DIRECT VISUALIZATION OF THE

BINDING AND INTERNALIZATION OF A FERRITIN

CONJUGATE OF EPIDERMAL GROWTH FACTOR

IN HUMAN CARCINOMA CELLS A-431

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ABSTRACT

We have prepared a conjugate of epidermal growth factor (EGF) and ferritin that retains substantial binding affinity for cell receptors and is biologically active. Glutaraldehyde-activated EGF was covalently linked to ferritin to produce a conjugate that contained EGF and ferritin in a 1:1 molar ratio. The conjugate was separated from free ferritin by affinity chromatography using antibodies to EGF. Monolayers of human epithelioid carcinoma cells (A-431) were incubated with EGF:ferritin at 4°C and processed for transmission electron microscopy. Under these conditions, $\sim 6 \times 10^5$ molecules of EGF:ferritin bound to the plasma membrane of each cell. In the presence of excess native EGF, the number of bound ferritin particles was reduced by 99%, indicating that EGF:ferritin binds specifically to cellular EGF receptors. At 37°C, cell-bound EGF:ferritin rapidly redistributed in the plane of the plasma membrane to form small groups that were subsequently internalized into pinocytic vesicles. By 2.5 min at 37°C, 32% of the cell-bound EGF:ferritin was localized in vesicles. After 2.5 min, there was a decrease in the proportion of conjugate in vesicles with a concomitant accumulation of EGF:ferritin in multivesicular bodies. By 30 min, 84% of the conjugate was located in structures morphologically identified as multivesicular bodies or lysosomes. These results are consistent with other morphological and biochemical studies utilizing ^{125}I-EGF and fluorescein-conjugated EGF.

KEY WORDS   hormone receptors · growth factor · endocytosis · lysosomes

Epidermal growth factor (EGF)[1] initiates a complex series of cellular events which ultimately

[1] *Abbreviations used in this paper:* DMEM/BSA, Dulbecco's Modified Eagle Medium containing 0.1% bovine serum albumin; EGF, epidermal growth factor; ^{125}I-EGF, ^{125}I-labeled epidermal growth factor; MVB, multivesicular body; PBS, 9.5 mM sodium phosphate (pH 7.4) containing 4.14 mM KCl and 0.137 M NaCl.

results in increased DNA synthesis and cell division (see references 6 and 7 for reviews). Cultured cells that are responsive to EGF contain specific, saturable plasma membrane receptors for this hormone (9, 19). As a first step in examining the biochemical mechanism by which EGF exerts its growth-promoting effects, we have investigated the metabolic fate of EGF using both ^{125}I-labeled EGF and fluorescein-conjugated EGF. Our studies (4, 17) have indicated that cell-bound EGF rapidly is internalized into endocytic vesicles and

eventually is degraded within lysosomes. Since exposure of cells to EGF results in an apparent decrease in the number of cell-surface receptors, it was postulated that the EGF receptor is internalized along with the hormone (4, 1). Das and Fox (12) have labeled a putative EGF receptor with a photoreactive derivative of ^{125}I-EGF and located degradation products of the receptor in the lysosomal fraction of the cell. These data provide direct experimental support for the proposal that EGF-induced down regulation of plasma membrane receptors involves internalization of the EGF:receptor complex. The internalization of hormone:receptor complexes has been proposed for a number of polypeptide hormones (3, 23, 18).

Although the proposed endocytic vesicles containing EGF have been visualized using biologically active fluorescent derivatives of EGF (17, 27), these experiments do not afford sufficient resolution to determine in detail the cell surface events that occur before the internalization of the hormone:receptor complex. Since ferritin-conjugated ligands have been useful tools in other ultrastructural studies of receptor ligand interactions (2, 13, 22), we have taken a similar approach in the study of EGF binding.

In this communication, we report the preparation of an EGF:ferritin conjugate that is univalent with respect to EGF, contains no ferritin dimers, is biologically active, and binds to cell surface EGF receptors in a specific manner. This conjugate has allowed us to visualize and quantitatively study at the molecular level the topography of the EGF:receptor complex on the plasma membrane and to determine the fate of cell-bound EGF as it is internalized and transported to lysosomes. A preliminary report of these results has been presented (11).

MATERIALS AND METHODS

Mouse EGF was isolated from the submaxillary gland (25). The immunoglobulin fraction of rabbit antiserum against mouse EGF (10) and ^{125}I-EGF (4) were prepared by published methods. Ferritin (cadmium-free, six times recrystallized) and an 8% solution of electron microscopy grade glutaraldehyde were purchased from Polysciences, Inc. (Warrington, Pa.); rabbit anti-horse ferritin was purchased from Miles Laboratories, Inc. (Kankakee, Ill.). Polylysine was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Synthesis of EGF:Ferritin

EGF was coupled to ferritin by a modification of the

general method of Kishida et al. (20). In a typical experiment, 9 mg of EGF containing a tracer amount of ^{125}I-EGF (final sp act $= 6.7 \times 10^5$ cpm/mg) was dissolved in 2.5 ml of 50 mM sodium phosphate buffer (pH 7.3) and activated by the addition of a 1,000-fold excess of glutaraldehyde (1.8 ml of an 8% solution). After stirring at 35°C for 3 h, the unreacted glutaraldehyde was removed by gel filtration on a 45 × 1.5 cm column of Sephadex G-25 (fine) that was equilibrated and eluted with 50 mM sodium phosphate (pH 7.3). In all experiments, the amount of EGF in a sample was calculated from the amount of radioactivity present, based on the assumption that native EGF reacted to the same extent as ^{125}I-EGF. Ferritin concentrations were determined by the absorbance at 280 nm using an extinction coefficient ($E_{cm}^{1\%}$) of 12.7. Molar concentrations of EGF and ferritin were calculated based on mol wt of 6,045 and 750,000, respectively.

Activated EGF from the excluded peak of the Sephadex G-25 column was immediately coupled to ferritin. In the experiment shown in Fig. 1, 780 mg of ferritin (in 7.8 ml of 0.15 M NaCl) was added to 6.3 mg of activated EGF in 14 ml of 50 mM sodium phosphate buffer (pH 7.3). After stirring at room temperature for 5 h and then at 4°C for 50 h, the solution was concentrated to 5 ml in an Amicon ultrafiltration cell (Amicon Corp., Scientific Sys. Div., Lexington, Mass.), using a UM-2 membrane, and unreacted EGF was removed by gel filtration on Bio-Gel A 1.5 m (Bio-Rad Laboratories, Richmond, Calif.). Fig. 1 A shows that 7.3% of the radioactive EGF applied to the column co-migrated with monomeric ferritin. The peak fraction of unreacted EGF eluted in tube 67. Some radioactivity eluted with the salt fraction but was found to be only partially dialyzable through a Spectrapore membrane (Spectrum Medical Industries, Inc., Los Angeles, Calif.) (mol wt cutoff ~3,000). The salt peak probably contained a mixture of ^{125}I$^-$ and other degradation products of ^{125}I-EGF.

