^7 cells suspended in 3 ml of lysis buffer (20 mM Tris × HCl, pH 8.4/150 mM NaCl/10 mM EDTA/1% Triton-X-100/1% Na-deoxycholate/0.1% Na-dodecyl sulfate/0.1 mM Na-vanadate/1 mM p-chloromercuriphenyl sulfonic acid/0.5 mM phenylmethylsulfonyl fluoride). After 15 min at 4°C, still adherent material was dislodged with a rubber policeman and the suspension subjected to ultrasonication in an ice bath for 3 × 5 sec (Branson sonifier; 50 W), followed by centrifugation at 5,000*g* for 15 min. The 3-ml supernatants were mixed with 0.3 ml of a 50% protein A–Sepharose suspension in lysis buffer to remove unspecifically binding components. After 30-min incubation at 4°C, the suspensions were centrifuged at 10,000*g* for 1 min, and the resulting supernatants were incubated with 40–60 μg of purified monoclonal antibody dissolved in the same volume of lysis buffer. After 2-h incubation at 4°C, a 50% suspension of protein A–Sepharose beads corresponding to one-tenth of the total lysate volume was added and further incubated at 4°C for 45 min under gentle rocking. Protein A–Sepharose beads with bound immune complexes were then sedimented at 10,000*g* for 1 min, and the sediment washed 5 times with lysis buffer. Elution of anti-EGFR immune complexes was achieved with 50 mM diethyl amine/20 mM octyl glucoside; in case of antiphosphotyrosine immune complexes, specific elution was achieved with 40 mM phenyl phosphate. For analysis by polyacrylamide gel electrophoresis (PAGE) [Laemmli, 1970], the proteins were eluted at 100°C for 5 min with sample buffer.

EGF Binding Assay

Quantitative determination of EGF binding to cells and inhibition of EGF binding by monoclonal antibodies was carried out with iodinated EGF according to the outlines given by Carpenter [1985]. Cells were cultured overnight in 24-well Costar plates starting with 10^5 cells/well. Then the cells were washed with PBS and further incubated at 37°C in serum-free RPMI 1640 medium supplemented with 0.2% bovine serum albumin (BSA) and 2 g glucose/L. In the case of inhibition assays, the cells were cultured with various concentrations of anti-EGFR monoclonal antibodies in the medium at 37°C for 45 min prior to the addition of ^{125}I-EGF (3.7 MBq/μg; Amersham, Braunschweig, FRG) at 4°C to yield a final EGF concentration of 1.65 nM. The cultures were then kept at 4°C for 2 h under gentle rocking, followed by 3 washes (each 5 min) with cold incubation medium and 2 washes with cold PBS/1 mM $CaCl_2$/1 mM $MgCl_2$. Thereafter, lysis was achieved by treatment with 0.5 ml 0.1 M NaOH/1% Triton-X-100 at 37°C for 15 min and bound radioactivity was determined in a LB 2101 Berthold gamma counter.

Immune Complex Kinase Assay and EGF- and mab 5-D43-Induced Phosphorylation

Autophosphorylation and phosphorylation of an exogenous peptide substrate was analyzed according to the outlines given by DiRenzo et al. [1986]. About 10^7 A431 cells were treated with 3 ml lysis buffer (20 mM Tris × HCl, pH 8.2/150 mM NaCl/1% Triton-X-100/1 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride) at 4°C for 15 min and immune precipitation was carried out as described above. The precipitates were washed 3 times in washing buffer (20 mM HEPES, pH 8.2/150 mM NaCl/0.1% Triton-X-100/10 mM $MgCl_2$/5 mM $MnCl_2$) and once in assay buffer (20 mM HEPES, pH 7.4/0.1% Triton-X-100/10 mM $MgCl_2$/5 mM $MnCl_2$). The immune precipitates were resuspended in 75 μl assay buffer, mixed with 5 μl EGF (0.1 μg/μl) and kept on ice for 10 min. Autophosphorylation was started by addition of 10 μl assay buffer containing 0.37 MBq γ^{32}P-ATP (370 GBq/mmol; Amersham International plc.). After 30-min incubation at room temperature, the reaction was stopped by the addition of Laemmli's sample buffer and heating at 100°C for 5 min, followed by electrophoretic separation of labeled components in sodium dodecyl sulfate (SDS)

polyacrylamide gels. Labeled components were visualized by autoradiography, using Kodak X-Omat paper. For quantification of incorporated radioactivity, labeled bands were excised and Cerenkov radiation was determined in a Beckman liquid scintillation counter.

Phosphorylation of exogenous substrate was performed under the conditions of the autophosphorylation assay but in the presence of 40 μg of a synthetic random copolymer consisting of glutamic acid, alanine, tyrosine 6:3:1 (molecular weight 20,000–50,000 Da; Sigma, Deisenhofen, FRG). The reaction was stopped by adding EDTA to a final concentration of 50 mM. For quantification of the incorporated radioactivity, 5-μl portions of the reaction mixture were transferred onto 1-cm^2 pieces of Whatman filter followed by 4 washings (each 30 min) with ice-cold 15% trichloroacetic acid/20 mM pyrophosphate. The filter pieces were then dryed and radioactivity was determined in a Beckman liquid scintillation counter.

In vivo autophosphorylation of EGFR induced by mab 5-D43 was studied with prelabeled A431 cells: Cell cultures in 6-well Costar plates containing 10^5 cells/cm^2 were incubated with phosphate-free RPMI + 0.5% FBS at 37°C overnight in the presence of 100 μCi ^{32}P-orthophosphate/ml (carrier-free; 370 MBq/ml, Amersham International plc.). The indicated amounts of mab 5-D43 or EGF (as a positive control) were then added directly to the labeling medium and incubated for a further 45 min at 37°C. The harvested cells were then lysed with lysis buffer, EGFR was precipitated with antiphosphotyrosine mab 3-365-10 and the washed sediment subjected to SDS–PAGE.

EGF-induced secondary tyrosine-phosphorylation of endogenous cellular proteins was studied in cell lysates. A431 cells were harvested with a rubber policeman, washed once with 150 mM NaCl, and portions of 5×10^6 cells resuspended in 500 μl of phosphorylation buffer (20 mM HEPES, pH 8.0/5 mM $MgCl_2$/2 mM $MnCl_2$) were lysed by ultrasonication. The nuclei were removed by sedimentation (1,000*g*; 10 min) and the supernatants incubated with 60 μg anti-EGFR or control antibodies at 4°C for 4 h. After the addition of 300 ng EGF/ml reaction mixture, phosphorylation was started by adding 0.185 MBq γ-^{32}P-ATP (370 GBq/mmol; Amersham, Braunschweig, FRG). The reaction was allowed to proceed at room temperature for 30 min and was then stopped by addition of 500 μl RIPA buffer, followed by a two-step immunoprecipitation: the first precipitate was obtained with anti-EGFR antibody and the second with antiphosphotyrosine antibody. The sediments were analysed by SDS polyacrylamide gel electrophoresis as described above and labeled proteins were visualized by autoradiography, using Kodak X-Omat paper.

