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Prelysosomal Divergence of Transferrin and Epidermal Growth Factor during Receptor-Mediated Endocytosis†

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ABSTRACT: The routes followed by epidermal growth factor and transferrin during their endocytosis by human epithelial cells were compared in double-label studies by using density gradient centrifugation of cell homogenates and fluorescence microscopy with intact cells. Gradient centrifugation studies of cells incubated with radioactively labeled epidermal growth factor and transferrin indicated that both ligands initially were associated with a class of vesicles having a density of 1.037 g/mL and then were rapidly transferred to a membrane compartment having a slightly higher density (1.039 g/mL). Subsequently, the two ligands diverged. Epidermal growth factor ultimately was transferred to a membranous compartment containing lysosomal enzymes (density 1.08 g/mL) where it was degraded. Transferrin was released intact from the cells; very little was transferred to lysosomes. Using fluorescently labeled ligands, it was observed that after cells were warmed to 37 °C for 5 min, transferrin and epidermal growth factor gave coincident, punctate fluorescent patterns, strongly suggesting they were localized within the same endocytic vesicles. Subsequently, the epidermal growth factor signal was observed in lysosomes whereas the transferrin signal became weaker and diffuse and did not coincide with the punctate epidermal growth factor fluorescence. The time course of the divergence of the radioactive and fluorescent ligands coupled with the previous morphologic studies on the pathway of epidermal growth factor internalization [Willingham, M. C., & Pastan, I. (1982) *J. Cell Biol. 94*, 207–212] suggests that the sorting process is prelysosomal and possibly Golgi associated.

Receptor-mediated endocytosis is the process involved in the cellular entry of certain hormones, plasma proteins, viruses, and bacterial toxins (Pastan & Willingham, 1981a,b; Goldstein et al., 1979). Ligands enter cells via clathrin-coated pits in the plasma membrane and are transferred to receptosomes, a term used to describe these endocytic vesicles to emphasize their role in receptor-mediated endocytosis (Pastan & Willingham, 1981a,b; Willingham & Pastan, 1980). Receptosomes are acidic (Tycko & Maxfield, 1982) and devoid of a clathrin coat Willingham & Pastan, 1980). Similar structures also have been termed endocytic vacuoles (Wall et al., 1980) or endosomes (Helenius et al., 1980). In double-label studies, many different ligands have been visualized entering cells together in the same coated pits and receptosomes, indicating that a common initial route of cellular entry exists for many receptor-bound ligands (Pastan & Willingham, 1981a,b; Dickson et al., 1981b; Via et al., 1982; Willingham et al., 1981, 1983a).

At present, two intracellular destinations for ligands entering cells by receptor-mediated endocytosis have been defined. Low-density lipoproteins, α_2-macroglobulin (α_2M),[1] asialo-

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[1] Abbreviations: α_2M, α_2-macroglobulin; EGF, epidermal growth factor; EGF–HRP, epidermal growth factor–horseradish peroxidase conjugate; PBS, Dulbecco's phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; TES buffer, triethanolamine-, EDTA-, and sucrose-containing buffer; IgG, immunoglobulin G; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; ER, endoplasmic reticulum; UDP-galactose, uridine diphosphogalactose; RH, rhodamine; FL, fluorescein.

glycoproteins, lysosomal enzymes, epidermal growth factor (EGF), and a variety of other ligands are eventually transferred to lysosomes (Pastan & Willingham, 1981a,b; Wall et al., 1980; Steer & Ashwell, 1980; Neufeld et al., 1977; Goldstein et al., 1979). Some viruses also enter cells via coated pits and receptosomes (Dickson et al., 1981b; Dales, 1973; Matlin et al., 1981; FitzGerald et al., 1983) but then rapidly appear in the cytosol. The kinetics of viral entry together with morphological studies suggest that their penetration into the cytosol occurs from receptosomes (FitzGerald et al., 1983). Some bacterial toxins may follow a similar route (FitzGerald et al., 1983; Keen et al., 1982).

The intracellular route followed by transferrin, the major iron carrier in the circulation of vertebrates, is not yet clear. Several reports have indicated that after endocytosis of mono- or diferric transferrin, iron-depleted intact transferrin is released back into the culture medium after several minutes (Karin & Minz, 1981; Octave et al., 1981, 1982; Renswoude et al., 1982; Van Brockxmeer & Morgan, 1979). These findings are of considerable interest since it has been reported that the protein portion of transferrin is susceptible to in vitro hydrolysis by a lysosomal fraction of rat liver (Charlwood et al., 1979). In addition, in a long time course study where rat embryo fibroblasts were continuously incubated with iron-loaded ^{3}H-transferrin for 24 h, some ^{3}H-transferrin was degraded. This observation together with the fact that transferrin-bound iron is acid dissociable and compounds such as chloroquine that raise the lysosomal pH inhibit the time-dependent cellular accumulation of iron has led to the proposal that iron was released from transferrin in the lysosome (Octave et al., 1981, 1982). On the basis of similar data with teratocarcinoma cells, a lysosomal role in the cellular processing of transferrin in those cells has also been proposed (Karin & Minz, 1981). Recent electron microscopic studies using developing erythroid cells from fetal rat liver have localized transferrin–ferritin conjugates in coated pits and in intracellular vesicles and "multivesicular bodies" that were described to be "lysosome-like" in morphology (Iacopetta et al., 1983). However, in recent studies using erythroleukemia cells, transferrin has been found in acidic vesicles that are not lysosomes, based on their enzymatic characterization after density gradient centrifugation (Renswoude et al., 1982). In light of all these findings, any conclusions about the role of lysosomes in the release of iron from transferrin need to be further evaluated. In addition, if transferrin does not enter lysosomes, the relationship of the transferrin pathway to that of other endocytosed ligands destined for lysosomal degradation needs to be elucidated.

