A431 Cell Variants Lacking the Blood Group A Antigen Display Increased High Affinity Epidermal Growth Factor-Receptor Number, Protein-Tyrosine Kinase Activity, and Receptor Turnover

L. H. K. Defize,* D. J. Arndt-Jovin,‡ T. M. Jovin,‡ J. Boonstra,§ J. Meisenhelder,ǁ
T. Hunter,ǁ H. T. de Hey,¶ and S. W. de Laat*

*Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht; The Netherlands;
‡Max Planck Institut für Biophysikalische Chemie, Department of Molecular Biology, D-3400 Göttingen, Federal Republic
of Germany; §Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands;
ǁMolecular Biology and Virology Laboratory, The Salk Institute, San Diego, California 92138; ¶Vrije Universiteit,
Department of Medical Chemistry, Boechorststraat 7, 1007 MC Amsterdam.

Abstract. The epidermal growth factor receptor (EGF-R) of human A431 cells bears an antigenic determinant that is closely related to the human blood group A carbohydrate structure. Labeling studies with blood group A reactive anti-EGF-R monoclonal antibodies and various lectins revealed that A431 cultures are heterogeneous with respect to blood group A expression. We have isolated clonal variants of these cells that either express (A431A⁺ cells) or completely lack (A431A⁻ cells) the blood group A specific N-acetyl-D-galactosamine (GalNAc) residue. We show that this difference is due to the absence of a UDP-GalNAc:Gal transferase activity in A431A⁻ cells.

Subsequently, we have compared EGF-R functioning in these cell lines. Scatchard analysis of EGF-binding shows that in A431A⁻ cells 6.3% of the EGF-R belongs to a high affinity subclass (K_d = 0.4 nM) while in A431A⁺ this subclass represents only 3.2% of the total receptor pool. The elevated level of high affinity receptors in A431A⁻ cells is accompanied by a parallel increase in receptor protein⁻ tyrosine kinase activity. In membrane preparations of A431A⁻ cells, receptor autophosphorylation as well as phosphorylation of a tyrosine-containing peptide substrate is 2–3-fold higher as compared with A431A⁺ cells. In intact A431A⁻ cells, the difference in receptor activity is measured as a 2–3-fold elevated level of receptor phosphorylation and a 2–3-fold higher abundance of phosphotyrosine in total cellular protein in A431A⁻ cells. In addition, [³⁵S]methionine pulse-chase experiments showed a ligand-independent increase in turnover of EGF-R in A431A⁻ cells: the receptor's half life in these cells is 10 h as compared with 17 h in A431A⁺ cells. Our results suggest a possible involvement of GalNAc residue(s) in determining EGF-R affinity, protein-tyrosine kinase activity and turnover in A431 cells. Furthermore, our results indicate that high affinity EGF-R are the biologically active species with respect to protein-tyrosine kinase activity.

THE human epidermal growth factor receptor (EGF-R)[1] is a structurally well-characterized transmembrane glycoprotein (for reviews see references 3, 9, and 31). The intracellular part of the receptor has a strong homology with the avian v⁻ erb-B oncogene (12) and contains a protein-tyrosine kinase activity which is activated after EGF binding, leading to tyrosine phosphorylation of intracellular substrates as well as of the receptor itself (auto-phosphorylation) (24). The extracellular part contains the EGF-binding domain and is heavily glycosylated. It has been estimated that of the 12 potential N-linked glycosylation sites, 11 are occupied by carbohydrate structures, adding 30–40 kD to the molecular mass of the receptor (27).

The human epidermoid carcinoma A431 cell line is a widely used model system in studies on the EGF-R, since it expresses an unusually high number of these receptors at its surface (i.e., 2–3 × 10⁶/cell) (13). Using the criteria of Childs et al. (5), at least six different receptor carbohydrate side chains display blood group relatedness in these cells. A number of monoclonal antibodies directed against the EGF-R have been produced using membrane preparations of A431 cells as immunogen. Most of these antibodies are directed against carbohydrate determinants which are reported to be present on other glycoproteins and glycolipids as well (11, 28). Measurements of binding of a number of the antibodies to receptor preparations in the presence of carbohydrates of

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1. *Abbreviations used in this paper:* EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; GalNAc, N-acetyl-D-galactosamine; DBA, dolichos Biflorus Agglutinin; UEA-1, Ulex Europaeus Agglutinin-1.

known composition have shown that the antigenic determinants recognized are closely related to (or identical with) the human blood group A antigen (18, 19, 28).

This apparently immunodominant determinant is characterized by a terminal N-acetyl-D-galactosamine (GalNAc) residue:

$$GalNAc\alpha1-3Gal\beta1-3/4GlcNAc-$$

GalNAcα1−3Galβ1−3/4GlcNAc−
2   2/4
↑   ↑
1   1
Fucα   Fucα.
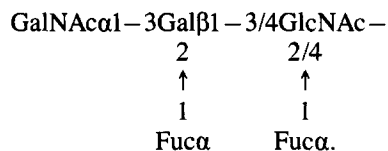
The role or influence of carbohydrates on receptor function is by no means clear. Experiments with glycosylation inhibitors have suggested that the receptor needs to be N-glycosylated for proper membrane insertion, EGF binding, and to serve as phosphate acceptor (35, 37). However, glycosylation as such seems not sufficient and additional processing steps are required for proper receptor functioning (36).

In the present study, we report the existence of A431 variants which are not recognized by blood group A reactive anti–EGF-R monoclonal antibodies and lectins. These variants comprise ∼40% of the total number of cells in the parental A431 line. We have subcloned the parental A431 cells and obtained a line that fully expressed (A431A$^+$) and a line that completely lacked (A431A$^-$) the A-antigen. We show that the difference in blood group A expression is due to the absence of UDP-GalNAc:Gal transferase activity from A431A$^-$ cells.

Comparison of EGF-R properties in the clones showed that in clone A431A$^-$ there is: (a) an elevated amount of high affinity EGF-R, (b) enhanced EGF-R tyrosine kinase activity, and (c) a shorter half-life of the receptor as measured by [^{35}S]methionine pulse-chase experiments. These results suggest that alterations in the composition of carbohydrates shared by EGF-R and other plasma membrane components may influence EGF-R function by affecting the amount of high affinity EGF-binding sites. Furthermore, our results indicate that an elevated high/low affinity EGF-R ratio leads to enhanced receptor protein-tyrosine kinase activity and turnover, even in the absence of any added EGF.

Materials and Methods

Cell Culture

The parental A431 cell line as well as the clones derived from it were grown in DME supplemented with 7.5% FCS and buffered with 44 mM NHCO$_3$ under a 7.5% CO$_2$ atmosphere.

Anti–EGF-R Antibodies

Monoclonal antibodies T1-5 and 29.1 were a gift of J. Schlessinger (Rehovot, Israel). Antibodies 2E9, 2D11, and 2G5 were obtained by immunizing mice with A431 plasma membrane vesicles, prepared as described (7). Antibodies were purified from ascites of hydridoma-injected mice by affinity chromatography, at pH 8.1, on a protein A Sepharose column (Pharmacia, Woerden, The Netherlands) as described (10). Antiserum 281-7 was prepared by immunizing rabbits with a synthetic EGF-R polypeptide sequence coupled to keyhole limpet haemocyanin (KLH). The sequence used was the same as described by Kris et al. (26).

Antibody–Glycolipid Recognition

A431 cells grown to confluency on roller bottles (Falcon, Becton and Dickinson Etten, Leur, The Netherlands) were scraped with a rubber policeman

into PBS. The cells were pelleted at 2,000 g and lysed by sonication in water at 0°C. The sonicated cells were extracted with 30 ml chloroform/methanol (2:1). The fluffy protein layer was reextracted with 30 ml chloroform/methanol/H$_2$O (1:2:0.1). The organic phases were combined and dried under N$_2$. The dried residue was taken up in 4 ml chloroform/methanol/H$_2$O (30:60:8) and kept under a N$_2$ atmosphere at −20°C. The isolated glycolipids were applied on aluminium foil high performance thin layer silicagel plates (Merck, Amsterdam, The Netherlands), 10 μl for each lane. Separation was by ascending chromatography in chloroform/methanol/water +0.02% CaCl$_2$2H$_2$O (60:35:8). After completion, the plates were dried under N$_2$ and the lanes were separated using a pair of scissors. Before incubation with antibodies, the lanes were dipped in a saturated solution of polyisobutyl-methacrylate (Janssen, Beerse, Belgium) in n-butanol. The lanes were treated subsequently with (a) 10% newborn calf serum in PBS (to saturate the lanes with protein), (b) a dilution of monoclonal antibody, (c) a 1,000-fold dilution of rabbit anti–mouse peroxidase (Nordic, Tilburg, The Netherlands), and (d) peroxidase substrate (3,3′, 5′, 5′-tetramethyl benzidine) (Sigma Chemical Co., St. Louis, MO) in citrate buffer, pH 5.0, containing dioctyl sodium sulfosuccinate.

