Sorting of Endocytosed Transferrin and Asialoglycoprotein Occurs Immediately after Internalization in HepG2 Cells

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Abstract. After receptor-mediated uptake, asialoglycoproteins are routed to lysosomes, while transferrin is returned to the medium as apotransferrin. This sorting process was analyzed using 3,3'-diaminobenzidine (DAB) cytochemistry, followed by Percoll density gradient cell fractionation. A conjugate of asialoorosomucoid (ASOR) and horseradish peroxidase (HRP) was used as a ligand for the asialoglycoprotein receptor. Cells were incubated at 0°C in the presence of both 125I-transferrin and 125I-ASOR/HRP. Endocytosis of prebound 125I-ASOR/HRP and 125I-transferrin was monitored by cell fractionation on Percoll density gradients. Incubation of the cell homogenate in the presence of DAB and H2O2 before cell fractionation gave rise to a density shift of 125I-ASOR/HRP-containing vesicles due to HRP-catalyzed DAB polymerization. An identical change in density for 125I-transferrin and 125I-ASOR/HRP, induced by DAB cytochemistry, is taken as evidence for the concomitant presence of both ligands in the same compartment. At 37°C, sorting of the two ligands occurred with a half-time of ~2 min, and was nearly completed within 10 min. The 125I-ASOR/HRP-induced shift of 125I-transferrin was completely dependent on the receptor-mediated uptake of 125I-ASOR/HRP in the same compartment. In the presence of a weak base (0.3 mM primaquine), the recycling of transferrin receptors was blocked. The cell surface transferrin receptor population was decreased within 6 min to 15% of its original size. DAB cytochemistry showed that sorting between endocytosed 125I-transferrin and 125I-ASOR/HRP was also blocked in the presence of primaquine. These results indicate that transferrin and asialoglycoprotein are taken up via the same compartments and that segregation of the transferrin–receptor complex and asialoglycoprotein occurs very efficiently soon after uptake.

Many serum macromolecules can be bound to the cell surface by specific receptors, and subsequently internalized via receptor-mediated endocytosis. Depending on the final destination of the ligand, distinct intracellular transport routes can be followed. The first events in these differing pathways seem to be identical: binding of the ligand to the receptor at the plasma membrane; clustering of receptor–ligand complexes in coated pits; internalization of the complex via coated vesicles; and transport to the compartment of uncoupling receptor and ligand (CURL) or endosomes. From there, different routes may be followed. Many ligands, such as asialoglycoproteins (ASGP) (reviewed in Breitfeld et al., 1985; Schwartz, 1984b) and low density lipoproteins (reviewed in Goldstein et al., 1985) are transported to the lysosomes where they are degraded, whereas their receptors recycle to the plasma membrane and can be re-utilized in a new endocytic cycle. Other ligands, such as polymeric IgA, are taken up at the basolateral plasma membrane and are not uncoupled from their receptors, but directed as ligand–receptor complexes to the apical plasma membrane in a process called transcytosis (Limet et al., 1982; Courtoy et al., 1985; Solari and Kraehenbuhl, 1984). Transferrin, a serum glycoprotein that plays an important role in iron delivery to cells, releases its iron intracellularly at acidic pH and recycles bound to its receptor to the plasma membrane (Klausner et al., 1983; Dautry-Varsat et al., 1983).

If uptake of various types of receptor–ligand complexes occurs through the same entry route, sorting among and between different types of ligands and receptors must occur intracellularly. By using double-label immunoelectron microscopy, we found that sorting between ASGPs and their receptors in rat liver occurred in a system composed of tubules and vesicles, termed CURL (Geuze et al., 1984). Furthermore, in rat hepatocytes, polymeric IgA and ASGP sorting was shown to occur in similar structures (Courtoy et al., 1985). The kinetics of this process has been studied in rat liver (Courtoy et al., 1985), using the 3,3'-diaminobenzidine (DAB) density shift method (Courtoy et al., 1984). Transferrin is an ideal marker for the study of receptor recycling, since it probably follows the same route as its recep-
tor during the entire process of recycling. As the recycling parameters for the transferrin and the ASGP receptor are very much alike, it is assumed that both receptors follow the same route in HepG2 cells (Ciechanover et al., 1983b). In the present study we have used the DAB-induced density shift principle (Courtoy et al., 1984) to address the questions of when and where transferrin and ASGP segregate. We conclude that both ligands are taken up via the same compartments. In addition, we show that the two ligands segregate shortly after uptake.

