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PRODUCTION AND CHARACTERIZATION OF ANTIBODY BLOCKING EPIDERMAL GROWTH FACTOR:RECEPTOR INTERACTIONS

HARRY T. HAIGLER [a,*] and GRAHAM CARPENTER [a,b,**]

[a] *Department of Biochemistry and* [b] *Division of Dermatology, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232 (U.S.A.)*

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Summary

Membranes were prepared from the human epithelioid carcinoma cell line A-431 which has approx. $2 \cdot 10^6$ epidermal growth factor receptors per cell. This membrane preparation which retained a high epidermal growth factor binding specific activity was used as an antigen to produce antisera in rabbits. Double-immunodiffusion experiments demonstrated that the immune serum contained precipitating antibodies to several components of detergent solubilized A-431 membranes.

The immonoglobulin G fraction of this immune sera inhibited ^{125}I-labeled epidermal growth factor binding to receptors in: (1) intact human and mouse cells; (2) membrane preparations from A-431 cells and human placenta, and (3) solubilized A-431 membranes. Inhibition of ^{125}I-labeled epidermal growth factor binding was observed with divalent and monovalent fragments of immunoglobulin G prepared from the immunoglobulin G fraction. Also, the immunoglobulin G fraction blocked growth factor binding to membranes at low temperature (5°C).

Anti-A-431 antibody blocked the induction of DNA synthesis in quiescent fibroblasts by epidermal growth factor in a manner similar to that of anti-epidermal growth factor antibody. Addition of either anti-A-431 or anti-epidermal growth factor antibodies to fibroblasts at times up to 5 h after the

* Present address: Laboratory of Molecular Biology, National Cancer Institute, Bethesda, MD 20014, U.S.A.

** To whom reprint requests should be sent.

Abbreviations: EGF, epidermal growth factor; IgG, immunoglobulin G; $F(ab')_2$, divalent fragments of immunoglobulin G; Fab', monovalent fragments of immunoglobulin G; FGF, fibroblast growth factor; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

addition of epidermal growth factor completely reversed the hormone's mitogenic potential. At later times (after 12 h) addition of either antibody was without effect on the stimulation of DNA synthesis by epidermal growth factor. Anti-A-431 antibody did not block the induction of DNA synthesis in fibroblasts by fibroblast growth factor or serum.

Introduction

Several aspects of the mechanism of action of epidermal growth factor (EGF), a potent mitogenic polypeptide for many cell types in vivo and in vitro (see Refs. 1—3 for recent reviews), have been elucidated recently. Subsequent to the binding of EGF to specific receptor molecules on the surface of target cells [4,5], the receptor-hormone complexes form small clusters [6,7] which are internalized by an endocytotic-like mechanism [6,8]. These intracellular hormone-receptor complexes rapidly reach lysosomes where the hormone and perhaps the receptor are degraded [6,8—10].

Studies of the interactions in vitro of EGF with membranes prepared from A-431 cells have shown that the formation of ^{125}I-labeled EGF-receptor complexes produces enhanced phosphorylation of endogenous proteins [11,12]. Experimentation with subcellular systems that remain responsive to polypeptide hormones offers an opportunity to explore the molecular biochemistry which couples the interaction of hormones and receptors to the production of a biochemical signal.

This report describes the production of antibodies to A-431 membrane components and indicates how these antibodies affect the binding and production of biologic activities by epidermal growth factor. Antisera to membrane receptors have recently proven to be valuable reagents in characterizing the mechanism of action of several polypeptide hormones [13,14].

Methods

Materials. Mouse EGF, prepared as described elsewhere [15], was generously supplied by Dr. Stanley Cohen (Vanderbilt University, Nashville, TN). Fibroblast growth factor (FGF) was obtained from Denis Gospodarowicz (University of California, San Francisco). ^{125}I-labeled EGF and ^{125}I-labeled IgG were prepared by published methods [8]. Goat anti-rabbit IgG was purchased from Miles Laboratories (Elkhart, IN) and *Staphylococcus aureus* protein A coupled to Sepharose CL-4B was purchased from Pharmacia (Uppsala, Sweden). [*Methyl*-^{3}H]thymidine, [γ-^{32}P]ATP and Na^{125}I were obtained from New England Nuclear (Boston, MA). Human fibroblasts and 3T3 mouse fibroblasts were cultured as previously described [16].

