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Involvement of Multiple Subcellular Compartments in Intracellular Processing of Epidermal Growth Factor

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The intracellular translocation and processing of epidermal growth factor (EGF) in 3T3 cells has been studied utilizing Percoll density gradients. EGF is internalized and rapidly becomes associated with two types of intracellular compartments. The extent to which EGF is delivered to these two compartments is apparently regulated depending upon the cell's physiological condition. In growth medium, an increased proportion of EGF is taken up into a Golgi-like element. Uptake through this pathway correlates with a decrease in degradation of the ligand. In the absence of serum and amino acids, an increased proportion of EGF is taken up into a component which has a density of 1.05. Uptake through this pathway correlates with increased degradation of the ligand. The ligand taken up through both pathways is transferred to dense vesicles which comigrate with lysosomes. In the presence of growth medium, however, dense vesicles containing EGF can be shown to be lysosomal enzyme-deficient upon further fractionation. In addition, in the presence of serum, a portion of the internalized EGF is apparently released from the cells, intact, and then re-bound. The processes described may be important in the production of a mitogenic response and the ability of cells to self-regulate their responsiveness to the growth factor.

Key words: hormone receptors, Golgi, lysosomes, Percoll gradient, endocytosis

We have previously demonstrated that, in the presence of serum, epidermal growth factor (EGF) is bound by 3T3 cells and internalized into a Golgi-like subcellular fraction and is subsequently processed into a lysosomelike fraction [1]. A large proportion of the EGF found in this lysosomelike fraction, however, failed to be degraded. It remained in an apparently intact state within this fraction for hours. In contrast, under serum-free conditions, a large proportion of EGF was internalized into an unidentified component and subsequently processed into lysosomes. It was then degraded and released from the cells.

These results suggested that EGF is taken up by 3T3 cells through two separate pathways which are regulated by serum components or the physiological state in which growth medium maintains the cells. In addition, these results suggested that

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the lysosomelike fraction consists of at least two components: one which has the ability to degrade internalized EGF and one which cannot degrade EGF but may function in some other capacity in receptor-mediated endocytosis or transmembrane signaling.

In this report we have extented our previous observations and found that indeed EGF is processed into both lysosomes and lysosomal enzyme-deficient cellular components.

MATERIALS AND METHODS

Cell Culture

Swiss/3T3 mouse fibroblasts (passages 2–15, after obtaining from ATCC) were grown in Dulbecco's modified Eagle medium (DME-medium) containing 10% fetal calf serum (FCS, GIBCO lot 29D1112), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% CO_2 and 100% humidity.

Iodination

EGF was iodinated using chloramine-T. The 100-μl reaction mixture contained 5.5 μg EGF (receptor grade from Collaborative Research), 0.5 mg/ml chloramine-T, 1 mCi sodium iodide (^{125}I, 13–17 mCi/μg, Amersham), and 0.25 M potassium phosphate (pH 7.5). The reaction was at room temperature for 50 sec. Free iodine was removed by filtration on Biogel P-4 equilibrated with Earle's balanced salt solution (EBSS). The final specific activity of EGF was 20–150 μCi/μg.

EGF Binding

Cells grown to confluency in 60-mm dishes were washed twice with cold EBSS containing 5 mM HEPES and 0.1% bovine serum albumin (EBSS-BSA, BSA is Sigma RIA grade). The cells were then covered with 2 ml of EBSS-BSA and floated on a 6°C water bath. After 5–10 min ^{125}I-EGF was added to a concentration of 1–2 ng/ml. After 1.5 hr the dishes were placed on ice and the unbound ^{125}I-EGF was removed by three rinses with EBSS-BSA. Three milliliters of either EBSS-BSA or DME-medium containing 10% FCS which had been conditioned for 1–4 days was added to each dish and the temperature was raised to 37°C in a CO_2 incubator. At the indicated times the cultures were placed on ice, the medium was removed, and trichloroacetic acid (TCA) was added to the medium to 5%. The cells were rinsed three times with EBSS-BSA, surface-bound EGF was removed by the method of Haigler et al [2], and then the cells were solubilized in 1N NaOH. Radioactivity was measured in a Beckman Gamma 4000 gamma counter.