Primaque, like other amines, neutralizes acidic compartments (Maxfield, 1982; Okhuma and Poole, 1978; Poole and Ohkuma, 1981; Tycko et al., 1983), and interferes both with the recycling of the ASGP receptor (Stiros et al., 1985) and the degradation of endocytosed ASGP (Schwartz et al., 1984a). In this paper we show that in the presence of 0.3 mM primaque the transferrin receptor recycling is blocked and that complete inhibition of sorting between endocytosed transferrin and the asialoorosomucoid/horseradish peroxidase complex (ASOR/HRP) is achieved.

Materials and Methods

Cells

The human hepatoma cell line HepG2 (Knowles et al., 1980) was cultured in monolayer in MEM supplemented with 10% decomplemented fetal bovine serum, and antibiotics (Schwartz et al., 1981). For experiments, 80% confluent cultures were used. The medium was refreshed 1 d before the experiment.

Preparation of the ASOR/HRP Conjugate

Orosomucoid was a gift from Dr. A. L. Schwartz (Children's Hospital, St. Louis, MO) and desialylated by incubating at 80°C for 60 min in 25 mM H2SO4 (Schwartz et al., 1980). The extent of desialylation was determined according to the procedure of Warren (1959), and exceeded 95%. ASOR was conjugated to HRP (type VI; Sigma Chemical Co., St. Louis, MO) essentially according to the method of Nakane and Kowaoi (1974), using equimolar quantities of ASOR and HRP. The conjugate was separated from free ASOR and HRP by Sephadex G-100 column chromatography. Fractions were analyzed by SDS-PAGE and by determining the ratio of adsorbances at 280 and 405 nm. 90% of the conjugation products from the pooled peak fractions consisted of one molecule ASOR and one molecule HRP (ASOR/HRP). The remaining 10% consisted of two ASOR and one HRP molecule (ASOR2/HRP). The peroxidase activity of ASOR/HRP was measured as described by the HRP manufacturer. The molar activities of the preparations used varied between 70 and 96%, compared with nonconjugated HRP.

Iron Saturation of Transferrin

Human transferrin (98% pure; Sigma Chemical Co.) was iron saturated using a modified method of Klausner and co-workers (1983): 6 mg transferrin was dissolved in 1 ml freshly prepared 0.25 M Tris/HCl, pH 8.0, 10 mM NaHCO3, 2 mM sodium nitritotriacetate, 0.25 mM FeCl3, and incubated for 30 min at room temperature, followed by dialysis against 3 x 1 liter 0.15 M NaCl, 20 mM Hepes, pH 7.2 at 4°C.

Ligand Iodination

 Portions of 200–1000 µg ASOR/HRP were iodinated in 500 µl PBS containing 1 mCi 125I (essentially carrier free; Amersham Corp., Arlington Heights, IL) and six iodobeads (Pierce Chemical Co., Rockford, IL). ASOR (100 µg) was iodinated in 500 µl PBS containing 0.5 mCi 125I and six iodobeads. Diferric–transferrin (300 µg) was iodinated in 500 µl 0.15 M NaCl, 20 mM Tris/HCl, pH 7.4, containing 1 mCi 125I (30 mCi/µg; New England Nuclear, Boston, MA) and six iodobeads, or alternatively 200 µg with 0.5 mCi 125I and three iodobeads. All iodinations were performed for 30 min at room temperature. Free 125I or 125I were removed by chromatography on a Sephadex G-25 column equilibrated in PBS. The specific activity and concentration of the iodinated proteins were determined by TCA precipitation before and after gel filtration. The specific activities were: 0.8 x 10⁴–3.4 x 10⁵ cpm/µg 125I-ASOR/HRP, 4.6 x 10⁴ cpm/µg 125I-ASOR, 0.7 x 10⁵ cpm 125I/µg transferrin, and 2.5 x 10⁵ cpm 125I/g transferrin.