Membrane preparations. Particulate membranes from A-431 cells were prepared by the procedures of Thom et al. [17] and have been characterized previously [12]. Detergent-solubilized EGF receptors were prepared from A-431 membranes as described by Carpenter [18]. Placenta membranes were isolated from term human placenta by the method of Cuatrecasas [19].

Immunoglobulin preparation and purification. A-431 membranes (4 mg

protein/ml) were suspended in 20 mM Hepes (pH 7.4) and added to an equal volume of Freund's complete adjuvant (Difco, Detroit, MI). An aliquot containing 2 mg protein of the adjuvant membrane solution was injected in the rear footpads and at three sites in the scapular region of each adult male white New Zealand rabbit. The rabbits received booster injections (800 μg protein) at approx. 5-week intervals and were bled from an ear vein approx. 5 weeks after each injection. The blood was allowed to clot and the serum was collected. Two rabbits were immunized by this procedure and both produced serum capable of inhibiting the binding of ^{125}I-labeled EGF to cellular receptors. The pooled serum from one rabbit was used for all the studies reported herein.

Protease-free IgG was isolated from serum by chromatography on DEAE-Affi gel blue (Bio-Rad, Richmond, CA). The serum was dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 28 mM NaCl, applied to a column containing DEAE-Affi gel blue, and eluted with the same buffer. Fractions containing IgG were pooled and concentrated to 10 mg/ml by ultrafiltration using an UM-2 membrane (Amicon Corp., Lexington, MA).

$F(ab')_2$ fragments of the anti-A-431 membrane antibody were produced by digesting the IgG with pepsin by established methods [20]. Fab' fragments were prepared by dialyzing $F(ab')_2$ against 0.6% 2-mercaptoethanol in phosphate-buffered saline (pH 8.0) followed by dialysis (2.5 h at room temperature) against 2.5 g/l iodoacetic acid in phosphate-buffered saline, pH 8.0 [20]. Both Fab' and $F(ab')_2$ fragments were purified by gel filtration on Sephadex G-150.

^{125}I-labeled EGF binding assays. The effect of antibody on the binding of ^{125}I-labeled EGF to receptors in intact cells was tested with human fibroblasts and Swiss-Webster 3T3 cells. Confluent monolayers in 60-mm dishes were washed twice with 4 ml of Hanks solution at 37°C. The cells were preincubated with antibody for 1 h at 37°C in 1.5 ml binding medium (Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin). A saturating level of ^{125}I-labeled EGF (10 ng/ml) was added to the dishes and the incubation was continued for 1 h at 37°C in the presence of antibody. Cell-bound radioactivity was determined by washing six times with a total of 12 ml of cold Hanks' solution containing 0.1% bovine serum albumin, solubilizing the cells with 0.5 N NaOH and determining the amount of ^{125}I radioactivity in a gamma spectrometer (Nuclear Chicago).

Binding of ^{125}I-labeled EGF to A-431 or placental membranes preparations was measured as previously described [12]. In brief, the binding reaction was carried out in a 200 μl reaction volume containing 20 mM Hepes buffer (pH 7.4), 0.1% bovine serum albumin, 12 μg (A-431) or 100 μg (placental) membrane protein and 15 ng of ^{125}I-labeled EGF. The amount of ^{125}I-labeled EGF bound to the membranes was determined by filtration and washing on Millipore EGWP membrane filters (0.2 μm pore size).

The binding of ^{125}I-labeled EGF to solubilized A-431 membrane receptor was determined as described by Carpenter [18]. Solubilized membrane was incubated for 30 min at room temperature in a 200 μl volume that contained 20 mM Hepes (pH 7.4), 0.1% bovine serum albumin, 0.2% Triton X-100 and 50 ng ^{125}I-labeled EGF. ^{125}I-labeled EGF-receptor complexes were precipitated

by poly(ethylene glycol) as previously described [18] and washed on Millipore EHWP membrane filters (0.5 μm pore size).

