Consumption of EGF by A431 Cells: Evidence for Receptor Recycling

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Abstract. We examined the extent of EGF consumption by EGFR in A431 cells. When 125I-EGF was added to A431 cell cultures at low or high density, at concentrations which corresponded to 10-fold excess of ligand over receptor on the cell surface, most of the 125I-EGF was consumed within 2 h. The amounts of 125I-EGF consumed were much greater than available EGFR on the A431 cells, by a factor of 6.5 in low-density cultures and 5.8 in high-density cultures. When the concentration of 125I-EGF was increased in low density cultures, further consumption of 125I-EGF by the A431 cells was greatly reduced, partially due to a rapid down regulation of EGFR. However, when higher concentrations of 125I-EGF were added to high density cultures, with reduced receptor down regulation, the cells continued to consume a large fraction of the EGF in the culture medium. The consumption of 125I-EGF by these cultures was in excellent agreement with the measured amount of ligand internalized into the cell. EGF consumption was far in excess of the number of EGFR down regulated or degraded. Only a minor portion of the EGFR could have been replaced during the assay period by synthesis of new EGFR or from the intracellular pool of EGFR, and the fluid-phase uptake of EGF is only temporarily increased by exposure to EGF. Our results demonstrate that EGFR in high density A431 cell cultures recycled many times. The apparent level of recycling was dependent upon the concentration of EGF and followed Michaelis-Menton kinetics for ligand concentrations as high as 215 nM. At this EGF concentration, high-density cultures consumed 45 EGF molecules per receptor over a period of 6 h.

EGF is a well-characterized growth factor that stimulates the proliferation of various types of cells in culture and in vivo. EGF also can inhibit the proliferation of some cultured cell lines which express high levels of EGF receptors (EGFR).1 When EGF binds to EGFR on the cell plasma membrane, it activates an intrinsic protein tyrosine kinase which causes autophosphorylation of the receptor and phosphorylation of intracellular substrates that induce a mitogenic response (4, 5, 44). Transforming growth factor alpha (TGF-α) also binds to EGFR and activates the same pathways (9). EGFR are distributed diffusely over the plasma membrane. Upon binding of EGF to its receptors, the EGF-EGFR complexes cluster into coated pits and are internalized into the endosomes. These vesicles fuse with lysosomes, where both the ligand and EGFR are degraded (6, 34, 37, 40). The continuous presence of EGF causes the loss of EGFR from the cell surface, a process known as down regulation, and may result in attenuation of the cellular response to the ligand. However, replenishing of surface receptors can be accomplished by the recycling of EGFR which has been reported by several investigators (12, 17, 25, 35, 38), or by synthesis and expression of new EGFR.

Some tumors, particularly squamous cell carcinomas, over-express EGFR (8, 23), and many of these tumors concomitantly produce TGF-α (10). Overexpression of EGF may render these cells more sensitive to the mitogenic effect of limiting concentrations of ligands, and thus provide these cells with a proliferative advantage by autocrine stimulation. A431 cells, a human squamous carcinoma cell line, have amplification of the EGFR gene and express high levels of EGFR on their plasma membranes (14, 30, 45, 48). For this reason A431 cells have been widely used for studying the binding of ligands to EGFR and the internalization and processing of EGF-EGFR complexes.

A431 cells show atypical behavior related to the high expression of EGFR. EGF in nanomolar concentrations inhibits the proliferation of these cells in culture (2, 16). Following the binding of EGF to its receptor on A431 cells, the EGF-EGFR complex is internalized through the coated pits as in other types of cells, but much of the complex may be internalized by an alternative process (19, 20). Furthermore EGFR endocytosis in A431 cells appears to be a saturable process, possibly due to a mechanism which limits internalization of a large number of receptors (47). Although EGF-
induced down regulation in A431 cells involves large numbers of receptors, the percent reduction in EGFR expression is less prominent than in most cells (40).

Our previous studies demonstrated that A431 cells show a dual mitogenic response to EGF: at low concentrations (<0.3 nM) EGF can stimulate the proliferation of A431 cells, but at higher concentrations it inhibits their growth (26). A431 cells synthesize and secrete TGF-α into the culture medium. We have provided evidence that the TGF-α produced by A431 cells activates the protein tyrosine kinase of receptors after they are expressed on the A431 cell surface membrane, and not during receptor biosynthesis or intracellular processing (46). Anti-EGFR mAbs, which block EGF binding and prevent activation of receptor tyrosine kinase, can inhibit the proliferation of A431 cells, both in culture and in human tumor xenografts (26, 33, 36). These data strongly support an autocrine role for TGF-α produced by A431 cells.

Recently we have determined that when A431 cell cultures grow logarithmically, at low cell density, EGF internalization, and EGF-induced catabolism of the EGFR are greater than with A431 cells growing near-confluency in high-density cultures (43). The goal of the present studies was to determine the extent to which EGFRs on the surface of A431 cells are internalized and recycled in response to ligand, in cultures growing at low and high density. We measured the consumption of EGF both at saturating concentrations and at levels comparable to the dissociation constant of EGF binding to EGFR, the extent of EGF uptake into the intracellular compartment, and EGFR degradation and down regulation under these conditions. Our results demonstrate that EGFR in A431 cell cultures at low density undergo more rapid down regulation compared with A431 cells at confluence. Furthermore, high density A431 cell cultures consume EGF molecules from the culture medium in amounts which indicate that there must be frequent recycling of EGFR under these growth conditions.