Ligand Binding

Semic confluent cultures were washed three times with binding medium (MEM, 0.85 g/liter NaHCO3, 20 mM Hepes/NaOH, pH 7.2), before a 30-min incubation period in binding medium at 37°C to deplete ASGP and transferrin receptors from their ligands. Cells were incubated for 1 h on a rocker in ice-cold binding medium supplemented with iodinated ligand(s), followed by two quick and two 5-min washes with binding medium at 0°C to remove excess ligand. Further processing is described in the text.

Gradient Centrifugation

Cells were scraped in homogenization buffer (0.25 M sucrose, 2 mM CaCl2, 10 mM Hepes/NaOH, pH 7.2), and homogenized with 50 strokes, using a dounce with a tight fitting pestle. Nuclei were removed by centrifugation for 10 min at 300 g in a minifuge (Heraeus Christ GmbH, Osterode, Federal Republic of Germany). 500 µl of the postnuclear supernatant was layered on top of 13 ml (25%) Percoll solution (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.25 M sucrose, 2 mM CaCl2, 10 mM Hepes/NaOH, pH 7.2. A density gradient was formed during 49.300 g centrifugation for 60 min in a superspeed centrifuge (Sorvall RC-5B; Dupont Co., Sorvall Instruments Div., Newtown, CT) using an SM 24 rotor, and fractionated by downward displacement in 0.5-ml fractions. 125I and 125I were counted in a gamma counter (PWE 4800; Philips Electronic Instruments, Inc., Mahwah, NJ). The density distribution in the gradient formed was measured using density marker beads (Pharmacia Fine Chemicals).

DAB Cytochemistry

A modification of the method, originally developed by Courtoy et al. (1984) was used. A 4.5-ml solution of DAB (Fluka AG, Buchs, Switzerland) in homogenization buffer was prepared, adjusted to pH 7.2 with 1 N NaOH, and filtered through a 0.22-µm filter (Millipore Corp., Bedford, MA). 250 µl postnuclear supernatant was mixed with 300 µl DAB solution and 3 µl 0.7 x 10⁶ cpm 125I-labeled transferrin, 0.25 x 10⁶ cpm 125I-labeled transferrin, and 2.5 x 10⁶ cpm 125I-labeled transferrin. A modification of the method, originally developed by Courtoy et al. (1984) was used. A 4.5-ml solution of DAB (Fluka AG, Buchs, Switzerland) in homogenization buffer was prepared, adjusted to pH 7.2 with 1 N NaOH, and filtered through a 0.22-µm filter (Millipore Corp., Bedford, MA). 250 µl postnuclear supernatant was mixed with 300 µl DAB solution and 3 µl 0.7 x 10⁶ cpm 125I-labeled transferrin, 0.25 x 10⁶ cpm 125I-labeled transferrin, and 2.5 x 10⁶ cpm 125I-labeled transferrin. A modification of the method, originally developed by Courtoy et al. (1984) was used. A 4.5-ml solution of DAB (Fluka AG, Buchs, Switzerland) in homogenization buffer was prepared, adjusted to pH 7.2 with 1 N NaOH, and filtered through a 0.22-µm filter (Millipore Corp., Bedford, MA). 250 µl postnuclear supernatant was mixed with 300 µl DAB solution and 3 µl 0.7 x 10⁶ cpm 125I-labeled transferrin, 0.25 x 10⁶ cpm 125I-labeled transferrin, and 2.5 x 10⁶ cpm 125I-labeled transferrin.

