Synthesis, Turnover, and Down-regulation of Epidermal Growth Factor Receptors in Human A431 Epidermoid Carcinoma Cells and Skin Fibroblasts*

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Epidermal growth factor (EGF) receptors extracted with Triton X-100 from human skin fibroblasts and A431 epidermoid carcinoma cells rapidly lose EGF-binding activity precipitable with polyethylene glycol. The presence of concanavalin A which can cross-link and, thereby, aggregate the receptors, allowed quantitative recovery of the lost EGF-binding activity. Scatchard analysis of EGF binding of Triton X-100-solubilized receptors showed that A431 cells and skin fibroblasts possess approximately 1.5 × 10⁶ and 7 × 10⁵ EGF-binding sites/cell, respectively, which exhibit similar affinities for the ligand. The heavy isotope density-shift method was employed to determine whether differences in rates of receptor synthesis or decay account for the large difference in number of receptors/cell between the two cell types. After shifting cells to medium containing heavy (¹⁵N, ¹³C, and ³²P) amino acids, light and heavy receptors, solubilized from total cellular membranes, were resolved by isopycnic banding on density gradients and then quantitated. It was demonstrated that A431 cells synthesize EGF receptors at a rate 12 times faster than skin fibroblasts and that the half-life for receptor decay of A431 cells is somewhat longer (t₁/₂ = 16 h) than that (t₁/₂ = 9 h) of fibroblasts.

Down-regulation of cell surface and total cellular EGF-binding capacity in A431 cells occurs with a t₁/₂ of 2-3 h and results in a 70–83% decrease in receptor level in 12 h. Scatchard analysis revealed that these changes in EGF binding were due to an alteration of receptor number and not EGF-binding affinity. Rates of EGF receptor synthesis and inactivation/decay were determined by the heavy isotope density-shift method. No change in the rate of receptor synthesis occurred as a consequence of EGF receptor down-regulation. Down-regulation, however, caused a decrease in receptor half-life from 16 to 4.5 h. These results indicate that EGF-dependent regulation of EGF receptor level in A431 cells involves an alteration of the rate of receptor inactivation.

The binding of epidermal growth factor to its specific receptors on the plasma membrane of target cells triggers an array of biological responses including both short term and long term effects. The short term effects which are initiated within minutes after binding include ruffling of the plasma membrane

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1 The abbreviations used are: EGF, epidermal growth factor; Con A, concanavalin A; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline.

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10^4 EGF-binding sites/cell, respectively, were employed. Two questions were addressed: 1) Do A431 cells maintain a higher steady state receptor level than fibroblasts because of a higher rate of receptor synthesis or because of a lower rate of receptor inactivation/decay? and 2) Is EGF-induced “down-regulation” of EGF receptor level the result of an altered rate of receptor synthesis or receptor inactivation/decay?

**EXPERIMENTAL PROCEDURES**

**Materials**

Human A431 epidermoid carcinoma cells were obtained from Dr. George Todaro, National Institute of Arthritis and Metabolic Fibroblasts from Dr. David Soffer, University of Illinois, and normal human fibroblasts from Dr. Diane Kingsley, University of Pittsburgh. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum in 60 mm dishes coated with 0.1% poly-L-ornithine and used for 4 days. Fibroblasts were cultured in the same medium in a 25 cm flask. A431 cells were also cultured in a Cellulator Model 281 cell counter following trypsinization and resuspension into 100 ml Falcon plastic dishes.

**Scanning of EGF Binding**

For preliminary studies, we isolated by the Chramption 1 method modified by Reu and Kornfeld (21) and for all further experiments reported herein by the lactoperoxidase method (17, 20). EGF labeling was performed in the absence of serum for 10 min at 25°C with 0.1 ng/ml of EGF. Incorporation of [3H]EGF was determined by scintillation counting with a Beckman LS-150 liquid scintillation system. Recombinant EGF (GIBCO) was used in a concentration of 2 ng/ml.

**Cell Surface '*I-EGF Binding Capacity**

Cell suspensions were incubated at 25°C with 2 nM '*I-EGF in medium containing 0.1% bovine serum albumin and 0.2% tritiated water. At the end of the incubation, the cells were washed 5 times with 0.5 N sodium hydroxide and transferred to 1 ml of 1% polyethylene glycol. See “Experimental Procedures” for details.