In all ^{125}I-labeled EGF binding studies the amount of nonspecific binding was determined by incubating parallel tubes or cultures with a large excess of unlabeled EGF (1.5—5.0 μg/ml) in addition to the ^{125}I-labeled EGF. All results are expressed as specific binding. Membrane protein was quantitated by the procedure of Bradford [21] using gamma globulin as a standard.

[^{3}H]thymidine incorporation. Confluent monolayers of human fibroblasts in 60-mm culture dishes were placed in Dulbecco's modified Eagle's medium containing 1—2.5% heat-inactivated calf serum and incubated for 48—72 h. The indicated mitogens were added and 20 h later [^{3}H]thymidine (final concentration 2.5 μM, 2 μCi/ml) was added. At 24 h the labeling was stopped by washing with cold saline and the incorporation of [^{3}H]thymidine into acid-insoluble material was determined as previously described [16].

Results

Immunodiffusion analysis

The IgG fractions from rabbits injected with A-431 membranes or from untreated rabbits were analyzed by double-immunodiffusion to detect the presence of precipitating antibodies against A-431 membrane components. The results (data not shown) indicated the presence of several species of antibodies to antigens present in A-431 membranes. Also, this antibody preparation formed precipitin lines when diffused against solubilized membranes from human placenta which has been shown to contain a high level of EGF receptor activity [22].

Interactions with cultured cells

Anti-A-431 IgG was tested for its capacity to affect the specific binding of ^{125}I-labeled EGF to membrane receptors on cultured human diploid fibroblasts and mouse 3T3 cells. Both of these cell types are responsive to the mitogenic activity of EGF and due to the wide species divergence should present different spectra of membrane antigens, with the possible exception of the EGF receptor and other conserved membrane components. The data in Fig. 1 show that, when monolayer cultures were preincubated for 1 h at 37°C with increasing concentrations of anti-A-431 IgG, there was a concentration-dependent reduction of ^{125}I-labeled EGF binding capacity. Control experiments (data not presented) showed that when unbound anti-A-431 IgG was removed by washing prior to the addition of ^{125}I-labeled EGF, binding was blocked. Therefore, the decrease in growth factor binding can be attributed to cell-bound IgG. Also, a high concentration (100 μg/ml) of normal rabbit IgG did not inhibit ^{125}I-labeled EGF binding (data not shown). These results indicate that the observed inhibition of ^{125}I-labeled EGF binding (Fig. 1) was due to the serum IgG fraction from the rabbit immunized with A-431 membranes. It should be mentioned that the anti-A-431 IgG (5 μg—1 mg/ml) did not interact with ^{125}I-labeled EGF in an immunoprecipitation assay using goat anti-rabbit IgG as the second antibody.

