

¹²⁵I-LABELED HUMAN EPIDERMAL GROWTH FACTOR

Binding, Internalization, and Degradation in Human Fibroblasts

GRAHAM CARPENTER and STANLEY COHEN

From the Department of Biochemistry, Vanderbilt University, Nashville, Tennessee 37232

ABSTRACT

¹²⁵I-labeled human epidermal growth factor (hEGF) binds in a specific and saturable manner to human fibroblasts. At 37°C, the cell-bound ¹²⁵I-hEGF initially may be recovered in a native form by acid extraction; upon subsequent incubation, the cell-bound ¹²⁵I-hEGF is degraded very rapidly, with the appearance in the medium of ¹²⁵I-monoiodotyrosine. At 0°C, cell-bound ¹²⁵I-hEGF is not degraded but slowly dissociates from the cell.

The data are consistent with a mechanism in which ¹²⁵I-hEGF initially is bound to the cell surface and subsequently is internalized before degradation. The degradation is blocked by inhibitors of metabolic energy production (azide, cyanide, dinitrophenol), some protease inhibitors (Tos-Lys-CH₂Cl, benzyl guanidobenzoate), a lysosomotropic agent (chloroquine), various local anesthetics (cocaine, lidocaine, procaine), and ammonium chloride.

After the binding and degradation of ¹²⁵I-hEGF, the fibroblasts are no longer able to rebind fresh hormone. The binding capacity of these cells is restored by incubation in a serum-containing medium; this restoration is inhibited by cycloheximide or actinomycin D.

Human epidermal growth factor (hEGF) is a polypeptide (mol wt approximately 5,400) that has been purified from human pregnancy urine (6). hEGF exhibits the biological activities ascribed to mouse-derived EGF (mEGF) and competes in a specific manner with ¹²⁵I-labeled mEGF for binding to human fibroblasts. However, the amino acid compositions and immunological and electrophoretic properties of human and mEGF are not identical. hEGF, therefore, appears to be an evolved form of the mouse-derived polypeptide, retaining the specificities required for binding to cells and for exerting its biological effect.

A new aspect of the biology of EGF recently has emerged with the publication of the amino

acid sequence and disulfide linkages of the human urinary hormone urogastrone (17). Of the 53 amino acid residues comprising the urogastrone and mEGF molecules, 37 are common to both peptides, and the three disulfide bonds are formed in the same relative positions. Furthermore, mEGF has been shown to possess gastric antisecretory activity, and urogastrone to possess the biological activity of EGF as judged by its ability to induce precocious eye opening in newborn mice. The results suggest that hEGF and urogastrone may be identical molecules.

hEGF is a potent mitogen for human fibroblasts in cell culture, stimulating both the synthesis of DNA and cell proliferation (3). Although the abil-

ity of mitogens to stimulate the transport of nutrients, the synthesis of cellular macromolecules, and cell division has been described (see references 4 and 28 for review), little is known about the initial interaction of these mitogens with the cell surface. Since the binding of macromolecular mitogens to the cell membrane is thought to be the first step necessary for their biological activity, it is important to define the biochemical events which occur during and subsequent to the interaction of mitogens with the cell surface. This report examines the binding and metabolic fate of the bound hormone.

MATERIALS AND METHODS

hEGF

hEGF was purified (6) from a urinary protein powder obtained from pregnant women.

Antiserum to hEGF

Rabbit antibody to hEGF was prepared, according to the procedure previously described (5), with a total of approximately 100 μg of hEGF. The gamma globulin fraction was purified by DEAE chromatography (12).

Iodination of hEGF and Antiserum

The iodination (22) of hEGF and of the antiserum (gamma globulin fraction) was carried out using a molar ratio of protein to Na^{125}I of approximately 2:1. hEGF (2 μg) was dissolved in 20 μl of 0.05 M phosphate buffer pH 7.5, and added to 20 μl of 0.5 M phosphate buffer, pH 7.5, containing 0.4 mCi of carrier-free Na^{125}I . Chloramine T (20 μg in 10 μl) was then added. The reaction was stopped in 25 s by the addition of sodium metabisulfite (40 μg in 10 μl). The labeled protein was separated from unreacted Na^{125}I by gel filtration through Sephadex G-25 with a buffer containing 0.05 M phosphate, pH 7.5, and 0.075 M sodium chloride. The labeled hEGF was stored frozen in the presence of 0.1% albumin. The specific activity of the hEGF was 120,000–145,000 cpm/ng and of the antibody, 3,500 cpm/ng.

Autoradiography

The cells, after incubation with ^{125}I -labeled hEGF, were washed free of unbound radioactivity and were fixed with 2% glutaraldehyde in phosphate buffer, pH 7.3, for 30 min at room temperature. After rinsing with water, the dishes were coated with NTB2 emulsion for autoradiography (1).

Cell Culture

Monolayer cultures of human foreskin fibroblasts (HF cells) were derived from explants of newborn foreskin and were grown in Dulbecco's Modified Eagle Medium

modified to contain 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 1.1 g/liter NaHCO_3 , and chlortetracycline (50 $\mu\text{g}/\text{ml}$). The final pH of this medium (DM medium) was 7.4. Stock cultures were maintained in the DM medium supplemented with 10% fetal calf serum.

To prepare experimental cultures, approximately 2×10^6 cells were inoculated into replicate 60-mm Falcon culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) which contained 5 ml of DM medium supplemented with 10% calf serum. The cultures were incubated at 37°C in a humidified CO_2 incubator until a confluent monolayer of cells was formed. Cell counts, as determined with a Coulter counter, showed that the confluent monolayers contained approximately 1×10^6 cells per dish and that there was a variance of 10% or less in cell numbers per dish in the replicate cultures used in a given experiment.

The human fibroblasts used in this study were between the 9th and 17th passages in culture (split ratio 1:4). Cells were subcultured by trypsinization (18).

^{125}I -hEGF Binding Assay

Confluent monolayer cultures of HF cells were washed twice with 4-ml portions of prewarmed Hanks' solution, and 1.4 ml of prewarmed binding medium were added to each dish. The binding medium consisted of DM medium plus bovine serum albumin (0.1%) and Gentamicin (50 $\mu\text{g}/\text{ml}$). Labeled hEGF or other components as specified in the text were added to a final vol of 1.5 ml. After incubation at 37°C, unbound radioactivity was removed by washing the cell monolayer eight times with a total of 13 ml of cold (0°–5°C) Hanks' solution containing bovine serum albumin (0.1%). To solubilize the cells, 1.5 ml of 0.5 N NaOH were added to each dish, and the dishes were incubated at 37°C for 1 h. The contents of the dishes were transferred to counting vials, and the radioactivity was measured with a Nuclear Chicago gamma-spectrometer (Nuclear-Chicago, Des Plaines, Ill.). Nonspecific binding, as determined by measuring the binding in the presence of excess unlabeled mEGF (20 $\mu\text{g}/\text{dish}$), amounted to less than 2% of the total label bound. All experiments were performed in duplicate or triplicate.

Gel filtration

To analyze the nature of radioactive material released into the binding medium, we lyophilized the cell-free medium and resuspended the material in 0.5 ml of water. Each sample was chromatographed on a column (1.5 \times 35 cm) of Bio-Gel P-10 (200–400 mesh) equilibrated with 0.1 M ammonium acetate. Fractions of approximately 2 ml were collected.