The present study is designed to compare the routes of intracellular transfer of EGF and transferrin in short-term, double-label ligand studies with cultured KB cells. We have chosen to carry out the studies in KB cells because a considerable amount is known about the pathway of endocytosis based on studies with EGF and adenovirus (Pastan & Willingham, 1981; FitzGerald et al., 1983; Willingham et al., 1983a). It has been shown that EGF enters these cells via coated pits, is carried to the Golgi apparatus in receptosomes, and then appears to traverse the transreticular portion of the Golgi apparatus and the coated pits of the Golgi apparatus on its way to lysosomes (Pastan & Willingham, 1981a; FitzGerald et al., 1983; Willingham et al., 1983a). Adenovirus, which also enters these cells via coated pits and receptosomes, rapidly escapes into the cytosol (FitzGerald et al., 1983). In the current study, we find that EGF and transferrin appear to enter cells together in the same vesicles. EGF is ultimately transferred to lysosomes, whereas substantial amounts of transferrin are returned undegraded to the medium without accumulation in lysosomes.

Materials and Methods

Cultured Cells. Human KB carcinoma cells, obtained from the American Type Culture Collection (Rockville, MD), were used in the present study. A time course of the morphologic pathway of endocytosis of an EGF–horseradish peroxidase conjugate (EGF–HRP) and fluorescent EGF has been previously documented in this cell line (Willingham & Pastan, 1982; Willingham et al., 1983a). Endocytosis is rapid, and subsequent intracellular transfer to lysosomes is markedly synchronous.

Approximately 10^{7} cells were plated 2 days before use in 150-mm culture dishes using Dulbecco–Vogt's-modified MEM (GIBCO) containing 10% calf serum (GIBCO). Nearly confluent monolayers were used in all experiments. Prior to experiments, monolayers were extensively washed and incubated in serum-free medium for 1 h.

Binding of ^{125}I- and ^{131}I-Labeled EGF and Transferrin by Cells: Analysis by Colloidal Silica Gradients. Washed cell monolayers were incubated with constant agitation for 1 h at 4 °C with radioactive EGF and/or transferrin in Dulbecco's phosphate-buffered saline (PBS). EGF was obtained from Bethesda Research Labs and iodinated by Meloy Labs with the chloramine T procedure (Haigler et al., 1980). The specific activity of ^{131}I-EGF was 27 μCi/μg while that of ^{125}I-EGF was 360 μCi/μg. Differic human transferrin was obtained from Calbiochem and radioiodinated by using the chloramine T procedure (Hunter & Greenwood, 1962) to 1 μCi/μg. Degradation studies of EGF and transferrin were carried out both in media and in 1% Triton X-100 solubilized cellular homogenates by using gel filtration on Sephadex G25 (P-10 columns from Pharmacia) in 0.1 M sodium acetate, pH 7.0. Radioactivity comigrating with the void volume was used as an estimate of intact ligand. Following gel filtration, recovery of radioactivity for all samples was 90% or greater.

For initial studies to characterize the binding sites, variable levels of ^{125}I-EGF or ^{125}I-transferrin were incubated with cells at 4 °C for 1 h. For double-label gradient fractionation studies, 3.8 mM ^{125}I-transferrin and 10 nM ^{131}I-EGF were added to 10 mL of Dulbecco's phosphate-buffered saline (PBS) and incubated with cells at 4 °C for 1 h. After incubation, cells were washed 4 times with ice-cold PBS, 37 °C, serum-free medium was added, and incubations were continued for various times at 37 °C in a cell culture incubator under 5% CO_2. To end incubations, media were removed, cells were cooled on ice, and cold PBS was rapidly added. Cells were removed from plates with a rubber scraper.

Cells were homogenized by using a tight Dounce homogenizer (Thomas Instruments) in 2 mL of TES buffer (10 mM triethanolamine, 1 mM EDTA, and 0.25 M sucrose, pH 7.5). Approximately 20 strokes were necessary for cellular disruption (>90%) with a minimum of nuclear disruption (monitored by trypan blue staining). Cytosol proteins were not removed because it was observed (unpublished results) that pelleting of endocytic vesicles released substantial amounts of ^{125}I-EGF and changed the equilibrium density sedimentation of the residual membrane-bound ^{125}I-EGF. After centrifugation of the homogenate at 3000*g* for 10 min to pellet nuclei and unbroken cells, 0.5 mL of the supernatant was layered over 9 mL of 20% isoosmotic Percoll (Pharmacia)–TES buffer. The bottom of the tube contained a 0.5-mL cushion of 2.5 M sucrose. After centrifugation (Beckman Ti 40 rotor) at 40000*g* for 1 h, the in situ generated density gradient was collected