To determine if divalent antibody to A-431 membranes was required for

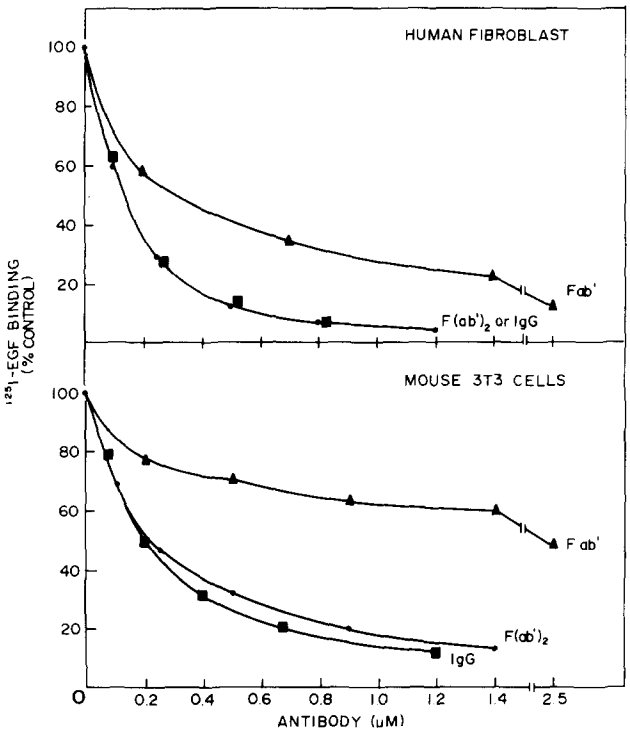

Fig. 1. Effect of anti-A-431 F(ab')$_2$ and Fab' fragments on binding of ^{125}I-labeled EGF to intact cells. Monolayers of either human fibroblasts (upper panel) or 3T3 cells (lower panel) were preincubated for 1 h with increasing concentrations of anti-A-431 IgG (■———■) or the F(ab')$_2$ (●———●) or Fab' (▲———▲) fragments prepared from this IgG fraction. 8 ng/ml ^{125}I-labeled EGF ($1.88 \cdot 10^5$ cpm/ng) were then added and the cells were incubated for 1 h at 37°C in the continuous presence of the antibody or antibody fragments. Cells were washed and specific binding determined as described in Methods.

reduction of ^{125}I-labeled EGF binding, F(ab')$_2$ and Fab' fragments were prepared and tested in the binding assay. The results shown in Fig. 1 demonstrate that while the F(ab')$_2$ fragment was quantitatively as effective as intact IgG for inhibition of ^{125}I-labeled EGF binding, the Fab' fragment also blocked ^{125}I-labeled EGF binding but was clearly less potent. This is not unexpected since the monovalent Fab' will have a greater dissociation rate than the divalent antibody [23]. A comparison of relative potencies of the antibody preparations on ^{125}I-labeled EGF binding in human and mouse cells (Fig. 1) indicates that both the intact IgG and divalent F(ab')$_2$ were quantitatively equivalent in both cell types in their capacities to block growth factor binding. However, the monovalent Fab' fragment was much more effective on human cells than mouse cells.

The inhibition of ^{125}I-labeled EGF binding by monovalent Fab' suggests that aggregation of cell surface components is not necessary for the antibody to block growth factor binding. The results in Fig. 2 demonstrate that anti-A-431 IgG is able to block ^{125}I-labeled EGF binding at both 5 and 37°C. These data further indicate that neither the aggregation of cell-surface components in response to IgG nor the endocytotic internalization of cell-bound IgG is required for the inhibition of growth factor binding by anti-A-431 IgG.

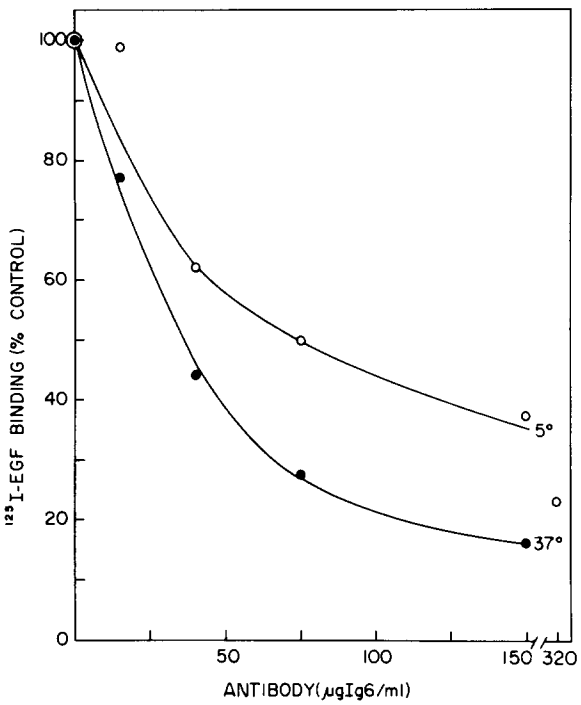

Fig. 2. Effect of temperature on the inhibition of ^{125}I-labeled EGF binding to intact cells. Monolayer cultures of human fibroblasts were preincubated with the indicated amounts of anti-A-431 IgG for 1 h at 37°C (●——●) or 2 h at 5°C (○——○). 10 ng/ml ^{125}I-labeled EGF were added to each dish and those cultures which had been preincubated at 37°C were incubated for an additional 1 h at 37°C whilst those which were preincubated at 5°C were reincubated at 5°C for 2 h. The cells were then washed and the level of nonspecific binding, relative to the dishes that received no IgG, was determined as described in Methods.