Inhibition of Tumor Growth In Vivo

Inbred BALB-c/nu-nu mice kept under pathogen-limited conditions were used for growth inhibition studies. The nude mice were inoculated subcutaneously at day 0 with 7×10^6 A431 tumor cells in 250 μl PBS, yielding palpable tumors in 100% of the animals within 10 days. Groups of 3 mice were serially treated with i.p. injections of distinct anti-EGFR mab (200 μg/200 μl PBS) starting at day 1 after tumor cell inoculation with further anti-EGFR injections on days 1, 3, 5, 8, 12, 16, and 19. Tumor growth was monitored by caliper measurements in three dimensions at days 2, 4, 9, 11, 15, 18, 22, 24, and 26, and tumor volumes were calculated using the formula $V = \pi/6\ (a \cdot b \cdot c)$.

RESULTS

Characterization of Anti-EGFR mab

Fusion experiments were performed with spleen cells derived from mice hyperimmunized with highly purified tyrosine-phosphorylated EGFR. Antibodies produced by hybridoma clones were screened for IgG subtypes and specific binding to EGFR. Most of them (80%) were members of the IgG1/kappa subclass. Immunofluorescence studies with fixed and fixed + permeabilized A431 cells demonstrated extracellular binding of 23/28 anti-EGFR mab, whereas 5/28 mab bound to the cytoplasmic domain of EGFR. Among the extracellularly binding mab, we recorded 5/23 defined by EGFR epitopes sensitive to 1% SDS in nonreducing buffers, i.e., these mab had conformational specificity and were especially suited to the study of the involvement of conformational shifts in signal transduction. The protein-specific anti-EGFR mab (19/23) also precipitated the nonglycosylated EGFR molecule (130 kDa) [Mangelsdorf-Soderquist and Carpenter, 1984] from lysates of A431 cells cultured with tunicamycin overnight. Only one of the extracellularly binding antibodies, mab 5-D43 (IgG1), induced 100% internalization within 30 min at 37°C after binding to viable A431 cells.

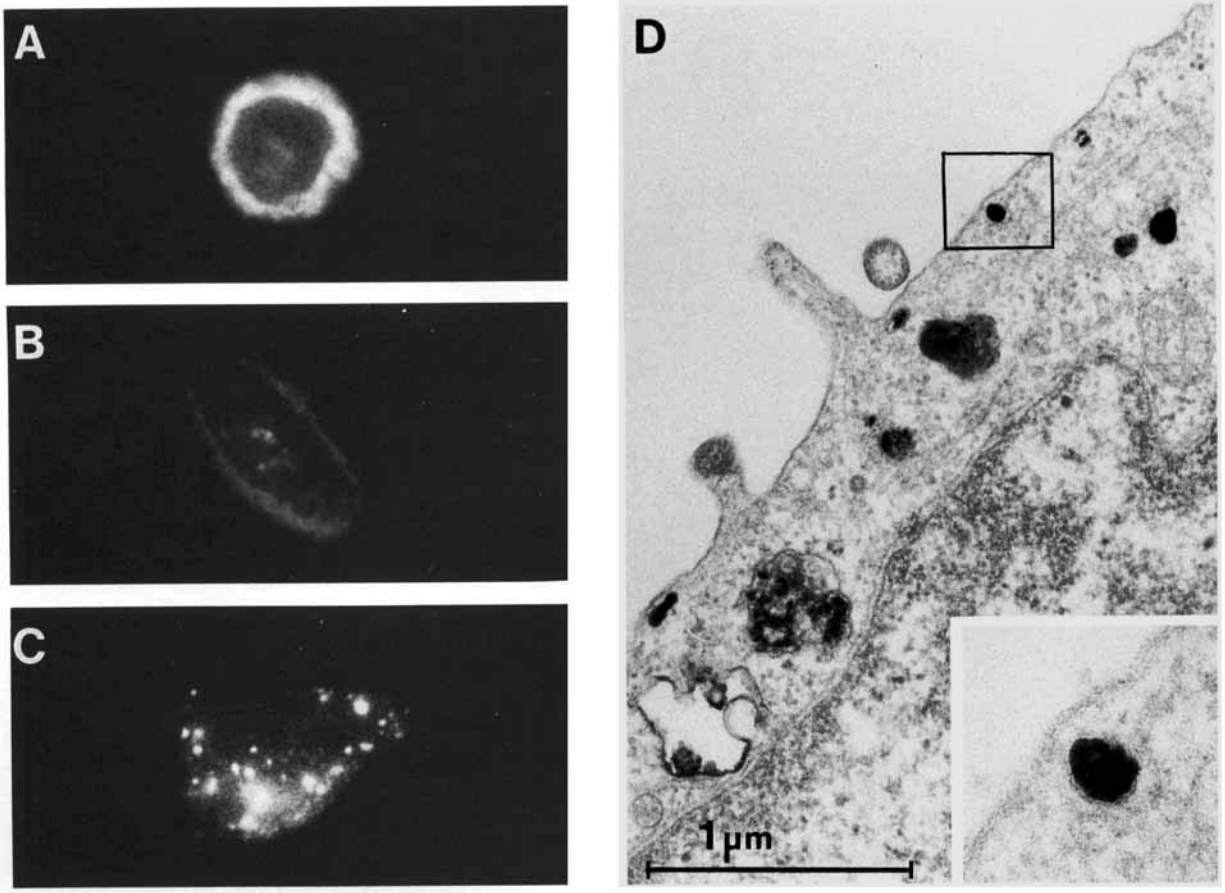

Fig. 1. Binding and internalization of anti-EGFR mab 5-D43 visualized by FITC- (A,B,C) and peroxidase-conjugated goat antimouse IgG(D) **A:** A431 cells were reacted with mab 5-43 at 4°C for 1 h, washed with ice-cold PBS, then incubated with second (FITC) antibody at 4°C for 1 h, washed again, and fixed with 3 5% formaldehyde for 15 min **B:** Analogous to A, but after the reaction with mab 5-D43 and washing, cells were kept at 37°C for 30 min prior to reaction with second antibody followed by fixation with formaldehyde **C:** Analogous to B but after incubation at 37°C, the cells were first fixed with formaldehyde and permeabilized with methanol/acetone (2 1) at −20°C for 5 min prior to reaction with the second antibody (×630) **D:** Electron microscopic visualization of internalized EGFR/mab 5-D43/second antibody complexes A431 cells were incubated with mab 5-D43 at 4°C for 1 h, washed with ice-cold PBS and water, further incubated with peroxidase-conjugated goat antimouse IgG at 4°C for 1 h, then washed with PBS and incubated at 37°C for 30 min prior to fixation with 2 5% glutaraldehyde for 1 h Peroxidase was allowed to react with diaminobenzidin (0 6 mg/ml) at room temperature for 20 min The next steps were postfixation with 1% osmium tetroxide in PBS for 1 h, washes with PBS and water, overnight staining with 1% uranyl acetate in water at 4°C, followed by dehydration with ethanol, embedding in Epon and preparation of ultrathin sections

After fixation and permeabilization of the mab 5-D43-treated cells, the antibody could be localized within the cells as randomly distributed punctual structures (Fig. 1A–C). Since mab 5-D43 bound to native A 431 cells was still internalized when reacted with the second antibody prior to shifting the temperature from 4° to 37°C, internalization could be also visualized by electron microscopy. Figure 1D shows prominently stained EGFR/mab 5-D43/second antibody complexes in cytoplasmic vacuoles and small heavily stained vesicles. This mab 5-D43 is defined by an SDS-sensitive EGFR epitope.