Fluorescent Labeling of Cells

For labeling experiments, cells were grown to near confluency on glass coverslips. Cells were fixed with 3% p-formaldehyde in PBS either before or after antibody/lectin incubations. Fluorescent-labeled lectins (E. Y., San Mateo, CA) were used at 25 μg/ml. For immunofluorescence, FITC- or rhodamine isothiocyanate–labeled rabbit anti–mouse antibodies (Nordic) were used as secondary reagents. After labeling, cells were mounted in polyvinyl alcohol (Moviol 7/88) containing medium and observed under a Zeiss microscope.

Subcloning of Cells

For subcloning, A431 cells were seeded at one cell per three wells on 96-well tissue culture clusters (Costar, Badhoevedorp, The Netherlands). Individual colonies were removed from the plates by trypsinization, replated, and screened for homogeneity by immunofluorescence. This cycle was repeated several times to obtain homogeneous clones. During the course of the experiments, clones were repeatedly checked for variants by immunofluorescence.

Measurements of N-acetyl Galactosamine Transferase Activity

Confluent A431 cells were scraped with a rubber policeman and spun down at 800 g. Cells were washed twice with PBS and lysed in a small volume of H$_2$O containing 0.01 mM each of the following protease inhibitors: N-tosyl-L-phenyl alanyl chloromethane (TPCK), N-tosyl-L-lysyl chloromethane (TLCK), phenylmethylsulfonyl fluoride (PMSF), and pepstatin. Lysis was enhanced by a Pjotter-Elvehjem system rotating at 1,200 rpm. 1 mg of cellular protein was added to an incubation mixture containing 100 mM MES (2[N-Morpholino] ethane sulfonic acid), adjusted to pH 6.7, with NaOH/20 mM MnCl$_2$/1% Triton X-100, 7 μM uridine diphosphate [^{14}C]GalNAc (60 Ci/mol sp act) and 0.255 mg A$^-$ porcine submaxillary mucin (PSM) as the acceptor substrate. Incubation was at 37°C for 1 h, after which period the PSM was precipitated by addition of 1 ml 1% phosphotungstic acid (PTA) in 0.5 M HCl at 0°C. The precipitate was washed twice with PTA and once with methanol, and radioactivity was measured in a Packard liquid scintillation counter.

Preparation of Total Cell Lysates

Total cell lysates were prepared by scraping confluent cells with a rubber policeman into PBS. Cells were collected by centrifugation at 500 g for 3 min and subsequently lysed by a 20-min incubation at 0°C in buffer containing 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol and 1 mM PMSF. Nuclei and debris were spun down at 12,000 g for 5 min at 4°C. The supernatants were collected and kept in small aliquots at −70°C.

Plasma Membrane Preparations

Plasma membrane preparations were prepared either by the method of Thom et al. (39), or by vesiculation of the cells as described by Cohen et al. (7).

EGF Binding to Intact Cells and Membrane Preparations

Monolayers of cells grown on 24-well tissue culture clusters (Costar) were washed three times with PBS. Mixtures of ^{125}I-labeled EGF (Amersham, Houten, The Netherlands, 100 μCi/μg sp act) and unlabeled EGF (receptor grade, Collaborative Research, Waltham, MA) were prepared in DME containing 0.1% BSA. For determination of high affinity binding, cells were preincubated with 300 nM of antibody 2E9 for 180 min at 20°C. 500 μl of mixture was added to the cells followed by an incubation for 120 min at 0°C. After this period, cells were washed five times with PBS, taken up in 0.5 N NaOH, and cell associated radioactivity was measured in a gamma counter. Nonspecific binding was determined by adding an excess of unlabeled EGF.

EGF binding to membrane preparations was determined by incubation of 50 μg membrane protein with different amounts of [^{125}I]EGF. Incubation was for 30 min at 20°C. After this period, the membranes were collected and washed by vacuum aspiration on cellulose acetate filters (Schleicher & Schuell, Hertogenbosch, The Netherlands, pore diameter 0.2 μm). The filters were dried and counted in a gamma counter.

EGF-R Tyrosine Kinase Activity Measurements In Vitro

EGF-R preparations (total cell lysates or plasma membranes) were incubated with or without EGF for 10 min at 20°C. The phosphorylation reaction was started by adding a mixture containing 20 mM Hepes, pH 7.4, 2 mM MnCl$_2$, 10 mM p-nitrophenylphosphate (PNPP), 40 μM Na$_3$VO$_4$, 0.01% BSA, 15 μM ATP and 1 μCi [γ-^{32}P]ATP (Amersham, UK, 3,000 Ci/mmol sp. act.) in a final volume of 60 μl. The reaction was allowed to proceed for 10 min on ice, and was subsequently stopped by boiling in Laemmli sample buffer for SDS-gel electrophoresis, or by application of the mixture to Whatmann 3-mm filters which were then soaked in a 10% TCA solution containing 10 mM Na$_4$P$_2$O$_7$ for determination of total endogenous membrane ^{32}P-incorporation. Phosphorylation of the tyrosine-containing peptide angiotensin I was essentially the same, except the incubation was in the presence of 0.2% Nonidet P-40 (NP-40) and for 5 min at 20°C. Phosphorylated membrane proteins were separated from the peptide by TCA precipitation. The phosphorylated peptide was collected by applying the supernatant to phosphocellulose paper (Whatman P81) which was subsequently washed with 7.5 mM H$_3$PO$_4$.

EGF-R and Total Cellular Phosphotyrosine Content in Intact Cells

Cells grown to subconfluency (150,000 cells/cm^2) on 35-mm dishes (Costar) were labeled with 1 mCi/ml [^{32}P]orthophosphate (Amersham) in phosphate-free DME (Gibco Breda, The Netherlands, Pharmacia, Woerdon, The Netherlands) for 16 h. EGF (50 ng/ml) or buffer were added to the same 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$_2$PO$_4$, 5 mM EDTA, 0.1 mM Na$_3$VO$_4$, 1% NP-40, 1% sodium desoxycholate, 0.1% SDS, 1 mM PMSF, 100 KIU ml Aprotinin). For receptor phosphoamino acid analysis, the receptor was precipitated using antiserum 281-7 and protein A-sepharose beads (Pharmacia). 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$_4$HCO$_3$/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$_2$. By this procedure, ∼70% of the counts present in the gel were recovered as measured by Cerenkov radiation. The pellets were resuspended in a small volume of formic acid and used for both phosphoaminoacid analysis and tryptic phosphopeptide mapping. Total cellular protein was extracted from RIPA lysates of parallel series of dishes by phenol extraction as described by Hunter and Sefton (22). Isolated receptor protein was hydrolysed in 6 M HCl at 110°C for 60 min. Total cellular protein was hydrolyzed in 6 M HCl at 110°C for 120 mins. The hydrolysed samples were applied to plastic foil thin layer cellulose plates (Merck, 5577) together with 60 ng each of unlabeled P-SER, P-THR, and P-TYR. Two-dimensional electrophoresis, at pH 1.9, and pH 3.5, were as described by Cooper and Hunter (8). 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 in a liquid scintillation counter.

Tryptic Phosphopeptide Mapping of EGF-R

Aliquots of the formic acid resuspended TCA pellets (see above) were dried in a Speed-Vac centrifuge (Savant, Hicksville, NY). Samples were subsequently treated with performic acid and trypsin as described by Beemon and Hunter (1). Tryptic phosphopeptides were separated on thin layer cellulose plates (Merck 5716) by electrophoresis, at 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$_2$O (75:50:15:60).

Receptor Turnover

Cells, grown to subconfluency on 12-well tissue culture clusters (Costar) were labeled for 16 h with 40 μCi [^{35}S]methionine (Amersham, 1,350 Ci/mmol sp act) per ml of methionine-free DME containing 0.5% FCS at $t = 0$. The labeling medium was replaced with DME/0.5% FCS containing 500 μg/ml methionine. At different time intervals, cells were scraped in RIPA buffer and the receptor was precipitated with antiserum 281-7. The precipitates were applied to a 5-15% linear SDS-polyacrylamide gel. Receptor was eluted from the gel after autoradiography as described under the previous heading. Radioactivity of the eluate was measured in a liquid scintillation counter.

