The Role of Intermediate Vesicles in the Adsorptive Endocytosis and Transport of Ligand to Lysosomes by Human Fibroblasts

MICHAEL MERION and WILLIAM S. SLY
The Edward Mallinckrodt Department of Pediatrics, Washington University School of Medicine, Division of Medical Genetics, St. Louis Children's Hospital, St. Louis, Missouri 63110

ABSTRACT Recent work from several laboratories has suggested the participation of intermediate structures in the delivery of adsorbed ligands from the plasma membrane to lysosomes. This report presents subcellular fractionation studies bearing on the role of these structures in adsorptive pinocytosis of epidermal growth factor (EGF), β-hexosaminidase, and low density lipoprotein (LDL) by human fibroblasts. Using a two-step Percoll density gradient fractionation, we identified newly internalized (5 min) EGF in two intermediate density structures that are essentially negative for plasma membrane marker, and more bouyant than secondary lysosomes. Continued incubation for 20 min resulted in transfer to (or conversion to) vesicles sedimenting with secondary lysosomes. Internalized β-hexosaminidase and LDL behaved similarly, appearing first in structures of intermediate density, and later appearing in association with secondary lysosomes. Two drugs, NH_4Cl and monensin, were found to inhibit ligand transfer to the secondary lysosome peak, although they did not inhibit entry of bound ligands into intermediate density structures. Upon removal of both inhibitors, internalized ligands were quickly transferred to the secondary lysosome peak. This "transfer process" was faster for EGF, than for the other two ligands studied. We interpret these data to indicate that the endocytosis of these three ligands, and their delivery to lysosomes in fibroblasts, proceeds through a common pathway, involving intermediate nonlysosomal structures.

Adsorptive pinocytosis of peptide hormones (1, 2), lysosomal enzymes (3), low density lipoprotein (LDL) (4), and asialoglycoproteins (5) utilizes high-affinity cell surface receptors specific for each ligand. Ligands bind to these receptors which, if not preclustered, rapidly cluster, following binding of ligand, into specialized regions of the plasma membrane called coated pits (4). Endocytosis occurs at this site, and newly internalized ligands appear first in coated vesicles that form as endocytic vesicles that pinch off from the plasma membrane. The clathrin coat is quickly lost, and the ligand-containing smooth membrane vesicles, which are cytochemically negative for the lysosomal activity aryl sulfatase (5), soon appear in the area of the Golgi apparatus, and later in secondary lysosomes (4). Morphological studies of the internalization of the ligands insulin, epidermal growth factor (EGF), and α_2 macroglobulin (6), as well as the lysosomal enzyme β-galactosidase (7), have led investigators to propose (8) a common nonlysosomal intermediate structure in the delivery of plasma membrane-bound ligand to secondary lysosomes. Recent studies suggest that this prelysosomal compartment is acidified (9).

Kinetic and morphological studies of the uptake of LDL (4), lysosomal enzymes (10), asialoglycoproteins (11), and high-mannose type glycoproteins (12) have indicated that their receptors are reutilized and participate in several rounds of ligand internalization. Exposure of cells to lysosomotropic amines such as NH_4Cl and chloroquine (10), as well as to the ionophore monensin (13), all of which have been shown to raise the pH of normally acidic intracellular compartments (9, 14), inhibits receptor recycling and results at least in part in the trapping of cell surface receptors within an internal pool. Concomitant incubation with ligand makes this effect even more pronounced (13).

Recently, it was found, using subcellular fractionation techniques (15), that the internalization of the plant lectin *Wistaria floribunda* agglutinin by murine fibroblasts, which is also mediated through coated regions of the plasma membrane (16),

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proceeds through two prelysosomal structures that can be demonstrated by subcellular fractionation utilizing a series of two Percoll gradients. These structures are localized in the area of the Golgi apparatus, vary in size from 0.2 to 0.4 μm, and are empty in appearance. Similar results were obtained in the study of the internalization of insulin by rat liver (17). In this paper we use similar subcellular fractionation techniques to show that at least three physiologically important ligands that are subject to receptor-mediated endocytosis by human fibroblasts share a common pathway in their internalization, and we conclude that intermediate vesicles play a role in the delivery of ligands from the cell surface to secondary lysosomes.

MATERIALS AND METHODS

Enzyme Assays: Lysosomal enzyme activities were determined fluorometrically (18). Substrates were purchased from Sigma Chemical Co. (St. Louis, MO). β-Glucuronidase was measured using 4-methylumbelliferyl-β-D-glucuronide at 10 mM in 0.1 M sodium acetate, pH 4.8. β-Hexosaminidase was assayed using 4-methylumbelliferyl-2-acetamide-2-deoxy-β-O-glucopyranoside at 5 mM in 0.08 M sodium phosphate-citrate, pH 4.4. Acid phosphatase activity was measured with the substrate 4-methylumbelliferyl phosphate at 1 mM in 0.1 M sodium citrate, pH 5.1. β-Galactosidase activity was determined using the substrate 4-methylumbelliferyl-β-D-galactoside at 1 mM in 0.08 M citrate-phosphate, pH 4.4. α-Mannosidase activity was determined using 4-methylumbelliferyl-β-D-mannoside at 1 mM in 0.05 M citrate, pH 4.0. Assays were carried out with the addition of 25 μl of enzyme to 100 μl of substrate, and incubation at 37° for 30 min. All reactions were carried out in the presence of 0.1% Triton X-100. Reactions were terminated with the addition of 1 ml of 50 mM glycine/NaOH at pH 10.5 containing 5 mM EDTA.