The radioactivity that remained bound to cells was extracted by adding 1.5 ml of 0.1 N HCl containing 0.1% bovine serum albumin to the monolayer of cells and incubating at room temperature for 90 min. The

acid-extractable material, approximately 80% of the total cell-bound radioactivity, was lyophilized, resuspended in 0.1 M ammonium acetate and chromatographed on the Bio-Gel column as described above.

Paper Chromatography

Ascending paper chromatography was carried out using Whatman No. 3MM paper, 42 cm in length. A ninhydrin spray (Nutritional Biochemicals Corp., Cleveland, Ohio) was used for detection.

Materials

Dulbecco's Modified Eagle medium, fetal calf serum, calf serum, trypsin, and chlortetracycline were obtained from Grand Island Biological Co., Grand Island, N. Y. Gentamicin and Hanks' balanced salt solution were purchased from Microbiological Associates, Inc., Bethesda, Md. Purified mouse-derived EGF was prepared as previously described (31). Bovine brain and pituitary fibroblast growth factors (FGF), multiplication stimulating activity (MSA), somatomedin C, and somatomedin B were generous gifts from Doctors Denis Gospodarowicz (The Salk Institute, San Diego, Calif.), Howard Temin (University of Wisconsin, Madison, Wis.), Judson Van Wyk (University of North Carolina, Durham, N.C.), and KABI Chemical Company, Sweden, respectively. Procaine hydrochloride and chloroquine were obtained from Sigma Chemical Co., St. Louis, Mo. Lidocaine hydrochloride was a product of ASTRA, and cocaine hydrochloride, a product of Mallinckrodt. Cycloheximide was purchased from Nutritional Biochemicals and

HEPES and Actinomycin D from Calbiochem, San Diego, Calif. Bovine serum albumin was purchased from Miles Laboratories, Inc. Na^{125}I was obtained from New England Nuclear Corp., Boston, Mass. Nuclear track emulsion Type NTB2, developer D-76, fixative, and chloramine T were products of Eastman Kodak Co., Rochester, N. Y.

RESULTS

Time-Course of Binding

The time-course of binding of ^{125}I -hEGF to human fibroblasts at 37°C and 0°C is shown in Fig. 1. Maximal binding was reached after incubations of 30–40 min at 37°C or approximately 2.5 h at 0°C. The maximal amount of hormone bound at 37°C was approximately twice that bound at 0°C. On continued incubation of the labeled hormone with HF cells at 37°C, the amount of cell-bound radioactivity decreased until a constant level of 15–20% of the initial maximal amount of cell-bound radioactivity remained associated with the cells. When the cells were incubated with the labeled hormone at 0°C, there was no net loss of cell-bound radioactivity.

The following experiments were conducted to determine the basis for the loss of cell-bound radioactivity during incubation of ^{125}I -hEGF with HF cells at 37°C. Labeled hormone was incubated with cells for 6 h at 37°C, the medium was re-

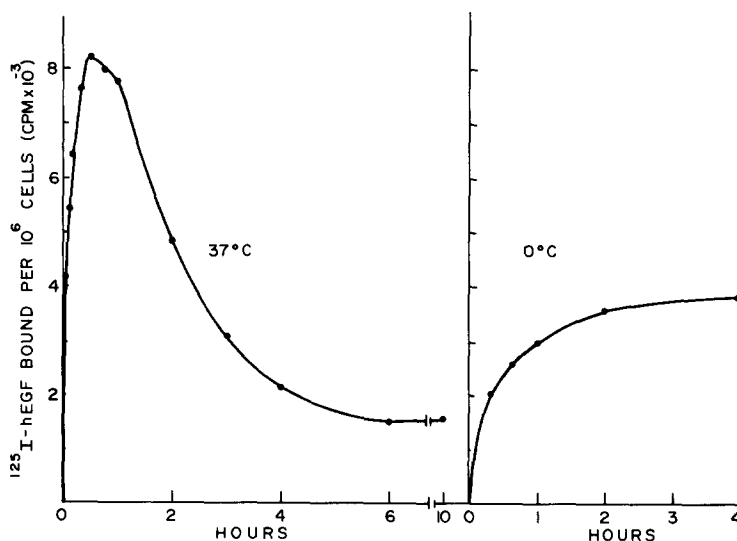


FIGURE 1 Time-course of ^{125}I -hEGF binding to human fibroblasts at 37° and 0°C. ^{125}I -hEGF (final concentration 4 ng/ml, 24,400 cpm/ng) was added to each culture dish containing the standard binding medium (1.5 ml for the 37°C experiments and 2.0 ml for the 0°C experiments). At the indicated time intervals, duplicate dishes were selected and the cell-bound radioactivity was determined as described under Materials and Methods.

moved and added to a monolayer of fresh cells, and the binding of the hormone in this "used" medium to fresh cells was determined (Table I, exp 4). The results showed that the hormone remaining in the medium was fully active in binding to fresh cells, indicating that extensive degradation or inactivation of free hormone in the binding medium had not occurred. In addition, cells that had been incubated for 6 h at 37°C with the hormone and then washed were unable to bind fresh hormone (Table I, exp 3). Control experiments showed only a slight decrease (10%) in hEGF-binding capacity during incubation in the binding medium in the absence of hEGF (Table I, exp 5). The decrease in cell-bound radioactivity which occurred at 37°C in the presence of the hormone was apparently due to the loss of a cellular function (plasma membrane receptors?) necessary for hormone binding.

The pattern of binding of ¹²⁵I-hEGF to HF cells was examined using modifications of the standard albumin-containing medium. A time-course similar to that illustrated in Fig. 1 was observed when the binding medium was altered to contain either 2% or 10% calf serum, although in 10% serum the final steady-state level reached was 35% of the initially bound radioactivity rather than the 15–20% attained in the standard medium (data not shown).

The addition of cycloheximide (20 μg/ml) to the standard binding medium depressed the maximal level of binding by 24% but did not otherwise significantly alter the pattern shown in Fig. 1. These results suggest that protein synthesis is not necessary for either the initial binding of ¹²⁵I-hEGF or the subsequent decrease in cell-bound radioactivity.

Effect of hEGF Concentration on Binding

The effect of increasing concentrations of hEGF on the binding of ¹²⁵I-hEGF to HF cells is shown in Fig. 2. These data show that the binding reaction is a saturable process with maximal binding at approximately 8 ng/ml (1.5×10^{-9} M) and half maximal binding at 1.8 ng/ml (3.3×10^{-10} M). At the lowest (0.04 ng/ml) and highest (20 ng/ml) concentrations of hEGF tested, 18.1% and 2.3%, respectively, of the hormone were bound under these conditions.

Specificity of hEGF Binding

It has been reported (2) that neither insulin, prolactin, growth hormone, follicle-stimulating

TABLE I
hEGF-Mediated Loss of Hormone-Binding Capacity

Exp no.	Cell-bound radioactivity <i>cpm/dish</i>
1	6,240
2	1,235
3	1,219
4	6,728
5	5,583

The binding of labeled hEGF to confluent HF cells was determined after ¹²⁵I-hEGF (4 ng/ml, 21,900 cpm/ng) was incubated with replicate cultures at 37°C for 40 min (exp 1) or 6 h (exp 2). After the 6-h incubation period in exp 2, the cells were washed, ¹²⁵I-hEGF (4 ng/ml, 21,900 cpm/ng) was added, and the cells were incubated for 40 min (exp 3). After the 6-h incubation with a second set of cultures in exp 2, the medium was transferred to fresh HF cells for a 40-min binding period (exp 4). Control cultures were incubated in the binding medium in the absence of hEGF for 6 h, and the capacity of the cells to bind ¹²⁵I-hEGF under standard (40 min) binding conditions was determined (exp 5).

hormone, thyrotropin, nor glucagon was able to compete with ¹²⁵I-labeled mouse EGF in the fibroblast receptor assay. Human EGF, however, has been shown to be an effective competitor of the mouse-derived hormone (6). The data in Table II show that, when human EGF is used as the ¹²⁵I-labeled ligand, none of the polypeptide mitogens tested (FGF, MSA, somatomedins B and C) was able to compete for the fibroblast receptor. As expected, mEGF was an effective competitor of the ¹²⁵I-labeled hEGF.

