Down Regulation of Epidermal Growth Factor Receptors: Direct Demonstration of Receptor Degradation in Human Fibroblasts

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ABSTRACT The metabolism of the receptor for epidermal growth factor (EGF) has been measured by labeling the receptor in vivo with radioactive amino acid precursors and then determining, by immunoprecipitation with specific anti-EGF receptor antisera, the rate of degradation of the receptor when the cells are placed in a nonradioactive medium. In human fibroblasts the rate of EGF receptor degradation ($t_{1/2}$ = 10.1 h) was faster than the rate of degradation of total cell protein. When EGF was added to the nonradioactive medium, the half-life of prelabeled receptor was decreased to 1.2 h in human fibroblasts. These data demonstrate by direct analysis of receptor protein that during "down regulation" the EGF receptor is rapidly degraded. Enhanced receptor degradation was observed 5–10 min after the addition of EGF. The EGF-induced degradation of the receptor was blocked by methylamine, chloroquine, iodoacetate, or incubation at 25°C. We have also shown that EGF-induced down regulation in human fibroblasts results in a decrease in the total amount of EGF receptor protein present.

The amount of EGF receptor protein has been quantitated by radiolabeling cellular protein and immunoprecipitation of the receptor. The EGF receptor constitutes ~0.0035% of the cellular protein in human fibroblasts.

Epidermal growth factor (EGF)[1] is a low molecular weight polypeptide that elicits mitogenic and nonmitogenic responses in a variety of cell types (reviewed in references 1 and 2). A specific membrane receptor for EGF has been identified and purified (3–5). The receptor is a 170,000-dalton glycoprotein having a single polypeptide chain. In addition to a binding site for EGF, the receptor has intrinsic protein kinase activity (5, 6) that is tyrosine specific (7) and activated by the binding of EGF (8). The protein kinase characteristics of the EGF receptor are similar to properties of protein kinase activities possessed by (*a*) receptors for other hormones that modulate cell growth, such as platelet-derived growth factor (9) and insulin (10), and (*b*) certain viral transforming proteins that are also membrane localized (11).

The binding of EGF to its receptors on the surface of cells produces, with continued incubation at 37°C, a decrease in the binding capacity of the cells for ^{125}I-EGF (12). This phenomenon has been observed for several polypeptide hormone-receptor systems and is referred to as "down regulation." Studies of EGF-induced down regulation have employed chemical (12) or morphological (13–17) techniques to determine the fate of cell-bound EGF labeled with ^{125}I-, fluorescein, or ferritin. These studies have demonstrated that immediately after the formation of EGF-receptor complexes on the cell surface, the labeled ligand is internalized with the formation of endocytotic vesicles containing the labeled ligand in the vesicle lumen. The ultimate fate of internalized EGF is degradation within lysosomes. The fate of the occupied receptor during these events has been examined only by indirect methodologies (12, 17–22).

To directly and quantitatively determine the fate of the EGF receptor during EGF-induced down regulation, we have employed an immunochemical approach that has been shown in previous studies of enzyme turnover to yield the least equivocal data (23). We show that in normal human fibroblasts EGF specifically and rapidly accelerates the degradation

[1] *Abbreviation used in this paper:* EGF, epidermal growth factor.

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of EGF receptor protein by a mechanism that seems similar to that involved in the degradation of internalized EGF.

MATERIALS AND METHODS

Cell Culture: Cultures of human foreskin fibroblasts (3) were prepared essentially as previously described. The cells were cultured in 60-mm tissue culture dishes (Falcon Labware, Oxnard, CA) and complete growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY) supplemented with 10% calf serum (Flow Laboratories, Inc., McLean, VA), 20 mM HEPES (Calbiochem-Behring Corp., San Diego, CA) pH 7.5, and Garamycin (M. A. Bioproducts, Walkersville, Maryland). Cell numbers were determined by electronic counting (Coulter Instruments, Inc., Hialeah, CA).

