Trafficking of the Epidermal Growth Factor Receptor and Transferrin in Three Hepatocytic Endosomal Fractions*

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Hepatocytes rapidly internalize epidermal growth factor (EGF) and transferrin by receptor-mediated endocytosis. Both EGF and its receptor are thought to be targeted for destruction in lysosomes, leading to down-regulation of the receptor, whereas transferrin, after unloading iron within the cell, is thought to recycle to the cell surface bound to its receptor. Previously, we isolated three endosomal fractions from livers of estradiol-treated rats and examined their roles in cellular trafficking of low density lipoproteins (LDL) and the LDL receptor, which cycles constitutively (Belcher, J. D., Hamilton, R. L., Brady, S. E., Hornick, C. A., Jackle, S., Schneider, W. J., and Havel, R. J., Proc. Natl. Acad. Sci. U. S. A. (1987) 84, 6785-6789). In the current study we have taken advantage of the distinct trafficking of the EGF receptor and transferrin to evaluate further the functions of these endosome fractions.

Intravenous injection of a saturating amount of EGF into estradiol-treated rats induced internalization of a single population of EGF receptors, which rapidly accumulated in the endosome fraction of intermediate density ("compartment of uncoupling of receptor and ligand" (CURL)) and subsequently in the low density endosome fraction (multivesicular bodies (MVBs)). The high density endosome fraction of estradiol-treated rats, which membranes contain a high concentration of recycling receptors (designated receptor-recycling compartment (RRC)), failed to accumulate EGF receptors after injection of EGF. In livers of rats not given exogenous EGF, EGF receptors were found in small but comparable concentrations in RRC, CURL, and MVB membranes, consistent with other evidence that targeting of the EGF receptor to lysosomes is mediated by ligand-induced phosphorylation.

Transferrin also accumulated first in CURL and later in MVBs, but it also accumulated rapidly in the RRC fraction, consistent with the proposed function of this fraction in receptor recycling. Since transferrin is not degraded during its endocytic cycle, these observations indicate that apotransferrin and its receptor recycle from late endosomes (MVBs) located at the apical pole of hepatocytes, as well as from early endosomes near the sinusoidal pole.

Receptor-mediated endocytosis is a general mechanism for the uptake of macromolecules including hormones, growth factors, and transport proteins (1). In research on the endocytosis of lipoproteins into hepatocytes of estradiol-treated rats, we have isolated three morphologically distinct endosomal fractions (2, 3); the lipid composition and major proteins of their membranes were similar and distinct from those derived from the Golgi apparatus. The fraction of lowest density is composed almost entirely of large (diameter ~0.55 μm) lipoprotein-filled multivesicular bodies (MVBs); the fraction of intermediate density of smaller lipoprotein-filled vesicles, in which intravenously injected radioiodinated low density lipoproteins (LDL) accumulated first; and the fraction of highest density mainly of membranes resembling appendages of the two vesicular fractions. 15 min after injection, we found the highest concentration of radioiodinated LDL in MVBs. The high density fraction contained very little LDL. All three fractions were enriched in receptors for LDL and asialoglycoproteins, but receptor concentrations were reduced in MVBs, which appear to represent the immediate prelysosomal compartment (4). This compartment appears to be analogous to the mannose 6-phosphate receptor-enriched, prelysosomal compartment described in cultured normal rat kidney cells and some cells in tissues by Griffiths and associates (5). We postulated that the fraction of intermediate density represents the compartment of uncoupling of receptor and ligand (CURL) described by Geuze et al. (6), and that the high density membranous fraction is highly enriched in a receptor-recycling compartment (RRC).

Lipoproteins and asialoglycoproteins are ultimately delivered to and degraded in lysosomes. After dissociation of the ligand, the LDL and asialoglycoprotein receptors find their way back to the cell surface from prelysosomal compartments independent of occupancy by ligand (7). Evidence from studies of cultured cells (8-12) and intact livers (13-16) indicates that the epidermal growth factor (EGF) receptor is delivered to lysosomes and degraded together with EGF. Unlike the LDL receptor, the EGF receptor is thought to be internalized only after it binds EGF (9, 17, 18). These characteristics are favorable for evaluation of the proposed function of the endosomal fractions that we have isolated from livers of intact rats.

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†† The abbreviations used are: MVBs, multivesicular bodies; LDL, low density lipoproteins; CURL, compartment of uncoupling of receptor and ligand; RRC, receptor-recycling compartment, EGF, epidermal growth factor; ψ-VLDL, ψ-very low density lipoproteins; TC, tyramine cellobiose.
Endocytosis of transferrin and iron has been studied in different cell lines (19-24) and in rat liver (25-28). Differrent transferrin is taken up by the transferrin receptor; at the reduced pH in endosomes, iron dissociates from transferrin and remains within the cell, but apotransferrin remains bound to the transferrin receptor and is recycled to the cell surface.