Autoradiography of Cell-Bound ¹²⁵I-hEGF

The binding of ¹²⁵I-labeled hEGF to HF cells after a 40-min incubation at 37°C in the presence and absence of a large excess of unlabeled mEGF is shown in Fig. 3. The binding reaction was specific as evident by the absence of radioactive grains in the cells incubated with unlabeled hormone (Fig. 3a). The pattern of cell-bound radioactivity presented in Fig. 3b was typical of all cells incubated with the labeled hormone. Although it was not possible from these data to determine whether the radioactivity was on the surface of the cell or internalized, the radioactivity appeared to be rather uniformly distributed, and all of the cells were labeled.

Nature of the Cell-Bound ¹²⁵I-hEGF

The cell-bound ¹²⁵I-hEGF, after acid extraction, was examined by gel filtration and tested for its

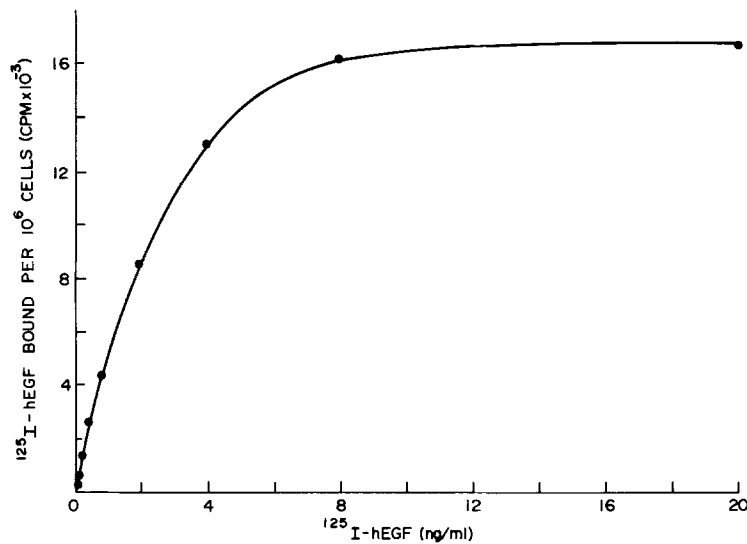


FIGURE 2 Effect of $^{125}\text{I-hEGF}$ concentration on binding to human fibroblasts. Indicated concentrations of labeled hEGF (25,300 cpm/ng) were added to culture dishes and the specific binding was determined after a 40-min incubation at 37°C , as described under Materials and Methods.

TABLE II
Specificity of Binding of $^{125}\text{I-hEGF}$

Addition	Concn. $\mu\text{g/ml}$	Specific binding of $^{125}\text{I-hEGF}$ cpm/dish
None		10,276
mEGF	1.0	146
FGF (brain)	6.7	10,792
FGF (pituitary)	6.7	10,822
Somatomedin B	1.0	9,857
Somatomedin C	1.4	9,680
MSA	10.0	9,522

The indicated polypeptides were added simultaneously with $^{125}\text{I-hEGF}$ (0.8 ng/ml, 53,700 cpm/ng) to HF cells in the standard binding assay (see Materials and Methods). The cell-bound radioactivity was determined after a 40-min incubation period at 37°C .

ability to rebind to fibroblasts. After a 40-min binding period at 37°C with labeled hEGF, the cells were extracted with 0.1 N HCl. 80% of the radioactivity was extracted by this procedure, and, of this amount, 98% was of the same molecular weight as native hEGF as determined by the gel filtration procedure (see Materials and Methods and reference 2). After a 30-min "washout period" at 37°C in binding medium lacking $^{125}\text{I-hEGF}$, approximately 92% of the remaining cell-bound radioactivity was still in the high molecular weight form. Thus, low molecular weight products

of $^{125}\text{I-hEGF}$ degradation do not appear to accumulate in the cell.

The ability of the cell-bound $^{125}\text{I-hEGF}$ to bind receptors on fresh fibroblasts was examined. In the first experiment, $^{125}\text{I-hEGF}$ was allowed to bind to fibroblasts for 15 min at 37°C before acid extraction; in the second experiment, after the 15-min binding period the hormone was removed from the medium and the cells were incubated for 35 min before extraction. The results (Table III) show that the $^{125}\text{I-hEGF}$ which bound to the fibroblasts in the initial 15-min binding period could be extracted from the cells and was able to rebind to fresh cells with an affinity almost identical to that of the native hormone (compare extract A with native $^{125}\text{I-hEGF}$). Upon longer incubation, the cell-bound radioactivity (extract B), although mainly in a high-molecular weight form, had a considerably reduced ability to bind to fresh fibroblasts. Therefore, some or all of the cell-bound high-molecular weight $^{125}\text{I-hEGF}$ was altered, in an as yet undetermined manner, during the degradation period.

Release and Degradation of Cell-Bound $^{125}\text{I-hEGF}$

To measure the release of cell-bound $^{125}\text{I-hEGF}$, we incubated monolayers of HF cells with labeled hormone for an indicated period of time and then extensively washed them to remove un-

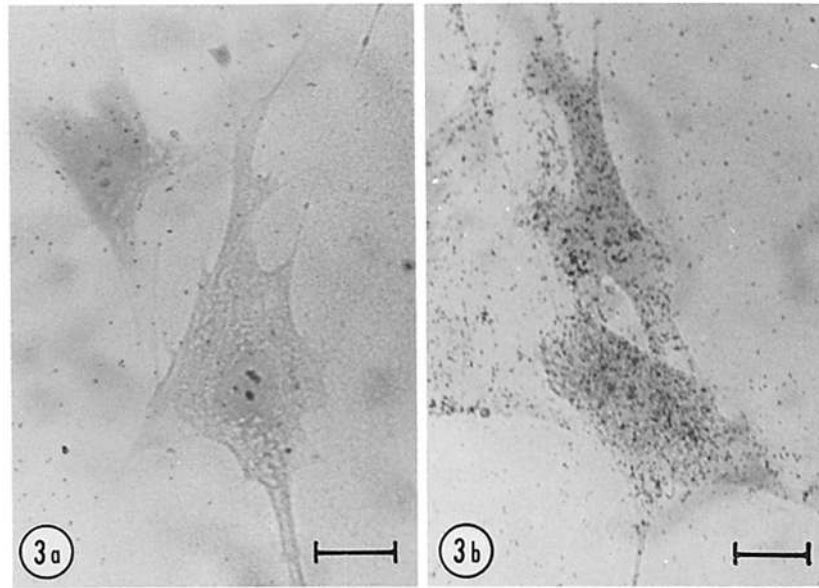


FIGURE 3 Autoradiography of cell-bound ^{125}I -hEGF. Labeled hEGF (3.5 ng/ml, 145,000 cpm/ng) was added to sparse cultures of human fibroblasts in the standard binding medium (a) in the presence and (b) in the absence of unlabeled mEGF (10 $\mu\text{g}/\text{ml}$). After a 40-min binding period at 37°C, the cells were washed, fixed with glutaraldehyde, covered with a layer of NTB-2 emulsion, and exposed for approximately 6 wk (see Materials and Methods). The bar represents 25 μm . $\times 400$.