Materials and Methods

Materials
Na $^{125}$I and $^{35}$S-cysteine (0.16 Ci/mmol) were purchased from New England Nuclear Research Products (Boston, MA). Mouse EGF was purchased from Collaborative Research (Bedford, MA). mAb 528 IgG2a against human EGFRs was produced as described in previous publications (26, 36, 42).

Analysis of Cell Proliferation
A431 cells were plated into 6-well plates containing 2 ml of a 1:1 (vol/vol) mixture of DME and Ham's F-12 medium (DME/F-12) supplemented with 5% newborn calf serum (NCS) and cultured at 37°C in an atmosphere of 5% CO$_2$/95% air. Cells were trypsinized and cell numbers were determined with a Coulter Counter (Coulter Electronics Inc., Hialeah, FL).

Consumption of $^{125}$I-EGF in Culture Medium
EGF was labeled with $^{125}$I by the chloramine T method as described previously (26, 36). A431 cells (1 x 10$^5$ cells/35-mm dish for high density cultures or 1 x 10$^4$/35-mm dish for low density cultures) were plated in DME/F-12 containing 5% NCS. 24 h later, cells were replenished with 1 ml of fresh medium containing $^{125}$I-EGF at various concentrations and incubated at 37°C. Aliquots (15 μl) of culture medium were removed and separated on 13.5% SDS-PAGE followed by autoradiography. The radioactivity in $^{125}$I-EGF, which was located by staining unlabeled EGF, was counted in a Packard gamma counter (Packard Instrument Co., Downers Grove, IL).

$^{125}$I-EGF Binding Competition Assay
EGF in the culture medium was measured as follows: 10 nM $^{125}$I-EGF was mixed with varying concentrations of unlabeled EGF in fresh culture medium, the binding of the EGF to A431 cell membranes was measured as described previously (26, 36), and a standard curve of $^{125}$I-EGF binding in competition with unlabeled EGF was constructed. In parallel, serial dilutions of the medium from cultures of A431 cells exposed to unlabeled EGF were mixed with 10 nM $^{125}$I-EGF under the same conditions and binding to A431 cell membranes was determined. The level of EGF in the culture medium was calculated by a comparison with the standard curve.

Measurement of Degradation of the EGF Receptor
A431 cells were suspended in 1 ml of cysteine-free DME containing 5% NCS and 100 μCi [35S]cysteine in 6-well plates, and incubated for 24 h at 37°C. The following day the medium was removed and the cell layer was rinsed with 0.15 M NaCl; 0.015 M Na phosphate, pH 7.4 (PBS) three times. The cells were incubated in 1 ml of fresh medium containing 15, 65, or 115 nM EGF for 6 h at 37°C. Then the medium was removed, and the cells were rinsed with cold PBS five times. The cells were then lysed with 500 μl of solubilization buffer (1% Triton X-100, 50 mM Hepes, pH 7.5, 250 mM NaCl, 10% Glycerol, 1.5 mM MgCl$_2$, 1 mM PMSF, 10 μg/ml Aprotinin, 1 mM EGTA, 10 mg/ml Leupeptin, 2 mM NaVO$_4$) at 4°C. After removal of insoluble material by centrifugation at 15,000 g for 10 min, 400 μl of the lysate was subjected to immunoprecipitation. After precipitation, the lysate was incubated with 10 μg of anti-EGF receptor mAb 528, then with 12 μg of rabbit anti-mouse IgG antisera, and precipitated at 4°C with 3 mg of protein A-Sepharose beads. The precipitate was washed with HNTG (20 mM Hepes, 150 mM NaCl, 0.1% Triton X, 10% glycerol) 3 times. [35S]-cysteine-labeled EGFR were isolated by SDS-PAGE, dried and subjected to autoradiography. The band of EGF receptors was excised and counted in a Beckman liquid scintillation counter.

Internalization of $^{125}$I-EGF
A431 cells were incubated in 1 ml of DME/F-12 containing 5% NCS with 3, 15, 65, 115, or 215 nM $^{125}$I-labeled EGF for 20 min at 37°C. Free ligand was removed by six washes with ice cold PBS. $^{125}$I-EGF associated with the cell surface was removed by washing with 0.2 M acetic acid (pH 3.0) containing 0.5 M NaCl for 6 min at 4°C (22). Internalized $^{125}$I-EGF was determined by solubilizing the cells in 2 ml of 1 N NaOH and measuring the radioactivity with a Packard gamma counter.

Down Regulation of the EGFR by EGF
A431 cells were incubated in 1 ml of DME/F-12 containing 5% NCS with 15 nM EGF for various periods of time (0-72 h) at 37°C. After cells were washed with cold PBS, EGF on the cell surface was removed with 0.2 M acetic acid as described above. Cells were incubated with 5 nM $^{125}$I-EGF for 2 h and maintained at 4°C to prevent the internalization of the $^{125}$I-EGF-EGFR complex. Unbound $^{125}$I-EGF was removed by five washes with cold PBS. $^{125}$I-EGF associated with receptors on the cell surface was assayed by dissolving the cells in 2 ml of 1 N NaOH and measuring the radioactivity with a Packard gamma counter.

