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Sorting and Recycling of Cell Surface Receptors and Endocytosed Ligands: The Asialoglycoprotein and Transferrin Receptors

Aaron Ciechanover, Alan L. Schwartz, and Harvey F. Lodish

Department of Biology, Massachusetts Institute of Technology, Boston, Massachusetts 02139 (A.C., H.F.L.) and Division of Pediatric Hematology/Oncology, Children's Hospital Medical Center, Dana-Farber Cancer Institute, and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115 (A.L.S.)

With few exceptions, receptor-mediated endocytosis of specific ligands is mediated through clustering of receptor-ligand complexes in coated pits on the cell surface, followed by internalization of the complex into endocytic vesicles. During this process, ligand-receptor dissociation occurs, most probably in a low pH prelysosomal compartment. In most cases the ligand is ultimately directed to the lysosomes, wherein it is degraded, while the receptor recycles to the cell surface.

We have studied the kinetics of internalization and recycling of both the asialoglycoprotein receptor and the transferrin receptor in a human hepatoma cell line. By employing both biochemical and morphological/immunocytochemical approaches, we have gained some insight into the complex mechanisms which govern receptor recycling as well as ligand sorting and targeting. We can, in particular, explain why transferrin is exocytosed intact from the cells, while asialoglycoproteins are degraded in lysosomes. We have also localized the intracellular site at which endocytosed receptor and ligand dissociate.

Key words: receptor recycling, asialoglycoproteins, transferrin, iron delivery

Many cells are capable of internalizing macromolecules by receptor-mediated endocytosis. The first step in this multiphase process involves binding of a ligand, such as a hormone, virus, plasma protein or toxin, to a specific receptor molecule functionally exposed at the cell surface. In most cases, these receptors are distributed

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diffusely over the cell surface. This has been demonstrated by visualization of fluorescent-labeled ligands such as α_2-macroglobulin, insulin, epidermal growth factor, transferrin, and asialoglycoproteins [1–3]. Binding of ligand is followed by rapid clustering of the surface ligand-receptor complexes into clathrin-coated pits in the plasma membrane and internalization into coated vesicles [4]. Thereafter, both the ligand and the receptor are found in uncoated vesicles. Many ligands, such as asialoglycoproteins, α_2-macroglobulin, low density lipoprotein, (LDL), and insulin, are then transported within membrane-limited compartments to lysosomes where they are rapidly degraded [5–12].

By contrast, the receptor almost invariably escapes degradation, recycles to the cell surface, and mediates the internalization of additional ligand molecules. Recycling of receptors was deduced from the early observation that cells continue to internalize receptor-bound ligands at a steady rate for many hours without depleting their surface receptors, even when synthesis of new receptor molecules is blocked by protein synthesis inhibitors. More direct evidence for recycling comes from the observation that the process can be inhibited by agents such as weak bases (eg, chloroquine, NH_4Cl) or carboxylic ionophores (eg, monensin) that disrupt proton gradients and raise the pH of acidic intracellular compartments. When cells take up ligands in the presence of these agents, the receptors do not return to the cell surface, and the number of receptors on the surface rapidly decreases. Such evidence that surface receptors recycle was obtained for receptors for asialoglycoproteins [8,13–16], mannose-6-phosphate-terminated proteins [17], mannose-terminal proteins [18], LDL [9], α_2-macroglobulin [19], insulin [20], and the chemotactic peptide [21].

An important development in understanding of the mechanism of receptor recycling was the demonstration by Tycko and Maxfield [22] that, following ligand internalization, endosomes rapidly become acidified. These investigators incubated cultured cells with fluorescein-labeled α_2-macroglobulin. Within 15 min of endocytosis, the fluorescein was located within an acidic compartment, as indicated by alteration of its emission spectrum. This interval was too short for the ligand to have reached the lysosomes, a conclusion confirmed by histochemical electron microscopy.

The finding of an acid pH in the endosome suggests a mechanism by which receptor-ligand dissociation may be initiated. Many ligands such as asialoglycoproteins [23], lysosomal enzymes [17], LDL [24] and insulin [25] rapidly dissociate from their respective receptors at pH values below 6. The low pH within the endosome would be expected to cause ligand-receptor dissociation, and thereby allow unoccupied receptors to return to the surface.

The assumption that the receptor-ligand dissociation occurs in a low pH, prelysosomal compartment accounts for two important features of receptor recycling. First, it explains how dissociation can occur so rapidly after internalization. The LDL receptor, for example, is estimated to return to the cell surface within 12 min after it enters the cell, a time too short to allow transit to the lysosome [9]. We found similarly short times ($\sim$8 min) for the asialoglycoprotein [8] and the transferrin receptors [26]. Second, this finding provides a mechanism whereby a receptor can make many trips into and out of the cell without sustaining proteolytic damage. By dissociating from their ligands prior to fusion with the lysosome, surface receptors are segregated from lysosomal proteases. Acidification of the endosome seems to be an obligate step in receptor-mediated endocytosis and occurs when many different ligands enter the cell: asialoglycoproteins [27], transferrin [28], and viruses [29]. The

process involves an ATP-dependent proton pump [30,31] similar (but probably not identical) to that described in lysosomes [32]. Still, many problems remain unsolved in understanding this complex round trip itinerary of cell surface receptors. What directs ligands only to lysosomes following their dissociation from receptor? What are the mechanisms and signals involved in segregation and recycling of the receptors to the cell surface? Why are certain ligands, such as transferrin, segregated from lysosomal proteases and returned to the cell surface intact?

In order to address some of these problems, we have studied the receptors for asialoglycoproteins and transferrin in a human hepatoma cell line (HepG2), using a combination of biochemical and immunocytochemical/electron micrographic approaches.

THE ASIALOGLYCOPROTEIN RECEPTOR

Recycling of the Asialoglycoprotein Receptor: Biochemical Evidence

We and others have been studying the receptor for galactose terminal carbohydrates of glycoproteins (asialoglycoproteins) which is localized to the hepatic parenchymal cell [33]. Endocytosis of asialoglycoproteins has been studied in considerable detail in whole liver in vivo, in perfused liver in situ, and in isolated rat hepatocytes [8,15,23,34,35]. There are as many as 500,000 high-affinity surface receptors in the rat hepatocyte [7,36]. In addition, this receptor has been isolated and purified from rabbit, rat, and human liver [37,38]. Recent studies have begun to elucidate the characteristics of receptor-mediated endocytosis in this system. Using electron microscopic techniques, Hubbard and colleagues have demonstrated the uptake of galactose terminal glycoproteins by rat hepatic parenchymal cells and followed their subsequent transfer through a series of endocytic vesicles to lysosomes [7,34,39]. Biochemical studies by Tolleshaug et al [40], Steer and Ashwell [15], and others, have provided evidence for a receptor-mediated uptake of asialoglycoproteins by isolated rat hepatocytes.

The human hepatoma cell HepG2 isolated by Knowles et al [41] contains abundant asialoglycoprotein receptors [42]. As seen in Table I, HepG2 cells specifically bind [^{125}I]asialoorosomucoid (ASOR). Binding studies were performed at 4°C in order to inhibit internalization of the ligand. Binding requires the presence of Ca^{++} and is not substantially affected by the presence of a 100-fold excess (by mass) of orosomucoid or asialoagalactoorosomucoid. Pretreatment of the cells with neuraminidase renders them incapable of binding [^{125}I]ASOR. There are 150,000–250,000 high-affinity ASOR-binding sites per cell surface. These data are consistent with the characteristics of the asialoglycoprotein receptor in rat hepatocytes. In addition, once bound to its receptor, [^{125}I]ASOR could be readily displaced by either a brief treatment at 4°C with edetate (EDTA) or N-acetylgalactosamine, but only minimally by galactose. N-acetylglucosamine or ASOR was without effect (Table II). The sensitivity of surface bound [^{125}I]ASOR to displacement by EDTA or N-acetylgalactosamine provides a sensitive and convenient assay for surface-bound ligand; internalized ligand is resistant to such treatments.

At 37°C, there is a linear increase in the amount of cell-associated [^{125}I]ASOR during the first 2 hr (Fig. 1). A constant level of cell-associated ligand is reached by 2 hr. There is little ^{125}I label in degradation products in the medium before 1 hr, and

TABLE I. Specificity of [^{125}I]ASOR Binding to Hepatoma Cells*

Addition	[^{125}I]ASOR bound (%)
None	100
EDTA	13
Asialoorosomucoid	22
Orosomucoid	93
Asialoagalactoorosomucoid	84
Neuraminidase preincubation	24

*Dishes were washed and incubated with [^{125}I]ASOR (2 μg ml^{-1}) in the absence or presence of additional agents in the standard manner (2 hr, 4°C; see [42]). Nonradioactive asialoorosomucoid, orosomucoid, and asialoagalactoorosomucoid were added at 200 μg ml^{-1}. EDTA was present at 5 mM. Neuraminidase preincubation was performed by incubation with 20 mU in 1 ml of phosphate buffered saline (PBS) for 15 min at room temperature. Results are expressed as the percentage of the total [^{125}I]ASOR bound compared with the control ("none") (adapted from Schwartz et al [5]).