Fractions 39–46 (Fig. 1 A), containing EGF:ferritin plus free ferritin were pooled and the ferritin was pelleted by ultracentrifugation (105,000 g, 2 h). 63.8% of the radioactivity pelleted with the ferritin. The pellet, which contained EGF and ferritin in a 1:17 molar ratio, was dispersed in 5 ml of 50 mM sodium phosphate buffer (pH 7.3), and applied to the Bio-Gel A 1.5 m column. As shown in Fig. 1 B, 95% of the radioactive EGF eluted as a single symmetrical peak with monomeric ferritin. Fractions 34–45 were pooled, and the ferritin was again pelleted in the ultracentrifuge. The pellet contained 94% of the radioactivity and was further purified by affinity chromatography.

Separation of EGF:Ferritin from Free Ferritin by Anti-EGF Affinity Chromatography

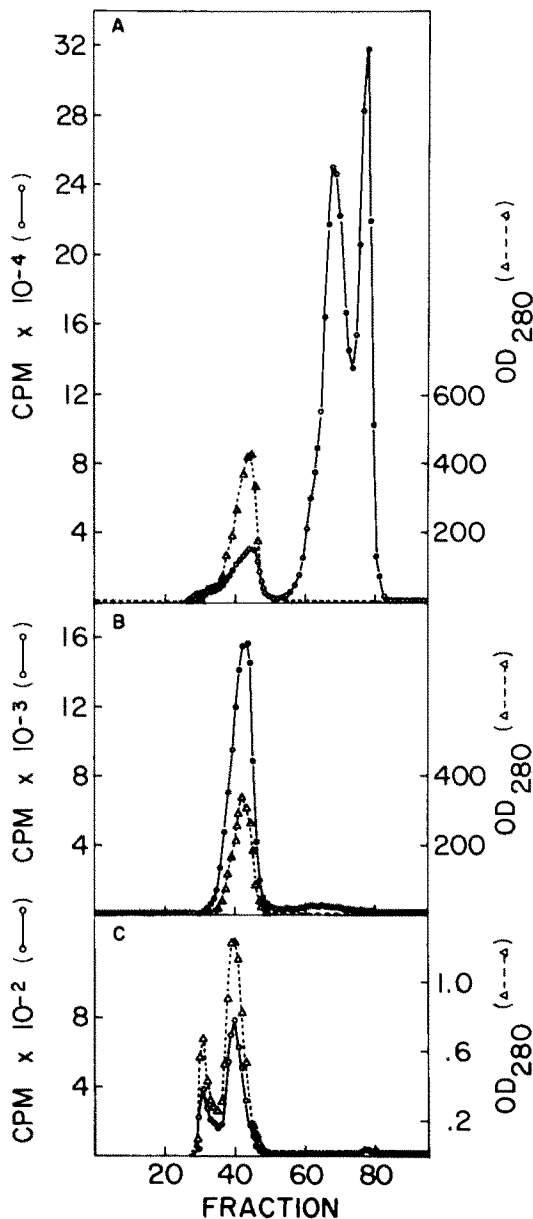An anti-EGF affinity column was prepared by coupling 36.3 mg of the IgG fraction of rabbit antiserum

against EGF to 10 ml of activated AcA22 ultragel by the method of Ternyck and Avrameas (30). To separate EGF:ferritin conjugate from free ferritin, an aliquot of pooled fractions 34-45 from Fig. 1 B was applied to the anti-EGF affinity column at room temperature in 5 ml of phosphate-buffered saline (PBS). The free ferritin and any nonimmunoreactive conjugate were eluted with 100 ml of PBS at room temperature. This unadsorbed protein did not compete with ^{125}I-EGF binding sites in the radioreceptor assay. The adsorbed protein then was eluted with 50 ml of 0.2 M HCl adjusted to pH 2.2 with 2 M glycine. Fig. 2 shows that 92% of the ferritin and 11% of the radioactivity appeared in the PBS wash. Fractions 110-124 (Fig. 2) containing the protein eluted by the HCl-glycine buffer were pooled and centrifuged (105,000 g, 2 h). The pellet was dispersed in 4 ml of PBS and applied to the Bio-Gel A 1.5 m column (Fig. 1 C). The acidic conditions used to elute the affinity column induced some aggregation of the conjugate as indicated by the appearance of a high molecular weight peak (fractions 29-33) of radioactivity and protein. 74% of the radioactivity and 72% of the protein eluted in a peak (fractions 37-45) with the same elution volume as monomeric ferritin. These fractions were pooled, concentrated by pelleting in the ultracentrifuge, and contained EGF and ferritin in approximately a 1:1.25 molar ratio. This preparation of monomeric conjugate was
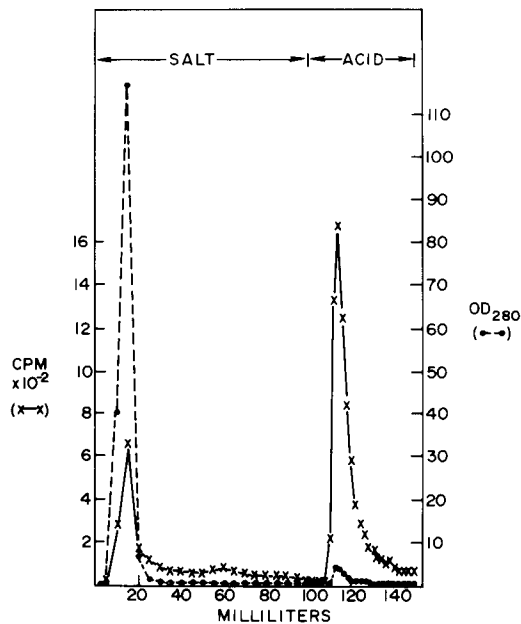
FIGURE 1 Gel filtration of EGF:ferritin. Samples (4-5 ml) were applied to a 100 × 1.4 cm column of Bio-Gel A 1.5 m equilibrated and eluted with 10 mM sodium phosphate buffer (pH 7.3). The flow rate was 17 ml/h and 2-ml fractions were collected. Standards of ferritin, EGF, and ^{125}I-Na eluted with peak values at fractions 41, 67, and 77, respectively. (A) Elution profile of the concentrated EGF-ferritin reaction mixture (see text for details). (B) Rechromatography of concentrated fractions 39-46 from Fig. 1A (184 µg of EGF and 403 mg of ferritin). (C) Elution profile of affinity purified EGF:ferritin (9.7 µg of EGF and 1.78 mg of ferritin) (see text for details).

FIGURE 2 Affinity chromatography of EGF:ferritin. An aliquot (25 µg of EGF and 54.5 mg of ferritin) of the EGF:ferritin from the A 1.5 m column described in Fig. 1 B was applied to the anti-EGF affinity column and eluted as described in Materials and Methods. 5- and 2-ml fractions were collected during the PBS (1-100 ml) and HCl/Gly (100-145 ml) elutions, respectively.

used in all subsequent experiments involving EGF:ferritin conjugate.

EGF:Ferritin Binding and Thymidine Incorporation Assays

The ability of EGF:ferritin to bind to cellular EGF receptors was tested using a ^{125}I-EGF competitive binding assay. Confluent monolayers of human foreskin fibroblasts (grown as previously described [4]) were washed twice with 4 ml of Hanks' solution at 37°C. The cells were incubated at 37°C in 1.5 ml of Dulbecco's Modified Eagle's Medium plus 0.1% bovine serum albumin (DMEM/BSA) containing varying amounts of either native EGF, EGF:ferritin, or EGF:ferritin preincubated with anti-ferritin. At 1 h, the cells were tested for their capacity to bind ^{125}I-EGF (12 ng/ml, sp act = 1.19×10^5 cpm/ng) as previously described (4).