Measurement of EGFR on the Cell Surface and in Intracellular EGFR Pools
A431 cells were labeled for 24 h with [35S]cysteine and the total content of labeled EGFR was measured as described above. To determine the amount of EGFR on the cell surface, a parallel culture of A431 cells was labeled with [35S]cysteine for 24 h, and saturating concentrations of 528 IgG were added to the cultures for 5 min. After washing off unbound 528 IgG by centrifugation, the cells coated with receptor-bound 528 IgG were pelleted and lysed. The lysate was subjected to immunoprecipitation without any additional 528 IgG, by adding rabbit anti–mouse IgG antisera, and protein A-Sepharose beads, as described above. The supernatant after immunoprecipitation was subjected to a second similar immunoprecipitation to determine the efficiency of the procedure, and the results showed that >99% of material had been immunoprecipitated. Having determined total [35S]-labeled EGFR and surface [35S]-labeled EGFR content by these two procedures, the intracellular [35S]-labeled EGFR was estimated as the difference between these two values.
Uptake of HRP

As a measure of fluid phase pinocytosis, the rate of uptake of HRP was measured as described by Haigler et al. (21). A431 cells were cultured, washed with PBS, and incubated for 15 min at 37°C in DME/F12 containing 0.1% BSA. This medium was replaced with 1.5 ml of the same medium containing 1.0 mg/ml HRP. The cultures were incubated at 37°C for 15 min, and rapidly washed two times with PBS/0.1% BSA followed by six washes at 4°C with cold PBS/0.1% BSA over 30 min. The cultures were lysed in 0.7 ml of 0.1% Triton X-100, and the amount of HRP in the lysate was determined. An aliquot of lysate was mixed with the buffer solution containing o-dianisidine and H2O2. The rate of reaction was measured by the change in absorption at 450 nm in a spectrophotometer (Beckman Instruments Inc., Palo Alto, CA). This rate was compared with a standard curve generated with known amounts of HRP.

Results

A431 Cell Growth

A431 cells, a human squamous carcinoma cell line, grow as a monolayer. To define A431 growth characteristics, the cells were cultured at low or high density in the presence or absence of 20 nM EGF, a saturating concentration, and the proliferation rate was determined. When cells were plated at a density of 7 x 10^4 cells per 35-mm dish, following a lag period of more than a day they grew actively and reached a confluent density of 2 x 10^5 cells after one day during the period of active growth. When A431 cells were plated at a density of 7 x 10^5 cells per dish, they doubled in one day, then grew at greatly reduced rates and reached a confluent density of 1 x 10^6 cells after less than one additional doubling. In the presence of 20 nM EGF, the cell growth was substantially inhibited at both high and low cell densities (Fig. 1). These results were used to design the culture conditions for subsequent experiments. For studies of growth in the logarithmic phase, at close to an optimal rate of proliferation, cells were plated at a "low density" of 1 x 10^5 per dish; for studies of cells near confluency and growing very slowly, the cultures were plated at a "high density" of 1 x 10^6 cells per dish.

Consumption of 125I-EGF from Culture Medium by A431 Cells

To initiate an analysis of the kinetics of EGF receptor internalization, we first measured the consumption of EGF from the culture medium, which should be equal to the amount of EGFR internalized by A431 cells. EGF was labeled with 125I and added to A431 cells in culture. Aliquots of medium were sampled at various times, and the level of 125I-EGF remaining in the culture medium was measured as described in Materials and Methods.

Because a major function of EGF is to regulate cell proliferation, we explored whether A431 cells growing rapidly at low density consume 125I-EGF at a different rate from those growing at high density. A431 cells were plated at high density and at low density, and 125I-EGF was added to give final concentrations of 15 and 1.5 nM, respectively. Under these conditions, the ratio of 125I-EGF molecules in the medium to EGFR molecules on cell surface membranes of A431 cells was the same in both cultures. The concentrations of 125I-EGF in the medium of the low density cultures (Fig. 2, curve B) and the high density cultures (curve A) decreased with time of incubation in an identical fashion. This is demonstrated in the transposed curve A', which superimposes curve A on curve B. The results of this experiment showed that when A431 cells are growing either in low density or high density cultures their EGF receptors can remove 125I-EGF from the culture medium at a similar rate.

The amounts of 125I-EGF consumed by A431 cells were surprisingly large compared to available EGFR on A431 cells. Cultures with 15 nM EGF contained 9 x 10^12 EGF molecules in the 1 ml of culture medium. When 15 nM 125I-EGF was added to the high-density A431 cell cultures and EGF consumption from the medium was assayed after various incubation times, the values for the number of molecules consumed were 6.4 x 10^12 (71%) after 2 h, 8.1 x 10^12 (90%) after 4 h, and 8.4 x 10^12 (93%) after 6 h (Table I, Experiment I). The initial total number of EGFR on all A431 cells in high-density cultures was 1.2 x 10^12 molecules, since we have shown that each cell bears 1.2 x 10^6 receptors under these growth conditions (43). Therefore, for each EGFR molecule initially present, the cells consumed 5.8, 7.4, and 7.6 molecules of 125I-EGF during 2, 4, and 6 h, respectively.