Results

Reactivity of Antibodies with A431 Antigens

The properties of anti-EGF-R monoclonal antibodies TL-5-IgG3 and 29.1-IgG1 have been described in detail elsewhere (19, 32, 33). We have raised a number of EGF-R reactive antibodies ourselves (10). Three of these: 2E9-IgG1, 2D11-IgG3 and 2G5-IgG2A have been used in the present study. Fig. 1 A shows that these antibodies precipitate autophospho-
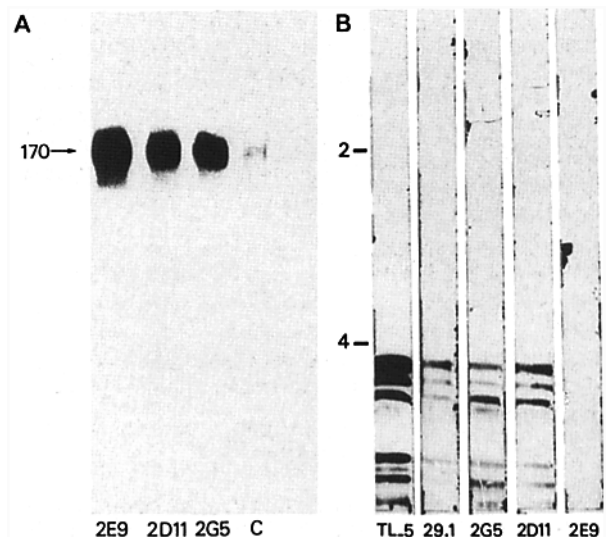

Figure 1. EGF-R precipitation and glycolipid recognition by monoclonal antibodies. (A) Autoradiograph of ^{32}P-labeled immunoprecipitates subjected to electrophoresis on SDS-PAGE. A431 plasma membrane preparations were treated with EGF and [^{32}P]γ-ATP, solubilized and immunoprecipitated with the indicated antibodies (C = nonimmune mouse serum, 100× diluted). (B) Immunoperoxidase staining of glycolipid extracts of A431 cells separated on silicagel plates and treated with the indicated antibodies. The numbers on the left side indicate glycolipid standards GL$_2$ and GL$_4$ (two and four sugar residues linked to ceramide respectively).

rylated EGF-R from A431 plasma membrane preparations. Antibodies 2D11 and 2G5 are, like TL-5 and 29.1 directed against blood group A–related carbohydrate determinants. These antibodies specifically haemagglutinate A-antigen–bearing erythrocytes. Furthermore, they are unable to precipitate biosynthetically labeled receptor from cells treated with tunicamycin. Antibody 2E9 does not haemagglutinate A-bearing erythrocytes and does recognize the unglycosylated 130-kD form of the receptor from tunicamycin treated A431 cells (Defize, L. H. K., J. Boonstra, and S. W. De Laat, manuscript in preparation). As was reported for other EGF-R antibodies reactive with carbohydrates (16, 20), the number of binding sites for 2D11 and 2G5 on A431 cells exceeds the number of EGF-R by about one order of magnitude (not shown). Antibody TL-5 on the other hand, despite its blood group A reactivity, was reported to recognize only $\sim$1.2 × 10^6 antigenic sites on A431 cells (32). Several blood group A reactive anti–EGF-R antibodies have been reported to cross-react with glycolipids (16, 28). This has not yet been reported for TL-5 and 29.1. Fig. 1 B shows that 2D11 and 2G5, as well as 29.1 and also TL-5 indeed recognize a number of complex glycolipids isolated from A431 cells. On the other hand antibody 2E9 has no such cross-reactivity (Fig. 1 B).

Immunofluorescence and Lectin Binding on A431 Cells

Immunofluorescence studies revealed that in A431 cells 30–40% of the cells were not recognized by any of the four anti–carbohydrate antibodies (shown for 2D11 in Figs. 2, A and B). This phenomenon was not restricted to the particular clone used in our laboratory (obtained from G. Todaro, Oncogene, Seattle, WA) since we observed the same differential labeling in A431 cells obtained from another source (J. Schlessinger, Rehovot, Israel). In contrast, fluorescein-ated EGF and antibody 2E9 labeled all cells with equal intensity (shown for 2E9 in Fig. 2, C and D). Thus, the nonreactivity of cells with 2D11, 2G5, TL-5, and 29.1 was not due to low levels or absence of receptor, but reflected a variance in the carbohydrate structure of the membrane. This variance was not correlated with the passage number of the cells, since the two cell types coexisted over prolonged periods of culture time, without any apparent change in relative numbers (up till 80 passages, not shown).

As reported earlier, antibody 2D11 mimics EGF in inducing rapid rounding up of A431 cells (10). As shown in Fig. 2, E and F, only the cells that are labeled by this antibody are rounded up, demonstrating that this effect is caused specifically by the binding of 2D11 and in addition that there is no variation between the antibody-reactive cells with respect to this phenomenon. EGF-induced morphological changes in all cells, eliminating a structural defect in the cytoskeleton of the unlabeled cells as a basis for their unresponsiveness to 2D11.

To learn more about the structural differences in membrane carbohydrates in the two cell types, a number of lectins were tested by double-labeling experiments together with the antibodies. Two lectins, Dolichos biflorus lectin (DBA) (an obligate exogalactosaminolectin [17]) and Ulex europaeus lectin (UEA-1) (an obligate exofucolectin [17]), gave unambiguous differential labeling of the two clones. Fluorescein-labeled derivatives of these lectins were added together with rhodamine-labeled antibody 2D11. As can be seen in Fig. 3,

D–F, only those cells which are recognized by 2D11 are also heavily labeled by DBA. In contrast, only those cells which are not recognized by 2D11 bind UEA-1 (Fig. 3, A–C).

These results suggested that the cells which do not bind blood group A reactive anti–EGFR monoclonal antibodies are missing the terminal N-acetyl galactosamine residue. Such a truncation would result in a blood group H-like structure, which has a terminal fucose residue, confirmed by the reactivity of the antibody/DBA negative cells with UEA lectin. Interestingly, Fredman et al. (16) and Willingham and Pastan (40) reported differential staining of A431 cells with an anti–EGF-R monoclonal antibody directed against a blood group H-like structure.

Cloning of A431 Subtypes

The A431 cells were subcloned by limiting dilution (see Materials and Methods) and clones were screened for reactivity with antibodies 2D11, 2G5, Tl-5, and 29.1. Clones which reacted seemingly uniform (positively or negatively) with the antibodies were recloned. All clones positive for the antibodies also reacted uniformly with Fl-DBA. Conversely, the negative cells were unable to bind Fl-DBA, but reacted uniformly with Fl-UEA (not shown). After several rounds of subcloning two particular clones, designated A431A$^+$ and A431A$^-$ respectively, were selected for further biochemical characterization. The differential A-antigen expression was a stable phenotypical trait for both clones and did not change even after prolonged periods of culture.

Biochemical Characterization of the Difference in Glycosylation Between the Clones

The lectin-binding studies suggested that in surface carbohydrates of A431A$^-$ cells only the terminal GalNAc residue is absent. Furthermore, there was no detectable difference in the molecular mass of EGF-R from the two clones (Fig. 5), excluding large truncations in receptor carbohydrates. An important clue to the nature of the A431A$^-$ phenotype came from measurements of UDP-GalNAc:Gal transferase activity in cell lysates of the clones. This enzyme converts A$^-$ carbohydrate structures into blood group A determinants by catalyzing an α1–3 glycosidic linkage of a GalNAc residue (34). We quantitated UDP-GalNAc:Gal activity in cell lysates by measuring incorporation of ^{14}C-labeled UDP-GalNAc into A$^-$ porcine submaxillary mucine (PSM, isolated from porcine submaxillary glands as described (34). The results of these measurements are given in Table I. The activity of UDP-GalNAc:Gal transferase is very high in clone A431A$^+$ as compared with clone A431A$^-$, strongly suggesting that the inactivity or absence of this enzyme in the latter cells constitutes the biochemical basis for their A$^-$ phenotype.