**Con A Binding Assay**

The possibility was considered that tetravalency of Con A, perhaps by promoting aggregation of soluble EGF-receptor complexes through cross-linking of their oligosaccharide moieties, it is evident that tetravalency is required for the Con A effect since succinylated Con A on '*I-EGF binding to and precipitation with solubilized receptor in the polyethylene glycol receptor precipitation assay. Triton X-100-solubilized extracts of total cellular membranes from Human A431 epidermoid carcinoma cells and fibroblasts. At least 90% of the '*I-EGF-binding activity originally present in the membrane is recovered in the 230,000 x g supernatant, leaving less than 5% in the pellet (results not shown). Soluble '*I-EGF-binding activity, measured by the method of Carpenter (22), decayed rapidly at 4°C (about 90% in 24 h) and somewhat more slowly at -20°C (about 85% in 2 days). Moreover, when subjected to equilibrium centrifugation in CsCl density gradients (initial CsCl concentration of 2.5 M), a procedure to isopically band the EGF receptor, all '*I-EGF-binding activity was lost. Since this procedure was needed to quantitate light and heavy receptors after labeling with heavy '*H), (22 H)-EGF, a modification of the '*I-EGF-binding assay was used to circumvent this problem.

It was discovered that the inclusion of Con A in the binding assay mixture restored '*I-EGF-binding activity lost during storage or isopycnic banding of solubilized EGF receptor. The concentration dependence of '*I-EGF binding by soluble EGF receptor from human A431 epidermoid carcinoma cells that had been stored at -20°C is shown in Fig. 1. Since maximal EGF binding occurred at the level of 100 pg of Con A, this amount was used in all subsequent studies.

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Receptor from A431 cells was incubated with \(^{125}\text{T}-\text{EGF}\) either in the presence or absence of 100 \(\mu\text{g}\) of Con A; \(^{125}\text{T}-\text{EGF}\)-receptor complexes were precipitated by polyethylene glycol only when Con A was present (Table I). When the supernatant from the sample without Con A (after polyethylene glycol addition and centrifugation) was subsequently treated with Con A, most of the \(^{125}\text{T}-\text{EGF}\)-receptor complexes were precipitated. Taken together these results indicate that Con A cross-links and aggregates EGF-receptor complexes, and thereby facilitates their precipitation by polyethylene glycol.

The dependence upon Con A of the precipitation of \(^{125}\text{T}-\text{EGF}\)-receptor complexes occurred over a wide range of EGF receptor concentrations in both human A431 and skin fibroblast extracts (Fig. 2, A and B). Since the curves are biphasic, all subsequent \(^{125}\text{T}-\text{EGF}\)-binding assays were conducted in the linear range, i.e. below 60 \(\mu\text{g}\) of soluble membrane protein from A431 cells (Fig. 2A) and below 20 \(\mu\text{g}\) of protein from fibroblasts (Fig. 2B). It is not understood why the receptor concentration dependence is biphasic, but it is possible that an inhibitor or competitor of EGF binding is present in the solubilized membrane preparations.

The characteristics of specific \(^{125}\text{T}-\text{EGF}\) binding to Triton X-100-solubilized receptors from human A431 cells and skin fibroblasts are compared in Fig. 3, A and B, respectively. The binding isotherms for the receptors of both cell types are similar, and the Scatchard plots are curvilinear in both cases. From the limiting slopes in the high affinity region of the respective Scatchard plots, the apparent equilibrium binding constants are estimated to be \(K_D = 1.5-2.5 \times 10^{-9}\) M. The total number of solubilized EGF-binding sites, determined by extrapolation to the x axis of the Scatchard plots, were 1.5 \(\times 10^6\) and 7 \(\times 10^5\) sites/cell for A431 cells and skin fibroblasts, respectively. The number of surface EGF-binding sites/cell (results not shown) represents 85-95% of the total number of sites/cell estimated from total detergent-solubilized receptor and is in good agreement with the number reported by others (23-25) for surface sites in these cell types. It appears, therefore, that the intracellular pool of active EGF receptors constitutes a small fraction, 5-15%, of the total number of receptors/cell.

### Table I

**Effect of concanavalin A on the precipitability of \(^{125}\text{T}-\text{EGF}\)-receptor complexes by polyethylene glycol**

| Treatment | Specifically bound \(^{125}\text{T}-\text{EGF}\) precipitated | % control |
|-----------|-------------------------------------|----------|
| A. Control: Con A, then PEG and centrifuge | 75 | 100 |
| B. PEG and centrifuge | 2 | 3 |
| C. PEG and centrifuge; Con A added to supernatant and recentrifuge | 51 | 68 |

![Fig. 2](image-url) **Dependence of \(^{125}\text{T}-\text{EGF}\) binding on the level of EGF receptor solubilized from total cellular membranes of A431 cells (A) and fibroblasts (B).** Soluble EGF receptor-binding assays were conducted with 2 nm \(^{125}\text{T}-\text{EGF}\) in the presence or absence of 100 \(\mu\text{g}\) of Con A/assay as described under "Experimental Procedures."

