Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1337–1341, March 1983
Cell Biology

Growth stimulation of A431 cells by epidermal growth factor: Identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody

(hormone receptors/mitogenesis)

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Communicated by Morris Friedkin, November 15, 1982

ABSTRACT **Epidermal growth factor (EGF) at 3 nM maximally inhibits the proliferation of A431 epidermoid carcinoma cells. We show that at lower concentrations, in the range of 3–100 pM, EGF has a mitogenic effect on A431 cells. In the presence of 100 nM anti-EGF-receptor monoclonal IgG (designated 528), which inhibits A431 cell proliferation and blocks >95% of EGF binding, EGF becomes mitogenic for A431 cells at concentrations up to 3 nM. These results suggest that a minor population of high-affinity EGF receptors may be involved in stimulation of A431 cell proliferation. Saturation binding assays with ^{125}I-labeled EGF indicate that ≈0.1–0.2% of receptors for EGF are high-affinity receptors that bind EGF with an estimated K_d of 7×10^{-11} M. This affinity is nearly 2 orders of magnitude higher than that of the remaining EGF receptors. Although A431 cell proliferation is maximally inhibited by nonsaturating amounts of EGF (3 nM), maximal inhibition by 528 IgG (≈70% of maximal inhibition by EGF) requires saturating concentrations of antibody (≈15 nM). Unlike EGF, rapid down-regulation is not observed with 528 IgG. These results indicate different mechanisms of growth inhibition of A431 cells by EGF and 528 IgG.**

Epidermal growth factor (EGF) promotes the growth of many cell types *in vitro* (1–3) and inhibits proliferation of several cell types—e.g., GH_4 rat pituitary tumor cells (4), A431 epidermoid carcinoma cells (5, 6), and certain human breast cancer cells (7). EGF initially binds to receptors homogeneously distributed on the cell surface. Subsequent events have been described by various investigators and include receptor phosphorylation, aggregation, internalization, and degradation in lysosomes (1). The mechanism by which these events induce DNA synthesis and cytokinesis is unknown.

It has been found that at least 6–8 hr of EGF exposure are required to stimulate DNA synthesis (8). Das and Fox have suggested that EGF-induced internalization and degradation of the EGF receptor are rate-limiting factors for EGF-induced mitogenesis (9, 10), perhaps through production of a second messenger. Recent studies showed enhancement of EGF stimulation of DNA synthesis by amine compounds, which inhibited clustering of receptors in coated pits (11), and by phorbol esters, which reduced both the affinity of EGF receptors for EGF and its subsequent degradation (12–14). These results suggest that EGF stimulation of cell growth might only require the presence of EGF–EGF receptor complexes at the cell surface in contradiction to the above hypothesis.

Shechter *et al.*, on the other hand, suggested that the stimulatory effect of EGF might be mediated by small amounts of high-affinity EGF receptors, which remain at the cell surface for more than 8 hr when occupied by EGF (15). King and Cuatrecasas also have suggested that the accumulation of stable intracellular complexes between high-affinity receptors and EGF are involved in growth stimulation, but the role of these high-affinity receptors in mitogenesis remains unclear (16).

A431 cells lend themselves to the study of EGF interactions with receptors because of their extremely high number of EGF receptors ($1–3 \times 10^6$ per cell) (1, 17, 18). They are atypical in that doses of EGF that are mitogenic in many other cell lines inhibit proliferation. In this paper we describe the stimulation of A431 proliferation with low levels of EGF and use a monoclonal antibody directed against EGF receptors to identify a population of high-affinity receptors that may be relevant to this stimulatory effect of EGF.

MATERIALS AND METHODS

Cell Culture. A431 cells were grown in 1:1 (vol/vol) Dulbecco's modified Eagle's medium/Ham's F12 medium (DME/F12 medium) containing 15 mM Hepes; 1.2 g of $NaHCO_3$, 40 mg of penicillin, 8 mg of ampicillin, and 90 mg of streptomycin per liter; and 0.5% newborn calf serum at 37°C in 5% CO_2/95% air.

