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Intracellular Processing of ^{125}I-Epidermal Growth Factor in Rat Embryo Fibroblasts

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The intracellular fate of endocytosed ^{125}I-epidermal growth factor was examined in Rat-1 fibroblasts. Cells were pulse-labeled for 5 min in ^{125}I-EGF and chased for 3 hr with an excess of unlabeled EGF. At various times after application of the cold chase, cells were harvested and processed for isopycnic gradient centrifugation on Percoll gradients. Within the period of the ^{125}I-EGF pulse, about 50% of the ^{125}I activity appeared in an organelle containing peak in the gradients. By 20 min after application of the cold chase, ^{125}I activity in the organelle peak began to decrease, and the decrease continued over the next few hours. The ^{125}I activity which exited from its organelle-associated location appeared to be present in the cytosol and was apparently not confined within organelles. Lysosomotropic amines inhibited the egress of ^{125}I activity from the organelle compartment. The ^{125}I activity from both organelle and nonorganelle compartments reacted as completely as authentic ^{125}I-EGF with anti-EGF antibodies and was similar in size to authentic ^{125}I-EGF. Little or no intracellular low molecular weight ^{125}I-containing compounds were detected, although they accumulated in the culture medium. Analytical isoelectric focusing revealed that the organelle-bound form of endocytosed ^{125}I-EGF was more acidic than authentic ^{125}I-EGF and, upon exiting from the organelle compartment, was processed to an even more acidic form. It was the second macromolecular form of processed ^{125}I-EGF that was ultimately degraded to low molecular weight compounds which were then externalized from the cells.

Key words: epidermal growth factor, intracellular processing, endocytosis, lysosomes, degradation, internalization.

When cultured cells are exposed to epidermal growth factor (EGF), DNA synthesis is induced after a lag of approximately 12 hr [1–3]. EGF labeled with fluorophores or ^{125}I has been employed as a probe in an attempt to understand the relationship between cellular binding and processing of EGF on the one hand and its biological activity on the other [see 4 for review]. Within 5 min after binding to high-affinity receptors situated on the cell surface, the EGF and its receptors undergo clustering and are ultimately endocytosed within vesicles. Within 30–45 min after exposure to cells, the ^{125}I-EGF is degraded, as determined by the release of ^{125}I-tyrosine into the culture medium. The ability of a variety of lysosomotropic amines to inhibit liberation of ^{125}I-tyrosine into the culture medium has suggested the involvement of lysosomes in the degradation process [5]; however, the accumulation of ^{125}I-EGF in lysosomes has not been demonstrated.

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The relationship of EGF binding, internalization, and degradation to the biological action of EGF remains unknown. It has recently been demonstrated that lysosomotropic amines, which inhibit lysosomal function by altering the intralysosomal pH [6], also inhibit EGF-stimulated mitogenesis [7,8] and induction of ornithine decarboxylase activity [9]. One explanation for these phenomena is that degradation of EGF or its receptor is necessary for the biological action of EGF.

In this paper we report the results of experiments in which cell homogenates have been separated on isopycnic gradients of Percoll at times after binding of ^{125}I-EGF to rat fibroblasts. When analyzed by gel filtration and isoelectric focusing, the ^{125}I-EGF was processed intracellularly into at least two successive products which were similar in size and immunoreactivity to EGF but which were more acidic. The first appearing of these processed products was present in an organellar fraction and was further processed to an even more acidic molecule which appeared not to be compartmentalized within a cellular organelle. There was little or no detectable intracellular ^{125}I-tyrosine, which did accumulate in the culture medium as the intracellular derivatives of ^{125}I-EGF decreased. The identification of these processed species of ^{125}I-EGF, coupled with their distinct intracellular compartmentalization, makes them attractive candidates as intracellular modulators of EGF activity.

MATERIALS AND METHODS

Cell Culture

The Rat-1 line used in these experiments refers to the F-2408 established line of Fischer rat embryo fibroblasts [10]. Cells were propagated in DMEM containing 10% calf serum (KC Biological Inc, Lenexa, Kansas) and 2% newborn calf serum (Biocell, Carson, California) at 37°C in a humidified 5% CO_2/95% air atmosphere.

Epidermal Growth Factor

EGF was prepared from mouse submaxillary glands as described by Savage and Cohen [11] and further purified to a single peak by high performance liquid chromatography [12]. The ultrapure EGF was iodinated using Na^{125}I and chloramine T [5]. The specific activity obtained was approximately 0.5m Ci/μg.

Isopycnic Centrifugation of Cell Extracts

Cell monolayers were incubated in binding medium (DMEM containing 1 mg/ml bovine serum albumin) containing ^{125}I-EGF as described in figure legends. Following incubation, culture dishes were rinsed six times in ice-cold Hank's buffered salt solution (HBSS) containing bovine serum albumin (1 mg/ml). Cells were scraped into 10 ml HBSS and pelleted at 800g for 5 min. Hypotonic buffer (1.3 ml; 1 mM $MgCl_2$, 0.4 mM $CaCl_2$, 0.5 mM dithiothreitol, 10 mM phenylmethyl sulfonyl fluoride, 25 mM Tris-HCl, pH 7.9) was added to the pelleted cells which were allowed to swell at 4°C for 15 min. The swollen cells were disrupted by 20 strokes of "B" pestle in a Dounce homogenizer (Kontes Glass Co, Vineland, New Jersey). The nuclei and undisrupted cells were pelleted by centrifugation at 2,000g for 10 min. Approximately 80–90% of the ^{125}I activity was recovered in the supernatant which was withdrawn and made 0.25 M in sucrose by addition of 2.5 M sucrose.

Cell disruption by homogenization in hypotonic buffer, as described above, was employed in all the experiments described in this paper. Cell disruption with a

nitrogen cavitation apparatus (Kontes), 100 psi for 30 min at 4°C in 0.25 M sucrose, produced similar Percoll gradient profiles as those obtained after hypotonic swelling of cells (data not shown). The organelle peak consistently appeared at fraction 17 or 18, and there was a similar flux of ^{125}I activity from the organelle peak to a nonsedimenting soluble form after incorporation of ^{125}I-EGF into the cells. The disadvantage of the nitrogen cavitation device is that samples must equilibrate under high nitrogen pressure for 30 min, and multiple samples representing time points could not be processed simultaneously. Similar results were also obtained by disrupting cells with several cycles of freezing-thawing or with sonication, although these two methods resulted in much organelle damage as evidenced by release of acid phosphatase. Furthermore, there was poor recovery of the acid phosphatase activity, much of which appeared in the nuclear pellet.

One milliliter of the ^{125}I-containing supernatant was carefully layered on 9.5 ml of a 21% Percoll solution (Pharmacia Fine Chemicals, Piscataway, New Jersey), in 0.25 M sucrose, in 16 × 76 mm centrifuge tubes. Tubes were centrifuged at 18,000 RPM (22,000g) in a type 40 angle rotor (Beckman Instruments, Fullerton, California) for 2 hr at 4°C. The $\omega^2 t$ for each run was 2.52×10^{10} rad^2 sec^{-1}. Gradients were fractionated volumetrically into 0.4-ml fractions by introducing mineral oil through the top of the stoppered gradient tubes in a Repipet (L/I Labindustries, Berkeley, California) and pumping the fraction through a needle inserted through the stopper to the bottom of the gradient. ^{125}I activity was measured in a Tracor model 1140 gamma counter.