Metabolic Labeling and Solubilization of Intact Cells: Slightly subconfluent ($\sim 5 \times 10^5$ cells/dish) fibroblasts in 60-mm dishes were metabolically labeled by incubating the cells for 3 d in complete growth medium containing 33 μCi/ml L-[^{35}S]methionine (Amersham Corp., Arlington Heights, IL). During this period the cell number doubled.

To prepare labeled cells for immunoprecipitation of the EGF receptor, the cells were washed three times with cold phosphate buffered saline and solubilized for 20 min at room temperature in RIPA buffer (10 mM Tris pH 8.5, 0.15 M NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 200 Kallikrein International Units (KIU)/ml Aprotinin). 1 ml of RIPA buffer was added for each milligram of cell protein in the culture dish. Insoluble material was removed by centrifugation at 140,000 *g* for 1 h. Approximately 10–15% of the total [^{35}S]methionine labeled cellular material remained in the pellet. The EGF-receptor was not detected in this pellet. Solubilized supernatants were either immediately subjected to immunoprecipitation or were frozen in dry ice-ethanol and stored at −70°C for later analysis.

Immunoprecipitation, Electrophoresis, and Fluorography: Solubilized cell extracts were centrifuged (8,000 *g*, 5 min) if previously frozen. The immunoprecipitation was initiated by the addition of 2 μl anti-EGF receptor antiserum (#451) per ml of solubilized extract containing 1 mg protein. After incubation at room temperature for 1 h, 20–40 μl of a 10% suspension of fixed *Staphylococcus aureus* cells (Immunoprecipitin, Bethesda Research Laboratories, Maryland) per μl of antiserum was added and the incubation continued for 15 min. The immune complexes were precipitated by centrifugation for 1 min at 8,000 *g* and the pellets were washed four times with 1 ml RIPA buffer. In all experiments replicate samples were processed in an identical manner with nonimmune rabbit serum.

The immunoprecipitated samples and molecular weight markers (BRL) were prepared for electrophoresis by boiling for 5 min in Laemmli buffer (24). The samples were electrophoresed on 7.5% acrylamide sodium dodecyl sulfate gels (24) and radioactivity in the gel was detected by fluorography (25). Preflashed Kodak X-Omat AR film was exposed for an appropriate time to the gel at −70°C. Under these conditions, there was a linear relationship between the amount of radioactivity in each protein band and the intensity of its image on the film (25). This was determined experimentally by employing dilutions of a standard radioactive marker protein. Dilutions of the radioactive molecular weight markers (BRL) were usually included in each gel to ascertain the true linearity of the assay. The developed films were densitometrically scanned with a Gilford spectrophotometer and gel scanner and the tracings corresponding to the EGF receptor location in the gel were cut out and weighed as a relative measure of the amount of radiolabeled receptor present.

Quantitation of Radioactivity from Sliced Polyacrylamide Gels: To determine the absolute amount of radioactivity present in the EGF receptor band, the gel was fixed, stained, and destained as previously described (4). After soaking in water for 1 h, the gel was sliced into 1-mm sections using an electric gel slicer (model 195, Bio-Rad Laboratories, Richmond, CA). Each slice was placed in a scintillation vial (Fisher Scientific Co., Pittsburgh, PA) and crushed with a glass rod. The radioactivity in each gel slice was eluted by adding 300 μl Soluene (Packard Instrument Co., Inc., Downers Grove, IL), incubating the closed vials for 1 h at 25°C, and then adding 10 ml Omnifluor (New England Nuclear, Boston, MA) and incubating the vials at 37°C for 24 h. The radioactivity in each slice was determined with a Beckman LS-3133P scintillation spectrometer (Beckman Instruments, Inc., Palo Alto, CA).