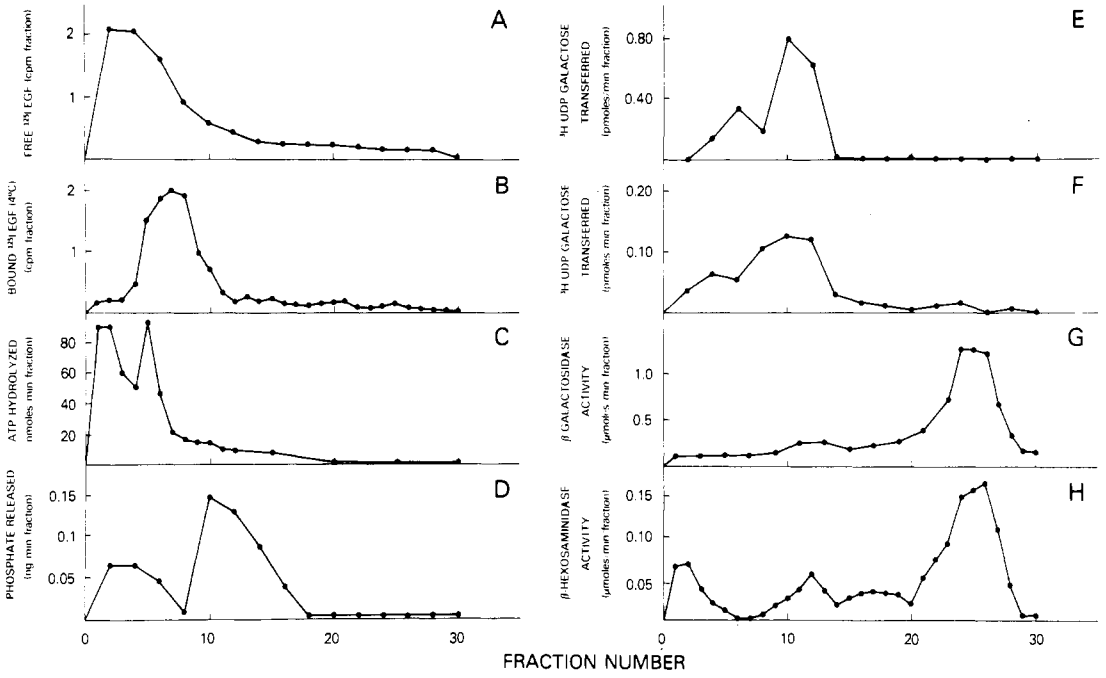

FIGURE 1: Characterization of the equilibrium density gradient used for KB cell fractionation. After the cells were homogenized, a 3000*g* supernatant was prepared and applied to a 20% Percoll gradient as described under Materials and Methods. Enzyme markers were determined to localize various cell membranes. (A) Free ^{125}I-EGF applied to the Percoll gradient indicating the sample loading zone and the probable position of soluble (cytosol) proteins. (B) Plasma membrane bound ^{125}I-EGF after cellular binding at 4 °C for 1 h (no warming of monolayers) as described under Materials and Methods. (C) (Na^+, K^+)-ATPase activity profile across the Percoll gradient. (D) 5′-Nucleotidase activity across the Percoll gradient. (E) Galactosyltransferase activity determined as the *N*-acetylglucosamine-dependent incorporation of [^{3}H]UDP-galactose. (F) Galactosyltransferase activity using ovalbumin as the acceptor for [^{3}H]UDP-galactose. (G) β-Galactosidase activity profile across the Percoll gradient. (H) β-Hexosaminidase activity profile across the Percoll gradient.

from the top. Fractions were collected and assayed for density by using density marker beads (Pharmacia) and for radioactivity by using an LKB mini-γ counter. High- and low-energy measurements were made and spillover corrections employed for each measurement.

Enzyme Assays. A variety of enzymes were assayed in gradient fractions to determine organelle and membrane distributions in KB cells (Figure 1). Sample amounts were chosen so as to yield enzyme activities within a linear range for each assay. All enzyme activities except 5′-nucleotidase were determined after solubilization of samples in 1% Triton X-100. Protein levels were determined by the method of Bradford (1976) using dye reagent from Bio-Rad; 70% of the protein was detected in fractions 1–7 (data not shown). Free ^{125}I-EGF was used to indicate the position of soluble proteins (Figure 1). For plasma membranes, 5′-nucleotidase was determined by using adenosine 5′-phosphate and paired sample incubations with β-glycerol phosphate to determine nonspecific phosphatase (Sigma kit) (Fiske & SubbaRow, 1925); (Na^+, K^+)-ATPase activity (Sigma Technical Bulletin No. 366-UV, 1980) was determined with K^+ stimulation and ouabain sensitivity as specificity controls. Binding of ^{125}I-EGF or transferrin to cell surfaces at 4 °C was also determined (Figure 1B–D). Cell-surface iodination (at 4 °C) catalyzed by lactoperoxidase (Der & Stanbridge, 1978) was also utilized to confirm the positions of cell-surface markers (data not shown). Hexosaminidase (Li & Li, 1972) and β-galactosidase (Rome et al., 1979) were assayed as previously described and used as markers for lysosomes (Figure 1G,H). *p*-Nitrophenyl β-D-glucosaminide (Sigma) was used as a substrate for hexosaminidase, and 4-methylumbelliferyl-β-D-galactose (Sigma) was used as a substrate for β-galactosidase. For fluorescence measurements, and Aminco-Bowman spectrophotofluorometer was used with excitation and emission wavelengths at 365 and 455 nm, respectively. Galactosyltransferase was assayed as a Golgi marker using [^{3}H]UDP-galactose (NEN) and *N*-acetylglucosamine (Sigma) or ovalbumin (Sigma) (Rome et al., 1979; Fleischer et al., 1969) as acceptors (Figure 1E,F). Substrate-dependent activity was taken as an indicator of this enzyme activity (Fleischer et al., 1969) which is thought to be specific for trans Golgi (stacks). Two additional confirmatory markers (not shown) were the following: acid phosphatase was assayed by using *p*-nitrophenyl phosphate (Sigma) as a substrate preferential for ER-Golgi and glycerol 2-phosphate (Sigma) as a substrate preferential for lysosomes (Dingle, 1977).