The densities of the Percoll gradient fractions were determined with Pharmacia density-gradient beads. The accuracy of the beads were checked by 1) determination of refractive indices of gradient fractions with a Bausch and Lomb refractometer, and 2) gravimetric analysis of 100-μl samples using a Kahn microbalance.

Gel Permeation Chromatography

Samples containing ^{125}I activity were applied in a volume of 0.5 ml or less onto columns of Sephadex G-25 (0.9 × 25 cm) or Sephadex G-75 (0.9 × 50 cm) equilibrated with HBSS. Columns were eluted with the same buffer.

Affinity Chromatography of ^{125}I Activity on Anti-EGF Columns

Rabbit antiserum to mouse EGF was prepared as described [13]. Ten milliliters of antiserum was passed through a 1-ml column containing 1 mg HPLC-purified EGF covalently attached to Affigel 10 (Bio Rad Laboratories, Richmond, California) according to the manufacturer's instructions. After extensive washing of the column with HBSS, the bound protein was eluted with 4 M guanidine. Following dialysis of the eluted anti-EGF antibody against HBSS, the antibody was coupled to 1 ml Affigel 10 according to the manufacturer's instructions.

Percoll gradient fractions of ^{125}I-labeled cell extracts were acidified by addition of 1/10 volume 1 N HCl. Five minutes later the material was neutralized and passed through the anti-EGF column. After the column had been rinsed with 20 volumes of HBSS containing bovine serum albumin, the bound ^{125}I activity was eluted with 4 M guanidine in 1-ml fractions. Peak ^{125}I-containing fractions were selected and dialyzed against H_2O using 3,500 MW cutoff dialysis membrane (Spectrum Industries, Los Angeles, California).

DEAE-Sepharose Chromatography

^{125}I-containing material was added to five volumes 0.02 M ammonium acetate buffer, pH 5.6. The material was then applied to a 1-ml column of DEAE-Sepharose which had been equlibrated with the same buffer. The column was rinsed with 5 ml of the same buffer followed by 7 ml of the buffer made 0.05 M in ammonium acetate and with 7 ml of the buffer containing 0.2 M ammonium acetate. One-milliliter fractions were collected during column elution.

Isoelectric Focusing

Isoelectric focusing was performed in slab gels, 145 × 205 × 1.5 mm, containing 1% (w/v) agarose (IsoGel, FMC) 12.5% (w/v) sucrose, 1% (w/v) Bio-Lyte 3/5 (BioRad Laboratories), 0.4% (w/v) Bio-Lyte 5/7, and 0.6% (w/v) Bio-Lyte 3/10. The anode electrode wick was soaked in 0.1 M H_3PO_4 and the cathode electrode wick in 1 M NaOH. The samples were either in double-distilled water or 2% (w/v) ampholytes, and were applied as droplets on the gel surface near the acidic wick. Focusing was at 10 W for 3.5 hr at 2°C. A micro-pH probe (Microelectrodes, Inc) was used for pH measurements in the cold gels. The gels were dried on a 80°C hot plate without prior fixation. The radioactive proteins were located by autoradiography with Kodak X-Omat AR film and Radelin T2 x-ray intensifying screens.

Acid Phosphatase Determination

To 0.15 ml of each Percoll gradient fraction was added 0.6 ml substrate buffer which contained 0.6 mM p-nitrophenyl-phosphate, 0.17% Triton X-100, and 0.07 M sodium acetate, pH 5.0. Samples were incubated at 37°C for 1 hr. One milliliter of 50 mM NaOH was added, and the samples were centrifuged at 1,500g and the supernatants decanted. To each supernatant was added 2 ml alkaline buffer (0.133 M glycine, 0.083 M Na_2CO_3, 0.067 M NaCl, pH 10.7). Absorbance was measured at 400 nm.

UDP-Galactosyl Transferase Activity

UDP-galactosyl transferase activity was measured as described [14], utilizing 0.1 ml of each Percoll gradient fraction in a total incubation mixture volume of 0.624 ml.

RESULTS

Subcellular Fractionation of Cells Following Incubation With ^{125}I-EGF After Cold-Chase Experiments

Culture dishes of Rat-1 fibroblasts were preincubated in binding medium (serum-free medium plus 1 mg/ml serum albumin) for 30 min in a humidified CO_2 incubator in order to achieve temperature and pH equilibration. At the end of that time, ^{125}I-EGF (10 ng/ml) was added in a small volume to each dish in the incubator to permit binding to cell surfaces. Five minutes later a 200-fold excess of unlabeled EGF was added in a small volume to serve as a cold chase. In a separate experiment, we determined that this concentration of unlabeled EGF reduced the binding of ^{125}I-EGF to less than 1% of that which occurred in its absence. At time intervals after addition of the unlabeled EGF, culture dishes were removed for harvesting and subcellular fractionation. Following cell disruption and low-speed centrifugation to remove nuclei

and undisrupted cells, each resulting supernatant was layered on top of a Percoll gradient for isopycnic centrifugation of organelles.

The ^{125}I activity in the Percoll gradients was confined primarily to a major peak located at density 1.038 and to a nonsedimenting portion that remained on top of the gradient (Fig. 1). The peak of ^{125}I activity at density 1.038 corresponded to the peak of acid phosphatase activity, a lysosomal marker enzyme, and UDP-galactosyl transferase, a marker for Golgi vesicles (Fig. 2). For ease of reference the peak at density 1.038 will be heretofore referred to as the organelle peak.

As early as 5 min after addition of ^{125}I-EGF to the culture medium, ^{125}I activity was distributed in the organelle peak and in the uppermost fractions of the gradient (Fig. 1A). Five minutes after addition of unlabeled EGF, the organelle peak still contained an amount of ^{125}I activity equivalent to that on top of the gradient (Fig. 1B), but by 20 min after addition of the cold EGF chase the proportion of total ^{125}I activity present in the organelle peak began to decrease (Fig. 1C) and continued to decrease over the next several hours (Fig. 1D–F). As the ^{125}I activity in the organelle