Blocking of EGF Binding

The EGF-binding assay with A431 tumor cells showed that mab 5-D43, which was readily internalized upon binding to EGFR at 37°C, did not block binding of ^{125}I-EGF (1.65 nM) at 4°C (Fig. 2A). This is an unexpected result, since the EGFR/mab 5-D43 complex was found to be internalized at 37°C; thus, we have to assume a rapid re-expression or recycling of EGFR. Confirmation of the inability of mab 5-D43 to block EGF binding was obtained from an experimental series in which both antibody binding and

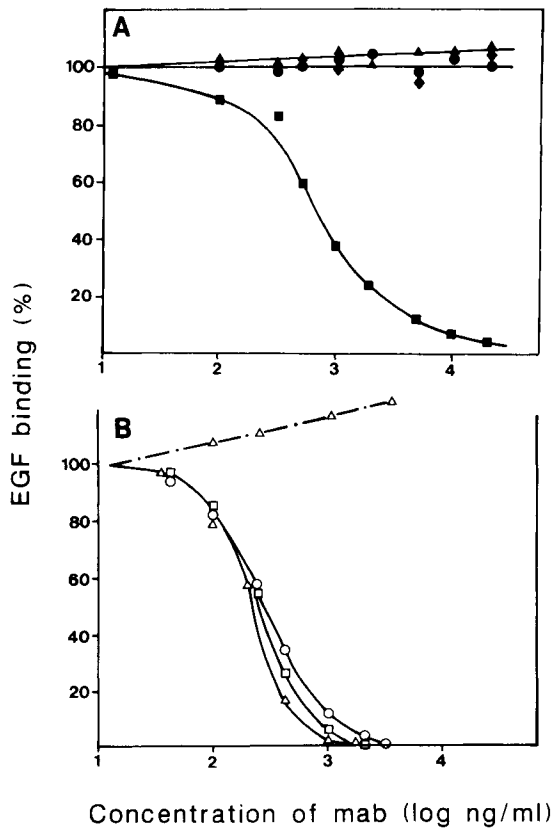

Fig. 2. **A:** Effects of some selected affinity-purified anti-EGFR mab on the specific binding of radiolabeled EGF to A431 cells. The cells were incubated with various concentrations of extracellularly binding mab for 45 min prior to addition of ^{125}I-EGF (final concentration 1.65 nM). The radioligand was allowed to bind to the cells for 2 h at 4°C in the presence of antibody. The effects of the following mabs are given in a semilogarithmic plot: 5-I25 (■) and 5-D43 (◆), both defined by SDS-sensitive epitopes; 3-162 (●) and 1-241 (▲), both defined by SDS-insensitive epitopes. **B:** Effects of mab 5-I25 on EGF-binding to glioblastoma cell lines N 28 (□), N31 (○) and N63 (△). The broken line represents EGF-binding of N 63 cells after pretreatment with mab 5-D43.

binding of EGF were performed at 4°C. Again, no inhibition of EGF-binding was observable up to antibody concentrations of 15 μg/ml. However, mab 5-I25 (IgG1), also defined by an SDS-sensitive EGFR epitope, induced complete blocking of EGF binding at a concentration of 10 μg mab/ml (Fig. 2A), but this mab appeared not to be internalized. Complete blocking of EGF-binding by mab 5-I25 was also recorded when the binding inhibition assay was performed at 4°C throughout. All other extracellularly binding mab, no matter whether defined by SDS-sensitive or SDS-insensitive EGFR epitopes, had no effects on EGF binding. The effect on EGF-binding curves of some of these mab are depicted in Figure 2A.

Mab 5-I25, which blocked EGF binding to A431 cells, also showed efficient blocking of EGF binding to glioblastoma cell lines N28, N31, and N63 (Fig. 2B). However, the mab concentrations required for 100% blocking were lower by an order of magnitude that correlated very well with data resulting from EGF-binding assays and with the yields of EGFR obtained from affinity purification of the corresponding cell lysates. The low-level expression of EGFR should render these cell lines more sensitive to a possible blocking effect of mab 5-D43 that might not have been observable with A 431 cells due to EGFR overexpression (Fig. 2A). However, when analogous studies of EGF binding were done with glioblastoma N63 cells and mab 5-D43, again no blocking effect by mab 5-D43 was recorded (Fig. 2B). In contrast, we observed a tendency of increased EGF binding with increased concentrations of mab 5-D43, an effect that again could result from a rapid antibody-induced re-expression or recycling of EGFR.

Inhibition of Cell Growth

The effect of various concentrations of anti-EGFR mab on the growth of A431 tumor cells was studied in 7-day cultures. The antibody concentration ranged from 1 to 100 nM. The initial cell number plated per well (day 0; 24-well plate) was 2×10^4. Antibody was added directly after plating. For antibodies affecting cell growth, controls with the antibody added one day after plating were also performed. In Figure 3 the dependence of cell growth from increasing antibody concentrations is plotted for some selected anti-EGFR mab. Mab 5-I25, which showed efficient blocking of EGF binding even at low antibody concentrations (see Fig. 2A), was also effectively inhibiting growth of A431 cells (Fig. 3). However, mab 1-241 (IgG2a), defined by an SDS-insensitive EGFR epitope, and that did not affect EGF binding, also showed some minor growth-inhibiting effects starting at an antibody concentration of 9 μg/ml (60 nM) (Fig. 3). An entirely different effect was obtained with mab 5-D43 which showed rapid internalization upon binding. This mab induced stimulation of cell growth in vitro up to 170% of control cultures (Fig. 3). The controls with mab 5-I25 and mab 5-D43 added 1 day after plating showed essentially similar growth inhibition or growth stimulation effects, respectively. All other anti-EGFR