![Fig. 3](image-url) **Dependence of \(^{125}\text{T}-\text{EGF}\) binding to soluble receptor upon EGF concentration.** EGF receptor, solubilized from the cellular membranes of 9 \(\times 10^5\) A431 cells (A) and of 1.5 \(\times 10^6\) fibroblasts (B), were used in each assay. Scatchard plots of the same binding data are shown in the insets. \(^{125}\text{T}-\text{EGF}\)-binding assays were conducted as described in the legend to Fig. 2.
in the gradient corresponding to a refractive index of 1.3675 (vertical dashed line H in Fig. 4). Of interest is the fact that both light and heavy EGF receptors banded at positions of lower density than the corresponding light and heavy forms of the insulin receptor ($\text{ICD} = 1.3635$ and 1.3690, respectively; Refs. 12, 14, and 16). Also, in addition to the main peak, a shoulder of greater density constituting $\leq 10\%$ of the total activity was consistently observed in gradients of A431 cell extracts.

An empirical relationship exists, for the insulin receptor (13, 14), between peak height and peak area for the $\text{I}'251$-EGF-binding activity. This allows quantitation of the relative amounts of light and heavy EGF receptors in CsCl density gradients. Fig. 4, A and B, shows that for EGF-binding activity of A431 cells and fibroblasts, peak area and height are linearly related; thus, peak area is determined by multiplying peak height by the slope, i.e. 7.1 and 6.5 for human A431 cells and fibroblasts, respectively. For gradients with both heavy and light receptor, peak height for the heavy receptor is used directly to determine peak area since there is minimal contribution of light EGF-binding activity to heavy EGF-binding activity at the peak point for heavy EGF receptor (Fig. 4, A and B, insets). Light EGF-binding activity is determined by subtracting heavy peak area from the total area under both peaks. This approach has been used successfully to estimate the relative amounts of heavy and light insulin receptors from several cell types on CsCl density gradients (13, 14, 16).

Heavy Isotope Density Shift Analysis of the Synthesis and Decay of EGF Receptors in Human A431 Cells and Skin Fibroblasts—To distinguish between newly synthesized and previously synthesized EGF receptors, confluent cell monolayers were shifted from medium containing light ($\text{^14N, ^12C, ^1H}$) amino acids to medium containing heavy ($\text{^15N, ^13C, ^2H}$) amino acids. At various time intervals after the shift to heavy medium (3, 6, 9, 12, and 16 or 20 h) heavy and light EGF receptors were solubilized from total cellular membranes with Triton X-100, resolved on CsCl density gradients, and quantitated. Typical profiles of heavy and light receptors from both cell types 3, 9, and 16 or 20 h after the shift are shown in Fig. 5 (A–F). The size of the light receptor peak decreased with time as the size of the heavy receptor peak increased concomitantly.

After correction for nonspecific $\text{I}'251$-EGF binding, the relative amounts of heavy and light EGF receptors were calculated from the peak areas as described above. These results, plotted in the form of progress curves, illustrate the rates of formation of newly synthesized heavy receptor and the decay of previously-synthesized receptors for human A431 cells (Fig. 6A) and skin fibroblasts (Fig. 6B). Replots of the light receptor decay data of Fig. 6, A and B, in semilogarithmic form show that the inactivation of EGF receptors in A431 cells and fibroblasts is a first order process (Fig. 6C). The half-lives for receptor decay differ somewhat, 16 h for A431 cells and 9 h for skin fibroblasts. The kinetic constants for the rates of synthesis and decay of EGF receptors in the two cell types are summarized in Table II. While the rates of receptor synthesis by A431 cells is more than 12-fold faster than by fibroblasts. These results were quite reproducible. The mean half-life for receptor decay was $18.4 \pm 2.2$ h (S.E.) for five experiments with A431 cells, and $10.0 \pm 1.2$ h for two experiments with fibroblasts. Together the differences in the rates of both processes, i.e. receptor synthesis and decay, account for the more than 20-fold higher level of EGF receptors in A431 cells than in skin fibroblasts in the steady state determined by Scatchard analysis (Fig. 3, A and B).

