

Recycling of the Asialoglycoprotein Receptor and the Effect of Lysosomotropic Amines in Hepatoma Cells

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ABSTRACT Receptor-mediated uptake and degradation of ^{125}I -asialoorosomucoid (ASOR) in human hepatoma HepG2 cells is inhibited by the lysosomotropic amines chloroquine and primaquine. In the absence of added ligand at 37°C , these amines induce a rapid ($t_{1/2}$ 5.5–6 min) and reversible loss of cell surface ^{125}I -ASOR binding sites as well as a rapid decrease in ^{125}I -ASOR uptake and degradation. There is no effect of these amines on the binding of ^{125}I -ASOR to the cell surface at 4°C or on the rate of internalization of prebound ^{125}I -ASOR. The loss of ^{125}I -ASOR surface binding at 37°C is not attributable to altered affinity of ligand-receptor binding. In the presence of added ligand at 37°C , there is a more rapid ($t_{1/2}$ 2.5–3 min) loss of hepatoma cell surface receptors. In addition, the amines inhibit the rapid return of the internalized receptor to the cell surface. We examined the nature of this loss of ^{125}I -ASOR surface binding sites by following the fate of receptor molecules after biosynthetic labeling and after cell surface iodination. At 37°C , chloroquine and primaquine induce a loss of asialoglycoprotein receptor molecules from the hepatoma cell surface to an internal pool.

The selective uptake of macromolecular ligands such as proteins via receptor-mediated endocytosis is a common feature of eucaryotic cells (1, 2). Receptor-mediated endocytosis involves the specific binding of macromolecular ligands (e.g., virus, toxin, transferrin, asialoglycoprotein) to specialized cell surface receptors and their internalization via a coated pit-coated vesicle pathway. Many ligands dissociate from their receptor in a prelysosomal compartment and allow the receptor to recycle back to the cell surface (1, 2). Often, these ligands are then transported in a still unknown way to the lysosomes, wherein they are degraded. The asialoglycoprotein receptor (ASGP-R),¹ localized to the hepatic parenchymal cell, provides a well-characterized system for examination of the mechanisms involved in these processes (3). A human hepatocyte-derived cell line, hepatoma HepG2, contains abundant ASGP-R (4). Using these cultured cells as a model, we have recently defined the kinetics of receptor-mediated endocytosis of asialoglycoprotein ligands and ASGP-R recycling (5, 6). Our studies indicate that a single ASGP-R can recycle from the cell surface into "endosomal sorting compartments" (compartment of uncoupling receptor and ligand

[CURL]) within the cell and back to the cell surface within 8 min (5–7).

The mechanisms responsible for ligand-receptor dissociation and the sorting and recycling of receptor molecules are not fully understood. Lysosomotropic agents (including the weak bases ammonium chloride and chloroquine) have been demonstrated to interfere with receptor-mediated uptake and degradation of numerous ligands, including asialoglycoproteins (8), mannose-6-phosphate-terminated ligands (9), alpha-2-macroglobulin-protease complexes (10), mannosylated albumin (11), and low-density lipoproteins (12). Both lysosomes (13) and endosomes (14, 15) are now known to be acidic, and the pH of both organelles can be raised by the presence of lysosomotropic agents (16). Studies following the fate of labeled ligand suggest that the lysosomotropic agents interfere with receptor recycling by sequestering receptor within intracellular compartments (17).

The hepatoma ASGP-R is a well-characterized system with which to examine the effects of the lysosomotropic amines on receptor recycling, since both well-defined parameters for ASGP-R recycling (5) and anti-human ASGP-R antibodies (18) are available. In the present study we define the effects of two lysosomotropic amines on the parameters of endocytosis of asialoglycoprotein in cloned hepatoma HepG2 cells.

¹ Abbreviations used in this paper: ASGP, asialoglycoprotein; ASGP-R, ASGP receptor; ASOR, asialoorosomucoid.

In the presence of the amines there is a rapid loss of cell surface binding sites. By following labeled receptor molecules, we demonstrate that the amines induce a loss of cell surface receptor to an internal pool. These data provide support for the constitutive recycling of ASGP-R in HepG2 cells.

MATERIALS AND METHODS

Materials: Human orosomucoid, provided by the American Red Cross, was desialylated and iodinated with ^{125}I as previously described (19). Chloroquine and primaquine were obtained from Sigma Chemical Company (St. Louis, MO) as the biphosphate salts, and monensin was obtained from Calbiochem-Behring Corp. (La Jolla, CA). All drug solutions were prepared fresh in Eagle's minimal essential medium (Gibco Laboratories, Grand Island, NY) for each experiment. The preparation and characterization of affinity-purified anti-human ASGP-R antibodies has been described (18).

Cells: The human hepatoma cell line HepG2 (clone a16) was used for all studies reported here. Maintenance of these cells has been detailed earlier (4, 18).

