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Hormone receptor topology and dynamics: Morphological analysis using ferritin-labeled epidermal growth factor

(endocytosis/lysosomes/lysosome inhibitors/multivesicular bodies)

JAMES A. MCKANNA*, HARRY T. HAIGLER†, AND STANLEY COHEN†

*Department of Anatomy and †Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

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ABSTRACT **Previous studies using a biologically active 1:1 conjugate of EGF and ferritin (F-EGF) have traced the binding and internalization of the hormone molecules. In the present report, we develop ultrastructural criteria for identification of the F-EGF·receptor complex, and, thereby, enable utilization of the F-EGF as an indirect marker to localize the receptor for this peptide hormone. The ferritin cores of bound F-EGF are situated 4–6 nm from the extracellular surface of the membrane. When cells were incubated for up to 30 min at 37°C, this characteristic spatial relationship was observed in all uptake stages (surface clustering, endocytosis, and incorporation into multivesicular bodies), indicating that the hormone·receptor complex remains intact through these steps. However, when incubation was continued for periods sufficient to allow hormone degradation (30–60 min), pools of free ferritin were observed in lysosomes. In the presence of various amine inhibitors of hormone degradation, internalization and multivesicular body incorporation proceeded, but hormone·receptor degradation was blocked as evidenced by preservation of the ferritin–membrane relationship; i.e., no pools of free ferritin were seen after 60 min. These data provide morphological support for the hypothesis that down-regulation of surface receptors involves internalization of intact hormone·receptor complexes. In addition, we have developed a method for viewing the surface of intact cells *en face*, allowing closer scrutiny of the clustering of F-EGF·receptor complexes in the plane of the membrane prior to internalization. The particles in the F-EGF clusters observed by this method are spaced at 12 nm center-to-center, serving to set upper limits on the packing dimensions of the EGF·receptor complex.**

Although investigations into the mechanisms of hormone action have demonstrated cell surface binding and subsequent internalization of a number of polypeptide hormones—including insulin, follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, and epidermal growth factor (EGF) (1–3)—a key question concerning the plasmalemmal hormone *receptors* remains unanswered: Are the *receptors* removed from the cell surface along with the hormone as part of the cellular response? Biochemical evidence that hormone binding capacity decreases after hormonal stimulation (down-regulation) suggests that receptors may be removed from the cell surface or modified to decrease binding capacity (4–7); however, the resolution of experimental techniques has been insufficient to differentiate clearly between these alternatives. Attempts to covalently link ^{125}I-labeled EGF to its putative membrane receptor and follow its metabolic fate have been reported (8–10). The results suggest receptor internalization; however, only a small fraction of the membrane-bound EGF was successfully crosslinked, and many questions remain concerning the membrane sites, stability, and mode of down-regulation for the majority of EGF receptors.

We recently have prepared an electron-dense derivative of EGF composed of monomeric ferritin coupled to EGF (F-EGF) that has provided improved resolution of hormone binding and uptake (11). Closer inspection of our micrographs and binding data suggested that F-EGF might also serve to indirectly label the EGF *receptor*. As reported in the present communication, we have developed ultrastructural criteria for identification of the membrane-bound F-EGF·receptor complex. The electron-dense ferritin core is situated 4–6 nm from the outer surface of the plasmalemma at the time of initial binding, and this characteristic relationship serves to identify the specific F-EGF·receptor complex at later time points. The 4- to 6-nm distance is maintained through the processes of surface clustering, pinocytic internalization, and incorporation into multivesicular bodies (MVBs), indicating that the specific F-EGF·receptor complex remains intact through these processes and, therefore, that the *receptors* are internalized with the hormone. Data also are presented demonstrating that (*i*) the characteristic ferritin–membrane relationship is disrupted in lysosomes 30–60 min after internalization; (*ii*) the ferritin–membrane relationship in MVBs is preserved in the presence of ammonia or amines, which presumably inhibit lysosomal degradation; and (*iii*) discrete cell surface clusters of F-EGF particles are observed in *en face* views of intact cells after incubation. The interparticle distances in such clusters define an upper limit for the molecular dimensions of the *receptors*.

MATERIALS AND METHODS

Mouse EGF was isolated from submaxillary glands according to the methods of Savage and Cohen (12), and F-EGF was synthesized as reported by Haigler *et al.* (11).