Protein Determinations: Total cell protein was quantitated in replicate dishes not containing radioisotope by the micro-Biuret method of Goa (1953). Radioactivity incorporated into total cell protein was determined by heating an aliquot of the cell extract to 90°C for 5 min to eliminate tRNA-aminoacyl complexes. Carrier protein (200 μg γ-globulin) was added to the sample and the protein was precipitated by incubation in 10% trichloroacetic acid overnight at 0°C. The precipitates were collected on glass fiber filters (Whatman Laboratory Products, Inc., Clifton, NJ), washed with ice cold 5% trichloroacetic acid, 95% ethanol, and acetone before drying at 60°C for 30 min. Subsequently, the filters were incubated for 30 min at 50°C after wetting them with 50 μl water and 300 μl Soluene. Omnifluor scintillation fluid was added and radioactivity was measured by scintillation counting. From these determinations the specific activity of radiolabeled cell protein was calculated as counts per minute per microgram of protein.

Materials: EGF, isolated from the mouse submaxillary gland as described by Savage and Cohen (1972), was kindly provided by Stanley Cohen, Vanderbilt University. Fibroblast growth factor was a generous gift of Denis Gospodarowicz, University of California at San Francisco and platelet-derived growth factor was donated by Russell Ross, University of Washington, Seattle. Bovine insulin, methylamine, chloroquine, and Aprotinin were purchased from Sigma Chemical Co. (St. Louis, MO). Iodoacetic acid was a product of Eastman Kodak and was recrystalized with ethanol. Rabbit antisera to the affinity-purified EGF-receptor from A-431 cells were prepared and characterized as described previously (26).

RESULTS

Immunoprecipitation and Quantitation of Labeled EGF Receptors

Before examining the metabolism of the EGF receptor, experimental procedures were adjusted to provide conditions for maximal immunoprecipitation of the EGF-receptor from solubilized fibroblast extracts. A ratio of 1 μl of antiserum per 1 mg total cell protein was determined to be necessary for immunoprecipitation of a maximal amount of EGF receptor protein under the conditions employed. The data in Fig. 1 show that maximal immunoprecipitation of the EGF receptor was obtained. In this experiment the immunoprecipitates were electrophoresed on SDS gels which were then sliced and counted. The region of gel surrounding the position of the EGF receptor at 170,000 daltons is shown. Fig. 1 *A* demonstrates the profile of the receptor precipitated from human fibroblasts that had been metabolically labeled with [^{35}S]-methionine. Fig. 1 *B* shows that when the supernatant solution from the initial immunoprecipitate was reprecipitated, additional receptor was not precipitated. Fig. 1 *C* demonstrates that no significant amount of radioactivity at the 170,000-

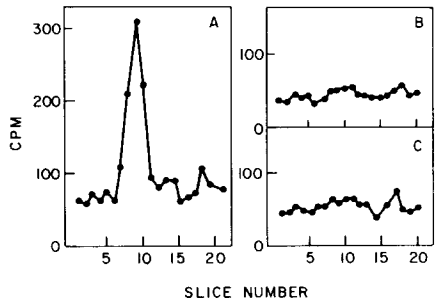

FIGURE 1 Quantitative immunoprecipitation of the EGF receptor. Fibroblasts were metabolically labeled with [^{35}S]methionine and solubilized as described in Materials and Methods. The EGF receptor was precipitated from 1 mg fibroblast extract using 2 μl antiserum and 40 μl immunoprecipitin. Immunoprecipitation, electrophoresis, gel slicing, and elution of radioactivity were performed as described in Materials and Methods. The panels show the radioactivity from gel slices in the region between the molecular weight markers myosin (200,000 daltons) and β-galactosidase (117,000 daltons). The EGF-receptor has an M_r = 170,000. *A* shows the radioactivity precipitated from solubilized fibroblasts by antireceptor serum #451, whereas C shows the results obtained with normal rabbit serum. *B* shows a reimmunoprecipitation of the supernatant solution derived from the immunoprecipitation shown in *A*.

dalton region of the gel was present in precipitates performed with normal serum. To ensure complete immunoprecipitation, at least a twofold excess of antiserum was used in the following experiments. Also a twofold excess of 10% wt/vol fixed *S. aureus* cells was used to completely precipitate antigen-antibody complexes.