TABLE III
Nature of Cell-Bound ^{125}I -hEGF

^{125}I -labeled material added	Radioactivity added	Cell-bound radioactivity	Percentage cell-bound
	cpm/dish	cpm/dish	%
Native ^{125}I -hEGF	12,900	1,350	10.5
	27,400	2,700	9.9
	57,800	5,330	9.2
Extract A	12,000	1,430	11.9
	24,200	2,360	9.8
Extract B	27,700	1,160	4.2

^{125}I -hEGF (3.2 ng/ml, 82,200 cpm/ng) was allowed to bind to confluent cultures of HF cells at 37°C for 15 min in the standard binding medium, and the cells were washed free of unbound radioactivity. In one set of cultures, the cell-bound radioactivity immediately was extracted by the HCl procedure (see Materials and Methods) with a 75% recovery. The extract (extract A) was lyophilized and then dissolved in standard binding medium. In a second set of cultures, fresh binding medium was added and the cell-bound ^{125}I -hEGF was allowed to degrade for 35 min at 37°C. The cell-bound radioactivity (56% of that initially bound) then was extracted with HCl with a recovery of 86%. This extract (extract B) was lyophilized and then dissolved in standard binding medium. The extract A material, the extract B material, and native ^{125}I -hEGF were assayed for their abilities to bind to HF cells during a standard binding reaction at 37°C for 40 min.

bound hormone. Binding medium without hEGF was added to the cell monolayer, and the amount of cell-bound radioactivity was determined at various times thereafter. The data in Fig. 4 (curve A)

show that, following incubation with ^{125}I -hEGF for 40 min at 37°C and removal of the unbound hormone in the medium, cell-bound radioactivity decreased rapidly ($t_{1/2} \cong 20$ min) during the subsequent incubation at 37°C. After 2-h incubation under these conditions, over 85–95% of the initial cell-bound radioactivity was not associated with the cells, and, upon the addition of fresh ^{125}I -hEGF, only a small fraction, 10–20%, of the original binding capacity could be detected (data not shown).

The data in Fig. 4 (curve B) show the loss of cell-bound radioactivity from HF cells preincubated with ^{125}I -hEGF for 2 h at 0°C, washed, and reincubated at 0°C. Under these conditions, the amount of cell-bound radioactivity slowly decreased until approximately 50–60% of the initial cell-bound material was released from the cells after 4–6 h.

The nature of the radioactive material released into the medium at both 37°C and 0°C (Fig. 4) was determined by gel filtration using Bio-Gel P-10 (2). Two peaks of radioactivity were detected: a high molecular weight fraction the elution volume of which corresponded to that of intact ^{125}I -hEGF, and a low molecular weight fraction with an elu-

tion volume corresponding to that of monoiodotyrosine. The distribution of the cell-released radioactivity in these two fractions under the varying experimental conditions is shown in Table IV. Essentially all of the radioactivity released at 0°C had the same elution volume as intact ^{125}I -hEGF; however, at 37°C most of the radioactivity released was of low molecular weight form. This low molecular weight material was identified as 80–85% [^{125}I]monoiodotyrosine and 5–10% [^{125}I]diiodotyrosine on the basis of co-chromatography on paper with mono- and diiodotyrosine standards in a butanol-acetic acid-water (210:63:180) solvent system. These data indicate that, whereas at 0°C cell-bound ^{125}I -hEGF dissociates from the cell as an intact molecule, at 37°C most of the cell-bound ^{125}I -labeled hEGF is rapidly degraded before the release of radioactivity from the cells. The degradation of cell-bound ^{125}I -hEGF at 37°C is similar to the results previously obtained with ^{125}I -labeled mEGF (2), although the rate of degradation of human EGF is more rapid.

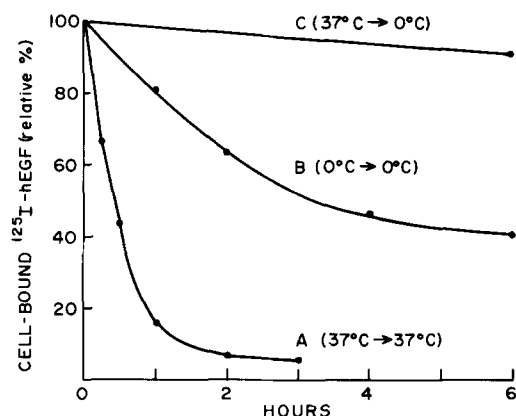


FIGURE 4 Dissociation of cell-bound ^{125}I -hEGF under varying temperature conditions. Curve A: ^{125}I -hEGF (6 ng/ml, 21,200 cpm/ng) was preincubated with fibroblasts for 40 min at 37°C. The cells then were washed, and 1.5 ml of standard binding medium were re-added. The cultures were incubated at 37°C, and at the indicated time intervals the cell-bound radioactivity was determined. Curve B: Identical to curve A, except that the initial binding period was 2 h at 0°C and the dissociation temperature was 0°C. Curve C: Identical to curve A, except that the dissociation temperature was 0°C. The results are expressed as the relative percentage of cell-bound radioactivity remaining at the indicated times, taking as 100% the amount of radioactivity present after the initial binding period.

TABLE IV
Nature of Cell-Bound ^{125}I -hEGF Released into Medium

Temperature	Time interval	Radioactivity released	
		High mol wt	Low mol wt
37°C	h	%	%
	0.0–0.5	32	68
	0.5–2.0	10	90
0°C	0.0–2.0	>99	<1
	2.0–8.0	>99	<1

^{125}I -hEGF (1.6 ng/ml, 24,700 cpm/ng) was allowed to bind to confluent cultures of HF fibroblasts at 37°C for 40 min or at 0°C for 60 min; approximately 5,000 or 1,000 cpm of ^{125}I -hEGF were bound, respectively. The culture dishes were then washed free of unbound hEGF with Hanks' solution, 2 ml of the standard binding medium were re-added, and the cultures were incubated at the indicated temperatures. The medium, containing the released radioactivity, was collected at the intervals indicated and lyophilized. The distribution of the radioactivity in each sample was examined by gel filtration (see text), using portions containing 400–5,000 cpm of ^{125}I .

Inhibition of Degradation of Cell-Bound ^{125}I -hEGF

To investigate the mechanisms involved in the rapid, cell-mediated degradation of ^{125}I -labeled hEGF, we attempted, by a variety of means, to inhibit this process. Previous studies (2) have shown that the trypsin inhibitors tosyl-L-lysine chloromethyl ketone and the benzyl ester of guanidobenzoic acid inhibited the degradation of ^{125}I -mEGF by human fibroblasts. The data in Table V (exp 1) show that these protease inhibitors also block the degradation of ^{125}I -hEGF.