TABLE II. Specificity of Release of Surface Bound ASOR From Hepatoma Cells*

Addition (concentration) (time)	[^{125}I]ASOR bound (%)
None	100
EDTA (5 mM; 3 min)	12
N-acetylgalactosamine	
(100 mM, 10 min)	11
(50 mM, 10 min)	24
Galactose (100 mM; 10 min)	62
N-acetylglucosamine (100 mM; 10 min)	100
ASOR (200 μg ml^{-1}; 300 min)	95

*Dishes were washed and incubated with [^{125}I]ASOR (2 μg ml^{-1}) in the standard manner (2 hr, 4°C; see [37]). After washing in PBS containing 1.5 mM $CaCl_2$, the indicated additions were made for the indicated time at 4°C. Thereafter, one further rinse in PBS with $CaCl_2$ was performed and the samples counted. Results are expressed as a percentage of the total [^{125}I]ASOR bound compared with the control ("none") (adapted from Schwartz et al [67]).

the linear increase in ^{125}I-degradation products begins by the second hour (Fig. 1). As expected, there is no detectable degradation of [^{125}I]ASOR when maintained for 6 hr at 37°C under identical conditions, but in the absence of cells (data not shown). As measured by the sum of cell-associated and degraded ^{125}I radioactivity, the overall rate of cellular uptake of ASOR is constant at 0.02–0.03 pmol min^{-1} per 10^6 cells for at least 6 hr (Fig. 1). In 28 independent experiments, the rate of [^{125}I] ASOR uptake at a concentration of 2 μg ml^{-1} at 37°C, assessed over the first 60 min, averaged 0.029 $\pm$ 0.001 pmol min^{-1} per 10^6 cells, as we have reported earlier [8,42].

Because the rate of ligand uptake (ligand flux) is dependent upon the total cell complement of functional receptors that participate in this process, we have determined the cell receptor distribution by destroying cell surface receptors with protease. As shown in Table III, single cell suspensions of HepG2, prepared by treatment of monolayer cultures with an EDTA solution at 4°C, bind at 4°C the same amount of [^{125}I]ASOR as do cells assessed under standard conditions in monolayer culture. The

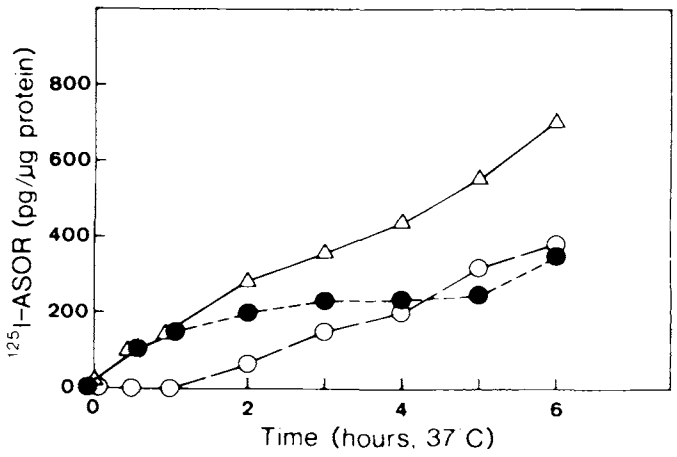


Fig. 1. Uptake and degradation of [^{125}I]ASOR in human hepatoma cells at 37°C. Tissue culture dishes containing 10^6 cells were washed and incubated with [^{125}I]ASOR (2 μg ml^{-1}) for various times at 37°C. At the appropriate times the media were removed and analyzed for ^{125}I degradation products. In addition, the cells were rinsed and analyzed for cell-associated [^{125}I]ASOR. The figures represent the mean and range of duplicate determinations of cell associated radioactivity (●—●), ^{125}I degradation products of the media (○—○), and the sum of the two previous values (△—△) (adapted from Schwartz et al [8]).

TABLE III. Surface Receptor Distribution and Uptake of [^{125}I)ASOR in Hepatoma Cells After Trypsin Treatment*

Treatment	Specific binding (pmol/10^6 cells)	Rate of [^{125}I]ASOR uptake (60 min) (pmol/10^6 cells/min)
Monolayer-EDTA	0.29 ± 0.04	0.023 ± 0.001
Suspension-EDTA	0.33 ± 0.04	0.023 ± 0.001
Suspension-EDTA/trypsin	0.02 ± 0.00	0.004 ± 0.000

*Dishes containing monolayer cells were rinsed and incubated with [^{125}I]ASOR (2 μg ml^{-1}) at either 4°C (specific binding) or 37°C (uptake) (42). Cell suspension prepared with either EDTA or EDTA/trypsin were incubated with [^{125}I]ASOR (2 μg ml^{-1}) at either 4°C (specific binding) or 37°C (uptake). Results (mean ± SE) are presented of duplicate (binding) or triplicate (uptake) values. The total fraction of cell associated receptors remaining after trypsin treatment is equal to the rate of uptake of [^{125}I]ASOR, relative to control cells (adapted in part from Schwartz et al [8]).

rate of ligand uptake at 37°C is also unimpaired. However, if trypsin is included in the EDTA solution at 4°C (as is used for dispersing monolayer cells), binding of [^{125}I]ASOR to cells is inhibited by over 90%, indicating that virtually all surface receptors are destroyed by this protease. When trypsin-treated cells are incubated at 37°C, uptake of [^{125}I]ASOR is linear with time (data not shown) but is only 20 ± 2% of that of control cells. Taking into account that only 94% of the surface receptors is actually destroyed by trypsin, the data in Table III indicate that in growing HepG2 cells approximately 88% of the functional receptors are on the surface, and 12% are internal. It should be pointed out however, that in some systems such as the mannose receptor in macrophages [18], the mannose-6-phosphate receptor in Chinese hamster

ovary (CHO) cells [43], and the receptor for asialoglycoproteins in rat hepatocytes [15], the bulk of the receptors are on the inside rather than on the cell surface.

Because the uptake and degradation of ligand continues at a steady rate of 15,000 molecules per cell per minute independent of new receptor synthesis for at least 6 hr, and because there are 150,000–200,000 binding sites per cell surface, either there must exist a large pool of previously synthesized receptors within the cell, or receptor reuse must occur to some extent. If no reuse occurs, then the functional receptor pool within the cell must be at least 30–60-fold greater than the number of surface receptors. However, in these HepG2 cells, 88% of all functional receptors are on the cell surface. As calculated from the total number of functional receptors per cell (225,000) and the rate of ligand uptake (15,000 molecules per minute at an ASOR concentration of 2 μg ml^{-1}), each receptor must recycle the ligand, on the average, every 15.9 min(= 225,000 ÷ 15,000 min^{-1}). These observations and calculated values were all obtained at a ligand concentration of 50 nM(2 μg ml^{-1}). Obviously, at higher ligand concentrations the total cycle time will decrease, as will the time required for ligand binding, until a point is reached at which binding is no longer rate limiting. The rate of ligand uptake and degradation at 10–20 μg of [^{125}I]ASOR ml^{-1} (ie 30,000 molecules per cell per minute) is double that 2 μg ml^{-1} and the cycle time at 10–20 μg ml^{-1} is about half that at 2 μg ml^{-1}, or 7.9 min.

At 2 μg ml^{-1} ASOR, binding of ligand to surface receptors requires a mean time of 8.7 min. Internalization of receptor ligand complexes requires a mean of 2.2 min, whereas a mean of 4.2 min is required for the internalized receptor to dissociate its ligand and return to the cell surface (Fig. 2, Table IV) [8]. Each of these rate constants was determined by two or more independent means; the sum of these times yields 15.1 min for the total cycle time of the asialoglycoprotein receptor (assessed at 2 μg ml^{-1} ligand).

Asialoglycoprotein (ASGP) ligands are therefore capable of being taken up and processed through to the lysosomes at a considerable rate (see also [33]), whereas the receptor is apparently spared degradation. Additional biochemical studies have demonstrated that the intracellular half life of ASGP ligand taken up by receptor-mediated endocytosis is about 15–20 min, whereas that of the receptor is probably greater than 40 hr [33].

Importantly, in HepG2 cells, degradation of internalized ligand begins only after 20–30 min, a time much longer than the total cycle time of the receptor (Fig. 1). Such studies suggest that receptor is not transferred to lysosomes, a conclusion substantiated by our morphological studies.

If the internalization of a surface receptor for a particular ligand is a specific process and if all surface receptors are internalized in synchrony, one should be able to show directly a significant and transient depletion of surface receptors, until the depleted pool is replaced. Even if the process of receptor internalization occurs continuously both in the presence and absence of bound ligand (thus making our assumption incorrect), it is likely that accelerated internalization or decreased externalization occurs in the presence of ligand, causing a transient depletion of surface receptors. This assumption was based on our previous finding that in the continuous presence of ASOR, there is a substantial redistribution of functional asialoglycoprotein receptors to intracellular sites. The number of functional intracellular receptors is doubled (13 to 28% of total) when HepG2 cells are continuously exposed to 2 μg ml^{-1} ASOR [8].