Properties of EGF-R in A431A$^+$ and A431A$^-$ Cells

EGF Binding. To determine whether the difference in receptor carbohydrate composition had any consequence for EGF-R activity, we compared EGF-R properties in the clones. First, the number of receptors per cell and the kinetics of ^{125}I-EGF binding were determined. Scatchard analysis of binding data (Fig. 4, left panel) revealed two receptor classes for each cell type, while the total amount of EGF-R was slightly higher in A431A$^-$ cells: 2.8 × 10^6 receptors com-
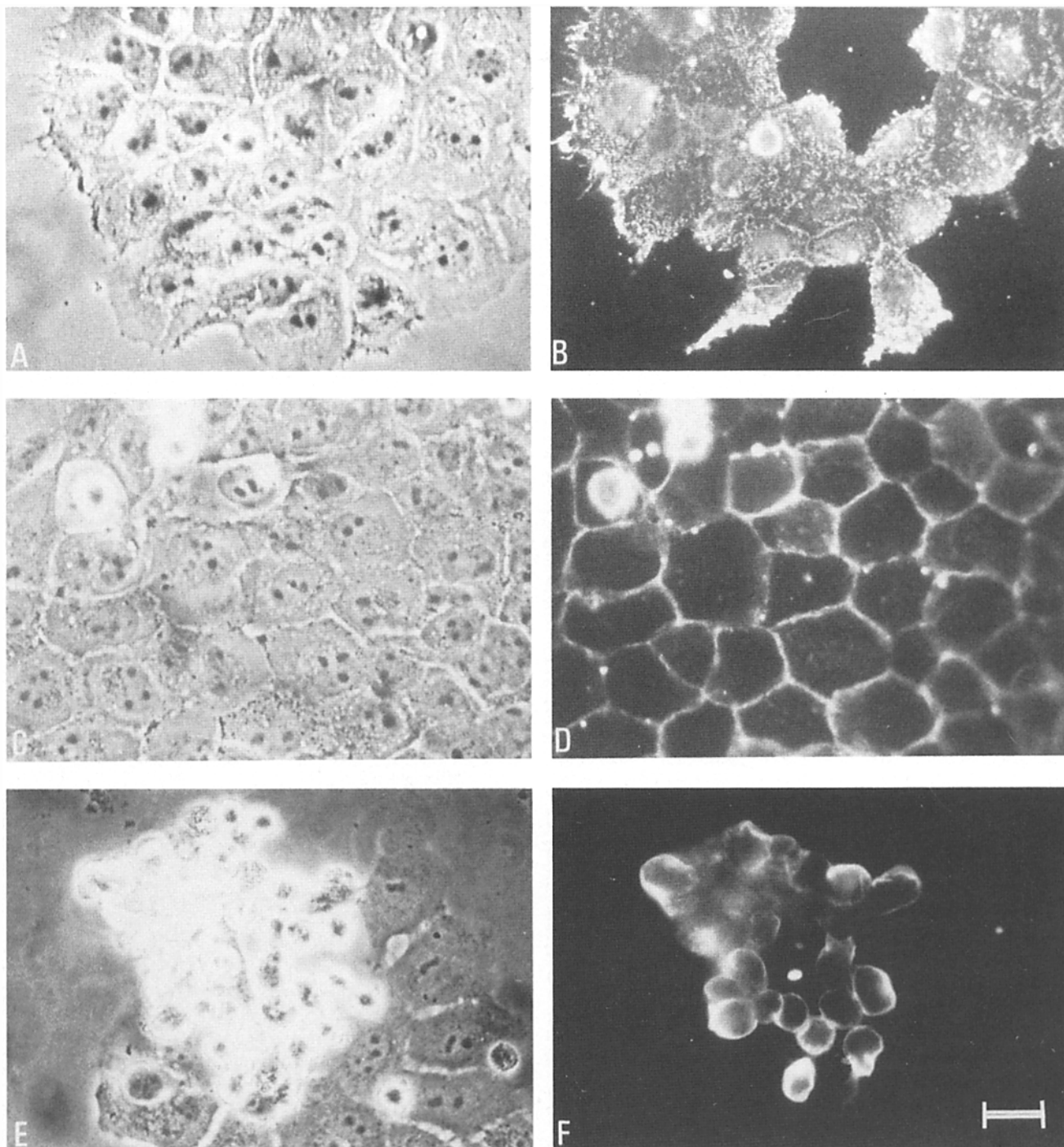
Figure 2. Phase contrast and immunofluorescence images of A431 cells with anti–EGF-R monoclonal antibodies. (*A* and *B*) 3% *p*-form-aldehyde fixed A431 cells were subsequently treated with monoclonal antibody 2D11 and a goat anti–mouse-FITC conjugate. (*A*) Phase-contrast image, (*B*) fluorescent image of the same field; (*C* and *D*) as in *A* and *B*, but with monoclonal antibody 2E9; (*E* and *F*) as in *A* and *B* but in unfixed cells at 37°C. Bar, 50 μM.

pared with 2.3×10^6 receptors in A431A$^+$ cells. The existence of two affinity classes of receptors on A431 cells has been reported by others (20, 23, 30). High affinity receptors constitute only about 5% of the total receptor pool, while the difference in dissociation constant between the two receptor classes is about one order of magnitude. Low abundance in combination with the small difference in dissociation con-

stant make it extremely difficult to quantitatively measure high affinity receptors in A431 cells by regular Scatchard analysis, since this is done by extrapolation of the initial part of the graph to the X-axis. Small errors in this area therefore lead to large errors in the calculated number of receptors. As will be reported elsewhere (Defize, L. H. K., J. Boonstra, and S. W. De Laat, manuscript in preparation), antibody 2E9
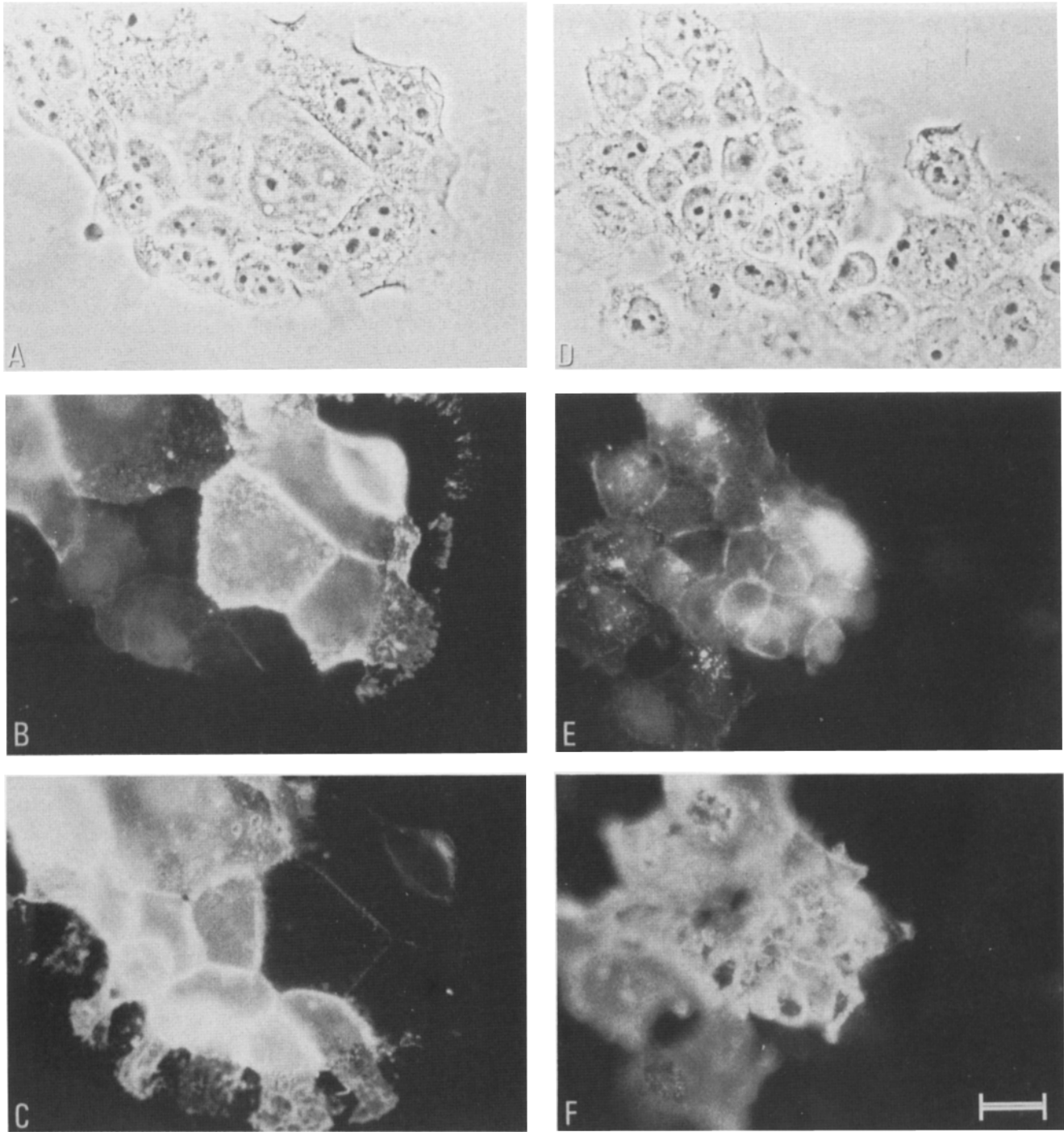
Figure 3. Phase-contrast and fluorescence images of A431 cells double-labeled with lectins and antibody 2D11. *(A-C)* 3% *p*-formaldehyde fixed A431 cells were treated simultaneously with antibody 2D11 and UEA lectin–FITC complex and subsequently with goat anti-mouse–TRITC complex. *(D-F)* as in *A-C*, but instead of UEA–FITC, DBA–FITC was used. *(A* and *D)* Phase-contrast images; *(B* and *E)* rhodamine fluorescence, *(C* and *F)* fluorescein fluorescence. Bar, 50 µM.

blocks EGF binding to low affinity EGF-R in a variety of cell types, while leaving high affinity EGF binding unperturbed. To quantitate high affinity receptors in the clones, we preincubated cells with a saturating amount of 2E9 (300 nM), thus blocking low affinity sites completely. Fig. 4 (right panel) shows a Scatchard analysis of EGF binding to these cells. Clearly, in both clones only a single, high affinity receptor

class (K_d 0.4 nM) is detected under these circumstances. Surprisingly however, the contribution of this subclass to the total receptor population is significantly higher in A431A⁻ cells as compared with A431A⁺ cells: 175,000 receptors per cell (6.3% of total) vs. 72,000 (3.2% of total) respectively.