Preparation of Anti-EGF Receptor Monoclonal Antibodies. BALB/c mice were immunized by intravenous administration of EGF receptors partially purified from A431 cells by EGF-affinity chromatography (unpublished data). Anti-EGF-receptor antibodies were detected by ^{125}I-labeled EGF (^{125}I-EGF) binding inhibition assays with A431 as target cells. EGF and IgG were iodinated by the chloramine-T method (19). Hybrid cells producing anti-EGF-receptor antibodies were cultured in modified serum-free medium as described (20). Secreted IgG was purified by protein A-agarose affinity chromatography (Calbiochem–Behring). The culture medium was applied directly to the column, the material was washed with 20 mM Hepes, pH 7.4/20 mM NaCl, and the IgG was eluted with 1.0 M acetic acid/0.1 M glycine (pH 3.0). After dialysis in the Hepes/NaCl buffer, the purity was confirmed by $NaDodSO_4$ gel electrophoresis.

^{125}I-EGF Binding Assay. A431 cells grown in DME/F12 medium without serum in 100 mm × 22 mm dishes ($\approx 1 \times 10^7$ cells per dish) were fixed with 0.2% paraformaldehyde for 10 min at room temperature to inhibit receptor internalization during incubations with EGF or antibody. This procedure does not alter

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Abbreviations: EGF, epidermal growth factor; DME/F12 medium, 1:1 (vol/vol) Dulbecco's modified Eagle's medium/Ham's F12 medium; NaCl/P_i, phosphate-buffered saline; NaCl/P_i/albumin, NaCl/P_i/bovine serum albumin.

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binding affinity (21). Cells were scraped off with a policeman, washed three times with phosphate-buffered saline ($NaCl/P_i$) (pH 7.4) containing 0.2% bovine serum albumin ($NaCl/P_i$/0.2% albumin) and resuspended in the same buffer at a cell density of 1×10^6 per ml. For binding assays, microtiter plates were used in which glass wool membrane filters overlay punctured well bottoms of Falcon microtiter plates (VP no. 107, V & P Enterprises, San Diego, CA). Cells are quantitatively collected upon the membrane filters by suction applied with an apparatus that holds the microtiter plates, obtained from the same vendor; 20 μl (2×10^4 cells) were collected onto membrane filters in 96-well plates and washed two times with 0.25% gelatin in $NaCl/P_i$/1% albumin to prevent nonspecific binding. ^{125}I-EGF (specific activity, 5×10^4 cpm/ng) was added with or without 100 nM anti-EGF-receptor monoclonal antibody (528 IgG) and incubated in 50 μl (total volume) of $NaCl/P_i$/0.2% albumin for 2 hr at room temperature (25°C). After incubation, the wells were washed five times with 0.25% gelatin in $NaCl/P_i$ containing 5% newborn calf serum, the filters were dried, and radioactivity was measured in a gamma counter. To examine more precisely the number of high-affinity EGF receptors and their K_d, 1×10^5 cells were seeded per well and ^{125}I-EGF (specific activity, 1.6×10^6 cpm/ng) binding assays were carried out in the presence of 100 nM 528 IgG by incubating at 37°C or 0°C for 3 hr.

Binding Assays with ^{125}I-Labeled 528 IgG (528 ^{125}I-IgG). Fixed A431 cells (2×10^4 per well) were incubated with 528 ^{125}I-IgG (specific activity, 6×10^4 cpm/10 ng) for 2 hr at 37°C and washed with gelatin in $NaCl/P_i$ as described above, and the filter-bound radioactivity was measured.

^{125}I-EGF Binding Inhibition Assay. A431 cells were seeded at 2×10^4 cells per well, and 100 pg of ^{125}I-EGF (1.6×10^6 cpm/ng) were added to each well with increasing concentrations of 528 IgG. The cells were incubated for 2 hr at 37°C and washed with gelatin in $NaCl/P_i$ as described above, and the filter-bound radioactivity was measured.

Effects of EGF and 528 IgG on Cell Proliferation. Cells were seeded at $1–2 \times 10^4$ cells per well in 24-well plates (Costar) in DME/F12 medium containing 0.5% newborn calf serum and incubated for 4–5 days with various concentrations of EGF with or without 100 nM 528 IgG. The proliferation of A431 and HeLa-S cells in response to 528 IgG was examined in a similar manner.

Internalization of ^{125}I-EGF and 528 ^{125}I-IgG in A431 Cells. A431 cells were seeded in 24-well plates (Costar), grown to confluence, washed with fresh DME/F12 medium once, and incubated with ^{125}I-EGF (2×10^5 cpm/4 ng) or 528 ^{125}I-IgG (2×10^5 cpm/10.6 ng) in 200 μl of DME/F12 medium per well for periods of between 0 and 8 hr at 37°C in 5% CO_2/95% air. The cells were chilled in an ice bath and incubated at 0°C for an additional hour. After three washes with cold DME/F12 medium, surface-bound ligand was eluted with acetic acid by the method of Haigler *et al.* (22). Radioactivity in the eluates was measured in a gamma counter.