Results

Binding of 125I-ASOR/HRP to the Asialoglycoprotein Receptor

125I-ASOR/HRP was used as a ligand for the ASGP receptor. The conjugation method used to prepare ASOR/HRP produces only low molecular weight conjugates, probably because of the limited accessibility of reactive amino groups of the heavy glycosylated ASOR molecule. Also, the relative high affinity of ASOR for ASGP receptors (Fig. 1) compared with other galactose terminal ligands such as asialofetuin and galactosylated bovine serum albumin makes the ASOR/HRP conjugate an ideal bifunctional molecule with respect to its receptor binding and peroxidase activity. To determine the specificity of binding and the number of binding sites for this ligand at the plasma membrane, HepG2 cells were incubated
in the presence of various concentrations of [125I]-ASOR/HRP for 1 h at 0°C (Fig. 1 A). At this temperature uptake is completely blocked, so that only surface receptors are labeled. Nonspecific [125I]-ASOR/HRP binding was measured in the presence of an excess of nonlabeled ASOR (400 μg/ml) and amounted to 30% of the total binding at 4 μg/ml ASOR/HRP. Excess of nonlabeled HRP (400 μg/ml) did not reduce ASOR/HRP binding. Thus, saturable binding of [125I]-ASOR/HRP was exclusively dependent on binding to the ASGP receptor and reached a plateau at a concentration of ~10 μg/ml [125I]-ASOR/HRP. The exact number of binding sites can only be roughly determined, since the ligand was not fully homogeneous (see Materials and Methods). Scatchard analysis (Scatchard, 1949) indicates that there are 240,000 ASGP receptors at the cell surface, postulating a homogeneous ASOR/HRPm population. Since the ASOR/HRP preparation also contained ~10% ASOR2/HRPm complexes, the real amount of surface ASGP receptors could probably be somewhat less than 240,000 per cell. As a control, a similar titration was performed using [125I]-ASOR (Fig. 1 B). Saturation binding was achieved at a concentration of ~5 μg/ml, which is about equimolar to the saturation concentration of ASOR/HRPm. Analysis of these data show that there are 200,000 plasma membrane ASGP receptors per cell saturated for 50% at 5.2 × 10-4 M ASOR after 1 h at 0°C. This number is well in agreement with that found for ASOR/HRP and close to the 150,000 ± 20,000 binding sites per cell surface reported elsewhere (Schwartz et al., 1981).

**Fractionation of Endocytosed [125I]-ASOR/HRP on Percoll Gradients**

To standardize the density distribution in the Percoll gradient of endocytosed ASGP, prebound [125I]-ASOR/HRP was allowed to enter the cell for various periods of time at 37°C. Subsequently, the cells were fractionated on Percoll density gradients (Fig. 2). If the cells were kept on ice, a single peak of [125I]-ASOR/HRP-containing vesicles with a mean density of ~1.045 g/ml was observed. This peak represented exclusively plasma membrane-bound ligand, as removal of Ca2+ or lowering of the pH to 5 before homogenization completely removed this peak (not shown). In the absence of Ca2+ ions or at pH 5, ASGP-receptor complexes are unstable (Schwartz et al., 1981). After 2 min of incubation at 37°C most of the prebound ligand was detected in vesicles focusing at a mean density of 1.050 g/ml (Fig. 2). Washing the cells in the presence of EGTA or at pH 5 removed the radioactivity present at a density of 1.045 g/ml, but did not affect the peak at 1.050 g/ml (not shown), indicating an intracellular localization. Prolonged incubation at 37°C gave rise to labeling of denser vesicles up to 1.09 g/ml after 30 min, while at the same time plasma membrane-bound label disappeared. After 60 min of incubation at 37°C, some label had left the cells, probably because of lysosomal degradation and release of the degradation products in the medium (Simmons and Schwartz, 1984). Label in the top fractions of the gradients represents nonmembrane-bound [125I]-ASOR/HRP. If excess of unlabeled ASOR was present during [125I]-ASOR/HRP binding, the nonspecific bound radioactivity was recovered at the top gradient fractions (Fig. 5 A). It was also possible that some endocytosed ligand originating from leaky vesicles contributed to the radioactivity present in these fractions.