Binding, Uptake, Degradation, and Internalization Assays: The binding of ^{125}I -asialoorosomucoid (ASOR) to HepG2 cells at 4°C has been described; specific binding is defined as the difference of binding of ^{125}I -ASOR in the absence and presence of excess unlabeled ASOR (4). Assays for the uptake and degradation of ^{125}I -ASOR at 37°C have been described (5). However, in the present study the medium used was Eagle's minimal essential medium/10% fetal calf serum/10 mM HEPES (pH 7.3). In general, uptake was assayed during a 2-h period at 37°C , a time period during which release of ^{125}I degradation products into the medium is minimal (5). Degradation was generally assayed at 4 h at 37°C .

Internalization was assessed by evaluating the cell-associated, EDTA-non-releasable ^{125}I -ASOR within cells (5). Cells were exposed to ^{125}I -ASOR at 4°C in order to saturate all surface receptors. Unbound ^{125}I -ASOR was removed and the cells were temperature-shifted to 37°C for various times and then returned to 4°C . The cells were then treated for 5 min at 4°C with 10 mM EDTA in PBS (pH 5). Both the ^{125}I -ASOR released by this treatment (cell surface ligand) and that sequestered within the cells (EDTA-nonreleasable) were determined (5).

Biosynthetic Labeling of Receptor: Biosynthetic labeling of human ASGP-R was performed using [^{35}S]methionine as detailed previously (18). Cells were labeled with 300 μCi of [^{35}S]methionine/ml for 60 min followed by a 2-h chase period in the presence of 400-fold excess unlabeled methionine.

Immunoprecipitation of ASGP-R and Analysis by SDS PAGE: After biosynthetic labeling and exposure to drugs, the cells were chilled to 4°C . For the analysis of total cell receptor, cells were solubilized in 1% Triton X-100/1 mM phenylmethylsulfonyl fluoride in PBS and immunoprecipitated with affinity-purified anti-human ASGP-R antibody as described previously (18). Immune complexes were isolated with Staphylococcal protein A, and the antigens and antibodies were separated by SDS-PAGE and prepared for fluorography with Enhance (New England Nuclear, Boston, MA) (18). Densitometry was performed using a Helena Laboratories (Beaumont, TX) Quickscan with integrator.

Immunoprecipitation of ASGP-R from the Cell Surface: As described previously (18), after the appropriate biosynthetic labeling, cells were chilled at 4°C , washed in PBS containing PMSF, and incubated for 3 h at 4°C with affinity-purified anti-human ASGP-R (5–60 $\mu\text{g}/\text{ml}$ in PBS/1 mM PMSF containing 0.1 mg/ml cytochrome *c*). Following removal of the unbound IgG, the cells were solubilized in 1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/PBS containing a 10-fold excess of unlabeled cell extract (to prevent surface IgG from binding intracellular labeled receptor, which became available during solubilization). This material was subjected to immunoprecipitation and analysis by SDS PAGE and fluorography (18).

Cell Surface Iodination: HepG2 cells were vectorally labeled with ^{125}I at 4°C as previously described (20). Briefly, 10^6 cells were washed with PBS and incubated for 15 min at 4°C with 1 mCi ^{125}I in the presence of lactoperoxidase and glucose oxidase. The cells were then washed, solubilized in 1% Triton X-100/1 mM phenylmethylsulfonyl fluoride/PBS, immunoprecipitated, and analyzed on SDS PAGE.

RESULTS

In the absence of added ligand, chloroquine (1 mM) induces a rapid loss of hepatoma ^{125}I -ASOR surface binding. At 37°C , the half-time for depletion of surface binding sites is 5–6 min

(Fig. 1). The effect is similar to the decrease of surface ligand binding of ASGP on isolated rat hepatocytes (8) and mannosylated BSA binding on alveolar macrophages (11). Identical kinetics of loss of ASGP surface binding was found with (0.2–1 mM) primaquine (data not presented). Chloroquine (20–200 μM) had minimal effects on the extent of ^{125}I -ASOR surface binding during a 2.5-h incubation at 4°C (Fig. 2a). However, there was a dose-dependent loss of surface binding in cells exposed to the drug at 37°C , which resulted in a 50% inhibition (I_{50}) at ~ 100 μM ; the I_{90} was ~ 400 μM (Fig. 2a). Similarly, primaquine had little if any effect on the saturation binding of ^{125}I -ASOR during incubation at 4°C . Primaquine also produced a dramatic effect at 37°C with an I_{50} of ~ 70 μM and an I_{90} of ~ 200 μM (Fig. 2a). Other primary amines, e.g., ammonium chloride and methylamine, were found to have similar but less potent effects (Table I). The carboxylic cationic ionophore monensin similarly decreased ^{125}I -ASOR binding to hepatoma cells (Table I).

We also examined the effect of these drugs on the uptake of ^{125}I -ASOR into hepatoma cells. We chose a period of 2 h at 37°C , a time by which little internalized ligand has been released into the media as degradation products (5). As seen in Fig. 2b, both chloroquine and primaquine induced a drug-dependent reduction of ^{125}I -ASOR uptake ($I_{50} \sim 80$ μM ; $I_{90} \sim 200$ μM). Similar to the effects on surface binding and uptake at 2 h, ^{125}I -ASOR degradation, assessed after 4 h at 37°C , was inhibited by both chloroquine and primaquine in a dose-dependent manner ($I_{50} \sim 40$ – 50 μM for chloroquine and 70 μM for primaquine; I_{90} 100 μM for chloroquine and 200 μM for primaquine) (Fig. 2c). Furthermore, identical effects of inhibition of uptake and degradation were seen in samples examined after 20 h of ligand uptake and degradation (data not presented). In addition, similar effects were seen after exposure to ammonium chloride, methylamine, and monensin (Table I).