To evaluate further the function of the three hepatoctic endosomal fractions, we have compared the intracellular trafficking of EGF, the EGF receptor, transferrin, and iron in livers of estradiol-treated rats. Sorting of transferrin was found to occur in MVBS as well as CRL; apotransferrin depleted of iron accumulated in the RCR fraction, but EGF and the EGF receptor did not. We conclude that cell surface receptors recycle via RCR from late as well as early endosomes in rat hepatocytes in vivo.

EXPERIMENTAL PROCEDURES

Reagents—EGF (isolated from mouse submaxillary glands by the method of Savage and Cohen (29)) was purchased from Collaborative Research Inc., Bedford, MA; 17α-ethinyl estradiol, human apotransferrin (iron free), and dipheric transferrin were from Sigma. Sodium [125I]iodide, sodium [131I]iodide (carrier-free), and [5Fe(III)] chloride were from Amersham/Searle.

Animals—Male Sprague-Dawley rats (250-300 g), treated for 3 days with 17α-ethyl estradiol as described (30), were fed Ralston Purina standard chow. Male New Zealand White rabbits weighing 2.5-3.0 kg were fed Purina Lab rabbit chow containing 1% cholesterol by weight for 3-4 weeks.

Preparation of Lipoproteins—Human LDL (1.025 < d < 1.060 g/ml) were isolated from blood of normolipidemic adult humans (31). Very low density lipoproteins (β-VLDL) (d < 1.006 g/ml) were isolated from the blood of cholesterol-fed rabbits (32).

Radiolabeling of Ligands—LDL and β-VLDL were labeled with [125I] or [131I] by a modification (33) of the method of McFarlane (34) to a specific activity of 100-200 cpm/pg. EGF was labeled with [125I] by a modification of the chloramine T method of Greenwood and Hunter (35). Unbound [125I] was separated on a column of Sephadex G-50. The specific activity of [125I]-EGF was 13-175 cpm/pg; 95-99% of the [125I] was precipitable by trichloroacetic acid. Dipheric transferrin was labeled with [131I] by the method of Greenwood and Hunter (35), as described (19), to a specific activity of 30-70 cpm/pg. Apotransferrin (iron free) was labeled with [5Fe(III)] using disodium nitrotriacetate (36) to a specific activity of 607 cpm/pg protein. For comparative studies, dipheric transferrin and LDL were alternately coupled to the intracellular trapped labels, [125I]- and [131I]-labeled tyramine cellobiose (37).

Kinetics of Transferrin and Iron in Blood Plasma—To study the removal of transferrin and iron, rats were anesthetized with diethyl ether and the radiolabeled ligand and unlated LDL (3-5 mg protein) were injected into a femoral vein. Blood samples were taken from the aorta.

Isolation of Organelles—Endosomes in liver homogenates from estradiol-treated rats were isolated as described (2). Routinely, three rats were anesthetized with diethyl ether, and the radiolabeled ligand and unlated LDL (3-5 mg protein) were injected into a femoral vein. 2 min before the designated time (2.5, 7.5, 15, and 30 min after injection), the abdomen was opened and the portal vein was cannulated; livers were thoroughly flushed at the designated times with 150 ml of ice-cold 0.15 M NaCl, removed, and homogenized. All steps of endosome isolation were carried out on ice or at 4 °C. Endosomes from livers of untreated rats were isolated as described (2) 15 min after intravenous injection of β-VLDL from cholesterol-fed rabbits (3 mg protein). Endocytic uptake of β-VLDL increases the size of early endosomes and yields fractions comparable to those obtained from estradiol-treated rats. For isolation of lysosomes from liver homogenates of estradiol-treated rats (38), food was withdrawn 16 h before the experiment. Rats were then anesthetized with diethyl ether and radiolabeled ligands were injected 60 min before livers were removed. Crude membranes were prepared from liver homogenates as described (39).

Isolation of Membranes—To separate the membranes of endosomes from their contents, each fraction was diluted to 4 ml with 1 mM

suramin* (final concentration; pH 5.5) and passed twice through a French pressure cell (SLM Aminco, Urbana, IL) at 16,000 p.s.i. (1 p.s.i. = 6.89 kilopascals). The membranes and contents were separated by centrifugation, and the membranes were washed as described (2).

Binding Assays—Binding of β-VLDL to endosome membranes was quantified in a direct binding assay with 2-3 μg of endosomal membrane protein in a volume of 50 μl as described (3) at 4 °C. EDTA-sensitive binding (a measure of the LDL receptor) was calculated by subtracting residual binding in the presence of 30 mM EDTA from total binding. Binding of EGF was measured by a modification of the method of O'Toole et al. (40). The assay was carried out at a final pH of 7.5 in 300 μl of buffer A (0.1% bovine serum albumin, 5 mM Tris-HCl, 10 mM MgCl2) with 6-9 μg of endosome membranes, 150 μg of crude liver membranes or 300 μg of liver homogenate, and various concentrations of labeled EGF in the presence or absence of a 100-fold excess of unlabeled EGF. After incubation for 60 min at 4 °C, bound and unbound ligand were separated by centrifugation (100,000 × g, 4 °C) of 100 μl of the incubation mixture for 30 min in a TI-422 rotor (Beckman Instruments). The supernatant was removed by aspiration, and the surface of the pellets was washed with 200 μl of buffer A. The tubes were again subjected to centrifugation (100,000 × g, 5 min). After removing the supernatant, the portion of the tubes containing the pellet was cut off with a razor blade and used for assay of 125I in a γ scintillation spectrometer. Specific binding was calculated by subtracting residual binding in the presence of excess unlabeled EGF from total binding. The amount of 125I-EGF bound was a linear function of the mass of membrane protein added, up to at least 450 μg of crude membranes. At 4 °C binding of 125I-EGF was maximal within 30 min.