If the degradation of ^{125}I -hEGF involves an energy-dependent form of internalization of the cell-bound growth factor, inhibitors of metabolic energy should prevent this process. Therefore, the effects of a number of inhibitors of metabolic energy production on the initial binding (40 min, 37°C) of ^{125}I -hEGF to HF cells and the subsequent loss of cell-bound radioactivity were examined. No appreciable effects on these parameters were noted when dinitrophenol and sodium fluoride were added to the standard binding medium which contained both glucose and amino acids (Table V, exp 2). In a medium lacking both glucose and amino acids, however, dinitrophenol, sodium azide, or sodium cyanide was effective in slowing the loss of cell-bound radioactivity but had little

TABLE V
Inhibition of the Release of Cell-Bound ¹²⁵I-hEGF

Exp. no.	Zero time radioactivity	Addition	Cell-bound ¹²⁵ I-hEGF after 2 h
	<i>cpm</i>		<i>% initial value</i>
1	6,511	None	9
	9,504	Tos-LysCH ₂ Cl (50 μg/ml)	57
	8,325	Tos-LysCH ₂ Cl (5 μg/ml)	46
	9,819	Benzyl guanidobenzoate (100 μg/ml)	53
	7,567	Benzyl guanidobenzoate (10 μg/ml)	12
2	6,159	None	7
	6,790	Dinitrophenol (0.5 mM)	10
	5,970	Sodium fluoride (1 mM)	8
	6,899	Dinitrophenol (0.5 mM) plus sodium fluoride (1 mM)	10
3	4,110	None	20
	4,131	Dinitrophenol (1 mM)	73
	4,041	Dinitrophenol (0.5 mM)	67
	4,200	Sodium azide (5 mM)	52
	3,996	Sodium azide (1 mM)	19
	4,581	Sodium cyanide (5 mM)	48
	4,062	Sodium cyanide (1 mM)	26
4	3,445	None	12
	3,741	Chloroquine (0.1 mM)	72
	3,966	Chloroquine (0.01 mM)	35
	2,712	Lidocaine (5 mM)	70
	3,213	Lidocaine (1 mM)	66
	3,278	Cocaine (1 mM)	78
	3,883	Procaine (1 mM)	64
	3,941	Procaine (0.1 mM)	15
	3,758	Ammonium chloride (10 mM)	98
	3,649	Ammonium chloride (2 mM)	67

Fibroblasts (0.8 to 1.0×10^6 cells/dish) were preincubated in 1.5 ml of binding medium* with the indicated compounds at 37°C. The preincubation period was 60 min for exp 1, 30 min for exp 2 and 3, and 15 min for exp 4. At the end of the preincubation period, ¹²⁵I-hEGF was added at a final concentration of 2–4 ng/ml for a 40-min binding period at 37°C. The cultures subsequently were washed eight times with a total of 15 ml of Hanks' solution containing 0.1% albumin, and 1.5 ml of the binding medium, with the indicated compounds, were added. The cell-bound radioactivity present at this time was taken as the zero-time control value. Then the culture dishes were incubated for 2 h at 37°C, washed four times with a total of 8 ml of cold Hanks' solution, and the cell-bound radioactivity was measured.

* The standard binding medium (see Materials and Methods) was employed in exp 1, 2, and 4. The binding and washing medium employed in exp. 3 consisted of glucose-free Hanks' solution containing 0.1% albumin and 20 mM HEPES buffer, pH 7.5.

influence on the amount of initial binding (Table V, exp 3). The results suggest that the degradation of ¹²⁵I-hEGF, under these experimental conditions, is dependent on the generation of metabolic energy.

To examine the possibility that the proteolysis required for the liberation of ¹²⁵I-labeled monoiodotyrosine from cell-bound ¹²⁵I-hEGF may be mediated by lysosomal hydrolases, we investigated the effect of chloroquine, which is reported to be

concentrated rapidly into lysosomes by fibroblasts and to inhibit lysosomal enzyme activities (9, 24, 36), on the binding and degradation of ¹²⁵I-hEGF. As shown in Table V (exp 4), 0.1 mM chloroquine produced a considerable amount of inhibition of degradation, suggesting that the proteolytic activity of lysosomal enzymes may indeed be involved in the cellular metabolism of hEGF.

Unexpectedly, we found that local anesthetics, such as procaine, lidocaine, and cocaine, which are reported to interact with cell-surface components (27, 29, 32), also inhibited the degradation of cell-bound ¹²⁵I-hEGF (Table V, exp 4). The inhibitory effects were observed at concentrations of these reagents which had no apparent effect on the morphological appearance of HF cells as seen by light microscopy. The possibility that these local anesthetics, which are tertiary amines, may inhibit degradation by a mechanism similar to that of chloroquine, which is also a tertiary amine, is considered in a later section.

In this regard, it is of interest that ammonium chloride at concentrations of 2–10 mM has been reported to inhibit protein degradation in isolated hepatocytes (33). Within the same range of concentrations, ammonium chloride was a potent inhibitor of the degradation of cell-bound ¹²⁵I-hEGF (Table V, exp 4).

Since the radioactivity bound to the cells after a 40-min incubation at 37°C was a result of the total amount of ¹²⁵I-hEGF bound during this period minus the amount of radioactivity lost from the cell due to degradation, it was expected that the presence of inhibitors of the degradation process would result in an increase in the initial level of radioactivity bound to the cell. The data in Table V show that, in general, the presence of inhibitors of degradation does result in an increase in the extent of initial binding of the labeled growth factor.

Internalization of Cell-Bound ¹²⁵I-hEGF

Three experiments were performed to provide evidence, albeit indirect, that cell-bound ¹²⁵I-hEGF is internalized before degradation. The rationale for these experiments is that, if internalization (endocytosis?) of membrane-bound hormone is minimized by lowering the temperature to 0°C, the accessibility of the membrane-bound hormone to reagents in the medium, such as trypsin or antibodies to hEGF, will be increased.

The experiment reported in Fig. 5 demonstrates

that ^{125}I -labeled antibodies to hEGF were able to bind to cells which had been pre-incubated with hEGF at 0°C . The capacity of these hEGF-treated cells to bind antibody was rapidly lost upon warming to 37°C for brief periods of time (1–8 min). Control experiments demonstrating that the hormone remained associated with the cells were carried out, under the conditions described for Fig. 5, with ^{125}I -labeled hEGF. The half-life for the disappearance of antibody binding was approximately 1–2 min.

The experiment shown in Table VI indicates that approximately 50% of the ^{125}I -hEGF bound at 0°C to the cells was liberated by trypsin, but only 10% of the ^{125}I -hEGF bound at 37°C to the cells was released by the same treatment.

The data in Fig. 4 demonstrate that, when the labeled hormone was bound to the cells at 37°C ,

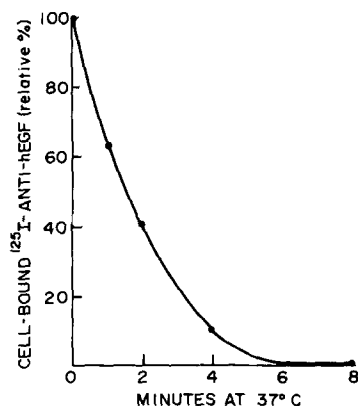


FIGURE 5 Binding of ^{125}I -labeled rabbit hEGF-antiserum to fibroblasts preincubated with hEGF. Unlabeled hEGF (6 ng/ml) was added to the culture dishes containing 2 ml of the binding medium at 0°C . After a 2-h incubation, the cells were washed free of unbound hEGF, and 2 ml of binding medium were re-added. ^{125}I -labeled antiserum to hEGF ($0.75\ \mu\text{g}$, $3 \times 10^6\ \text{cpm}/\mu\text{g}$) was added and the cultures were re-incubated for 90 min at 0°C . Then the cultures were washed and the cell-bound radioactivity was determined; the amount of radioactivity present (corrected for "nonspecific" binding) was taken as 100%. Replicate cultures, taken just before the addition of antiserum, were incubated at 37°C for the indicated time intervals, re-chilled, and then tested for their ability to bind the ^{125}I -labeled antiserum. The radioactivity which was cell-bound in the absence of added hEGF was considered to be the "nonspecific blank" and was subtracted from the experimental values obtained. In separate experiments, this "blank" value of approximately 3,000 cpm/dish amounted to 25–50% of the value obtained in the presence of hEGF.