In most experiments we used A-431 human epithelioid carcinoma cells because of their capacity to bind much larger quantities of EGF than human fibroblasts (13). A-431 cells were propagated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum to near confluence in 35-mm culture dishes. Cultures were washed twice with 4°C Hanks' solution followed by incubation for 40 min at 4°C in 1.5 ml of the modified Eagle's medium plus 0.1% bovine serum albumin containing 100 nM F-EGF. (Such times and concentrations have been shown to reach the asymptotic region of the binding curve—approximately 80% saturation.) After binding, 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. They were either fixed at this time or further incubated at 37°C in modified Eagle's medium/bovine serum albumin for given experimental intervals. Fixation was achieved by addition of either cold or room temperature 5% (wt/vol) glutaraldehyde buffered to pH 7.2 with 0.1 M sodium cacodylate containing 1 mM calcium chloride. Except for whole mounts, which were dehydrated and

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Abbreviations: EGF, epidermal growth factor; F-EGF, epidermal growth factor labeled with ferritin; MVB, multivesicular body.

dried without osmium tetroxide treatment (14), cultures were rinsed in buffer and postfixed with 1.3% osmium tetroxide in 0.1 M cacodylate. After dehydration in ethanol solutions, the monolayers were released from the polystyrene by propylene oxide and embedded in Epon 812.

RESULTS

Identification of the F-EGF·Receptor Complex. After incubation with F-EGF at 4°C, the surface of A-431 cells was studded with ferritin particles in a dispersed array (Fig. 1*A*). Each particle seemed to be associated individually with the membrane; however, the proximity of the particles to the membrane appeared somewhat variable, especially in regions where the membrane passed obliquely through the plane of section (Fig. 1*B*, asterisk). Our interpretation of the basis for such variability is illustrated in Fig. 2. In a 50-nm-thick section, when the F-EGF and receptor are situated on obliquely sectioned regions of the membrane, the particle appears either farther from (Fig. 2 center) or closer to (Fig. 2 right) the image of the membrane, which is created predominantly by portions of the membrane normal to the plane of section. In regions where the entire section of the membrane is normal to the plane of section (Fig. 2 left), the plasmalemmal image is more distinct and the membrane–particle relationships seem more uniform. We have measured the distance from particle to membrane in regions of the plasmalemma similar to those shown in Fig. 1 *A* and *B* (arrows). For 400 particles from five different experiments, we obtained 5.03 ± 1.25 nm (mean ± SD) as the dimension of the electron-lucent space separating the edge of the dense ferritin core (6-nm diameter) from the surface of the noncytoplasmic dense lamina of the membrane. [Adopting freeze-fracture terminology (15), we refer to this membrane surface as the "ES face."]