Ligand-induced Down-regulation of EGF Receptor in A431 Cells—Exposure of A431 epidermoid carcinoma cells to EGF causes a reduction, i.e. down-regulation, in the level of cell surface EGF receptors. In the presence of 50 nM EGF, cell surface EGF-binding capacity is reduced to 30% of its original level in 12 h, the $t_{1/2}$ for the process being 2–3 h (Fig. 7A). The rate of loss of receptor extractable with Triton X-100 from total cellular membranes (i.e. internal as well as cell surface membranes) follows similar kinetics, dropping to 30% of control cells in 12 h (Fig. 7A). Down-regulation is concentration-
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To determine whether this modulation of EGF-binding capacity was the result of a change in absolute number of receptors, affinity of the receptor for EGF, or both, the EGF-binding activities of total soluble receptors from control and down-regulated cells were compared. Cells were incubated for 12 h either in the absence or in the presence of 10 or 50 nM EGF to induce down-regulation. The EGF-binding isotherms for receptors extracted with Triton X-100 from total cellular membranes are compared in Scatchard plots in Fig. 8. It is evident that the loss of EGF-binding capacity during down-regulation is due to a reduction in the number of receptors with little or no change in the affinity of the receptors for the ligand. Receptor levels for A431 cells down-regulated with 10 and 50 nM EGF, estimated from the Scatchard plots (Fig. 8), were 40 and 17%, respectively, of untreated controls.

Fig. 6. Kinetics of heavy receptor synthesis and light receptor decay in A431 cells (A and C) and human fibroblasts (B and C). The amounts of heavy and light receptor at each time point were calculated as described in the text using the data shown and described in the legend to Fig. 5. C shows semilogarithmic plots for the decay of light receptor in both cell types using the results from A and B. The synthetic and degradative rate constants for the two cell types determined from these results are summarized in Table II.

To assess the effect of EGF-induced down-regulation on the rates of synthesis and turnover of EGF receptors, the heavy isotope density-shift method was employed. After reaching confluence, A431 cells were exposed to medium containing 50 nM EGF for 12 h to induce the down-regulated state. The cells were then shifted to medium containing heavy (15N, 13C, 2H) amino acids with or without 50 nM EGF. At various times (0, 3, 6, 9, 12, and 18 h) after the density shift, receptor was extracted from total cellular membranes and banded isopycnically in CsCl density gradients, and light and heavy receptor were quantitated as described above. Typical banding profiles for light and heavy EGF receptors from control and EGF

Fig. 7. Kinetics and EGF concentration dependence of EGF-induced down-regulation of the 125I-EGF-binding capacity of A431 cells. In A, cell monolayers were exposed to 50 nM EGF for the time indicated and were then subjected to the rapid acidic wash procedure to remove any bound EGF. 125I-EGF binding to cell surface or total cellular Triton X-100-solubilized receptor was determined as described under “Experimental Procedure.” The experiment shown was repeated three times with similar results. In B, cells were exposed to the indicated concentrations of EGF for 12 h after which 125I-EGF binding to total cellular Triton X-100-solubilized receptor was determined as described above.
DISCUSSION

When maintained under comparable cell culture conditions different cell types have been found to express widely differing levels of EGF receptor (Refs. 23-25 and Table II), ranging from 50,000 to several million receptor sites/cell. In addition, the steady state level of EGF receptor for a particular cell type can be modulated by changes in nutritional/physiological state. For example, exposure of cells to EGF causes ligand-induced down-regulation of EGF receptor level (Figs. 7 and 8 and Refs. 8-10). Differences such as these in the level of cell surface receptors are thought to be an important factor in determining the responsiveness of the cell to EGF. Few investigations, however, have focused on the mechanism(s) by which the level of active EGF receptors is varied. At steady state the total number of functional EGF receptors in a cell will be determined by the relative rates of synthesis and decay, $k_s$ and $k_d$, of active receptor. Thus, a change in either rate constant will produce an alteration of cellular receptor level.

The specific objectives of the present investigation were to ascertain: 1) how the levels of EGF receptors are maintained at 1.5 million/cell in human A431 cells but at only 70,000/cell in human fibroblasts when cultured under comparable conditions and 2) whether EGF-induced down-regulation of EGF receptor number in A431 cells is due to altered rates of synthesis or degradation of receptor.

The heavy isotope density-shift method, employed in these studies to measure rates of active receptor synthesis and degradation, has several important attributes. Labeling of cells with heavy amino acids to measure simultaneously the synthesis and turnover of receptor is a benign procedure and does not require the use of inhibitors. Although inhibitors of protein synthesis are commonly used to follow receptor decay in the absence of receptor synthesis, the inhibition of protein synthesis is known to cause other impairments of cellular function. We have shown (26), for example, that inhibitors of protein synthesis, such as cycloheximide or puromycin, reduce the rate of insulin receptor inactivation in 3T3-L1 adipocytes by 3-4-fold. It appears that the continued synthesis of a short-lived protein(s) is required for the rate-limiting step in the turnover of the insulin receptor (28).