When EGF was added at 1.5 nM to A431 cultures at low density, the ratio of EGF molecules to EGFR molecules can be calculated as in the cultures described above. There were 9 x 10^11 EGF molecules in the medium. The initial total

![Figure 1. Growth of A431 cells in the presence and absence of EGF. Cells plated at high density in presence of 20 nM EGF (o); and in absence of EGF (•). Cells plated at low density in the presence of 20 nM EGF (△); in the absence of EGF (△).](image)

![Figure 2. Consumption of 125I-EGF from medium by A431 cells. A431 cells were plated at low and high densities and 125I-EGF was added at concentrations of 15 or 1.5 nM. Aliquots of medium were measured as described in Materials and Methods. (A) High density, nearly confluent cells, 15 nM 125I-EGF; (B) cells, at low density in logarithmic phase, 1.5 nM 125I-EGF. A' is the curve of A that is transposed and superimposed for comparison between A and B. In another experiment (C) cells were at low density in 15 nM 125I-EGF. In another experiment (D) 15 nM unlabeled EGF was added to high density cultures, and the levels of EGF remaining in the medium were measured by a binding competition assay as described in Materials and Methods.](image)
density was further explored under the conditions described above, with initial ligand concentrations of 65, 115, and 215 nM in the culture media. With EGF at these three concentrations, the cells consumed 16, 26, and 49 × 10² EGF molecules in 6 h and 31, 55, and 70 × 10² molecules in 24 h (Table I, Experiment II). It is readily calculated that during 6 h of incubation with these three concentrations of EGF, the A431 cells consumed 15, 24, and 45 EGF molecules per EGFR initially present. This represents consumption of ~40% of the EGF present in the culture medium, regardless of the initial concentration of ligand.

When comparable studies using high concentrations of EGF were performed with low density A431 cell cultures, the results were different from those observed with high density cultures. When the ¹²⁵I-EGF concentration in low-density cultures was raised tenfold, from 1.5 to 15 nM, the consumption of EGF was <10% over 24 h (Fig. 2, curve C).

### Kinetics of EGF Consumption in High-density Cultures

The number of EGF receptors available for occupation in the high-density cultures during the 6-h incubation period depended upon the initial receptor number expressed on the plasma membrane, the rate of receptor internalization, and the rate of entry of receptors into the plasma membrane, due either to recycling of internalized receptors or addition of new receptors from a precursor pool. In exploring the role of ligand concentration in receptor-ligand interaction, we made the assumption (as a first approximation) that levels of receptor expression remain constant. It will be shown below that this is reasonable in the case of high density cultures. If, for purposes of this calculation, we assume that the initial rates of EGF consumption, then the rate can be plotted by the Michaelis-Menten equation. The rate of uptake is found to be linear for ligand concentrations up to 215 nM (Fig. 3).

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**Table I. Consumption of ¹²⁵I-EGF from the Medium by A431 Cells at High Density**

| Experiment | Initial concentration (nM) | Time (h) | Concentration in medium (nM) | Percent consumed (%) | Number of EGF molecules consumed (×10⁻⁹) | Number of EGF molecules consumed per EGFR |
|------------|---------------------------|----------|-----------------------------|---------------------|----------------------------------------|----------------------------------------|
| I          | 15                        | 0        | 15                          | 0                   | 0                                      | 0                                      |
|            |                            | 2        | 4.3                         | 71                  | 6.4                                    | 5.8                                    |
|            |                            | 4        | 1.5                         | 90                  | 8.1                                    | 7.4                                    |
|            |                            | 6        | 1.0                         | 93                  | 8.4                                    | 7.6                                    |
|            |                            | 24       | 0.4                         | 97                  | 8.8                                    | 8.0                                    |
| II         | 65                        | 0        | 65                          | 0                   | 0                                      | 0                                      |
|            |                            | 6        | 38                          | 42                  | 16                                     | 15                                     |
|            |                            | 24       | 14                          | 78                  | 31                                     | 28                                     |
|            | 115                       | 0        | 115                         | 0                   | 0                                      | 0                                      |
|            |                            | 6        | 72                          | 37                  | 26                                     | 24                                     |
|            |                            | 24       | 24                          | 79                  | 55                                     | 50                                     |
|            | 215                       | 0        | 215                         | 0                   | 0                                      | 0                                      |
|            |                            | 6        | 133                         | 38                  | 49                                     | 45                                     |
|            |                            | 24       | 49                          | 54                  | 70                                     | 64                                     |

¹²⁵I-EGF was added to high-density cultures of A431 cells at various concentrations. Aliquot of medium were sampled at the stated time and the level of ¹²⁵I-EGF was measured as described in Materials and Methods. The consumed ¹²⁵I-EGF was converted to the number of ¹²⁵I-EGF molecules and divided by the total number of EGFR initially present on the cell surface membrane to estimate the number of EGF molecules consumed by one EGFR molecule. Each value is an average of two measurements from one of the depicted experiments (I and II). Measurements agreed within 10%.

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number of EGFR on A431 cells in low-density cultures was 1.8 × 10¹ molecules, since each cell bears 1.8 × 10⁹ receptors under these growth conditions (42). The cells at low density consumed the same quantity of ¹²⁵I-EGF molecules for each EGFR molecule as in the high density cultures: 6.5, 6.9, and 7.0 during 2, 4, and 6 h, respectively.

