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Epidermal growth factor: Morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts

(receptors/endocytosis/autoradiography/lysosomes)

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ABSTRACT **Using a quantitative electron microscopic autoradiographic technique, we have localized the initial binding step of ^{125}I-labeled epidermal growth factor (^{125}I-EGF) to the plasma membrane of the human fibroblast. After initial binding, labeled EGF is internalized progressively by the cell in a time- and temperature-dependent fashion; when cell-associated radioactivity comes to steady state, approximately 1/3 of the autoradiographic grains are related to the plasma membrane and approximately 2/3 have been internalized. Under these conditions the internalized grains are almost exclusively related to lysosomal structures. When ^{125}I-EGF associates with the cells for 2 hr at 4° or 2 min at 37°, 34% of grains localize to coated regions of the membrane. These coated regions make up less than 2% of the membrane surface. These data directly confirm kinetic studies and suggest that saturable binding of EGF is followed by adsorptive pinocytosis and cellular degradation of the ligand and possibly its cell surface receptor.**

Epidermal growth factor (EGF) is a polypeptide that has been isolated from mouse salivary gland (1) and human urine (2). EGF has certain unique biologic effects in epidermal tissues of newborn animals, but in addition, like other so called "growth factors" (3), it is a potent mitogen for fibroblasts (4, 5). Further studies have demonstrated that EGF shares other features with the growth factors and insulin. It binds to specific plasma membrane receptors (4–6), and this is thought to be the first step in its biologic action. Recent studies have demonstrated that, when ^{125}I-labeled EGF (^{125}I-EGF) is incubated with cultured human fibroblasts at 37°, the cell-bound ligand becomes progressively less accessible to surface-active agents such as trypsin or antibodies; in addition, steady-state binding of ^{125}I-EGF can be maintained for longer periods of time and degradation can be inhibited when agents thought to inhibit lysosomal function are included in the incubation media (5). These studies suggested that, after binding, labeled EGF is internalized by the cell and undergoes degradation, possibly lysosomal (5).

By using a direct visual probe of ^{125}I-insulin binding to isolated rat hepatocytes it has been demonstrated that, after initial binding to specific plasma membrane receptors, labeled material is internalized in a time- and temperature-dependent fashion and becomes associated with lysosomes (7–9).

In the present study we have: (*i*) localized the initial binding step of ^{125}I-EGF to human fibroblasts by quantitative electron microscopic autoradiography, (*ii*) followed the fate of the labeled hormone from initial binding through steady-state binding, (*iii*) studied the effect of agents thought to inhibit lysosomal function on the distribution and localization of autoradiographic grains.

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MATERIALS AND METHODS

Reagents and Cells. Mouse-derived EGF was iodinated as previously described (5). Mouse EGF and human EGF have identical biologic properties, similar immunochemical properties, and a high degree of structural homology. The mouse-derived material is somewhat more stable under higher incubation temperatures, and steady-state binding can be maintained for a longer period than for human EGF. For the purposes of the present study we have considered the behavior of mouse-derived EGF to be a valid indicator of the behavior of human EGF (4, 5).

Incubation and Fixation Conditions. Confluent monolayer cultures of human fibroblasts containing approximately 8×10^5 cells per 6-cm culture dish were washed three times with 3-ml portions of prewarmed Hanks' solution. Cells were then incubated in the culture dish at 37° (pH 7.4) for various time periods in the presence of 1.4 ml of Dulbecco's medium containing 0.1% bovine serum albumin and 0.1 ml of ^{125}I-EGF (final concentration of 10 ng/ml). After the appropriate period of incubation, cells were washed six times with 2-ml portions of ice-cold Hanks' solution containing 0.1% bovine serum albumin and one additional time with Hanks' solution without the albumin (5).