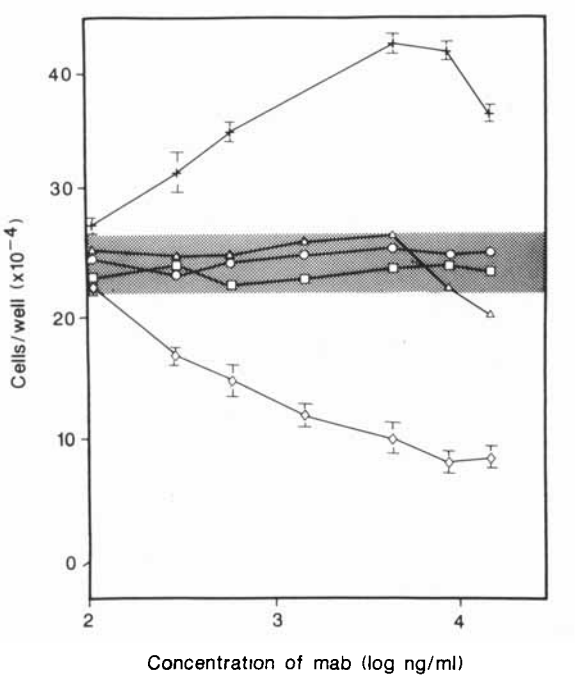

Fig. 3. Effects of some selected affinity-purified anti EGFR mab on the in vitro growth of A431 tumor cells. The cells were cultured for 7 days in the presence of different concentrations of mab The initial number of cells/well was 2×10^4 (day 0) Antibodies were added directly after plating. After 7 days of culture, the cell numbers/well were determined. Growth curves obtained in the presence of the following mabs are given in semilogarithmic plots. 5-I25 (◇) and 5-D43 (+) defined by SDS-sensitive epitopes, 3-162 (□), 1-241 (△), and 1-594 (○) all defined by SDS-insensitive epitopes. Mab 1-594 binds to the cytoplasmic domain of EGFR and was used as a control. The shaded area represents the average number of cells/well grown in the absence of antibodies for 7 days

mab showed no growth-inhibiting effects in the concentration range tested.

Experiments performed to obtain information on tumor development in vivo in the presence of anti-EGFR antibodies yielded first results that confirm the in vitro findings. Growth inhibition in vivo was studied with nude mice inoculated subcutaneously with 7×10^6 A431 cells at day 0 yielding 100% tumor take in the controls. The animals received 7 injections of anti-EGFR mab within 19 days after inoculation, i.e., a total of 1.4 mg per mouse. Tumor growth was followed by caliper measurements and average tumor volumes of developing tumors are listed in Table I. Mab 5-I25, the antibody that showed blocking of EGF binding and growth inhibition in vitro, also blocked A431 tumor growth in the nude mouse. Treatment with mab 1-241, the antibody inhibiting cell growth in vitro only at high mab concentrations, resulted in a strong general depression of tumor growth and induced tumor regression in one mouse whereas two mice died in spite of bearing only very small tumors. Surprisingly, treatment with mab 5-D43, the antibody that stimulated cell growth in vitro, induced strong retardation of tumor development in vivo during the initial 14 days, followed by a phase of very rapid tumor growth. Death occurred simultaneously with the first death in the untreated control group. Mab 3-162, which had no effect on cell growth in vitro, also showed no effect on tumor development in vivo.

Blocking of EGFR Autophosphorylation by Binding to the Cytoplasmic EGFR Domain

In a first set of experiments, anti-EGFR mab defined by epitopes of the cytoplasmic domain were screened for blocking autophosphorylation using an immune complex kinase assay. The corresponding EGFR immune complexes precipitated from A431 cell lysates were treated with EGF prior to initiation of phosphorylation with γ-^{32}P-ATP. Mab 1-594 (IgG1), which is specific for a site of the cytoplasmic EGFR domain, showed complete blocking of EGF-induced autophosphorylation, either by blocking activation and/or by blocking accessibility of the cytoplasmic kinase site of EGFR (Fig. 4D) The other mab with specificity for the cytoplasmic EGFR domain had no effect on autophosphorylation (e.g., Fig. 4C). An additional prove for the presence of EGFR in all immunoprecipitates was obtained by immunodetection with anti-EGFR mab 3-162 after electrophoretic transfer to nitrocellulose membranes (Fig. 4b–e) and specificity of autophosphorylation by immunodetection with the phosphotyrosine-specific mab 3-365-10 (not shown).

Under saturation conditions blocking of EGFR autophosphorylation by mab 1-594 binding to a cytoplasmic domain, also abolished the ability to phosphorylate the tyrosine residues of GAT, a synthetic 6:3:1 random copolymer of glutamic acid/alanine/tyrosine. GAT peptide chains range between 20- and 50-kDa molecular mass and are highly suitable exogenous substrates to prove secondary tyrosine phosphorylation in the EGFR immune complex kinase assay. All other anti-EGFR mab that did not block EGFR autophosphorylation in the respective immune complexes did neither block GAT phosphorylation in the immune complex kinase assay.

TABLE I. Average Tumor Volume of Tumor-Bearing Mice Determined Various Days After Inoculation s.c. of 7 × 10^6 A431 Cells at Day 0

Treatment mab	Average tumor volume (mm^3) at day								
	2	4	9	11	15	18	22	24	26
—	28	67	75	108	335	512	851	1,173	1,389
3-162	31	56	59	85	225	847	973	1,374	1,516
1-241	58	82	36	36	38	136	115	—[a]	—
5-I25	24	62	56	36	16	8	10	12	6
5-D43	13	12	10	27	66	171	313	586	983

[a]Death of all three mice between day 22 and 25; one of these 3 mice was tumor free.

Extracellularly Binding Anti-EGFR mab and the Mechanism of Signal Transduction

Anti-EGFR mab defined by extracellular EGFR epitopes did not influence EGF-induced autophosphorylation in the immune complex kinase assay (e.g., mab 3-162) (Fig. 4B) with the exception of two mab. Mab 5-I25 that efficiently could block EGF binding (see Fig. 2A), partially allowed autophosphorylation of EGFR in the corresponding immune complexes in the presence of EGF (Fig. 4E). This can be explained by the possibility that, under the conditions of the immune complex kinase assay, the EGF-binding site must have become partly accessible, most likely due to dissociation of the immune complexes and loss of mab saturation conditions during washing of precipitates because, in the presence of additional mab 5-I25 during the kinase assay, no autophosphorylation of EGFR was detectable. The reaction of mab 5-I25 with EGFR without subsequent EGF treatment did not trigger autophosphorylation.

The second anti-EGFR mab influencing autophosphorylation was mab 5-D43, which showed conformational specificity to an extracellular EGFR domain and did not block EGF binding (see Fig. 2) but was rapidly internalized upon binding to A431 cells. This mab was capable to induce EGFR autophosphorylation in the absence of EGF using the following assay conditions: 1 × 10^6 A431 cells prelabeled with ^{32}P orthophosphate were incubated with varying concentrations (5–300 μg/ml) of mab 5-D43 at 37°C for 45 min; cells were then lysed; EGFR was precipitated by antiphosphotyrosine mab 3-365-10 and subjected to SDS polyacrylamide gel electrophoresis (Fig. 5). Treatment of cells with 50–300 μg/ml mab 5-D43 induced a high degree of EGFR autophosphorylation, with a maximum effect at 150 μg/ml mab 5-D43 corresponding to about 40% of EGF-induced EGFR autophosphorylation at EGF saturation condition (77 ng EGF/ml).