We used an independent assay method to confirm the consumption of large amounts of EGF by A431 cells. Unlabeled EGF was added at a concentration of 15 nM to the high density cultures. Aliquots of culture medium were removed at various times, and the levels of EGF in the medium were measured by an ¹²⁵I-EGF binding competition assay as described in Materials and Methods. The levels of EGF remaining in the culture medium were 5.5, 3.3, and 2.0 nM after 2, 4, and 6 h of incubation, respectively. These values are plotted in curve D of Fig. 2, and are in good agreement with the data obtained by measuring the disappearance of ¹²⁵I-EGF from the medium (curve A in Fig. 2).

These results demonstrate that high density A431 cell cultures initially consumed EGF at high rates when EGF was added to the culture medium at 15 nM, under which conditions the number of EGF molecules initially present was eightfold in excess of total EGFR on A431 cells. In these cultures, the majority of the EGF in the medium was consumed in less than 2 h, and by 4 h the ¹²⁵I-EGF levels were reduced to 1.5 nM, comparable with the dissociation constant. While further uptake of EGF continued with incubation, the rate of uptake was greatly reduced at the 6 and 24 h time points due to the reduced amount of ligand available (Table I, Experiment I).

We wished to examine the uptake of labeled EGF under conditions where ligand in the culture medium fulfilled two conditions: (a) concentration of more than 10× the dissociation constant of EGF binding to the EGF receptor; and (b) number of EGF molecules more than 10× the number of EGFR. The consumption of ¹²⁵I-EGF by A431 cells at high density was further explored under the conditions described above, with initial ligand concentrations of 65, 115, and 215 nM in the culture media. With EGF at these three concentrations, the cells consumed 16, 26, and 49 × 10² EGF molecules in 6 h and 31, 55, and 70 × 10² molecules in 24 h (Table I, Experiment II). It is readily calculated that during 6 h of incubation with these three concentrations of EGF, the A431 cells consumed 15, 24, and 45 EGF molecules per EGFR initially present. This represents consumption of ~40% of the EGF present in the culture medium, regardless of the initial concentration of ligand.

When comparable studies using high concentrations of EGF were performed with low density A431 cell cultures, the results were different from those observed with high density cultures. When the ¹²⁵I-EGF concentration in low-density cultures was raised tenfold, from 1.5 to 15 nM, the consumption of EGF was <10% over 24 h (Fig. 2, curve C).
These results show that at high EGF concentrations, A431 cells growing in high density cultures can consume far greater numbers of EGF molecules than the total number of EGFR initially available on the cell surface. The fact that each receptor is taking up so many EGF molecules suggests that EGFR on A431 cells are recycling. The possible role of an additional contribution from intracellular pools of stored or newly synthesized receptor will be considered below.

**Internalization of 125I-EGF**

The above measurements of EGF depletion from the culture medium were complemented by direct assays of ligand binding and internalization into A431 cells. Studies of high density cultures incubated with 15 nM 125I-EGF, a saturating concentration, demonstrated that binding was rapid, reaching maximal levels within 5 min (Fig. 4). Internalization of 125I-EGF followed, and intracellular ligand eventually exceeded the level of externally bound EGF (Fig. 4). Internalization followed nearly linear kinetics during the initial 20 min of incubation. To explore the role of EGF concentration in the rate of ligand uptake, the internalization of 125I-EGF into A431 cells was measured 20 min after the addition of 125I-EGF. At 15, 65, 115, and 215 nM the internalization of 125I-EGF was 1.0, 1.4, 1.7, and 1.6 picomoles per dish in 20 min (Table II). This corresponds to 6 × 10^11, 8 × 10^11, 10 × 10^11, and 10 × 10^11 EGF molecules, respectively. These values are in good agreement with the number of 125I-EGF molecules consumed from the culture medium in 20 min (Table II). The main discrepancy is the lower apparent intracellular levels of labeled EGF in the 215 nM culture; this may be due to enhanced catabolism of internalized ligand under these conditions.

**Effect of EGF on the Degradation of the EGFR**

The number of EGF molecules consumed by an EGFR molecule can also be estimated by comparing the number of EGF molecules consumed from the medium with the number of receptor molecules degraded during incubation with EGF. A431 cells were incubated with [35S]cysteine overnight. This period of incubation permitted nearly all of the labeled receptor to reach the cell surface membrane, where activation by EGF could occur (46). EGF was added at concentrations of 15, 65, and 115 nM, and the amount of EGF degraded after 6 h of incubation was measured, as described in Materials and Methods (Table III, Experiment A). Labeled EGFR was degraded by only 30, 50, and 62% under these conditions (Table III, column 2). Thus, at each EGF concentration examined, the number of EGF molecules consumed was far greater than the number of EGFR molecules degraded over a 6-h interval of cell culture (Table III, column 3).

**Down Regulation of the EGFR by EGF**

Binding of EGF to the EGFR is followed by internalization of the EGF-EGFR complex and, eventually, by degradation of EGF and EGFR in lysosomes, resulting in down regulation of EGFR. If the EGFR recycles during these processes, the loss of EGFR due to the down regulation of the receptor should be less than predicted from the consumption of EGF from the culture medium.