Since high affinity EGF-R have been implicated by others to be the biologically active species with respect to signal

Table I. Activity of UDPGal:GalNAc Transferase in Cell Lysates

Clone	Activity
	pmol/mg⁻¹ per h
A431A⁺	267 ± 17 ($n = 3$)
A431A⁻	17 ± 5 ($n = 3$)

Cell lysates were incubated with A⁻PSM (see text) and UDP⁻[¹⁴C]GalNAc as described in Materials and Methods. GalNAc transferase activity is expressed in pmoles GalNAc incorporated per milligram of cellular protein per hour. Values are corrected for background (no A⁻PSM in the incubation mixture).

transduction (23, 30), we next compared the EGF-R protein-tyrosine kinase activity in the clones.

EGF-R Protein-Tyrosine Kinase Activity in Membrane Preparations

Receptor protein-tyrosine kinase activity was measured with [γ³²P]ATP as phosphate donor, either in total cell lysates or in plasma membrane preparations of the clones. The autoradiograms of total cell lysates prepared from equal amounts of cells are shown in Fig. 5 *A*. In clone A431A⁻ the level of receptor phosphorylation both with and without EGF was higher than in clone A431A⁺. Since this difference could be due to substrate competition, given the higher level of phosphate incorporation into other proteins present in the cell lysate of A431A⁺ cells (notably a protein with a M_r of 94 kD) we repeated the experiments with plasma membrane preparations from the clones. Also here, the difference in receptor autophosphorylation was apparent (Fig. 5 *B*). Receptor autophosphorylation with and without EGF, as well as the phosphorylation of several endogenous proteins, were about threefold higher in A431A⁻ cells as measured by densitometric scanning of the autoradiogram depicted in Fig. 5 *B*. Importantly, the EGF-R content of the membrane preparations (total number of receptors per milligram of protein), as checked by EGF-binding assays (see Materials and Methods), was equal for both clones. The heavily labeled 94-kD protein in total cell lysates of A431A⁺ cells (Fig. 5 *A*), was found to a much lesser extent in the plasma membrane preparations. Phosphoamino acid analysis showed that in these ex-
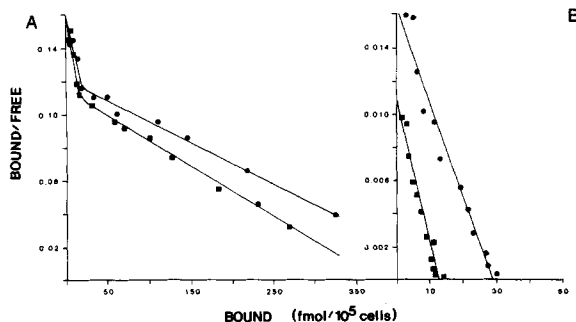

Figure 5. Autoradiographs of phosphorylated total cell lysates and membrane preparations of A431A⁺ and A431A⁻ cells. (*A*) Total cell lysates prepared from 28 cm² dishes as described in Materials and Methods, or (*B*) plasma membranes prepared according to Thom (39) were preincubated with or without EGF for 10 min at 20°C. The phosphorylation reaction was started by adding ³²P-phosphorylation mix as described in Materials and Methods. Phosphorylation was for 10 min at 0°C (all lanes except lanes *3* and *6* in *B*) or for 20 min at 0°C (lanes *3* and *6* in *B*). The samples were boiled in Laemmli sample buffer and separated on linear 5–15% SDS-polyacrylamide gradient gels.

periments the receptor was phosphorylated exclusively on tyrosine residues, while tryptic phosphopeptide analysis revealed no differences in the sites of receptor autophosphorylation (results not shown).

The differences in incorporation of radioactive phosphorus could be the result of a difference in the activity of phosphatases rather than of enhanced kinase activity. Therefore, the time dependent ³²P incorporation into the total TCA precipitable fraction from plasma membranes was measured in the presence or absence of EGF. In a parallel experiment, excess cold ATP was added to the incubation mixtures to stop ³²P incorporation and test for possible phosphatase activity. The results of these experiments are depicted in Fig. 6. Clearly, no such activity was measurable in the membrane preparations, while the total ³²P incorporation was again significantly higher over the total time course in A431A⁻ plasma membranes.

Finally, phosphorylation of the tyrosine-containing peptide angiotensin I was measured in the absence or presence

Figure 4. Scatchard analysis of [¹²⁵I]EGF binding to A431A⁺ (*squares*) and A431A⁻ (*circles*) cells, either untreated (*A*) or pretreated for 180 min at 20°C with 300 nM of antibody 2E9 to block low affinity EGF binding (*B*). Cells (density 50,000 cells/cm²) were treated for 120 min at 0°C with [¹²⁵I]EGF (0.5 ng/ml) and increasing concentrations of unlabeled EGF. Cells were washed five times with ice cold PBS, taken up in 0.5 M NaOH and cell associated radioactivity was determined in a gamma counter.
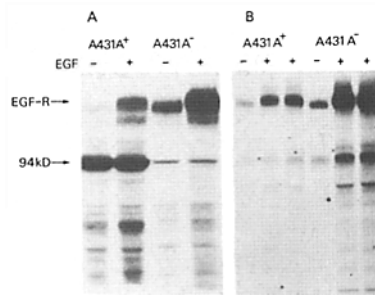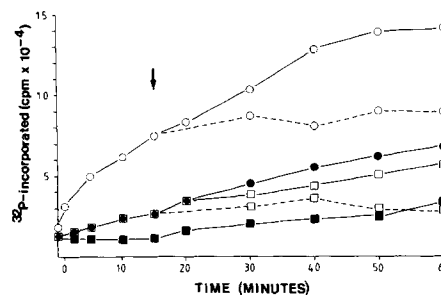

Figure 6. Incorporation of ³²P into the TCA precipitable fraction of A431A⁺ and A431A⁻ plasma membrane vesicles. A431A⁺ (*squares*) or A431A⁻ (*circles*) plasma membrane vesicles (25 μg protein) were incubated at 0°C with [³²P]γ-ATP in the presence (*open symbols*) or absence (*closed symbols*) of EGF. At $t = 15$ (marked by arrow) a 300-fold excess of unlabeled ATP was added to some of the samples (*dotted lines*). Incorporation of ³²P in the TCA precipitable fraction was determined as described in Materials and Methods.
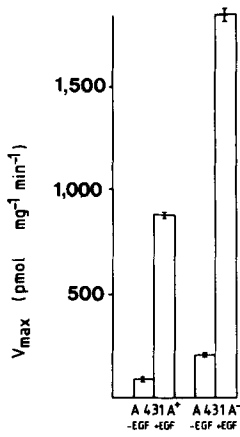
Figure 7. Phosphorylation of angiotensin I by A431A$^+$ and A431A$^-$ plasma membranes. Plasma membranes (25 µg of protein) were incubated with [^{32}P]γ-ATP and angiotensin I (20 mM) in the presence or absence of EGF as indicated. Incorporation of ^{32}P into the peptide was determined as described in Materials and Methods.

of EGF. As shown in Fig. 7, these experiments showed that also towards an exogeneous substrate, the A431A$^-$ receptor has a higher tyrosine kinase activity. Whereas the relative stimulation by EGF is roughly the same in both preparations (9.6 times the basal level), the A431A$^-$ preparations gave a 1.6-fold higher level of phosphorus incorporation into angiotensin I.

Measurements of Receptor Activity in Intact Cells

To measure the activity of the receptor in intact cells, cells were labeled with ^{32}P for 18 h and subsequently treated with EGF or buffer for a further 30 min. After this period the cells were scraped and lysed in RIPA buffer (see Materials and Methods) and the receptor was precipitated. In a parallel series of cells the total cellular phosphotyrosine content was measured without receptor precipitation. The outcome of these experiments, as shown in Fig. 8 and Table II, confirmed the results from the previous experiments. Both the relative cellular phosphotyrosine content (Table II) as well as the absolute amount of phosphotyrosine present in the precipitated receptor (Fig. 8 *B*) were significantly higher in unstimulated as well as in EGF-stimulated A431A$^-$ cells than in A431A$^+$ cells. Interestingly, quantitative determination of the relative amounts of receptor phosphoaminoacids revealed no differences between the clones (Table II). Accordingly, tryptic phosphopeptide maps of the precipitated receptor were identical (Fig. 8 *C*), also confirming that there are no major structural EGF-R differences between the clones.