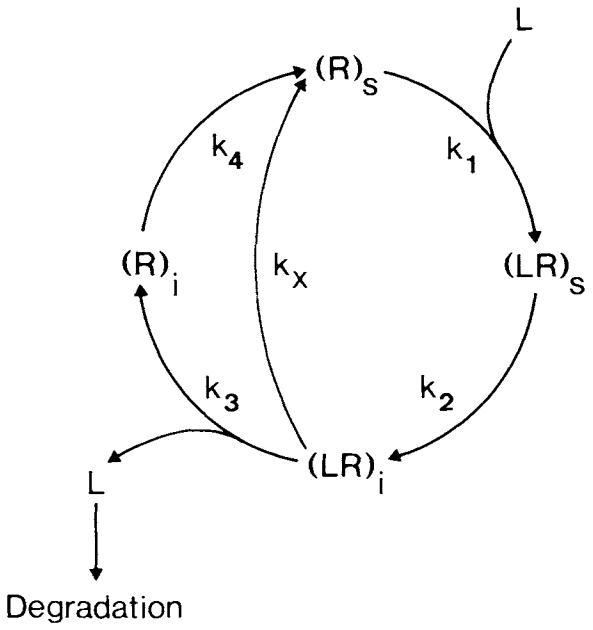


Fig. 2. A kinetic model for receptor-mediated endocytosis of asialoglycoprotein receptor. L, ligand; $(R)_S$, unoccupied surface receptors; $(LR)_S$, occupied surface receptors; $(LR)_i$, occupied internal receptors; $(R)_i$, unoccupied internal receptors; k_1, rate constant for binding; k_2, rate constant for internalization; k_3 rate constant for dissociation of ligand and receptor within the cell; k_4, rate constant for reappearance of receptor to cell surface; k_x, overall rate constant for dissociation of receptor-ligand complex and return of internal receptor to cell surface($1/k_x = 1/k_3 + 1/k_4$) (adapted from Schwartz et al [8]).

Indeed, we were able to demonstrate a 55% reduction in cell surface asialoglycoprotein binding sites after saturating the cell surface sites with ASOR at 4°C and then warming the cells to 37°C (Fig. 3). Cells were incubated at 4°C for 2 hr in the presence of excess unlabeled ASOR (40 μg ml^{-1} K_d = 0.4 μg ml^{-1} [42]), and thereafter washed free of unbound ligand. The cells were then warmed to 37°C in the absence of added ligand for various times ranging from 0.5–11 min, then quickly rechilled to 4°C (warming and rechilling require approximately 5 sec each). Surface receptors unoccupied by ligand were quantified by binding [^{125}I]ASOR to the cells under saturating conditions at 4°C. To measure the total number of receptors present on the cell surface, both those occupied and unoccupied with ligand, we first stripped the cells of surface-bound ASOR by incubation for 3 min at 4°C in ice-cold phosphate-buffered saline (PBS) containing 5 mM EDTA. Replicate dishes of cells were then incubated under saturating conditions at 4°C with [^{125}I]ASOR.

After internalization of ASOR, the total number of surface receptors dropped to 45–55% by 2 min, and then returned to its original value within the next 8 min (Fig. 3B). Initially, all of the surrface receptors were occupied with ligand. All of the receptors that reappeared on the cell surface after one cycle of endocytosis lacked bound ligand (Fig. 3B). Following a lag of about 1 min, unoccupied receptors reappeared on the surface with a half-time of about 3.5 min. Since control studies

TABLE IV. Parameters of Endocytosis of Transferrin and Asialoglycoprotein*

Parameter	Receptor	
	Transferrin	Asialoglycoprotein
A. Binding of ligand[a]		
k_1 (mol $^{-1}$min^{-1})	3.02×10^6	2.23×10^6
Mean time (min)[b]	4.3	8.7
B. Dissociation of ligand		
k_{-1} (min^{-1})	0.09/0.106[c]	< 0.00
Mean time (min)	11.1/9.4[c]	—
C. Internalization of surface receptor		
k_2 (min^{-1})	0.20/0.30[c]	0.46
Mean time (min)	5.0/3.3[c]	2.2
D. Return of receptor to surface		
k_x (min^{-1})	0.14	0.24
Mean time (min)	7.19	4.2
E. Dissociation of apotransferrin		
k_o (min^{-1})	2.6	—
Mean time (min)	0.38	—
F. Cycle time (T_c) (min)		
Measured form the rates of iron or asialoorosomucoid uptake	15.8	15.9
G. Sum of $1/(K_1L) + 1/(k_2) + 1/(K_x) + (1/(K_o))$	16.9	15.1

*Adapted from Schwartz et al [8] and Ciechanover et al [26]. The rate constants are defined in Figures 2 and 15.

[a]Experiments were performed at 50 nM asialoorosomucoid and 77 nM transferrin.

[b]Equals (k_1^{-1}) (molar concentration of ligand)$^{-1}$; the mean time required for a surface receptor to bind a ligand at the concentration employed: 50 nM asialoorosomucoid *or* 77 nM transferrin.

[c]The first measurement was carried out using [^{125}I]ferrotransferrin and the second using [^{59}Fe]transferrin. The former values are used in all subsequent calculations.

(data not shown) demonstrated no loss of prebound ligand to the medium after 15 min incubation at either 4°C or 37°C, and since new receptor synthesis was totally abolished by the presence of cycloheximide throughout the experiment, we feel that all of the measured surface ligand binding sites at the end of the study originated from those which were originally on the surface and recycled, or from receptors which were internal at the start of the study. It is possible that kinetics of reappearance of the receptors on the cell surface (Fig. 3B) is slightly different, since some receptor-ligand complexes may reappear on the cell surface and, perhaps, remain undetected.

If ASOR ligand is not prebound to the cell surface at 4°C, there is no alteration in the number of surface ASOR receptors subsequent to warming to 37°C (Fig. 3B). We conclude that both the loss and reappearance of surface receptors is a consequence of ligand binding and internalization.

This experiment directly shows that many surface receptors recycle back to the cell surface. At the start of the 37°C incubation, only 13% of the total population of receptor was internal, while 87% was on the surface [8], yet 45–55% of the cell surface receptors disappeared and then reappeared. At least 34% of the receptors on the surface at the end of the study must have been those that were originally on the surface and then internalized and recycled (see [44]).

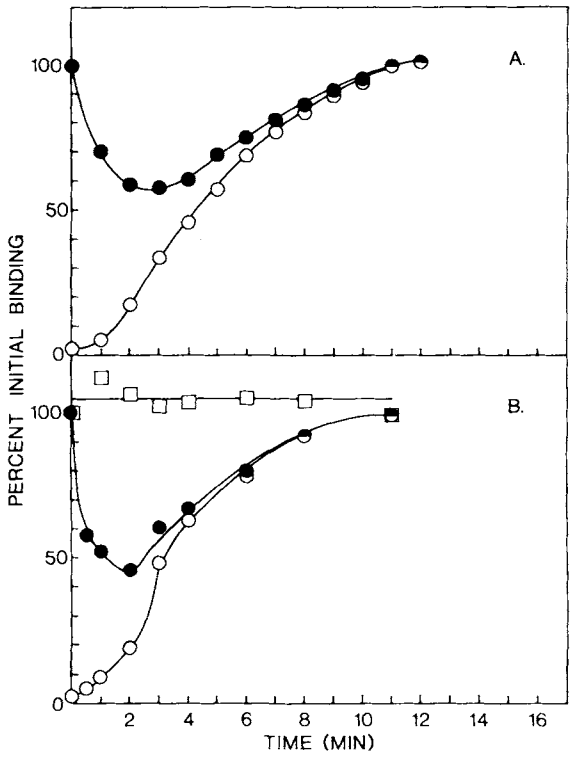


Fig. 3. Calculated and observed recycling kinetics of the asialoglycoprotein receptor in HepG2 cells. A. Calculated curve: The differential equation [8] describing our model of receptor internalization and cycling was solved making the following assumptions: At t = 0, $(LR)_s = 0.87$, $(LR)_i = 0.13$, and $R_s = 0$; $k_1L = 0$(ie, the absence of free ligand precludes the binding of additional ligand to the cell); $k_2 = 0.47\ min^{-1}$; and $k_x = 0.23\ min^{-1}$. The total number of surface receptors (●—●)($[LR]_s + R_s$) is plotted, normalized to the value of 1.00 at t = 0. Since we assume here that $k_1L = 0$, at the end of the experiment all of the receptors will be on the surface ($R_s = 1.00$) and thus there will be slightly more surface receptors than at t = 0. The number of surface receptors free of ligand (R_s) is also plotted (○—○). B. Experimental curve: HepG2 cells were preincubated for 30 min at 37°C in binding medium containing 0.4 mM cycloheximide, and chilled. They were incubated for an additional 2 hr at 4°C with (○—○, ●—●) or without (□—□) 0.5 μM of unlabeled ASOR in the presence of 0.4 mM cycloheximide. The binding medium was removed and the cells were washed three times in PBS (containing 1.7 mM $CaCl_2$) at 4°C. The cells were than incubated with 1 ml of prewarmed binding medium (containing 0.4 mM cycloheximide) at 37°C. At the indicated times, the medium was quickly removed and the cells chilled immediately to 0°C by immersion in ice-cold PBS (containing 1.7 mM $CaCl_2$). The cells were then treated for 3 min at 4°C in PBS containing 5 Mm EDTA (●—●) to release the surface-bound ligand) or in PBS alone (○—○, □—□). Binding of [^{125}I]ASOR at 4°C was performed in a medium contained 0.4 mM cycloheximide (adapted from Ciechanover et al [44]).