We measured the down regulation of EGFR after addition of 15 nM EGF to A431 cell cultures at low and high densities. The number of EGFR on A431 cells in high density cultures was 92, 83, and 61% of the original number at 2, 6, and 24 h, respectively (Fig. 5). After 72 h the number of EGFR had decreased to less than half of the original number. The number of EGFR on A431 cells in the low-density cultures decreased more rapidly and reached levels as low as 15% within 24 h (Fig. 5). This may have contributed to the low consumption of 125I-EGF by A431 cells at low density, when added at 15 nM, as described above (Fig. 2, curve C).

To compare these data on down regulation with the data on receptor turnover and on EGF consumption that were obtained in earlier experiments, down regulation was assayed over a period of 6 h in high-density cultures incubated with EGF at concentrations of 15, 65, 115, and 215 nM. As shown in Table III, Experiment B, the receptor numbers expressed

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**Figure 3. Analysis of maximal rate of consumption by confluent A431 cells.** 

**Figure 4. Internalization of 125I-EGF into A431 cells.** 15 nM 125I-EGF was added to A431 cells. The amounts of 125I-EGF on the cell surface (O), and internalized (●), were measured as described in Materials and Methods.
Table III. Amount of EGF Consumed by Degradation or Down Regulation of EGFR

| EGF (nM) | (1) Number of EGF molecules consumed from medium* | (2) Number of EGFR molecules degraded | (3) EGF molecules consumed per EGFR molecule degraded | (4) Number of EGFR molecules down regulated | (5) EGF molecules consumed per EGFR molecule down regulated |
|----------|-------------------------------------------------|--------------------------------------|--------------------------------------------------|------------------------------------------|--------------------------------------------------|
| 0        | (x 10^-12)                                      | 0.11 (9%)                            | 23                                              | 0.00                                     | -                                                |
| 15       | 8.4                                             | 0.36 (30%)                           | 23                                              | 0.25 (23%)                              | 34                                               |
| 65       | 16                                              | 0.60 (50%)                           | 27                                              | 0.48 (44%)                              | 33                                               |
| 115      | 26                                              | 0.74 (62%)                           | 35                                              | 0.52 (47%)                              | 50                                               |
| 215      | 49                                              | -                                    | -                                               | 0.64 (58%)                              | 77                                               |

* Column 1 presents data taken from Table I.

Experiment A. Effect of EGF on degradation of EGFR in A431 cells: A431 cells in high-density culture were labeled with [35S]cysteine overnight. The cultures were replenished with fresh medium containing EGF at the stated concentrations and incubated at 37°C for 6 h. EGF was immunoprecipitated and quantitated as described in Materials and Methods. The degradation of EGF was expressed as the percent of labeled receptor no longer detectable, and then converted to the number of EGFR molecules degraded using the initial EGFR number, 1.2 x 10^12 EGFR molecules per cell in high-density culture.

Experiment B. Effect of EGF on down regulation of EGFR in A431 cells: A431 cells in high-density cultures were fed with fresh medium containing EGF at the stated concentrations and incubated at 37°C for 6 hrs. Down regulation of EGF was quantitated as described in Materials and Methods. The down regulation of EGFR was expressed as the percent of surface receptor no longer detectable, and then converted to the number of EGFR molecules down regulated using the initial EGFR number.

on the cell surface decreased by 23, 44, 47, and 58% under these conditions (Table III, column 4). This represents a reduction of up to 0.64 x 10^12 receptor molecules on the A431 cells. One can calculate the number of EGF molecules consumed from the culture medium per receptor down regulated, and the results for the respective concentrations of EGF are 34, 33, 50, and 77 (Table III, column 5). These values are in close agreement with the calculated number of EGF molecules consumed from the culture medium per receptor degraded (Table III, column 3). This is consistent with the conclusion that while down-regulated EGFR may recycle, they eventually are degraded. Clearly the number of EGF molecules consumed cannot be accounted for by the degradation or internalization/down regulation of its receptor, since under all conditions in columns 3 and 5 of Table III, the values are far greater than 1.0.

EGFR on the Cell Surface and in the Intracellular Pool in A431 Cells

To test whether replenishment of receptors from a large intracellular pool could explain the high levels of EGF consumption from the culture medium, we measured the amount of EGFR that was present on the cell surface and the EGFR contained in the intracellular pool, as described in Materials and Methods. 73% of EGFR was on the cell surface and only 27% was found to be in the intracellular pool. This result demonstrated that the intracellular pool of EGFR could not be a major factor contributing to the consumption of EGF.

Fluid-phase Pinocytosis of EGF by A431 Cells

Fluid-phase pinocytosis in A431 cells is known to be stimulated by EGF, and this could have contributed to the large amounts of EGF consumption by A431 cells in the presence of high concentrations of ligand. To assess this possibility, we measured the fluid-phase uptake of HRP, as described in Materials and Methods. The uptake during the initial 15 min was increased by an average of 61% in cultures exposed to saturating concentration of EGF, but the levels of uptake after 2 and 6 h of culture were close to the control values observed in the absence of EGF. These results are in accordance with previous studies, and they demonstrate that increased fluid-phase pinocytosis in A431 cells does not account for the increased consumption of EGF in the presence of high amounts of the ligand.