TABLE VI
Effect of Cold Trypsinization on the Release of Cell-Bound ^{125}I -hEGF

Binding temperature	Exp no.	Percentage of radioactivity	
		Cell pellet	Supernate
		%	%
37°C	1	90	10
	2	90	10
0°C	1	46	54
	2	61	39

^{125}I -hEGF (6 ng/ml, 33,000 cpm/ng) was allowed to bind to confluent cultures of HF fibroblasts at 37°C for 40 min or at 0°C for 60 min. The culture dishes then were chilled on ice and washed free of unbound ^{125}I -hEGF using cold Hanks' solution. To each dish, 1 ml of 0.25% trypsin (in GIBCO solution A containing 0.1% albumin) was added, and the dishes were incubated for 30 min on ice. Serum-containing DM medium (0.5 ml) then was added, and the detached cells were suspended and centrifuged. The supernatant fluid was carefully aspirated, and the radioactivity present in both the cell pellets and supernates was measured.

the expected release of the cell-associated radioactivity, either as iodotyrosine or undegraded hormone, did not occur when the temperature was lowered to 0°C (compare curve C with either curve A or B). All of these results are consistent with a mechanism in which ^{125}I -hEGF initially is bound to the cell surface and subsequently is internalized prior to degradation.

Recovery of hEGF Binding Capacity

The results of previous experiments indicated that following the binding and degradation of hEGF, human fibroblasts were capable of re-binding only a small quantity of fresh hormone (approximately 10–20% of the initial value). The following experiment was performed to examine the kinetics of and the metabolic requirements for the recovery of hEGF-binding capacity by these cells. The binding sites on replicate cultures of HF cells were saturated with unlabeled EGF, unbound hormone was removed, and the cell-bound EGF was allowed to degrade. The time-course of the recovery of hEGF-binding capacity by these cells, in the presence and absence of serum and in the presence of cycloheximide or actinomycin D, was examined (Fig. 6). The results indicate that: (a) approximately 10 h of incubation in serum-containing medium were required for the complete recovery of the initial hEGF binding capac-

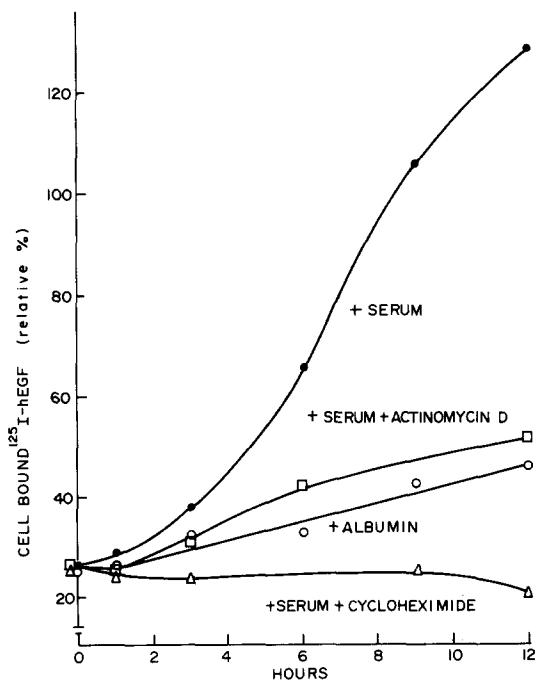


FIGURE 6 Recovery of hEGF binding capacity in fibroblasts. An excess of unlabeled mEGF ($1 \mu\text{g/ml}$) was added to replicate culture dishes containing 1.5 ml of the standard binding medium at 37°C . After a 1-h period, the cells were washed to remove unbound mEGF, 1.5 ml of the binding medium were re-added, and the cultures were incubated for 4 h at 37°C to permit degradation of the bound hormone. Then the medium was replaced with one of the following: (a) DM plus 0.1% albumin (○—○), (b) DM plus 10% calf serum (●—●), (c) DM plus 10% calf serum plus cycloheximide ($20 \mu\text{g/ml}$) (Δ — Δ), or (d) DM plus 10% calf serum plus actinomycin D ($1 \mu\text{g/ml}$) (□—□). At the indicated times, duplicate dishes from each group were selected, washed, and assayed for their binding capacity for ^{125}I -hEGF (6 ng/ml) in the standard binding medium (40 min at 37°C). The extent of ^{125}I -hEGF binding in replicate cultures, not exposed to mEGF, was taken as 100%, and the results are expressed as the relative percentage of cell-bound radioactivity.

ity; and (b) the synthesis of both protein and RNA was required for the reappearance of hEGF binding in these cells.

DISCUSSION

Many of the characteristics of the interaction of ^{125}I -labeled hEGF with human fibroblasts are similar to those previously reported for ^{125}I -labeled mouse EGF (2). Both polypeptides bind rapidly to HF cells at 37°C , appear to compete for the same

binding sites on these cells, and subsequently are degraded upon prolonged incubation. The concentrations of each polypeptide required to achieve maximal binding were similar ($1.5\text{--}2 \times 10^{-9} \text{ M}$) and were approximately fourfold higher than the concentrations required ($3.7 \times 10^{-10} \text{ M}$ for hEGF and $8 \times 10^{-10} \text{ M}$ for mEGF) for the maximal stimulation of ^3H thymidine incorporation into confluent cultures of fibroblasts (7). These values are in reasonable agreement with those reported for mEGF by Hollenberg and Cuatrecasas (20), using a different strain of human fibroblasts and slightly different experimental conditions ($1.3 \times 10^{-9} \text{ M}$ and $3.3 \times 10^{-10} \text{ M}$ for saturation of binding and maximal stimulation of thymidine incorporation, respectively). The data indicate that only a fraction (less than 25%) of the available EGF-binding sites need to be occupied for cells arrested in the G_1 phase of the cell cycle to enter the S phase.

After a brief incubation of ^{125}I -hEGF with HF cells at 37°C , acid extraction of the cells yields radioactive material which seems to be identical to native ^{125}I -hEGF, based on the binding affinity to fresh cells and on gel filtration studies. It appears, therefore, that the native form of the ^{125}I -hEGF molecule binds to the cell surface receptors and that its structure is not detectably altered in an irreversible manner by the binding process. Mouse-derived EGF bound to liver and placental membranes *in vitro* has been extracted and found to rebind to fresh membranes (25). The extraction of other polypeptide hormones bound to intact cells (8, 11, 23, 35) or plasma membrane preparations (13, 30) has yielded similar results.

Experiments reported herein indicate that, after the binding of hEGF to the cell surface at 37°C , the hormone is rapidly internalized and degraded. This process most likely occurs via the endocytotic formation of pinosomes containing membrane-bound hEGF, fusion of the pinosomes with lysosomes, and degradation of hEGF by lysosomal proteases.

The ability of ^{125}I -labeled mouse EGF to bind in a reversible manner to cell membrane preparations has been reported (25). The reversible binding of ^{125}I -labeled hEGF is also demonstrable with intact human fibroblasts, provided the temperature is kept at 0°C . In contrast, most of the cell-bound radioactivity is released as monoiodotyrosine during incubation at 37°C .