Making use of a simple kinetic model for asialoglycoprotein receptor function (Fig. 2 and [8]), we can calculate a theoretical curve (Fig. 3A) for the number of surface receptors following ligand internalization.We have used the rate constants for

internalization of surface receptor-ligand complexes (k_2, 0.47 min^{-1}; mean time of 2.1 min) and for dissociation of internalized receptor-ligand complexes and return of the receptor to the surface (k_x, 0.23 min^{-1}; mean time = 4.2 min), which we calculated previously [8]. We regard the agreement of the experimental results in Figure 3B with our prediction (Fig. 3A) as marked confirmation both of our model and of the calculated values of k_2 and k_x. We conclude that surface asialoglycoprotein receptor is internalized in parallel with ligand, with a first-order rate constant of about 0.47 min^{-1}, and that receptor returns to the surface with a rate constant of about 0.23 min^{-1} (the experimental value is 0.32 min^{-1}, taken from Fig. 3B).

Recycling of the Asialoglycoprotein Receptor: Immunoelectron Microscopy During Receptor Mediated Endocytosis

In collaboration with H.J. Geuze at the Center for Electronmicroscopy, Utrecht, The Netherlands, we have used the recently developed double-labeling immunocytochemical electron microscopic technique [45], with antibodies against both asialoglycoproteins ligand and receptor to visualize the compartment in which dissociation of the ligand receptor complex occurs. Asialofetuin (1–6 mg) was administered in 1-ml physiological saline containing 1.5 mM $CaCl_2$ to adult rats by continuous infusion into a tail vein over 30–60 min, followed by perfusion fixation with 2% formaldehyde-0.5% glutaraldehyde. Cryosectioning and immunolabeling with colloidal gold adsorbed to staphylococcal protein A were essentially as described by Geuze et al [3, 45]. Affinity-purified monospecific rabbit antibodies against the purified rat liver asialoglycoprotein receptor [38] and against purified asialofetuin were employed.

Both ligand and receptor are taken up from the sinusoidal cell surface in clathrin-coated vesicles, which deliver the complexes to vesiculotubular structures [5]. Both receptor and ligand were found associated with the membrane of small clathrin-coated endocytic vesicles close to the cell surface. Little or no free ligand occurred within the lumen of these vesicles.

We also identified other larger vesicles found at some distance from the plasma membrane, which contain ligand accumulated within the lumen. The membranes of these latter vesicles contained little receptor, but receptor was concentrated in detached tubular extensions that were largely free of ligand (Fig. 4). No significant receptor labeling was ever found within the vesicle lumen. Interestingly, receptor was not uniformly distributed along the membrane of these larger vesicles, but was either dispersed in clusters along the vesicle membrane or appeared as accumulations at the poles, where vesicles and thin membranous tubules approximated each other or were continuous. In most such vesicles, receptor labeling was either low compared with the connected or adjacent tubules or was absent. It is in these vesicles that, we believe, the ligand is uncoupled from the receptor; the tubular membranous structures could be intermediates in the recycling of receptor to the cell surface. This double labeling pattern strongly suggests that these curl-tailed vesicles represent the Compartment of Uncoupling of Receptor and Ligand. The acronym CURL has been suggested to identify this compartment of dissociation [5].

THE TRANSFERRIN RECEPTOR

General Properties

Transferrin is a serum glycoprotein which plays an important role in iron transport and delivery to cells. It has two binding sites for ferric ions (reviewed in

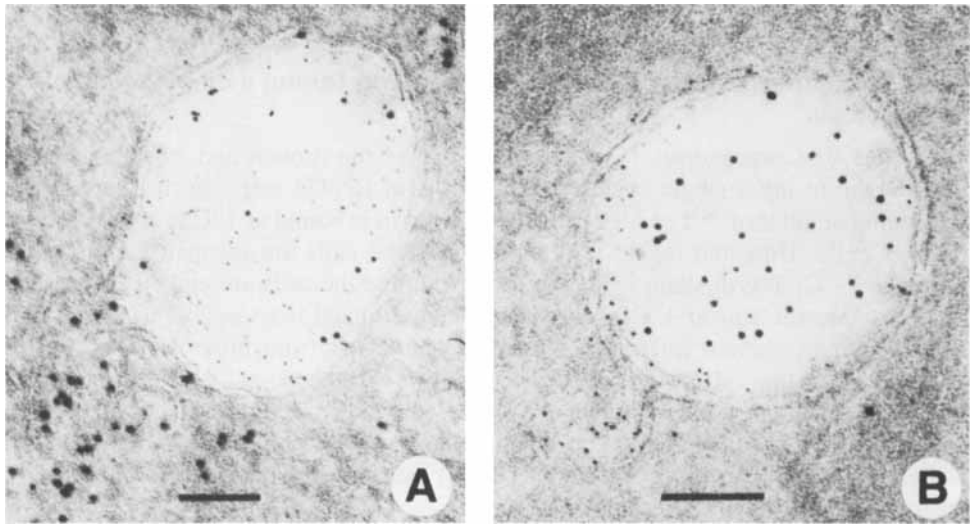


Fig. 4. A. Immunocytochemical electron micrograph of ultrathin cryosections from perfusion-fixed rat liver during continuous infusion of asialofetuin. Ligand was labeled first with antiasialofetuin antibody and them with 5 nm colloidal gold protein A. Thereafter asialoglycoprotein receptor was immunolabeled with antibody and then with 8 nm colloidal gold protein A. Free ligand can be seen in the lumen of the vesicular portion of this sorting vesicle, which also shows scarce and heterogeneous receptor distribution. Receptor labeling is intense over the connecting tubules. Bar = 0.1 μm. B. Similar to A except that receptor is labeled with 5 nm gold, whereas ligand is labeled with 8 nm gold. Receptor is located predominantly at the fold where a tubule with heavy receptor labeling is connected. Most of the ligand is present free within the vesicle lumen (adapted from Geuze et al [5]). Bar = 0.1 μm.

[46]) and it binds to a specific membrane receptor which appears to be the first step in the complex process of iron uptake [47]. This receptor glycoprotein is found on many cells and has recently been purified [48,49]. All cells require iron as a constituent of respiratory and other heme-containing proteins [50]. Studies of iron uptake by cells and the role of the transferrin receptor have led to some disagreement about the events that occur following binding of transferrin to the cell surface receptor. According to one model, iron enters the cell together with transferrin by receptor-mediated endocytosis. The iron then dissociates from transferrin, probably in a low pH prelysosomal compartment [28,51], and is delivered in a yet unknown way to the iron storage protein, ferritin [52]. The apotransferrin recycles to the cell surface and is released to the medium to be reutilized as an iron carrier [26,51–53].

According to an alternative model, iron is removed from the transferrin at the cell surface and the iron alone is internalized by an as yet, undefined cellular process [54–56]. It is possible that different cells employ different mechanisms of iron uptake.

We became interested in the process of receptor-mediated endocytosis of transferrin because of the many puzzling features of the system. While most ligands endocytosed by a receptor-mediated mechanism are transported to lysosomes and are degraded, apotransferrin (after delivery of iron to the cell) is exocytosed intact into the medium [52; Ciechanover et al, unpublished data]. Is apotransferrin dissociated from its receptor within the cell as are other ligands? If so, how does it escape degradaton by the lysosome and how is it secreted into the medium? Or does

transferrin remain bound to its receptor in endocytic vesicles? If so, how and when is apotransferrin released from its receptor into the culture medium?

The Fate of the Transferrin Polypeptide and Iron During a Single Cycle of Endocytosis

Our first experiments focused on the fate of the protein and iron moieties of transferrin during a single cycle of endocytosis in HepG2 cells. In these studies, a saturating amount of ^{125}I or ^{59}Fe diferric transferrin is bound at 4°C to the surface of HepG2 cells. Unbound ligand is removed, and the cells are incubated for various times at 37°C. The medium is quickly removed, and the cells are chilled and treated with Pronase for 1 hr at 4°C. Only surface-bound ligand is accessible to the proteolytic enzyme, whereas internalized ligand is protected from proteolysis and is recovered with the cell pellet. At least 39% of surface bound [^{125}I]transferrin is internalized within 5 min, and then is exocytosed into the medium (Fig. 5). All of the exocytosed ^{125}I radioactivity is in intact transferrin, as shown by sodium dodecyl sulfate-gel electrophoresis [Ciechanover et al, data not shown]. Similar results have been reported by others [52, 54, 57–60].