Protein was determined fluorometrically using fluorescamine according to the method of Rome et al. (19). Blank values were determined for each fraction by assaying an identical Percoll gradient to which no sample was applied.

NADH reductase activity was determined spectrophotometrially using cytochrome c as an acceptor according to the method of de Duve et al. (20).

Lactoperoxidase-catalyzed Iodination: Lactoperoxidase-catalyzed iodination of cell surface proteins was performed as previously described (15). Cell viability was determined to be 95% following labeling by trypan blue exclusion.

Pentamannosyl Phosphate: Pentamannosyl phosphate was prepared as described previously (21).

Ligands: Epidermal growth factor (EGF) was purchased from Sigma Chemical Co. (St. Louis, MO). Low density lipoprotein (LDL) was generously donated by Dr. G. Shoenfeld (Lipid Research Center, Washington University). Partially purified β-hexosaminidase was prepared as previously described (22).

Bovine Serum Albumin-Pentamannosyl Phosphate Conjugation: BSA was conjugated to pentamannosyl phosphate (BSA-PMP) by a modification of the method of Schwartz and Gray (23). To 1 ml of 50 mM bicine pH 8.6 was added 5 mg of BSA (Sigma Chemical Co., St. Louis, MO), pentamannosyl phosphate to 0.2 M, and 5 mg of $NaCNBH_3$ (Sigma Chemical Co., St. Louis, MO). The mixture was incubated for 24 h at 37°. An additional 5 mg of $NaCNBH_3$ was then added and the incubation continued for an additional 24 h at 37°C. The solution was then extensively dialyzed against 20 mM sodium phosphate, 0.12 M NaCl, pH 7.0.

Ligand Iodination: All ligands were iodinated using insolubilized lactoperoxidase and glucose oxidase (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's recommendations. ^{125}I was obtained from New England Nuclear (Boston, MA) as carrier-free Na ^{125}I.

Cells and Cell Culture: Normal human lung fibroblasts IMR 91 (Institute for Medical Research, Camden, NJ) and β-hexosaminidase-deficient human skin fibroblasts WG 98/EW (Repository for Mutant Human Cells, Montreal, Canada) were used between passages 12 and 18. Cells were cultured in 10-cm diameter petri dishes (Scientific Products Co., Evanston, IL) in Eagle's minimum essential medium (MEM) plus 15% heat-inactivated fetal bovine serum at 37°C and pyruvate, penicillin, and streptomycin in an atmosphere of 5% CO_2. All cells were used 2–4 d after reaching confluence.

Internalization Studies: Cells were incubated with ligands under varying conditions in 2.5 ml of MEM. Incubations with EGF were conducted at 20 ng/ml at a specific activity of 20,000 cpm/ng. Incubations with ^{125}I-LDL were conducted at 1 μg/ml at a specific activity of 100,000 cpm/μg. β-Hexosaminidase uptake was conducted at 4000 U/ml. Incubations with BSA-PMP were conducted at 10 μg/ml at a specific activity of 50,000 cpm/μg.

When NH_4Cl was employed, it was added to cells at 10 mM in 2.5 ml of MEM 15 minutes before addition of the ligand. In studies employing 50 μM monensin, ligand and drug were added to the cells simultaneously.

Cells used for studies of LDL internalization were incubated 24 h before use in Waymouth's medium without serum.

Subcellular Fractionation: Fractionation was conducted using the self-forming gradient medium Percoll (Pharmacia Inc., Piscataway, NJ) by using the method of Merion et al. (15). Cell monolayers were washed three times in 5 ml of 0.25 M sucrose plus 1 mM EDTA (HM) adjusted to pH 7.0 with 1 M KOH. Cells were then scraped from the dish with a rubber policeman in HM and homogenized with a Polytron (Brinkman, Luzern, Switzerland) in 1.0 ml of HM until ~50% of the cells were disrupted as judged by phase contrast microscopy. The resulting homogenate was then subjected to centrifugation at 800 *g* for 10 min, the supernatant reserved, and the pellet washed in HM. The supernatants were combined (1.5 ml) and layered on 9 ml of Percoll solution in HM with a density of 1.07 g/ml. Samples were subjected to centrifugation at 33,000 *g* for 50 min with a 50 Ti rotor in a Beckman L2-ultracentrifuge (Beckman Instruments, Palo Alto, CA). Fractions (0.4 ml) were collected by upward flow displacement in a Buchler fractionator (Buchler Instruments Inc., Fort Lee, NH). Aliquots (0.3 ml) of fractions 8–13 from this first gradient were combined and 0.5 ml of HM was added. This sample was applied to 8 ml of a Percoll solution in HM with a density of 1.044 g/ml and subjected to centrifugation at 33,000 *g* for 20 min to produce a second gradient. Fractions (0.4 ml) were collected as above. All experiments were repeated at least three times, and representative experiments are presented in all figures.

Gamma Determination: ^{125}I was determined with a Packard model 5260 autogamma counter (Packard Instrument Co., Downers Grove, IL).