It should be emphasized that the density-shift method follows the rate at which EGF-binding activity of the receptor is lost and, thus, measures the rate-limiting step in the receptor inactivation process. This step is of particular physiological importance in the pathway leading to the degradation of receptor protein, since it is at this point that receptor function is lost. It remains to be determined whether EGF receptor inactivation is synchronous with degradation of receptor protein. This does not seem likely, however, since blocking lysosomal proteolysis with chloroquine,4 which causes the intracellular accumulation of the ligand-binding activity of certain other receptors (27, 28), has virtually no effect on cellular EGF-binding capacity. These results are similar to those observed with the insulin receptor in chick hepatocytes (29).

The success of the density-shift technique is dependent upon quantitative assay of the EGF receptor. Two major problems were encountered in preliminary experiments, however. First, the EGF-binding activity was found to be unstable in crude cellular extracts, possibly due to proteolysis of receptor. Secondly, detectable activity was greatly reduced in the presence of a variety of salts, including CsCl, and thus it seemed that the isopycnic banding procedure could not be applied for the EGF receptor. We observed, however, that both of these problems could be circumvented by including Con A in the EGF-binding assay mixture. This modification of the immobilized-lectin assay of Nexo et al. (30) allowed for the quantitative estimation of EGF-binding activity, even in the presence of CsCl. A number of experiments indicated that Con A probably served to increase the precipitability of the
125I-EGF-receptor complexes. It is of interest that in whole cells, both Con A (31) and succinylated Con A (32) were effective inhibitors of EGF binding to the receptor. Thus, the lectin, whether in solution or immobilized (30), evidently exerts opposite effects on the EGF receptor depending upon whether the receptor is in the cell membrane or in solution.

The results of our present investigation on the control of the synthesis and inactivation/decay of the EGF receptor using the heavy isotope density-shift technique are summarized in Table II. The 20-fold greater number of cellular EGF receptors in human A431 carcinoma cells than in human fibroblasts was found to be due both to a 12-fold higher rate of receptor synthesis and to a slightly lower, ~1.8-fold, rate of receptor inactivation. The estimate of the synthetic rate constant, $k_s$, was determined either by measurement of the limiting slopes of the progress curves (Fig. 6, A and B) for heavy receptor synthesis (6,300 and 72,000 site/h/cell) or was calculated from the equation $R = k_s/k_p$ (5,320 and 64,500 sites/h/cell). Although the absolute values obtained by the two methods of calculation are slightly different, both methods imply that the A431 cells synthesize receptor at a 12-fold faster rate than the fibroblasts.

Several investigators have reported turnover rates for the EGF receptor. Aharonov et al. (8) estimated that EGF-binding activity in 3T3 fibroblasts decays with a half-life of 6 h when protein synthesis is blocked by cycloheximide. Carpenter (33) has shown that in human fibroblasts starved for histidine in medium supplemented with $\alpha$-histidinol, the EGF receptor turns over with a half-life of 14.5 h. Bhargava and Makman (34) observed a receptor half-life of 6 h in IMR-90 human lung fibroblasts when protein synthesis was blocked with cycloheximide or when glycosylation was inhibited with tunicamycin. Treatment of the cells with either actinomycin D or captoide in the presence of inhibitors is used to control receptor decay with a 23-h half-life. Thus, down-regulation occurs with a time course similar to the A431 cells. The extent of down-regulation in 3T3 fibroblasts decays with a half-life of 6 h when protein synthesis is blocked (8). The results of our present investigation on the control of receptor synthesis and to a slightly lower, ~1.5-fold, time course of the A431 cells. The results of our present investigation on the control of receptor inactivation are shown in Table II. The 20-fold greater number of cellular EGF receptors in human A431 carcinoma cells than in human fibroblasts was found to be due both to a 12-fold higher rate of receptor synthesis and to a slightly lower, ~1.8-fold, rate of receptor inactivation. The estimate of the synthetic rate constant, $k_s$, was determined either by measurement of the limiting slopes of the progress curves (Fig. 6, A and B) for heavy receptor synthesis (6,300 and 72,000 site/h/cell) or was calculated from the equation $R = k_s/k_p$ (5,320 and 64,500 sites/h/cell). Although the absolute values obtained by the two methods of calculation are slightly different, both methods imply that the A431 cells synthesize receptor at a 12-fold faster rate than the fibroblasts.

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