Percoll Gradient Fractionation

Cells were grown to confluence in 100-mm dishes. Binding and 37°C incubation were carried out exactly as detailed above for most experiments. In some experiments ^{125}I-EGF was added directly to the growth medium of the cultures and binding was carried out at 37°C in a CO_2 incubator. After the 37°C incubation the cultures were placed on ice and rinsed twice with EBSS-BSA. The cells were then scraped from the dish in 5 ml of SAT buffer (0.25 M sucrose, 10 mM acetic acid, 10 mM triethanolamine, pH 7.4) and pelleted at 800 rpm for 10 min. The cell pellet was then suspended in 1 ml of SEAT buffer (0.25 M sucrose, 1 mM EDTA, 10 mM acetic acid, 10 mM triethanolamine, pH 7.4) and lysed by pipetting 100 times with a P1000 Gilson

pipetman. Nuclei were pelleted by centrifuging for 10 min at 1,000 rpm. The postnuclear supernatant was then layered on a 9-ml suspension of 20%, 34.6%, or 45% Percoll in SEAT buffer in a 5/8 × 3-in polyallomer tube (Beckman). A gradient was formed by spinning at 16,000 rpm for 90 min in an SS34 rotor in a Sorvall RC-5B centrifuge. The gradients were fractionated from the top by injecting 70% glycerol into the bottom of the tube. The density of the fractions was determined using an Abbe refractometer.

Enzyme Assays

All marker enzymes were assayed exactly as described previously [1].

RESULTS

Figure 1 shows the sedimentation profile of ^{125}I-EGF at several time points following a temperature shift from 6°C to 37°C (see Methods). The cells used in this experiment were grown to confluency, at which time the medium was changed and conditioned for 4 days. The sedimentation profiles of marker enzymes for the various subcellular organelles were described in detail in a previous publication [1]. The arrows in Figure 1A indicate the peak fractions of markers for the following: PM, plasma membranes determined by ^{125}I-EGF binding at 0°C and cell surface iodination using lactoperoxidase; ER, endoplasmic reticulum determined by NADPH cytochrome C reductase activity; L, lysosomes determined by acid phosphatase, β-galactosidase, and β-hexosaminidase. Figure 1E shows the sedimentation profile of ^{125}I-EGF after binding at 6°C. At this temperature very little internalization of ligand occurs and it therefore is found associated with the plasma membrane fraction. Five minutes after raising the temperature to 37°C there is a dramatic shift of label from the plasma membrane fraction into two other subcellular fractions (Fig. 1D). One of these fractions has a peak density of 1.046 and cosediments with Golgi marker enzymes. The second fraction has a peak density of 1.05 and does not correspond to any of the marker enzymes for which we have tested. The extent of uptake into this fraction appears to correlate with rapid degradation of EGF and release of degradation products into the medium (see Figs. 2, 3; [1]). After 12 min at 37°C there is a further shift of count out of the plasma membrane region with corresponding increase in Golgi-like elements (Fig. 1C). There is no apparent increase or decrease in the heterogeneous component with a peak density of 1.05 but label has begun to appear in the lysosome region of the gradient. By 30 min at 37°C the majority of cell bound ^{125}I-EGF cosediments with lysosomal enzyme markers (Fig. 1B).