After the wash procedure, 2.5 ml of 4% glutaraldehyde in 0.1 M Na phosphate buffer, pH 7.4, was added to the culture dish and allowed to fix at room temperature for 4 hr. The fixative was then aspirated from the dish and the cells were gently scraped from the dish with a spatula and transferred to an Eppendorf microcentrifuge tube. The contents of two culture dishes were transferred to a single Eppendorf tube and centrifuged at 12,000 rpm for 1 min (Eppendorf microcentrifuge 3200). The pellet was washed once with 0.1 M sodium phosphate buffer (pH 7.4) and then stored in 0.1 M phosphate buffer until further processed for electron microscopy.

Identical culture dishes were incubated and prepared as previously described for determination of cell-associated radioactivity (5). Nonspecific binding (cell-associated radioactivity in the presence of unlabeled EGF at 20 μg/ml) was less than 2% of the total radioactivity.

Preparation for Electron Microscopy and Autoradiography. Cell pellets were washed, dehydrated in alcohol, postfixed in osmium tetroxide, embedded, cut into 60- to 80-nm thin sections, and placed on copper grids exactly as previously described (10). The grids were coated with emulsion, incubated, developed, stained with uranyl acetate and lead citrate, and examined on a Philips EM 300 electron microscope as previously described (10). For each time point of incubation two

Abbreviation: EGF, epidermal growth factor.

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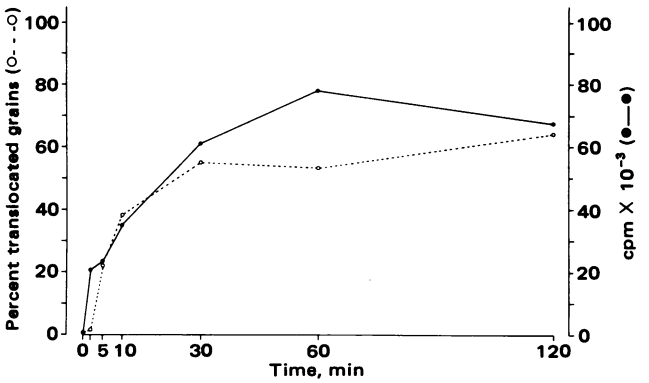

FIG. 1. Relationship of EGF binding to translocation of autoradiographic grains. The percent of translocated grains is expressed as a function of our best estimate of localization of grains to the plasma membrane. Plasma membrane localization is best represented by the localization of ^{125}I-insulin to cultured human lymphocytes at 2 min of incubation at 15°. This is almost identical to the localization of ^{125}I-EGF at 2 min of incubation at 37° (see Fig. 4 *left*). The details of the calculation are given in ref. 10.

blocks were prepared and four sections were cut from each block. Thus for each time point of incubation eight separate grids were examined and from 200 to 300 photographs were taken of developed grains. Developed grains were photographed sequentially from all cells judged to be morphologically intact.

RESULTS

Qualitative Analysis. When ^{125}I-EGF is incubated with human fibroblasts, binding is approximately 30% of maximal after only 2 min of incubation at 37°. Approximate steady-state binding is reached by 30–60 min of incubation and maintained for up to 2 hr at 37° (Fig. 1). Cells are typically well isolated and assume an elongated configuration. When cells are prepared for autoradiography, developed grains on morphologically well-preserved cells are easily detected (Fig. 2).

Quantitative Analysis. Because of the problem of radiation scatter, the apparent localization of individual silver grains has no particular meaning; a quantitative analysis is, therefore, essential. We have assumed that the earliest time point of incubation at the lowest temperature should most closely simulate the initial ligand receptor interaction. We previously established that, when ^{125}I-insulin is incubated with cultured human lymphocytes for 2 min at 15°, the distribution of autoradiographic grains is consistent with localization to specific plasma membrane receptors (8, 11). Similarly, when labeled EGF is incubated with human fibroblasts for 2 hr at 4°, the distribution of autoradiographic grains is consistent with predominant plasma membrane localization (11).