Thymidine incorporation assays were performed on quiescent confluent monolayers of human foreskin fibroblasts by published procedures (5).

Localization of Cell-Bound EGF:Ferritin by Electron Microscopy

Human epithelioid A-431 cells were propagated in 10% fetal calf serum (17). To label cells at 4°C with EGF:ferritin for electron microscopy, monolayer cultures of A-431 cells in 60-mm tissue culture dishes were washed twice at 4°C with Hanks' solution. DMEM/BSA (1.5 ml at 4°C) containing EGF:ferritin (100 nM) was added and the cultures were incubated for 40 min at 4°C. Cultures were washed five times with a total of 10 ml of cold Hanks' solution containing 0.1% bovine serum albumin followed by two 3-ml washes with cold Hanks' solution. The cells were either fixed at this time or further incubated at 37°C in DMEM/BSA for the indicated time intervals, and the cultures were rinsed at 4°C with Hanks' solution and fixed. Fixation was achieved by adding cold 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 1 mM $CaCl_2$ and incubating for 2 h at room temperature. The cultures were rinsed with buffer and postfixed with 1.3% osmium tetroxide in the same buffer for 2 h and dehydrated into ethanol. Monolayers were released from the polystyrene dishes with propylene oxide. The pelleted cells were embedded in Epon 812, and 60-nm sections were examined unstained in a Hitachi 11E microscope.

In certain experiments, monolayers of A-431 cells were prefixed with formaldehyde at either 4° or 23°C before binding EGF:ferritin. To prefix cells at 4°C, cultures were washed twice at 4°C with PBS, then incubated for 20 min at 4°C with 1% formaldehyde in PBS. The cells were washed with cold PBS, quenched with 0.1 M glycine in PBS, and washed with cold PBS. To prefix cells at 23°C, the above steps were performed at room temperature and 0.15% formaldehyde was used. EGF:ferritin was incubated with prefixed cells, and the cells were processed for electron microscopy as

described. These light prefixations did not significantly reduce the binding of EGF:ferritin nor was the nonspecific binding increased.

The binding of EGF:ferritin to monolayers of A-431 cells at 37°C was also examined. After washing with warm Hanks' solution, DMEM/BSA (1.5 ml at 37°C) containing EGF:ferritin (100 mM) was added. After incubating at 37°C for 30 s, 2 min, or 10 min, the cultures were rapidly washed five times with a total of 10 ml of warm Hanks' solution containing 0.1% bovine serum albumin followed by a wash with warm Hanks' solution. The cells were fixed at room temperature with 5% glutaraldehyde and processed for electron microscopy as described above.

Quantification of Cell-Bound EGF:Ferritin

The amount of EGF:ferritin associated with various cellular organelles (plasma membrane, endocytic vesicles, and lysosomes) was determined by counting ferritin particles in randomly selected unstained sections. For each determination, at least 750 ferritin particles were counted, and the percentage of particles bound to the plasma membrane and the percentage inside endocytic vesicles and lysosomes was calculated. Lysosomes were identified morphologically and, for the purpose of this tabulation, all multivesicular bodies were scored as lysosomes.

The degree of grouping of EGF:ferritin bound to the cell surface was also determined. If an EGF:ferritin particle was >30 nm away from adjacent EGF:ferritin particles, it was scored as a singlet. Ferritin particles within 30 nm of adjacent particles were arbitrarily defined as a group, and the number of particles in the group was recorded.

RESULTS

Characterization of the EGF:ferritin Synthetic Reaction

The procedure for the synthesis of EGF:ferritin was designed to yield a conjugate containing EGF and ferritin in a 1:1 molar ratio. In the first synthetic step, EGF was activated with a large molar excess of glutaraldehyde to prevent dimer formation. Since EGF contains only one free amino group (26), a monoactivated EGF should be formed. In a control experiment, glutaraldehyde-activated EGF was chromatographed on the Bio-Gel A 1.5 m column. All the EGF eluted with the same volume as native EGF, indicating that no detectable polymerization occurred.

To evaluate the percentage of EGF that was activated by excess glutaraldehyde, glutaraldehyde-treated EGF was prepared, separated from residual glutaraldehyde, and reacted with a 17-fold molar excess of polylysine (mol wt ~13,000)

under the same conditions used in the activated EGF-ferritin reaction. We consistently found that ~10% of the total EGF was coupled to polylysine as judged by gel filtration of the reaction products on Sephadex G-75. We interpret this to mean that only 10% of the EGF is activated by glutaraldehyde under these conditions. Such results are consistent with previous reports of low reactivity of the NH_2-terminal amino group of EGF (17).

In the second synthetic step, monoactivated EGF was reacted with ferritin. Since <10% of the EGF was coupled to ferritin (see Materials and Methods and Fig. 1A), the molar ratio of ferritin to reactive EGF exceeds 10:1. Therefore, on the basis of statistical considerations, we estimate that <5% of the conjugate will contain more than one EGF molecule per ferritin particle. The above calculations are consistent with the observation that, when free ferritin was removed from the reaction product by affinity chromatography, the conjugate contained EGF and ferritin in approximately a 1:1 molar ratio.

To confirm that the conjugate contained only monomeric ferritin, a dilute solution of affinity purified conjugate was applied to parlodion-coated grid and examined directly with the electron microscope. Greater than 95% of the ferritin particles observed were monomeric.

The covalent linkage of EGF to ferritin was stable for at least 2 mo when stored in PBS at 4°C.

Binding of EGF:Ferritin to Cellular Receptors

The ability of EGF:ferritin to bind to cellular receptors was evaluated by measuring the reduction of ^{125}I-EGF binding by a 1-h preincubation of human fibroblasts with increasing concentration of EGF:ferritin (Fig. 3). The data show that EGF:ferritin reduced the binding of ^{125}I-EGF ~8% as efficiently as native EGF. When EGF:ferritin was preincubated with anti-ferritin the conjugate did not inhibit ^{125}I-EGF binding, thereby confirming that no free EGF was present and that the observed inhibition is due to EGF conjugated to ferritin. The addition of anti-ferritin alone or anti-ferritin plus ferritin did not alter the binding of ^{125}I-EGF.