Anti-A-431 IgG inhibited ^{125}I-labeled EGF binding at all concentrations of EGF tested. Incubation of human fibroblasts with 2, 5, 10 and 70 ng ^{125}I-labeled EGF/ml and 20 μg/ml anti-A-431 IgG, under the conditions described in Fig. 1, resulted in inhibitions of growth factor binding of 44%, 42%, 39% and 39%, respectively. Therefore, the effect of the antibody cannot be overcome by the addition of higher concentrations of EGF.

Since anti-A-431 IgG effects the binding activity of the EGF receptor, we examined the influence of the antibody on the induction of DNA synthesis in quiescent monolayers of human fibroblasts. The data in Fig. 3 show that anti-A-431 IgG was able to inhibit the induction of DNA synthesis by EGF, but not by FGF or fresh calf serum. It should be noted that it has not been possible, in the absence of labeled FGF, to determine whether the A-431 membranes contain FGF receptors. Nevertheless, the capacity of cells to respond to FGF in the presence of anti-A-431 IgG demonstrates that the antibody preparation does not have a nonspecific inhibitory or toxic effect on human cells. The addition of 150 μg/ml anti-A-431 IgG alone did not stimulate DNA synthesis. In a separate experiment (data not shown) varying concentrations of anti-A-431 IgG (10, 20, 30, 45, 60, 75, 90, 120 and 150 μg/ml) were tested and no stimulation of DNA synthesis was observed.

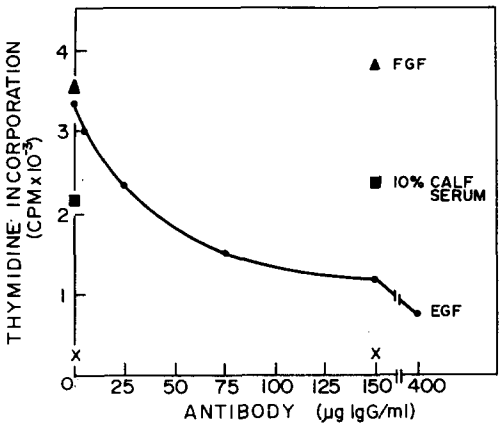

Fig. 3. Effect of anti-A-431 IgG on the stimulation of DNA synthesis by different mitogens. Quiescent monolayers of human fibroblasts were preincubated for 1 h at 37°C with increasing concentrations of anti-A-431 IgG. Mitogens were then added as follows: ●———●, 5 ng/ml EGF; ▲, 50 ng/ml FGF; ■, 10% (v/v) calf serum; X, no addition. 20 h later the cells were pulsed for 4 h with [^{3}H]thymidine and the amount of radioactivity incorporated into acid-insoluble material was determined as described in Methods.