Internalization of surface-bound ^{125}I -ASOR was unaffected by 200 μM primaquine or chloroquine (Fig. 3). The $t_{1/2}$ of 2–2.2 min is identical to that reported earlier (5). However, the extent of internalization of surface-bound ^{125}I -ASOR may be reduced by 10% at longer incubation times (e.g., 10 min) (Fig. 3).

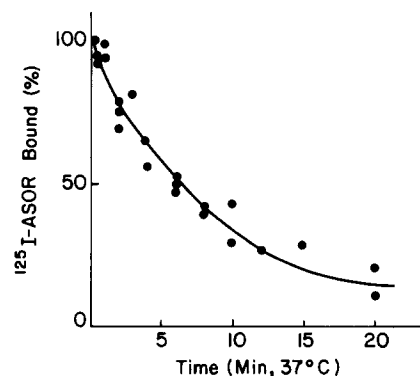


FIGURE 1 Time course of effect of chloroquine on ^{125}I -ASOR surface binding to hepatoma cells. Hepatoma cells were rinsed in fresh binding media and thereafter incubated at 37°C in the presence of 1 mM chloroquine. At the appropriate time, cells were rapidly (<5 s) chilled to 4°C , washed, and incubated with ^{125}I -ASOR in the absence or presence of excess unlabeled ASOR to determine specific binding. The results are expressed as percent of specific binding by control samples that were not exposed to the drug; each point represents the mean of 3–4 samples from each of four independent experiments. The SEM was less than $\pm 15\%$.

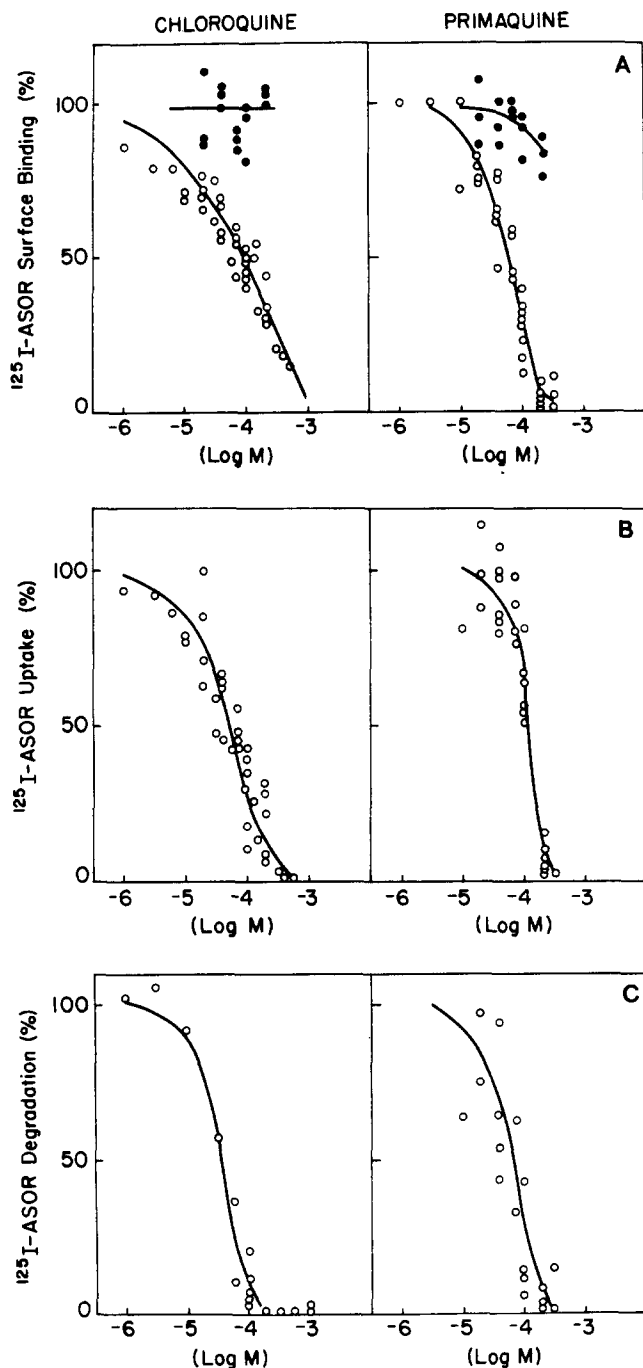


FIGURE 2 Effect of chloroquine and primaquine on ^{125}I -ASOR surface binding, uptake and degradation in hepatoma cells. (A) Hepatoma cells were rinsed and incubated at either 4°C (●) or 37°C (○) for 30 min with various concentrations of drugs. Thereafter binding of ^{125}I -ASOR was performed at 4°C in the absence or presence of excess unlabeled ASOR to determine specific binding as described in the text. The results are expressed as percent of specific binding by control samples that were not exposed to drug; each point represents the mean of two to four samples from a total of five experiments. (B) Hepatoma cells were rinsed and incubated at 37°C for 30 min with various concentrations of drugs. Thereafter ^{125}I -ASOR was added in absence or presence of excess unlabeled ASOR and the incubation continued for 2 h. Total cell-associated ^{125}I was determined as described in the text. The results are expressed as in A. (C) Hepatoma cells were prepared and treated as in B, except that incubation with ^{125}I -ASOR was continued for 4 h. ^{125}I -degradation products in the media were determined as described in the text. The results are expressed as in A.