To answer the question of whether associated membrane components are involved in mab 5-D43-induced EGFR autophosphorylation or whether the EGFR kinase site is activated by a mab 5-D43-induced conformational shift in the EGFR structure, the effects of mab 5-D43 were studied in specific EGFR immune precipitates prepared with antibody mab 3-162 immobilized on protein A–Sepharose beads. The EGFR immune complexes contained no associated proteins (Fig. 6). Stimulation of EGFR immune complexes with EGF or with anti-EGFR mab 5-D43 for 10 min in the presence of ATP induced extensive tyrosine phosphorylation (Fig. 6B,C), a reaction already observed in EGFR immune complexes obtained with mab 5-D43 immobilized on protein A–Sepharose beads (Fig. 6D), but not in EGFR immune complexes with immobilized anti-EGFR mab 3-162 (Fig. 6A). EGF and anti-EGFR mab 5-D43-induced EGFR autophosphorylation in immobilized EGFR immune complexes was demonstrated with the "enhanced chemiluminescence" detection system (Amersham International plc.) as well as directly by autoradiography when the reaction was performed in the presence of γ-^{32}P-ATP (20 μCi/ml).

Endogenous Cellular Substrates of EGFR Kinase Activity

Blocking of the EGFR kinase site by mab 1-594 opens up the possibility of obtaining information on direct cellular substrates of EGFR. EGF-induced phosphorylation of A431 cell lysates was studied after 4 h preincubation with or without the kinase-site-blocking antibody. After addition of EGF, the phosphorylation reaction was initiated by addition of γ-^{32}P-ATP and was allowed to proceed for 30 min. Thereafter,

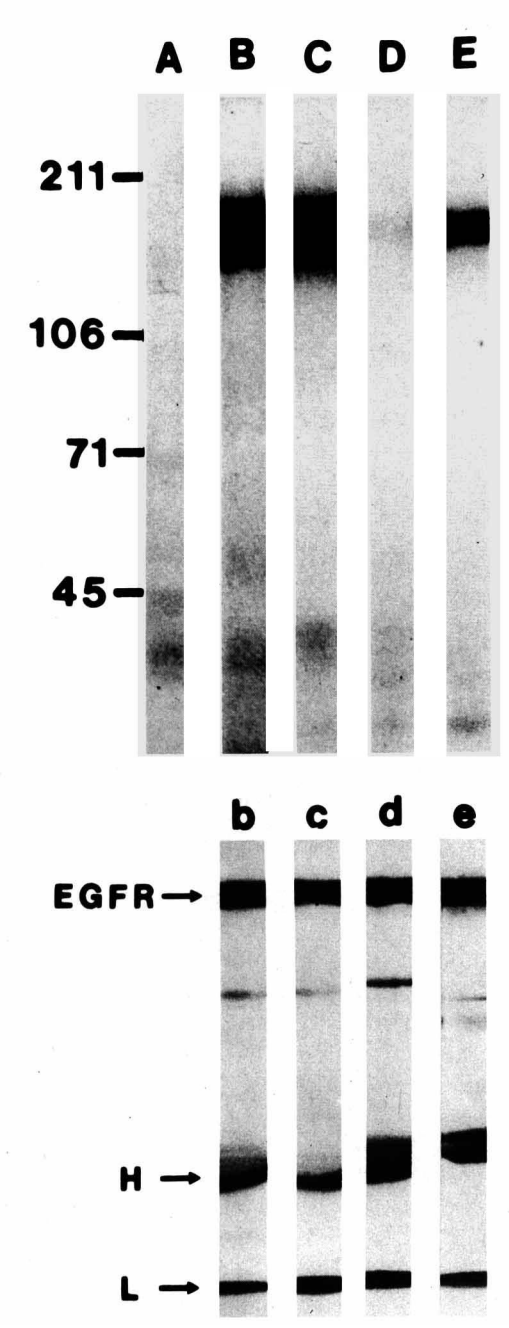

Fig. 4. Electrophoretic patterns of anti-EGFR immune complexes obtained with different anti-EGFR mab, showing different degrees of EGF-induced autophosphorylation in the presence of γ-^{32}P-ATP (immune complex kinase assay). **A:** Control mab, not EGFR-specific. **B:** Mab 3-162, defined by an extracellular SDS-insensitive EGFR epitope, +EGF. **C:** Mab 1-353, defined by a cytoplasmic SDS-insensitive EGFR epitope, +EGF. **D:** Mab 1-594, defined by a cytoplasmic SDS-insensitive EGFR epitope, +EGF. **E:** Mab 5-I25, defined by an extracellular SDS-sensitive EGFR epitope, +EGF. *b,c,d,e:* immune detection of B–E using anti-EGFR mab and then antimouse IgG-H/L chain antiserum. Reference molecular masses are given as kDa.

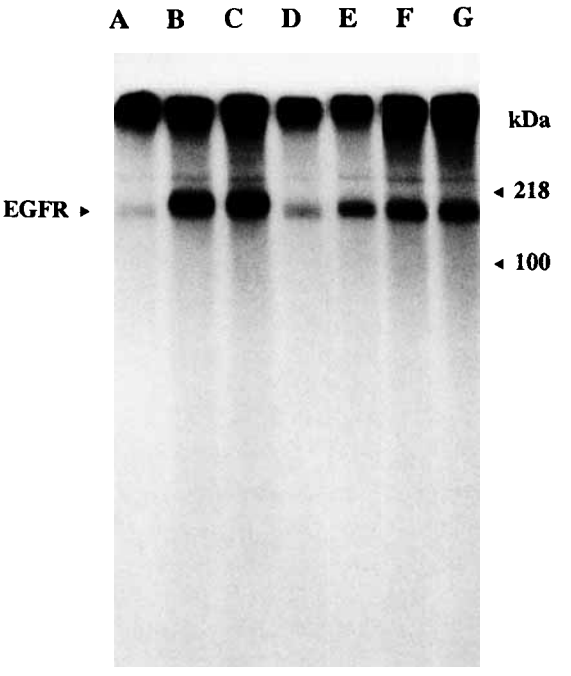

Fig. 5. Autoradiography of the electrophoretic pattern of EGFR autophosphorylation in ^{32}P-orthophosphate-labeled A431 cells induced by 45-min treatment of cells with 38 (**B**) and 77 (**C**) ng/ml EGF or with 5 (**D**), 50 (**E**), 150 (**F**), and 300 (**G**) μg/ml anti-EGFR mab 5-D43. Controls were performed with labeled cells without any induction (**A**). EGFR was precipitated from the cell lysates by antiphosphotyrosine mab 3-365-10 immobilized on protein A–Sepharose beads, followed by SDS–PAGE.