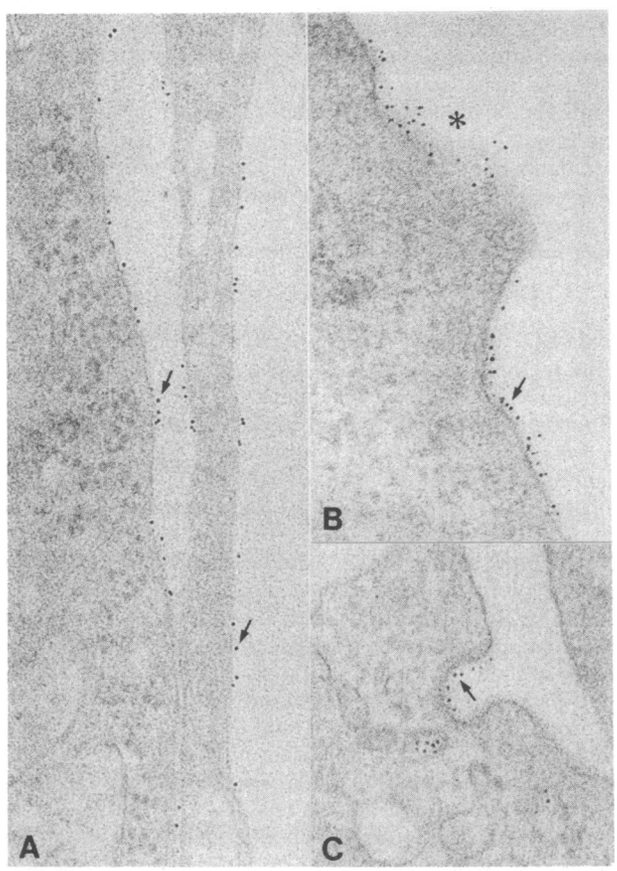

FIG. 1. Cells exposed to F-EGF for 40 min at 4°C and fixed either immediately at 4° (*A*) or after warming to 37°C for 150 sec (*B*) or 30 sec (*C*). In oblique regions (*), the exact spatial relationship of ferritin and membrane is unclear; however, in regions where the membrane is normal to the plane of section, the characteristic 4- to 6-nm separation is apparent (arrows) both in the native dispersed distribution (*A*) and after clustering (*B*, *C*). (×80,000.)

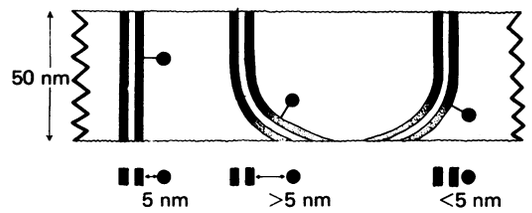

FIG. 2. Diagrammatic transverse plane of a 50-nm thin section illustrating "plane-of-section" artifacts. When membrane is normal to plane of section (left), image of particle appears 5 nm from membrane. When membrane curves within the section (center or right), apparent distance in the image is artifactual.

Upon washing to remove unbound F-EGF and warming to 37°C, the A-431 cells rapidly redistributed the dispersed particles as reported previously (11). Throughout such lateral movement, the 4- to 6-nm gap between the particles and the ES face of the membrane remained obvious in appropriate planes of section (Fig. 1 *B* and *C*).

The binding of native ferritin particles to the ES face of the plasmalemma in A-431 cells is nil. Furthermore, we have demonstrated that F-EGF binding is >99% inhibited by excess native EGF. Therefore we are certain that virtually every ferritin particle observed at the cell surface is uniquely conjugated to an EGF molecule that is specifically bound to a plasmalemmal receptor. We assume that the consistent spatial relationship of ferritin particles to the ES face of the membrane provides a reliable anatomic characteristic for identification of the F-EGF·receptor complex under subsequent circumstances.

Dimensions and Packing Density of the F-EGF·Receptor Complex. In an attempt to examine the nature of the electron-lucent space, the A-431 cells were prepared with ruthenium red, a stain for extracellular polysaccharides (16). As shown in Fig. 3, a rich glycocalyx, 5–15 nm thick, is present on the plasmalemma. Because the saccharide portions of glycoproteins do not stain with normal osmium tetroxide procedures, it is likely that such substances occupy some of the space between ferritin and membrane and may compose part of the receptor.

In addition to confirming the persistence of the F-EGF·receptor complex, closer examination of the clustering phenomenon provided evidence regarding the packing and dimensions of the complex in the plane of the membrane. As reported previously, an average number of seven particles per cluster was observed in thin sections, a number we interpret to represent groups of ten or more when the third dimension is considered (11). To directly test this interpretation, F-EGF-labeled cells were incubated at 37°C for 2.5 min and examined

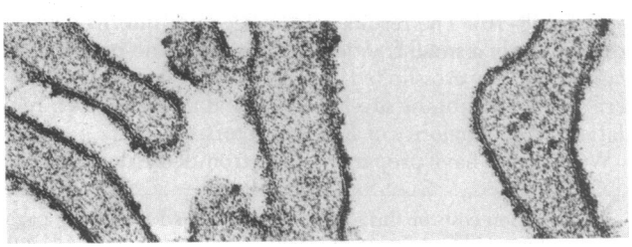

FIG. 3. A-431 cells stained with ruthenium red to reveal surface glycocalyx. Not exposed to F-EGF. (×80,000.)