RESULTS

First Percoll Density Fractionation

In order to examine the interaction of ligands with human fibroblasts, we employed the Percoll density gradient fractionation seen in Fig. 1. In a density range of 1.035–1.080 g/ml, lysosomal enzyme activities are distributed within three peaks. A small amount of activity is found at the top of the gradient (fractions 1–3) where soluble substances are localized (15, 19). A second peak of activity is localized at a peak modal density of 1.040 g/ml (fractions 8–13) and has been suggested by Rome et al. (19) to contain GERL-derived structures. Lysosomal enzyme activity in this peak may also be due to enzyme associated with endoplasmic reticulum, Golgi and plasma membrane, since plasma membrane and endoplasmic reticulum markers also migrate at a peak modal density of 1.040 g/ml (fractions 8–13) and previous work (15, 19) has shown that the Golgi marker galactosyl transferase is also localized here. The majority of lysosomal enzyme activity is localized at a peak modal density of 1.070 g/ml (fractions 21–25), and has been shown to contain typical secondary lysosomes (15, 19).

Internalization of EGF and Transfer to Secondary Lysosomes

In order to examine EGF binding to human fibroblasts without internalization, we incubated ^{125}I-EGF (20 ng/ml) with monolayer cultures at 4°C. Under these conditions EGF binds to cell surfaces, and the binding was >90% inhibitable in the presence of a 100-fold excess of unlabeled ligand (data not shown). ^{125}I-EGF previously bound to cell surfaces at 4°C was >85% elutable by briefly washing cells at 4°C with buffer at pH 3.0 while retaining cell viability (data not shown) as reported previously by Haigler et al. (24). When cells were incubated with ^{125}I-EGF for 10 min at 37°C, and then subjected to an acid wash, only 30% of cell-associated radioactivity was removed. Thus, >60% of the cell-associated activity after 10 min at 37°C is internalized EGF. Fig. 2 presents the radioactivity profiles from cells fractionated with the first Percoll gradient (as in Fig. 1) 10–30 min after exposure to ^{125}I-EGF. In Fig. 2*A* (10 min), a small amount of radioactivity is found at

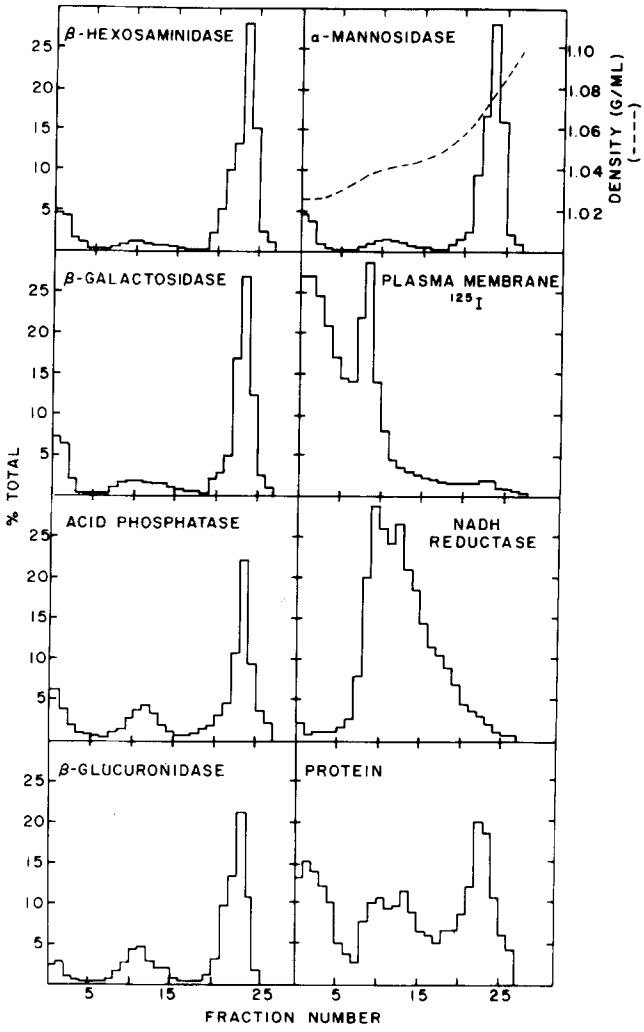

FIGURE 1 First Percoll density gradient fractionation. Normal human fibroblasts in monolayer culture were iodinated using soluble lactoperoxidase at 4°C. A postnuclear supernatant (PNS) was then prepared and subjected to density gradient separation as described in Materials and Methods. Fractions (0.4 ml) were collected by upward flow displacement and activities determined. The density gradient varies from 1.035 to 1.080 g/ml.

the top of the gradient (fractions 1–3) where soluble substances are localized, but the majority of the remaining radioactivity is localized at a peak modal density of 1.040 g/ml (fractions 8–13). When incubation was continued for 20 min (Fig. 2*B*) EGF was seen in these two positions of the gradient (as in Fig. 2*A*), and also in a new peak of radioactivity at a peak modal density of 1.070 g/ml (fractions 21–25), migrating with secondary lysosomes. Continued incubation of ^{125}I-EGF with cells for 30 min (Fig. 2*C*) shows a progressive increase in the fraction of internalized ^{125}I-EGF associated with secondary lysosomes. Prior studies of the internalization of the lectin *Wistaria floribunda* agglutinin by murine fibroblasts using similar subcellular fractionation techniques have also shown that the internalized ligand becomes associated with secondary lysosomes within 30 min of incubation at 37°C. Morphological analysis of fractions containing secondary lysosomes from cells incubated with a ferritin-labeled lectin for 30 min at 37°C shows that WFA is contained within structures with the apparent morphology of secondary lysosomes (15, 16).