The results presented thus far indicate that: (*a*) A431A$^-$ cells display an elevated level of protein-tyrosine kinase activity. Given the results of the in vitro experiments, this is
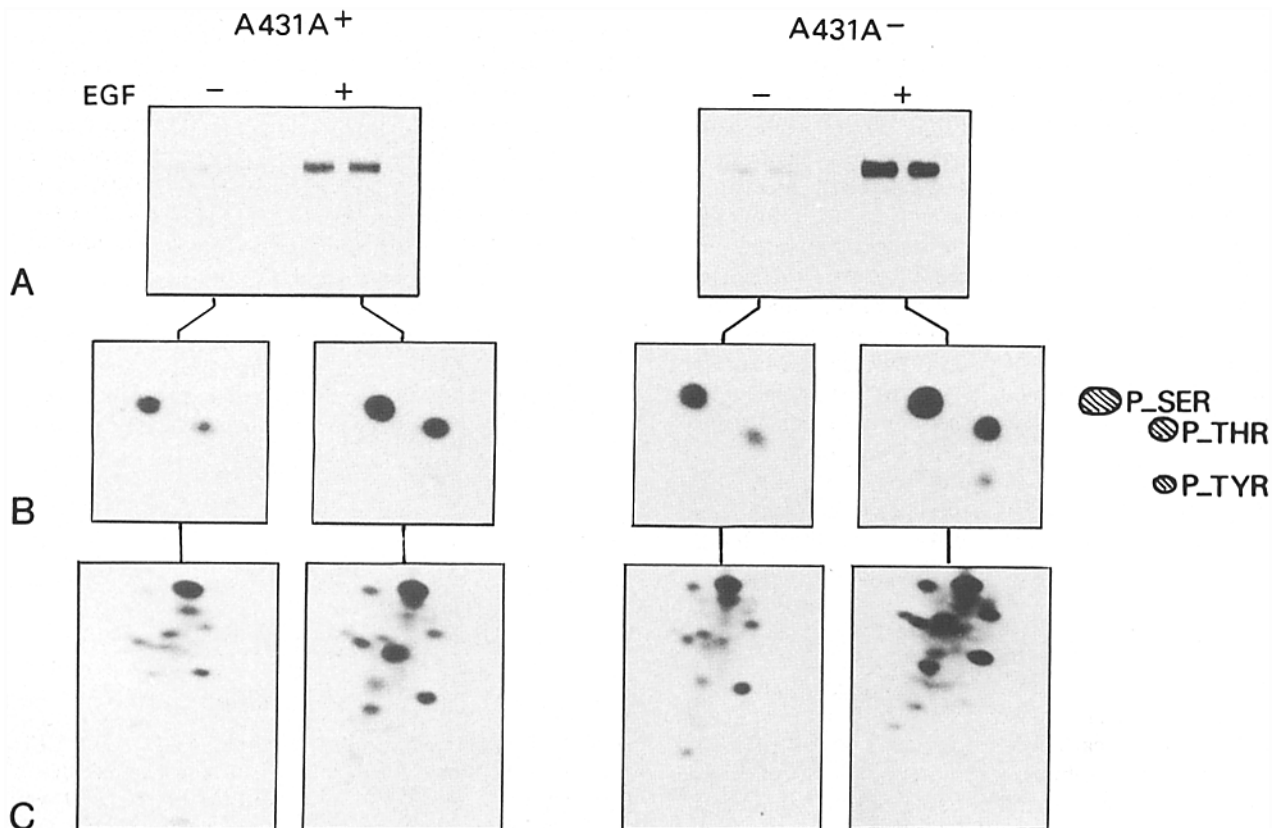

Figure 8. EGF-R phosphorylation in intact cells. ^{32}P labeled A431A$^+$/A431A$^-$ cells were treated for 30 min with EGF (50 ng/ml) or buffer, lysed in RIPA buffer, and the EGF-R was immunoprecipitated as described in Materials and Methods. The immunoprecipitates were applied to linear 5–15% SDS-polyacrylamide gels. (*A*) Autoradiograph of EGF-R bands in the dried gel. Receptor bands were cut out from the dried gel, the receptor protein was eluted and (*B*) subjected to phosphoamino acid analysis and (*C*) tryptic phosphopeptide mapping as described in Materials and Methods. For autoradiography, preflashed Kodak XAR5 films were used in combination with Kodak X-Omatic regular intensifying screens. cpms Loaded were from left to right: (*B*) 400 cpm; 1,100 cpm; 600 cpm; 1,400 cpm, (*C*) 2,250 cpm; 5,000 cpm; 3,100 cpm; 7,300 cpm. Exposure was for 24 h at −70°C.

Table II. Relative Phosphoamino Acid Contents in Total Cellular Protein and EGF-R from ^{32}P-labeled Cells

Total cellular protein		A431A$^+$		A431A$^-$	
		−EGF	+EGF	−EGF	+EGF
	P-SER	92.04	92.33	92.33	92.61
	P-THR	7.94	7.62	7.65	7.30
	P-TYR	0.015	0.060	0.035	0.090
EGF-R	cpm total	24,656	59,427	39,569	72,188
	P-SER	58.59	52.43	66.07	59.43
	P-THR	37.17	28.56	29.53	20.76
	P-TYR	4.23	19.00	4.40	19.81

For total cellular phosphoamino acid (PAA) analysis, lysates from ^{32}P-labeled A431 cells were treated as described in Materials and Methods. From parallel series of lysates, the EGF-R was immunoprecipitated, subjected to SDS-gel electrophoresis, and analyzed for PAA content. The PAAs were separated by two-dimensional high-voltage thin layer electrophoresis as described in Materials and Methods. The radioactivity in the PAA spots was determined by liquid scintillation counting as described in Materials and Methods. Numbers represent the radioactivity present in individual PAAs as the percentage of radioactivity present in the three PAAs together. For the immunoprecipitated receptor the number of cpm's in the gel band as measured by Cerenkov radiation are also indicated.

most probably attributable to the EGF-receptor in these cells, (*b*) in A431A$^-$ cells there is no increase in the relative amount of phosphotyrosine present in the EGF-R as compared with A431A$^+$ cells, which indicates that the elevated level of tyrosine autophosphorylation of the receptor in A431A$^-$ cells is accompanied by an increase in phosphorylation of the receptor on serine and threonine residues, probably by other cellular kinases. Such phosphorylations could play a regulatory role in receptor functioning, for instance by providing (a) signal(s) for receptor internalization and breakdown.

Receptor Turnover in A431$^+$ and A431A$^-$ Cells

The turnover of EGF-R is increased considerably after activation by EGF (38). If there is a direct relationship between receptor tyrosine kinase activity and receptor breakdown as recently suggested by Honegger et al. (21), the turnover of EGF-R protein should be faster in A431A$^-$ cells. To determine the half-life of the receptor in the clones, we labeled cells metabolically with [^{35}S]methionine for 16 h. After this the cells were chased with excess cold methionine, the receptor was isolated from the cells at various time intervals, and its radioactive content measured as described in Materials and Methods and the legend Fig. 9. While the turnover of [^{35}S]methionine in total cell protein was essentially the same (not shown), the half life of the receptor in A431A$^-$ cells was significantly shorter: ∼10 h vs. ∼17.5 h in A431A$^+$ cells (Fig. 9). Under identical experimental conditions the half-life of the receptor in uncloned A431 cells was 14 h (not shown).

Discussion

In the present study, we report that the human epidermoid carcinoma cell line A431 consists of at least two different cell types which differ in carbohydrate composition of the EGF-R and other plasma membrane constituents. These cell types coexist for a prolonged period of tissue culture without any apparent change in relative numbers. Furthermore, we report that stable clones of each cell type can be obtained from the parental cells, showing that the observed differential labeling of the uncloned cells is not due to some cell cycle phenomenon as was suggested by others who reported similar observations (5, 40).