RESULTS

Stimulation of A431 Cell Growth by Low Concentrations of EGF. It has been reported (5, 6) that the growth of A431 cells was inhibited by EGF at a concentration of ≈3 nM, which stimulated proliferation of other types of cells (HeLa-S, human foreskin fibroblasts). This observation was confirmed in our initial experiments (Fig. 1). However, we observed stimulation of proliferation at low concentrations (3 pM) of EGF in DME/F12 medium containing 0.5% newborn calf serum (Fig. 1) or in serum-free medium (data not shown). The increase in cell number of >25% was statistically significant ($P < 0.05$) and was observed in three separate experiments.

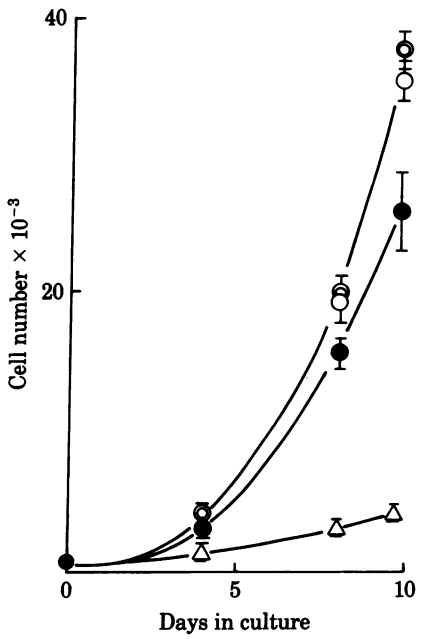

FIG. 1. Effect of EGF on A431 cell growth. Cells (5×10^4) were seeded per 100 × 22 mm dish in 5 ml of DME/F12 medium containing 0.5% newborn calf serum and incubated at 37°C in 5% CO_2/95% air. Additions: none (●), 1.7 pM EGF (○), 3.4 pM EGF (⊙), 3.4 nM EGF (△). Fresh medium (5 ml) was added on days 4 and 7. Each point represents mean cell counts ± SEM.

Characterization of Monoclonal 528 IgG and Its Inhibitory Effect on A431 Cell Growth. Anti-EGF-receptor monoclonal antibodies were obtained by fusing spleen cells from mice immunized with EGF receptor and NS-1 mouse myeloma cells. Antibody 528 IgG was in competition with EGF for binding to A431 and HeLa-S cells. It inhibited the mitogenic effect of EGF on HeLa-S cells, and it inhibited the stimulation of EGF receptor phosphorylation by EGF (unpublished data). 528 IgG alone inhibited the growth of A431 cells *in vitro* by >50% (Fig. 2). Maximum binding of 528 IgG to A431 EGF receptors occurred at 15 nM (Fig. 3), the concentration at which growth inhibition was maximal (Fig. 2). Another monoclonal IgG, 455,

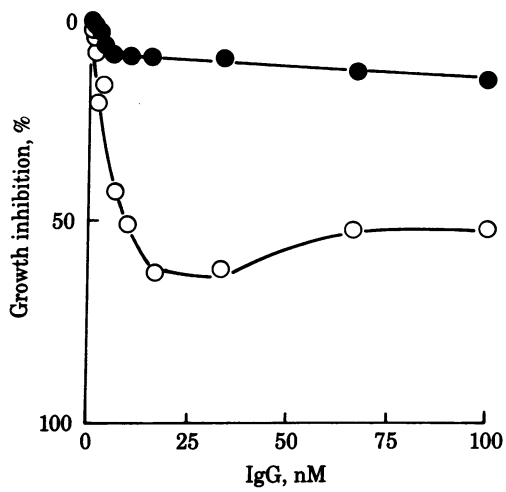

FIG. 2. Effect of 528 IgG and 455 IgG on A431 cell proliferation. A431 cells (1×10^4 cells per well) were seeded into 24-well plates and incubated for 5 days at 37°C in 5% CO_2/95% air with DME/F12 medium containing 0.5% newborn calf serum and either 528 IgG (○) or 455 IgG (●). Each point represents the mean of duplicate wells. Control cell number in the absence of any Ig was 2×10^5 per well on day 5.