The experiments discussed above showed substantial transfer of ^{125}I-EGF to secondary lysosomes within 30 min at 37°C. We next examined both the temperature dependence of this transfer and the effects of two drugs known to affect receptor mediated endocytosis. Fig. 3*A* and *B* show that no transfer to the lysosomal peak occurs during a 30-minute incubation at either 4°C (Fig. 3*A*) or 17°C (Fig. 3*B*). Thus, the transfer is temperature-dependent. A similar inhibition of transfer was observed for the transport of internalized asialoglycoproteins to lysosomes by liver hepatocytes (5). Fig. 3*C* shows that very little transfer occurs in 30 min at 37°C in the presence of

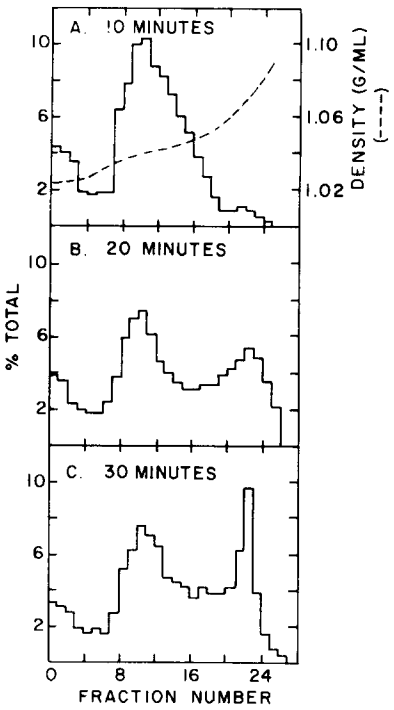

FIGURE 2 Internalization of ^{125}I-EGF in the first density gradient fractionation. Normal human fibroblasts were incubated with ^{125}I-EGF (20 ng/ml) in serum-free media at 37°C for: (*A*) 10 min; (*B*) 20 min; (*C*) 30 min. Cells were then subjected to density gradient separation as in Fig. 1 and radioactivity was determined.

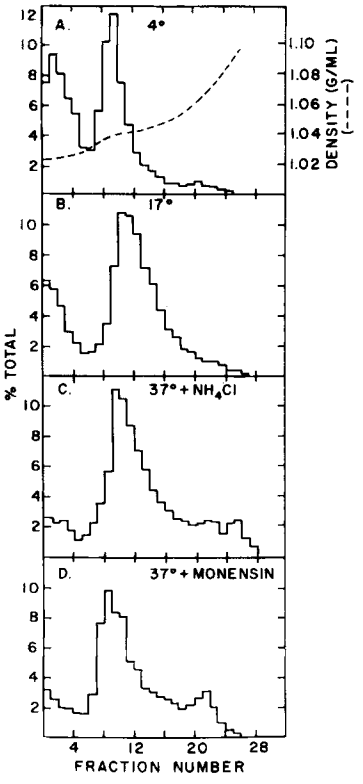

FIGURE 3 Normal human fibroblasts were subjected to density gradient fractionation as in Fig. 1 following incubation with ^{125}I-EGF under varying conditions. (*A*) 4°C; (*B*) 17°C; (*C*) 37°C in the presence of 10 mM NH_4Cl; (*D*) 37°C in the presence of 50 μM monensin.

NH_4Cl. Under these conditions, the rate of accumulation of cell-associated EGF was not affected, but the ligand localized in lighter density fractions. Similarly, incubations of cells with ^{125}I-EGF for 30 min at 37°C in the presence of monensin resulted in the inhibition of transfer of ligand to the lysosomal peak. None of these treatments significantly affected the lysosomal enzyme distribution in these gradients.

To determine whether ^{125}I-EGF internalized in the presence of NH_4Cl and monensin could be transferred from these compartments to secondary lysosomes when the drugs were removed, we allowed cells to accumulate ^{125}I-EGF for 30 min in the presence of the drugs and fractionated them at different time intervals following their removal. However, to eliminate cell surface bound EGF, we washed the cells briefly at pH 3.0 before the postincubation. This treatment removed 30% of cell-associated radioactivity in both cases, indicating that at least 60% of cell-associated radioactivity was contained within some intracellular compartment. Cells were then harvested immediately (Fig. 4*A* and *B*) or following postincubation in serum-free media for 15 (Fig. 4*A* and *B*), and 30 (Fig. 4*A* and *B*) min. Cells harvested immediately following incubation with EGF and drugs showed that very little ligand had entered the lysosomal peak as was the case in the experiment seen in Fig. 3. However, 15 min following the removal of the drug and the ligand, ~50% of cell-associated radioactivity was localized with secondary lysosomes (fractions 21–25). After 30-min incubation following removal of the drugs and the ligand, ~70% of cell-associated radioactivity was localized with secondary lysosomes. Thus, the inhibition of transfer of EGF from intermediate vesicles to lysosomes by these two drugs is reversible.