In an attempt to best estimate the source of developed grains

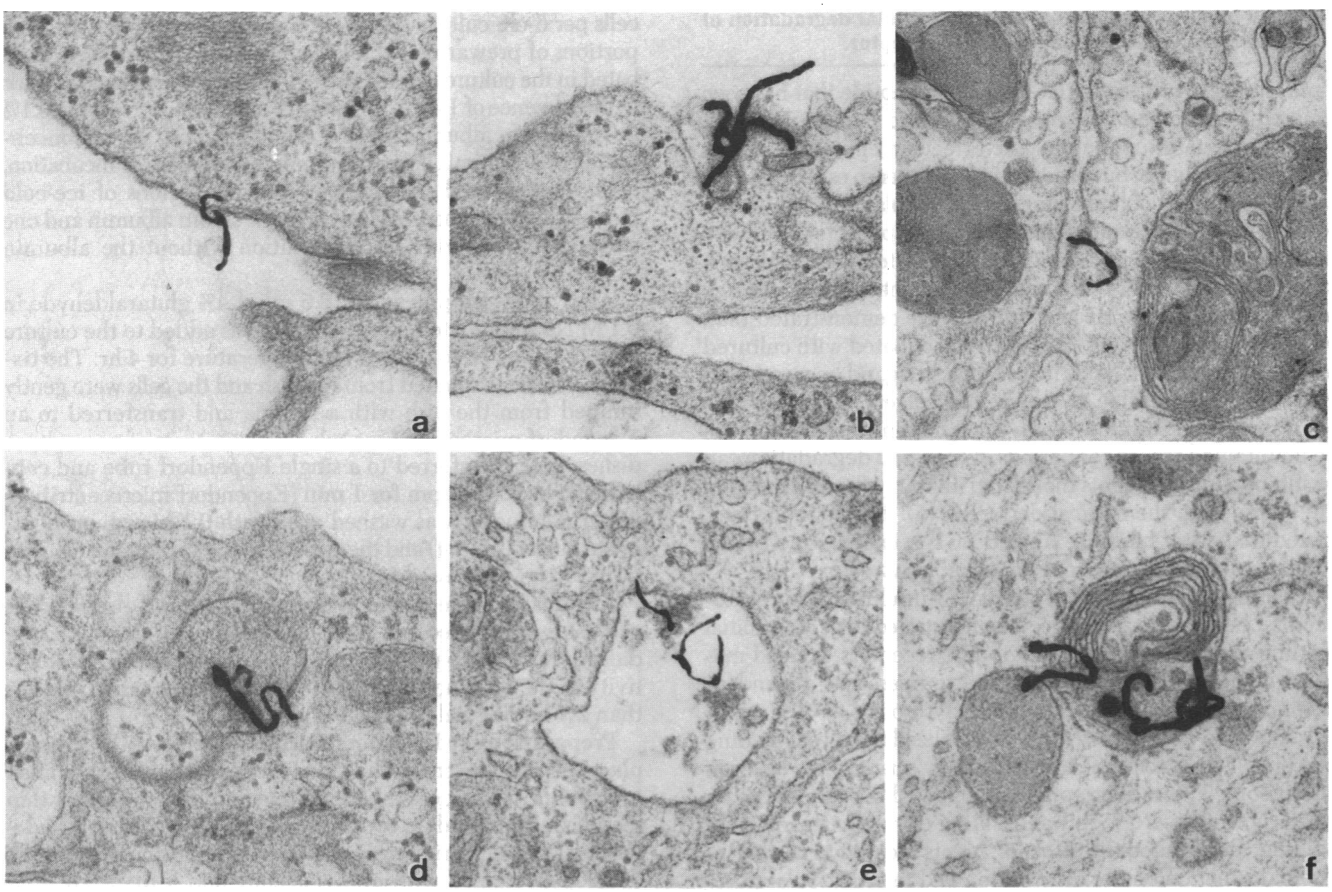

FIG. 2. Selected images of developed autoradiographic grains. (×39,000.) (*a*) Grain overlying noncoated segment of membrane. Such grains constitute 66% of grains related to the membrane (Table 2). These segments of the membrane are typically straight or slightly scalloped (i.e., noninvaginated). (*b*) Grain overlying coated segment of the membrane. Such grains constitute 34% of grains related to the membrane (Table 2). These segments are always invaginated but of variable size and shape. Note the fine filamentous material at the base of the invagination that morphologically identifies the "coat." (*c*) Grain near small noncoated invagination. These images are infrequent and constitute less than 4% of all grains related to noncoated segments of the membrane. (*d*) Grain overlying lysosomal structure that appears to be fusing with a coated vesicle. (*e*) Grains overlying multivesicular body. (*f*) Grains overlying dense body containing myelinic structures. Lysosomal structures referred to in Figs. 3 and 4 have the appearance of the structures shown in *d*, *e*, and *f*. In addition, they sometimes appear as autophagosomes.

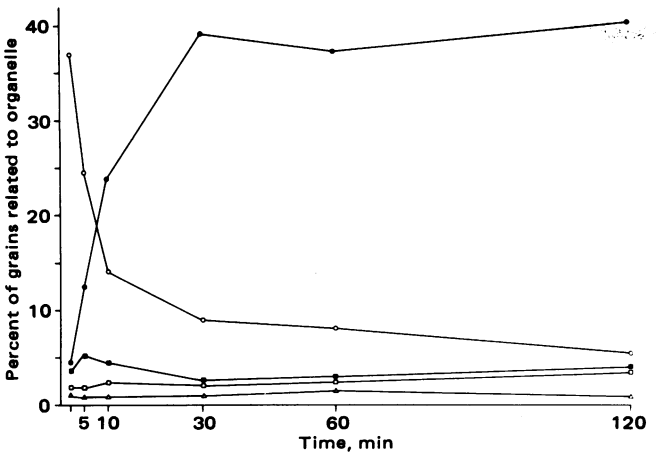

FIG. 3. Relationship of autoradiographic grains to cellular organelles. For this analysis a circle of diameter of 500 nm was superimposed and centered on each developed grain. Each structure that was encompassed by the circle was given a fraction of a point; if the circle fell on or beyond the plasma membrane this structure was scored. (For example, if a circle superimposed the plasma membrane, a lysosome, and a mitochondrion, each structure was given a score of 1/3.) The data were also analyzed by another variation of the probability circle. In this case five points were placed equidistantly around the perimeter of the circle and the circle was superimposed on grains as above. Each structure that touched a point was scored 1/5. For example, if 2 points fell outside the plasma membrane, 2 points on a lysosome, and 1 point on a mitochondrion, the score = 2/5 plasma membrane, 2/5 lysosome, and 1/5 mitochondrion. The results of both types of analysis were essentially identical. For a description of what is included as a lysosomal structure see legend to Fig. 2. ●, Lysosomes; ○, plasma membranes; ■, vesicles; □, rough endoplasmic reticulum; △, mitochondria.