TABLE I
Effect of Ammonium Chloride, Methylamine, and Monensin on Surface Binding and Uptake of ^{125}I -ASOR by Hepatoma Cells

Drug	Concentration mM	Surface Binding		
		4°C preincubation %	37°C preincubation %	2-h uptake 37°C preincubation %
None	—	100 ± 5	100 ± 5	100 ± 6
Chloroquine	1	103 ± 10	34 ± 2	14 ± 4
	0.1	94 ± 6	79 ± 8	54 ± 4
NH_4Cl	10	93 ± 18	62 ± 8	48 ± 3
	1	—	85 ± 11	—
	0.1	—	96 ± 10	—
Methylamine	10	111 ± 17	64 ± 8	53 ± 5
	1	—	89 ± 13	—
	0.1	—	92 ± 10	—
Monensin	0.1	100 ± 31	47 ± 7	21 ± 1
	0.03	—	53 ± 8	—

Cells were washed and preincubated with appropriate drugs for 30 min at either 4 or 37°C . Thereafter, in the continued presence of drug, surface binding (2 h \times 4°C) or uptake (2 h \times 37°C) was determined. Nonspecific binding and uptake was determined for each experimental condition and the results of specific binding or uptake are presented as percent control \pm SEM for three determinations.

This inhibitory effect of chloroquine and primaquine on the uptake of ^{125}I -ASOR in hepatoma cells is rapidly reversible. As seen in Table II, the removal of $200 \mu\text{M}$ primaquine from the media after a 30-min preincubation at 37°C allowed the uptake of ^{125}I -ASOR to return to control values (94%) by 2 h at 37°C , while cells maintained with $200 \mu\text{M}$ primaquine at 37°C demonstrated only 8% control uptake. Furthermore, replacement of primaquine with chloroquine at equimolar concentrations during the uptake incubation demonstrated that chloroquine was not as effective in maintaining the inhibition of ^{125}I -ASOR uptake, which is consistent with the effects on surface binding and uptake seen in Fig. 2.

Since chloroquine and primaquine promote a loss of surface ligand binding sites, in addition to reducing ligand degradation (Fig. 2), we examined which effect is observed first at lower drug concentrations. In order to test this, cells were incubated with various concentrations of primaquine (20–200 μM) for 2 or 4 h at 37°C . Thereafter, we determined binding (at 4°C), degraded ligand in media, total cell-associated ligand, and internalized ligand. A dose-dependent decrease in surface ligand binding and ligand degradation at 4 h was found (Table III) and was similar to that found earlier (Fig. 2). It should be noted that the determination of surface ligand binding is a static measurement and reflects only the number of binding sites at the cell surface at the time of assay, in this instance after a 4-h incubation at 37°C with primaquine. In contrast, the total cell-associated ^{125}I -ASOR and degradation reflect the overall uptake and processing of ligand during 4 h. The total amount of ligand internalized decreased only slightly to 93% of control in the presence of $40 \mu\text{M}$ primaquine and to 83% of control in the presence of $70 \mu\text{M}$ primaquine (Table III). Even at $100 \mu\text{M}$ primaquine, <30% reduction in total ligand processed was found. However, at primaquine concentrations of 20–70 μM , there was a substan-