Fluorescence Microscopy. Fluorescence microscopy of endocytosed ligands was carried out by using indirect antibody localization in fixed, permeabilized cells. A 1:1 conjugate of EGF with horseradish peroxidase (HRP) (Willingham & Pastan, 1982; Dickson et al., 1981a) was used to follow EGF entry; the route of entry of this ligand has been extensively studied at the electron microscopic level, and the antigenicity provided by HRP for subsequent binding of the fluorescent antibody makes detection feasible. The cellular binding of this conjugate can be entirely blocked with excess native EGF (Willingham & Pastan, 1982; Willingham et al., 1983a). Previous studies with a variety of fluorescence, electron microscopic, and radioactive methods of EGF detection have all demonstrated that EGF is transferred to lysosomes after approximately 15–30 min (Willingham & Pastan, 1982; Willingham et al., 1983a; Haigler et al., 1979). Unmodified human transferrin (Calbiochem) was also used. Fluorescein–anti-HRP and rhodamine–goat anti-rabbit IgG (Cappel Laboratories) were prepared as previously described (Pastan et al., 1977) and purified by using HRP– and rabbit IgG–Sepharose affinity columns. Cell monolayers were first incubated with PBS (+Ca^{2+} and Mg^{2+}) and 2 mg/mL bovine serum albumin (Sigma) for 15 min at 4 °C. Then 100 nM EGF–HRP and 125 nM transferrin were added at 4 °C for 1 h with constant

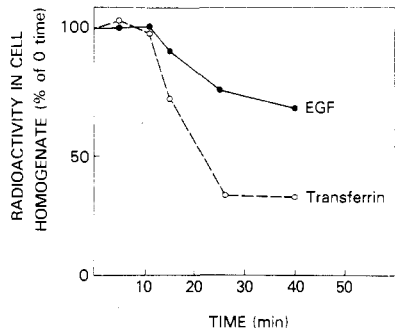

FIGURE 2: Kinetics of release of EGF and transferrin from KB cells. Cells were incubated with ^{125}I-EGF or ^{125}I-transferrin at 4 °C, washed, and warmed to 37 °C for the indicated period of time. Total radioactivity in the cell homogenates was determined for each time. These data are averages from three experiments.

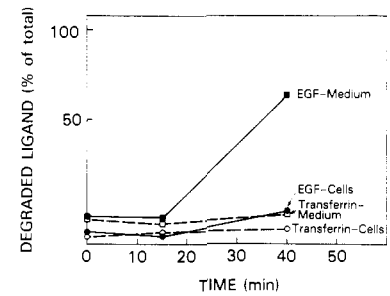

FIGURE 3: Kinetics of degradation of EGF and transferrin. Radioactivity in all homogenates and media (Figure 2) was chromatographed on Sephadex G-25 (Pharmacia PD-10 columns). Radioactivity eluting at the void volume was used to estimate the amount of intact ligand at each time point. Data are averages of three experiments.

agitation. Cells were washed at 4 °C, and the temperature was raised to 37 °C for various times. Incubations were ended by fixation in 3.7% formaldehyde (in PBS) in 10 min. Cells were permeabilized with 0.1% Triton (in PBS) for 10 min and then incubated with 4 mg/mL normal rabbit globulin, 0.1% Saponin, 1 mM EGTA, and PBS. Then 100 μg/mL affinity-purified rabbit anti-HRP–fluorescein and 100 μg/mL affinity-purified goat anti-transferrin were added for 15 min each at 23 °C. This was followed by incubation in 100 μg/mL affinity-purified rabbit anti-goat IgG–rhodamine for 15 min at 23 °C. All antibody incubations were performed in a buffer consisting of the same diluent (normal rabbit globulin, Saponin, EGTA, and PBS) as used above. Each step was followed by four washes in PBS. Following PBS washing, monolayers were embedded in 5% *n*-propyl gallate in glycerol (Giloh & Sedat, 1982) and viewed by rhodamine and fluorescein epifluorescence optics. Photographs were directly taken at constant exposure on Kodak Tri-X film and developed in Diafine.

Results

Characterization of Binding Sites for EGF and Transferrin. KB cells possess binding sites for both EGF and transferrin. We determined that the clone of KB cells we have employed has approximately 80 000 transferrin receptors per cell with an apparent K_D of 2.9 nM when measured on cell monolayers at 4 °C. Binding reaches steady state by 45 min. The same cells have approximately 180 000 EGF receptors per cell with an apparent K_D of approximately 1 nM. Binding of EGF reaches steady state by 4 h at 4 °C (M. Ruff and I. Pastan, unpublished results). The affinity and capacity of ^{125}I-EGF receptors are similar to those previously reported for KB cells (King et al., 1980). Binding parameters of transferrin receptors were also comparable to those previously reported in a variety of cell types (Hamilton et al., 1979).

Release and Degradation of EGF and Transferrin from Cells at 37 °C. The release and degradation of cell-bound EGF (10 nM) and transferrin (3.8 nM) were compared after binding these ligands at 4 °C and then incubating the cells for various times at 37 °C. Figure 2 demonstrates that transferrin is more rapidly released than EGF from cells that were warmed to 37 °C. At 15 min, a small portion of both EGF and transferrin was released. By 40 min, 33% of the EGF and 69% of the transferrin were released. Using chromatography on Sephadex G-25 (see Materials and Methods), we evaluated the degradation of EGF and transferrin (Figure 3). At 40 min, the majority of EGF released into the medium was degraded, whereas very little degradation of transferrin was evident. These data confirm previous observations in a variety of cell types that in contrast to EGF, transferrin is not markedly degraded (Karin & Minz, 1981; Octave et al., 1981, 1982; Renswoude et al., 1982; Van Brockxmeer & Morgan, 1979). These observations also suggest that transferrin and EGF may proceed through different intracellular pathways in KB cells.