RESULTS

Trafficking of EGF and Its Receptor—Initially, we measured the hepatic uptake of EGF in estradiol-treated rats for up to 30 min after injection of 125I-EGF together with unlabeled human LDL (3-5 mg of protein). After injection of 0.5 μg of 125I-EGF, 38% of the 125I was found in the livers taken 15 min later. To achieve maximal internalization of the EGF receptor, we injected a saturating amount (20-21 μg of 125I-EGF (41); the mean hepatic recovery of 125I was 11.6 ± 5.6, 11.2 ± 4.7, 12.9 ± 2.9, and 8.9 ± 2.7% at 2.5, 7.5, 15, and 30 min after injection (mean ± S. D., n = 3).

The high purity of the endosomal fractions allowed measurement of high affinity binding of EGF to endosomal membranes as an estimate of EGF receptors with very low nonspecific binding (with crude membranes, nonspecific binding was about 50% of total binding at the highest concentration (13 nm) of 125I-EGF tested. Data from an experiment with MVB membranes isolated 15 min after injection of EGF are shown in Fig. 1. At all times after injection, only a single class of binding sites was detected (Fig. 2). The affinity of these sites for EGF was essentially the same in all three endosome fractions at all times after injection. The mean dissociation constant was 5.7 ± 3.0, 5.9 ± 3.1, and 5.2 ± 2.1 nm for MVBs, CURL, and RRC, respectively (n = 18 for each). Nonspecific binding of EGF was also unchanged in all fractions after injection of EGF.

Data on the concentration of EGF receptors in endosomal membranes from estradiol-treated rats are summarized in Fig. 3. The concentration of receptors in CURL membranes increased 3.7-fold 2.5 min after injection of EGF, whereas that in MVB membranes was unchanged. At 7.5 min after injection, the receptor concentration was similar in CURL and MVB membranes; both were increased 7-fold. At this time interval after injection, the receptor concentrations were 69- and 67-fold higher in CURL and MVB, respectively, than that found in crude membranes before injection of EGF. Receptor concentration was maximal in MVB membranes after 15 min, whereas the concentration in CURL membranes

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*Suramin has been shown to dissociate EGF from its receptor (69). Dissociation of EGF is also promoted by reducing the pH to 5-6 (70).
EGF, LDL, and the LDL receptor, we isolated the three endosomal fractions 15 min after simultaneous injection of 
\(^{125}\text{Tl-EGF}\) and \(^{125}\text{Tl-LDL}\). The labeled ligands were measured in the vehicle contents, and concentration of the EGF was estimated from binding isotherms. As shown in Table I, MVBs contained the highest mass of EGF, LDL receptors, and LDL, but a lower mass of LDL receptors than CURL and RRC. RRC membranes contained the lowest amount of EGF, LDL receptors, and LDL, but the highest mass of LDL receptors. The ratio of LDL to LDL receptor 15 min after injection differed in MVBs and RRC by a factor of 66, whereas that for EGF differed by a factor of only 1.5. Thus, EGF and LDL seem to follow a similar pathway: the EGF receptor is transferred with its ligand to MVB, whereas the LDL receptor is transferred mainly to the RRC fraction.

To determine whether the pathway for EGF and its receptor demonstrated in estradiol-treated rats is also present in untreated rats, we measured the uptake of \(^{125}\text{Tl-EGF}\) into livers of rats not given estradiol. Recovery of \(^{125}\text{Tl}\) in the liver 15 min after injection was 22.2 ± 4.9% in untreated rats (n = 7), as compared with 12.9 ± 2.9% (n = 5) in estradiol-treated rats (p < 0.005). The number of binding sites for EGF in control liver membranes from untreated rats was almost twice that of estradiol-treated rats (Fig. 4). The dissociation constant was the same, and only a single high affinity site was detected in crude membranes as well as in CURL membranes.