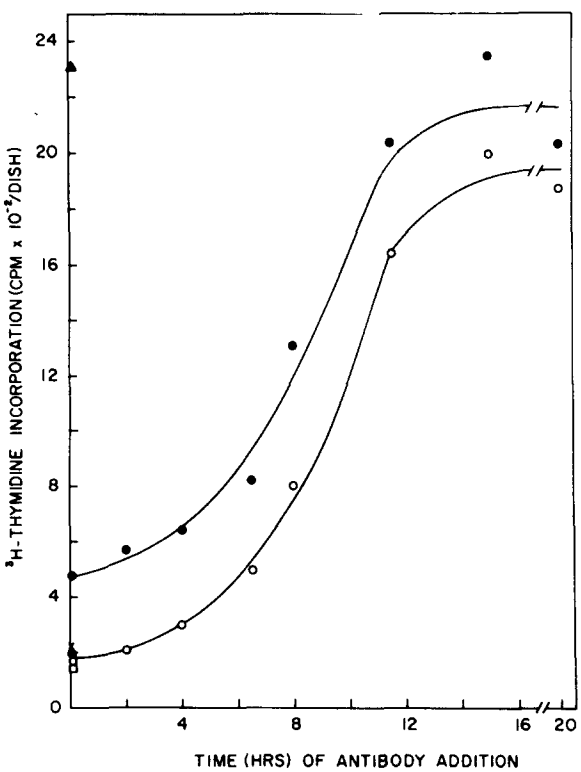

Fig. 4. Effect of the addition of anti-A-431 IgG or anti-EGF IgG, at various times, on the stimulation of DNA synthesis by EGF. 10 ng/ml EGF were added to confluent quiescent monolayers of human fibroblasts and at the indicated times 65 µg/ml of anti-EGF IgG (○———○) or 380 µg/ml anti-A-431 IgG (●———●) were added. For zero time additions the antibodies were added 1 h prior to the EGF. Control cultures received: ▲, EGF but no antibody; ■, no addition; X, anti-A-431 IgG but no EGF; □, anti-EGF but no EGF. All the cultures were labeled with [^{3}H]thymidine for 4 h at 20 h after the addition of EGF. The amount of radioactivity incorporated into acid-insoluble material was determined as described in Methods. The addition of anti-EGF or anti-A-431 IgG fractions in the absence of EGF did not increase [^{3}H]thymidine incorporation above the unstimulated level.

Previous studies [16] have shown that if EGF is removed from the media of stimulated fibroblasts at any time up to approx. 5 h after its addition, there is no subsequent increase in DNA synthesis. The experiment described in Fig. 4 was performed to examine the effect of interrupting EGF-receptor interactions by the addition of either anti-A-431 IgG or anti-EGF IgG at various times after the addition of EGF to quiescent cultures of human fibroblasts. The data in Fig. 4 show that, independently, each of the antibodies has a similar effect on the ability of exogenous EGF to commit quiescent fibroblasts to DNA synthesis. No stimulation of DNA synthesis occurred if either of the antibodies was added during the first 5 h after addition of EGF. Neither antibody inhibited the commitment to DNA when added 12 h or more after EGF.

Effect of antibody in subcellular receptor preparations

If the anti-A-431 IgG contains an antibody to the EGF receptor, then ^{125}I-labeled EGF binding should be blocked in subcellular receptor preparations where there are fewer indirect effects than in intact cells. The data in Table I demonstrate that anti-A-431 IgG blocks ^{125}I-labeled EGF binding in different subcellular receptor preparations to a quantitatively similar extent. Receptor activity in particulate membrane fractions from both cultured A-431 cells and human placenta was inhibited by approx. 83% in the presence of 30 μg (150 μg/ml or 1.0 μM) anti-A-431 IgG. Significantly, the inhibitory effect of anti-A-431 IgG also was observed with Triton X-100-solubilized A-431 membranes. Since the solubilized EGF receptor has been separated from many other membrane components by detergent treatment, this provides the strongest evidence that the anti-A-431 IgG actually contains antibodies to the EGF receptor.

It has been reported that antibodies against the insulin receptor can precipitate solubilized insulin-receptor complexes [24]. We have tested the anti-A-431 IgG to determine if the antibodies present are capable of precipitating soluble

TABLE I

INHIBITION OF ^{125}I-LABELED EGF BINDING BY ANTI-A-431 IgG TO SUBCELLULAR RECEPTOR PREPARATIONS

Human placental membranes (100 μg), particulate A-431 membranes (12 μg), or solubilized A-431 membranes (30 μg) were preincubated in the presence of the indicated amounts of anti-A-431 IgG for 30 min at room temperature in a total volume of 200 μl. ^{125}I-labeled-EGF (29 000 cpm/ng) was then added at final concentrations of 15 ng/200 μl for particulate membranes and 50 ng/200 μl for the solubilized membranes. After incubation for 30 min at room temperature, the level of specific ^{125}I-labeled-EGF binding was determined as described in Methods. The values in parentheses are percentages of binding activity relative to the binding obtained in the abscence of antibody.