Density Gradient Centrifugation of Cell Fractions on Colloidal Silica. As a first step in analyzing the intracellular location of EGF and transferrin at various times after entry, the cells were homogenized and the membranes fractionated on gradients of colloidal silica (Percoll) (Miskimins & Shimizu, 1982). Colloidal silica was chosen because of the ease, rapidity, and reproducibility of gradient formation and because of the separations obtainable between lysosomes at high density and other cellular membranes at low density. Figure 1 shows the location of various markers for subcellular fractions using 20% Percoll gradients.

Experiments comparing the subcellular fractions containing EGF and transferrin were carried out in two ways. ^{125}I-EGF (10 nM) or ^{125}I-transferrin (3.8 nM) was separately bound to monolayers of cells at 4 °C, and then the cells were washed and warmed to 37 °C for various times. Cells were homogenized at each time point and analyzed on colloidal silica gradients. Experiments shown in Figure 4 were carried out by a variation of the protocol described above using ^{125}I-transferrin and ^{131}I-EGF bound to the same cell monolayers and analyzed by using double-label counting conditions. Results using single- and double-label procedures were indistinguishable.

Figure 4 demonstrates that initially before warmup to 37 °C, each ligand was concentrated near the top of the gradient. Of the total cell-associated radioactivity at this time, 50–70% was recovered in the low-speed supernatant prior to layering over colloidal silica gradients. The 30–50% of the EGF and transferrin which was lost was probably bound to large plasma membrane sheets which were sedimented and discarded prior to being layered on colloidal silica. After cells were incubated for 5 min or longer, 70% or more of ^{131}I-EGF or ^{125}I-transferrin was recovered in the low-speed supernatant. When the cell-associated radioactivity from a 5-min point was analyzed on a colloidal silica gradient, EGF and transferrin had nearly identical sedimentation profiles; there was a broad peak of radioactivity with a peak at 1.039 g/mL which returned to a base line of 1.070 g/mL density. After 15 min, the transferrin lost from this peak was not present in other fractions but began to appear in the medium (Figure 4). In contrast, the peak of EGF broadened and shifted to a density of 1.052 g/mL. By 40 min, very little cell-associated transferrin remained. In contrast, most of the EGF was retained in the cells and had moved to a peak of 1.08 g/mL (Figures 4 and 5).

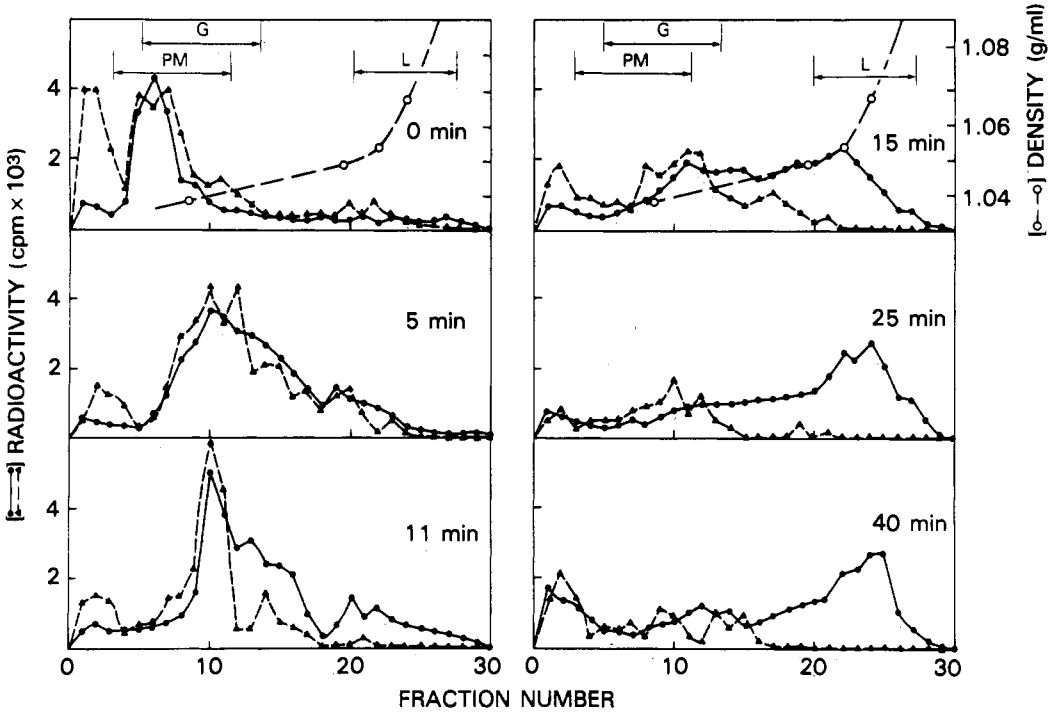

FIGURE 4: Kinetics of transfer of ^{125}I-transferrin and ^{131}I-EGF between intracellular compartments as determined by equilibrium density centrifugation. In a double-label study, cells were incubated with EGF or transferrin at 4 °C. Cells were washed and warmed to 37 °C for various times. After homogenization and fractionation on Percoll gradients, the amounts of ^{125}I and ^{131}I were determined by using double-label counting conditions. The radioactivity associated with each ligand is plotted in the figure. Density marker beads were used to determine gradient density. The solid line indicates EGF while the broken line indicates transferrin. Abbreviations for the gradient regions are as follows: PM, plasma membrane [composite of the results obtained for (Na^+, K^+)-ATPase activity, 5′-nucleotidase activity, surface lactoperoxidase catalyzed iodination, and 4 °C ^{125}I-EGF binding]; G, Golgi (galactosyltransferase activity); L, lysosomes (β-hexosaminidase and β-galactosidase activities).