Competitive binding experiments in which EGF:ferritin was added simultaneously with ^{125}I-EGF were performed also. In these experiments, the conjugate was again ~8% as efficient as native EGF in competing for ^{125}I-EGF-binding sites.
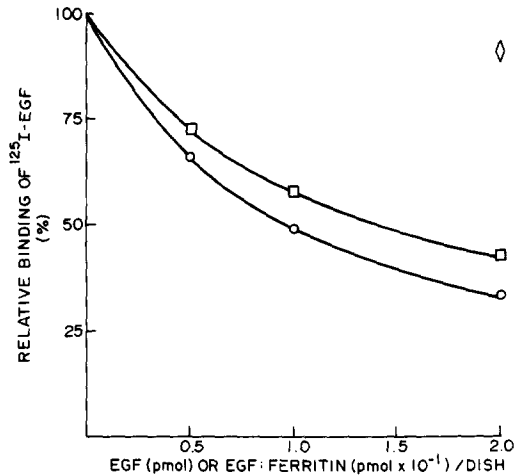

FIGURE 3 Competition of either native EGF or EGF: ferritin and ^{125}I-EGF for binding to human fibroblasts. Cells were incubated with increasing concentrations of either EGF, EGF:ferritin, or anti-ferritin-treated EGF: ferritin for 1 h at 37°C, washed, and reincubated with ^{125}I-EGF (see Materials and Methods for details). The results are expressed as specific binding relative to the binding of ^{125}I-EGF alone. The values represent the average of duplicate cultures which varied by <10%. O, EGF; □, EGF:ferritin; ◊, EGF:ferritin preincubated with anti-ferritin. EGF:ferritin (containing 116 μg of ferritin and 725 ng of EGF) was incubated with 1.09 mg of anti-ferritin in 225 μl of PBS for 2 h at room temperature, then for 24 h at 4°C. The precipitate was removed by centrifugation, and an aliquot of the supernate that would correspond to the indicated concentration of conjugate if no precipitation had occurred was added to the cells.

Biological Activity of EGF:Ferritin

The relative ability of EGF and EGF:ferritin to stimulate the synthesis of DNA in quiescent human fibroblasts was compared. Fig. 4 shows the effect of increasing concentrations of EGF or EGF:ferritin conjugate on the incorporation of radioactive thymidine. One-half maximal stimulation occurred at 0.9×10^{-10} M EGF and 1.2×10^{-10} M conjugate; therefore, the EGF:ferritin is ~75% as effective as native EGF in this assay. EGF:ferritin that had been preincubated with either anti-ferritin or anti-EGF did not stimulate thymidine incorporation above control levels. Anti-ferritin did not reduce the mitogenicity of native EGF.

It is not possible to define the reasons for the different efficiencies observed for EGF:ferritin in the competitive binding and thymidine incorpora-
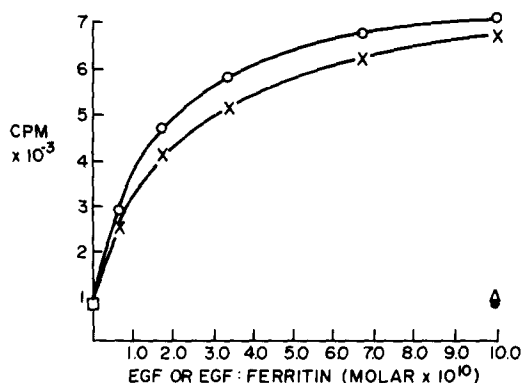
FIGURE 4 Stimulation of DNA replication as a function of EGF or EGF:ferritin concentration. Confluent quiescent fibroblasts were stimulated at t = 0 with the indicated concentration of EGF, EGF:ferritin, or EGF:ferritin preincubated with either anti-EGF or anti-ferritin. The incorporation of [^{3}H]thymidine (27 mCi/mg, 1 μCi/ml) was measured during a 4-h interval beginning at 20 h. The data represent the average of duplicate cultures which varied by <10%. O, EGF; ×, EGF-ferritin; ●, EGF-ferritin preincubated with anti-ferritin; △, EGF-ferritin preincubated with anti-EGF. EGF:ferritin was preincubated with anti-ferritin as described in the legend of Fig. 3. The anti-EGF preincubation was identical except that 0.83 mg of anti-EGF was substituted for the anti-ferritin.

tion assays because the relationship between the binding of EGF to its receptor and subsequent metabolic alterations is complex and biochemically undefined. However, the presence of "spare" receptors (4, 1) and the large difference in time of incubation in the two assays probably contributed to the discrepancy in the assays.

Observation of Cell Surface-Bound EGF:Ferritin by Electron Microscopy

Binding of EGF:ferritin to cellular EGF receptors was visualized directly with the electron microscope. In these studies, we used human epithelioid carcinoma cells (A-431) because of their capacity to bind much larger quantities of EGF compared to human fibroblasts (17, 14). Biochemical studies have shown that A-431 cells internalize and degrade ^{125}I-EGF in a manner similar to fibroblasts (17). Subconfluent cultures of these epithelioid cells grow as monolayers with a cobblestone-like appearance. Numerous membrane folds were observed on the apical surfaces, and the plasma membranes of adjacent cells were interdigitated and joined by desmosomes.

Monolayers of A-431 cells were incubated with

EGF:ferritin (100 nM) at 4°C for 40 min; electron micrographs of unstained sections (Fig. 5) show ferritin particles associated with the plasma membrane. EGF:ferritin was detected on the plasma membrane of all cells examined, and the density of ferritin particles varied less than twofold between different cells. While some binding was observed on the bottom of the cell, the density of ferritin particles was greater on the apical surface and at cell-cell borders.

Averaged over the entire cell surface, ~12 ferritin particles were bound per μm of plasma membrane in 60-nm thick sections. To establish that EGF:ferritin was binding to specific and saturable EGF receptors, an excess (20 μg/ml) of native EGF was added together with EGF:ferritin. In sections of these control cultures, one ferritin core was observed per 20 μm of membrane; therefore, nonspecific binding was ~1%. The specific binding observed in the above experimental cultures corresponds to 200 particles per μm^2. On the basis of our estimation that the surface area of these 30-μm-Diam cells is 2,800 μm^2, we calculate that each cell bound ~6 × 10^5 molecules of EGF:ferritin. Under the conditions used to bind EGF:ferritin (4°C, 40 min), these cells bind ~1 × 10^6 molecules of ^{125}I-EGF/cell at saturating concentrations of ^{125}I-EGF.

Although no large patches or large clusters of EGF:ferritin were detected on the cell surface, binding was not entirely random. As can be seen in Fig. 5 and Table I, cell-bound EGF:ferritin was frequently located in small groups. The degree of grouping was not reduced if the cells were lightly fixed with formaldehyde at either 4° or 23°C (see Materials and Methods) before binding the conjugate, thereby suggesting that the pattern seen in Fig. 5 was not due to clustering after binding at 4°C.