The uptake of EGF by two separate pathways as described above and in the Introduction was studied under two physiological conditions. In this experiment cells were grown to confluence, at which time fresh growth medium was added to the cultures. On the following day ^{125}I-EGF binding was carried out at 6°C and unbound ligand was removed. The cells were then covered with conditioned growth medium (DMEM-FCS) or EBSS-BSA and the temperature was shifted to 37°C. The cells were processed for Percoll gradient analysis at the times indicated (Fig. 2). For analysis the gradients were divided into four regions as indicated in Figure 1A. Region I is from the top of the gradient to a density of 1.045 and includes plasma membrane fractions and any EGF which dissociates from the plasma membrane during preparation. Region II is from densities 1.045–1.049 and includes the Golgi elements. Region III is from densities 1.049–1.062 and includes the heterogeneous

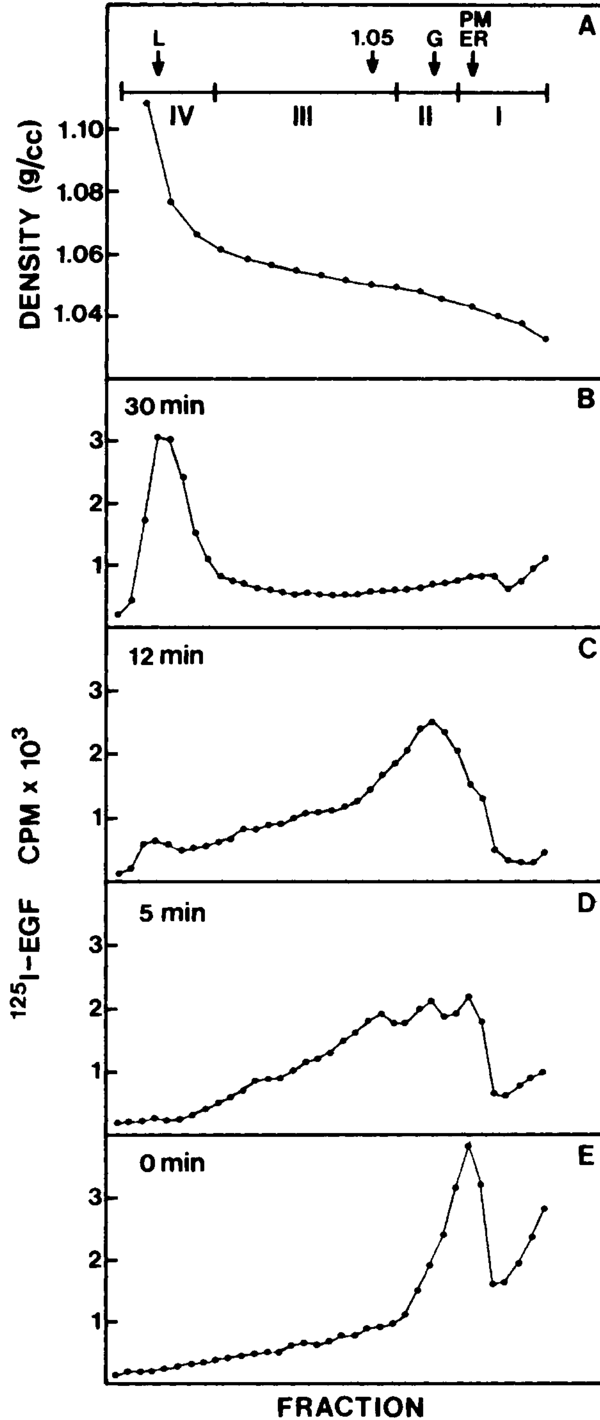

Fig. 1. Subcellular distribution of ^{125}I-EGF on Percoll density gradients. Swiss/3T3 cells were incubated at 6°C in the presence of ^{125}I-EGF for 1.5 hr. Unbound EGF was then removed, the cells were covered with growth medium (conditioned for 4 days), and the temperature was raised to 37°C. The postnuclear lysate was fractionated on Percoll gradients (see Methods). A) Density, arrows indicate the peak fraction of marker enzymes for plasma membrance (PM), endoplasmic reticulum (ER), Golgi apparatus (G), and lysosomes (L). Minutes after shifting the temperature to 37°C: (B) 30 min, (C) 12 min, (D) 5 min, and (E) 0 min.