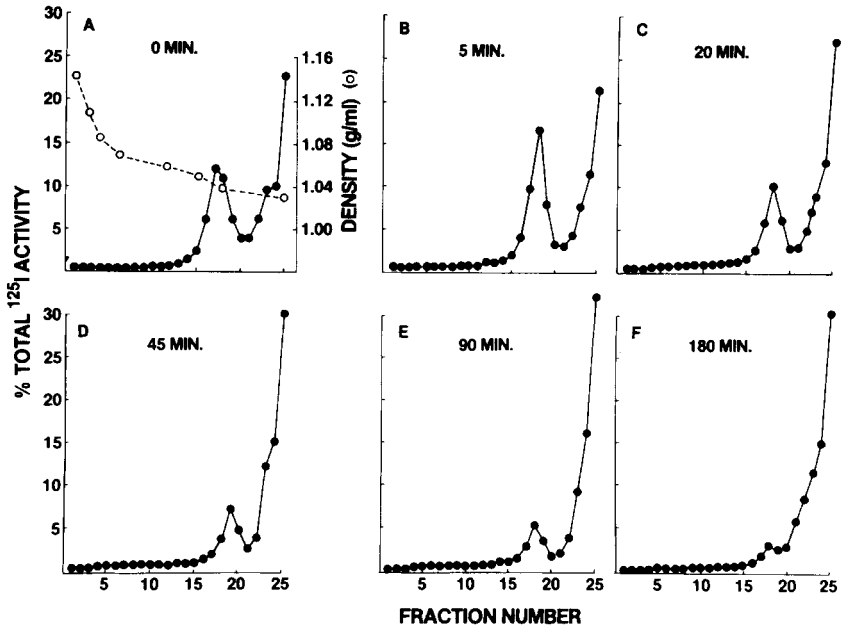

Fig. 1. Pulse chase of ^{125}I-EGF in Rat-1 cells analyzed by isopycnic centrifugation on Percoll gradients. Rat-1 fibroblasts were plated into 10-cm culture dishes and cultured for 2 days to yield 10^7 cells per dish at the time of the experiment. Cells were rinsed once in serum-free medium and incubated in 5 ml binding medium (serum-free DMEM plus 1 mg/ml serum albumin) at 37°C in a humidified CO_2 incubator for 30 min to achieve equilibration of temperature and pH (7.2). At that time, 50 ng ^{125}I-EGF in 100 μl binding medium were added to each culture dish. After 5 min of incubation (the interval of the ^{125}I-EGF pulse), 10 μg unlabeled EGF (as the cold chase) was added in 25 μl binding medium to each culture dish (T = 0). At T = 0 and at times up to T = 180 min, after application of the cold chase, dishes were harvested and processed for Percoll gradient centrifugation as described in Materials and Methods. Panels A through F represent Percoll gradient profiles of ^{125}I activity at times after addition of the cold EGF chase. The density of a parallel gradient containing density-gradient marker beads is also shown in panel A.

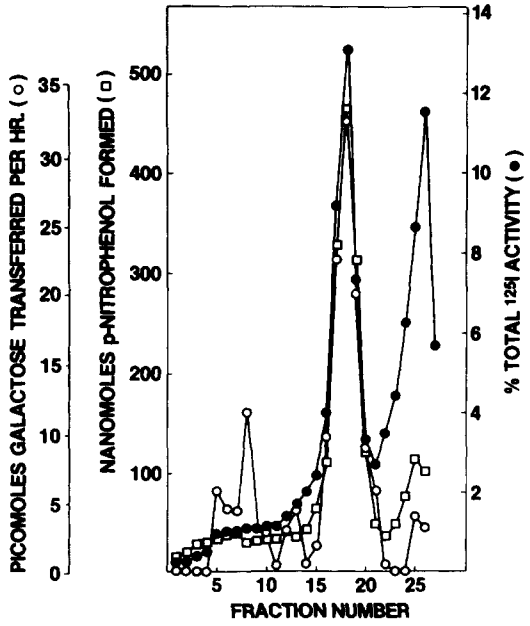

Fig. 2. Location of acid phosphatase and UDP-galactosyl transferase activity on isopycnic Percoll gradient. Rat-1 cells in one 10-cm culture dish (1×10^7 cells total) were labeled with 5 ml 0.5 ng/ml ^{125}I-EGF for 30 min at 37°C. The cells were rinsed well in HBSS and pooled with cells from five similar but unlabeled culture dishes. The cells were processed and centrifuged on a single Percoll gradient as described in Materials and Methods. After determination of ^{125}I activity in each 0.4-ml fraction (●), 100 μl was removed for determination of UDP-galactosyl transferase activity (○), and 150 μl was removed for determination of acid-phosphatase activity (□). A parallel tube containing density marker beads for density calibration was essentially identical to the density graph shown in Figure 1 (data not displayed).

peak decreased, the nonsedimentable ^{125}I activity correspondingly increased on top of the gradients (Fig. 1A–F).

The decrease in organelle peak ^{125}I activity in the experiment shown in Figure 1 is compared to total cell-bound ^{125}I activity after a cold EGF chase (Fig. 3). Whereas the organelle peak ^{125}I activity had decreased by more than 50% 30 min after initiation of the cold chase, there was no detectable decrease in total cellular activity at that time. By 1 hr after initiation of the cold EGF chase, a decrease in cell-bound ^{125}I activity began and continued over the next several hours. Analysis of the culture medium by G-25 Sephadex chromatography demonstrated that the ^{125}I activity released into the medium appeared as low molecular weight metabolites (see Fig. 5B).

Effect of Methylamine on Intracellular Distribution of ^{125}I-EGF

Treatment of cells by methylamine and other lysosomotropic compounds such as ammonium chloride, procaine, and chloroquine results in the cellular accumulation of ^{125}I-EGF and a concomitant reduction of ^{125}I-containing metabolites in the culture medium [5]. To test whether the transit of ^{125}I activity from the organelle peak to the upper fractions on Percoll gradients depended on lysosomal activity, cells were

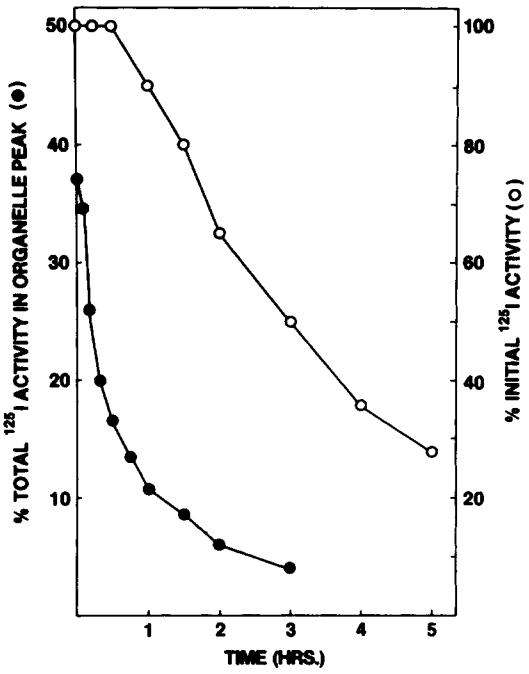

Fig. 3. Loss of ^{125}I activity after application of cold chase to cells labeled with ^{125}I-EGF. From the experiment described in Figure 1, the amount of ^{125}I activity remaining in the cells at times after the cold chase is expressed as the percent intially bound ^{125}I activity (○). The initially bound ^{125}I activity was 70,000 cpm. Also shown is the percent of ^{125}I activity of each Percoll gradient from the experiment in Figure 1, which was recovered in the "organelle" peak (●). These values were obtained by planimetry.

preincubated in 10 mM methylamine prior to exposure to ^{125}I-EGF in the presence of methylamine at 4°C. Thirty minutes after incubation in ^{125}I-EGF, cells were rinsed several times and shifted to 37°C. Percoll gradients from cells harvested 30 min after the temperature shift demonstrated similar profiles for cells treated or untreated with methylamine (Fig. 4A). Two hours after the temperature shift, gradients from control cells showed an increase in the nonsedimenting ^{125}I activity present in the organelle peak (Fig. 4B). In contrast, methylamine-treated cells exhibited a similar profile of ^{125}I activity in the gradient at 2 hr as at 30 min; the ^{125}I activity in the upper portion of the gradient did not increase at the expense of the organelle peak (Fig. 4B). In other similarly conducted experiments we demonstrated that when methylamine was added to one of the untreated cultures 60 min after the temperature shift, the ^{125}I activity that was present in the organelle peak at that time ceased to decrease over the next 2 hr (results not shown).