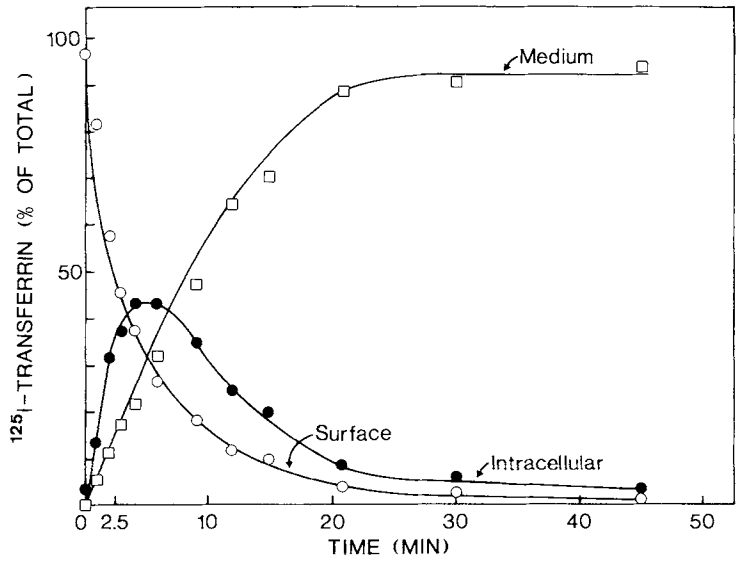


Fig. 5. Diacytosis of [^{125}I]transferrin in HepG2 cells. Transferrin was bound to HepG2 cells at 4°C. After washing off excess unbound ligand, the cells were incubated at 37°C following addition of prewarmed binding medium containing 128 nM unlabeled transferrin. At the indicated times, the medium was quickly removed and the cells chilled in ice-cold PBS (containing 1.7 mM $CaCl_2$) and treated with Pronase. The radioactivity in the Pronase-resistant (internalized) fraction (●—●), Pronase-sensitive (cell surface) fraction (○—○), and the medium (□—□) was determined (adapted from Ciechanover et al [26]).

The fate of the iron moiety of transferrin is different. As can be seen from Figure 6, 63% of the iron of surface-bound [^{59}Fe]transferrin is subject to endocytosis and remains within the cell. Twenty three percent of the ^{59}Fe is lost from the cell surface and released directly into the medium as intact [^{59}Fe]transferrin.

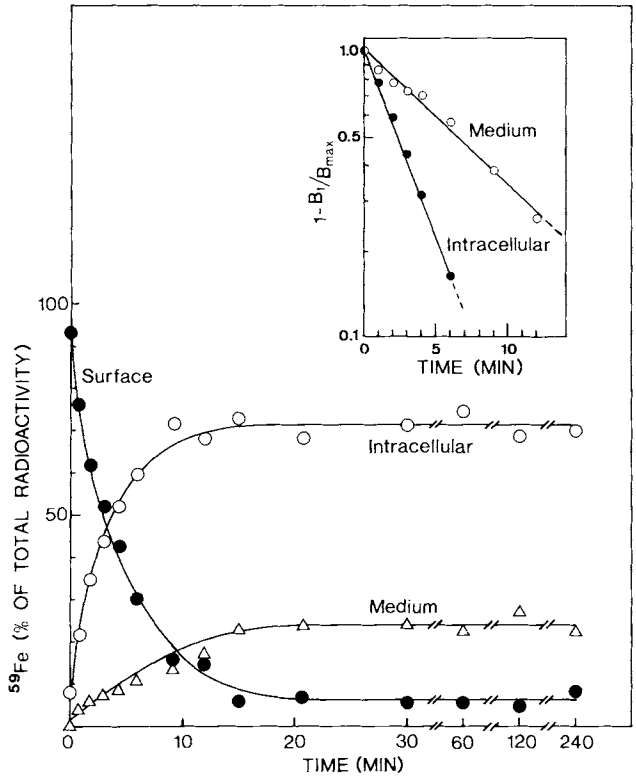


Fig. 6. Single cycle receptor-mediated endocytosis of [^{59}Fe]transferrin in HepG2 cells. The experimental details are as delineated in the legend to Figure 5 except that [^{59}Fe]transferrin was bound to the cells instead of [^{125}I]transferrin. Inset: The accumulation of [^{59}Fe] in the cell and the dissociation of [^{59}Fe]transferrin into the medium were replotted semilogarithmically as $1-(B_t/B_{max})$ versus time, where B_{max} is the maximum amount in the compartment, and B_t is the amount at time t. From the intracellular values, 8% was subtracted to correct for the background of pronase-resistant radioactivity at zero time (adapted from Ciechanover et al [26]).

pH and the Recycling of Transferrin and the Transferrin Receptor During Receptor Mediated Endocytosis

Next, we examined the mechanism(s) involved in the dissociation of Fe and transferrin from the transferrin receptor. Since endocytic vesicles which contain α_2-macroglobulin [22], transferrin [28], and asialoglycoproteins [27] are acidic, we examined the effect of low pH on dissociation of various ligands from their respective receptor. As can be seen in Figure 7, the stability of the transferrin-receptor complex was not affected by pH, whereas both insulin and asialoorosomucoid are dissociated from their respective receptors at pH's of 5 or less. This result underscores the marked differences between the transferrin-receptor complex and other ligand-receptor complexes.

Because the pH of the endocytic vesicle is approximately 5 [22,28], we examined the possibility that iron dissociates from receptor-bound transferrin at low pH, and more importantly, that the resultant apotransferrin does not dissociate from its

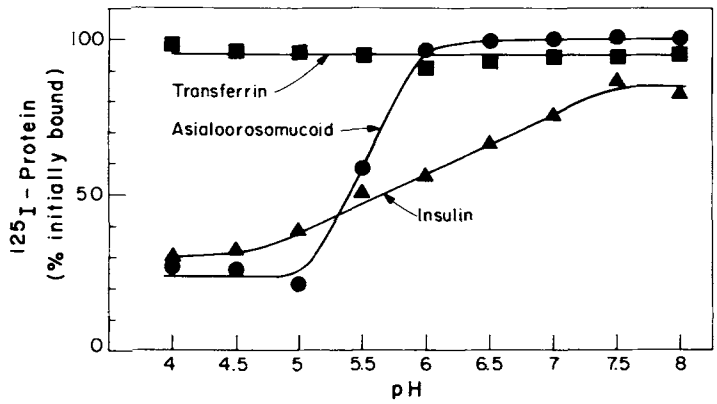


Fig. 7. Effect of pH at 4°C on dissociation of transferrin, insulin, and asialoorosomucoid from HepG2 cells. [^{125}I]insulin (▲—▲), transferrin (■—■), and [^{125}I]asialoorosomucoid (●—●) were bound to HepG2 cells at 4°C. After washing off unbound ligand, the cells were incubated at 4°C for 5 min at different pH's (adapted from Dautry-Varsat et al [51]).

receptor under these conditions. As can be seen in Figure 8, treatment at acid pH in the presence of the iron chelator desferrioxamine released the iron associated with receptor-bound transferrin, while the transferrin protein itself remained tightly bound to its receptor.

The above experiment suggested that apotransferrin has a high affinity for its receptor at low pH. The experiment in Figure 9 shows this directly. Scatchard analysis of the data gave a similar number of cell surface binding sites for holotransferrin at both pH 5 (not shown) and neutral pH (44; approximately 50,000 sites/cell with K_d of about 10^{-8} M), suggesting that apotransferrin is tightly bound to the transferrin receptor molecule at low pH.

Importantly, apotransferrin shows very little detectable specific binding to cell surface receptors at pH 7.3. At 30 nM, the saturating concentration for holotransferrin [44], the binding of apotransferrin is at most 5% of that found for holotransferrin. In another experiment (Fig. 10), we showed that apotransferrin, bound to its receptor at pH 5.4, is rapidly dissociated with a half-time of 16 sec (Fig. 10) when the pH is raised to 7.4. Under the same conditions, differic transferrin dissociates with a half-time of 7.5 min (not shown).