**DAB-induced Density Shift**

If HRP-containing microsomes are incubated with DAB and H2O2 before sucrose density fractionation, a density shift can be obtained due to HRP-catalyzed polymerization of...
vesicles did not change upon DAB incubation (Fig. 3 B). Our conclusion is that the density shift of \(^{125}\text{I}-\text{ASOR/HRP}\)-containing vesicles upon DAB cytochemistry is entirely dependent on peroxidase activity.

**The Rate of Sorting of Endocytosed Transferrin and ASOR/HRP**

The DAB density shift principle was used to distinguish between vesicles containing both transferrin and ASOR/HRP, and vesicles containing only one of the ligands. Cells were incubated with \(^{125}\text{I}\)-transferrin (4 \(\mu\)g/ml) and \(^{125}\text{I}-\text{ASOR/HRP}\) (4 \(\mu\)g/ml) at 0°C. Under these conditions the plasma membrane transferrin binding sites were nearly saturated with transferrin (Ciechanover et al., 1983a) and less than half of the 200,000 ASGP binding sites were occupied by ASOR/HRP (Fig. 1). After ligand binding at 0°C, excess ligand was removed, and the cells were incubated at 37°C for 2, 5, or 10 min. After 2 min at 37°C, the density distribution of endocytosed \(^{125}\text{I}\)-transferrin is nearly identical to that of \(^{125}\text{I}-\text{ASOR/HRP}\) (Fig. 4, upper left panel). In contrast to \(^{125}\text{I}-\text{ASOR/HRP}\) containing vesicles, \(^{131}\text{I}\)-transferrin containing vesicles did not increase in density upon prolonged incubation periods at 37°C. After 10 min at 37°C a significant portion had already completed the endocytic cycle and was released from the cells, presumably as apotransferrin. This can be concluded from the decreased total amount of label present at a density of 1.045 g/ml compared with earlier time points. At each time point the \(^{125}\text{I}\)-ASOR/HRP-containing vesicles could be shifted towards densities of \(\sim\)1.09 g/ml after DAB incubation (Fig. 4, right panels). To quantitate the density shift of \(^{125}\text{I}\)-ASOR/HRP-containing vesicles, the ratio of shifted and total endosomal \(^{125}\text{I}\)-ASOR/HRP radioactivity was calculated and used as a measure for calculating...
Sorting of $\text{I}^{125}$I-ASOR/HRP and $\text{I}^{125}$I-transferrin during single cycle endocytosis. Tissue culture dishes (9 cm) were treated as described in Fig. 1 to deplete cell surface receptors from their ligands. Subsequently, the cells were incubated for 60 min at 0°C in medium containing 4 $\mu$g/ml of both $\text{I}^{125}$I-ASOR/HRP (solid circle) and $\text{I}^{125}$I-transferrin (open circle). Excess of ligands was washed away and the cells were incubated for 2, 5, or 10 min at 37°C. Equal aliquots of the postnuclear supernatant were incubated with (+), or without (-) DAB before fractionation. The densities in the gradient were as in Fig. 2.

The degree of $\text{I}^{125}$I-transferrin shift after 2, 5, and 10 min incubation at 37°C was similarly determined, and is given as a percentage of the degree of shift of $\text{I}^{125}$I-ASOR/HRP at 2 min. After 2 min at 37°C, 60% of the intracellular $\text{I}^{125}$I-transferrin coshifted with $\text{I}^{125}$I-ASOR/HRP to denser fractions, implicating a localization of most endocytosed $\text{I}^{125}$I-transferrin in vesicles in which ASOR/HRP was also present. After 5 min, 40% of internalized $\text{I}^{125}$I-transferrin shifted together with $\text{I}^{125}$I-ASOR/HRP, and after 10 min only 20% $\text{I}^{125}$I-transferrin shifted towards denser fractions. These data show that after internalization, most, if not all, $\text{I}^{125}$I-transferrin initially resides in ASOR/HRP-containing vesicles, and that the two ligands are sorted with a half-time of $\sim$2 min, after warming to 37°C.