Internalization of EGF:Ferritin

The fate of cell-bound EGF:ferritin was investigated by binding the conjugate to monolayers at 4°C, removing unbound EGF:ferritin, and then warming the cultures to 37°C (see Materials and Methods). As shown in Table I, the amount of EGF:ferritin located in groups containing five or more particles increased from 27 to 44% when cells were warmed to 37°C for 30 s. The binding pattern at 30 s is illustrated in Fig. 6. Although some of the ferritin clusters were located in coated pits, the majority were located on areas of the membrane with no distinguishing features.
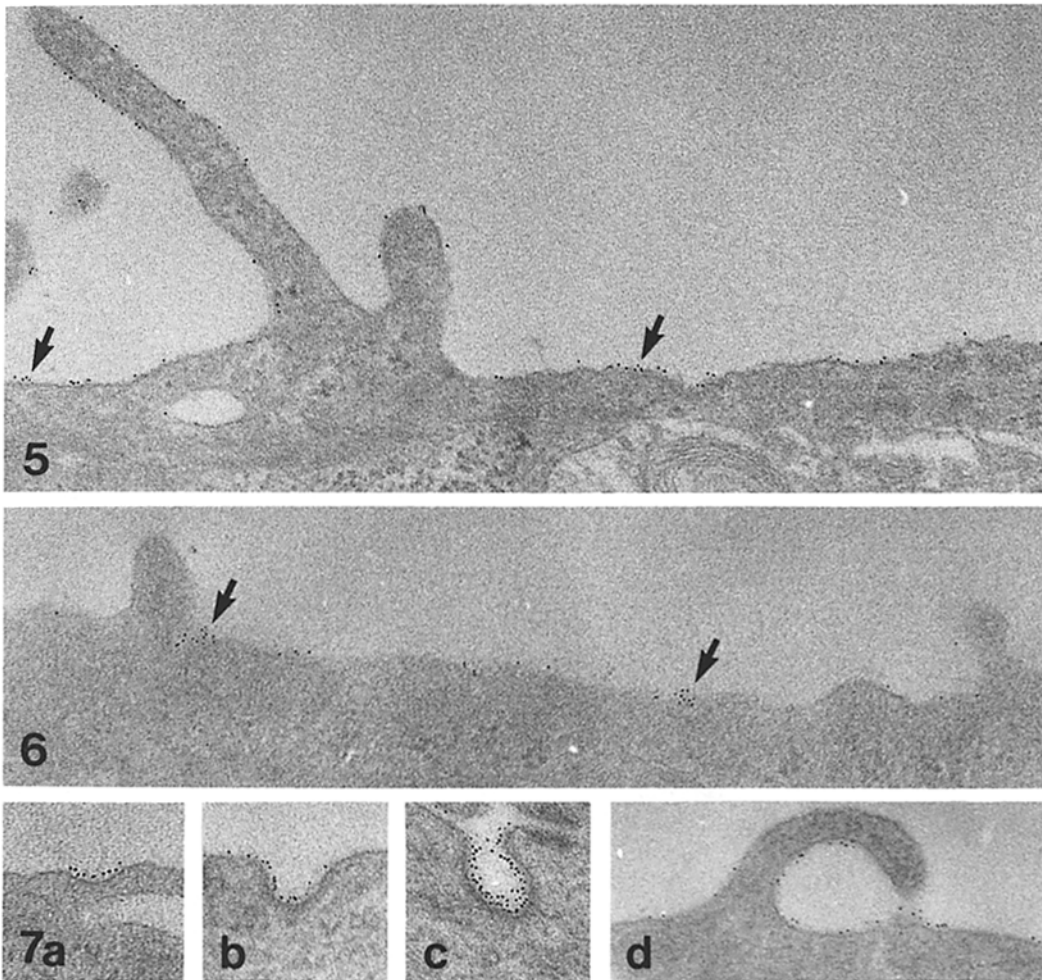
FIGURE 5   Binding of EGF:ferritin at 4°C. Monolayers of A-431 cells were incubated with EGF:ferritin (100 nM) for 40 min at 4°C, washed, and prepared for electron microscopy as described in Materials and Methods. Conjugate bound to the plasma membrane is predominantly dispersed, but some small clusters are present (arrows). × 66,000.

FIGURE 6   Redistribution of cell-bound EGF:ferritin at 37°C. Cultures were preloaded with conjugate at 4°C as in Fig. 5 and warmed to 37° C for 0.5 min (see Materials and Methods). EGF:ferritin was frequently located in clusters (arrow). × 66,000.

FIGURE 7   Internalization of EGF:ferritin. Cultures were preloaded with conjugate at 4°C as in Fig. 5 and warmed to 37°C for 2.5 min. Clusters of EGF:ferritin are seen apparently in the process of being internalized into small endocytic vesicles (a–c). EGF:ferritin is seen inside a membrane fold that appears to be fusing with the plasmalemma (d) to form a large vesicle. × 66,000.

The distribution of EGF:ferritin in cells after 2.5 min at 37°C is shown in Figs. 7 and 8. Ferritin particles can clearly be seen inside cytoplasmic vesicles with a diameter of ~120 nm (Fig. 8). These vesicles were most likely formed by adsorptive pinocytosis. Fig. 7 (a–c) shows a series of progressively deeper plasmalemmal indentations and illustrates the probable sequence of events by which groups of EGF:ferritin are internalized into pinocytic vesicles. In this instance, the invaginated pits are coated; however, most conjugate-containing endocytic vesicles and many plasma membrane

TABLE I

Binding of EGF:Ferritin at 4°C and Quantitation of the Redistribution after Warming to 37°C

| | Location of cell-bound EGF:ferritin | | | | | | |
| | Cell surface | | | | | Internalized | |
Time at 37°C	Singlet	Doublet	Triplet	Quadruplet	5 or more	Vesicle	Lysosome*
min			%				%
0	29	19	15	10	27 (7.0)	0	0
0.5	15	11	19	11	44 (5.7)	0	0
1.0	16	13	15	11	45 (7.2)	<5	0
2.5	13	15	12	10	18 (6.5)	32 (8.0)	0
15.0	4	4	4	2	3 (5.6)	22 (7.7)	61 (63.3)
30.0	1	1	1	1	2 (8.5)	10 (7.6)	84 (54.5)

Monolayers of A-431 cells were incubated with EGF:ferritin (100 nM) for 40 min at 4°C, unbound conjugate was removed, and the cells were warmed to 37°C for the indicated times and processed for electron microscope observation. The amount and distribution of EGF:ferritin on the cell surface and located inside the cell was quantitated (see Materials and Methods) and the results are expressed as percent of total cell-bound conjugate. The numbers in parentheses indicate the average number of ferritin particles associated with that particular structure. In this experiment, an average of 12 ferritin particles (range = 10.6–14.7) was observed per μm of cell membrane or underlying cytosol in these 60-nm sections.
* For the purpose of this tabulation, all multivesicular bodies were scored as lysosomes.

invaginations did not have the thickened membrane characteristic of coated pits. EGF:ferritin was occasionally observed in vesicles with a diameter of ~400 nm. These could have been formed by fusion of the tip of a plasma membrane fold with the membrane as shown in Fig. 7d. However, it is possible that these apparent vesicles are actually exterior to the cell and appear as vesicles due to sectioning artifacts. Because of these uncertainties, these 400-nm vesicles (a small minority of the total number of vesicles) were not included in the quantitation in Table I. Experiments are in progress to determine their true nature.

As shown in Table I, 32% of the cell-bound EGF:ferritin was in endocytic vesicles within 2.5 min at 37°C. In 60-nm sections, each vesicle profile contained an average of 8.0 EGF:ferritin particles; 73% of the hormone was located in vesicles that contained between 5 and 13 particles per vesicle. It is of interest to note the similarity between the number of particles in vesicles at 2.5 min (8.0) and the number of particles in cell surface groups (7.2) at 1 min. Also, note that the decrease in the number of cell surface groups between 1 and 2.5 min quantitatively corresponds to the amount of EGF:ferritin incorporated into vesicles during the same time period. These quantitative similarities together with the direct observation of invaginations (Fig. 7a-c) strongly suggest that, during the 37°C incubation, small clus-

ters of EGF:ferritin-receptor complexes are segregated in the plane of the plasma membrane and that these groups are rapidly endocytosed into 120-nm vesicles.