**TABLE I**

| Mass of EGF and LDL and their receptors in endosomal compartments 15 min after injection of the ligands |
|-------------------------------------------------------------|
| Values are mean of two experiments and represent total mass recovered in endosome fractions |

| Ligand | MVB | CURL | RRC |
|--------|-----|------|-----|
| EGF system | 33.51 | 19.95 | 4.55 |
| Receptor (pmol) | 13.01 | 9.82 | 2.70 |
| Ligand/receptor | 2.58 | 2.03 | 1.69 |
| LDL system | 215.5 | 81.5 | 7.1 |
| Receptor (μg of protein) | 4.1 | 7.8 | 8.9 |
| Ligand/receptor | 52.9 | 10.4 | 0.8 |

- Estimated from binding of \(^{125}\text{Tl-EGF}\) at \(B_{\text{max}}\) (assumes univalent binding of EGF).
- Estimated as mass of \(\beta-VLDL\) bound to EDTA-sensitive sites at \(B_{\text{max}}\).

The concentration of EGF receptors in the high density (RRC) fraction changed little after injection of EGF (see also Fig. 2). The concentration of EGF receptors in CURL and MVB membranes before injection of EGF was only slightly higher than in RRC membranes. These values, however, are 7–10-fold higher than those found in crude liver membranes.

To compare the pathway for the EGF receptor with that of
**Endosomal Trafficking of EGF Receptor and Transferrin**

**Trafficking of Transferrin and Iron**—The hepatic uptake of transferrin and iron was measured at intervals up to 60 min after injection into estradiol-treated rats, 30 min after intravenous injection of 25–2,000 μg of 125I-labeled diferric transferrin, 91% of the 125I was found in blood plasma (Fig. 5A). At transferrin concentrations below that at which transferrin receptors are saturated (0.5–1 μM), the liver takes up iron from diferric transferrin predominantly by receptor-mediated endocytosis via the transferrin receptor. We therefore injected subsaturating amounts (<500 μg) (27, 42–44) of 125I-transferrin and 59Fe-transferrin. At 2.5 and 7.5 min after injection, the hepatic concentrations of transferrin and iron were similar. Transferrin reached a plateau after 15 min and then fell, despite the continuing high concentration of transferrin in plasma (Fig. 5B); after injection of 59Fe-labeled transferrin, however, the hepatic concentration of 59Fe increased almost linearly for up to 45 min.

The kinetics of 125I in endosomal fractions was followed from 2.5 to 30 min after injection of labeled transferrin. At 2.5 min after injection of 1–3 μg of 125I-transferrin, 5.82% of the hepatic 125I was found in CURL, 1.06% in MVBS, and 3.85% in the hepatic fraction (total in three fractions = 10.72%). At this time after injection, 125I in CURL was enriched 89-fold over liver homogenate (Fig. 6). At 7.5 min after injection, the enrichment of 125I-transferrin in MVBS exceeded that in CURL, where its concentration had changed little. After 30 min, the enrichment was similar in all three fractions (Fig. 6A).

At all times after injection, the recovery and enrichment of 59Fe in isolated endosomes was much lower than that of transferrin. Only 1.33% of the hepatic 59Fe was recovered in the sum of MVBS, CURL, and RRC 2.5 min after injection of 59Fe-labeled transferrin. The total endosomal content of 59Fe fell to 0.6, 0.17, and 0.11% of the hepatic 59Fe after 7.5, 15, and 30 min, respectively. Enrichment was least in MVBS at all times after injection (Fig. 6B).

To characterize further the intracellular pathway of transferrin, we compared the recovery of transferrin and LDL in lysosomes isolated from livers of estradiol-treated rats 60 min after simultaneous injection of 125I-labeled transferrin and 131I-labeled LDL. To compare the extent of degradation of injected transferrin and LDL, separate experiments were performed in which radiolabeled tyramine cellulose (TC), a residualizing trapped label for substances entering lysosomes, was covalently coupled to each ligand. Recovery of TC-LDL in the liver was 94% greater than that of uncoupled LDL 60 min after injection, whereas the content of TC-transferrin was only 18% higher than that of uncoupled transferrin (Table II). In the lysosomes, 11.1-fold more of the injected 125I was recovered than of the injected 131I after injection of 125I-LDL and 131I-transferrin, and 18.7-fold more than 131I-TC than 125I-TC after injection of 125I-TC-LDL or 125I-TC-transferrin (Table II).

**DISCUSSION**

The current experiments demonstrate that all three of our endosomal fractions (MVBS, CURL, and RRC) contain distinct compartments of the receptor-mediated endosomal pathway. The CURL fraction contains predominantly early endosomes and the MVBS fraction, almost exclusively the immediate prelysosomal compartment (4), late endosomes. Both CURL and MVBS take part in sorting receptors and ligands, evidently generating the nonvesicular membranes of the RRC,
in which recycling receptors and transferrin are returned to the cell surface. The predominant membranes in the RRC fraction indeed appear to be derived from the membranous appendages of vesicular endosomes (2), consistent with the latter concept. We took advantage of the limited recycling of the EGF receptor and the predominant recycling of the transferrin receptor in hepatocytes in vivo to evaluate the role of RRC membranes; the EGF receptor was largely excluded from the putative recycling fraction, but transferrin, which remains bound to the transferrin receptor, was not.