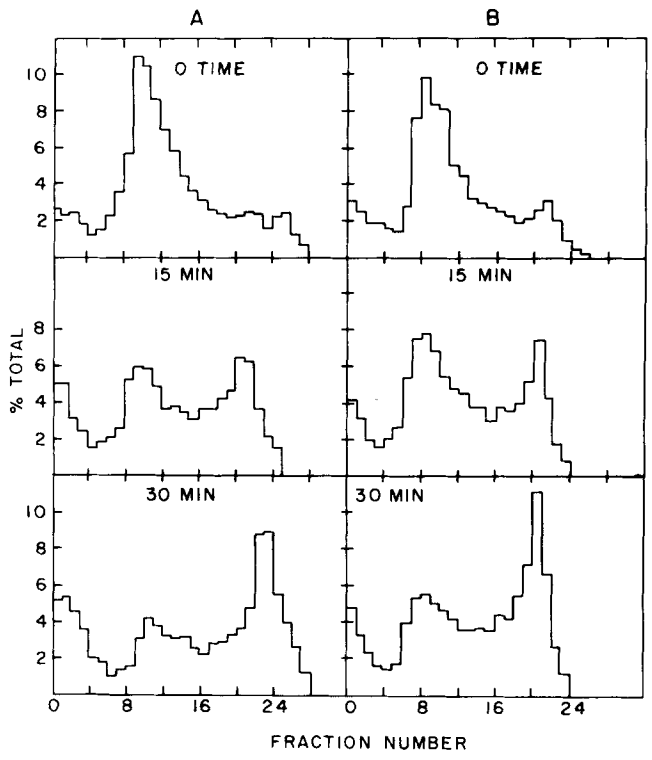

FIGURE 4 Transfer of ^{125}I-EGF to secondary lysosomes following removal of inhibitors. Normal human fibroblasts were incubated with ^{125}I-EGF (20 ng/ml) in serum-free media at 37°C in the presence of 10 mM NH_4Cl (*A*) and 50 μM monensin (*B*). Cultures were then washed in buffer at pH 3.0 for 5 min at 4°C to remove plasma membrane-bound ligand and either harvested immediately (0 time postincubation), or incubated at 37°C in serum-free media for 15 (15 min postincubation) and 30 (30 min postincubation) min. Cells were then subjected to density gradient separation as in Fig. 1 and radioactivity was determined. In each frame the time, postincubation, is given.

Transfer of β-hexosaminidase, ^{125}I-BSA-PMP, and ^{125}I-LDL into Secondary Lysosomes

In order to examine other ligand receptor systems, we selected the internalization of β-hexosaminidase by β-hexosaminidase-deficient fibroblasts and ^{125}I-BSA-PMP by normal human fibroblasts for study of the phosphomannosyl receptor system. β-Hexosaminidase internalization was chosen because it represents the pathway of a physiological ligand. ^{125}I-BSA-PMP internalization was chosen to examine the same pathway in normal (i.e., not β-hexosaminidase deficient) fibroblasts. ^{125}I-LDL internalization by normal human fibroblasts was also chosen for study because this pathway represents the best characterized of receptor-mediated endocytosis pathways. In these experiments an approach similar to that used for the study of EGF internalization was employed. Cells were incubated with β-hexosaminidase (Fig. 5*A*), ^{125}I-BSA-PMP (Fig. 5*B*), and ^{125}I-LDL (Fig. 5 *C*) for 30 min at 37°C in the presence of 10 mM NH_4Cl and either fractionated immediately following the incubation (Fig. 5, 0 time postincubation), or following a postincubation in serum-free media at 37°C for 15 (Fig. 5, 15 min. Postincubation) or 30 (Fig. 5, 30 min. Postincubation) minutes. In all cases ligand was found with secondary lysosomes (fractions 21–25) at 30-, but not 15-min postincubation period. Identical results were obtained when 50 μM monensin was substituted for NH_4Cl (data not shown). These results show three things: (*a*) Both NH_4Cl and monensin block the accumulation of ^{125}I-EGF within typical secondary lysosomes. (*b*) Transfer of internalized ligands to lysosomes occurs following removal of drug inhibitors. (*c*) Transfer to lysosomes of EGF following removal of drug inhibitors occurs more quickly than for the other studied ligands.

Simultaneous Internalization of ^{125}I-EGF and β-Hexosaminidase

In order to verify the differential rate of transfer of EGF and β-hexosaminidase to secondary lysosomes, we incubated the two ligands simultaneously with β-hexosaminidase-deficient fibroblasts for 30 min at 37°C in the presence of 10 mM NH_4Cl. Cells were then either harvested immediately following this incubation (Fig. 6*A*), or following postincubation in serum-free media for 15 (Fig. 6*B*) or 30 (Fig. 6 *C*) min. Postincubation for 15 min results in EGF localization within secondary lysosomes (Fig. 6*B*, fractions 21–25), while a 30-min postincubation is required for the appearance of β-hexosaminidase in these fractions (Fig. 6 *C*, fractions 21–25).

Ligand Internalization Into Intermediate Structures

In order to examine more closely the initial steps of ligand internalization, we conducted further fractionation of fractions 8–13 from the first Percoll gradient (Fig. 1) on a second Percoll gradient. The enzymatic profile of this gradient is seen in Fig. 7. Plasma membrane marker is unimodal in distribution at a peak modal density of 1.035 g/ml (fractions 4–7). NADH reductase activity is distributed in a broad peak between 1.037 and 1.041 ml (fractions 4–13), as well as in a distinct peak at