These results led us to suggest a novel model for the dissociation of iron in cells, and for cycling of transferrin Fig. 11 [51]. Iron-loaded transferrin binds to its receptor on the cell surface at neutral pH; under these conditions binding of apotransferrin is negligible. After binding, diferric transferrin is internalized by receptor-mediated endocytosis. The transferrin-receptor complex moves to an acidic prelysosomal compartment. There, perhaps in the presence of an iron-chelating component, iron is released from transferrin, and is transported in a yet undefined pathway to the iron storage protein, ferritin [52]. Apotransferrin remains bound to its receptor, and together they are recycled to the cell surface. Upon reaching neutral pH, (either at

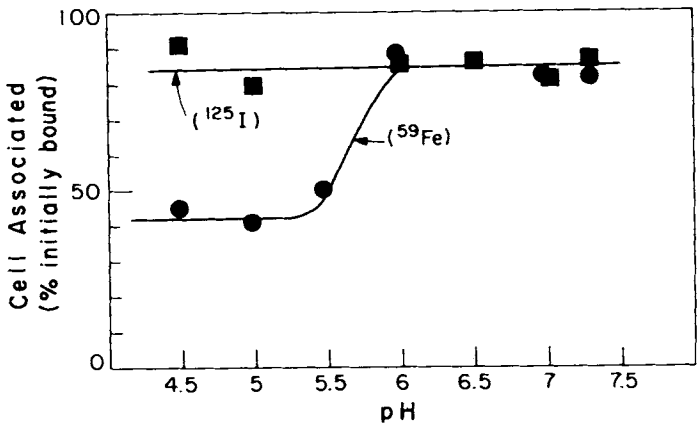


Fig. 8. Effect of pH at 37°C on dissociation of transferrin from HepG2 cells. [^{125}I]transferrin (■—■) and [^{59}Fe]transferrin (●—●) were bound at pH 7.2 and 4°C to HepG2 cells, in binding medium containing inhibitors of ATP generation. Excess ligand was removed and to each dish incubation buffer was added. The buffer contained inhibitors of ATP generation to prevent internalization, and the iron chelator desferrioxamine. The prewarmed medium was rapidly added while the dishes were transferred to a 37°C water bath. After 2 min at 37°C, the buffer was collected; the cells were counted for radioactivity as well as the buffer (adapted from Dautry-Varsat et al [51]).

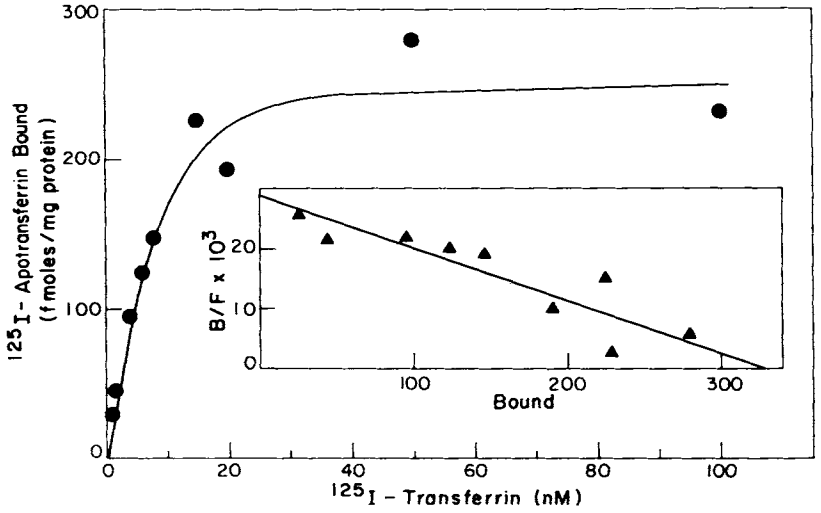


Fig. 9. Binding of apotransferrin at 4°C, pH 5.4, to HepG2 cells. [^{125}I]apotransferrin binding was measured at the indicated concentrations in minimal essential medium buffered at pH 5.4 in the presence of 5×10^{-5} M desferrioxamine (adapted from Dautry-Varsat et al [51]). Inset: Scatchard analysis of the same data.

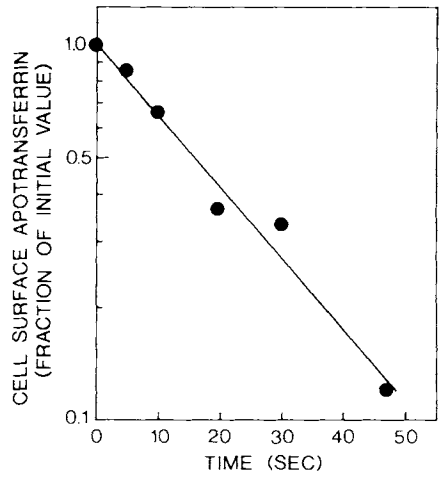


Fig. 10. Dissociation of apotransferrin from the surface receptor of HepG2 cells at pH 7.3. [^{125}I]ferrotransferrin was bound to cells at 4°C in the presence of energy inhibitors and excess unbound ligand was removed. One ml of pH 5 buffer containing energy inhibitors and desferrioxamine was added for 5 min at 4°C. The medium was aspirated and replaced by prewarmed Hank's solution (pH 7.3) containing an excess of unlabeled transferrin, inhibitors of ATP generation and desferrioxamine. At the indicated time points, the medium was removed, and the cell-associated radioactivity was quantified (adapted from Ciechanover et al [26]).

the cell surface or just prior to it in an intracellular vesicle) apotransferrin rapidly dissociates from its receptor. The free receptor at the cell surface is available for another cycle of receptor-mediated endocytosis. The released apotransferrin will be transported in the blood to a loading site where two Fe^{3+} ions will be rebound.

A similar model has also been recently proposed by Klausner and coworkers [53]. The two models are consistent, although they differ in the equilibrium-binding constants for apotransferrin to the receptor at neutral pH. Similar findings were also recently reported by Harding and Stahl [57].

In order to further probe the role of pH in the dissociation of iron from transferrin and its delivery to the cell, we have utilized several lysosomotropic agents. These are weak bases that increase the intralysosomal [61] and the endosomal pH [62]. As was reported recently, these agents perturb the pH-dependent dissociation of asialoorosomucoid from its receptor and its delivery to the lysosome [6]. One such agent, NH_4Cl, decreases iron uptake into cells [52; Ciechanover et al (data not shown)]; however, the fate of iron internalized via transferrin was not determined. These agents do not inhibit binding of transferrin to cell surface receptor, nor do they inhibit internalization of the receptor-ligand complex [52; Ciechanover et al (data now shown)]. However, it is evident from Figure 12, they block the retention of iron in the cell (compare to Fig. 7). All of the endocytosed Fe is secreted into medium as intact diferric transferrin (Fig. 13).

The principal effect of NH_4Cl, therefore, is the inhibition of dissociation of iron from the endocytosed transferrin receptor complex, presumably because it raises the

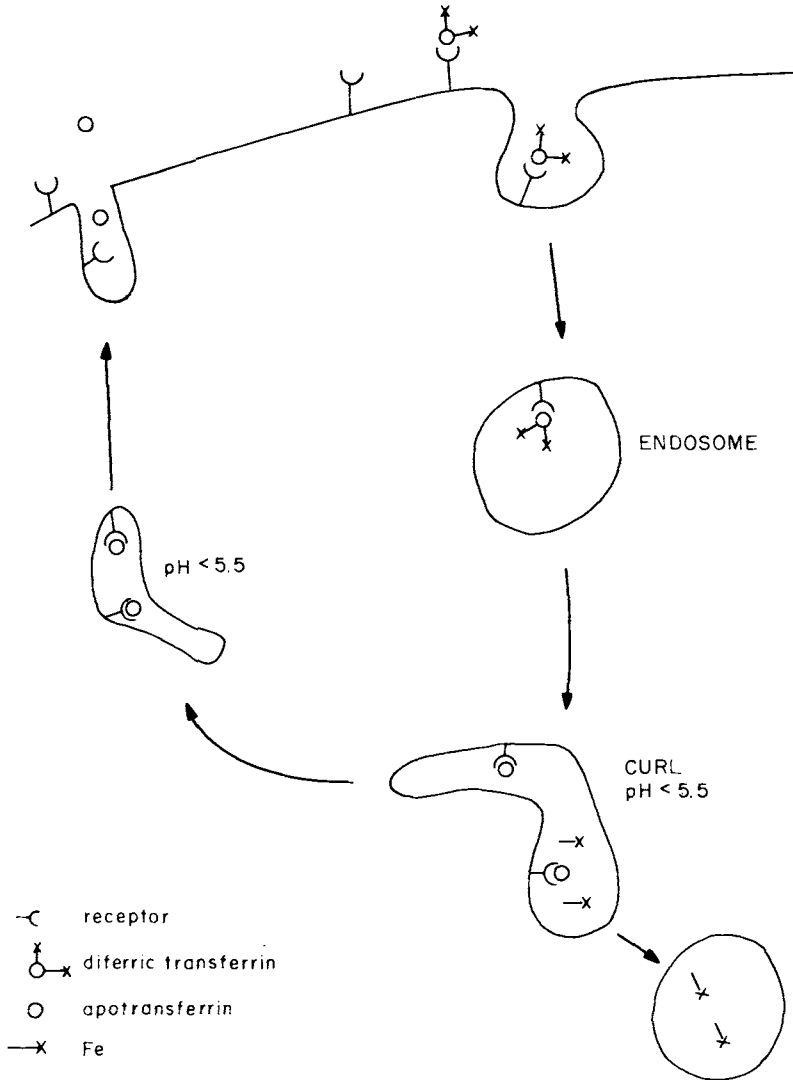


Fig. 11. The transferrin cycle. See text for details (adapted from Dautry-Varsat et al [51]).