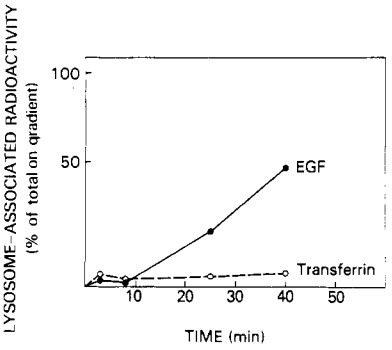

FIGURE 5: Kinetics of the appearance of EGF and transferrin in lysosomes. Using ^{125}I-EGF or ^{125}I-transferrin, we quantified the amount of radioactivity cosedimenting with lysosomes in Percoll gradients for each ligand and expressed it as a percent of the total amount of ligand recovered from the gradient.

The dense EGF peak clearly cosediments with lysosomal enzyme activities. These data for EGF confirmed previous data with Percoll gradients indicating that EGF is eventually transferred to lysosomes (Miskimins & Simizu, 1982) and emphasize how differently EGF and transferrin are processed.

A low concentration (3.8 nM, used above) and a high concentration (125 nM) of ^{125}I-transferrin were compared for their processing by cells (data not shown). Cells were incubated at 4 °C for 1 h with either concentration of transferrin, washed, and warmed to 37 °C for 5, 11, 15, and 40 min. At both concentrations, the binding was receptor specific because excess unlabeled transferrin competed for binding. Analysis of cellular fractions on Percoll demonstrated that over the time course studied, there was no significant difference between the sedimentation profiles at either transferrin concentration. At neither concentration was there significant accumulation of radioactive transferrin in the lysosomal region of the gradient.

In other studies, the sedimentability of radioactivity from various gradient regions (Figure 4) was evaluated by high-speed ultracentrifugation. Gradient fractions were diluted in TES buffer and centrifuged for 30 min at 100000*g*. At the 5-min time point, 90% or more of the ^{131}I-EGF and ^{125}I-transferrin in fractions 1–5 was not sedimentable. This indicates that the ligand in this region neither was within an organelle nor was bound to a receptor. In a pool from fractions 10–15, 80% or more of the EGF and transferrin was sedimentable, indicating that the ligands either were in an organelle or were bound tightly to a receptor. At the 40-min time point, 70% of the EGF from a pool of fractions 20–25 was sedimentable.

Fluorescence Microscopy. The above data demonstrate that after a 5-min incubation of cells with ^{131}I-EGF and ^{125}I-transferrin, the vesicles containing the two ligands cosedimented. To determine if both ligands might be contained in the same endocytic vesicles or different vesicles with the same density at the different time periods of incubation, a fluorescence experiment in living cells was carried out. Cells were preincubated at 4 °C with EGF–HRP or transferrin, warmed to 37 °C for various times, fixed, and permeabilized, and their location was determined with fluorescein- or rhodamine-labeled antibodies directed against the two different ligands (see Materials and Methods).

Figure 6 shows a time course of the double-label study. After 1 min at 37 °C, both transferrin fluorescence and EGF fluorescence were in a diffuse pattern over entire cell images with a higher apparent intensity between adjacent cells. This indicates binding at the plasma membrane. The brighter RH–transferrin signal allowed resolution of a fine punctate pattern possibly consistent with transferrin being concentrated in coated pits. Five minutes after being warmed to 37 °C, both ligands were in a bright punctate pattern suggestive of re-

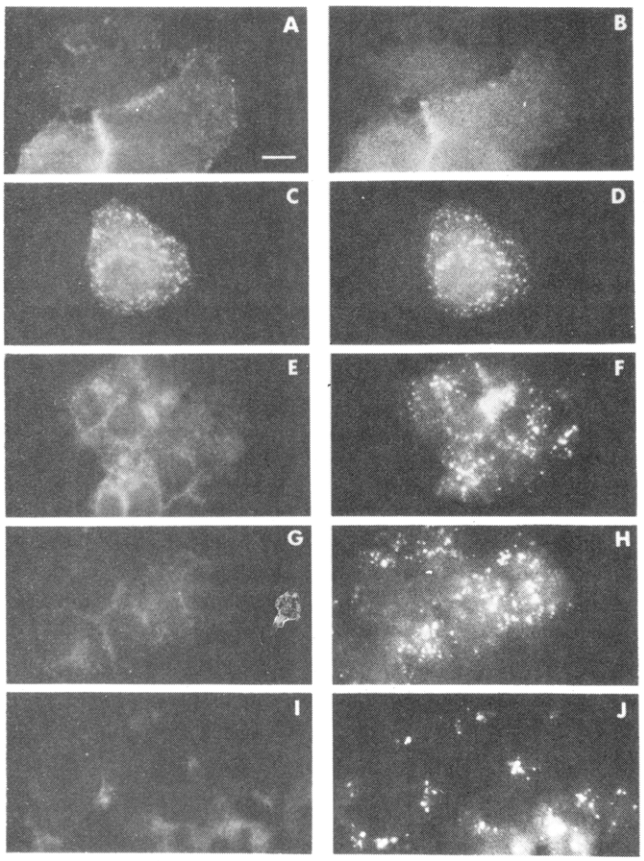

FIGURE 6: Kinetics of intracellular transfer of EGF and transferrin as determined by immunofluorescence. Cells were incubated with transferrin and EGF–HRP at 4 °C, washed, and warmed to 37 °C for various lengths of time. Cells were then fixed and permeabilized. Ligands were visualized with fluorescein-labeled antibodies (directed against HRP) and with indirect rhodamine-labeled antibodies against transferrin. Cell were directly photographed by using fluorescence microscopy with filters for selective detection of rhodamine or fluorescein. Panels A, C, E, G, and I show rhodamine–transferrin, while panels B, D, F, H, and J show fluorescein–EGF. Times after warmup are as follows: 1 (A and B), 5 (C and D), 10 (E and F), 20 (G and H), and 30 min (I and J). The bar represents 10 μm.