EGFR and phosphotyrosine proteins were isolated by immunoprecipitation in separate sets of experiments. For phosphotyrosine-specific precipitation, mab 3-365-10 (Fig. 7B,D). and for EGFR-specific precipitation mab 3-162 (Fig. 7A,C), an antibody that had no effect on EGF binding and kinase activity, defined by an extracellular SDS-insensitive EGFR epitope, was used. After preincubation with the blocking mab 1-594 (Fig. 7A,B), the phosphorylation pattern of the precipitate obtained with anti-EGFR 3-162 showed strongly reduced ^{32}P incorporation into EGFR compared with ^{32}P incorporation in the absence of mab 1-594 (Fig. 7C,D). The weak phosphorylation observed in the EGFR-specific precipitate (Fig. 7A) predominantly resulted from non-EGFR-mediated Ser/Thr phosphorylation since in the phosphotyrosine-specific precipitate only a trace band of labeled EGFR could be detected (Fig. 7B). Blocking of the EGFR kinase site also completely abolished tyrosine phosphorylation of other cellular proteins (Fig. 7B). Preincubation of A431 cell lysates in the absence of the blocking mab 1-594 yielded strong

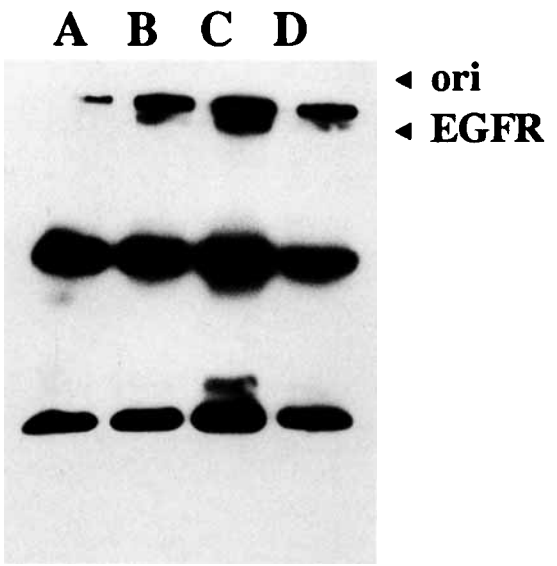

Fig. 6. Electrophoretic patterns of anti EGFR immune complexes obtained with anti-EGFR mab 3-162 (**A–C**) or mab 5-D43 (**D**) immobilized on Sepharose beads. Washed immune complexes were treated with 10 μmoles/ml ATP (A–D) and 1 μg/ml EGF (C) or 300 μg/ml mab 5-D43 (B) for 10 min at 37°C. The electrophoretic pattern was visualized with anti phosphotyrosine mab 3-365-10, using the "enhanced chemiluminescence" (ECL) detection system (Amersham International plc.), which uses antimouse immunoglobulin H and L chain antiserum.

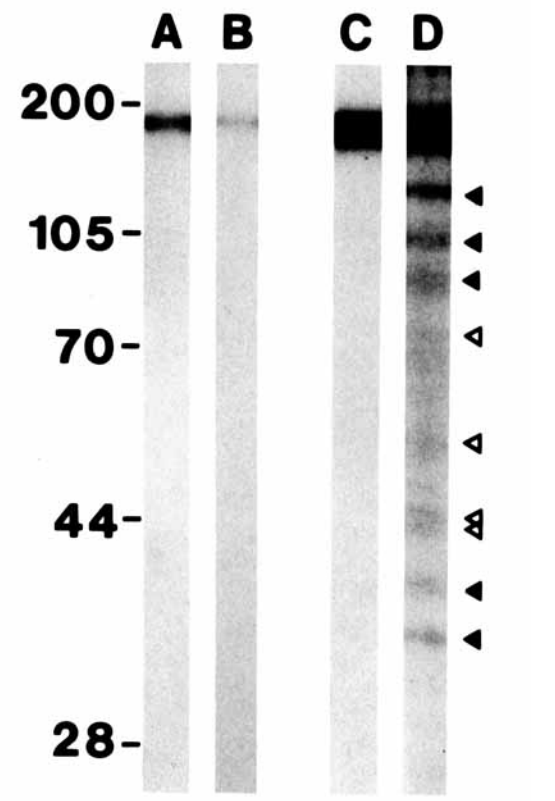

Fig. 7. Autoradiography of electrophoretic patterns of immunoprecipitates prepared from pretreated A431 cell lysates with anti-EGFR mab 3-162 (**A,C**) or antiphosphotyrosine mab 3-365-10 (**B,D**). Prior to precipitation, A431 cell lysates were preincubated with (A,B) or without (C,D) kinase-blocking mab 1-594 for 4 h, followed by addition of EGF (300 ng/ml) and phosphorylation with γ-^{32}P-ATP at room temperature for 30 min. Reference molecular masses are given as kDa.

phosphotyrosine-specific phosphorylation of EGFR and of five major cellular proteins with molecular masses of 145, 97, 85, 37, and 32 kDa (Fig. 7D). In addition, minor proteins with phosphorylated tyrosine residues were frequently detectable in these immunoprecipitates having molecular masses of 72, 54, 45, and 42 kDa (Fig. 7D).

DISCUSSION

The use of highly purified native EGFR as an immunizing antigen yielded several anti-EGFR monoclonal antibodies with conformational specificity. The epitope characteristics of the anti-EGFR antibodies used in the study described in this paper are listed in Table II. Since the immunizing EGFR sample was isolated in its tyrosine-phosphorylated form [Steinhilber et al., 1990], the conformational specificity of these mab is confined to the conformation of the activated EGFR molecule. One of these antibodies, mab 5-D43, characterized in our present investigations, showed an exceptional capability to induce most details of EGF-like signal transduction. Mab 5-D43 is defined by a site of the extracellular domain of EGFR but does not block EGF binding. Upon binding to viable untreated A431 cells, the mab 5-D43/EGFR complex is readily internalized in full analogy to the EGF ligand/receptor complex formation. However, internalization is not a prerequisite for mab 5-D43-induced activation of the EGFR kinase site and subsequent autophosphorylation, since mab 5-D43-induced activation of the EGFR kinase site also occurred in specific EGFR immune complexes with the antibody immobilized on protein A–Sepharose beads indicating that this activation is an intramolecular process. This finding is in agreement with the observation of kinase-active/internalization-defective EGFR in transformed cells [Chen et al., 1989]. The mechanism of this type of activation of the EGFR kinase site must be based on the alteration of distinct structural criteria enforced by the predefined specificity of mab 5-D43, implying a transmembrane conformational shift, shifting the conformation of the inactive kinase site to

TABLE II. Properties of the Anti-EGFR-Antibodies Used in This Study

mab	Sublass IgG	SDS-Sensitive epitope[a]	Blocking of EGF binding	Internalization	Activation of tyrosine kinase site	In vitro growth of A431	
						Inhibition	Proliferation
3-162	1κ	–	–	–	–	–	–
1-241	2Aκ	–	–	–	–	–	–
1-594	1κ	–	–	–	–	–	–
5-D43	1κ	+	–	+	+	–	+
5-I25	1κ	+	+	–(+)	–	+	–

[a]All mab recognize protein epitopes.

the active state. This mechanism might also apply to EGF-induced activation of the EGFR kinase site, at least in cells with kinase-active but internalization-defective EGFR. Moreover, binding of mab 5-D43 to EGFR of intact cells inducing an increase of the proliferation rate of cells in vitro in spite of internalization, is not necessarily in contradiction to the hypothesis that ligand-induced internalization serves to terminate or attenuate the mitotic signal [Chen et al., 1989]. An antibody-mediated acceleration of tumor growth in vivo and enhancement of autophosphorylation in the immune complex kinase assay was also reported for a monoclonal antibody against the ERBB2 gene product [Stancovski et al., 1991].