pH of the endocytic vesicle. Iron is dissociated from the transferrin receptor complex only at pH values less than 6. When the pH is perturbed, iron is not dissociated and an intact diferric transferrin is exocytosed. These results suggest that the low pH of the endocytic vesicle is essential for dissociation of iron from the transferrin receptor complex, but is not essential for the recycling of the transferrin polypeptide back to the cell surface. Recently, Klausner et al [63] have characterized mutants defective in acidification of the endosome. In these cells, they noted similar behavior of transferrin to that seen in NH_4Cl-treated wild-type cells—diacytosis of diferic transferrin com-

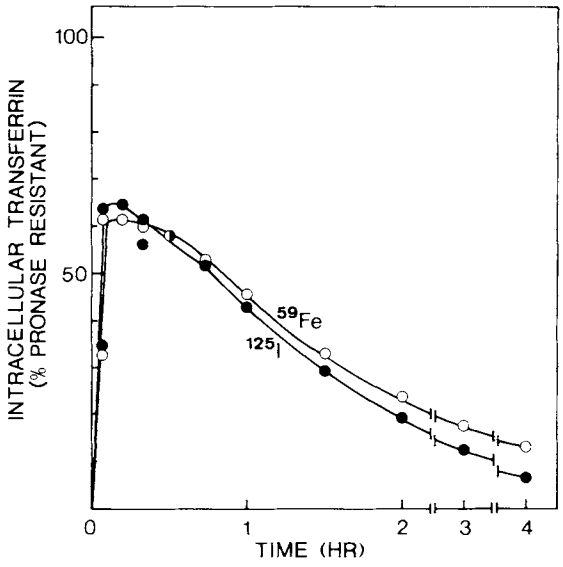


Fig. 12. Effect of NH_4Cl on the endocytic cycle of transferrin. Experimental details are the same as described in legends to Figures 5 and 6 except that 20 mM NH_4Cl was added to all the solutions. Shown are percentages of radioactivity in cell-associated material that is Pronase resistant (ie internalized). ^{125}I (●—●), ^{59}Fe (○—○) (adapted from Ciechanover et al [26]).

pared to apotransferrin. This finding supports our contention that the low pH is required mainly for removal of Fe from transferrin.

Determination of the Cycle Time of Transferrin Receptor

As shown earlier (Figs. 5 and 6), the uptake kinetics of iron in a single endocytic cycle is different from that of the transferrin protein moiety. While iron remains within the cell, the protein diacytoses through the cell, and is exocytosed as apotransferrin [26, 52]. Thus, the rate of transferrin-mediated iron uptake is a measure of the total rate of transferrin endocytosis. At 4°C, the maximum amount of [^{59}Fe]transferrin that could be associated with the cell was similar to that obtained with [^{125}I]ferrotransferrin, as expected, since both bind to the same surface receptors. In contrast, at 37°C, there is a time-dependent uptake of iron which is linear for almost 4 hr (Fig. 14). The rate of uptake is about 1.9×10^4 iron ions/cell/min. Since each transferrin binds two irons, this represents 9.5×10^3 transferrin molecules which cycle through the cell per minute. Taking into account that there are about 1.5×10^5 functional transferrin binding sites/cell ([26]; data not shown) and that the experiment is carried out in the presence of cycloheximide to block protein synthesis, and therefore synthesis of new receptor molecules, we calculate that the time required for each receptor molecule to traverse an endocytic cycle is $1.5 \times 10^5 \div 9.5 \times 10^3 = 15.8$ min.

The cycle time was also determined from an additional independent series of experiments. In these experiments we measured separately each step of the endocytic

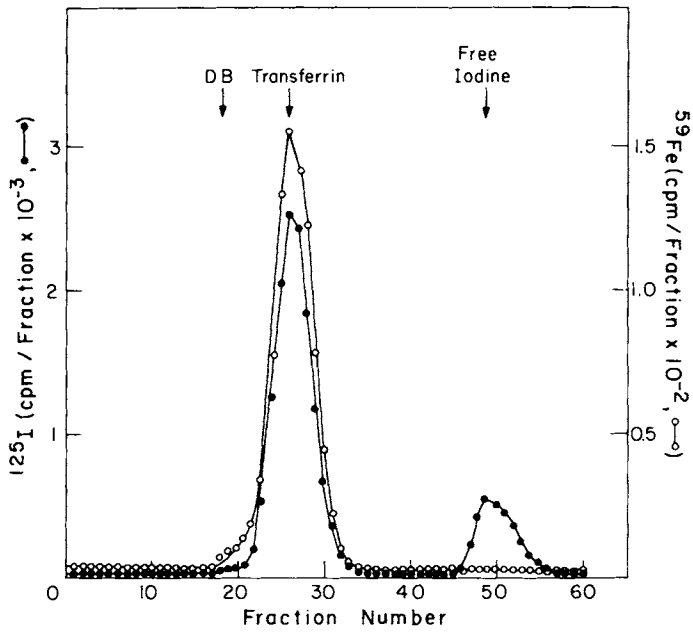


Fig. 13. Exocytosis of intact [^{125}I] and [^{59}Fe] transferrin from HepG2 cells in the presence of NH_4Cl. The experiment was performed essentially as described in the legend to Figure 12. After 10 min at 37°C, the medium was removed and quickly replaced with fresh medium also containing 20 mM NH_4Cl and 5 × 10^{-4}M desferrioxamine. At this time, 87% of the cell-associated radioactivity is resistant to Pronase. The incubation was continued for an additional 230 min in the presence of NH_4Cl and desferrioxamine. The medium was collected and concentrated under vacuum. It was then chromatographed on a Sephadex G-150 column (1 × 50 cm). Markers are dextran blue (DB) and labeled transferrin. Note that all of the exocytosed ^{59}Fe-chromatographs with intact transferrin, even though the medium contains sufficient desferrioxamine to prevent binding of any exocytosed free iron to apotransferrin (adapted from Ciechanover et al [26]).

cycle as depicted in Figure 15 ([26]; experimental details are not shown). The sum of the mean time for each separate step of the cycle is 16.9 min (see also Table IV). As can be seen from Figure 16, there is a close agreement between the experimental data (Fig. 16B) and the theoretical curve for the behavior of transferrin during endocytosis (Fig. 16A) as calculated and computer-modeled from the rate constants of each step (Fig. 15).

It is of interest to compare the kinetic parameters for endocytosis of transferrin to those for asialoglycoprotein in the same cell line (Table IV). As can be seen, the various parameters of the endocytic cycle are similar, though there are some differences. We note that the cycling time of both the transferrin and the asialogylcoprotein receptors is rather short, about 16 min. The time necessary for internalization of the receptor ligand complex into the low pH endocytic vesicles and the dissociation of the ligand or the coligand (in the case of transferrin) is even shorter. Dissociation of asialoglycoprotein from its receptor probably occurs in the low pH endocytic vesicle designated CURL by Geuze et al [5]. However, degradation of many ligands in

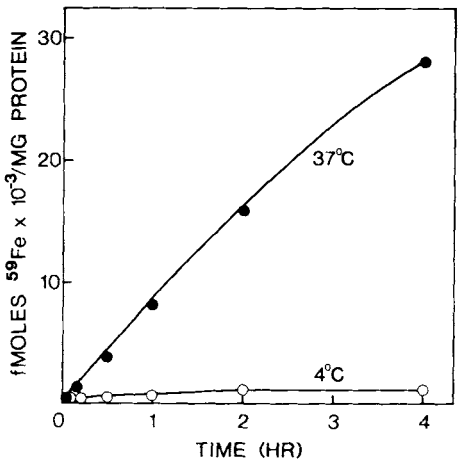


Fig. 14. Time course of transferrin-mediated ^{59}Fe uptake into HepG2 cells. [^{59}Fe]transferrin was bound to cells for the indicated times. 4°C (○—○), 37°C (●—●) (adapted from Ciechanover et al [26]).

lysosomes starts only after a lag period of about 30 min [8, 64]. This delay is probably due to slow delivery of the ligand to the lysosome or to a delayed action of the lysosomal proteases, but not to any steps in which the receptor is involved.

Are Cell Surface Receptors Internalized and Recycled Independently?

Most cells contain more than one type of cell surface receptor. Does internalization of one ligand cause internalization of other cell surface receptors or do cell surface receptors internalize and recycle independently of one another? Specifically, are receptors for asialoglycoproteins, transferrin, and insulin internalized and recycled independently?