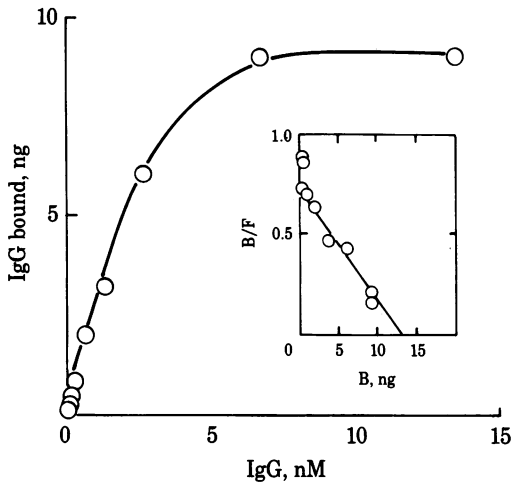

FIG. 3. 528 ^{125}I-IgG binding to A431. Fixed A431 cells (2×10^4) were plated into each well of a microtiter plate with glass wool filters. After washing twice with 0.25% gelatin in NaCl/P_i/1% albumin, 528 ^{125}I-IgG (6×10^4 cpm/10 ng) was added in 50 μl of NaCl/P_i/0.2% albumin. The cells were incubated for 2 hr at 37°C. Nonspecific binding (blank) was determined in the presence of a 100-fold excess of unlabeled 528 IgG. Each point represents the mean of duplicate determinations after subtracting the blank value. (*Inset*) Scatchard analysis of the IgG binding data. B, bound; F, free.

which bound to A431 with high affinity but could not compete with EGF, did not inhibit the growth of A431 cells (Fig. 2). 528 IgG bound to A431 cells with an apparent K_d of 2.5×10^{-9} M, which was similar to that of EGF (apparent K_d, $\approx 5 \times 10^{-9}$ M) (Figs. 3 and 4*a*).

These studies were carried out in the presence of 0.5% fetal calf serum, which optimizes growth of A431 cells. An identical experiment was performed in serum-free medium to examine the possibility that 528 IgG prevented proliferation by blocking access of low amounts of EGF in the 0.5% serum supplement. The results were nearly identical to the data in Fig. 2: proliferation was inhibited to a level of 50% of controls, at a concentration of 10 nM IgG (data not shown). Therefore, the antibody was not inhibiting growth by preventing binding of endogenous EGF in the serum.

^{125}I-EGF Binding to A431 Cells in the Presence of a High Concentration of 528 IgG. After examining individually the binding of EGF and of 528 IgG to the receptor, we next investigated the interaction between EGF and 528 IgG when presented to A431 cells simultaneously. In the presence of 100 nM 528 IgG, there was uptake of small amounts of ^{125}I-EGF, with an apparent plateau at a concentration of <0.2 nM (Fig. 4*b*). This was further explored by using ^{125}I-EGF with higher specific activity, larger numbers of A431 cells, and more data points in the range of 0–3 nM EGF (Fig. 5). ^{125}I-EGF binding to A431 cells in the presence of a higher-than-saturating concentration (100 nM) of 528 IgG showed a biphasic binding curve, which confirmed the presence of a small number of high-affinity EGF receptors. These receptors represent about 0.1–0.2% of total EGF receptors, and they bind EGF with an apparent K_d of 7×10^{-11} M, estimated as 50% of the saturating concentration.

It is conceivable, however, that the presence of apparent biphasic EGF binding (i.e., the presence of high- and low-affinity receptors) may be due to the possibility of covalent binding of ^{125}I-EGF to EGF receptors (23). To eliminate such a possibility, A431 cells were incubated with unlabeled EGF for 2.5 hr after they had been incubated with ^{125}I-EGF for 2 hr. It was anticipated that if covalent binding of ^{125}I-EGF to the receptors had occurred, a significant amount of radioactive EGF should have been retained on the cells, even after incubating the cells with an excessive amount of unlabeled EGF. The results showed that covalent binding of ^{125}I-EGF to the receptor was negligible (data not shown).