Discussion

It is generally accepted that upon binding of EGF, the complex of EGF with its receptor is internalized, and both the ligand and receptor are degraded in lysosomes, which, in turn, results in reduced surface expression or down regulation of the receptor. If this concomitant degradation of ligand and receptor occurs stoichiometrically, the number of EGFR molecules consumed would be equal to the number of EGF molecules degraded. However, this is not the case with A431 cells. Our observation that >90% of 125I-EGF was consumed in 6 h when 1.5 nM EGF was added to low-density cultures, or when 15 nM EGF was added to high density cultures, demonstrates that many EGF molecules are consumed by each EGFR.

When 125I-EGF was added at graded concentrations of 65, 115, and 215 nM to the high-density cultures, the A431...
cells consumed $\sim 40\%$ of the ligand from the culture medium in 6 h regardless of the initial concentration of ligand (Table I), demonstrating that A431 cells consume increasing amounts of EGF with the addition of higher concentrations of the ligand under these culture conditions. At the initial $^{125}\text{I}-\text{EGF}$ concentrations of 65, 115, and 215 nM, each EGFR molecule initially present on these cells consumed 15, 24, and 45 molecules of EGF from the culture medium during a period of 6 h (Table I). The results of our quantitative assays of EGF internalized from the culture medium give similar results (Table II).

When $^{125}\text{I}-\text{EGF}$ was added to the low-density cultures at an initial concentration of 15 nM the ratio of $^{125}\text{I}-\text{EGF}$ molecules to EGFR molecules increased to 50-fold, which is equivalent to 95 nM in the high-density cultures. By extrapolating from the data on EGF consumption in high density cultures (Table I) we predicted that each EGFR molecule in the low density A431 cell cultures would consume 45 molecules of $^{125}\text{I}-\text{EGF}$ during 24 h of culture, if the rate of $^{125}\text{I}-\text{EGF}$ consumption did not change with a 50-fold ratio of ligand to receptor. If this were the case, the levels of $^{125}\text{I}-\text{EGF}$ in the medium would have decreased from the initial 15 to 1.7 nM, during the 24 h, representing consumption of 13.3 nanomoles per liter. Actually, the consumption of $^{125}\text{I}-\text{EGF}$ was only $\sim \frac{1}{2}$ nanomole per liter over 24 h (Fig. 2, curve C). For each EGFR molecule which was initially present on the cell surface only about three molecules were consumed during 24 h. However, due to rapid down regulation of the EGFR in low-density A431 cell cultures, the average number of EGFR on the cell surface during this period was only $\sim 30\%$ of the initial number (by calculating from an integral of the curve of down regulation in Fig. 5). Therefore, during the 24-h period, each EGFR molecule in low density cultures consumed about 10 molecules of $^{125}\text{I}-\text{EGF}$. This calculation suggests that some, but by no means all, of the difference in EGF consumption by high and low density cultures in the presence of high concentrations of ligand is attributable to the more rapid receptor down regulation at low-culture density.

The number of EGFR on A431 cells that are available for consuming EGF from the culture medium, are affected by: (a) EGFR expressed initially on the cell surface membrane; (b) loss of receptors by internalization and degradation; and (c) entry of receptors from intracellular pools, due to recycling or to synthesis of new receptors. The data in Table III show that the limited catabolism of receptors initially present in the cell can not possibly account for the demonstrated quantity of EGF consumed over a 6-h period, unless either receptor recycling prior to catabolism or major levels of receptor replacement occurred. Our studies as well as others showed that most EGFR in A431 cells exist on the cell surface membrane, with only a minor intracellular reservoir of receptors (27, 40). Although addition of the ligand induces synthesis of EGFR in several cell lines (3, 7, 13, 28), ligand-induced stimulation of EGFR synthesis has not been observed in A431 cells (27, 41). We have determined that the half-life of EGFR is about 20 h in A431 cells cultured in the absence of exogenous ligand (43). Based on this value, the newly synthesized EGFR in A431 cells during a culture period of 6 h would be $\sim 30\%$ of the total EGFR initially expressed on the cells. Therefore, in consuming the observed quantities EGF from the medium during a 6-h period, the contribution by EGFR other than molecules that existed initially on the surface membranes of the A431 cells would be minor. To account for the observed levels of EGF consumption, there must be recycling of EGFR to the plasma membrane, after internalization of the receptor–ligand complex and deposition of ligand inside the cell for degradation. It has been known that EGF stimulates fluid-phase entry of A431 cells, but this phenomenon was temporary and did not account for the continuous consumption of EGF by A431 cells over a period of 6 h.

Our experiments have ruled out the possibility that replenishment of receptors from a large intracellular pool or EGF entry by fluid-phase pinocytosis could provide an alternative explanation for EGF uptake. In addition, we considered the possibility that non-specific binding EGF to the A431 cells could account for apparent EGF uptake. In experiments examining binding of $^{125}\text{I}-\text{EGF}$ to A431 cell receptors, non-specific binding is estimated by adding 100-fold excess unlabeled EGF. In such experiments, non-specific binding increases with increased concentrations of EGF, but at the high concentration of 1 $\mu$M EGF the non-specific binding of EGF reached a level of only $\sim 50\%$ of the specific binding to EGFR (data not shown). Therefore, apparent uptake of EGF through non-specific binding cannot explain the amount of EGF consumption by A431 cells under our experimental conditions.