By 15 min at 37°C, 84% of the EGF:ferritin had been internalized (Table I). 27% of the internalized EGF:ferritin was in endocytic vesicles located throughout the cytoplasm. EGF:ferritin-containing vesicles located near multivesicular bodies (MVBs) were smaller (65 nm Diam) than those nearer the plasma membrane (120 nm Diam). 73% of the internalized EGF:ferritin was in structures morphologically identified as MVBs with a diameter of ~500 nm (Fig. 9). Profiles of these MVBs contained an average of 63 EGF: ferritin particles. Most of the EGF:ferritin in MVBs was bound to the convex surface of the internal vesicles or located in small clusters on the limiting membrane of the MVBs (Figs. 9 and 10). The vesicles inside the MVBs were approximately the same size (65 nm Diam) as the EGF:ferritin-containing cytoplasmic vesicles located near the MVBs, and suggest that the internal vesicles were derived from the cytoplasmic vesicles. Since the ferritin particles were bound to the inside of cytoplasmic vesicles and to the outer surface of the MVB vesicles, the mchanism by which cytoplasmic vesicles and MVBs fuse probably produces an eversion of the sideness of the membrane of the vesicle.
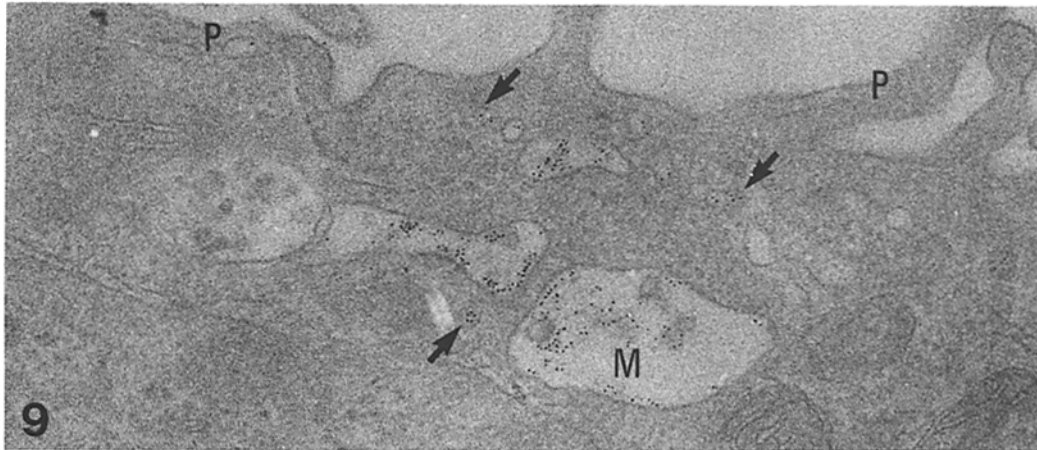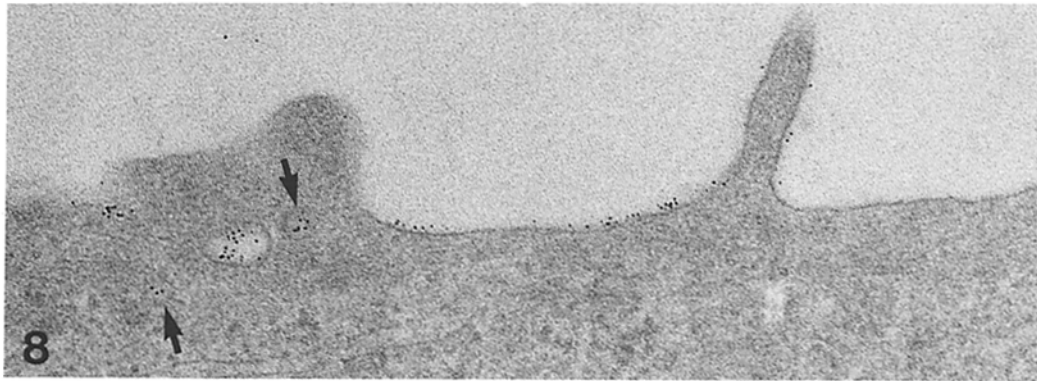
FIGURE 8   Endocytic vesicles containing EGF:ferritin. Cultures were preloaded with conjugate at 4°C as in Fig. 5 and warmed to 37°C for 2.5 min. Arrows indicate endocytic vesicles containing EGF:ferritin. × 66,000.

FIGURE 9   Multivesicular bodies containing EGF:ferritin. Cultures were preloaded with conjugate at 4°C as in Fig. 5 and warmed to 37°C for 15 min. The plasmalemma (P) is nearly depleted of EGF:ferritin while cytoplasmic vesicles (arrows) and multivesicular bodies (M) contain most of the cell-bound conjugate. × 66,000.

Internalized EGF:ferritin represented 94% of the cell-bound EGF:ferritin in cells that had been warmed to 37°C for 30 min (Table I). 84% of the internalized EGF:ferritin was present in MVBs or lysosomes. These structures were frequently located adjacent to the cell nucleus (Fig. 10). EGF: ferritin was never observed in the nucleus or in the stacked Golgi cisternae. Occasionally, a few ferritin cores were observed associated with the rough endoplasmic reticulum.

The data presented in Table I are summarized in Fig. 12 and show the temporal relationship between the binding of EGF:ferritin to the plasma membrane, clustering within the plane of the plasma membrane, internalization into endocytic

vesicles, and accumulation of hormone in lysosomes or MVBs.