Effects of EGF on A431 Cell Growth in the Presence of 528 IgG. Fig. 6 shows the effect of EGF on A431 cell growth in the presence and absence of 100 nM 528 IgG. In both cases, addition of EGF stimulated growth, with a maximum at ≈3 pM. Maximal growth inhibition was observed at 3 nM EGF in the absence of 528 IgG and at 100 nM EGF in the presence of a saturating concentration of 528 IgG. Thus, the presence of this amount of anti-receptor antibody shifted the EGF concentration causing inhibition of proliferation to the right on the curve by >1 order of magnitude. Under the conditions of maximum

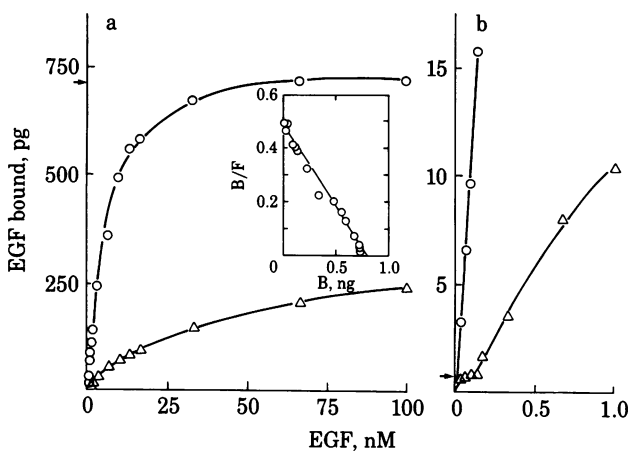

FIG. 4. ^{125}I-EGF binding to A431. (*a*) Fixed A431 cells (2×10^4) were plated per well and washed with 0.25% gelatin in NaCl/P_i/1% albumin. ^{125}I-EGF (5×10^4 cpm/ng) was added without (○) and with (△) 100 nM 528 IgG in 50 μl of NaCl/P_i/0.2% albumin and incubated for 2 hr at room temperature. Each point represents the mean of duplicate determinations from which was subtracted the blank value. The blank value was determined by incubating in the presence of a 100-fold excess of unlabeled EGF. (*Inset*) Scatchard analysis of EGF binding data in the absence of 528 IgG. B, bound; F, free. (*b*) Enlargement of *a* at low concentrations of EGF. The arrow on the ordinate axis indicates the apparent saturation of high-affinity binding sites.

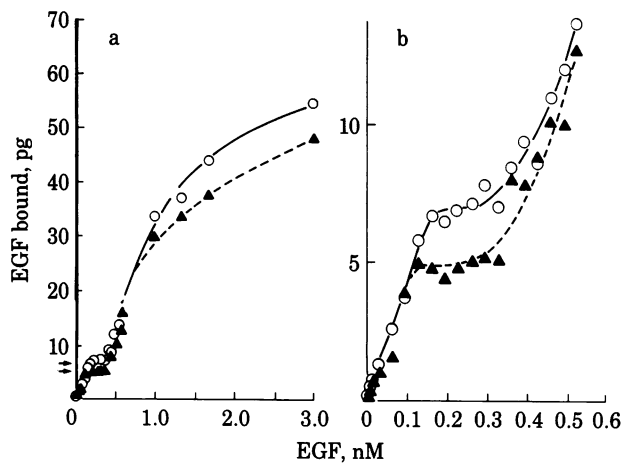

FIG. 5. ^{125}I-EGF binding on A431 cells in the presence of 100 nM 528 IgG. (*a*) To define the number and affinity of the high-affinity EGF receptors more precisely, 1×10^5 fixed A431 cells were seeded per well, and ^{125}I-EGF (1.6×10^6 cpm/ng) was added in the presence of 100 nM 528 IgG and incubated at 0°C (▲) or 37°C (○) for 3 hr. Arrows show the saturation level of the high-affinity EGF receptor at 0°C and 37°C. The blank values (100-fold unlabeled EGF) have been subtracted for each point. (*b*) Enlargement of *a* for low concentrations of EGF.