To determine the abundance of EGF receptor protein in human fibroblasts an experiment similar to that shown in Fig. 1 was performed. Based on the radioactivity present in the 170,000-dalton EGF receptor band and the specific activity (counts per microgram cell protein) of the original cell extract, we calculate that the EGF receptor comprises ~0.0035% of the total cell protein in human fibroblasts. Since a protein may contain a disproportionate amount of methionine, we repeated this experiment using a mixture of eight different ^{14}C-labeled amino acids (New England Nuclear). The results were not substantially different from those cited above, suggesting that the receptor contains an average number of methionine residues.

Metabolism of the EGF Receptor in Fibroblasts

Since human fibroblasts respond mitogenically to EGF and the down regulation of EGF receptors by EGF has been extensively characterized, we have investigated receptor metabolism in these cells. Cell protein was radiolabeled by incubating the cells in growth medium containing [^{35}S]methionine. The effect of EGF on the degradation of the EGF receptor was then determined by quantitating the amount of radioactive receptor remaining during a "chase" incubation in nonradioactive medium. The results in Fig. 2 demonstrate the difference in the amount of labeled receptor remaining during an 8 h chase in the absence or presence of EGF. The data demonstrate that there is a slight decrease in the amount of immunoprecipitable labeled receptor from cells incubated in the absence of EGF (lane *B*) compared with the amount of receptor present at the start of the chase (lane *A*). Lane *C* shows the amount of receptor present when EGF was added with the chase medium. Densitometric scanning of the fluorograms indicated that after the 8-h chase, the EGF-treated cultures contained 14% of the receptor level present in the cells incubated in the chase medium without EGF. Degradation products of the receptor were not detectable in cultures incubated either in the presence or absence of EGF. In contrast to the accelerated degradation of the EGF receptor in the presence of EGF, the growth factor had no detectable influence on the degradation of other cellular proteins (lanes *D-F*).

A quantitative analysis of EGF receptor turnover was begun by determining the half-life of the receptor under normal culture conditions, i.e., in the absence of added EGF. The data presented in Fig. 3 show that the basal half-life of the EGF receptor is ~10.1 h, whereas the half-life of total fibroblast protein is about 51 h. Similar values were obtained when a mixture of ^{14}C-labeled amino acids was used instead of [^{35}S]methionine to label the cells. The linearity of a semilog plot of the degradation curves indicates that the turnover of both the EGF receptor and total cell proteins can be described by a first-order function. Several groups (27–29) have reported, based on measurement of ^{125}I-EGF binding capacity, a shorter half-life (<6 h) of the EGF receptor when cells are incubated with cycloheximide. Other assessments of the decay of EGF receptor activity during incubation in the presence of other macromolecular synthesis inhibitors, such as histidinol