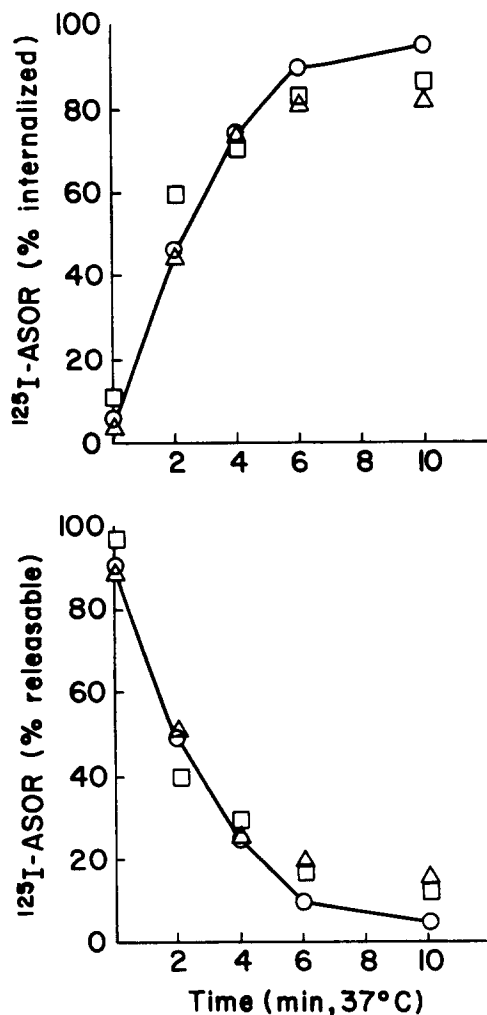


FIGURE 3 Internalization of ^{125}I -ASOR by hepatoma cells in the presence of chloroquine and primaquine. Hepatoma cells were rinsed and incubated for 2 h at 4°C with a saturating concentration of ^{125}I -ASOR (50 nM) in the presence of no drug (O), 200 μM chloroquine (Δ), or 200 μM primaquine (\square). Appropriate control samples contained excess unlabeled ASOR. Unbound ^{125}I -ASOR was removed by washing and the cells were rapidly temperature-shifted to 37°C in the presence of the appropriate drugs for the indicated times. Thereafter, the cells at 4°C were incubated at 4°C with 10 mM EDTA to release surface-bound ^{125}I -ASOR. Both EDTA-nonreleasable ^{125}I (upper panel) as well as EDTA-releasable ^{125}I (lower panel) were determined. The results have been corrected for nonspecific binding and represent the mean of triplicate determinations.

TABLE II
Reversibility of the Effects of Primaquine on Uptake of ^{125}I -ASOR by Hepatoma Cells

Preincubation drug mM	Uptake incubation drug mM	2-h uptake %
None	None	100 ± 5
Primaquine (0.2)	Primaquine (0.2)	8 ± 5
Primaquine (0.2)	None	94 ± 5
Primaquine (0.2)	Primaquine (0.02)	101 ± 10
Primaquine (0.2)	Chloroquine (0.2)	29 ± 2

Cells were washed and preincubated for 30 min at 37°C in the absence or presence of 0.2 mM primaquine. After washing, the cells were incubated for 2 h at 37°C with ^{125}I -ASOR in the presence of 0.02 or 0.2 mM primaquine or 0.2 mM chloroquine or no drug. Nonspecific uptake was determined and the results are expressed as percent control specific binding ± SEM for three determinations.

TABLE III
Simultaneous Effects of Primaquine on Surface Binding, Uptake, and Degradation of ^{125}I -ASOR by Hepatoma Cells at 4 h

Primaquine μM	^{125}I -ASOR (ng/well)			
	Surface bound	Total cell-associated	Internal*	Degraded (media)
0	6.4	29.3	22.9 (1.00)*	13.8 (1.00)*
20	4.8	39.3	34.5 (1.51)	10.4 (0.75)
40	3.9	40.4	36.5 (1.59)	7.3 (0.53)
70	3.8	38.1	34.3 (1.50)	4.6 (0.33)
100	2.1	33.8	31.7 (1.38)	2.0 (0.14)
200	0.1	2.7	2.6 (0.11)	1.2 (0.09)

Cells were rinsed and incubated at 37°C for 30 min with various concentrations of primaquine. Thereafter, in the continued presence of the primaquine, saturation binding of ^{125}I -ASOR at 4°C or uptake (total cell-associated) and degradation of ^{125}I -ASOR at 4 h, 37°C, was assessed as described in the text. Each value is the mean of triplicate determinations with a range ±10%.

* Internal = (total cell-associated - surface bound).

* The numbers in parentheses are normalized such that 0 M primaquine equals 1.00.

tial increase in internal ligand (150% control) (Table III). Additional studies performed at 20 h demonstrated a similar increase (150% control) in internal ^{125}I -ASOR in the presence of 60 μM primaquine (data not presented). These studies demonstrate an accumulation of undegraded ligand and suggest that the major effect of these agents is on inhibition of ligand degradation.

We could directly assess the mechanism(s) responsible for the loss of ^{125}I -ASOR surface binding. Hepatoma cells were incubated at 37°C with chloroquine or primaquine in order to reduce surface binding to ~30% of the control value. Under these conditions the affinity (K_d) of ^{125}I -ASOR binding was unaltered (data not presented). These data suggest that the loss of surface binding was not the result of altered affinity of receptor-ligand binding.

To examine directly whether the loss of cell surface binding of ASOR was the result of inactivation of surface ASGP-R (i.e., it was no longer capable of binding ligand) or whether surface ASGP-R were no longer present at the cell surface, we assessed the effect of the drugs by two independent methods: immunoprecipitation of cell surface receptors after biosynthetic labeling and immunoprecipitation of total cell receptors after cell surface iodination. HepG2 cells were labeled with [^{35}S]methionine by incubation for 60 min, followed by a 2-h chase with unlabeled methionine. These conditions allow the newly labeled receptor molecules to reach the cell surface (18). The cells were then treated for 30 min at 37°C with 200 μM chloroquine or primaquine. In one set of dishes, the total receptor from the entire cell lysate was immunoprecipitated with anti-receptor antibody. As seen in Fig. 4a, there was no difference in the amount of total cell receptor immunoprecipitated by anti-human ASGP-R antibody in the drug-treated or control cells. Approximately 95% of the labeled receptor migrated at an apparent molecular weight of 46,000, which is the molecular weight of the mature receptor, whereas <5% of the labeled receptor was of an apparent molecular weight of 40,000, the predominant intracellular precursor (18). After only the surface receptor was immunoprecipitated, as seen in Fig. 4b, a radiolabeled receptor of 46,000 mol wt was identified in the control cells. There was a substantial reduction in the immunoprecipitable surface receptor from cells treated with either 200 μM chloroquine (~20% control