Our experiments on the intracellular trafficking of the EGF receptor and transferrin and their ligands also permitted us to evaluate the role of early and late endosomes in receptor recycling. In earlier research, we measured the concentrations of two recycling receptors, the LDL receptor and the asialoglycoprotein receptor, in three endosomal fractions (2, 3); these receptors are, however, constitutively endocytosed, independent of ligand binding, and thus are not well suited for study of their intracellular movement under physiological conditions. Before exposure to injected EGF, EGF receptors associated with hepatocytes appeared to be limited primarily to the sinusoidal plasma membrane. After injection of a saturating amount of EGF, virtually all of these surface receptors presumably bind EGF, become tyrosine-phosphorylated, and rapidly undergo endocytosis (13, 15, 45). Consistent with this notion, we found that approximately 2.5 µg of EGF (of 20–21 µg injected) was associated with the liver 2.5 min after injection and that this mass remained constant during the next 15 min.

At the early time interval of 2.5 min, virtually all of the increment in endosomal EGF binding activity was in a single endosomal fraction (CURL). The EGF receptor was then transferred to the MVB fraction between 2.5 and 7.5 min after injection. In the steady state, MVBs are found mainly at the bile canalicular pole of hepatocytes (4, 46). On the basis of previous observations of the movement of radiiodinated LDL (46) and of EGF (16, 41) and its receptor (15) within hepatocytes, translocation of endocytosed ligands and the EGF receptor to the apical pole of the cell is probably extensive by 15 min, a time at which we found the concentration of EGF receptors in the MVB fraction to reach its peak.

The RRC fraction is highly enriched in LDL receptors (2), asialoglycoprotein receptors (2), and the LDL receptor-related protein (47). At no time after injection of EGF, however, was there any accumulation of the EGF receptor in RRC membranes (Fig. 3). After injection of a saturating amount of EGF, Lai and associates (13) found virtually no recycling of the EGF receptor in rat liver. EGF and its receptor are thought to be targeted for destruction in lysosomes, leading ultimately to clearance of the ligand and receptor down-regulation (13, 15). Recycling of the EGF receptor in hepatocytes after exposure to EGF was suggested by Dunn et al. (15, 41), because in their studies, degradation of EGF exceeded the loss of EGF binding sites. Dunn et al. (15, 41) postulated that a latent pool of low affinity receptors (Kd ~200 nM) mediates the uptake of additional EGF after lysosomal degradation of the high affinity receptors. This receptor population was observed only after injection of EGF and in the presence of the detergent Brij 35. The suggestion of a cryptic pool of low affinity receptors is not supported by our studies and the findings of Lai and associates (13). We have observed only a single population of high affinity receptors under our assay conditions, as have others in rat liver membranes (40, 48) and on rat hepatocytes (49, 50).

The dynamics of the EGF receptor after ligand binding have permitted us to carry out a type of pulse-chase experiment in vivo. However, our results suggest that some EGF receptors are endocytosed in the absence of EGF injection. Thus, we found the concentration of EGF receptors in all endosomal fractions from rats not injected with EGF to be ~7-10-fold higher (on a protein basis) than that found in crude liver membranes. This observation, coupled with our finding that the concentration of EGF receptors was as high in RRC membranes as in CURL membranes before injection of EGF, suggests that a much larger fraction of endocytosed receptors normally undergoes recycling, in contrast to the situation that obtains after injection of a saturating amount of EGF. These data are consistent with those of Lai and associates (13). They found, after injection into rats of <1 µg of EGF/100 g body weight, that most endocytosed EGF receptors returned to the plasma membrane, whereas after injection of >5 µg of EGF/100 g body weight, the majority of surface receptors underwent down-regulation. These investigators found autophosphorylation activity of EGF receptors in their Golgi/endosome fraction to be increased after injection of a saturating amount of EGF (45). As suggested by several investigators (45, 51, 52), phosphorylation may mediate down-regulation of EGF receptors. Our observations in un.injected rats are consistent with rapid constitutive endocytosis of unoccupied receptors at the cell surface, as suggested by Gladhag and Christiansen (53), but do not exclude the possibility that binding of small amounts of EGF present in the space of Disse to the receptor triggers endocytosis.

Hepatocytes take up iron from iron-loaded transferrin very efficiently. The hepatic content of iron increased almost linearly after injection of diferric transferrin for at least 45 min, but the hepatic content of transferrin did not increase beyond 7.5 min after injection (Fig. 5). These data give evidence for recycling of transferrin after unloading iron within the cell as postulated for different cell lines (19–24, 54), hepatocytes (26, 27, 55), and intact rat liver (25–28). Recycling of most transferrin is further supported by the low recovery of transferrin