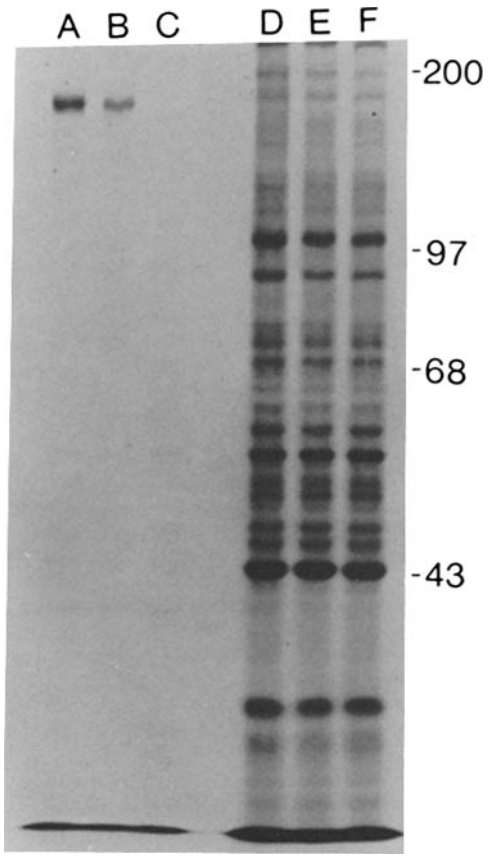

FIGURE 2 Effect of EGF on the degradation of the EGF receptor in fibroblasts. Fibroblasts were metabolically labeled with [^{35}S]methionine as described in Materials and Methods. To begin the chase the cell monolayers were washed three times with Hanks' balanced salt solution and the medium was replaced with DME-containing 0.1% BSA and 600 μg/ml unlabeled L-methionine plus EGF as indicated, and the cells were incubated for 8 h at 37°C. Solubilization of the cells, immunoprecipitation, electrophoresis, and fluorography were performed as described in Materials and Methods. Immunoprecipitations of the EGF-receptor are shown in lanes *A–C* and 5-μg aliquots of the solubilized cells are shown in lanes *D–F*. Lanes *A* and *D*, no chase; lanes *B* and *E*, 8-h chase in medium without EGF; lanes *C* and *F*, 8-h chase in medium containing 10 ng/ml EGF. Values at right equal the molecular weight × 10^3.

(30) or Actinomycin D (28), have indicated half-life values of ~15 h. Therefore, we have measured the half-life of EGF receptor protein in the presence of cycloheximide. The results (data not presented) showed that the half-life of the EGF receptor is increased in the presence of cycloheximide to ~20 h. The capacity of protein synthesis inhibitors to decrease the rate of protein degradation has been observed in other systems (23).

The influence of EGF on the extent and kinetics of receptor degradation is shown in Fig. 4. Within 5–10 min EGF produced a decrease in the amount of labeled receptor remaining in the cells. A semilog plot of the data shows first-order decay of the receptor in control cells and a biphasic decay of the receptor in EGF-treated cells. The half-life of the EGF receptor in control cells in this experiment was determined to be 9.1 h. In the EGF-treated cells, the half-life of the receptor during the first 2 h following the addition of EGF was calculated to be 1.1 h and after 2 h the half-life was 5.3 h. The biphasic decay that was observed in the EGF-treated cells is

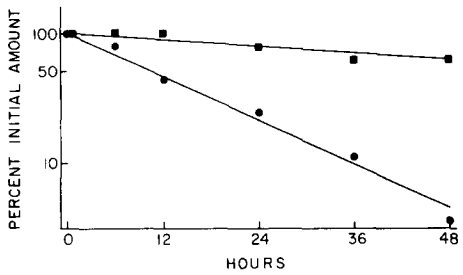

FIGURE 3 Comparison of turnover rates of the EGF receptor and total cellular proteins in fibroblasts. Fibroblasts were labeled with [^{35}S]methionine as described in Materials and Methods. To begin the chase the cell monolayers were washed three times with Hanks' balanced salt solution and the medium was replaced with complete growth medium containing 600 μg/ml methionine. At the indicated times cultures were solubilized as previously described and assayed for radioactivity present in total trichloroacetic acid precipitable protein and the EGF receptor. Solubilization of the cells, immunoprecipitations, electrophoresis, fluorography, trichloroacetic acid precipitation of labeled protein, and chemical protein determinations were performed as described in Materials and Methods. ■, log of the radioactivity (counts per minute) present in trichloroacetic acid-precipitable total cell protein. ●, log of the area under the densitometrically scanned receptor peak.

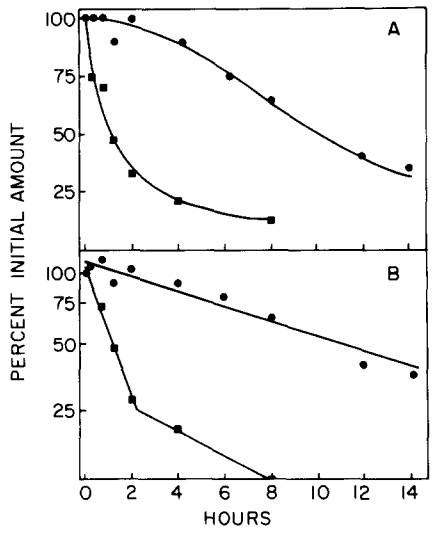

FIGURE 4 Effect of EGF or the kinetics of EGF receptor degradation in human fibroblasts. Fibroblasts were labeled with [^{35}S]methionine as described in Materials and Methods. The cell monolayers were washed three times with Hanks' balanced salt solution and the medium was replaced with Dulbecco's Modified Eagle's Medium containing 0.1% BSA and 600 μg/ml methionine, plus 10 ng/ml EGF where indicated. At the indicated times, cells incubated in the chase medium in absence (●) or presence of EGF (■) were solubilized and assayed for the amount of radioactive EGF receptor remaining as described in Materials and Methods.