To ascertain that nonspecifically bound ASOR/HRP did not interfere with the density shift of endocytosed $\text{I}^{125}$I-transferrin, a control experiment was performed in which an excess of nonlabeled ASOR was added to the binding medium to prevent receptor binding of $\text{I}^{125}$I-ASOR/HRP. After 2 min of incubation of prelabeled cells at 37°C, almost no $\text{I}^{125}$I-ASOR/HRP was endocytosed. Only very little $\text{I}^{125}$I-label was recovered in membrane fractions after Percoll gradient fractionation (Fig. 5 A). Under these conditions no endocytosed $\text{I}^{125}$I-transferrin shifted after incubation of the homogenate with DAB (Fig. 5 A, right panel).

To show that the density of $\text{I}^{125}$I-transferrin-containing vesicles was not influenced by the presence of ASOR/HRP in different vesicles during DAB cytochemistry, a mixing experiment was performed. Two cell culture plates were labeled with $\text{I}^{125}$I-ASOR/HRP or $\text{I}^{125}$I-transferrin, respectively, and incubated for 2 min at 37°C. The cells were scraped, combined, homogenized, and DAB cytochemistry was performed. No $\text{I}^{125}$I-transferrin could be shifted to a higher density upon DAB incubation, indicating that intravesicular colocalization of the two ligands was an absolute requirement for co-shifting. We conclude that sorting between receptor-mediated endocytosed transferrin and ASOR/HRP is an intracellular event, occurring within minutes after internalization.

Figure 5. Specificity of the DAB-induced density shift procedure. (A) A 9-cm culture dish was treated as described in Fig. 4, except that besides $\text{I}^{125}$I-ASOR/HRP (solid circle) and $\text{I}^{125}$I-transferrin (open circle), 400 $\mu$g/ml ASOR was also present during ligand binding at 0°C to prevent specific $\text{I}^{125}$I-ASOR/HRP binding. The cells were incubated for 2 min at 37°C before fractionation. (B) Two culture dishes (6 cm) were treated as described in Fig. 4. One culture dish was incubated in medium supplemented with 4 $\mu$g/ml $\text{I}^{125}$I-ASOR/HRP, and the other one in medium with 4 $\mu$g/ml $\text{I}^{125}$I-transferrin for 1 h at 0°C. Excess of ligands were removed and the cells were incubated for 2 min at 37°C. The two cell cultures were scraped, pooled, and homogenized together. The distribution of $\text{I}^{125}$I-ASOR/HRP (solid circle), and $\text{I}^{125}$I-transferrin (open circle) was measured after incubation with (+) or without (-) DAB and Percoll gradient fractionation. The densities in the gradients were as in Fig. 2.
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The incubation period.

ted in a percentage of the binding sites present at the beginning of pH 7.2 in binding medium, respectively. Then the amount of sur-
mm NaCl, 50 gM desferrioxamine, 5 mM CaCl2), and 10 min at

Figure 6. The effect of primaquine on transferrin receptor recycling. Culture dishes (35 mm) were incubated at 37°C for various periods of time in medium supplemented with 40 ng/ml unlabeled transferrin with (solid circle), or without 0.3 mM primaquine (open circle). The cell surface-bound transferrin was removed at 0°C by incubating the cells for 10 min at pH 4.5 (25 mM Na acetate, pH 4.5, 150 mM NaCl, 50 uM desferrioxamine, 5 mM CaCl2), and 10 min at pH 7.2 in binding medium, respectively. Then the amount of surface transferrin receptors was measured by incubating the cells in the presence of 4 gM 125I-transferrin at 0°C. Nonspecific binding, determined in the presence of 100-fold excess unlabeled transferrin, was subtracted from all values. The specific binding is plot-
ing, determined in the presence of 100-fold excess unlabeled trans-
the ceils for 10 min at pH 4.5 (25 mM Na acetate, pH 4.5, 150