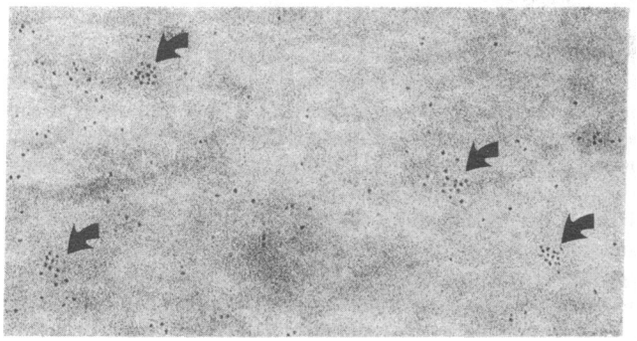

FIG. 4. *En face* view of intact A-431 cells exposed to F-EGF at 4°C and warmed to 37°C for 2.5 min. Discrete clusters of ferritin particles are observed on the plasmalemma (arrows). (×80,000.)

intact. Peripheral regions that were thin enough to permit penetration of the electron beam revealed surface-bound particles situated predominantly in clusters of ten or more (Fig. 4). In a cluster, the ferritin cores were separated by center-to-center distances of 12–16 nm, corresponding to a hexagonal close-packing pattern for the 12-nm-diameter ferritin molecules and defining an upper limit of 12 nm for the lateral dimensions (diameter) of the F-EGF·receptor complex. It also should be noted that these clusters were discrete and uniform in diameter, and are presumed to be the sites of micropinocytosis. Their relationship to the patches or caps observed in other systems (17–19) is unclear.

Internalization of the Hormone·Receptor Complex. After clustering, the F-EGF·receptor complex is taken into the cell predominantly by micropinocytosis. Macropinocytosis, which is transiently stimulated by EGF, also accounts for some uptake (20). Cytoplasmic vesicles containing F-EGF were observed within 30 sec after warming to 37°C (Fig. 1*C*). It is difficult to document the characteristic ferritin–membrane relationship through the process of micropinocytosis because the small radius of membrane curvature in 50- to 100-nm-diameter vesicles minimizes the opportunity for examining the membrane in transverse profile. In 150-nm-diameter and larger sized pinocytic vesicles, however, the ferritin was always observed associated with the vesicle membrane. These data suggest that the F-EGF·receptor complex is endocytosed intact and that the ligand is not released to lie free in the vesicle lumen.

Incorporation of the F-EGF·Receptor Complex into MVBs. Within 10 min after warming, ferritin was present in MVBs,

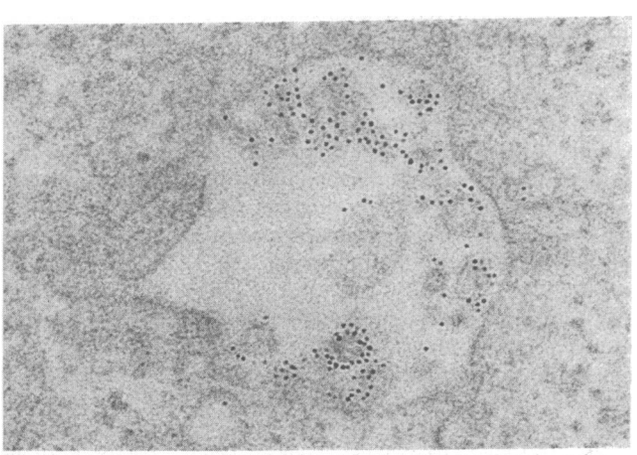

FIG. 5. A-431 cells exposed to F-EGF at 4°C and incubated at 37°C for 30 min, illustrating transport of the hormone to MVBs. F-EGF is attached to the convex surface but excluded from the lumen of MVB vesicles. (×80,000.)

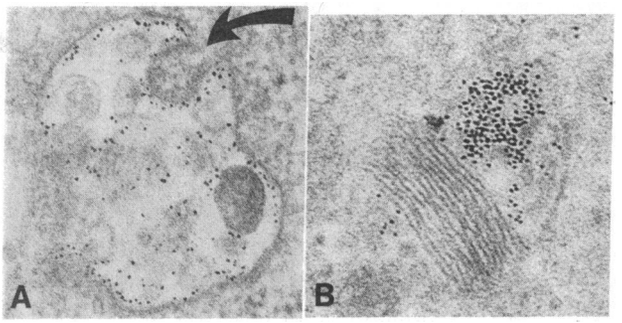

FIG. 6. (*A*) Limiting membrane of an MVB, with membrane-attached ferritin particles, captured in the process of invaginating to form a vesicle (arrow). (*B*) Typical image of a lysosome from cells treated as in Fig. 5 and fixed 30–60 min after warming to 37°C. Myelin figures and free ferritin particles are observed. (×80,000.)

which became increasingly loaded with label at the expense of the ferritin-containing vesicle population (11). Close examination revealed that the ferritin initially was not free in the lumen of the MVB but remained associated with the ES faces of both the MVB limiting membrane and MVB vesicles (Fig. 5). The characteristic 4- to 6-nm gap was apparent in transverse sections, indicating that the F-EGF·receptor complex, carried via the pinocytic vesicles, reaches the MVBs intact.