probably the result of at least two independent events, the EGF-induced accelerated rate of receptor degradation and the basal rate of receptor degradation, which occur simultaneously. A biphasic decay of Fc receptors during receptor-mediated phagocytosis in macrophages likewise has been observed (31). No accelerated loss of EGF receptors was observed when cells were incubated with other mitogens such as fibroblast growth factor, platelet-derived growth factor, or insulin. The percentage of labeled EGF-receptors degraded depended on the concentration of EGF in the medium. Following an 8 h-chase, cultures treated with EGF at 0.3, 1.0, 3, and 10 ng/ml contained, respectively, 56, 44, 14, and 9% of the amount of radiolabeled EGF receptor protein present in a control culture not exposed to the growth factor.

An alternative explanation for the observed increase in receptor degradation in the presence of EGF is that the growth factor decreases the reutilization of [^{35}S]methionine derived from protein turnover. This explanation is unlikely, as the rate of degradation of total cell protein in these experiments was unaffected by the presence of EGF. Alternatively, an apparent loss of receptor protein in EGF-treated cells could be obtained if antibody recognition of the receptor was affected by the binding of EGF. Control experiments however, show that this is not the case. For example, after incubation of cells with EGF in the presence of inhibitors that block receptor degradation but not EGF binding, the receptor was completely precipitable (Table I). Likewise, when cells were incubated with EGF at 5°C, the receptor remained fully precipitable.

The preceding experiments demonstrate that EGF causes a rapid and extensive loss of prelabeled EGF receptors. Down regulation, however, implies a net decrease in the total number of EGF receptors. The following experiment was performed to determine whether EGF does, in fact, produce a net decrease in the total EGF receptor population. Cells were prelabeled with [^{35}S]methionine, EGF was added to several cultures without removing the radioactive precursor, and 24 h later the amount of EGF receptor remaining was determined. The results shown in Fig. 5 demonstrate that compared with a parallel culture incubated in the absence of EGF, the growth factor-treated cultures showed decreases of 25 and 84% (1 and 10 ng EGF/ml, respectively) in the total amount of receptor present. Since the label was present throughout the experiment, these decreases represent net decreases in the total EGF receptor population based on immunoprecipitable receptor protein. Similar quantitative reductions in EGF bind-

TABLE I

Influence of Inhibitors on Degradation of the EGF Receptor during Down Regulation in Fibroblasts

Experiment no.	Additions	EGF receptor level*
		%
1	None	100
	EGF (10 ng/ml)	13
	Methylamine (10 mM) + EGF	103
	Iodoacetate (0.1 mM) + EGF	39
	Monensin (20 μM) + EGF	71
	25°C + EGF	133
2	None	100
	EGF (10 ng/ml)	29
	Chloroquine (0.1 mM) + EGF	105
	Iodoacetate (1 mM) + EGF	99
	Colcemid (0.5 μM) + EGF	50

Human fibroblasts were metabolically labeled with [^{35}S]methionine as described in Materials and Methods. The medium was replaced with nonradioactive DME containing 600 μg/ml methionine and 0.1% BSA. The inhibitors were added at the indicated concentrations and the cultures were incubated for 1 h at 37°C (or 25°C where indicated). Down regulation was then initiated by the addition of EGF (10 ng/ml) to the indicated cultures and the incubation at 37°C (or 25°C as shown) was continued for 2 h. The amount of labeled receptor was then determined as described in Materials and Methods.

* Values represent percent of untreated control.