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**Table II**

Recovery of 125I and 131I 60 min after injection of 125I-transferrin with 131I-LDL and after injection of 125I-TC-transferrin with 131I-TC-LDL

|                | Experiment A | Experiment B |
|----------------|--------------|--------------|
| 125I-Transferrin|              |              |
| Liver          | 2.95 ± 0.05  | 11.11 ± 2.44 |
| (100)          | (100)        | (100)        |
| Lysosomes*     | 0.045 ± 0.006| 0.50 ± 0.08  |
| (1.5)          | (4.5)        | (5.7)        |
| 131I-LDL       |              |              |
| Liver          | 11.11 ± 2.44 | 21.54 ± 1.99 |
| (100)          | (100)        | (100)        |
| Lysosomes*     | 0.20 ± 0.021 | 3.74 ± 0.74  |
| (5.7)          | (17.2)       |              |

* Lysosomes were isolated in fractions 1–3 of the metrizamide gradient (38).
in isolated lysosomes, as compared with LDL, where it is degraded. The uptake of injected transferrin, estimated from hepatic accumulation of \(^{59}\)Fe, was 31.4% in 45 min (Fig. 5). Of this, only 0.63% (3.48/51.4 \times 5.7\%) based upon the fraction of TC-labeled transferrin taken up that was actually retained in the liver and the lysosomal content of TC-labeled LDL, as shown in Table II) was in isolated lysosomes 60 min after injection, as compared with 17.2% of hepatic TC-labeled LDL. Transferrin thus recycles almost entirely to the cell surface, and is therefore a highly suitable marker of the recycling pathway in rat hepatocytes.

Like LDL (2) and the EGF receptor, transferrin accumulated first in the CURL fraction. At 2.5 min after injection, transferrin was enriched 3.5 times more in CURL than in MVBS (Fig. 6). Although a minor fraction of transferrin and LDL (2) was found in the MVB fraction 2.5 min after injection, there was no increase in the content of the EGF receptor in the MVB fraction at this time after injection of EGF, suggesting that endocytosis of the EGF receptor is slower than that of the transferrin and LDL receptors.

The enrichment of transferrin in the RRC fraction is almost 10-fold higher than that of LDL, VLDL, and chylomicron remnants (2, 3), and that of EGF and the EGF receptor (Table I). This fraction is highly enriched in recycling receptors: the LDL receptor (2), the asialoglycoprotein receptor (2), and LDL receptor-related protein (47). The high concentration of recycling receptors and transferrin and the depletion of ligands and the EGF receptor, which undergo lysosomal degradation, give strong evidence for the function of the RRC fraction in receptor recycling. Although MVBS contain lower concentrations of LDL and asialoglycoprotein receptors than CURL and RRC, membranes, they are still manyfold enriched in these receptors as compared with plasma membranes or Golgi membranes (2); MVBS contain a lower fraction of appendage membrane relative to vesicular membrane than do CURL vesicles (2). MVBS thus seem to be involved in the recycling of the LDL and asialoglycoprotein receptor to a lesser extent than the vesicles in our CURL fraction. The MVB fraction, however, was highly enriched in transferrin, which therefore presumably recycles to the cell surface from MVBS at the apical pole of hepatocytes. There is broad evidence that transferrin and the transferrin receptor are transported to MVBS and recycled through the Golgi/lysosomal paranuclear region in several cell lines (23, 24, 54, 56–61). From the short recycling time of transferrin in different cell lines (20, 21, 62), it has, however, been concluded that only early endosomes near the plasma membrane are involved in transferrin recycling. Schmidt et al. (63) found transferrin only in early and not in late endosomes, isolated from Chinese hamster ovary cells by free flow electrophoresis. These two populations of isolated endosomes contain markedly different protein compositions, unlike CURL and MVBS isolated from livers of estradiol-treated rats (2). Given our strong evidence that late endosomes (MVBS) in rat hepatocytes in vivo are a sorting site for transferrin, the late endosomal fraction from Chinese hamster ovary cells evidently is functionally different. Yamashiro and Maxfield, reviewing their work on endocytosis of transferrin and \(\alpha\)-macroglobulin in Chinese hamster ovary cells, have distinguished early endosomes from “sorting endosomes” containing recycling receptors and “late prelysosomal endosomes,” which lack these receptors (64). The latter organelles may resemble vesicles transitional between typical MVBS and “early” lysosomes, which constitute a minor fraction of the apical vesicles in our MVBS fraction (4), whereas our CURL fraction probably contains mainly sorting endosomes located near the basolateral pole of hepatocytes.

Low concentrations of transferrin-bound iron are thought to be taken up almost exclusively by the transferrin receptor via receptor-mediated endocytosis (20–22, 55, 65). After injection of subsaturating amounts of iron and transferrin, both ligands are first found in the same intracellular compartment (27); iron dissociates from transferrin within endosomes (66), traverses the endosomal membrane, and is finally incorporated into ferritin (25–27). We found almost no enrichment of \(^{59}\)Fe in MVBS at any time up to 30 min after injection of \(^{59}\)Fe-labeled transferrin; enrichment of \(^{59}\)Fe in CURL and RRC was about 10-fold lower than that found for transferrin (Fig. 6). The enrichment of \(^{59}\)Fe decreased substantially in all three endosomal fractions between 2.5 and 30 min after injection. From these findings, we conclude that iron dissociates from transferrin at an early step in the transferrin cycle and is transported rapidly through the endosomal membrane into the cytosol, as demonstrated in partially purified endosomes from K562 cells (65). This transport requires an acidification system (67), an iron reduction system, and a ferrous iron transporter (68).