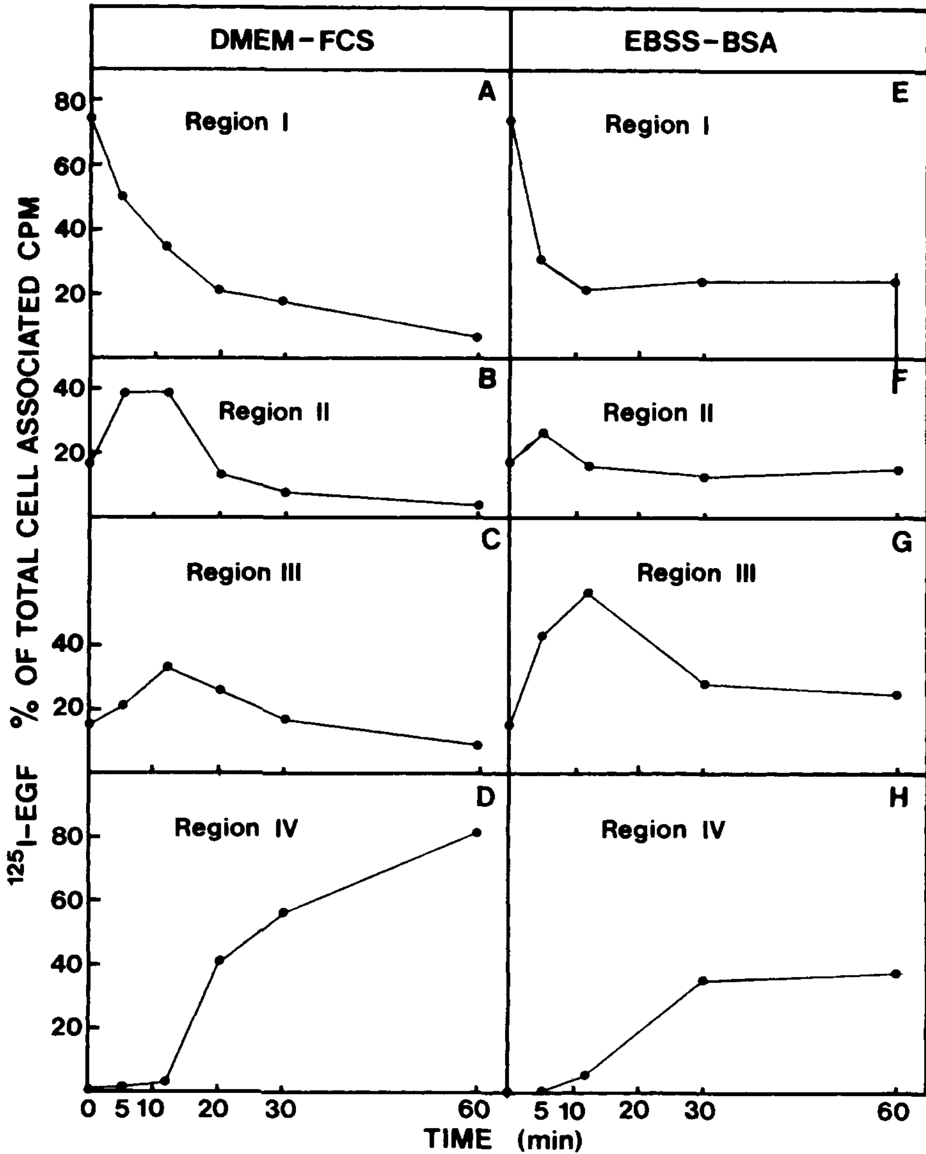

Fig. 2. Time course of ^{125}I-EGF association with subcellular fractions. Cells were incubated at 6°C in the presence of ^{125}I-EGF. Unbound EGF was removed, the cells were covered with either 1-day conditioned growth medium (A–D) or EBSS-BSA (E–H), and the temperature was raised to 37°C. For analysis the gradients were divided into four regions as shown in Fig. 1A. Region I includes plasma membranes and any EGF which dissociates from the plasma membrane during preparation. Region II includes Golgi elements. Region III contains an unidentified component with which a portion of internalized EGF becomes associated (see text). Region IV includes lysosomes.