Chromatography of Intracellular ^{125}I Activity on Columns of G-25 Sephadex

We tested for the presence of intracellular low molecular weight degradation products by chromatography on columns of G-25 Sephadex. ^{125}I-EGF was recovered in the excluded column volume, whereas low molecular weight metabolites of ^{125}I-

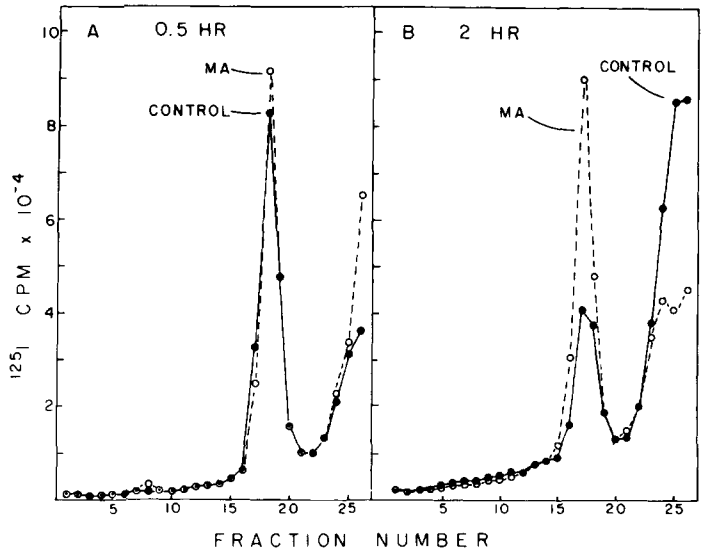

Fig. 4. Effect of methylamine on Percoll gradient profiles of cells incubated with ^{125}I-EGF. Culture dishes of Rat-1 cells (10^7 cells per 10-cm dish) were incubated in binding medium with or without 10 mM methylamine for 30 min at 4°C in an atmosphere of 5% CO_2. The dishes were rinsed six times with ice-cold HBSS containing 1 mg/ml serum albumin and incubated in 10 ml per dish of fresh binding medium, with or without 10 mM methylamine, at 37°C for 1.5 hr (panel A) and 2 hr (panel B). Cells were harvested and the extracts subjected to Percoll gradient centrifugation as described in Materials and Methods. Methylamine-treated cells, ○; control cells, ●.

EGF were eluted in later column fractions [15]. The Percoll gradient peak fraction from the organelle peak was selected from cells incubated in ^{125}I-EGF for 30 min at 37°C (Fig. 5A). A gradient fraction containing the nonsedimentable ^{125}I activity was selected from cells incubated initially as above but postincubated in ^{125}I-free medium for an additional 2 hr (Fig. 5A). The selected gradient fractions were made 0.05 N in HCl to release organelle-bound ^{125}I activity, which was then applied to columns of G-25 Sephadex. Most of the ^{125}I activity from both organelle and upper gradient fractions were recovered from the columns in the excluded column volume (Fig. 5B). In contrast, of the ^{125}I activity which the cells released into the culture medium during the postincubation, less than 10% appeared in the excluded column volume; the remainder appeared in column fractions at a position that coincided with mono- and diiodotyrosine [15]. This experiment demonstrated that most of the intracellular ^{125}I activity in both the organelle peak and in the upper gradient fractions was not in the form of low molecular weight ^{125}I-containing metabolites.

Chromatography of Intracellular ^{125}I Activity on Columns of G-75 Sephadex

We further investigated the relative sizes of the intracellular ^{125}I-labeled molecules by chromatography on columns of G-75 Sephadex. In an experiment similar to that described in Figure 5, ^{125}I activity from organelle and upper Percoll gradient fractions was applied to the G-75 Sephadex columns immediately after gradient fractionation (Fig. 6). Approximately 30% of the ^{125}I activity from the organelle peak appeared in the void volume (peak at fraction 13), with the remainder appearing as a peak at fraction 28 (Fig. 6A). When the fraction was first made acidic to permit release of organelle-bound ^{125}I activity prior to chromatography, more than 90%

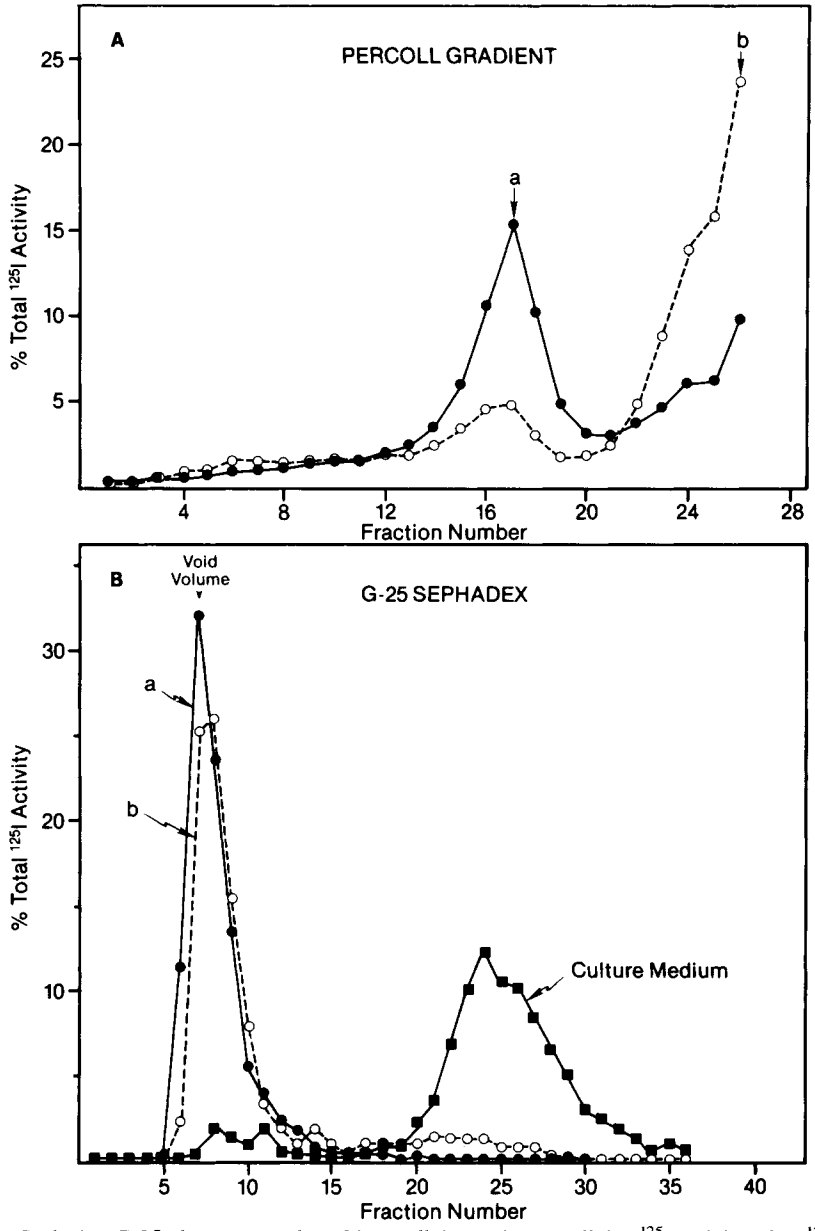

Fig. 5. Sephadex G-25 chromatography of intracellular and extracellular ^{125}I activity after ^{125}I-EGF binding. Rat-1 cells in 10-cm culture dishes were seeded to produce 10^7 cells per dish at the time of the experiment. Cells were incubated in binding medium containing 25 ng/ml ^{125}I-EGF for 30 min at 37°C. One set of four plates was rinsed, pooled, harvested and processed for Percoll gradient centrifugation (gradient No. 1) as described in Materials and Methods. A similar set of plates was refilled with fresh binding medium after rinsing, and was returned to the 37°C incubator for a 2-hr postincubation. The culture medium from the second set of dishes was collected and saved, and the cells were processed for Percoll gradient centrifugation (gradient No. 2). Panel A) The Percoll gradient profiles obtained after centrifugation of the cell extract from cells labeled for 30 min and harvested (●) and from cells incubated for 30 min and postincubated for 2 hr before harvesting (○). Fraction 17 from gradient No. 1 (labeled *a*) and fraction 24 from gradient No. 2 (labeled *b*) were made 0.1 N in HCl, neutralized, and applied to a column of Sephadex G-25 equilibrated with HBSS. The column was run with the same buffer. One-milliliter fractions were collected. Panel B) Column profiles of fractions *a* (●) and *b* (○) (from panel A), and a 0.5 sample of the culture medium collected after postincubation of ^{125}I-EGF-labeled cells as described above (■).