Because the attachment of F-EGF to the convex surface of vesicles inside the MVB implies that the cytoplasmic vesicles somehow evert as they enter the MVB lumen, we have searched for profiles to illustrate the events making up vesicle addition to the MVBs. Because the diameter of vesicles in the vicinity of the MVBs approximates the thickness of a thin section (50–60 nm), satisfactory transverse profiles are rare. It is possible, however, to record images suggesting the fusion of vesicles to the cytoplasmic surface of the MVB and the invagination of the MVB limiting membrane to form lumenal vesicles (Fig. 6*A*). The characteristic attachment of ferritin particles to the membrane ES face implies the persistence of the membrane-bound F-EGF·receptor complex throughout the fusion and vesiculation processes.

Lysosomal Digestion of the F-EGF·Receptor Complex. Incubation at 37°C for longer periods (30–60 min) allowed further accumulation of ferritin in multivesicular bodies. At these time points, some of the ferritin-containing cytoplasmic vacuoles and MVBs were identified as secondary lysosomes on the basis of morphological characteristics. They contained myelin figures and amorphous condensed regions resembling

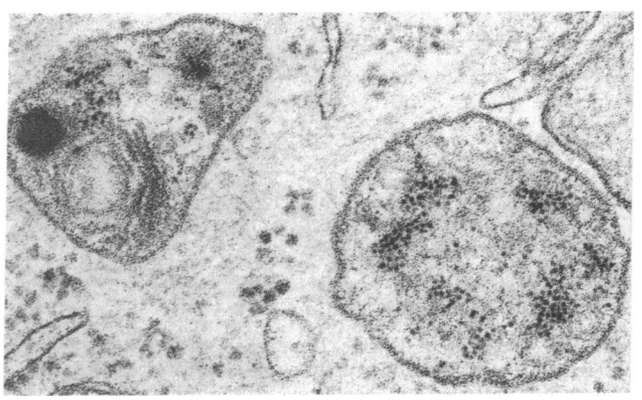

FIG. 7. Detection of lysosomal degradation in a section similar to Fig. 6*B* was improved by bismuth staining (21), which enhances ferritin density. One lysosome (left) contains a myelin figure and dense body, another (right) contains amorphous debris. Both contain free non-membrane-bound ferritin particles. (×80,000.)

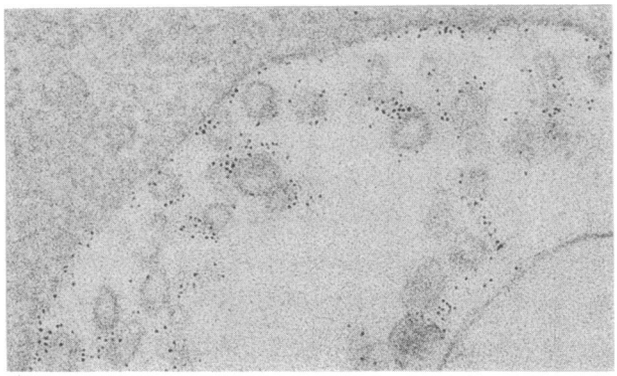

FIG. 8. Distribution of ferritin in lysosomes after 60-min incubation with F-EGF (100 nM) at 37°C in the presence of 2 mM cocaine. MVB is greatly enlarged relative to noninhibited specimens. All ferritin is membrane bound, indicating persistence of the F-EGF·receptor complex when degradation is inhibited. The population density of particles on the vesicles is approximately equal to that on the limiting membrane. (×80,000.)

lysosomal dense bodies (Figs. 6*B* and 7). In these digestive lysosomes, the ferritin was frequently observed forming pools free in the lumen. We interpret these images to indicate disruption of the F-EGF·receptor complex due to digestion of one or several components.