To address this problem, we first characterized specific insulin receptors on the cell surface of HepG2 cells ([44]; data not shown). We then made use of the experimental protocol described in the legend to Figure 3B. As can be seen in Figure 17, binding of ASOR causes a rapid and transient reduction in the number of surface asialoglycoprotein receptors. However, there is no alteration in the number of surface binding sites for either transferrin (Fig. 17A) or insulin (Fig. 17B). We conclude that binding of ASOR induces a highly specific internalization only of its own receptor.

However, there is an alternative explanation for our results. It is possible that internalization of asialoglycoprotein receptor occurs at the same rate whether or not ligand is bound. The recycling of receptor to the surface could be slowed dramatically if ligand is bound to it, perhaps because a different pathway is used. It should be noted however, that we were unable to see depletion of cell surface receptors even after very short times of warming (0.5 min) without prebinding of a ligand. In contrast to our findings, a portion of the LDL [65] and the mannose [66] receptors

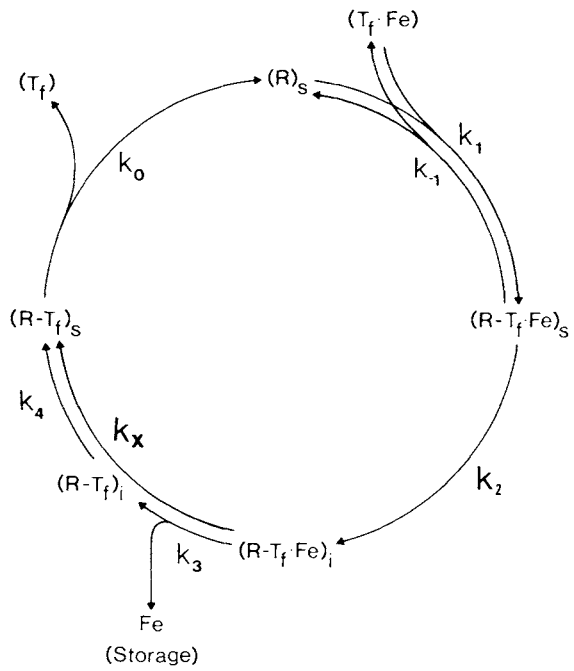
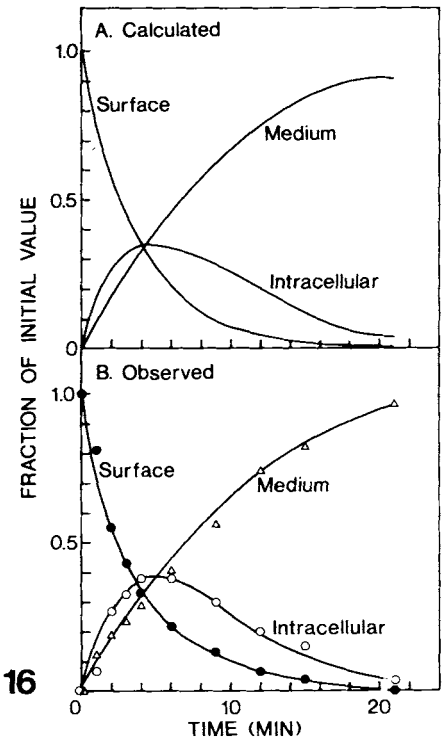


Fig. 15. A kinetic model for receptor-mediated endocytosis of transferrin. Tf·Fe, ferrotransferrin; $(R)_s$, unoccupied surface receptor; $(R\text{-}Tf\cdot Fe)_s$, surface ferrotransferrin receptor complex; $(R\text{-}Tf\cdot Fe)_i$, intracellular ferrotransferrin-receptor complex; $(R\text{-}Tf)_i$ intracellular apotransferrin receptor complex; $(R\text{-}Tf)_s$ surface apotransferrin-receptor complex; Tf apotransferrin; k_1 rate constant for binding of ferrotransferrin to surface receptors; k_{-1}, rate constant for dissociation of ferrotransferrin fromcell surface receptors; k_2, rate constant for internalization of surface ferrotransferrin-receptor complex; k_3, rate constant for dissociation of iron from internalized ferrotransferrin-receptor comlexes; k_4, rate constant for movement of the receptor-apotransferrin complex to the surface; k_x, overall rate constant for dissociation of iron and movement of the apotransferrin-receptor complex to the cell surface; k_o, rate constant for dissociation of apotransferrin from cell surface receptor (adapted from Ciechanover et al [26].

appear to cycle independently of ligand binding. Perhaps the pathway and parameters for recycling of these receptors with or without bound ligand is the same.

CONCLUDING REMARKS

The studies described herein, together with many other studies on receptor-mediated endocytosis and intracellular membrane traffic, have defined a circulatory system for the cell. By circulating proteins and lipids components within this endocytic system, the cell can regulate its critical functions: ingest and degrade macromolecules, secrete and maintain the composition of its organelle. Furthermore, our studies have defined important modifications of the general pathway of receptor-mediated endocytosis: by differing in their response to a low pH environment, various receptor-ligand complexes may be sorted and differentially directed within the cell.



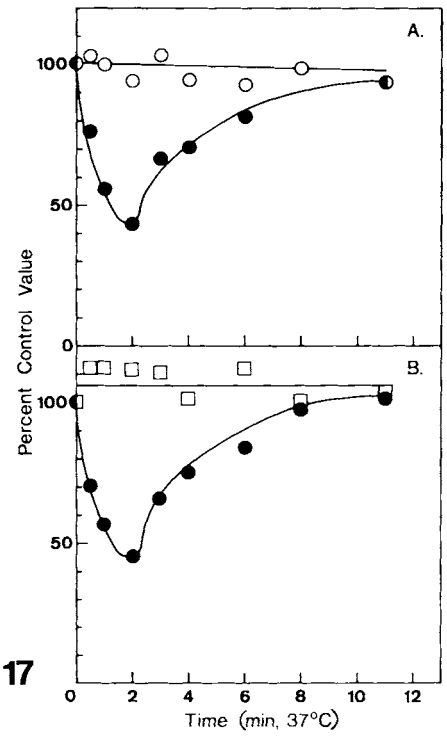


Fig. 16. Calculated and observed recycling kinetics of the transferrin receptor in HepG2 cells. A. Calculated curve: The differential equation describing our model of receptor internalization and recycling (Fig. 15; [8, 26]) was solved for a single endocytic cycle making the following assumptions: At t = 0, $(R\text{-}Tf.Fe)_s = 1$, $(R\text{-}L)_i = 0$, $R_s = 0$; $k_1L = 0$ (ie, the absence of free ligand precludes the binding of additional ligand to the cell); $k_2 = 0.20\ min^{-1}$, $k_x = 0.14\ min^{-1}$ and $k_0 = 2.6\ min^{-1}$. The number of surface ligands, intracellular ligands, and ligand released to the medium is plotted, normalized to the value of 1.0 at t = 0. B. Experimental curve: The experiment was similar to that described in the legend to Figure 5. At t = 0, 6% of the cell-associated radioactivity could not be removed by Pronase. This background value was subtracted from all values of cell-associated radioactivity. The amount of cell surface radioactivity was then normalized to a value of 1.0 at t = 0 min (adapted from Ciechanover et al [26]).

Fig. 17. Independent internalization of receptors for asialoglycoprotein, transferrin, and insulin. Cells were treated as described in the legend to Figure 3B. After "stripping" of the noninternalized ASOR with EDTA, binding of labeled ASOR, transferrin and insulin were measured as described [44]. (In A, zero time values for ASOR were 1,147 and 209 fmol/mg protein for total and nonspecific binding, respectively, and 391 and 47 for transferrin. In B, the values were 1,096 and 21 for ASOR and 374 and 69 for insulin, respectively.) A. ASOR (●—●), transferrin (○—○). B. ASOR (●—●), insulin (□—□). All data shown represent averages of quadruplicate determinations and were corrected for nonspecific binding (adapted from Ciechanover et al [44].

However, much is still not known of the complex itinerary of recycling receptors. In particular, the signals which determine the movement of the receptors from one compartment to another are unknown. On the cell surface these proteins mingle with other proteins that are permanent residents of the plasma membrane. What signals differential segregation, such that only the appropriate receptors and not other molecules will be directed to the coated pits? What signals internalization of receptor-ligand complexes and their transport to CURL vesicles? How are receptor and ligand segregated from each other following their dissociation? And what signals recycling of the receptor to the cell surface? It is likely that there are signals within the migrant proteins themselves that ticket them for inclusion into transport vesicles to move them to the next location. Since many cell surface receptors probably share the same route, it is not inconceivable that they also share common regulatory signals. Attempts to elucidate these signals will include comparative studies of the primary structure, synthesis, and processing of these proteins, together with functional characterization of coated pits and CURL vesicles, the two compartments at which segregation occurs. What now appears simplest to understand are the pH-dependent mechanisms involved in receptor-ligand dissociation, a step which is crucial to receptor recycling.

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