Data suggesting recycling of EGFR has been reported by several investigators. The studies of Murthy et al. (35) provided evidence of receptor recycling in A431 cells. Sorkin et al. (38) found that when A431 cells were loaded intracellularly with $^{125}\text{I}-\text{EGF}$ and further incubated, internalized labeled EGF returned to the cell surface membrane, where it could be trapped by cross-linking agents, providing evidence for recycling of both EGF and the EGFR. Recycling of the EGF–EGFR complex has also been shown in 3T3 cells transfected with human EGFR (39). Dunn et al. (11) fused $^{125}\text{I}-\text{EGF}$ into isolated rat liver and found that four-fold excess $^{125}\text{I}-\text{EGF}$ was degraded compared with the loss of EGF expressed on cell surfaces in this tissue. They concluded that there must be recycling of EGFR in the process of EGF binding, internalization and degradation, and they suggested that recycling of the EGFR in the resting hepatic cells might serve some metabolic functions, perhaps in the removal and degradation of EGF/TGFα from the blood and tissues (11, 12). EGFR recycling in hepatocytes has also been reported by Gladhaug et al. (17). However, this was not the conclusion of Jackle et al. (25) who examined the role of three endosomal membrane fractions in rat liver to explore the trafficking of EGFR. After injection of EGF into the animals, EGFR were rapidly internalized. EGF accumulated in the endosomal fractions with intermediate density, and subsequently in the low density endosomes (multivesicular bodies). However, EGF did not accumulate in the high density endosomes, which contain high concentrations of the known recycling receptors for transferrin asialoglycoprotein, and low-density lipoprotein. These data supported the conclusion that EGF receptors in rat hepatocytes do not recycle. Lai et al. (29) also studied rat liver in vivo and concluded that hepatocyte EGFR can recycle when exposed to low levels of ligand, but do not recycle in the presence of saturating concentrations of EGF. Our observations in A431 cell cultures provide a quantitative estimate of receptor recy-
cycling, and characterize its dependence upon ligand concentration and cell density. If recycling of EGF also occurs, then our calculations underestimate the level of EGFR recycling.

Recent studies of EGFR with site-directed mutations provide further information about receptor internalization and degradation. Glenney et al. (18) found that kinase-deficient EGFR mutants were internalized at much slower rates and therefore showed little down regulation in response to EGF. However, Honegger et al. (24) showed with a similar kinase-deficient mutant that EGFR internalize at a similar rate to the wild-type EGFR, but recycle rapidly back to the cell surface membrane. They proposed that the kinase activity is indispensable for targeting the EGF–EGFR complex to lysosomes. By comparing the trafficking pathway of internalized EGF–EGFR complex for wild-type and mutated EGFR, they concluded that the sorting of the complex for either lysosomal degradation or recycling to the cell surface occurs through spatial segregation with multivesicular bodies and that this sorting is controlled by protein tyrosine kinase activity of EGFR (15).

Wiley and his colleagues (31) developed a quantitative method to evaluate the interaction of cell surface receptors and the endocytic apparatus. According to their model, internalization of EGFR occurs through two pathways: one is an EGF-induced pathway through coated pits and has a relatively small capacity, and the other is a constitutive pathway which has a large capacity. They reported that in A431 cells EGFR are internalized by both pathways, and internalization by the constitutive pathway can reach a significant rate when EGFR number is very large and the ligand concentration is high (47), as under our experimental conditions. In their model they analyzed the initial phase of internalization, but the most significant finding in our studies is the recycling of EGFR after internalization. It is possible that under our experimental conditions, a large number of the EGF–EGFR complexes are internalized through both the EGF-induced pathway and the constitutive pathway. In this situation, the sorting of the complex to lysosomes for degradation of EGF and EGFR might become saturated and could become the rate limiting step in receptor trafficking. The excess complex might then become dissociated, with EGF undergoing eventual degradation and EGFR returning to the cell surface membrane.

The recycling of EGFR in high density A431 cell cultures may explain why ligand-induced down regulation of EGFR is not prominent in these cells. Our preliminary results show that Caski human squamous carcinoma cells, expressing $1 \times 10^5$ EGF, consumed much less $^{125}$I-EGF from the culture medium compared to A431 cells. This suggests that EGFR-induced down regulation of EGFR without extensive recycling can occur efficiently in a cell line that expresses large numbers of EGFR (unpublished observations). Further studies comparing characteristics of different cell lines will clarify this issue. It is interesting that the recycling of EGFR with consumption of large amounts of EGF from the medium occurred in nearly confluent A431 cell cultures, while in growing, low-density A431 cell cultures EGFR are rapidly down regulated with much less consumption of EGF. Presently, we do not know whether the recycling of EGFR in confluent A431 cell cultures has a specific regulatory function.

Recycling of the insulin receptor is generally accepted, and it has been proposed that insulin receptors degraded in lysosomes and insulin receptors recycled have different functions (1, 32). Because the insulin receptor and the EGFR have common structural and functional characteristics, recycling of EGFR may turn out to be a more general and functionally important phenomenon when these studies are extended to other types of cells.

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