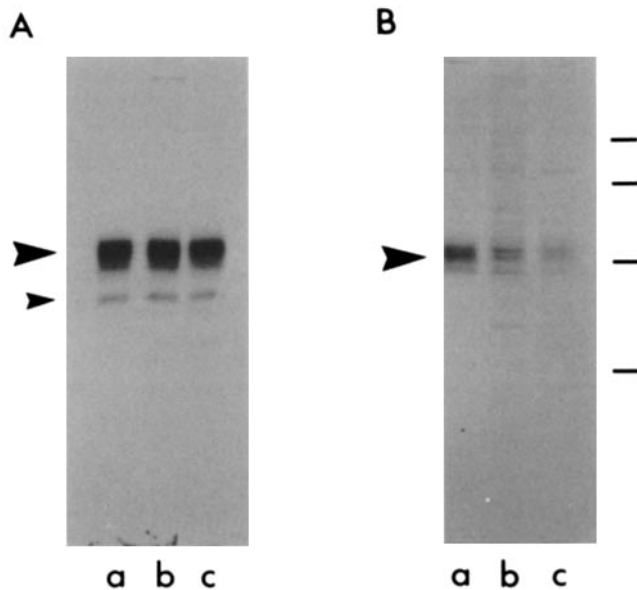


FIGURE 4 Effect of chloroquine and primaquine on hepatoma cell surface and total cell ASGP-R. Hepatoma cells were labeled with [³⁵S]methionine for 1 h and chased with excess unlabeled methionine for 2 h. Sets of dishes were then incubated for 30 min at 37°C with no drug (a), 200 μM chloroquine (b), or 200 μM primaquine (c). Thereafter, some dishes were solubilized and total cell receptor was isolated by immunoprecipitation (A), or cell surface receptor was isolated by binding antireceptor antibody to the cell surface at 4°C as described in the text (B). Equal aliquots of cell extract were processed identically in A and B, however the concentration of affinity purified antibody used for surface binding was 40% of maximal (15 μg/ml). The immunoprecipitates were analyzed by SDS PAGE and autoradiography. Large arrow, 46,000 daltons; small arrow, 40,000 daltons. Molecular weight markers are indicated at right (top to bottom: 93, 68, 46, and 30 kdaltons).

level) or 200 μM primaquine (~10–15% control level). As expected, there was no surface receptor of 40,000 mol wt.

Immunoprecipitation of surface receptors allows a quantitative determination of receptor at the cell surface. As seen in Fig. 5, after primaquine incubation there is a decrease in immunoprecipitable surface receptor that parallels the loss of ligand binding sites (Fig. 2). This is seen at anti-receptor antibody concentrations of 5–60 μg/ml (K_d 20–30 μg/ml) (Figs. 4b and 5; unpublished data). An independent assessment of the presence of surface receptor after incubation with primaquine is seen in Fig. 6. After preincubation in the absence or presence of 200 μM primaquine for 30 min at 37°C, cell surface proteins were labeled with ¹²⁵I at 4°C. Analysis of total cell receptor after immunoprecipitation reveals a single receptor species at 46,000 mol wt in the control cells, while cells preincubated with primaquine display a dramatic reduction in the 46,000-mol wt receptor (Fig. 6).

In HepG2 cells there is a marked ligand-induced depletion of cell surface receptors (6). In the presence of ASGP, there is a rapid depletion of surface ASGP-R. This is followed by a rapid reappearance of surface receptors that originate for the most part from the pool of surface receptors that had internalized and recycled (6). Therefore, we examined the effect of chloroquine and primaquine on this ligand-induced recycling of the ASGP-R. As seen in Fig. 7, in the presence of ASOR at 37°C there is a very rapid loss ($t_{1/2}$ 2.5–3 min) of cell surface binding in the presence of chloroquine. This initial rate (first 2 min) of loss of surface binding is unaltered by the