Anti-A-431 IgG added (μg)	^{125}I-labeled EGF bound (cpm)		
	Particulate placenta membranes	Particulate A-431 membranes	Solubilized A-431 membranes
0	11 089 (100)	6403 (100)	7107 (100)
1.3	9 609 (87)	5469 (85)	5773 (81)
6.6	7 480 (67)	3851 (60)	4579 (64)
30.0	1 800 (16)	1168 (18)	711 (10)

TABLE II

EFFECT OF ANTI-A-431 IgG ON THE STIMULATION OF MEMBRANE PHOSPHORYLATION BY EGF

A-431 membranes (22 μg) were preincubated with 80 μg of each IgG in a 60 μl reaction mixture containing Hepes buffer (20 mM, pH 7.4), 1 mM $MnCl_2$ and 7.5 μg bovine serum albumin for 10 min at 0°C. 13 ng EGF were added to the indicated tubes and the incubation continued at 0°C for an additional 10 min. The reaction was initiated by the addition of 15 μM [γ-^{32}P]-ATP ($8 \cdot 10^5$ cpm). After a 10 min incubation at 0°C in the presence of labeled ATP, the reaction was terminated and the amount of radioactivity incorporated into endogenous membrane proteins was determined as described previously [12].

Additions	^{32}P incorporated (cpm)	
	Basal	Plus EGF
None	703	1856
Normal IgG	743	1736
Anti-A-431 IgG	634	925
Anti-EGF IgG	740	739

^{125}I-labeled EGF-receptor complexes. The results demonstrated that after reaction mixtures containing ^{125}I-labeled EGF (10 ng, 17 300 cpm/ng) and solubilized A-431 membranes were incubated for 30 min at room temperature, rapid poly(ethylene glycol) treatment precipitated 2.9% of the total counts (5151 cpm), while sequential incubations with anti-A-431 IgG and protein A precipitated 0.9% or 1499 cpm. Control treatments with normal IgG and protein A, or anti-A-431 IgG alone precipitated less than 0.03% or approx. 50 cpm. Also, in the absence of solubilized membranes, less than 50 cpm of ^{125}I-labeled EGF was precipitated by anti-A-431 IgG and protein A. Although immunoprecipitation of the solubilized ^{125}I-labeled EGF-receptor complex by anti-A-431 IgG and protein A was not as efficient as the more rapid precipitation accomplished with the poly(ethylene glycol) procedure, significant levels of solubilized receptor-hormone could be precipitated by the antibody.

The formation of EGF-receptor complexes has been shown to increase the level of endogenous protein phosphorylation in A-431 membrane preparations in the presence of [γ-^{32}P]ATP [11,12]. Table II shows that preincubation of A-431 membranes with anti-A-431 IgG decreased the basal level of phosphorylation by only 10%, but blocked the EGF-increased phosphorylation by 75%. The anti-A-431 IgG preparation, therefore, does not appear to contain inactivating antibody to the protein kinase present in A-431 membranes.

Discussion

The human epithelioid carcinoma cell line A-431 has approx. $2 \cdot 10^6$ EGF receptors per cell [25]. Membranes prepared from these cells and used as an antigen in this study bind about 16 pmol of ^{125}I-labeled EGF/mg of membrane protein [12]. Experiments with mouse 3T3 cells [26] and human placenta [27] indicate that membrane-bound EGF can be covalently cross-linked to putative receptor molecules having molecular weights of 195 000 and 180 000, respectively. Assuming that one molecule of EGF binds to each receptor mole-

cule, we calculate that approx. 0.3% of the protein in the A-431 membrane preparation is EGF receptor. Each injection of the membrane preparation (2 mg) into rabbits for antibody production, therefore, contained approx. 6 μg of EGF receptor to serve as an antigen. Although this is a small amount of antigen, the data in this report indicate it may well be sufficient to elicit an immune response to the human EGF receptor.