Lysosomal Inhibition Maintains F-EGF·Receptor Complex. To investigate further the genesis of free ferritin pools as observed in mature lysosomes after 30- to 60-min incubation at 37°C, we examined the morphologic effects of substances that have been shown to inhibit lysosomal digestion of ^{125}I-labeled EGF (4). Monolayers of A-431 cells were preincubated for 10 min at 37°C with inhibitor followed by incubation at 37°C with F-EGF plus inhibitor. In these experiments, chloroquine (2 mM), ammonium chloride (10 mM), ammonium acetate (10 mM), methylamine (30 mM), cocaine (2 mM), or lidocaine (2 mM) gave essentially identical results. Binding, aggregation, pinocytosis, and transport to the MVBs appeared to proceed normally. However, after 40- to 60-min incubation at 37°C in the presence of inhibitors, the ferritin particles were localized predominantly in MVBs, attached to the ES faces of vesicle and MVB membranes (Fig. 8); no pools of free ferritin were observed. In contrast, parallel cultures that were incubated without lysosomal inhibitors showed large pools of free ferritin indicative of F-EGF·receptor complex degradation.

A diagrammatic summary of our results is presented in Fig. 9.

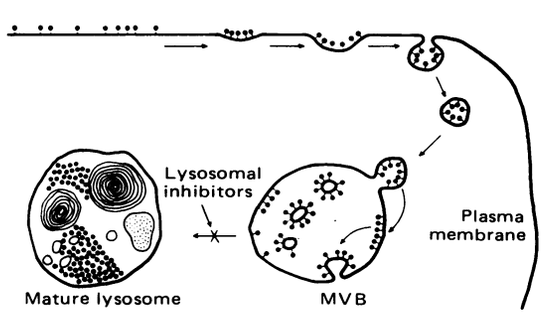

FIG. 9. Diagram of F-EGF interaction with A-431 cells. F-EGF·receptor complexes, identified by the characteristic spatial relationship of particles and membrane (4- to 6-nm separation), are apparent at initial binding and are preserved through the processes of clustering, pinocytosis, and incorporation into MVBs. Further incubation at 37°C allows disruption of the F-EGF·receptor complex (attested by pools of free ferritin), a process blocked by the presence of amines.

DISCUSSION

As documented in detail previously (11), our methods of F-EGF preparation and the specificity of EGF binding to A-431 cells provide assurance that each ferritin particle visualized at the cell surface uniquely identifies an EGF molecule specifically bound to a plasmalemmal receptor. Thin sections enable identification of the F-EGF·receptor complex; however, without supporting data, they are insufficient to define the dimensions and architecture of the EGF·receptor complex. For example, the 4- to 6-nm gap between ferritin and membrane need not necessarily contain recognition regions of the EGF or receptor molecules; the receptor conceivably could be a perpendicularly extended glycoprotein with its EGF binding site in a midmolecular region 5–15 nm from the membrane. When the *en face* views of clusters in whole mounts are taken into consideration, however, the likelihood of such an extended configuration seems small. In such images, the ferritin cores approach each other to a tightly packed configuration spaced at 12 nm center-to-center, leaving virtually no room for an intervening extended receptor. These data suggest that both hormone and receptor molecules are situated in the 1- to 3-nm space between the ferritin molecule and membrane ES face. Furthermore, the tight-packing array of the F-EGF viewed *en face* places an upper limit of 12 nm on the diameter of the EGF·receptor complex. It should be recognized, however, that our data allow no strong inferences regarding the absolute minimum dimensions of EGF·receptor complexes. Also, the clustering properties of the hormone·receptor complex in the absence of ferritin may differ considerably from those observed in the presence of a relatively huge label ($M_r > 500,000$).

The transport of intact membrane-bound F-EGF·receptor complexes into MVBs offers improved resolution of the process of MVB genesis and growth. F-EGF reaches the MVBs solely via pinocytosis; and, therefore, the presence of F-EGF bound to the MVB limiting membrane clearly shows that pinocytic vesicles do not immaculately enter the MVB lumen. Our data suggest that the pinocytic vesicles (membrane including receptors and ligand) fuse with the MVB and are incorporated into its limiting membrane, which subsequently gives rise to vesicles as illustrated in Figs. 6*A* and 9.