The acidic environment is likely to be a prerequisite for
somal degradation are blocked in the presence of 0.3 mM
Ciechanover et al., 1983a). Subsequently, the number of specific
in intracellularly after 2, 5, and 10 min. Taking into account that the half-time of secretion of endocytosed transferrin is ~5 min (Ciechanover et al.,

The Effect of Primaquine on the Sorting of Transferrin and ASOR/HRP

Since primaquine blocks the recycling of transferrin and ASGP receptors, as well as the dissociation of ASGP from its receptor, we have also tested the effect of primaquine on the sorting of the two ligands. If 0.3 mM primaquine was present during a 10-min incubation of cells at 37°C, prela-
beled with 125I-transferrin and 125I-ASOR/HRP at 0°C, a broad endosomal peak with a mean density of 1.05 g/ml was observed for both labeled ligands after Percoll density gradient fractionation (Fig. 7 A). The primaquine-induced endosom-
al swelling, as determined by morphological studies (Geuze et al., 1984), is a possible explanation for the altered density distribution of ligand-containing vesicles in the Per-
coll gradient. DAB treatment of the homogenate resulted in a density shift of 125I-ASOR/HRP to a density comparable to experiments in which no primaquine was used (Fig. 7 B). In addition, all 125I-transferrin exhibited a similar density distribution upon DAB cytochemistry. Our conclusion is that the 125I-transferrin is localized in ASOR/HRP-containing vesicles, at least up to 10 min at 37°C in the presence of primaquine.

Discussion

Our results show that transferrin is sorted from ASGPs within minutes after internalization. The DAB density shift procedure used to monitor this process is a modification of the method originally developed by Courtoy et al. (1984). Plasma membranes, endosomes, and density-shifted material labeled with 125I-ASOR/HRP were separated with a very high reproducibility by single-step Percoll gradient centrifugation. A shift in density of 125I-transferrin-containing vesicles upon DAB cytochemistry indicated a colocalization with ASOR/HRP. If prelabeled cells were incubated for 2 min at 37°C, the density shift after DAB treatment of 125I-transferrin–containing vesicles did not entirely equal that of 125I-ASOR/HRP. An explanation for this phenomenon is that the observed peak of 125I-transferrin after DAB cyto-
chemistry is composed of 125I-transferrin–containing vesicles with and without ASOR/HRP. The percentages of sorted cell-associated 125I-transferrin are 60, 40, and 20% of total radioactive transferrin present intracellularly after 2, 5, and 10 min. Taking into account that the half-time of secretion of endocytosed transferrin is ~5 min (Ciechanover et al.,

Figure 7. The effect of primaquine on the sorting of 125I-transferrin and 125I-ASOR/HRP. A 9-cm culture dish was treated exactly as described in Fig. 4, except that after binding of 125I-ASOR/HRP (solid circle) and 125I-transferrin (open circle) the cells were in-
cubated at 37°C for 10 min in the presence of primaquine. Equal aliquots of the postnuclear supernatant were incubated with (+) or without (−) DAB before fractionation. The densities in the gra-
dients were as in Fig. 2.

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1983a), it can be calculated that prebound transferrin and ASOR are sorted with a half-time of 2 min.

The mean time of internalization at 37°C in HepG2 of prebound 125I-ASOR is 2.2 min (Schwartz et al., 1982). The half-time of internalization of surface-bound transferrin is reported to be 3.5 min (Ciechanover et al., 1983a). Both receptors have nearly similar internalization kinetics (Ciechanover et al., 1983b). Taken together with our results, it can be concluded that both ligands are sorted immediately after internalization. Using Percoll gradient cell fractionation, we found distributions of both ligands between plasma membranes and intracellular locations that are fully in agreement with the data mentioned above. Our results clearly show that the divergence in routing of transferrin and ASGP occurs intracellularly and not at the plasma membrane. In isolated rat hepatocytes (Bridges et al., 1982) and HepG2 cells (Simmons and Schwartz, 1984) ASGPs remain associated with their receptor for several minutes during the process of internalization, as demonstrated by ammonium sulphate precipitation and saponin permeabilization, respectively. Thus, sorting between ASGPs and their receptor and between ASGPs and transferrin occurs within the same time span, and, therefore, possibly in the same compartment. Using double-label immuno-electron microscopy, Geuze et al. (1983a) demonstrated in rat liver that sorting of ASGP and its receptor occurs in a prelysosomal tubulovesicular network, termed CURL. Therefore, CURL is indeed the most likely candidate for transferrin-ASGP sorting. This, however, still needs morphological confirmation.