resulting from the incubation of ^{125}I-EGF with human fibroblasts at 37°, we have analyzed data from various time points of incubation by two different methods.

We first used a probability circle to determine, generally, which structures were labeled (12, 13). Using this method of analysis, we found that at 2 min of incubation grains primarily localized to the plasma membrane (Fig. 3); the percentage of grains related to the plasma membrane progressively declined as a function of time until 30 min of incubation, when the effect gradually plateaued. By contrast, at 2 min there were very few grains associated with lysosomal structures, but these structures were progressively labeled until 30 min, when the effect plateaued. Very few grains were associated with rough endoplasmic reticulum or mitochondria over the entire course of incubation and there was no fluctuation as a function of time. There was a slight increase in the labeling of small vesicles from 2 to 10 min and a plateau by 30 min (Fig. 3).

When the data were analyzed according to a line source model of irradiation (i.e., plotting the distribution of developed grains as a function of the distance of the grain center from the plasma membrane), the distribution of grains at 2 min of incubation was consistent with predominant plasma membrane localization (Fig. 4) (8, 10–12). If the 2-min time point represents predominantly plasma membrane localization, it can be seen that there was a progressive internalization of labeled EGF as a function of time until about 30 min of incubation, when the effect appeared (Fig. 4 *left*) to plateau. If the distribution of grains around the lysosomal membrane, instead of around the plasma membrane, was plotted, it could be seen that grains progressively localized to lysosomal structures until 30 min, when the effect predominantly plateaued (Fig. 4 *right*).