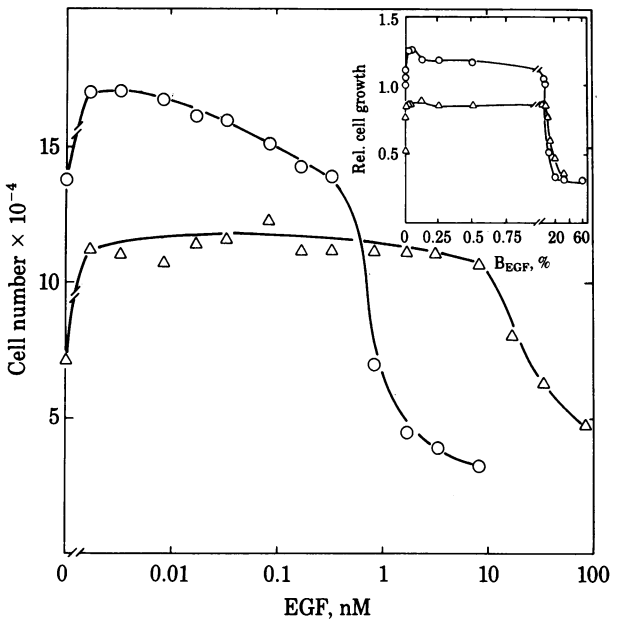

FIG. 6. Effect of EGF on A431 cell growth in the presence or absence of 100 nM 528 IgG. A431 cells (2×10^4) were seeded per 35-mm dish and incubated at 37°C in 5% CO_2/95% air for 4 days with 2 ml of DME/F12 medium containing 0.5% newborn calf serum without (○) or with 100 nM 528 IgG (△). Each point represents the mean of duplicate wells. (*Inset*) Bound EGF (B_{EGF}, % of total) at each concentration was calculated from the data in Fig. 4; 100% EGF binding was the saturation level in the absence of 528 IgG. Cell number on day 4 is expressed relative (Rel.) to that obtained in the absence of both EGF and 528 IgG (1.4×10^5 cells per dish).

growth inhibition, 20–30% of the EGF receptor sites were occupied by EGF, both in the presence and absence of 528 IgG (Fig. 6 *Inset*).

Down-Regulation of A431 Cell EGF Receptors by ^{125}I-EGF or 528 ^{125}I-IgG. No reduction in surface-bound 528 ^{125}I-IgG was apparent in an 8-hr incubation at 37°C, although ≈70% of bound ^{125}I-EGF was internalized within 3 hr (Fig. 7).

DISCUSSION

A431 epidermoid carcinoma cells are atypical because of the growth inhibition shown in response to EGF at doses that are mitogenic in other cell lines (5, 6). Until now there have been

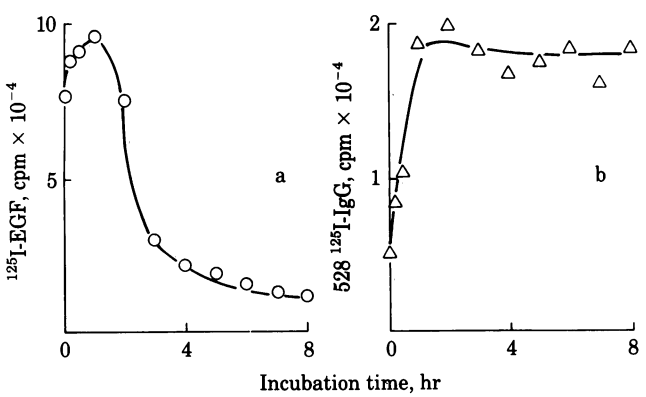

FIG. 7. Internalization of EGF and 528 IgG by A431 cells. Confluent cultures of A431 cells were incubated at 37°C in the continuous presence of ^{125}I-EGF (*a*) or 528 ^{125}I-IgG (*b*). At the indicated times, the cells were washed, bound ligand was eluted, and radioactivity in the eluates was counted. Each point represents the mean of duplicate assays.

no reports of A431 growth stimulation by EGF. However, we observe stimulation of A431 cell proliferation at extremely low concentrations (3 pM) of EGF (Fig. 1). This response suggests that EGF receptors may be heterogeneous, with a high-affinity component being relevant to growth stimulation. Our data support this hypothesis. 528 IgG inhibits the binding of ^{125}I-EGF to A431 cells by >95% (unpublished data). However, EGF maximally stimulates A431 cell growth in the presence of a 33,000-fold excess (100 nM) of 528 IgG (Fig. 6). In addition, in the presence of 100 nM 528 IgG, a small number of high-affinity EGF binding sites were detected in saturation binding experiments (Figs. 4 and 5). These high-affinity sites represent 0.1–0.2% of the total EGF binding sites per cell, and they bind EGF with an apparent K_d of 7×10^{-11} M. In contrast, EGF binds to the vast majority of receptor sites with a K_d of 5×10^{-9} M. The data suggest that, in the case of the high-affinity receptor sites, 528 IgG competes poorly with EGF for binding. The high-affinity sites may well be responsible for the mitogenic effect of EGF on A431 cells because the dissociation constant of these sites is about 20-fold higher than the concentration of EGF that causes maximum stimulation of growth.