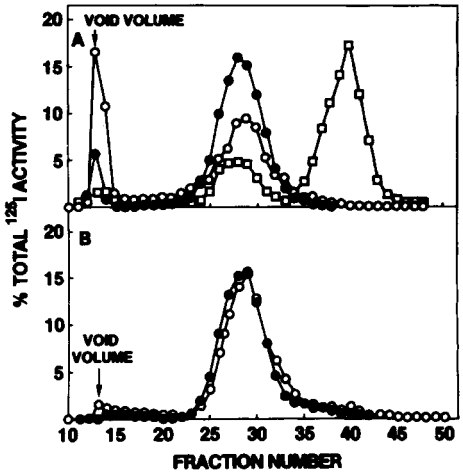

Fig. 6. Sephadex G-75 chromatography of ^{125}I-containing extracts from Percoll gradients. Rat-1 cells (10^7 cells per 10-cm dish) were incubated for 30 min at 37°C in 5 ml binding medium containing 25 ng/ml ^{125}I-EGF. One set of cells (four dishes per set) was pooled, harvested, and processed for Percoll gradient centrifugation (gradient A). An identical set was rinsed six times in warm HBSS containing 1 mg/ml serum albumin and postincubated in fresh binding medium at 37°C for 2 hr. The latter set of dishes was likewise pooled and processed for Percoll gradient centrifugation (gradient A). Fractions 17, 18, and 19 from gradient A (organelle peak) were pooled, and 0.5 ml was loaded onto a 1 × 50 cm column of Sephadex G-75 equilibrated with HBSS. One-milliliter fractions were collected (panel A, ○). To an identical 0.5-ml fraction, 0.05 ml 1N HCl was added. After 10 min the sample was neutralized with NaOH and applied to a Sephadex G-75 column as above (panel A, ●). The uppermost four fractions from gradient B were pooled and treated similarly to the pooled fractions of gradient A. The pooled fraction was analyzed on Sephadex G-75 with (panel B, ●) and without (panel B, ○) HCl treatment. Also shown in panel A is the Sephadex G-75 profile of 0.5 ml of the culture medium obtained after postincubation of the second set of ^{125}I-labeled cells for 2 hr. Panel A) Organelle peak before (○) and after (●) acidification. Culture medium 2 hr after postincubation of labeled cells (□). Panel B) Upper gradient fractions before (○) and after (●) acidification.

appeared at fraction 28 with the remainder in the void volume. Authentic ^{125}I-EGF also appeared with a peak at fraction 28 on a column of G-75 Sephadex (Fig. 8B). There was no detectable ^{125}I activity from the organelle peak which eluted at the same position as the low molecular weight metabolites recovered from the culture medium (peak at fraction 40).

When the ^{125}I activity from the uppermost portion of Percoll gradients was immediately applied to G-75 Sephadex, less than 5% appeared in the void volume (Fig. 6B). Approximately 90% of the ^{125}I activity eluted with a peak at fraction 28. Acidification of the upper gradient fraction prior to column application had little, if any, effect on the elution profile.

Comparison of "Top-Layered" vs "Mixed" Percoll Gradient

The appearance of virtually all the ^{125}I activity from upper Percoll gradient fractions at the same G-75 column positions as authentic ^{125}I-EGF suggests that the ^{125}I activity recovered from the upper portion of the gradients is present in free (vs organelle-bound) form. To test this possibility, we thoroughly mixed one ^{125}I-labeled cell supernatant sample with the Percoll prior to centrifugation and compared the ^{125}I

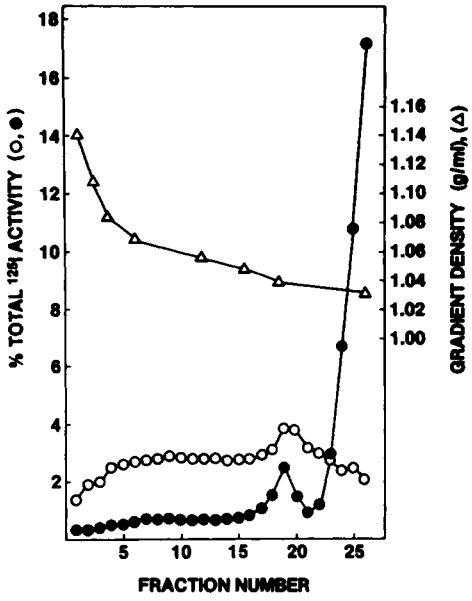

Fig. 7. Percoll gradient profiles of ^{125}I-EGF-labeled cell extracts either layered on gradient or thoroughly mixed with gradient. Two dishes of Rat-1 cells (10^7 cells per dish) were labeled with 5 ml ^{125}I-EGF, 25 ng/ml, for 15 min at 37°C. At that time 10 μg unlabeled EGF was added in a small volume to each dish. Thirty minutes later the cells from the dishes were pooled, harvested, and processed for Percoll gradient centrifugation as described in Materials and Methods. One milliliter of the cell extract was layered on one Percoll solution in the centrifuge tube (●), whereas an identical 1 ml sample was thoroughly mixed with the Percoll solution in another centrifuge tube (○). The density gradient generated by density-gradient marker beads is shown (△).

activity distributed in the gradient to the distribution produced by an identical sample which instead was layered on top of the Percoll solution (Fig. 7). The large peak of ^{125}I activity which remained on top of the "layered" gradient was homogeneously distributed throughout the "mixed" gradient. The organelle peak which characteristically appeared in the "layered" gradient was also present in the "mixed" gradient. The nonsedimentable ^{125}I activity which remained on top of the Percoll gradients after layering was therefore apparently not organelle-bound; its position on top of the gradient did not result from its inclusion in an organelle with a low buoyant density.