Relationship of Internalization to Binding. As shown by two different analyses, ^{125}I-EGF binding to human fibroblasts

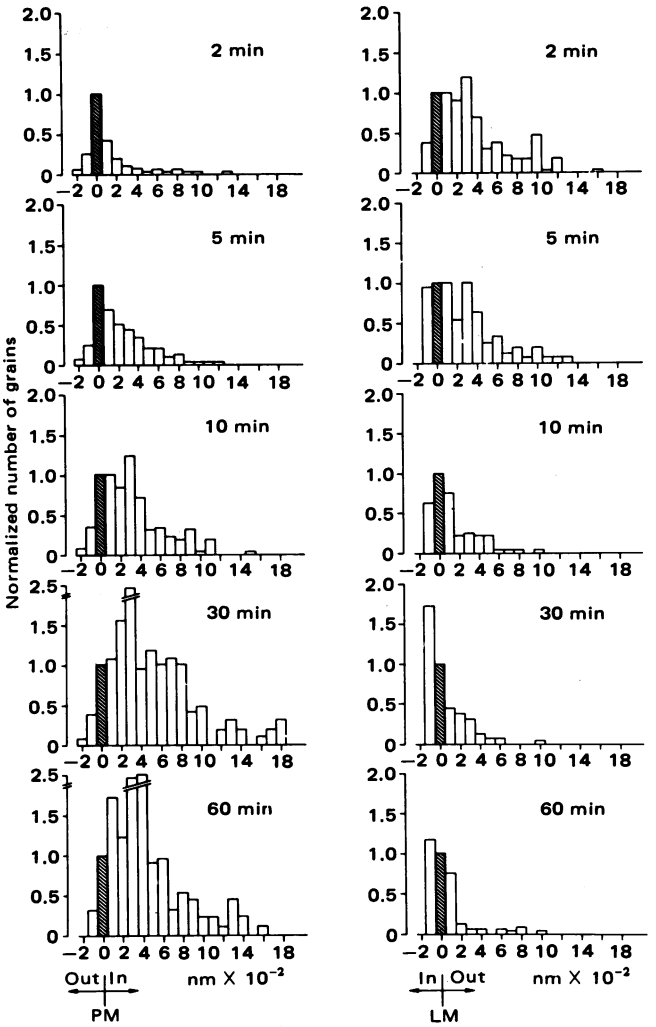

FIG. 4. Distribution of autoradiographic grains around the plasma membrane or lysosomal membrane. All incubations were at 37°. (*Left*) The normalized number of grains is plotted as a function of the distance of the grain center from the plasma membrane (PM). The 2-min time point conforms predominantly to a line source model of irradiation (8, 10–12). (*Right*) The normalized number of grains is plotted as a function of the distance of the grain center from the lysosomal membrane (LM) (see legend to Fig. 2). Beginning at 30 min, the distribution of grains conforms to a small solid disc model or hollow band source of irradiation (12).

initially localizes to the plasma membrane. With increasing time of incubation at 37°, however, there is a progressive internalization of the labeled EGF; under steady-state conditions approximately 2/3 of the labeled material is intracellular and approximately 1/3 is on the plasma membrane. The internalization process remains a constant function of binding from 2 to 120 min of incubation (Fig. 1). There is a progressive association of the internalized grains with lysosomal structures, so that, at steady state, grains predominantly localize to lysosomal structures. We find no evidence for labeling of other identifiable intracellular structures such as rough endoplasmic reticulum, mitochondria, or nuclei. Thus there is a strong positive correlation between the percent maximal binding and percent maximal translocation of grains (Fig. 1).