Binding and Internalization of EGF:Ferritin at 37°C

The quantitative data presented in Table I on the binding and endocytosis of EGF:ferritin are based on experiments in which cells preloaded with the conjugate at 4°C were subsequently warmed to 37°C. To eliminate the possibility that the incubation at 4°C induced an unphysiological result, we did a similar study entirely at 37°C. Monolayers were incubated at 37°C with EGF: ferritin (100 nm) for 30 s, 2 min, or 10 min, rapidly rinsed at 37°C, and fixed at room temper-
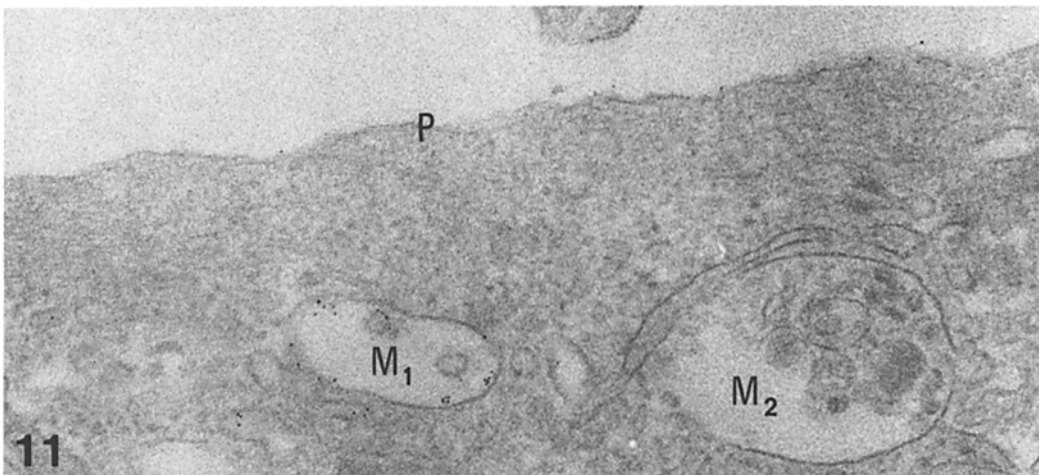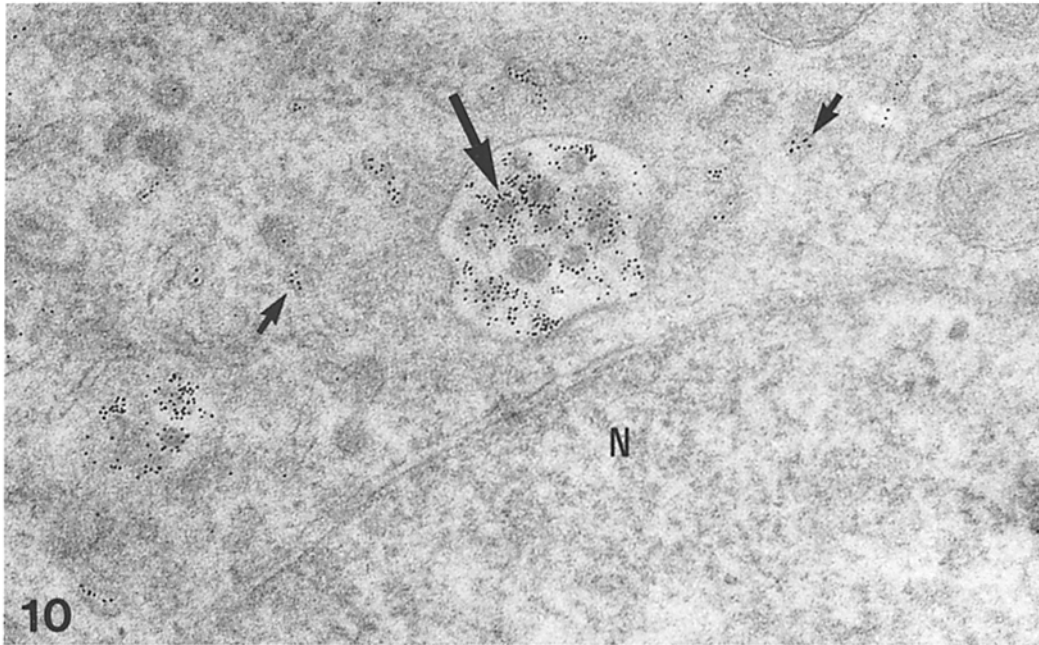
FIGURE 10   Accumulation of EGF:ferritin in multivesicular vesicles. Cultures were preloaded with conjugate at 4°C as in Fig. 5 and warmed to 37°C for 30 min. EGF:ferritin was observed inside multivesicular bodies located primarily on the convex surface of internal vesicles (large arrow) and also in cytoplasmic vesicles (small arrows). N, nucleus. × 66,000.

FIGURE 11   Binding of EGF:ferritin at 37°C. Cultures were incubated with EGF:ferritin (100 nM) at 37°C for 10 min, washed, and prepared for electron microscopy as described in Materials and Methods. A multivesicular body containing EGF:ferritin (M_1) is seen beside a multivesicular body devoid of conjugate (M_2). At this time, some of the hormone is bound to the plasmalemma (P).

ature (see Materials and Methods). The results are summarized in Table II.

The amount of EGF:ferritin bound increased with time at approximately the same rate as the binding of ^{125}I-EGF. At 0.5 min, none of the EGF:ferritin was located in groups containing greater than three particles and probably is a reflection of low binding density after this brief
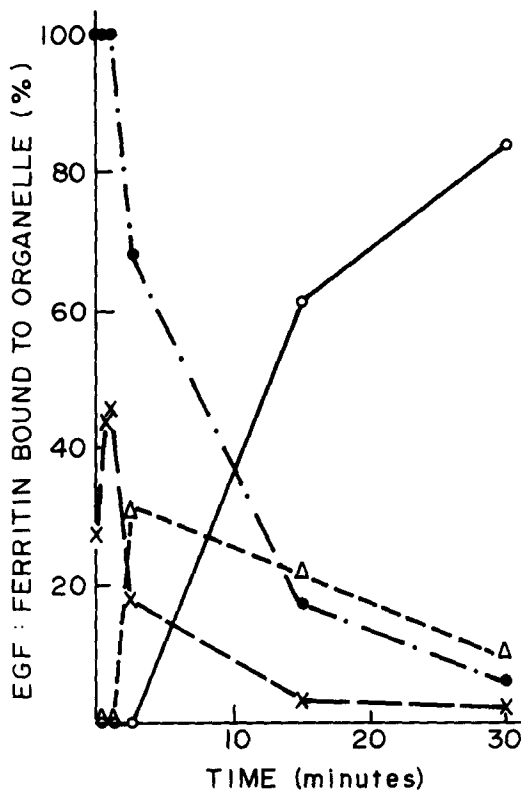
Figure 12 Fate of cell-bound EGF:ferritin. The data presented in Table I are summarized to show the percentage of the total cell-bound conjugate that was associated with the plasma membrane (●), cell surface groups containing five or more ferritin particles (x), cytoplasmic vesicles (△), or lysosomes (○) as a function of the time of incubation at 37°C.

exposure. At the 2-min point, clusters of the conjugate that contained an average of nine particles were detected on the cell surface. At the same point, 14% of the cell-bound EGF:ferritin was located in 120-nm-diameter vesicles that contained an average of 8.6 particles. These vesicles appeared identical to the 120 nm EGF:ferritin-containing vesicles seen in cells preloaded at 4°C and then warmed to 37°C (see Fig. 8). At 10 min, 74% of the bound EGF:ferritin had been internalized. One-half of the internalized EGF was in 120-nm vesicles that contained an average of 8.6 particles. The other half of the internalized EGF: ferritin was located in MVBs (Fig. 11) that contained an average of 17.6 particles. These structures appear similar to the 500-nm MVBs seen in cells preloaded with EGF:ferritin at 4°C and warmed to 37°C for 15 min (see Fig. 10).

DISCUSSION

A 1:1 conjugate of EGF:ferritin has been prepared that retains substantial binding activity for cellular EGF receptors and is biologically active. The number of EGF:ferritin particles bound per cell is in good agreement with the number of cellular EGF receptors as determined biochemically using ^{125}I-EGF. Each EGF:ferritin particle observed on the cell membrane corresponds to a single EGF-binding site because: (a) there is no multimeric ferritin in the conjugate, (b) <5% of the conjugate contains more than one molecule of EGF per ferritin particle, and (c) the nonspecific binding of EGF:ferritin is only 1% of the total binding.

Table II

Quantitation of the Binding and Internalization of EGF:Ferritin at 37°C

	Location of cell-bound EGF:ferritin						
	Cell surface					Internalized	
Incubation time	Singlet	Doublet	Triplet	Quadruplet	5 or more	Vesicle	Lysosome*
min			%				%
0.5	50	27	23	0	0	0	0
2.0	47	13	8	4	14 (9.0)	14 (8.6)	0
10.0	2	7	8	5	4 (6.0)	36 (8.6)	38 (17.6)

Monolayers of A-431 cells were incubated with EGF:ferritin (100 nM) for the indicated times at 37°C, washed to remove unbound hormone, and processed for electron microscope observation. The amount and distribution of EGF:ferritin on the cell surface and located inside the cell was quantitated (see Materials and Methods), and the results are expressed as percentage of total cell-bound conjugate. The numbers in parentheses indicate the average number of ferritin particles in that particular structure. In this experiment, 1.2, 3.9, and 8.6 ferritin cores were observed per μm of membrane or underlying cytosol at 0.5, 2, and 10 min, respectively. Binding was reduced by ~99% if an excess (20 μg/ml) of native EGF was added to the binding media.