According to our data, signal transduction by a conformational shift activating the EGFR kinase site appeared to be restricted to at most two different conformational sites of the extracellular EGFR domain: one site is defined by mab 5-D43 and the other by the site of EGF-binding. However, the conformational epitope of EGF-binding was not affected by binding of mab 5-I25, which blocks subsequent EGF binding, as well as both sites were not affected by all the other extracellularly binding anti-EGFR mab characterized by us.

Our data do not support the assumption that ligand-induced activation of the EGFR kinase domain is mediated by receptor dimerization or oligomerization [Schlessinger, 1988; Williams, 1989], which appears to be initiated by ligand binding and a subsequent conformational alteration of the extracellular EGFR domain. On the other hand, our data do not exclude in situ requirement of dimerization for EGFR activation. Since we could show that the signal for activation of the EGFR kinase site was also transduced in immobilized EGFR immune complexes, i.e., immobilized EGFR, when treated with EGF or anti-EGFR mab 5-D43, multiple pathways of signal transduction have to be assumed.

Mab 5-I25-induced blocking of EGF binding consequently resulted in blocking of EGF-induced autophosphorylation of EGFR and in inhibition of cell proliferation in vitro and tumor growth in vivo. Similar blocking effects were described for monoclonal antibodies No. 425 [Murthy et al., 1987], defined by a conformation-dependent epitope common to both high- and low-affinity EGFR, mab 528 [Kawamoto et al., 1983; Wu et al., 1989], mab LA 22, LA 58, and LA 80 [Wu et al., 1989] essentially defined by a sequence of 14 amino acids from Ala 351 to Asp 364 of the mature human EGFR and mab 96, 108 [Lax et al., 1989] both binding to epitopes also located in domain III of human EGFR. However, inhibition of tumor growth by our mab 1-241, defined by an SDS-insensitive extracellular EGFR epitope, is not well understood. This mab did neither block EGF binding nor EGF-induced autophosphorylation. The critical point of this phenomenon may be seen in its IgG2a isotype which was reported to specifically inhibit growth of human tumors in nude mice via IgG2a-dependent macrophage-mediated cytotoxicity whereas all antitumor antibodies belonging to the other immunoglobulin classes (IgG1, IgG2b, IgG3, IgM, and IgA) showed no inhibition of tumor growth [Herlyn and Koprowski, 1982]. This mechanism might also apply to tumor-inhibiting effects of some other anti-EGFR mab of the IgG2a isotype reported by other groups [Masui et al., 1984; Masui et al., 1986; Rodeck et al., 1987; Aboud-Pirak et al., 1988]. In case of our IgG2a-isotype anti-EGFR mab 1-241, it is still open as to why part of the animals remained tumor free and another part died in spite of only very small tumors.

Mab 1-594 that blocks the kinase site of EGFR opened up the possibility to obtain information on cellular substrates of EGFR by comparing

EGF-induced phosphorylation of cell lysates in the presence and absence of the blocking antibody. A variety of tyrosine-phosphorylated components could be correlated with the EGF-induced EGFR-mediated reaction comprising the already known substrates phospholipase C-γ [Margolis et al., 1989; Meisenhelder et al., 1989; Wahl et al., 1990; Nishibe et al., 1990; Kim et al., 1990] and MAP-kinase [Rossomando et al., 1989; L'Allemain et al., 1991]. However, our data are the result of a 30-min phosphorylation reaction and are hampered by the possibility that during this time rapid desensitization of the kinase activity [Countaway et al., 1992] or dephosphorylation might have occurred. Therefore, the investigation of phosphorylation kinetics are a goal for further studies.

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REFERENCES

Aboud-Pirak E, Hurwitz E, Pirak ME, Bellot F, Schlessinger J, Sela M (1988): Efficacy of antibodies to epidermal growth factor receptor against KB carcinoma in vitro and in nude mice. JNCI 80:1605–1611.

Braun S, Raymond WE, Racker E (1984): Synthetic tyrosine polymers as substrates and inhibitors of tyrosine-specific protein kinases. J Biol Chem 259:2051–2054.

Carpenter G (1985): Binding assays for epidermal growth factor. Methods Enzymol 109:101–110.

Chen WS, Lazar CS, Poenie M, Tsien RY, Gill GN, Rosenfeld MG (1987): Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. Nature 328:820–823.

Chen WS, Lazar CS, Lund KA, Welsh JB, Chang CP, Walton GM, Der CJ, Wiley HS, Gill GN, Rosenfeld MG (1989): Functional independence of the epidermal growth factor receptor from a domain required for ligand-induced internalization and calcium regulation. Cell 59:33–43.

Cooper J, Sefton B, Hunter T (1983): Detection and quantification of phosphotyrosine in proteins. Methods Enzymol 99:387–405.

Countaway JL, Nairn AC, Davis RJ (1992): Mechanism of desensitization of the epidermal growth factor receptor protein-tyrosine kinase. J Biol Chem 267:1129–1140.

Czech MP, Klarlund JK, Yagaloff KA, Bradford AP, Lewis RE (1988): Insulin receptor signaling. J Biol Chem 263: 11017–11020.

DiRenzo MF, Ferracini R, Naldini L, Giordano S, Comoglio PM (1986): Immunological detection of proteins phosphorylated at tyrosine in cells stimulated by growth factors or transformed by retroviral-oncogene-coded tyrosine kinases. Eur J Biochem 158:383–391.

Downward J, Yarden Y, Mayes E, Scarce G, Toffy N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD (1984): Close similarity of epidermal growth factor receptor and v-erbB oncogene protein sequences. Nature 307:521–527.

Ellis C, Moran M, McCormick F, Pawson T (1990): Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. Nature 343:377–381.

Engvall E (1980): Enzyme immunoassay ELISA and EMIT. Methods Enzymol 70:419–439.

Fabricant R, DeLarco J, Todaro G (1977): Nerve growth factor receptors on human melanoma cells in culture. Proc Natl Acad Sci USA 74:565–569.