Preparation and Chromatography of Immunoreactive Intracellular Products of ^{125}I-EGF

The ^{125}I-containing activity from ^{125}I-EGF-labeled cells was tested for immunoreactivity by affinity chromatography on columns containing covalently bound rabbit anti-mouse EGF immunoglobulin. Organelle peak fractions and upper soluble fractions were removed from Percoll gradients prepared from ^{125}I-EGF-labeled cells in an experiment similar to that described in Figure 6. After acidification and subsequent neutralization of the pooled fractions, the ^{125}I-containing material was passed through the anti-EGF columns, which were then rinsed extensively to remove nonbound ^{125}I activity. Elution of specifically bound ^{125}I activity was achieved by addition of 4 M guanidine (Fig. 8A). Of the total ^{125}I activity present in the applied

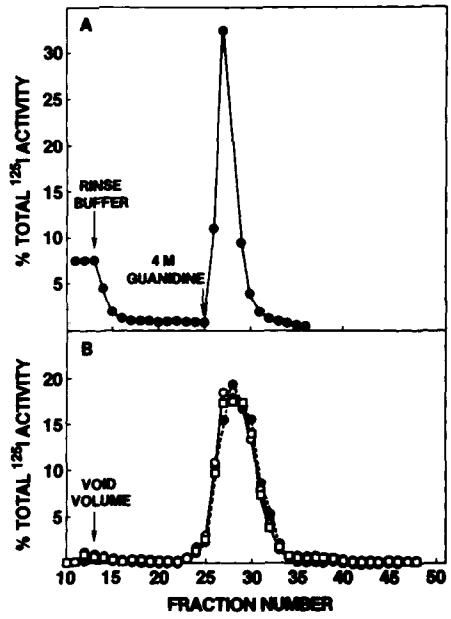

Fig. 8. Preparation and Sephadex G-75 chromatography of immunoreactive ^{125}I-EGF and intracellular ^{125}I activity. In an identical experiment to the one described in Figure 6, the ^{125}I-containing Percoll gradient fractions from the organelle peak (fractions 17–19) of gradient A and from the uppermost four fractions (fractions 23–26) of gradient B were passed through affinity columns of immobilized anti-EGF antibody. Also bound to the columns was "surface-bindable" ^{125}I-EGF, prepared by incubating A-431 carcinoma cells with ^{125}I-EGF at 4°C and eluting the surface-bound ^{125}I activity by incubation with 0.5 N NaCl, pH 2.5, at 4°C [16]. After column loading, the columns were rinsed with 20 volumes of HBSS containing 1 mg/ml serum albumin. Immunoreactive ^{125}I activity was eluted with 4 M guanidine. Panel A shows the elution profile of bound ^{125}I activity extracted from the organelle-containing peak of Percoll gradient A. To peak fractions of 4 M guanidine-containing eluted ^{125}I material, 200 μl HBSS containing 1 mg/ml serum albumin was added, and fractions were dialyzed against distilled H_2O at 4°C with 3,500 MW cutoff dialysis tubing. The dialysate was applied to Sephadex G-75 columns as described in the legend to Figure 6. The column profiles represent immunoreactive ^{125}I activity from 1) Percoll gradient "organelle" fractions (○); 2) Percoll gradient uppermost fractions (●); and 3) "surface-bindable" EGF (□), as described above.

material, 76% of the organelle and 78% of the upper soluble gradient fractions bound specifically to the anti-EGF columns and were eluted by guanidine. By comparison, 82% of the ^{125}I-EGF used to label cells bound to the anti-EGF columns.

After elution from anti-EGF columns, the ^{125}I-containing material was applied to columns of G-75 Sephadex (Fig. 8B). The immunoreactive ^{125}I activity from both the organelle and upper soluble Percoll gradient fractions were eluted from the G-75 Sephadex column at a position coinciding with cell-surface-bound ^{125}I-EGF. The latter material had been prepared by binding ^{125}I-EGF at 4°C to the surface of A431 cells, eluting the bound ^{125}I activity by brief rinsing of the cells with pH 2.5 acetic acid saline [16] and subsequently purifying the ^{125}I activity by anti-EGF affinity chromatography. These data indicate that the intracellular ^{125}I activity, both organelle-contained and soluble, are approximately as immunoreactive as and similar in size to the ^{125}I-EGF used for binding.

Isoelectric Focusing of ^{125}I-EGF and Internalized ^{125}I Activity

The immunoreactive ^{125}I-containing material purified from the organellar and upper Percoll gradient fractions were examined by isoelectric focusing in agarose gels (Fig. 9). ^{125}I-EGF purified from stock ^{125}I-EGF by cell-surface binding and anti-EGF affinity chromatography revealed two major bands of isoelectric points 4.5 and 4.9, which correspond to similar bands present in the stock ^{125}I-EGF (Fig. 9). The immunoreactive ^{125}I activity recovered from the Percoll gradient organelle peak appeared as a major band at pI 4.2 with a minor band at pI 3.8. The immunoreactive ^{125}I-containing material from the upper soluble fractions of the Percoll gradient appeared as a major band at pI 3.9 with a minor band at pI 4.2, which corresponded to the major band of the organelle peak.

That the two intracellular forms of ^{125}I activity were more acidic than the ^{125}I-EGF from which they were derived was apparent following ion exchange chromatography on DEAE-Sepharose (Fig. 10). Cell-surface-bound ^{125}I-EGF and the intracellular species of ^{125}I-EGF described in Figure 9 were bound to DEAE-Sepharose in 0.02 M ammonium acetate at pH 5.6. The column was then rinsed with 0.05 and 0.2 M ammonium acetate. Whereas the majority of cell-surface-bound ^{125}I-EGF was eluted soon after application of 0.05 M ammonium acetate, the two intracellular forms of ^{125}I-EGF isolated from Percoll gradient fractions eluted soon after application of 0.2 M ammonium acetate. Attempts at separating the forms of ^{125}I activity

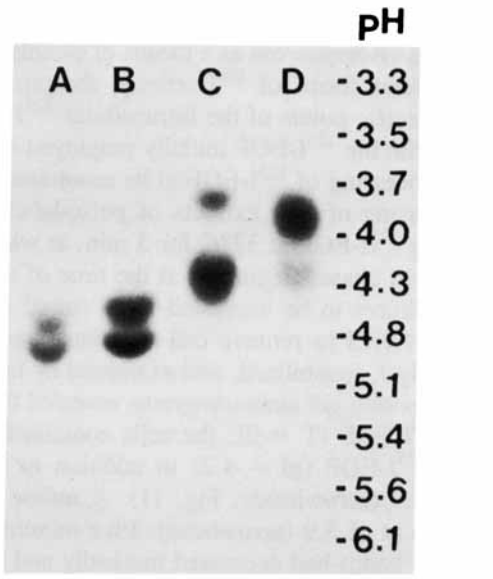

Fig. 9. Isoelectric focusing autoradiograms of ^{125}I-EGF and intracellular ^{125}I-containing macromolecules. ^{125}I-EGF and intracellular ^{125}I-containing immunoreactive material prepared as described in the legend to Figure 8 were separated by isoelectric focusing on agarose gels as described in Materials and Methods. A) ^{125}I-EGF; B) "surface-bindable" ^{125}I-EGF prepared as described in the legend to Figure 8; C) immunoreactive ^{125}I-containing material from Percoll gradient "organelle" fractions prepared as described in the legend to Figure 8; D) immunoreactive ^{125}I-containing material from uppermost Percoll gradient fractions prepared as described in the legend to Figure 8.