All of our experiments are consistent with the hypothesis that membrane-bound ^{125}I -hEGF is in-

ternalized before degradation. When the hormone is bound to the cells at 0°C, much of it is released by trypsin; when the hormone is bound at 37°C, very little is released by trypsin (Table VI). Further, cells preincubated with hEGF at 0°C are able to bind antibodies to hEGF; the ability of these hEGF-treated cells to bind antibodies is lost rapidly upon warming, with a half-life of 1–2 min (Fig. 5). Finally, cell-bound hEGF neither dissociates nor is degraded when the hormone initially is bound at 37°C and the temperature subsequently is lowered to 0°C (Fig. 4).

These results indicate that hEGF bound to cells at low temperature (which inhibits endocytosis) exists as an intact molecule primarily on the cell surface, and that both internalization and degradation are temperature dependent. We also have demonstrated that the degradation of cell-bound hEGF requires the production of metabolic energy (Table V, exp 3); it is unlikely that plasma membrane-mediated proteolysis would be energy dependent. Endocytosis has been shown by others to be an energy- and temperature-dependent process (34).

The evidence presented in this paper indicates that concomitant with the internalization of cell-bound hEGF, most of the membrane receptors for this polypeptide are inaccessible to added fresh hormone. The data in Fig. 1 show that, in the continuous presence of nanogram quantities of ¹²⁵I-hEGF at 37°C, the capacity of the cells to bind the hormone decreases to approximately 15% of the initial value after 6 h of incubation. Similar results were obtained (Fig. 6) when the cells were preincubated with a saturating amount of unlabeled mEGF, washed, incubated for 4 h to allow membrane-bound hormone to be degraded, and tested for their ability to bind fresh ¹²⁵I-hEGF. Binding capacity was reduced to 26% of the initial value. The decreased ability of the cells to bind ¹²⁵I-hEGF either in the continuous presence of the hormone or following binding and degradation of the unlabeled hormone suggests that either the entire hormone-receptor complex has been removed from the membrane or that the receptor has been inactivated or masked. We have not been able, as yet, to distinguish experimentally between these alternatives. The decreased ability of the cells to bind ¹²⁵I-hEGF is a hormone-dependent phenomenon; binding capacity is not reduced by incubation of the cells in the binding medium for 6 h. The return of binding capacity in the presence of serum and the inhibition of this

recovery by cycloheximide or actinomycin D (Fig. 6) suggest that the receptor is resynthesized after endocytosis of the hormone-receptor complex. The possibility that protein and RNA synthesis are required for the recycling of "old" receptors has not been excluded. Evidence for the regulation of the concentration of membrane receptors by insulin (14), thyrotropin-releasing hormone (19), α -bungarotoxin (10), concanavalin A (26), and *Ricinus communis* agglutinin (26) has been reported.

The major product of degradation of ¹²⁵I-labeled hEGF which appears in the medium is [¹²⁵I]moniodotyrosine. Similar results have been reported for the degradation of other cell-bound ¹²⁵I-labeled proteins (10, 15) including mEGF (2). That the proteolytic process occurs following the fusion of endocytotic pinosomes with lysosomes is indicated by inhibition of degradation by certain agents whose mode of action is suspected to be the inhibition of lysosomal enzymes. As shown in Table V, chloroquine inhibits the degradation of ¹²⁵I-labeled hEGF. The lysosomotropic action of chloroquine recently has been discussed by De Duve et al. (9). Lie and Schofield (24) have shown that chloroquine blocks the lysosomal degradation of mucopolysaccharides in human fibroblasts, and Goldstein et al. (16) have reported that chloroquine inhibits the degradation of ¹²⁵I-labeled low density lipoproteins by human fibroblasts. Wibo and Poole (36) have shown that chloroquine is concentrated rapidly within rat fibroblasts and inhibits the degradation of cellular protein. These authors also demonstrated that chloroquine inhibits the enzymatic activity of cathepsin B₁ in vitro.

Recently, Seglen (33) has reported that protein degradation in rat hepatocytes is inhibited by ammonium chloride. The data in Table V show that ammonium chloride is a potent inhibitor of the degradation of ¹²⁵I-labeled hEGF. We also have found that local anesthetics such as cocaine, procaine, and lidocaine inhibit the degradation of hEGF.

Although there is no evidence to indicate the manner in which protein degradation is inhibited by ammonia or local anesthetics, it is possible that their mode of action may be similar to that suggested for chloroquine. All of these inhibitors are lipid soluble and permeable to cellular membranes. The data of Wibo and Poole (36) show that chloroquine is concentrated several hundred fold within lysosomes, and Papahadjopoulos et al. (27) have shown that local anesthetics have a high

partition coefficient between phospholipid membranes and aqueous buffer. In un-ionized form, ammonia would also be able to permeate cell membranes. A unifying hypothesis for the inhibition of degradation of ^{125}I -labeled hEGF by these various agents is suggested by the fact that at physiological pH these compounds contain amine groups that are not fully protonated. If these agents are able to permeate the lysosomal membrane, the intralysosomal concentrations would increase as the amino groups become protonated due to the low pH inside the lysosome. Presumably, the protonated species would then be trapped inside the lysosome and the internal pH of the organelle would be increased, resulting in decreased activity of hydrolytic lysosomal enzymes. This hypothesis has been introduced by Home-wood et al. (21) and reviewed by De Duve et al. (9) to explain the lysosomotropic activity of chloroquine. We suggest that ammonia and local anesthetics may also act in this manner. Poste et al. (29) have concluded that local anesthetics influence the activity of peripheral membrane proteins and that the addition of Ca^{++} negates the effects of local anesthetics in their experimental systems. However, we do not find that Ca^{++} affects the inhibition of ^{125}I -labeled hEGF degradation (data not shown), which suggests for local anesthetics a mode of action different from that observed in other systems.

Although the experiments reported here indicate that human fibroblasts are capable of binding, internalizing, and degrading hEGF, it is not known whether internalization, with or without degradation, is required in order to elicit an increase in the synthesis of DNA or is simply a mechanism for the inactivation of the hormone. Any molecular explanation for the mitogenic effect of hEGF must take into account the observation that hEGF must be present in the medium for an extended period of time, much longer than the time required to saturate the binding sites, in order to observe a stimulation of DNA synthesis (3). If there are transient alterations in metabolic parameters as a consequence of the initial binding of the hormone by the fibroblasts, these would not appear to be sufficient for the initiation of a round of DNA replication. The exposure of the fibroblasts to hEGF appears to result in the removal of most of the surface receptors for this mitogen. After approximately 6 h at 37°C (Fig. 1), a steady-state equilibrium is apparently achieved in which 10–20% of the receptor sites, initially available for

binding, remain on the surface. Whether this is a result of the recycling of old receptors, the synthesis of new receptors, or the inability of some of the receptors to be internalized is not known. In an experiment similar to that described in Fig. 1, we have examined the metabolic fate of the cell-bound ^{125}I -hEGF remaining after 6 h of incubation; upon removal of unbound hormone by the addition of fresh medium, 70–80% of the bound ^{125}I -hEGF was still degradable to monoiodotyrosine, albeit at a somewhat slower rate ($t_{1/2}$ approximately 1 h, data not shown). Thus, the cell-bound ^{125}I -hEGF which is detectable after 6 h of incubation degrades in a manner essentially similar to that of the hormone initially bound. These data suggest that the low steady-state level of bound hormone observed after 6-h incubation at 37°C is not due to a unique class of receptors which are unable to internalize bound hormone.