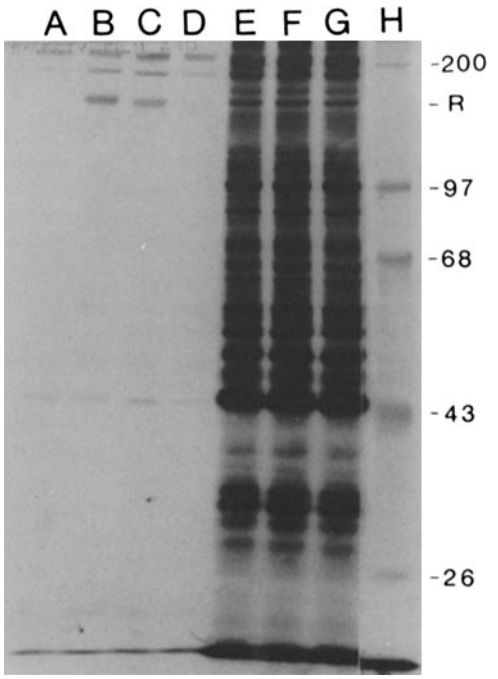

FIGURE 5 Changes in total EGF receptor level in human fibroblasts following down regulation by EGF. Fibroblasts were metabolically labeled with [^{35}S]methionine for 3 d as described in Materials and Methods. Without removing the radioactive medium separate cultures received no EGF (lanes *A*, *B*, and *E*), 1 ng per ml EGF (lanes *C* and *F*), or 10 ng per ml EGF (lanes *D* and *G*) and the incubation was continued for 24 h. The cultures were then solubilized and the amount of radiolabeled EGF receptor present in each culture was determined by immunoprecipitation (lanes *A–D*) as described in Materials and Methods. The precipitation in lane *A* was performed with normal rabbit serum. Aliquots (5 μl) of the total cellular protein are shown in lanes *E–G*. Values at right equal the molecular weight × 10^3.

ing capacity were obtained in human fibroblasts incubated with or without EGF as described in Fig. 5, washed with low pH buffer to remove surface-bound ligand, and incubated with ^{125}I-EGF (10 ng/ml) for 3 h at 4°C (data not shown).

Inhibitors of Receptor Degradation

The results shown in Table I demonstrate the effect of several types of inhibitors on the EGF-stimulated degradation of the EGF receptor. Inhibition of receptor degradation was achieved by general inhibitors of lysosome activity, chloroquine, or methylamine (12), and by agents that block intracellular vesicle traffic, monensin (40) and reduced temperature, i.e., 25°C (41). In cultures treated with methylamine, but not chloroquine, we have consistently noted the presence of a 150,000-dalton receptor band. It is not clear whether this is a natural degradation product of the receptor or whether it is induced by the presence of inhibitors. Iodoacetate was a potent inhibitor of receptor degradation and may suggest the participation of sulfhydryl proteases in receptor turnover. Interestingly, two groups (32, 33) have described an iodoacetate-sensitive protease in cell extracts that converts the native 170,000-dalton EGF receptor to a 150,000-dalton species. Control experiments, in which we used the low pH-wash procedure of Ascoli (34) to differentiate surface and internalized ^{125}I-EGF, have demonstrated that iodoacetate does not block the binding or internalization of ^{125}I-EGF-receptor complexes (data not shown).

DISCUSSION

We have directly quantitated the levels of EGF receptor protein in cultured human cells—normal diploid skin fibroblasts. We calculate from the relative abundance of EGF receptor protein, that human fibroblasts have an average of ~2 × 10^5 molecules of receptor per cell. The reported number of receptors per cell based on ^{125}I-EGF binding capacity is ~1 × 10^5 receptors for human fibroblasts (3). Our data, therefore, may suggest the presence of slightly more EGF receptors than can detected by binding assays. Given the limitations in precision of each method for determining receptor number, however, we do not interpret the difference as evidence of an intracellular pool of EGF receptors.