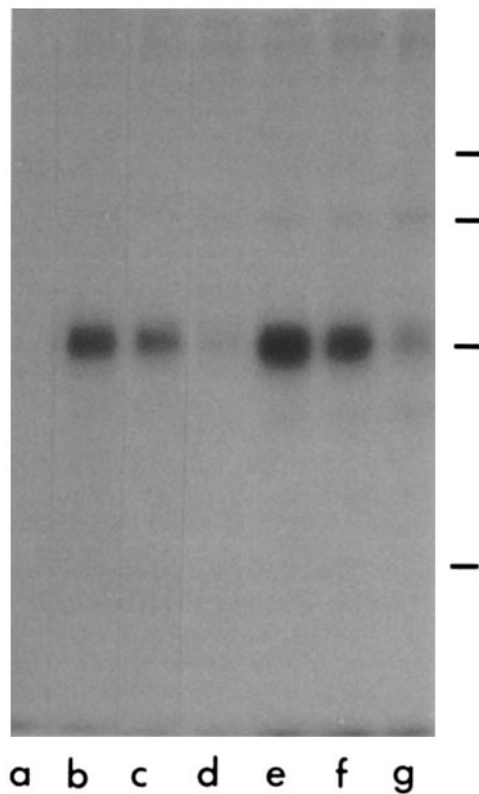


FIGURE 5 Effect of primaquine concentration on cell surface ASGP-R. Hepatoma cells were labeled with [³⁵S]methionine for 1 h and chased with excess methionine for 2 h. Cells were then incubated with various concentrations of primaquine at 37°C for 30 min. Thereafter, cell surface receptor was isolated by binding anti-receptor antibody (lanes b–d, 20 μg/ml; lanes e–g 40 μg/ml; lane a, 20 μg/ml normal rabbit IgG) to the cell surface at 4°C as described in the text. Lanes a, b, and e = 0 μM primaquine; lanes c and f = 80 μM primaquine; lanes d and g = 200 μM primaquine. Molecular weight markers are indicated at right (top to bottom = 93, 68, 46, and 30 kdaltons).

presence of the amine (Fig. 7). However, the rapid return of surface binding to control levels is abrogated by the presence of amine (Fig. 7), i.e., there is a continued rapid loss of cell surface ASGP-R in the presence of amine. The loss of surface binding in the presence of amine and ligand is more rapid than that seen in the presence of amine alone (Figs. 1 and 7).

DISCUSSION

The lysosomotropic agents chloroquine and primaquine inhibit receptor-mediated endocytosis of ASGPs in human hepatoma HepG2 cells. In studies performed in the absence of added ASGP, there is a dose-dependent and reversible loss of cell surface ligand binding sites, ligand uptake, and degradation. However, there is no effect of these agents on binding of ligand to surface receptor or on internalization of surface-bound ligand. These observations are consistent with earlier studies on the effects of lysosomotropic agents on the receptor-mediated endocytosis of ASGPs in isolated rat hepatocytes (8, 21), as well as lysosomal enzymes, mannosyl-terminated proteins, low-density lipoproteins, alpha-2-macroglobulin-protease complexes, chorionic gonadotropin, and insulin in a variety of cell types (9–12, 17, 22–24).

The concentrations of chloroquine and primaquine used for the most part in the present study (20–200 μM) are similar to those required to achieve significant reduction in receptor-

mediated endocytosis of many ligands (e.g., low-density lipoprotein [LDL], mannosyl-terminated albumin, alpha-2-macroglobulin-protease) (10–12).

The mechanism of action of the lysosomotropic agents has been the subject of intensive investigation. This class of drugs inhibits lysosomal cathepsins and protein degradation (25, 26), neutralizes lysosomal pH (13, 27), and neutralizes endosomal pH (14, 16). Many of the actions of these agents may be directly attributable to protonation within acidic intracellular compartments (27).

Intracellular accumulation of ligand was found at intermediate concentrations of primaquine (20–70 μM), concentrations at which ligand degradation was reduced by 75%, while surface binding was reduced only 40% (Table III). Basu et al. (12) have also reported similar effects of chloroquine on intracellular accumulation of LDL in fibroblasts. Indeed, they demonstrated that at 50–200 μM chloroquine, intracellular ligand accumulated to 150–200% of control values, while degradation decreased to <5% control. Cell surface ligand binding decreased to <50% control. These observations sug-

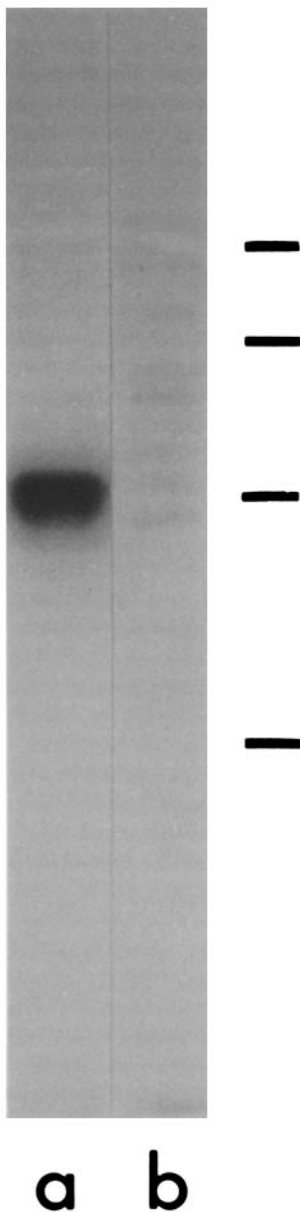


FIGURE 6 Effect of primaquine on cell surface ASGP-R following vectorial ^{125}I iodination. Hepatoma cells were incubated for 30 min at 37°C in media in the absence (lane a) or presence (lane b) of 200 μM primaquine. Following rinsing in PBS at 4°C , the cells were labeled with ^{125}I at 4°C and solubilized as described in the text. The total cell extract was immunoprecipitated with antireceptor antibody and analyzed by SDS PAGE. Molecular weight markers are indicated at right (top to bottom; 93, 68, 46, and 30 kdaltons).