At 37°C, prebound transferrin is secreted, with a half-time of 5 min, as apotransferrin into the medium (Ciechanover et al., 1983a). Electron microscopic studies demonstrate the appearance of ASGP in lysosomes as early as 5 min after internalization at 37°C, whereas the majority of ligand is localized in secondary lysosomes after 15 min in both HepG2 (Geuze et al., 1983b) and rat hepatocytes (Wall et al., 1980; Geuze et al., 1983a). These data are in agreement with our results, which show that after 10 min at 37°C the total amount of cell-bound 125I-transferrin decreased and the intracellular transferrin retained its localization in the gradient, whereas the endocytosed 125I-ASOR/HRP migrated to heavier fractions, compared with earlier time points.

Both ASGP and transferrin routing are not unidirectional processes. In both rat hepatocytes (Weigel and Oka, 1984) and HepG2 cells (Simmons and Schwartz, 1984), a large slowly dissociating pool of endocytosed ligand-receptor complexes returns to the cell surface. In HepG2 50% of initially internalized ASGP returns receptor-bound to the cell surface in a half-time of 24 min. Up to 28% of internalized 125I-ASOR returns undegraded non-receptor-bound to the incubation medium in a half-time of 84 min. The intracellular route of the transferrin receptor complex is also not unidirectional. Several data indicate that at least part of this complex recycles through Golgi compartments. Morphologically, surface-labeled transferrin receptor has been shown capable of entering the Golgi complex (Hopkins, 1983; Willingham et al., 1984; Woods et al., 1986). Resialylation of surface-desialylated transferrin receptor occurs in a half-time of 2 h (Snider and Rogers, 1985), implicating receptor passage through a sialyltransferase-containing compartment. A possible involvement of the Golgi in transferrin receptor recycling is likely to be only partial because of the low efficiency of resialylation of the transferrin receptor as observed by Snider and Rogers (1985). Our data on the rapid sorting between transferrin receptors and ASGPs are in agreement with the receptor recycling kinetics reported by Ciechanover et al. (1983a). Since this process is almost completed after 10 min at 37°C (Fig. 4) it is not likely that transferrin recycling occurs through the different ASGP routes referred to above. However, the possibility that identical routes, albeit at different rates, are followed cannot be excluded.

The effect of lysosomotropic agents on membrane flow has been reviewed (Dean et al., 1984). They inhibit lysosomal protein degradation (Carpentier and Cohen, 1976; Wibo and Poole, 1974) and neutralize the acidic environment of lysosomes and endosomes (Maxfield, 1982; Ohkuma and Poole, 1978; Poole and Okhuma, 1981; Tycko et al., 1983). Recently the dose-response characteristics of the lysosomotropic agent primaquine on ASGP receptor recycling in HepG2 cells has been published (Strous et al., 1985; Schwartz et al., 1984a). Receptor recycling is completely blocked in the presence of 0.3 mM primaquine. Similar effects have been reported for the ASGP receptor in rat hepatocytes (Tolleshaug and Berg, 1979; Berg and Tolleshaug, 1980). The effect of weak bases on the recycling of the transferrin receptor is not clear. In K562 cells up to 0.1 mM chloroquine did not affect transferrin recycling (Stein et al., 1984), whereas ammonium chloride has been demonstrated to slow down transferrin receptor recycling in HepG2 cells (Ciechanover et al., 1983a). Our results show that transferrin receptor recycling is inhibited in the presence of 0.3 mM primaquine. Similar effects have been reported for the ASGP receptor in rat hepatocytes (Tolleshaug and Berg, 1979; Berg and Tolleshaug, 1980).

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