Our data indicate that the basal rate of EGF receptor turnover has a half-life of ~10 h in human fibroblasts. Fibroblasts would, in the absence of added EGF, degrade EGF receptors at a rate of ~10,000 receptors per cell per hour. When exogenous EGF was added, there was an immediate increase in the rate of EGF receptor degradation. The relative magnitude of this change was approximately 8.3-fold, indicating the degradation of ~85,000 receptors per cell per hour immediately after the addition of EGF.

The data presented in this report are difficult to reconcile with recycling or inactivation of the EGF receptor during down regulation. Rather, the clearest interpretation is that EGF receptors are rapidly degraded in the presence of EGF. We cannot, however, from these data determine whether there is a one to one stoichiometry between ligand degradation and receptor degradation. It is interesting, however, that the published data for the degradation of ^{125}I-EGF indicate that it has a half-life of ~1 h in human fibroblasts (3), equivalent to that calculated for the EGF-accelerated turnover of the receptor in these studies.

The speed of the EGF-induced accelerated receptor degradation was not anticipated. Although we have observed accelerated receptor degradation within 5–10 min, published data show that ^{125}I-EGF degradation (as measured by the appearance of degradation products) is not detectable in the first 15 min. Two alternative explanations can be offered for this discrepancy. EGF is reported to bind to surface receptors and very rapidly induce their internalization (35). Internalization of the ligand should easily occur within the time that we have observed stimulated degradation of the receptor. Once internalized, the hormone is localized within the lumen of an endocytic vesicle and is segregated from the cell cytoplasm. The EGF receptor, however, is most likely a transmembrane protein and would, at least in part, be exposed to the cell cytoplasm. At this point preferential degradation of the receptor by cytoplasmic enzymes could occur.

Alternatively, the EGF receptor may simply be more sensitive to lysosomal enzymes than EGF and for that reason degraded more quickly. The morphological data of Gorden et al. (14) demonstrate that when ^{125}I-EGF is added to human fibroblasts at 37°C, autoradiographic grains begin to accumulate in the lysosomal compartment after 2 min. It would appear, therefore, that both EGF and its receptor reach the lysosome within the times that we have observed receptor degradation. That EGF-accelerated receptor degradation occurs within lysosomes is indicated by the fact that either methylamine and chloroquine, lysosomotropic agents that are

routinely employed to block EGF degradation (12), or conditions that interfere with the movement of endosomes, i.e., monensin or reduced (25°C) temperature, inhibited receptor degradation.

Although the significance of EGF-receptor internalization and degradation to the production of cellular responses to the hormone is not clear, the degradation of the receptor has one definite consequence for the mitogenic activity of EGF. Data from our laboratory (36, 37) and others (38) have shown that EGF must remain in the medium and be able to bind to surface receptors in a continuous fashion for at least 6 to 8 h before quiescent cells are committed to enter the S phase of the cell cycle. Since EGF receptors are rapidly degraded in the presence of EGF, there would seem to exist a requirement for the constant synthesis of new EGF receptors to replace those that are degraded. Continuous receptor synthesis would be required for 6 to 8 h after EGF addition for stimulation of DNA synthesis to occur. It has been recognized for some time that continuous protein synthesis is necessary for quiescent cells to become proliferative (39). Our data would indicate that the synthesis of specific membrane receptors constitutes part of this requirement.

The EGF receptor is, by several experimental approaches, a glycoprotein that contains not only a ligand binding site, but also a tyrosine-specific protein kinase activity that is activated by EGF binding. In view of the intrinsic association of this kinase activity with the receptor, the production of lower molecular weight degradation products during receptor turnover would be of interest. However, we do not detect any degradation intermediates. Putative degradation products at very low levels were detected in studies utilizing covalent ^{125}I-EGF-receptor complexes (20–21). Of course our assay will only detect fragments that are antigenically recognized by the antibodies we have employed. Proteolytic fragments of the EGF receptor, produced by papain treatment in vitro, as small as 45,000 daltons were recognized by these antisera (unpublished data). Although these antisera do recognize the ^{125}I-EGF binding site as well as other surface-exposed portions of the receptor, we do not know whether they detect the protein kinase domain (26).

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