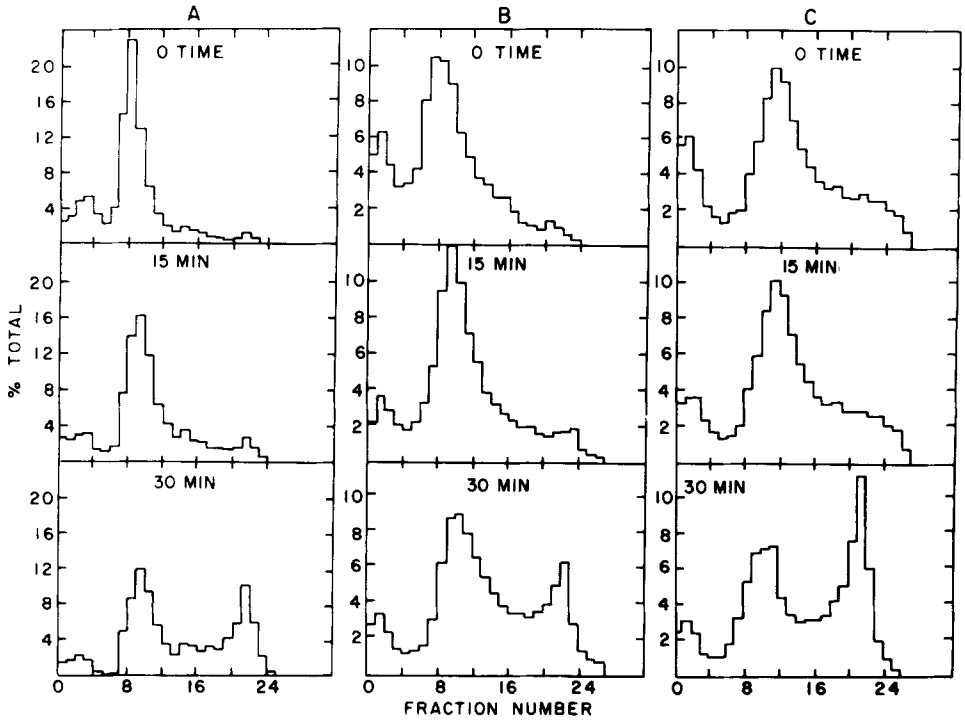

FIGURE 5 Transfer of β-hexosaminidase, ^{125}I-BSA-PMP, and ^{125}I-LDL to secondary lysosomes. (*A*) β-hexosaminidase-deficient human fibroblasts were incubated with β-hexosaminidase (4,000 U/ml) at 37°C for 30 min in the presence of 10 mM NH_4Cl. (*B*) Normal human fibroblasts were incubated with ^{125}I-BSA-PMP (10 μg/ml) at 37°C for 30 min in the presence of 10 mM NH_4Cl. (*C*) Normal human fibroblasts were incubated with ^{125}I-LDL (1 μg/ml) at 37°C for 30 min in the presence of 10 mM NH_4Cl. Cultures were then harvested immediately or incubated at 37°C in serum-free media for 15 or 30 min. All cells were then subjected to density gradient separation as in Fig. 1. In each frame the time, postincubation, is given.

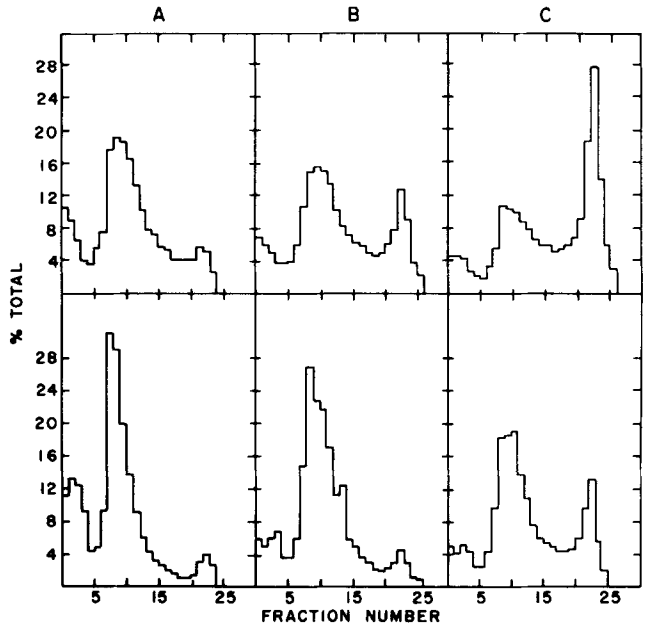

FIGURE 6 Transfer of ^{125}I-EGF and β-hexosaminidase to secondary lysosomes. β-Hexosaminidase-deficient human fibroblasts were incubated with ^{125}I-EGF (20 ng/ml, top panels) and β-hexosaminidase (4,000 U/ml, bottom panels) at 37°C in serum-free media for 30 min in the presence of 10 mM NH_4Cl. Cultures were than either fractionated immediately (*A*) as in Fig. 1 or incubated at 37°C in serum-free media for 15 (*B*) or 30 (*C*) min and then fractionated as in Fig. 1. In these experiments, no pH stripping was employed. Radioactivity and β-hexosaminidase activity were then determined.

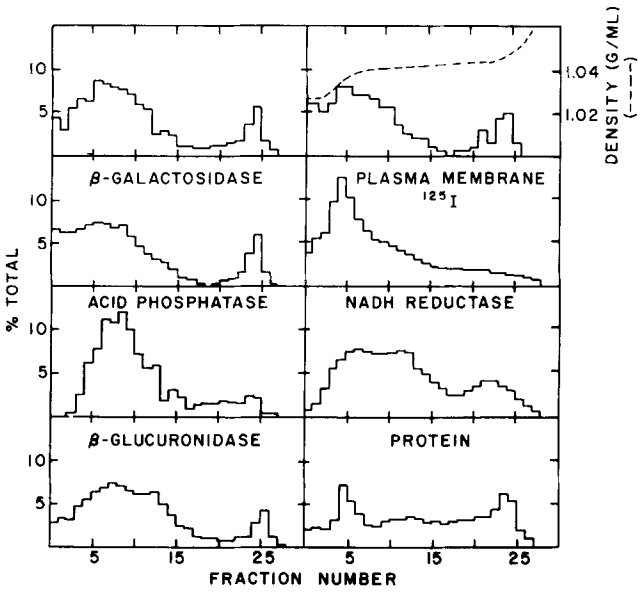

FIGURE 7 Second Percoll density gradient fractionation. Fractions 8–13 from the first gradient as presented in Fig. 1 were combined and subjected to density gradient separation as described in Materials and Methods. Fractions (0.4 ml) were collected by upward flow displacement and activities determined. Densities vary between 1.025 and 1.050 g/ml. Left column, from top to bottom: β-hexosaminidase; β-galactosidase; acid phosphatase; β-glucuronidase. Right column, from top to bottom: α-mannosidase; plasma membrane ^{125}I; NADH reductase; protein.