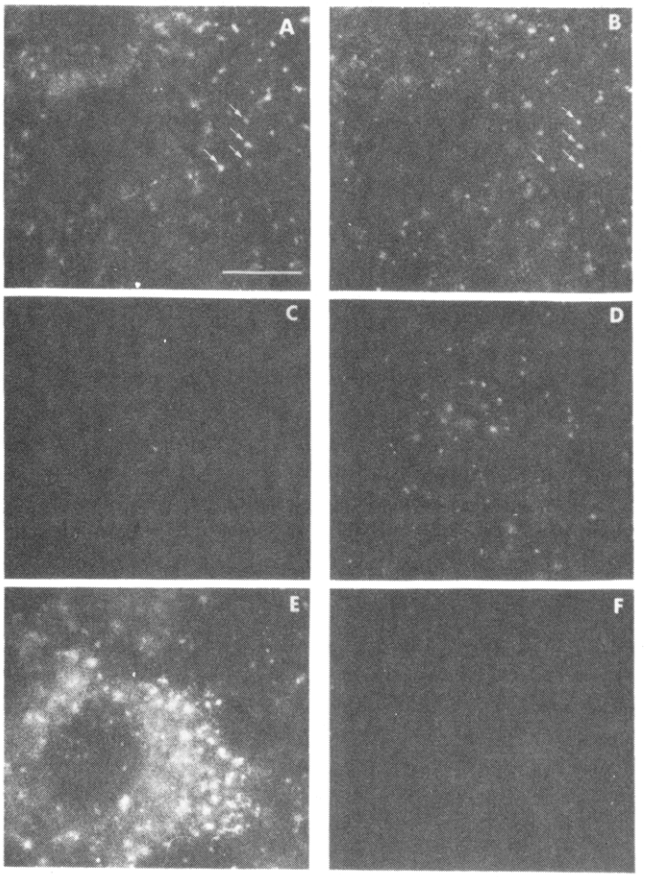

FIGURE 7: Analysis of the extent of coincidence of EGF- and transferrin-containing vesicles in cells. Cells were incubated at 4 °C to allow for ligand binding, washed, and then warmed to 37 °C for 5 min to allow interalization to occur. Fluorescence visualization was carried out as described above for EGF and transferrin. In panels E and F, EGF was deleted and in panels C and D transferrin was deleted as controls for fluorescence crossover. The bar represents 10 μm. Arrows in panels A and B indicate coincident localization of EGF and transferrin fluorescence.

ceptosomes (Dickson et al., 1982). Ten minutes after being warmed, EGF was still brightly punctate, with many cells showing a perinuclear concentration of the bright foci. The transferrin pattern was also punctate, but with an additional diffuse or granular appearance. Fluorescence was also often concentrated in perinuclear regions. Twenty minutes after warming, the transferrin fluorescence was markedly diminished; only low levels of perinuclear fluorescence were noted. At 20 and 30 min, the EGF fluorescence was still markedly punctate.

It has been previously shown that 5 min after entry, EGF is found in the receptosomes of KB cells (Willingham & Pastan, 1982; FitzGerald et al., 1983; Willingham et al., 1983). To determine if transferrin was in the same vesicles as EGF, photographic prints of the 5-min point were enlarged and analyzed for the presence of EGF and transferrin in the same vesicle (Figure 7A,B). From the micrographs, it appears that the coincidence of transferrin and EGF was nearly complete after 5 min. To quantify the coincidence, Fl–EGF spots were plotted on a transparent overlay and then compared with RH–transferrin spots. Of 274 spots examined, 266 were coincident, and 8 were not. This is a correspondence of 97%. Although light (or fluorescence) microscopy is of insufficient resolution to define images of endocytic vesicles or coated pits (Willingham & Pastan, 1980), it is likely that the fluoresence coincidence of two ligands may be interpreted as colocalization in the same population of organelles. The fluorescence coincidence has been previously used to demonstrate initial colocalization (4 °C) of receptor-bound rhodamine–α_2M and fluorescein anti-clathrin, followed by their rapid divergence after warming of cells to 37 °C. That α_2M and clathrin initially colocalized at the electron microscopic level was also verified [see Dickson et al. (1982) and unpublished results]. In addition, double-label pairs of fluorescent and electron-dense labeled ligands have been previously studied in a variety of systems (Via et al., 1982; Willingham et al., 1981, 1983). In each of these studies, colocalization in receptosomes of ligands (α_2M, EGF, low-density lipoproteins, and lysosomal enzymes) by fluorescence coincidence was verified at the electron microscopic level. Figure 7C–F demonstrates that deletion of either transferrin or EGF does not change the fluorescence pattern of the other ligand. This indicates that there is very little crossover of fluorescence from the rhodamine to the fluorescein channel.

Discussion

We have used two different methods to compare the intracellular route followed by transferrin and EGF during receptor-mediated endocytosis of these ligands. It is already known (Willingham & Pastan, 1982; FitzGerald et al., 1983; Willingham et al., 1983) from morphologic studies of KB cells that at 5 min EGF is present in receptosomes, at 10–12 min it begins to appear in the transreticular portion of the Golgi

elements, and a few minutes later it begins to accumulate in lysosomes. The density gradient studies shown here indicate that at the time EGF is known from these previous studies to be in receptosomes (5–10 min) and Golgi elements (10–14 min), it is present in a membrane fraction with a density of about 1.04. At 15 min, EGF began to appear in organelles with the density of lysosomes.