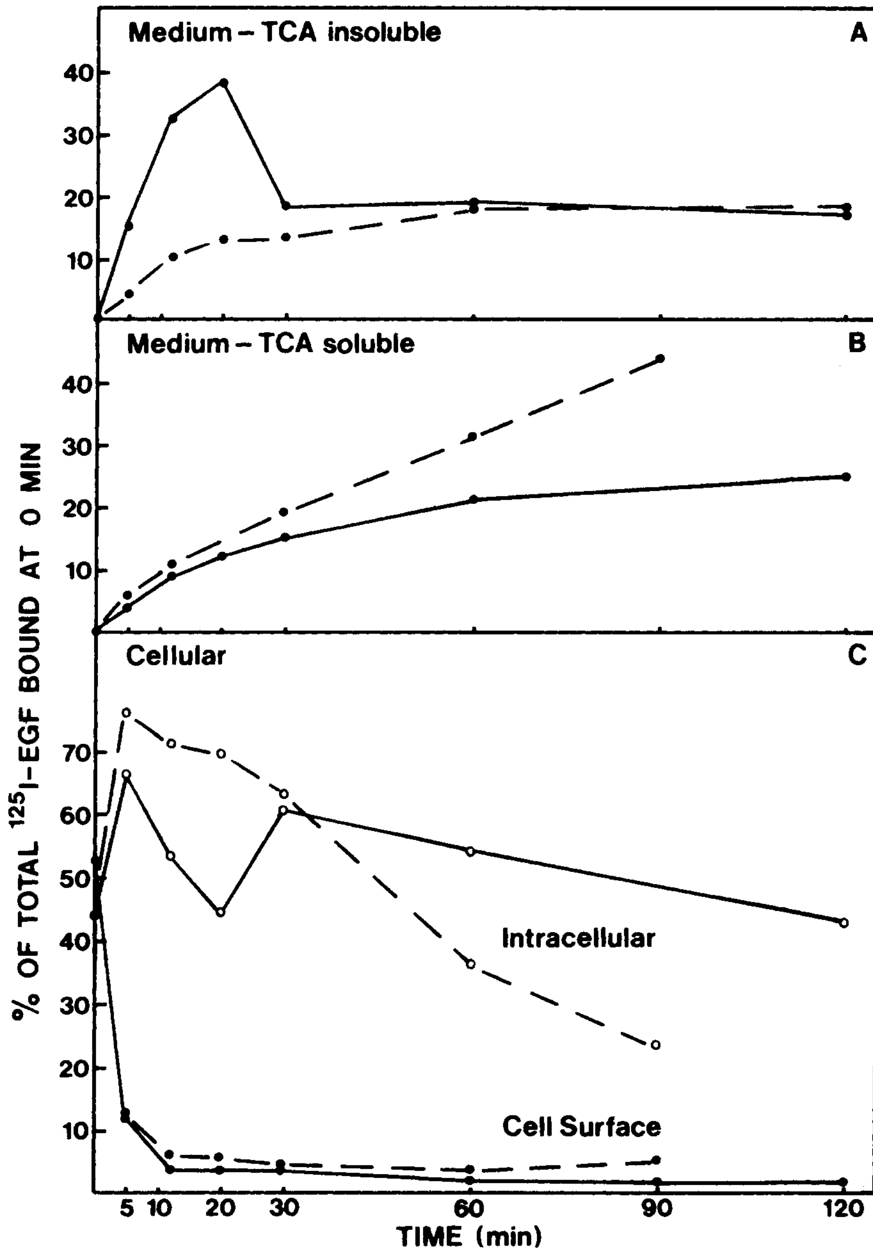

Fig. 3. ^{125}I-EGF internalization, dissociation, and degradation. Binding was carried out at 6°C for 1.5 hr. Unbound EGF was then removed, the cells were covered with either 1-day conditioned growth medium (—) or EBSS-BSA (---), and the temperature was raised to 37°C. At the indicated time, the medium was removed and TCA was added to the medium to 5%. The cells were thoroughly washed and surface bound EGF was separated from internalized EGF by the method of Haigler et al [2]. A) TCA-insoluble counts in the medium. B) TCA-soluble counts in the medium. C) Intracellular counts (○) and cell surface counts (●).

component with peak density at 1.05. Region IV is from a density of 1.062 to the bottom of the gradient and includes lysosomes. This analysis is not absolute since there is obvious overlap between the various fractions, but it provides a useful indication of time and extent of processing of EGF through the various cellular components. As shown in Figures 2A,E, under both incubation conditions, count in region I is maximal at 0 min and declines rapidly during the first 10 min at 37°C. This loss of count in the plasma membrane fractions correlates with the appearance of ^{125}I-EGF in regions II and III (Fig. 2B,C,F,G). The extent of uptake into these two components varies with the incubation conditions. In the presence of growth medium (Fig. 2B,C) uptake of EGF into region II, which contains the Golgi apparatus, is increased. While in the presence of EBSS-BSA, which is an extreme case of serum and amino acid deprivation (Fig. 2F,G), uptake into region III is accentuated. Loss of EGF from both region II and region III appears to correlate with the appearance of count in region