Cell-Associated Radioactivity in the Presence of Agents that Inhibit ^{125}I-EGF Degradation. Various amine-containing agents appear to inhibit the intracellular degradation of labeled EGF, and the most potent thus far identified is NH_4Cl (5). When 10 mM NH_4Cl is included in the incubation medium, cell-associated radioactivity is increased by about 40% after 2

Table 1. Effect of ammonium chloride on cell-associated radioactivity

Incubation	Cell-associated radioactivity, cpm	Intracellular grains, %
Without NH_4Cl	67,443	63.8
With NH_4Cl	94,364 (40.3%)*	63.7 (0%)*

Incubation was for 120 min at 37°; NH_4Cl was 10 mM when present.
* Percent increase over control.

hr of incubation (Table 1). Under these circumstances about ⅔ of the cell-associated radioactivity is intracellular and essentially all associated with lysosomes (data not shown, but essentially identical to that of figure 2 lower of ref. 11). Note, however, that the proportion of grains on the plasma membrane and intracellularly is essentially the same in cells incubated with or without NH_4Cl (Table 1). It might be expected that if the lysosome is responsible for EGF degradation and this function is inhibited by NH_4Cl, there would be an accumulation of grains intracellularly. If, however, binding is increased by the same amount that degradation is inhibited, at steady state, the proportion of grains on the membrane and intracellularly would be unchanged. This is consistent with our hypothesis that the rate of binding controls the rate of internalization, but there may be many other control points in this sequence.

Relationship of Autoradiographic Grains to Coated Regions of the Membrane. Specializations in the plasma membrane of fibroblasts known as coated pits or invaginations are identified in thin sections by a bristle-like filamentous material that covers the cytoplasmic surface of the plasma membrane (Fig. 2) (14–17). These membrane specializations containing the specific protein clathrin (18) and possibly other proteins (19) are throught to be involved in different types of vesicular transfer processes.

To determine the relationship of autoradiographic grains to coated regions of the membrane, photographs were examined from incubations carried out at 4° for 2 hr and at 37° for 2 min. Approximately ⅓ of the developed grains were associated with coated regions of the membrane (Fig. 2; Table 2). It has been reported for the cultured human fibroblast that less than 2% of the total plasma membrane comprises coated regions (15), and our findings are consistent with this observation (Table 2). On the other hand, when membrane is selected for the presence of grains, there is a 3-fold increase in the proportion of coated regions (Table 2). Thus in absolute terms there is no preferential association of grains to coated regions of the membrane (i.e., ⅔ off coated regions and ⅓ on coated), but when expressed in terms of the small proportion of membrane that is coated there is a preferential association (i.e., ⅓ of grains on about 2% of the membrane[§]). Further, there is an increased proportion of coated segments of membrane in regions selected for the presence of autoradiographic grains.

Table 2. Relationship of autoradiographic grains to coated regions of the plasma membrane

Grains*	
Associated with coated regions	34%
Unassociated with coated regions	66%
Coated membrane†	
Selected for grains	7.7%
Unselected	2.5%

* For this determination 177 photographs were selected from incubations of 4° for 120 min and 37° for 2 min. The criteria for selection were: (*i*) the grain center was ±200 nm from the plasma membrane; and (*ii*) the plane of the section was appropriate for a clear identification of the plasma membrane. If the grain center was nearest to a coated region (as shown in Fig. 2) it was assumed to have originated from a coated region, and if it was nearest to a noncoated region (Fig. 2) it was assumed to have originated from a noncoated region.
† The above 177 photographs were all taken at the same magnification. The length of the total plasma membrane on the photograph in the region of a developed grain was measured by planimetry. The portion of that membrane that was coated was also measured. The membrane length coated divided by the total membrane length equals the percent membrane coated. Other cells in the same photographs did not contain developed grains and are designated as unselected.