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REFERENCES

1. BASERGA, R., and D. MALAMUD. 1969. *Autography: Techniques and Applications*. Harper and Row, New York. 19–21.
2. CARPENTER, G., K. J. LEMBACH, M. M. MORRISON, and S. COHEN. 1975. Characterization of the binding of ^{125}I -labeled epidermal growth factor to human fibroblasts. *J. Biol. Chem.* **250**:4297–4304.
3. CARPENTER, G., and S. COHEN. 1976. Human epidermal growth factor and the proliferation of human fibroblasts. *J. Cell Physiol.* **88**:227–237.
4. CLARKSON, B., and R. BASERGA, editors. 1974. *Control of Proliferation in Animal Cells*. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.
5. COHEN, S. 1962. Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal. *J. Biol. Chem.* **237**:1555–1562.
6. COHEN, S., and G. CARPENTER. 1975. Human epidermal growth factor: isolation and chemical and biological properties. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1317–1321.
7. COHEN, S., G. CARPENTER, and K. LEMBACH. 1975. Interaction of epidermal growth factor (EGF) with cultured fibroblasts. In *Advances in Metabolic Disorders*. R. Luft and K. Hall, editors. Academic Press, Inc., New York. **8**:265–284.
8. CUATRECASAS, P. 1971. Insulin-receptor interactions in adipose tissue cells: direct measurement and properties. *Proc. Natl. Acad. Sci. U. S. A.* **68**:1264–1268.
9. DE DUVE, C., T. DEBARSY, B. POOLE, A. TROUET,

- P. TULKENS, and F. VAN HOOF. 1974. Lysosomotropic agents. *Biochem. Pharmacol.* **23**:2495-2531.
10. DEVREOTES, P. N., and D. M. FAMBROUGH. 1975. Acetylcholine receptor turnover in membranes of developing muscle fibers. *J. Cell Biol.* **65**:335-358.
 11. DUFAU, M. L., K. J. CATT, and T. TSURUHARA. 1972. Biological activity of human chorionic gonadotropin released from testis binding-sites. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2414-2416.
 12. FAHEY, J. L. 1967. Chromatographic separation of immunoglobulins. In *Methods in Immunology and Immunochemistry*. C. Williams, editor. Academic Press, Inc., New York. 1:321-332.
 13. FREYCHET, P., R. KAHN, J. ROTH, and D. NEVILLE, JR. 1972. Insulin-interactions with liver plasma membranes. Independence of binding of the hormone and its degradation. *J. Biol. Chem.* **247**:3953-3961.
 14. GAVIN, J. R., III, J. ROTH, D. M. NEVILLE, JR., P. DEMEYTS, and D. N. BUELL. 1974. Insulin-dependent regulation of insulin receptor concentrations: a direct demonstration in cell culture. *Proc. Natl. Acad. Sci. U. S. A.* **71**:84-88.
 15. GOLDSTEIN, J. L., and M. S. BROWN. 1974. Binding and degradation of low density lipoproteins by cultured human fibroblasts. *J. Biol. Chem.* **249**:5153-5162.
 16. GOLDSTEIN, J. L., G. Y. BRUNSCHEDE, and M. S. BROWN. 1975. Inhibition of the proteolytic degradation of low density lipoprotein in human fibroblasts by chloroquine, concanavalin A, and Triton WR 1339. *J. Biol. Chem.* **250**:7854-7862.
 17. GREGORY, H. 1975. Isolation and structure of urogastrone and its relationship to epidermal growth factor. *Nature (Lond.)*. **257**:325-327.
 18. HAYFLICK, L. 1973. Subculturing human diploid fibroblast cultures. In *Tissue Culture: Methods and Applications*. P. F. Kruse, Jr. and M. K. Patterson, Jr., editors. Academic Press, Inc., New York. 220-223.
 19. HINKLE, P. M., and A. H. TASHJIAN, JR. 1975. Thyrotropin-releasing hormone regulates the number of its own receptors in the GH₃ strain of pituitary cells in culture. *Biochemistry*. **14**:3845-3851.
 20. HOLLENBERG, M. D., and P. CUATRECASAS. 1975. Insulin and epidermal growth factor: human fibroblast receptors related to deoxyribonucleic acid synthesis and amino acid uptake. *J. Biol. Chem.* **250**:3845-3853.
 21. HOMEWOOD, C. A., D. C. WARHURST, W. PETERS, and V. C. BAGGALEY. 1972. Lysosomes, pH and the anti-malarial action of chloroquine. *Nature (Lond.)*. **235**:50-52.
 22. HUNTER, W. M., and F. C. GREENWOOD. 1962. Preparation of ¹³¹I-iodine-labeled growth hormone of high specific activity. *Nature (Lond.)*. **194**:495-496.
 23. LEFKOWITZ, R. J., J. ROTH, and I. PASTAN. 1971. ACTH-receptor interaction in the adrenal: a model for the initial step in the action of hormones that stimulate adenylyl cyclase. *Ann. N. Y. Acad. Sci.* **185**:195-209.
 24. LIE, S. O., and B. SCHOFIELD. 1973. Inactivation of lysosomal function in normal cultured human fibroblasts by chloroquine. *Biochem. Pharmacol.* **22**:3109-3114.
 25. O'KEEFE, E., M. D. HOLLENBERG, and P. CUATRECASAS. 1974. Epidermal growth factor: characteristics of specific binding in membranes from liver, placenta, and other target tissues. *Arch. Biochem. Biophys.* **164**:518-526.
 26. OLIVER, J. M., T. E. UKENA, and R. D. BERLIN. 1974. Effects of phagocytosis and colchicine on the distribution of lectin-binding sites on cell surfaces. *Proc. Natl. Acad. Sci. U. S. A.* **71**:394-398.
 27. PAPAHAJIOPOULOS, D., K. JACOBSON, G. POSTE, and G. SHEPHERD. 1975. Effects of local anesthetics on membrane properties. Changes in the fluidity of phospholipid bilayers. *Biochim. Biophys. Acta.* **394**:504-519.
 28. PARDEE, A. B. 1975. The cell surface and fibroblast proliferation. Some current trends. *Biochem. Biophys. Acta.* **417**:153-172.
 29. POSTE, G., D. PAPAHAJIOPOULOS, K. JACOBSON, and W. J. VAIL. 1975. Effects of local anesthetics on membrane properties. II. Enhancement of the susceptibility of mammalian cells to agglutination by plant lectins. *Biochim. Biophys. Acta.* **394**:520-539.
 30. RODBELL, M., M. J. KRAUS, S. L. POHL, and L. BIRNBAUMER. 1971. The glucagon-sensitive adenylyl cyclase system in plasma membranes of rat liver. III. Binding of glucagon: method of assay and specificity. *J. Biol. Chem.* **246**:1861-1871.
 31. SAVAGE, C. R., JR., and S. COHEN. 1972. Epidermal growth factor and a new derivative: rapid isolation procedures and biological and chemical characterization. *J. Biol. Chem.* **247**:7609-7611.
 32. SEEMAN, P. 1972. The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24**:583-655.
 33. SEGLEN, P. O. 1975. Protein degradation in isolated rat hepatocytes is inhibited by ammonia. *Biochem. Biophys. Res. Comm.* **66**:44-52.
 34. STEINMAN, R. M., J. M. SILVER, and Z. A. COHN. 1974. Pinocytosis in fibroblasts. Quantitative studies in vitro. *J. Cell Biol.* **63**:949-969.
 35. TERRIS, S., and D. STEINER. 1975. Binding and degradation of ¹²⁵I-insulin by rat hepatocytes. *J. Biol. Chem.* **250**:8389-8398.
 36. WIBO, M., and B. POOLE. 1974. Protein degradation in cultured cells. II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin B₁. *J. Cell Biol.* **63**:430-440.