* For the purpose of this tabulation, all multivesicular bodies were scored as lysosomes.

EGF:ferritin initially is localized exclusively on the plasma membrane (Fig. 6). Most of the binding occurs on the apical surface and on the interdigitated membrane folds at cell-cell borders. The observed binding capacity of these numerous membrane folds at cellular borders is in agreement with and explains the prominent staining of this region by fluorescein-conjugated EGF (17).

Singer (28) has proposed that clustering is a prerequisite for endocytosis of receptor-bound ligands. Although it clearly has been established that multivalent ligands induce clustering of their receptors (29, 13), the fate of univalent ligands has not been extensively investigated. In this report, we have used the univalent ligand EGF: ferritin to study cell surface events that occur before endocytosis. At 4°C or in lightly prefixed cells, distribution of the plasma membrane-bound EGF:ferritin was not entirely random (Fig. 5). Initially, ~25% of the EGF receptors appear to be located in groups of five or more (Table I). When unfixed cells were warmed to 37°C, the conjugate rapidly redistributed in the plane of the membrane such that nearly 50% was located in small groups (Table I). The redistribution was not dependent on metabolic energy since it occurred in glucose-free media containing sodium azide and dinitrophenol (unpublished results). The essential features of surface binding and redistribution of EGF:ferritin have been confirmed in another dimension by *en face* examination of whole mounted cells (21). A hypothesis consistent with these results is that the binding of EGF to its receptor alters the configuration of the receptor in such a way as to predispose it to either (*a*) associate with other EGF:receptor complexes or (*b*) bind to a specific area of the membrane predetermined to be the site of clustering. The initial nonrandom distribution of EGF receptors may reflect a limited tendency of unoccupied receptors to self associate.

Time-course studies revealed that the clusters of EGF:ferritin on the cell surface rapidly were pinocytosed into vesicles (average Diam = 120 nm). The internalization occurred at both coated and noncoated regions of the membrane. The formation of endocytic vesicles required metabolic energy since no EGF:ferritin-containing vesicles were observed when cells were incubated with conjugate in glucose-free media containing sodium azide and dinitrophenol (unpublished results). With use of horseradish peroxidase as a marker, it has been shown biochemically that

native EGF stimulates the rate of endocytosis in A-431 cells by approximately fivefold (unpublished results). Therefore, it is possible that cell-bound EGF stimulates its own internalization into endocytic vesicles. The recent observation that EGF directly enhances the ability of A-431 membranes to phosphorylate endogenous proteins in vitro (8) raises the possibility that the phosphorylation of specific membrane proteins may be involved in clustering and/or pinocytosis.

EGF:ferritin-containing vesicles apparently serve to shuttle the hormone to multivesicular bodies. Although histochemical studies will be required to conclusively identify the multivesicular bodies as secondary lysosomes, the rapidity with which ^{125}I-tyrosine is released into the media subsequent to the binding of ^{125}I-EGF (4) suggests that lysosomal degradation begins at approximately the same time that EGF:ferritin is observed to accumulate in multivesicular bodies (Fig. 12).

EGF:ferritin was not observed to associate with any other cellular organelles to a significant extent, although a small amount was seen in rough endoplasmic reticulum. The significance, if any, of this association is unknown.

Since coupling of EGF to ferritin results in large changes in the physiochemical properties of the hormone, results obtained with EGF:ferritin must be analyzed with caution. Nonetheless, the following independent observations suggest that the metabolic fate of EGF:ferritin is an accurate marker of the physiological process by which EGF is internalized. First, EGF:ferritin is univalent with respect to EGF; therefore, artifacts due to valence of the ligand were avoided. Second, the conjugate retained nearly complete biological activity (Fig. 4). Third, the kinetics of the binding and internalization of EGF:ferritin observed by electron microscopy are similar to those inferred from biochemical studies of the interaction of ^{125}I-EGF with fibroblasts and A-431 cells (4, 17). Fourth, the kinetics of binding and the cellular location of internalized fluorescein-conjugated EGF observed by fluorescence microscopy in A-431 cells (17) is consistent with the results reported herein. Fifth, Gordon et al. (16) have used quantitative electron microscope autoradiography to investigate the fate of ^{125}I-EGF in human fibroblasts. They found that the hormone initially localized to the plasma membrane. With incubation at 37°C, they observed a decrease in membrane-bound hormone with a concomitant in-

crease in the amount of ^{125}I-EGF associated with lysosomes. The observed accumulation of EGF: ferritin in lysosomes in A-431 cells (Tables I and II) is in agreement with the temporal sequence of internalization reported by these investigators. Since the cellular fate of EGF:ferritin was very similar to the fate of both ^{125}I-EGF and fluorescein-conjugated EGF, it is probable that these derivatives of EGF accurately reflect the physiological interaction of EGF with its target cell.

Recent evidence suggests that coated pits (24, 15) on the plasma membrane are involved in the process of adsorptive endocytosis. Using a ferritin conjugate of low density lipoprotein, Anderson et al. (2) showed that this complex is rapidly internalized in cultured fibroblasts. They obtained convincing evidence that low density lipoprotein initially was bound to coated pits on the plasma membrane and that subsequently the coated pit regions were endocytosed into coated vesicles. The electron microscope autoradiography experiments of Gordon et al. (16) suggest that ^{125}I-EGF also binds with some preference for coated pit regions of human fibroblasts. Although EGF:ferritin did bind to coated pits (Fig. 7) in A-431 cells, much of the cell-bound EGF:ferritin seemed to be internalized by a process that did not involve coated pits or coated vesicles. Additional experiments will be required to determine quantitatively the role of coated pits in this process.

Schlessinger et al. (27) have investigated the fate of cell-bound rodamine-labeled EGF in monolayers of living 3T3 fibroblasts, using a sensitive video intensification microscope system. In contrast to the results obtained with fluorescent EGF (17) in A-431 cells, they found that rodamine-conjugated EGF formed patches before internalization. The data of Schlessinger et al. (27) and the ^{125}I-EGF autoradiography experiments showing EGF binding preferentially to coated pits (16) raise the possibility that details of the cell surface distribution of EGF receptors may be different in fibroblasts and A-431 cells. Investigation of the topography of the EGF:ferritin binding to fibroblasts should determine whether these observed differences are the consequence of cell morphology (fibroblastic or epithelioid), the state of transformation, and/or the number of EGF-receptors per cell, or are due to the limitations in the methods of visualization.

It is now clear that, subsequent to the initial binding of EGF to the plasma membrane, the hormone, probably together with its receptor, is internalized rapidly in endocytotic vesicles and eventually degraded in lysosomes (4, 12, 17, 27, and Fig. 12). However, we can only speculate (7) concerning the stage or stages of this process at which the signals for the many observed metabolic alterations are generated.

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