IV, which includes lysosomes (Fig. 2D,H). Our previous work [1] has shown that in the presence of serum, EGF remains associated with this fraction for hours in an apparently intact form. In EBSS-BSA, however, EGF is rapidly lost from this fraction due to degradation (see Fig. 3).

The cellular and extracellular pools of ^{125}I-EGF from a similar experiment are shown in Figure 3. In the presence of both growth medium and EBSS-BSA, EGF is rapidly lost from the cell surface following the temperature shift to 37°C (Fig. 3C). This loss is due largely to internalization (Fig. 3C) and, to a lesser extent, dissociation into the medium (Fig. 3A). In the presence of EBSS-BSA, the loss of intracellular ^{125}I-EGF corresponds to the appearance of TCA-soluble count in the medium (Fig. 3B). The appearance of TCA-soluble material in the medium is much retarded in growth medium (Fig. 3B) while nearly 50% of the ^{125}I-EGF bound at 0 min remains inside the cells after 2 hr (Fig. 3C). This intracellular EGF is greater than 90% intact as determined by gel filtration on Sephadex G-75 [1]. The appearance of TCA-insoluble ^{125}I-EGF in the presence of growth medium (Fig. 3A) seems somewhat anomalous. A rapid increase in TCA-precipitable EGF over the first 20 min (Fig. 3A) after the shift to 37°C correlates with a decrease in intracellular ligand (Fig. 3C). This suggests the possibility that, in the presence of serum, a portion of bound EGF is internalized and subsequently released into the medium and re-bound. A more detailed analysis is needed to prove such a phenomenon, but it is interesting to note that Tolleshaug et al [3] have observed a similar endocytic pathway for asialotransferrin in rat liver in vivo, which they term diacytosis.