DISCUSSION

Quantitative electron microscopic autoradiography combined with iodinated ligand binding to specific receptors provides a direct visual probe of the events involved from the initial ligand–cell interaction through steady-state binding (7–11). Using this method and nanogram concentrations of labeled EGF, we have drawn the following conclusions from the present study: (*i*) Labeled EGF initially localizes to the plasma membrane of human fibroblasts (i.e., for 2 min of incubation at 37° or for up to 2 hr at 4°) (11). (*ii*) Grains preferentially localize to coated segments of membrane. (*iii*) After the initial binding step, labeled EGF is progressively internalized by the fibroblast in a time- and temperature-dependent fashion. (*iv*) The proportion of labeled EGF internalized is directly proportional to the proportion bound at all time points from 2 min to 2 hr at 37°. (*v*) The internalized EGF progressively associates with lysosomal structures as a function of time and temperature. (*vi*) NH_4Cl in the incubation medium increases the amount of EGF associated with the cell at 2 hr of incubation at 37°, but does not change the distribution of autoradiographic grains at this time.

These findings directly confirm the predictions Carpenter and Cohen (5) made on the basis of their binding studies.

The results of the present study are strikingly similar to results obtained using the same methodology to study insulin binding. We have observed that labeled insulin initially localizes to the plasma membrane of cultured human lymphocytes (10) and isolated rat hepatocytes (8). After binding, the labeled material is progressively internalized in a time- and temperature-dependent fashion, and in the rat hepatocyte it becomes progressively associated with lysosomes (9).

If the internalization occurs by adsorptive pinocytosis, a small transfer vesicle should be involved. While we find grains qualitatively associated with small coated or noncoated vesicles, the quantitative relationship of grains to these small vesicles is more difficult to assess. There is, however, a small increase in the labeling of these small vesicles in the early phase of EGF association to the cell (Fig. 3); during initial association approximately twice as many small vesicles could be a source of radioactivity, compared to the period of steady-state binding.[¶] We must assume, however, that at 37° this transfer process from the plasma membrane receptor to the lysosome is extremely rapid.

Internalization of the ligand could provide a mechanism for degradation of the ligand. This is further suggested by lysosomal association and inhibition of EGF degradation by agents

§ The relationship of developed grains to coated and noncoated regions of the membrane can be given as an arbitrary ratio: i.e., 34% of grains on 2% of membrane is 34/2 or 17, whereas 66% of grain on 98% of membrane is 66/98 or 0.7. Thus grains appear on coated segments approximately 17 times more frequently than would be expected from random localization.
¶ Gorden, P., Carpentier, J.-L., Cohen, S. & Orci, L., (1978) *Proceedings of the 60th Annual Meeting of the Endocrine Society*, p. 82.

thought to inhibit lysosomal function (5). NH_4Cl does not prevent the association of EGF with the lysosome, but it does appear to inhibit further breakdown. Thus for both EGF (5) and insulin (20) there appears to be a receptor-linked degradative process that proceeds by way of ligand-induced adsorptive pinocytosis (8–11). The amount of degraded hormone associated with the cell at any point in time is small for both EGF (5) and insulin (8). This must mean that degraded material is released from the cell very rapidly, and, as previously pointed out, autoradiographic grains are primarily derived from intact labeled molecules (8, 10).

Another common feature of insulin (21) and EGF (5) is that both agents induce the loss of their specific receptor. Again, as previously discussed (8), ligand-induced adsorptive pinocytosis is likely to result in the internalization of the specific receptor. The membrane vesicle containing the receptor could then be degraded by lysosomes or in some other way, or the membrane segment could be recycled and inserted back into the membrane (22). It is of note that EGF at concentrations in the low nanogram range, as used in this study, induces loss of EGF receptor in human fibroblasts (5).

Our data provide no specific information on how the binding of the ligand results in a biologic response for either EGF or insulin, but again ligand-induced endocytosis could provide a mechanism by which the specific polypeptide or its metabolic product gains access to a particular constituent of the intracellular milieu.

There are apparent analogies between the binding and internalization of EGF and low-density lipoprotein (LDL) by human fibroblasts (15–17). Both appear to bind preferentially to coated regions of the membrane, though preliminary data suggest that this binding is quantitatively more significant for low-density lipoprotein.

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