We have reported previously that the membranes of our CURL, MVB, and RRC fractions share major proteins detected by gel electrophoresis (2). Given the distinct functional characteristics of these fractions defined here, it is evident that further research on their membrane proteins may help to define distinct characteristics related to receptor recycling and the biogenesis of lysosomes.

REFERENCES

1. Goldstein, J. L., Anderson, R. G. W., and Brown, M. S. (1979) Nature 279, 679–685
2. Belcher, J. D., Hamilton, R. L., Brady, S. E., Hornick, C. A., Jackle, S., Schneider, W. J., and Havel, R. J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6785–6789
3. Jackle, S., Brady, S. E., and Havel, R. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 86, 1880–1884
4. Jost-Vu, E., Hamilton, R. L., Hornick, C. A., Belcher, J. D., and Havel, R. J. (1986) Histochemistry 85, 457–466
5. Griffiths, G. (1988) J. Cell Sci. Suppl. 11, 139–147
6. Genev, H. J., Belcher, J. D., Strous, G. J. A., Lodish, H. F., and Schwartz, A. L. (1983) Cell 32, 277–287
7. Brown, M. S., Anderson, R. G. W., and Goldstein, J. L. (1983) Cell 32, 663–667
8. Carpenter, G., and Cohen, S. (1976) J. Cell Biol. 71, 159–171
9. Haigler, H. T., McKanna, J. A., and Cohen, S. (1979) J. Cell Biol. 81, 382–395
10. McKanna, J. A., Haigler, H. T., and Cohen, S. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5689–5693
11. Beguinot, L., Lyall, R. M., Willingham, M. C., and Pastan, I. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2384–2388
12. Cohen, S., and Fava, R. A. (1983) J. Biol. Chem. 260, 12351–12358
13. Lai, W. H., Cameron, P. H., Wada, I., Doherty, J.-J., Il, Kay, D. G., Posner, B. I., and Bergeron, J. J. M. (1989) J. Cell Biol. 109, 2741–2749
14. Burwen, S. J., Barker, M. E., Goldman, I. S., Hradek, G. T., Raper, S. E., and Jones, A. L. (1984) J. Cell Biol. 99, 1299–1305
15. Dunn, W. A., Connolly, T. P., and Hubbard, A. L. (1986) J. Cell Biol. 102, 24–36
16. Kay, D. G., Lai, W. H., Uchihashi, M., Khan, M. N., Posner, B. I., and Bergeron, J. J. M. (1986) J. Biol. Chem. 261, 8475–8480
17. Cohen, S., and Fava, R. A. (1983) J. Biol. Chem. 260, 12351–12358
18. Miller, K., Beadmore, J., Kanety, H., Schlessinger, J., and Hopkins, C. R. (1986) J. Cell Biol. 102, 500–509
19. Ciechanover, A., Schwartz, A. L., Dautry-Varsat, A., and Lodish, H. F. (1983) J. Biol. Chem. 258, 9681–9699
20. Dautry-Varsat, A., Ciechanover, A., and Lodish, H. F. (1983) J. Biol. Chem. 258, 9681–9699
21. Dautry-Varsat, A., Ciechanover, A., and Lodish, H. F. (1983) J. Biol. Chem. 258, 9681–9699
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Proc. Natl. Acad. Sci. U. S. A. 80, 2258–2262
22. Klausen, R. D., Ashwell, G., Van Renswoude, J., Harford, J. B., and Bridges, K. R. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2263–2266
23. Willingham, M. C., Hanover, J. A., Dickson, R. B., and Pastan, I. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 175–179
24. Storrie, W., Geuze, H. J., Griffith, J. M., and Strous, G. J. (1988) J. Cell Biol. 106, 1821–1829
25. Miloslom, J. P., and Batey, R. G. (1979) Biochem. J. 182, 117–125
26. Young, S. P., Roberts, S., and Bomford, A. (1985) Biochem. J. 232, 819–823
27. Manug, E. H., Smith, G. D., and Peters, T. J. (1986) Biochem. J. 237, 163–173
28. Sibille, J.-C., Octave, J.-N., Schneider, Y.-J., Troutet, A., and Crichton, R. (1986) Eur. J. Biochem. 155, 47–55
29. Savage, C. R., Jr., and Cohen, S. (1972) J. Biol. Chem. 247, 7609–7611
30. Chao, Y., Windler, E. E., Chen, G. C., and Havel, R. J. (1979) J. Biol. Chem. 254, 11360–11366
31. Havel, R. J., Eder, H. A., and Bradlon, J. H. (1955) J. Clin. Invest. 34, 1345–1353