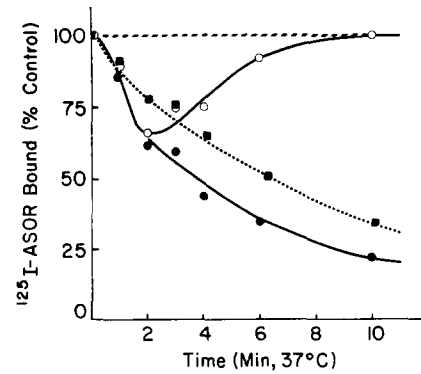


FIGURE 7 Effect of chloroquine on the surface binding of ^{125}I -ASOR to the hepatoma ASGP-R in the presence of ligand. Hepatoma cells were rinsed and incubated 2 h at 4°C in the absence (dashed lines) or presence of 2 μg unlabeled ASOR/ml (solid lines). Thereafter unbound ASOR was removed and the cells incubated at 37°C in the absence (O) or presence (●, ■) of 1 mM chloroquine. At the appropriate time, the cells were rapidly (<5 s) chilled to 4°C . Surface ASOR was removed by incubation for 5 min at 4°C in PBS/10 mM EDTA. Cell surface binding sites were then saturated (2 $\mu\text{g}/\text{ml}$, $4^\circ\text{C} \times 2$ h) with ^{125}I -ASOR. Each point is the mean of triplicate determinations of specific binding.

gest that at least two processes are involved in inhibition of receptor-mediated uptake and degradation by primaquine. The effect of these agents on the lysosomal degradation of ligands appears to be the more sensitive process, while loss of cell surface binding occurs at higher drug concentrations. It is presently unclear whether neutralization of lysosomal pH alone is sufficient to account for the reduction in ligand degradation observed with these agents.

The loss of cell surface binding sites seen in the presence of chloroquine or primaquine and in the absence of ligand may have resulted from (a) altered affinity of ligand-receptor binding, (b) inactivation (i.e., loss of binding activity) of receptor while still at the cell surface, or (c) loss of the receptor from the cell surface. The first possibility appears unlikely since there was no apparent alteration in receptor K_d . The latter two possibilities were examined directly using receptor-labeling (both cell surface ^{125}I -iodination and biosynthetic labeling) and anti-receptor antibodies. It was found that in the presence of chloroquine or primaquine there was a loss of ASGP-R from the cell surface, while the total cell ASGP-R was unaltered (Figs. 4–6). Thus, surface receptor is sequestered within the cell. The intracellular site of receptor sequestration is not presently known; however, the availability of antireceptor antibodies and colloidal gold immunocyto-electron microscopy (7) will allow this question to be directly examined.

These results are not fully consistent with the findings of Fiete et al. (28). These authors were unable to demonstrate a loss of ASGP-R from the cell surface after treatment with monensin, although ligand binding activity decreased. However, the loss of ASGP-R from the cell surface of hepatoma cells in the presence of lysosomotropic agents is similar to that observed by Harford et al. (29) in rat hepatocytes treated with monensin. They demonstrated intracellular (endosomal) accumulation of ligand binding sites coincident with loss of ASGP-R from the cell surface.

The notion that constitutive recycling of the ASGP-R occurs in hepatoma cells is supported by two observations. First, in the absence of ligand the lysosomotropic amines induce a loss of surface receptor into an internal pool. Second, the

kinetics of surface receptor loss are rapid both in the absence and in the presence of ligand. These studies suggest that the constitutive rate of ASGP-R recycling may be ~35–50% ($t_{1/2}$ 5.5–6 min vs. 2.5–3 min) that seen in the presence of ligand (Fig. 7). However, it is not known whether in the absence of ligand these agents induce receptor loss by increasing the rate of unoccupied receptor internalization or by decreasing the rate of receptor return to the surface or both. Nonetheless, the presence of ligand itself induces a rapid and transient loss of surface ASGP-R in hepatoma HepG2 cells (Fig. 7) (6). The presence of lysosomotropic amines has no effect on the very rapid ligand-induced loss of surface receptors, but it inhibits the rapid return of receptors to the cell surface (Fig. 7). These findings demonstrate that endocytosis of the ASGP-R itself is not altered by the amines, but that these agents diminish or prevent the reappearance of internalized receptors.

Thus, it appears that the lysosomotropic agents' effect on ASGP-R recycling most likely involves a substantial decrease in the rate of receptor return from an intracellular pool. Similarly, Ciechanover et al. (30) have recently demonstrated that ammonium chloride decreases the rate of return to the cell surface of transferrin and the transferrin receptor in HepG2 cells.

The concept of constitutive recycling of receptors involved in receptor-mediated endocytosis has evolved from the studies of Tolleshaug and Berg (8), Kaplan and Keogh (10), Tietze et al. (17), and Brown et al. (31). In general, these studies have used various agents, including the amines and carboxylic ionophores, to demonstrate loss of surface ligand binding sites in the absence of ligand. In addition, Brown et al. (31) provided evidence for the intracellular trapping of LDL receptors in studies with anti-LDL receptor antibody and immunofluorescence. The present studies with the ASGP-R support these observations and provide evidence that the amines interrupt constitutive receptor recycling by diminishing the rate of reappearance of internalized receptor. Although the mechanism responsible for this observation is not known, acidification within endosomal compartments (16, 32) may be obligatory for receptor reappearance at the cell surface.

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