1.043 g/ml (fractions 20–24). Lysosomal enzyme activities are distributed in a broad peak from 1.035 to 1.041 g/ml (fractions 4–12) and, with the exception of acid phosphatase, in a second peak at 1.045 g/ml (fractions 24–26) that contains between 10 and 15% of the total activity on the second gradient.

This fractionation was employed to examine the initial steps in the internalization of EGF by human fibroblasts. ^{125}I-EGF was incubated with normal human fibroblasts at 37°C for 1, 3, and 5 min. Cells were fractionated as in Fig. 1, and fractions 8–13 were pooled and fractionated as in Fig. 7. The profiles of radioactivity for the second gradient fractionation are seen in Fig. 8. Following a 1-min incubation, radioactivity is unimodal in distribution at a density of 1.035 g/ml (fractions 4–7) and migrates in the position seen for plasma membrane marker (Fig. 7). Following a 3-min incubation, in addition to the peak

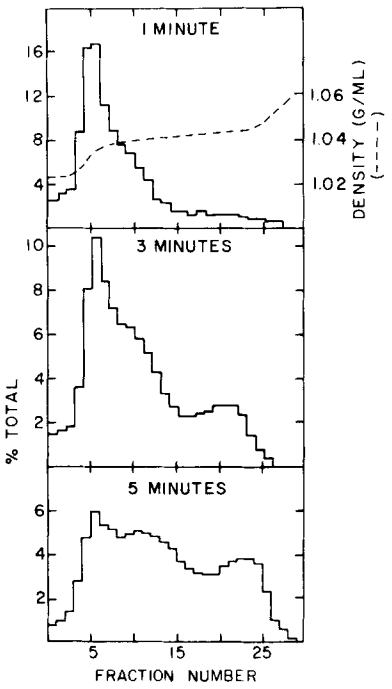

FIGURE 8 Analysis of internalization of ^{125}I-EGF on the second Percoll density gradient. Normal human fibroblasts were incubated with ^{125}I-EGF (20 ng/ml) in serum-free media at 37°C for 1, 3, and 5 min. In these experiments, no pH stripping was employed. Cells were then subjected to gradient separation as in Fig. 1 (not shown). Fractions 8–13 were then pooled and subjected to further fractionation as in Fig. 7, and radioactivity was determined. *Top*, 1 min; *middle*, 3 min; *bottom*, 5 min.

at 1.035 g/ml (fractions 4–7), radioactivity also appears on a shoulder of this peak that extends to 1.041 g/ml (fractions 8–13), and in an additional peak at 1.043 g/ml (fractions 20–23). Following 5-min incubation three distinct radioactive peaks are seen at densities of 1.035 g/ml (fractions 4–7), 1.041 g/ml (fractions 10–13), and at 1.043 g/ml (fractions 21–25). The peak of radioactivity localized at 1.043 g/ml was consistently two to three fractions less dense than the peak of lysosomal enzyme activity localized at 1.045 g/ml. When cells were incubated with ^{125}I-EGF for 30 min at 17°C and fractionated as described above, radioactivity peaks were also detected at densities of 1.035, 1.041, and 1.043 g/ml (data not shown).

To determine whether the other ligands studied are also internalized into intermediate nonlysosomal structures labeled with EGF, we treated cells with ligand for 30 min at 37°C in the presence of 10 mM NH_4Cl. Cultures were then fractionated as in Fig. 1, and fractions 8–13 pooled and fractionated again as in Fig. 7. Activity profiles of this gradient fractionation are seen in Fig. 9. Figure 9*A* shows the distribution of cell-associated β-hexosaminidase from β-hexosaminidase-deficient human fibroblasts allowed to internalize the purified enzyme. β-Hexosaminidase activity is localized at 1.035 g/ml (fractions 4–7), at 1.041 g/ml (fractions 8–12), and at 1.043 g/ml (fractions 19–23). Fractionation of normal human fibroblasts incubated with ^{125}I-EGF (Fig. 9*B*) shows the same three peaks of radioactivity as with β-hexosaminidase (Fig. 9*A*). When cells are washed briefly at an acid pH before fractionation (Fig. 9*C*), the peak of radioactivity at 1.035 g/ml (fractions 4–7) where plasma membrane marker is localized (Fig. 7) is removed. Fractionation of normal human fibroblasts incubated with ^{125}I-LDL is seen in Fig. 9*D*. Again, ligand is localized at densities of 1.035, 1.041, and 1.043 g/ml. Thus, all three ligands appear to enter and accumulate in both of the intermediate compartments in the presence of NH_4Cl.

DISCUSSION

These studies illustrate four important points that merit discussion: (*a*) The three physiological ligands β-hexosaminidase, EGF, and LDL are internalized by human fibroblasts into two prelysosomal intermediate structures that can be isolated by density gradient subcellular fractionation. (*b*) Ligands are transferred from these intermediate structures to secondary lysosomes. (*c*) This "transfer" is inhibited in the presence of both NH_4Cl and monensin and is reversible upon removal of the drug. (*d*) Upon removal of drug inhibitors, EGF is transferred from intermediates to lysosomes faster than the other studied ligands.