The results detailed above and in our previous work [1] show that, in growth medium, EGF is processed into a subcellular compartment which cosediments with lysosomal enzyme markers. However, only a portion of the ligand in this fraction is degraded and released. This suggests a degree of heterogeneity within this fraction due to the presence of nonlysosomal organelles or the presence of lysosomes which are unable to degrade EGF. To further explore these possibilities we fractionated ^{125}I-EGF-treated cells on Percoll gradients designed to separate vesicles in the density range of lysosomes. In the experiment shown in Figure 4, 3T3 cells were incubated in conditioned growth medium in the presence of 2 ng/ml ^{125}I-EGF for 3 hr at 37°C. The cell lysate was divided and analyzed on Percoll gradients of two different starting densities (see Methods). Figure 4D shows the profile of EGF in a gradient identical to those in Figure 1. The majority of count cosediments with lysosomal enzyme markers. However, on a denser gradient the same sample is fractionated into at least three components (Fig. 4C). Furthermore, the profile of ^{125}I-EGF does not corre-

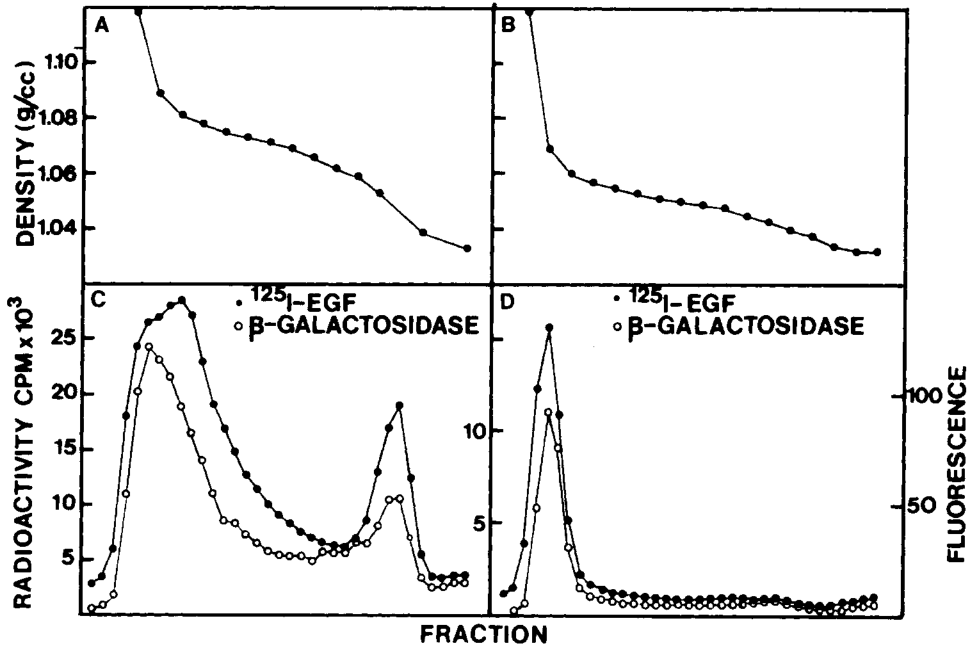

Fig. 4. Distribution of subcellular fractions containing EGF on Percoll gradients of different densities. ^{125}I-EGF was added directly to the growth medium and the cells were incubated at 37°C for 3 hr. The cells were lysed and the sample was divided (3:1) and layered on two different gradients. A and B) Density. C and D) ^{125}I-EGF (●) and β-galactosidase (○).

spond to that of lysosomal enzymes. In particular, a component between densities 1.075 and 1.090 is clearly separated from the peak of lysosomal enzyme activity. In an attempt to improve the resolution of these components a third, denser gradient was utilized. Figure 5B shows the distribution of radioactivity after incubation of cells for 3 hr at 37°C with labeled ligand. There is clear separation of vesicles containing EGF (Fig. 5B) from the peaks of lysosomal enzyme activity (Fig. 5C).

DISCUSSION

An endocytic pathway in which EGF binds to receptors on the cell surface followed by clustering of receptor-ligand complexes, formation of endocytic vesicles, lysosomal fusion, and degradation has been well documented [4–9]. Additionally, we have provided evidence for a second intracellular pathway for EGF processing which involves uptake into Golgi-like elements [1]. The data described here further document the existence of multiple intermediate states of EGF processing and uptake into lysosomal enzyme-deficient vesicles.