32. Kovarne, P. T., Brown, M. S., Basu, S. K., Biemer, D. W., and Goldstein, J. L. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 1396–1400
33. Sigurdsson, G., Neil, S.-P., and Havel, R. J. (1978) J. Lipid Res. 19, 628–634
34. McFarlane, A. S. (1958) Nature 182, 53–54
35. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) Biochem. J. 89, 114–123
36. Van Renswoude, J., Bridges, K. R., Harford, J. B., and Klausen, R. D. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6186–6190
37. Pittman, R. C., and Taylor, C. A., Jr. (1986) Methods Enzymol. 129, 612–628
38. Wattiaux, R., Wattiaux-De Connick, S., Ronveaux-Dupal, M.-F., and Dubois, F. (1978) J. Cell Biol. 78, 349–368
39. Kovarne, P. T., Brown, M. S., and Goldstein, J. L. (1979) J. Biol. Chem. 254, 11367–11373
40. O’Keefe, E., Hohenberg, M. D., and Cuatrecasas, P. (1974) Arch. Biochem. Biophys. 164, 518–526
41. Dunn, W. A., and Hubbard, A. L. (1984) J. Cell Biol. 98, 2148–2159
42. Cole, E. S., and Glass, J. (1983) Biochem. Biophys. Acta 762, 102–110
43. Page, M. A., Baker, E., and Morgan, E. H. (1984) Am. J. Physiol. 246, G26–G33
44. Thorstensen, K., and Romansio, I. (1988) J. Biol. Chem. 263, 8844–8850
45. Lai, W. H., Cameron, P. H., Doherty, J.-J., II, Posner, B. I., and Bergeron, J. M. (1989) J. Cell Biol. 109, 2751–2760
46. Chao, Y.-s., Jones, A. L., Hradek, G. T., Windler, E. E. T., and Havel, R. J. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 597–601
47. Lund, H., Takahashi, K., Hamilton, R. L., and Havel, R. J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9318–9322
48. Hoath, S. B., Pickens, W. L., Bucuvalas, J. C., and Suchy, F. J. (1987) Biochim. Biophys. Acta 930, 107–113
49. Moriarty, D. M., and Savage, C. R., Jr. (1980) Arch. Biochem. Biophys. 203, 506–518
50. Rush, G. F., and Alberts, D. (1987) Life Sci. 40, 679–685
51. Beguinot, L., Hanover, J. A., Ito, S., Richert, N. D., Willingham, M. C., and Pastan, I. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 2774–2778
52. Honegger, A. M., Dull, T. J., Felder, S., Van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ulrich, A., and Schlessinger, J. (1987) Cell 51, 199–209
53. Gladhaug, I. P., and Christoffersen, T. (1988) J. Biol. Chem. 263, 12199–12203
54. Stein, B. S., and Sussman, H. H. (1986) J. Biol. Chem. 261, 10319–10331
55. Thorstensen, K., and Romansio, I. (1984) Biochim. Biophys. Acta 784, 393–397
56. Harding, C., Heuser, J., and Stahl, P. (1983) J. Cell Biol. 97, 329–339
57. Stein, B. S., Bensch, K. G., and Sussman, H. H. (1984) J. Biol. Chem. 259, 14762–14772
58. Snider, M. D., and Rogers, O. C. (1985) J. Cell Biol. 100, 826–834
59. Pan, B.-T., Teng, K., Wu, C., Adam, M., and Johnstone, R. M. (1985) J. Cell Biol. 101, 942–948
60. Salzman, N. H., and Maxfield, F. R. (1988) J. Cell Biol. 106, 1083–1091
61. Dunn, K. W., McGraw, T. E., and Maxfield, F. R. (1989) J. Cell Biol. 109, 3303–3314
62. Klausen, R. D., Van Renswoude, J., Ashwell, G., Kempf, C., Schechter, A. N., Dean, A., and Bridges, K. R. (1983) J. Biol. Chem. 258, 4715–4724
63. Schmid, S. L., Fuchs, R., Male, P., and Mellman, I. (1988) Cell 52, 73–85
64. Yamashiro, D. J., and Maxfield, F. R. (1988) TIPS 9, 190–193
65. Young, S. P., and Aisen, P. (1981) Hepatology 1, 114–119
66. Bakkeren, D. L., De Jeu-Jaspars, M. H., Kroos, M. J., and Van Eijk, H. G. (1987) Int. J. Biochem. 19, 179–186
67. Van Dyke, R. W., Hornick, C. A., Belcher, J., Scharschmidt, B. F., and Havel, R. J. (1985) J. Biol. Chem. 260, 11021–11026
68. Núñez, M.-T., Gaete, V., Watkins, J. A., and Glass, J. (1990) J. Biol. Chem. 265, 6688–6692
69. Coffey, R. J., Jr., Leuf, E. B., Shipley, G. D., and Moses, H. L. (1987) J. Cell. Physiol. 132, 143–148
70. Sorkin, A. D., Teslenko, L. V., and Nikolsky, N. N. (1988) Exp. Cell Res. 175, 192–205