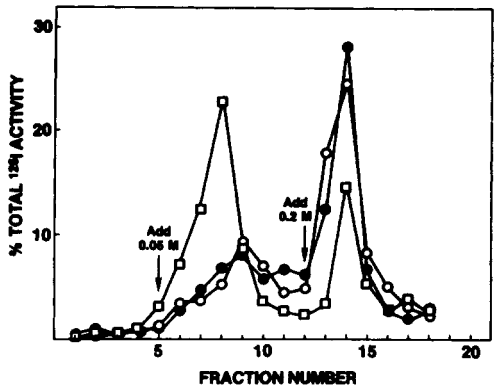

Fig. 10. DEAE-Sepharose ion-exchange chromatography of ^{125}I-EGF and intracellular ^{125}I-containing macromolecules. ^{125}I-containing immunoreactive samples from experiments described in the legends to Figures 8 and 9 were chromatographed on DEAE-Sepharose as described in Materials and Methods. "Surface-bindable" ^{125}I-EGF (□); Percoll gradient "organelle" fraction (○); uppermost Percoll gradient fractions (●).

with a continuous ammonium acetate gradient were unsuccessful; the two more acidic intracellular forms of ^{125}I-EGF activity began to elute from the column slowly but completely in the presence of 0.05 M ammonium acetate. Whereas we were unsuccessful at employing DEAE-Sepharose as a means of cleanly separating bindable ^{125}I-EGF from the intracellular forms of ^{125}I-activity, the profiles shown in Figure 10 confirm the lower isoelectric points of the intracellular ^{125}I-containing derivatives of EGF when compared with the ^{125}I-EGF initially employed in binding to the cells.

The sequential processing of ^{125}I-EGF to its more acidic derivatives was examined by isoelectric focusing of acid extracts of pulse-labeled cells (Fig. 11). Cells were incubated in 25 ng ^{125}I-EGF at 37°C for 5 min, at which time 10 μg unlabeled EGF was added as a cold chase. Beginning at the time of addition of cold EGF and continuing for 2 hr, cultures to be harvested were rinsed extensively in HBSS and then exposed to 0.05 N HCl to remove cell-associated radioactivity. Acid extracts were dialyzed against H_2O, lyophilized, and examined by isoelectric focusing.

The isoelectric focusing gel autoradiograms revealed that as early as 5 min after exposure of cells to ^{125}I-EGF (T = 0), the cells contained the organelle-associated species of processed ^{125}I-EGF (pI = 4.2) in addition to the two major ^{125}I-EGF species at pI 4.5 and 4.9 (arrowheads, Fig. 11). A minor band contaminant of the ^{125}I-EGF was also seen at pI 3.9 (arrowhead). Five minutes after application of the cold chase the ^{125}I-EGF bands had decreased markedly and had virtually disapperaed by 30 min. The decrease in ^{125}I activity in the EGF bands was accompanied by an increase in the organelle-associated ^{125}I-containing species which appeared as a doublet band at pI 4.2. By 30 min after application of the cold chase, the latter bands had begun to decrease in intensity as the ^{125}I activity increased in the band at pI 3.8, which corresponded to the major ^{125}I-containing product found in the uppermost fractions of Percoll gradients (see Fig. 9). At 45 min this species began to disappear and had decreased considerably by 2 hr after application of the cold chase.

DISCUSSION

Centrifugation of cell homogenates on isopycnic Percoll gradients was used to follow the intracellular compartmentaliztion of ^{125}I activity after exposure to cells to ^{125}I-EGF at 37°C. Within 5 min after exposure of cells to ^{125}I-EGF, the ^{125}I activity appeared in gradient fractions which contained lysosome and Golgi marker enzymes. That the ^{125}I activity was contained in or bound to cellular organelles is demonstrated by 1) the appearance of the ^{125}I activity at the same density position in the gradient after thorough premixing of the cell homogenate with the Percoll prior to centrifugation; 2) the appearance of a significant portion of the ^{125}I activity in the excluded volume of Sephadex G-75 columns; and 3) the release of the ^{125}I activity by brief exposure to 0.1 N HCl.

The ^{125}I activity that appeared in the organelle peak after application of the cold EGF chase was not surface-displaced ^{125}I-EGF that had not yet been internalized because 1) surface elution of ^{125}I activity using acidic saline [16] at times after the

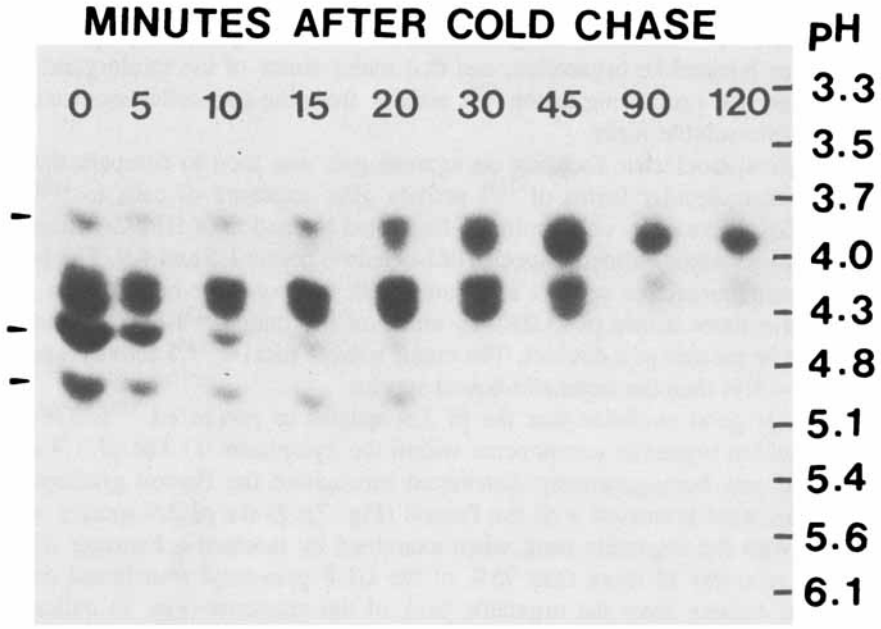

Fig. 11. ^{125}I-EGF pulse-chase experiment employing agarose gel isoelectric focusing. Rat-1 cells were plated into 35-mm dishes 2 days prior to the experiment to yield 10^6 cells per dish. To each dish was added 1 ml binding medium (37°C) containing 25 ng/ml ^{125}I-EGF for 5 min (the time of the ^{125}I-EGF pulse). At that time (T = 0), 10 μg unlabeled EGF in 25 μl binding medium was added to each plate to provide a cold chase. At times from T = 0 to T = 120 min, culture dishes were removed from the incubator, rinsed six times in HBSS containing 1 mg/ml serum albumin, and extracted in 1 ml 0.05 N HCl for 30 min at room temperature. This procedure removed more than 95% of the cell-bound ^{125}I activity. The acid extracts were dialyzed against distilled water, evaporated to dryness, and applied to an agarose gel for isoelectric focusing and subsequent autoradiography as described in Materials and Methods. Arrowheads on the left border indicate the bands present in the ^{125}I-EGF employed in the cell binding. The uppermost of the ^{125}I-EGF bank at pI = 3.8 was present in low amounts in the ^{125}I-EGF sample, and consistently focused to a position above the intracellular pI 3.9 band that appeared at later times.

cold chase (Fig. 11) demonstrated that by 15 min, more than 95% of the ^{125}I activity was intracellular (unpublished); and 2) the absence of intact ^{125}I-EGF (pI 4.5 and 4.9) from the cell 15 min after application the cold chase argues against the presence of unmodified ^{125}I-EGF. Interestingly, when intact ^{125}I-EGF was added to cell homogenates and incubated at 37°C for 1 hr there was no detectable conversion of the ^{125}I-EGF to its more acidic processed forms (unpublished).