EGF is simultaneously transferred from the plasma membrane into at least two intracellular components which differ in density (Fig. 1D,E). One of these components comigrates with the Golgi apparatus and seems to correlate with processing through a nondegradative pathway (Figs. 2,3). The second component does not cosediment with any marker enzyme which has been tested but does correlate with rapid degradation of the ligand (Figs. 2,3). Furthermore, the extent of uptake into

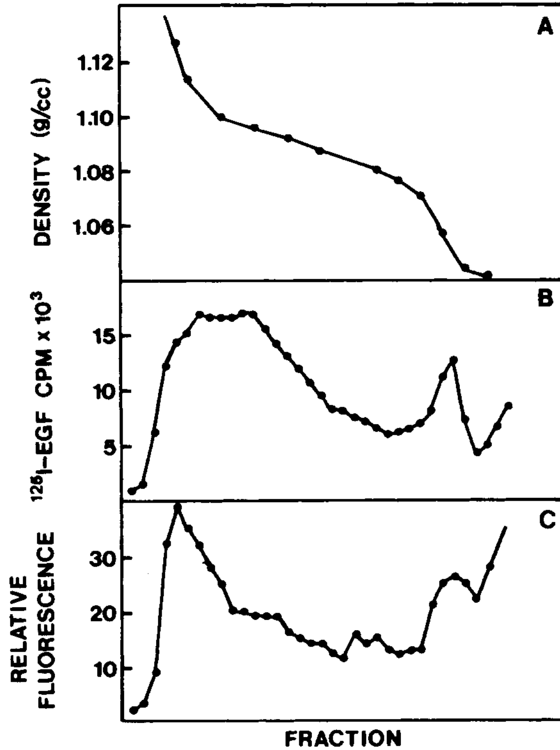

Fig. 5. Distribution of EGF-containing vesicles and lysosomes on a dense Percoll gradient. Experimental details are the same as Figure 4 except that a denser gradient was utilized to improve the resolution of the various components. A) Density. B) ^{125}I-EGF. C) β-Galactosidase.

these components is apparently regulated according to the physiological state of the cell (Fig. 3). Uptake into a Golgi-like component is enhanced under conditions which can fully support cell growth. Uptake through a pathway which leads to rapid degradation is enhanced under conditions of serum or amino acid deprivation.

We have also demonstrated in this report that in the presence of growth medium, EGF is delivered into a lysosomelike but enzyme deficient compartment (Figs. 4,5). Under several fractionation conditions, components carrying internalized EGF are clearly separable from the peaks of lysosomal enzyme activity. We are attempting to morphologically identify and evaluate the physiological role of these structures. One possibility is that this component represents a prelysosomal compartment en route to lysosomal fusion. A second possibility is that these vesicles play a more direct role in the production of EGF's mitogenic signal. Interestingly, Khan et al [10, 11] have recently reported the uptake of insulin into purified rat liver Golgi fractions and have observed a transfer of ligand from Golgi vesicles of low density to Golgi-like, nonlysosomal elements of high density. This is an interesting parallel to the results described here for EGF and suggests a similar intracellular pathway for both ligands.

Finally, our results suggest that, in the presence of growth medium, a portion of the internalized EGF may be subsequently returned to the medium, intact, and rebound by the cell. Such a pathway has been described for asialotransferrin in rat

liver [3]. Further work is necessary to establish the existence of this phenomenon for EGF and its relationship to the interesting possibility that it is correlated with the intracellular pathways described above and mitogenic activity of the ligand.

Thus, it is becoming apparent that the intracellular processing of EGF is much more complex than previously appreciated. The uptake of EGF by various pathways and the delivery of the ligand to multiple destinations suggests interesting possibilities for the mechanism by which a mitogenic signal is produced and the means by which cells self-regulate their responsiveness.

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