Double immunodiffusion of A-431 membrane components against the IgG fraction of the immune sera shows the presence of precipitating antibodies to several species of A-431 membrane antigens. Several lines of evidence presented in this report indicate that antibody to the EGF receptor is present. ^{125}I-labeled EGF binding to human fibroblasts and mouse 3T3 cells was reduced to similar extents at the same concentrations of antibody. Binding was reduced to 50% by either intact IgG and F(ab')$_2$ at concentrations of 0.15 μM in human cells and 0.20 μM in mouse cells. The monovalent Fab', however, was considerably less effective on mouse as opposed to human cells. This may suggest a slight antigenic difference in the EGF receptors of these two species.

Inhibition of ^{125}I-labeled EGF binding to intact cells by monovalent Fab' indicates that aggregation of cell-surface components which would be produced by IgG or F(ab')$_2$ is not a mechanism for the inhibition of binding. This conclusion is further supported by the ability of the IgG to block binding at 5°C (where the lateral mobility of cell-surface molecules in the plane of the membrane is limited). Also, internalization of the cell-surface components, including EGF receptors, in response to IgG binding cannot account for the decreased binding as internalization is minimal at low temperatures.

That the anti-A-431 IgG blocks EGF binding in a direct manner is best evidenced by the inhibition of ^{125}I-labeled EGF binding to detergent-solubilized receptor preparations at IgG concentrations similar to those used with intact cells. Also, solubilized ^{125}I-labeled EGF-receptor complexes could be precipitated by addition of anti-A-431 IgG followed by the addition of protein A.

The biologic effects of the anti-A-431 IgG described in this report are specific in that the antibody blocked stimulation of DNA synthesis in human fibroblasts by EGF but not by FGF or calf serum. It has been reported that some antisera to receptors which affect the interaction of particular hormones and their receptors also are able to mimic the biological activity of the respective hormone, i.e., insulin [28,29], thyroid-stimulating hormone [30]. The anti-A-431 IgG described herein did not, at various concentrations, stimulate DNA synthesis in human fibroblasts as did EGF. This antibody has not been tested for its ability to evoke other EGF responses, particularly those which occur early in the mitogenic response. However, the antibody did not mimic the effect of EGF in enhancing protein phosphorylation in vitro.

Maximal binding of EGF to human fibrobalsts is achieved within 1 h after addition of the growth factor [5,8]; however, induction of DNA synthesis is not observed until 12 h and is maximal at 20—24 h [16]. Previous experiments in which EGF was removed, either by the addition of anti-EGF IgG [16] or by washing [31], at various times after its addition to quiescent cells, indicate that the growth factor must be continuously present in the media for many hours (5—8 h) for a significant stimulation of DNA synthesis to occur.

In the experiment described in Fig. 4 we have compared the consequence of

interrupting EGF-receptor interactions, either by removing EGF with anti-EGF IgG or by blocking the EGF receptors with anti-A-431 IgG, at various times after the addition of EGF to quescent human fibroblasts. The data show that if growth factor-receptor interactions are blocked by either method, an identical effect on the induction of DNA synthesis is produced.

Although ^{125}I-labeled EGF is rapidly internalized and degraded [8], not all the cell-bound radioactivity is lost when cells are incubated for extended times (20 h) in the continuous presence of the growth factor. After 6 h of incubation with human fibroblasts, a steady-state level of hormone binding is reached which does not decrease further and which represents approx. 20% of the maximal binding. Since 70—80% of the cell-bound ^{125}I-labeled EGF is still being rapidly internalized and degraded at these later (after 6 h) times [8], it appears that newly synthesized or 'recycled' receptors are constantly being inserted into the membrane at a rate which permits a constant but decreased level of ^{125}I-labeled EGF binding. It is significant, therefore, that anti-A-431 IgG blocked EGF-stimulated thymidine incorporation when added at times up to approx. 6 h after the growth factor. These observations suggest that the antibody is able to block EGF binding to receptors which are constantly being inserted into the membrane and that EGF must occupy these receptors to stimulate DNA synthesis. The initial interaction of EGF with its receptor, therefore, is not sufficient to enhance DNA synthesis, but rather persistent occupancy of the newly synthesized or recycled receptors is required.

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