The number of EGF receptors in A431 cells is extremely high ($1–3 \times 10^6$ per cell)—≈10–100 times more than on most other cell lines (1, 17, 18). However, the estimated number of high-affinity receptors ($3–6 \times 10^3$ per cell) may well be within the normal range of other cell types (16, 24–26).

At high concentrations, both EGF and 528 IgG inhibit the proliferation of A431 cells. Our data suggest that the inhibitory effects on A431 cell growth occur by different mechanisms. EGF shows maximal inhibition at ≈20–30% receptor occupancy (Fig. 6 *Inset*). 528 IgG displays maximum inhibition only at concentrations approaching saturation (Figs. 2 and 3). In addition, 528 IgG does not appear to induce either phosphorylation of EGF receptors (data not shown) or down-regulation of receptors (up to 8 hr of incubation) on A431 cells (Fig. 7). Inhibition of A431 growth by EGF might be caused by the huge energy expenditure necessary for receptor phosphorylation, internalization, degradation (9, 10, 27, 28), and new receptor synthesis (29), which must occur on a large scale in comparison to other cell lines with fewer receptors. Alternatively, monoclonal 528 IgG bound to EGF receptors might perturb the normal fluidity and function of the membrane surface, thus blocking spontaneous internalization and degradation of EGF receptors and causing growth inhibition as a result.

Recently, Buss *et al.* (30) suggested that high levels of tyrosine-specific protein kinase activity mediated the inhibitory effect of EGF on A431 cell growth. This hypothesis might explain our observation of a stimulatory effect of EGF in the presence of 528 IgG at concentrations at which both are normally inhibitory (Fig. 6). 528 IgG competes with EGF for the majority of EGF receptors (low-affinity sites) but does not stimulate the associated protein kinase. The lower level of total phosphorylation in the presence of excess 528 IgG may abrogate the inhibitory effect of EGF, resulting in the stimulation of growth, mediated, presumably, by the high-affinity receptors. EGF only becomes inhibitory in the presence of 528 IgG when it is present in concentrations high enough to compete effectively for access to the low-affinity receptors.

Very recently, Fox *et al.* (31) observed that high-affinity EGF receptors appear after pretreatment of 3T3 cells with EGF followed by incubation at 37°C. They also observed the presence of a low number of high-affinity receptors (<0.3%) on A431 cells (K. Iwata and C. F. Fox, personal communication). These observations agree with our results obtained by using monoclonal 528 IgG. On the other hand, our results show a clear presence of these high-affinity receptors on A431 cells with incubation at

0°C, even without EGF pretreatment. These results indicate the constant presence of high-affinity EGF receptors on the surface membranes of the A431 cells. Further experiments need to be done with our method to see if preincubation with EGF enhances the number of high-affinity receptors as has been suggested (16, 31).

At this time, it is not clear whether the high-affinity EGF receptors are converted from the major population of low-affinity receptors, as reported for insulin receptors (24, 25), nerve-growth-factor receptors (26), and EGF receptors of human KB cells (16). Growth stimulation by a low concentration (3 pM) of EGF with a saturating concentration (100 nM) of 528 IgG during days of continuous culture suggests the probability of *de novo* synthesis of high-affinity EGF receptors on the proliferating A431 cells. Further evaluation of the role of the high-affinity receptor in the mitogenic response should be possible by studying other cell lines that respond to EGF and A431 cell variants that are not inhibited by EGF (30).

There has been one previous report in which investigators have raised monoclonal antibodies against the receptor for EGF (32). We have used our anti-receptor antibody to explore the characteristics and biological roles of high- and low-affinity receptors. This approach can be used to study other hormone–receptor systems, such as insulin, transferrin, and nerve growth factor.

We wish to thank Elaine Hanson for technical assistance and M. A. Zurbach for her patience in typing this manuscript. This work was supported by National Institutes of Health Grants CA 33397 and GM 17702 to G.H.S. and Grants CA 09290 and CA 23052 to J.M. This research was conducted in part by the Clayton Foundation for Research, California Division; J.M. is a Clayton Foundation Investigator.

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