At 5 min after entry, transferrin was found associated with membranes of the same density as EGF. Fluorescence experiments provided strongly suggestive evidence that transferrin and EGF were in the same vesicles (Figures 6 and 7). At 10–11 min, both ligands were still cell associated (Figure 2) and still in a low-density membrane fraction although some heterogeneity was observed (Figure 4, 11 min). At 10–15 min after entry, EGF is known from electron microscopic studies to be entering Golgi elements (Willingham & Pastan, 1982). The precise location of transferrin at this time remains to be established. Starting at the 15-min point (Figure 4), the divergence of EGF and transferrin, as observed by subcellular fractionation, was striking. The amount of transferrin found in the cell diminished, and it appeared undegraded in the medium; little transferrin was detected in the lysosomal fraction of the gradient (Figures 2–5). On the other hand, EGF was beginning to accumulate in the lysosomal fraction at 15 min (Figures 4 and 5). With time, the majority of the EGF accumulated in lysosomes where it was degraded, whereas transferrin accumulated in the medium.

The above studies indicate that EGF and transferrin undergo a prelysosomal divergence after being internalized in receptosomes. One possible mechanism for this divergence is that both ligands enter the Golgi system (Willingham & Pastan, 1982) and sorting occurs in the reticular portion of the Golgi system. Another mechanism is that transferrin proceeds from receptosomes back to the cell surface in some other organelle. Electron microscopy should help to clarify this point. A recent study from our laboratory has localized transferrin–HRP first in endocytic vesicles and then in trans-Golgi-associated tubules and vesicles which appear to participate in its return to the plasma membrane (Willingham et al., 1983b). It is of interest that in vivo in rat liver, asialotransferrin is endocytosed (following binding, presumably, to the asialoglycoprotein receptor), resialated, and then released back into the circulation undegraded. Resialation presumably involved modification in Golgi elements (Regoeczi et al., 1982; Debanne et al., 1982). In addition, a variety of other ligands have been observed in association with Golgi structures following their endocytosis by cells (Willingham et al., 1980, 1981; Kahn et al., 1982; Herzog & Farquhar, 1977; Ottosen et al., 1980).

The molecular signals and events in the intracellular divergence of EGF and transferrin are also not known. It has been reported that the EGF–receptor complex is acid dissociable (Haigler et al., 1980), whereas the transferrin–receptor complex is not (Wada et al., 1979). Thus, in acidic receptosomes, it is likely that EGF would dissociate from its receptor while transferrin remains receptor bound. Therefore, receptor-bound transferrin and soluble EGF could be separated upon Golgi (or other intracellular compartment) transfer. Since the initial submission of this paper, three studies have been published which may have begun to shed light on the sorting process (Geuze et al., 1983; Klausner et al., 1983; Dautry-Varsat et al., 1983). The latter two of these studies provide support for the concept that transferrin, in contrast to asialoglycoprotein, probably does not dissociate from its receptor in acidic endocytic vesicles. Following iron release (promoted by low pH), transferrin is returned to the cell surface where the near-neutral pH and a low extracellular ligand concentration favor its dissociation and release into the media. The intracellular routing of the receptors for EGF and transferrin is not yet known. To date, only the asialoglycoprotein–receptor complex has actually been demonstrated to dissociate from its receptor intracellularly in a prelysosomal compartment (Bridges et al., 1982). However, a recent double-label electron microscopic study on the intracellular localization of asialoglycoprotein and its receptor in hepatocytes may shed light on the fate of receptors as well as the sorting process of transferrin and EGF (Geuze et al., 1983). While endocytosed asialoglycoprotein was localized in endocytic vesicles and lysosomes, only its receptor appeared to be highly concentrated in tubular extensions of some of these endocytic vesicles and in an extended tubular system. The receptor-rich, tubular network (termed CURL for compartment of uncoupling of receptor and ligand) was proposed as an intermediate in receptor recycling. Since the endocytic vesicles are also known to eventually transfer some of their contents to lysosomes (Goldstein et al., 1979; Pastan & Willingham, 1981b), it was proposed that the endocytic vesicles with receptor-rich tubular extensions represent evidence of a sorting compartment. Confirmation of this hypothesis awaits detailed time course studies. It is possible that sorting of transferrin from EGF occurs through a similar system in KB cells. CURL appears very similar to a system of vesicles and tubules containing transferrin–horseradish peroxidase prior to plasma membrane delivery (Willingham et al., 1983b).

We believe the present study provides the first direct examination of the site of intracellular sorting of two physiologically important ligands taken into cells in the same vesicles during receptor-mediated endocytosis. A previous study (Abrahamson & Rodenwald, 1981) has reported that in newborn rat intestinal epithelium, Fc receptor bound IgG and horseradish peroxidase taken in by "fluid phase" endocytosis were present in the same vesicles. Following interlization, horseradish peroxidase was transferred to apical lysosomes, while intact IgG was transferred to the lateral plasma membrane and released into the circulation. Interestingly, like transferrin, the IgG–receptor complex is reported to be acid stable. In this system, however, divergence of the two markers was only partial; approximately 50% of the endocytosed IgG was transferred to lysosomes and degraded. The intracellular site of divergence of IgG and horseradish peroxidase was not identified. Preliminary studies in another laboratory have suggested an analogous divergence of asialoglycoproteins from polymeric IgA in hepatocytes (Quintart et al., 1983).

In summary, using fluorescence microscopy and gradient fractionation, we demonstrate for the first time that two physiological ligands can be independently sorted after endocytosis in a common class of prelysosomal vesicles. The time course of divergence of the two ligands is consistent with a prelysosomal and possibly Golgi-associated sorting process.

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