Within minutes after entrance of the ^{125}I activity into the organelle compartment, the ^{125}I activity appeared to leave that compartment, presumably into the cytosol. The ^{125}I activity was nonsedimentable on Percoll gradients, and appeared in the included volume on Sephadex G-75 columns at the same position as ^{125}I-EGF. Similar results were obtained using nitrogen cavitation disruption of cells instead of homogenization of hypotonically swollen cells (see Materials and Methods for further discussion). Methylamine, a lysosomotropic amine which acts by altering the intralysosomal pH [6], and which inhibits degradation of cell-bound ^{125}I-EGF [7,8] prevented the egress of the organelle-bound ^{125}I activity. Methylamine also inhibited the processing of the pI 4.2 species to the pI 3.9 species (unpublished). The correspondence of organelle-bound ^{125}I activity with lysosomes on gradients coupled with the effects of methylamine on release of ^{125}I activity suggests that the ^{125}I activity was incorporated into lysosomes or lysomelike organelles, and that maintenance of the intraorganellar pH was necessary for processing of the ^{125}I activity from the organelle-associated form to the cytosolic-soluble form.

Analytical isoelectric focusing on agarose gels was used to compare the intracellular macromolecular forms of ^{125}I activity after exposure of cells to ^{125}I-EGF. The ^{125}I-EGF prepared by chloramine T–facilitated iodination of HPLC-purifed EGF consisted of two major iodinated species of isoelectric points 4.5 and 4.9. The primary iodinated immunoreactive species associated with the organelle-bound form of ^{125}I activity had a more acidic pI (4.2) than either of the major ^{125}I-EGF species, and appeared to be present as a doublet. The major soluble form of ^{125}I activity was more acidic (pI = 3.9) than the organelle-bound species.

There is good evidence that the pI 3.9 species of processed ^{125}I-EGF is not contained within organelle components within the cytoplasm: 1) The pI 3.9 species of ^{125}I-EGF was homogeneously distributed throughout the Percoll gradient when homogenates were premixed with the Percoll (Fig. 7); 2) the pI 3.9 species was not associated with the organelle peak when examined by isoelectric focusing (Fig. 9); and 3) the recovery of more than 95% of the UDP galactosyl transferase and acid phosphatase activity from the organelle peak of the gradients (Fig. 2) indicated an insignificant amount of destruction and leakage of Golgi and lysosomal vesicles. However, the containment of the pI 3.9 species in a different, more fragile species of intracellular vacuole cannot be excluded as a possibility.

Pulse-chase experiments using ^{125}I-EGF strongly suggest that soon after incorporation of ^{125}I-EGF into the cell the EGF was processed to a more acidic form. The altered behavior on analytical isoelectric focusing gels may result from covalent modification such as by phosphorylation or deamidation or by a proteolytic cleavage of a small fragment. It has been well documented that EGF and other polypeptide hormones bind to receptors on the cell surface at 4°C, and, upon warming cells to 37°C, EGF appears in endocytic vesicles [17–19]. These vesicles have been termed "receptosomes" [20]. It has recently been shown that these vesicles have an acidic pH similar to that of lysosomes [21]. The temporal coincidence of the appearance of

^{125}I-EGF in endocytic vesicles with the appearance of the acidic organellar form of processed ^{125}I-EGF suggests that entrance of EGF into the endocytic vesicle may be coupled with processing of EGF to the more acidic form. These data also suggest that the ^{125}I activity associated with the lysosome-Golgi peak on Percoll gradients within 5 min after exposure to ^{125}I-EGF may be associated with endocytic vesicles. These organelles may respond to lysosomotropic agents in view of their acidic intraorganellar pH.

Pastan and his colleagues have shown that 30–45 min after exposure of cells to the α_2 macroglobulin, the material initially present in endocytic vesicles appear in the Golgi region of the cell [22]. It is at that time that we have observed processing of the organellar form of ^{125}I-EGF to a more acidic product which appears to be present in the cytoplasm unassociated with an organelle. It has been postulated that because of the association of the endocytic vesicles with the Golgi endoplasmic reticulum lysosome, or GERL, system of organelles [23], there is a transfer of the EGF to these organelles preparatory to degradation [24]. We suggest that, after the appearance of the endocytic vesicle-bound modified EGF in the GERL region, the compound undergoes a second processing step which renders the EGF more acidic, and which results in liberation of the EGF from its organelle-bound compartment into the cytoplasm. The soluble macromolecular form of the ^{125}I may subsequently be degraded by typical lysosomes or by some other cellular proteolytic system.

The relationship of the series of EGF processing steps described above to the mitogenic action of EGF remains unknown. However, there are a number of reasons to believe that internalization of EGF may represent more than a mechanism for the cell to utilize in disposing of EGF. The several processing steps that EGF undergoes in the cell and the appearance of the final processed form of EGF in a soluble form in the cytoplasm suggest that one of the processed forms of EGF may be involved in the generation of an intracellular signal. Furthermore, the inhibition of both DNA synthesis [8,9] and of induction of ornithine decarboxylase activity [9] by lysosomotropic drugs suggest that processing of EGF from the organelle-bound form to the more acidic soluble form of EGF may be necessary for the biological activity of EGF. Alternatively, the internalized EGF receptor may supply the mitogenic signal. Further characterization and testing of the intracellular forms of EGF and of the intracellular processing of the EGF receptor may provide answers to these questions.

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REFERENCES

1. Cohen G, Carpenter G, Lembach KJ: Adv Metab Dis 8:625, 1975.
2. Lindgren A, Westermark B: Exp Cell Res 99:357, 1976.
3. Matrisian LM, Bowden GT, Magun BE: J Cell Physiol 108:417, 1981.
4. Pastan IH, Willingham MC: Science 214:504, 1981.
5. Carpenter G, Cohen S: J Cell Biol 71:159, 1976.

6. Ohkuma S, Poole B: Proc Natl Acad Sci USA 75:3327, 1978.
7. Michael JH, Bishayee S, Das M: Febs Lett 117:125–130, 1980.
8. King AC, Hernaez-Davis L, Cuatrecases P: Proc Natl Acad Sci USA 78:717, 1981.
9. Widelitz RB, Russell DH, Magun BE (submitted).
10. Prasad I, Zouzias D, Basilico C: J Virol 18:436, 1976.
11. Savage CR Jr, Cohen S: J Biol Chem 247:7609, 1972.
12. Matrisian LM, Larsen BR, Finch JS, Magun BE: Anal Biochem 125:339, 1982.
13. Cohen S: J Biol Chem 237:1555, 1962.
14. Fleischer B: In Fleisher S, Packer L (eds): "Methods in Enzymology, Biomembranes Part A" Vol 31, 1974, p 180.
15. Magun BE, Matrisian LM, Bowden GT: J Biol Chem 255:6373, 1980.
16. Haigler HT, Maxfield FR, Willingham MC, Pastan I: J Biol Chem 255:1239, 1980.
17. Haigler H, Ash JF, Singer SJ, Cohen S: Proc Natl Acad Sci USA 75:3317, 1978.
18. McKanna JA, Haigler HT, Cohen S: Proc Natl Acad Sci USA 76:5689, 1979.
19. Schlessinger J, Schechter Y, Willingham M, Pastan I: Proc Natl Acad Sci USA 75:2659, 1978.
20. Willingham MC, Pastan I: Cell 21:67–77, 1980.
21. Tycko B, Maxfield FR: Cell 28:64, 1982.
22. Pastan IH, Willingham MC: Ann Rev Physiol 43:239, 1981.
23. Novikoff AB, Novikoff PM: Histochem J 9:525, 1977.
24. Pastan IH, Willingham MC: Science 214:504, 1981.