In previous studies, stimulation of endocytosis in the presence of extracellular tracer substances such as ferritin or horseradish peroxidase resulted in accumulation of tracer and vesicles in MVBs (22). The tracer in these experiments is usually situated free in the MVB, excluded from the vesicle lumens. Although exceptions have been described (discussed in ref. 23), these data have been interpreted to indicate a generalized scheme in which the cytoplasmic vesicles evert as the MVB limiting membrane invaginates to form MVB vesicles. In A-431 cells, we have never detected ferritin in the lumen of MVB vesicles, suggesting that all MVB vesicles in this system form by invagination of the MVB limiting membrane. Our data further indicate that the polarity of the membrane is maintained through this process. The F-EGF·receptor complex remains on the ES face even though in cytoplasmic vesicles the ES face is highly concave whereas in MVB vesicles the ES face is highly convex.

In addition, our data suggest some factors influential in determining which areas of the MVB limiting membrane will invaginate to form the vesicles. In preliminary quantitative studies using a semi-automated image analysis instrument ("MOP," distributed by Karl Zeiss), we have measured the number of ferritin particles per unit membrane length (particle density) for MVB vesicles to be seven times greater than the particle density on limiting membranes of MVBs in F-EGF-labeled cultures after 30 min at 37°C (see Fig. 5). In striking

contrast, the particle density is approximately equal on the vesicle and MVB membranes of cells incubated in the presence of ammonium chloride, ammonium acetate, or cocaine (Fig. 8). It seems likely that the anomalous distribution of receptors in inhibited MVBs is related to alterations of lysosomal structure and function. In the normal MVBs, on the other hand, we interpret the apparently greater density of F-EGF·receptor complexes on the MVB vesicles relative to limiting membranes to indicate either preferred invagination of newly fused vesicle membrane (invaginating before lateral diffusion disperses the hormone·receptor complexes in the plane of the MVB membrane) or else a preferred invagination of MVB membrane regions occupied by high concentrations (clusters) of bound ligand. The latter possibility would resemble an everted duplication of aggregation and pinocytosis at the cell surface.

Experiments at later time points provide a test of our hypothesis that ferritin observed 4–6 nm from the MVB membranes identifies specific F-EGF·receptor complexes (rather than coincidental juxtaposition due to fixation). We have reasoned that if cells labeled with F-EGF were incubated under conditions permitting lysosomal degradation of the hormone (30–60 min at 37°C), then ferritin cores might be liberated from the membranes. As shown in Figs. 6*B* and 7, pools of free ferritin (not membrane associated) appeared in cytoplasmic vacuoles containing myelin figures and dense amorphous material. We interpret these images to represent mature lysosomes containing ferritin liberated by digestion of the hormone or receptor.

The hypothesis that the F-EGF·receptor enters the MVBs intact is supported further by experiments involving ammonium and amine compounds, which have been shown to inhibit EGF degradation (4). In cells incubated 40–60 min at 37°C in the presence of inhibitors, the F-EGF is internalized and transported to MVBs, but the ferritin remains membrane associated, indicating that the F-EGF·receptor complex is protected by the presence of the amines. Such results are predictable on the basis of work by Gordon *et al.* (2), who radioautographically demonstrated accumulation of ^{125}I-labeled EGF in the lysosomes of fibroblasts incubated 2 hr with 10 mM NH_4Cl. Because our data for amine-inhibited A-431 cells were fully consistent with previous work in fibroblasts (2, 4), we attempted to reconcile our results with a conflicting report that some amine inhibitors of EGF degradation function primarily by preventing clustering and internalization of bound ligand (monitored using fluorescent derivatives of EGF) (24). We have repeated amine inhibitor experiments with human fibroblast cultures, and we find that the general mechanism of F-EGF uptake in human fibroblasts parallels that observed in A-431 cells (unpublished results). After incubation with F-EGF for 40 min at 37°C, ferritin is present in the MVBs of human fibroblasts in both the presence and absence of amine inhibitors, but the ferritin appears to remain membrane associated when the incubation is carried out in the presence of amine inhibitors of lysosomal digestion.

Although we are unable to comment at the present time on the possibilities that inhibitors increase surface binding (2) or slow the rate of clustering and internalization (24), our experiments clearly demonstrate that hormone·receptor complexes are internalized and appear to be maintained in an undegraded form in the presence of a variety of ammonium and amine inhibitors.

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