The two-step Percoll density gradient separation permitted us to demonstrate EGF quite clearly in two prelysosomal intermediates. These two structures differ slightly in density and are localized in areas of the density gradient that are essentially negative for plasma membrane marker. Both structures are also more bouyant than secondary lysosomes. The heavier of the two intermediates is almost completely lacking in lysosomal enzyme markers, while the area of the gradient containing the lighter intermediate contains a small amount of lysosomal enzyme activity. This activity may be due to the association of lysosomal enzymes with co-migrating endoplasmic reticulum and/or GERL. Alternatively, lysosomal enzymes may be contained within intermediate structures, which could be involved in the transport of newly synthesized enzymes to lysosomes. These structures were also seen to contain internalized β-hexosaminidase, a ligand for the phosphomannosyl receptor, and internalized LDL. Thus, although we do not show directly that ligands are internalized within the same vesicles, the internalization of three physiological ligands appears to proceed through a common pathway. Previous studies on the internalization of the plant lectin *Wistaria floribunda* agglutinin (WFA) have shown that this ligand also shares this endocytic pathway (15). In that study, morphological exami-

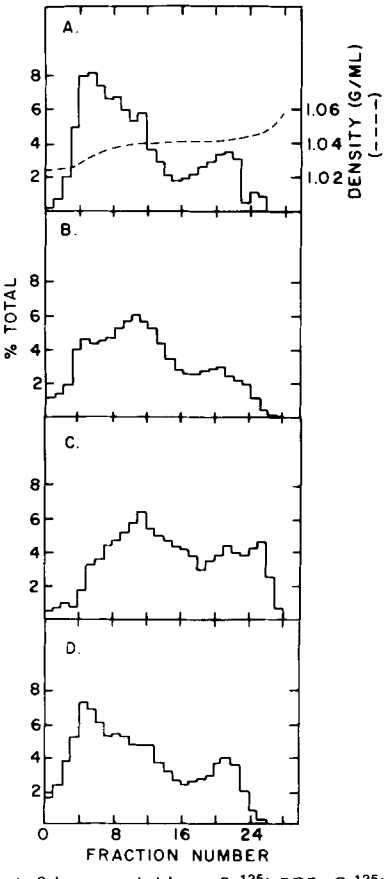

FIGURE 9 Second Percoll density gradient analysis of internalization of β-hexosaminidase, ^{125}I-EGF, and ^{125}I-LDL into intermediate structures. β-Hexosaminidase-deficient human fibroblasts were incubated with β-hexosaminidase (4,000 U/ml) (*A*), and normal human fibroblasts were incubated with ^{125}I-EGF (20 ng/ml) (*B* and *C*), and ^{125}I-LDL (1 μg/ml) (*D*) at 37°C for 30 min in the presence of 10 mM NH_4Cl. Cells in C were then washed at 4°C in buffer at pH 3.0 for 5 min. All cultures were then subjected to density gradient separation as in Fig. 1 (not shown). Fractions 8–13 from these fractionations were then combined and subjected to further fractionation as in Fig. 7. β-Hexosaminidase and radioactivity were then determined. *A*, β-hexosaminidase; *B*, ^{125}I-EGF; *C*, ^{125}I-EGF plus Gly/HCl; *D*, ^{125}I-LDL.

nation of fractions containing internalized lectin-ferritin conjugate revealed that ligand-containing vesicles, derived from an area of the density gradient containing intermediate structures, were bound by a single smooth membrane and varied in diameter from 0.2 to 0.4 μM (16).

These structures appear to be true intermediates in receptor-mediated endocytosis and delivery of ligand to lysosomes. This was clearly demonstrated by removing the ligand associated with the cell surface and showing that the internalized ligands in the intermediates moved to the lysosomal peak on subsequent incubation.

Treatment of cells with ligand in the presence of NH_4Cl or monensin, drugs known to raise the pH of normally acidic intracellular compartments (10, 13, 14), inhibits the appearance of ligand within the dense peak of lysosomal enzyme activity. This may be due to (*a*) inhibition of fusion of intermediate structures with lysosomes, or (*b*) inhibition of a subsequent step in which the fusion product of the intermediate and lysosomes, which is presumably more buoyant than lysosomes, fails to undergo the reduction in volume and/or increase in density required to sediment with secondary lysosomes, or (*c*) failure of the ligand to dissociate from the receptor in the prelysosomal compartment, with consequent failure to segregate with soluble content markers into lysosomes. The results presented here do not distinguish these alternatives.

The slower rates of transfer of LDL and acid hydrolases to lysosomes than are seen for EGF were unexpected. This may reflect a requirement for these ligands to first dissociate from their receptors to be delivered to lysosomes. This dissociation would be both time and pH dependent. Such dissociation from the receptor may not be required for EGF delivery to lysosomes, as this receptor does not recycle back to the cell surface following internalization in most mammalian cell types (2). If this interpretation were correct, it would imply that the dissociation of LDL and acid hydrolases from their pinocytosis receptors is a prelysosomal rather than a lysosomal event and that the site of this dissociation may be the intermediates demonstrated in this study.

Isolation of intermediate structures that participate in the delivery of bound ligand from the plasma membrane to lysosomes provides the opportunity to examine in detail the process of ligand-receptor dissociation, as well as the factors that regulate the recycling and degradation of receptors.

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