Fitzpatrick SL, Brightwell J, Wittliff JL, Barrows GH, Schultz GS (1984): Epidermal growth factor binding by breast tumor biopsies and relationship to estrogen receptor and progestin receptor levels. Cancer Res 44:3448–3453.

Galfré G, Milstein C (1981): Preparation of monoclonal antibodies: Strategies and procedures. Methods Enzymol 73:3–46.

Hawkes K, Niday E, Gordon J (1982): A dot-immunobinding assay for monoclonal and other antibodies. Anal Biochem 119:142–147.

Hendler FJ, Ozanne B (1984): Squamous-cell lung cancer express increased epidermal growth factor receptors. J Clin Invest 74:647–651.

Herlyn D, Koprowski H (1982): IgG2a monoclonal antibodies inhibit human tumor growth through interaction with effector cells. Proc Natl Acad Sci USA 79:4761–4765.

Kawamato T, Sato JD, Le A, Polikoff J, Sato GH, Mendelsohn J (1983): Growth stimulation of A 431 cells by epidermal growth factor: Identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. Proc Natl Acad Sci USA 80:1377–1341.

Kim JW, Sim SS, Kim UH, Nishibe S, Wahl MI, Carpenter G, Rhee SG (1990): Tyrosine residues in bovine phospholipase C-γ phosphorylated by the epidermal growth factor receptor in vitro. J Biol Chem 265:3940–3943.

Laemmli UK (1970): Cleavage of structural proteins during assembly of the heads of bacteriophage T4. Nature 227: 680–685.

L'Allemain G, Sturgill T, Weber MJ (1991): Defective regulation of mitogen-activated protein kinase activity in a 3T3 cell variant mitogenically nonresponsive to tetradecanoyl phorbol acetate. Mol Cell Biol 11:1002–1008.

Lax I, Bellot F, Howk R, Ullrich A, Givol D, Schlessinger J (1989): Functional analysis of the ligand binding site of EGF-receptor utilizing chimeric chicken/human receptor molecules. EMBO J 8:421–427.

Liberman TA, Nusbaum H, Razen N, Kris R, Lax I, Soreq H, Whittle N, Waterfield MD, Ullrich A, Schlessinger J (1985): Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. Nature 313:144–147.

Mangelsdorf-Soderquist A, Carpenter G (1985): Glycosylation of the epidermal growth factor receptor in A431 cells. J Biol Chem 259:12568–12594.

Margolis B, Li N, Koch A, Mohammadi M, Hurwitz DR, Zilberstein A, Ullrich A, Pawson T, Schlessinger J (1990): The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC-γ. EMBO J 9:4375–4380.

Margolis B, Rhee SG, Felder S, Mervic M, Lyall R, Levitzki A, Ullrich A, Zilberstein A, Schlessinger J (1989): EGF induces tyrosine phosphorylation of phospholipase C-II: A potential mechanism of EGF receptor signalling. Cell 57: 1101–1107.

Masui H, Kawamoto T, Sato JD, Wolf B, Sato G, Mendelsohn J (1984): Growth inhibition of human tumor cells in athymic mice by anti epidermal growth factor receptor monoclonal antibodies. Cancer Res 44:1002–1007.

Masui H, Moroyama T, Mendelsohn J (1986): Mechanism of antitumor activity in mice for anti epidermal growth factor receptor monoclonal antibodies with different isotypes. Cancer Res 46:5592–5598.

Meisenhelder J, Suh PG, Rhee SG, Hunter T (1989): Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. Cell 57;1109–1122.

Murthy U, Basu A, Rodeck U, Herlyn M, Ross AH, Das M (1987): Binding of an antagonistic monoclonal antibody to an intact and fragmented EGF-receptor polypeptide. Arch Biochem Biophys 252:549–560.

Nishibe S, Wahl MI, Wedegaertner PB, Kim JJ, Rhee SG, Carpenter G (1990): Selectivity of phospholipase C phosphorylation by the epidermal growth factor receptor, the insulin receptor and their cytoplasmic domains. Proc Natl Acad Sci USA 87:424–428.

Peterson GL (1977): A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356.

Rodeck U, Herlyn M, Herlyn D, Molthoff C, Atkinson B, Varello M, Steplewski Z, Koprowski H (1987): Tumor growth modulation by monoclonal antibody to the epidermal growth factor receptor: Immunologically mediated and effector cell-independent effects. Cancer Res 47:3692–3696.

Rosen OM (1987): After insulin binds. Science 237:1452–1458.

Rossomando AJ, Pyne DM, Weber MJ, Sturgill TW (1989): Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. Proc Natl Acad Sci USA 86:6940–6943.

Rylatt DB, Parish CR (1982): Protein determination on an automatic spectrophotometer. Anal Biochem 121:213–214.

Schlessinger J (1988): Signal transduction by allosteric receptor oligomerization. Trends Biochem Sci 13:443–447.

Schlessinger J, Schreiber AB, Levi A, Lax I, Liberman TA, Yarden Y (1983): Regulation of cell proliferation by epidermal growth factor. Crit Rev Biochem 14:93–111.

Shulman M, Wilde CD, Koehler G (1978): A better cell line for making hybridomas secreting specific antibodies. Nature 276:269–270.

Stancovski I, Hurwitz E, Leitner O, Ullrich A, Yarden Y, Sela M (1991): Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth. Proc Natl Acad Sci USA 88:8691–8695.

Stavrou D, Keiditsch E, Schmidberger F, Bise K, Funke I, Eisenmenger W, Kurrle R, Martin B, Stocker U (1987): Monoclonal antibodies against human astrocytomas and their reactivity pattern. J Neurol Sci 80:205–220.

Steinhilber G, Sproll MW, Wolff JM, Anderer FA (1990): Highly specific characterization of tyrosine phosphoproteins in tumor cells based on monoclonal antibodies defined by conjugated phosphotyramine. Anticancer Res 10: 907–912.

Ullrich A, Schlessinger J (1990): Signal transduction by receptors with tyrosine kinase activity. Cell 61:203–212.

Wahl MI, Nishibe S, Suh PG, Rhee SG, Carpenter G (1989): Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase C-II independently of receptor internalization and extracellular calcium. Proc Natl Acad Sci USA 86:1568–1572.

Williams LT (1989): Signal transduction by the platelet-derived growth factor receptor. Science 243:1564–1570.

Wong AJ, Bigner SH, Bigner DD, Kinzler KW, Hamilton SR, Vogelstein B (1987): Increased expression of the epidermal growth factor receptor gene in malignant gliomas is variably associated with gene amplification. Proc Natl Acad Sci USA 84:6899–6903.

Wu D, Wang, Sato GH, West KA, Harris WR, Crabb JW, Sato JD (1989): Human epidermal growth factor (EGF) receptor sequence recognized by EGF competitive monoclonal antibodies. J Biol Chemn 264:17469–17475.

Yarden Y, Ullrich A (1988): Growth factor receptor tyrosine kinases. Annu Rev Biochem 57:443–478.