The EGF- and 2E9-binding data indicate that the total

number of receptors is roughly equal for both clones. Thus, the differential reactivity of blood group A reactive anti-EGF-R antibodies toward these lines is not due to a difference in receptor expression. The lectin-binding data and the measurements of UDPGalNAc:Gal transferase activity indicate that A431A$^-$ cells differ from A431A$^+$ cells in that GalNAc residues are absent from carbohydrates in the former cells. Interestingly, Willingham and Pastan (40) showed differential reactivity of A431 cells with an antibody (MC101) directed against a blood group H-like antigen, which can be derived from the A-antigen by removal of the terminal GalNAc residue. This antibody thus probably only recognizes A431A$^-$ cells. Our data of course do rule out the possibility of other deletions or substitutions in carbohydrate residues in A431A$^-$ cells. The fact that the molecular mass of the receptor in the clones is essentially the same however argues against large deletions. The complete unreactivity of anti-blood group A EGF-R antibodies with one clone shows that
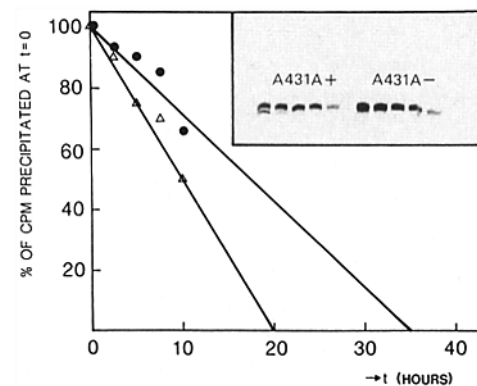

Figure 9. EGF-R degradation in A431A$^+$ and A431A$^-$ cells. Cells were labeled for 16 h with [^{35}S]methionine. Labeling medium was replaced by fresh medium containing 300 mM methionine. At various time points up till 10 h after the medium change, cells were lysed, the EGF-R was immunoprecipitated and the precipitate was subjected to SDS-PAGE. After autoradiography, receptor bands were cut out from the gel and their radioactive content was determined as described in Materials and Methods. (●) A431A$^+$ cells; (△) A431A$^-$ cells. (*Inset*) Autoradiograph of 170-kD region of polyacrylamide gel. Time of cell lysis from left to right: 0, 2.5, 5, 7.5, and 10 h after medium change.

in A431 cells the transferase uses both glycoproteins and glycolipids as substrates since these antibodies recognize both types of molecules (16, 28, Fig. 1). We do not know whether the glycosylation variants were already present in the tumor from which the A431 cell line was derived, or whether the appearance of the variants occurred spontaneously at some later time point, nor do we have any clue about the genetic background of the variation. Clones A431A$^+$ and A431A$^-$ both have an aneuploid karyotype in which all human chromosomes are present, and both clones contain the chromosome 7 translocation typical for A431 cells (Geurts van Kessel, A., personal communication).

Since our results show that A431 cells are inhomogeneous with respect to at least one structural feature of their EGF-R and given the fact that these cells are widely used as a model system to study EGF–EGF-R interactions, we decided to compare functional EGF-R properties in the clones. A possible role for carbohydrates in receptor regulation has been reported by others. Podskalny et al. (29) have studied insulin receptor function in two Chinese hamster ovary (CHO) cell variants, and reported that the defects in carbohydrate processing in these cells lead, interestingly, to either a large increase or a decrease in the receptors' affinity for insulin. Kingsley et al. (25), also using CHO glycosylation mutants, showed that impaired carbohydrate processing leads to a diminished low density lipoprotein (LDL) receptor stability. To our knowledge, the present study is the first to deal with EGF-R function in glycosylation variants. The fact that the differences in EGF-R functioning between the clones were found, as will be discussed below, of course does not provide a direct proof for a regulatory role of receptor carbohydrates. Other phenotypic parameters, not detected by our screening methods, could equally well be responsible for the observed phenomena. To obtain such a proof would require a rigorous statistical analysis of quite a number of clones. Experiments in which the enzyme N-Acetyl-galactosaminidase was used to remove the A-antigen and look for possible effects on receptor function failed, since the optimal conditions for this enzyme to work proved devastating to EGF-R stability (data not shown).

The finding that in A431A$^-$ cells as compared with A431A$^+$ cells, a higher percentage of EGF-R is of a high affinity subclass was rather unexpected. Total absence of receptor glycosylation has a debilitating effect on its function (37). How the absence of one sugar residue could lead to a shift in the distribution of high and low affinity sites might be explained by a model presented by Childs et al. (5) and Feizi and Childs (15). These authors suggest that interactions between carbohydrate determinants shared by glycoproteins and glycolipids, for instance mediated by endogenous lectin-like molecules, play a regulatory role. In A431 cells, the A-antigen could perform such a function, while in other cell types, which do not express the A antigen different carbohydrates and lectins might be involved. Absence of A-antigen in A431A$^-$ cells could lead to an altered mobility and/or a change in the conformation of EGF receptors. This in turn could lead to an enhanced ability of receptors to aggregate, resulting in a shift in the high vs. low affinity equilibrium. Evidence for allosteric EGF-R activation by aggregation has recently been presented by Yarden and Schlessinger (41, 42). Based upon experiments with purified EGF-R preparations, these authors also propose that the high affinity, aggregated receptor population has enhanced protein-tyrosine kinase

activity even without EGF (41). Fanger et al. (14) have also presented evidence that tyrosine kinase activity is primarily associated with high affinity EGF-receptors in Hela cells. In accordance with this, our results clearly demonstrate a parallel increase in high affinity receptor number and protein-tyrosine kinase activity, both in membrane preparations as well as in intact cells.

The role of EGF-R tyrosine (auto)phosphorylation in the cascade of events following EGF binding still remains obscure. Recently, Honegger et al. (21) found that a point mutation at the ATP-binding site of the EGF-R which abolishes its tyrosine kinase activity, prevents receptor break down. These authors suggest that tyrosine (auto)phosphorylation provides a signal for receptor degradation, whereas unphosphorylated receptors are continually recycled back to the plasma membrane. In line with this, Chen et al. (4) reported that receptor protein – tyrosine kinase activity is required for the induction of EGF-R down regulation by EGF. While these results demonstrate that absence of protein – tyrosine kinase activity prevents receptor breakdown, our results indicate that an elevated receptor (auto)phosphorylation is associated with enhanced receptor breakdown. Even in A431A$^-$ cells, high affinity EGF-R comprise only a small fraction of the total receptor population. The increased turnover of the total receptor pool could be explained by (a) "cross"-phosphorylation of low affinity EGF-R by high affinity receptors, or (b) continuous replacement of internalized high affinity receptors by new receptors recruited from the low affinity pool. At present, we cannot distinguish between these two possibilities.

An effect of EGF is increased biosynthesis of its own receptor (6). Since the total number of receptors is equally high in both clones, receptor biosynthesis must be increased in A431A$^-$ cells. That indeed this is the case can be inferred from the higher amount of radioactivity present in the receptor from [^{35}S]methionine-labeled cells (see Fig. 9, inset).

The elevated tyrosine autophosphorylation of EGF-R in A431A$^-$ cells is accompanied by a parallel increase in serine and threonine receptor phosphorylations, most probably by other protein kinases. Whether these additional modifications play a role in the elevated activity of A431A$^-$ EGF-R or are simply a consequence of the increased receptor turnover remains to be established. A431A$^+$ variants with altered responses to EGF have been isolated by Buss et al. (2). In these cells, which have a decreased number of EGF-R, receptor kinase activation by EGF is decreased in comparison with "wild-type" A431 cells, an effect which is correlated with absence of growth inhibition by EGF in these mutants. In our clones, receptor kinase activity is equally well stimulated by EGF, while EGF inhibits growth of A431A$^+$ and A431A$^-$ cells to a similar extent (unpublished observations).

In conclusion, our results demonstrate the existene of a substantial amount (40%) of stable glycosylation variants within the A431 cell line, which in its uncloned form is by far the most extensively used system to measure EGF-R functioning. The isolation of these variants enabled us to show that the difference is caused by the absence or presence of UDPGalNAc:Gal transferase activity, leading to absence of blood group A antigens on the EGF-R as well as on other glycoproteins and glycolipids in one variant (A431A$^-$), while these antigens are fully expressed in another (A431A$^+$). Furthermore we report a substantial difference in EGF-R ac-

tivity between these clones: the absence of A-antigen in A431A⁻ cells is correlated with an elevated ratio of high over low affinity EGF-R. We have discussed this phenomenon in the context of recent ideas about receptor affinity regulation. Since the relative increase in high affinity EGF-R number in A431A⁻ cells is paralleled by both an increase in receptor protein-tyrosine kinase activity as well as receptor turnover, our results support the notion that high affinity receptors are the biologically active species with respect to EGF signal transduction.

References

1. Beemon, K., and T. Hunter. 1978. Characterization of Rous sarcoma virus SRC gene products synthesized in vitro. *J. Virol.* 28:551–566.
2. Buss, J. E., J. E. Kudlow, C. S. Lazar, and G. N. Gill. 1982. Altered epidermal growth factor (EGF)-stimulated protein kinase activity in variant A431 cells with altered growth responses to EGF. *Proc. Natl. Acad. Sci.* 79:2574–2578.
3. Carpenter, G. 1987. Receptors for epidermal growth factor and other polypeptide mitogens. *Annu. Rev. Biochem.* 56:881–914.
4. Chen, W. S., C. S. Lazar, M. Poenie, R. Y. Tsien, G. N. Gill, and M. Rosenfeld. 1987. Requirement for intrinsic protein tyrosine kinase in the immediate and late action of the EGF-receptor. *Nature (Lond.).* 328:820–823.
5. Childs, R. A., M. Gregoriou, P. Scudder, S. J. Thorpe, A. R. Rees, and T. Feizi. 1984. Blood group-active carbohydrate side chains on the receptor for epidermal growth factor of A431 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2227–2233.
6. Clark, A. J. L., S. Ishii, N. Richert, G. T. Merling, and I. Pastan. 1985. Epidermal growth factor regulates the expression of its own receptor. *Proc. Natl. Acad. Sci. USA.* 82:8374–8378.
7. Cohen, S., H. Ushiro, C. Stoschek, and M. Chinkers. 1982. A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. *J. Biol. Chem.* 257:1523–1531.
8. Cooper, J. A., and T. Hunter. 1981. Changes in protein phosphorylation in Rous sarcoma virus-transformed chicken embryo cells. *Mol. Cell Biol.* 1:165–178.
9. Defize, L. H. K., and S. W. de Laat. 1986. Structural and functional aspects of epidermal growth factor (EGF) and its receptor. In *Prog. Brain Res.* 69:169–182.
10. Defize, L. H. K., W. H. Moolenaar, P. T. van der Saag, and S. W. de Laat. 1986. Dissociation of cellular responses to epidermal growth factor using anti-receptor monoclonal antibodies. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1187–1192.
11. Defize, L. H. K., C. L. Mummery, W. H. Moolenaar, and S. W. de Laat. 1987. Antireceptor antibodies in the study of EGF-receptor interaction. *Cell Differ.* 20:87–102.
12. Downward, J., Y. Yarden, E. Mayes, G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, and M. D. Waterfield. 1984. Close similarity of epidermal growth factor receptor and verbB oncogene protein sequences. *Nature (Lond.).* 307:521–527.
13. Fabricant, R. N., J. E. de Larco, and G. J. Todaro. 1977. Nerve growth factor receptors on human melanoma cells in culture. *Proc. Natl. Acad. Sci. USA.* 74:565–569.
14. Fanger, B. O., K. S. Austin, H. S. Earp, and J. A. Cidlowski. 1986. Crosslinking of epidermal growth factor receptors in intact cells: detection of initial stages of receptor-clustering and determination of molecular weight of high-affinity receptors. *Biochemistry.* 25:6414–6420.
15. Feizi, T., and R. A. Childs. 1985. Carbohydrate structures of glycoproteins and glycolipids as differentiation antigens, tumour associated antigens and components of receptor systems. *Trends in Biochem. Sci.* 10:24–29.
16. Fredman, P., N. D. Richert, J. L. Magnani, M. C. Willingham, J. Pastan, and V. Ginsburg. 1983. A monoclonal antibody that precipitates the glycoprotein receptor for epidermal growth factor is directed against the human blood group H type 1 antigen. *J. Biol. Chem.* 258:11206–11210.
17. Gallagher, J. T. 1984. Carbohydrate-binding properties of lectins: A possible approach to lectin nomenclature and classification. *Biosci. Rep.* 4:621–623.
18. Gooi, H. C., J. K. Picard, E. F. Hounsell, M. Gregoriou, A. R. Rees, and T. Feizi. 1985. Monoclonal antibody (EGR/G49) reactive with the epidermal growth factor receptor of A431 cells recognizes the blood

group Al₍ᵇ and Al₍ʸ structures. *Mol. Immunol.* 22:689–693.
19. Gooi, H. C., J. Schlessinger, I. Lax, Y. Yarden, T. A. Libermann, and T. Feizi. 1983. Monoclonal antibody reactive with the human epidermal growth factor receptor recognizes the blood-group-A antigen. *Biosci. Rep.* 3:1045–1052.
20. Gregoriou, M., and A. R. Rees, 1984. Properties of a monoclonal antibody to epidermal growth factor receptor with amplications for the mechanism of action of EGF. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:929–937.
21. Honegger, A. M., T. J. Dull, S. Felder, E. V. Obberghen, F. Bellot, D. Szapary, A. Schmidt, A. Ullrich, and J. Schlessinger. 1987. Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. *Cell.* 51:199–209.
22. Hunter, T., and B. M. Sefton. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc. Natl. Acad. Sci. USA.* 77:1311–1315.
23. Kawamoto, T., J. D. Sato, A. Le, J. Polikoff, G. A. Sato, and J. Mendelsohn. 1983. Growth stimulation of A431 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.* 80:1337–1341.
24. King, L. E., G. Carpenter, and S. Cohen. 1980. Characterization by electrophoresis of Epidermal Growth Factor stimulated phosphorylation using A431 membranes. *Biochemistry.* 19:1524–1528.
25. Kingsley, D. M., K. F. Kozarsky, L. Hobbie, and M. Krieger. 1986. Reversible defects in O-linked glycosylation and LDL-receptor expression in a UDP-Gal/UDP-GalNAc 4-epimerase deficient mutant. *Cell.* 44:749–459.
26. Kris, R. M., J. Lax, W. Gullick, M. D. Waterfield, A. Ullrich, M. Fridkin and J. Schlessinger. 1985. Antibodies against a synthetic peptide as a probe for the kinase activity of the avian EGF receptor and erbB protein. *Cell.* 40:619–625.
27. Mayes, E. L. V., and M. D. Waterfield. 1984. Biosynthesis of the epidermal growth factor receptor in A431 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:531–537.
28. Parker, P. J., S. Young, W. J. Gullick, E. L. V. Mayes, P. Bennett, and M. D. Waterfield. 1984. Monoclonal antibodies against the human epidermal growth factor receptor from A431 cells. Isolation, characterization and use in the purification of active epidermal growth factor receptor. *J. Biol. Chem.* 259:9906–9912.
29. Podskalny, J., A. McElduff, and P. Gorden. 1984. Insulin receptors on Chinese hamster ovary (CHO) cells: altered insulin binding to glycosylation mutants. *Biochem. Biophys. Res. Comm.* 125:70–75.
30. Rees, A. R., M. Gregoriou, P. Johnson, and P. B. Garland. 1984. High affinity epidermal growth factor receptors on the surface of A431 cells have restricted lateral diffusion. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1843–1847.
31. Schlessinger, J. 1986. Allosteric regulation of the epidermal growth factor receptor kinase. *J. Cell Biol.* 103:2067–2072.
32. Schlessinger, J., I. Lax, Y. Yarden, H. Kanety, and T. Libermann. 1984. Monoclonal antibodies against the membrane receptor for Epidermal Growth Factor: a versatile tool for structural and mechanistic studies. In Monoclonal antibodies to receptors: probes for receptor structure and function. In Receptors and Recognition, M. F. Greaves, editor. Chapman and Hill, London. Series B. Vol. 17. 280–301.
33. Schreiber, A. B., T. A. Libermann, I. Lax, Y. Yarden, and J. Schlessinger. 1983. Biological role of epidermal growth factor-receptor clustering. Investigation with monoclonal anti-receptor antibodies. *J. Biol. Chem.* 258:846–853.
34. Schwyzer, M., and R. L. Hill. 1977. Porcine A blood group specific N-Acetylgalactosaminyl transferase. II. Enzymatic properties. *J. Biol. Chem.* 252:2346–2355.
35. Slieker, L. J., and M. D. Lane. 1985. Post-translational processing of the epidermal growth factor receptor. Glycosylation-dependent acquisition of ligand-binding capacity. *J. Biol. Chem.* 260:687–690.
36. Slieker, L. J., T. M. Martensen, and M. D. Lane. 1986. Synthesis of epidermal growth factor receptor in human A431 cells. Glycosylation-dependent acquisition of ligand binding activity occurs post-translationally in the endoplasmic reticulum. *J. Biol. Chem.* 261:15233–15241.
37. Soderquist, A. M., and G. Carpenter. 1984. Glycosylation of the epidermal growth factor receptor in A431 cells. The contribution of carbohydrate to receptor function. *J. Biol. Chem.* 259:12586–12594.
38. Stoscheck, C. M., and G. Carpenter. 1984. Characterization of the metabolic turnover of epidermal growth factor receptor protein in A431 cells. *J. Cell Physiol.* 120:296–302.
39. Thom, D., A. J. Powell, C. W. Lloyd, and D. A. Rees. 1977. Rapid isolation of plasma membranes in high yield from cultured fibroblasts. *Biochem. J.* 168:187–194.
40. Willingham, M. C., and I. Pastan. 1985. In An atlas of immunofluorescence in cultured cells. Academy Press, London. 30–33.
41. Yarden, Y., and J. Schlessinger. 1987. Self-phosphorylation of epidermal growth factor receptor: evidence for a model of intermolecular allosteric activation. *Biochemistry.* 26:1434–1442.
42. Yarden, Y., and J. Schlessinger. 1987. Epidermal growth factors induces rapid, reversable aggregation of the purified epidermal growth factor receptor. *Biochemistry.* 26:1443–1451.