Ligand- and Weak Base-induced Redistribution of Asialoglycoprotein Receptors in Hepatoma Cells

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Abstract. The receptor for asialoglycoproteins (ASGPR) was localized in human hepatoma Hep G2 cells by means of quantitative immunoelectron microscopy. Without ligand added to the culture medium, we found 34% of the total cellular receptors on the plasma membrane, 37% in compartment of uncoupling receptor and ligand (CURL), and 21% in a trans-Golgi reticulum (TGR) that was defined by the presence of albumin after immuno-double labeling. A small percent of the ASGPR was associated with coated pits, the Golgi stacks, and lysosomes. After incubation of the cells with saturating concentrations of the ligand asialo-orosomucoid (ASOR), the number of cell surface receptors decreased to 20% of total cellular receptors, whereas the receptor content of CURL increased by a corresponding amount to 50%. The ASGPR content of TGR remained constant. In contrast, after treatment of the cells with 300 µM of the weak base primaquine (PMQ), cell surface ASGPR had decreased dramatically to only 4% of total cellular receptors whereas label in the TGR had increased to 42%. ASGPR labeling of CURL increased only to 47%. The labeling of other organelles remained unchanged. This affect of PMQ was independent of the presence of additional ASOR. Implications for the intracellular pathway of the ASGPR are discussed.

Liver parenchymal cells possess cell surface receptors that function in the clearance of galactose-terminated glycoproteins from the circulation (1, 17). In addition, these asialoglycoprotein receptors (ASGPR) are present in abundance on the human hepatoma cell line Hep G2 that contains ~225,000 high affinity ligand-binding sites per cell (22, 23). In Hep G2 cells not exposed to ligand a considerable fraction of the functional ligand-binding sites is located on the cell surface (21). However, the presence of ligand (e.g., asialoorosomucoid [ASOR]) in the extracellular fluid promotes a relative increase in the fraction of receptors located intracellularly (4, 23).

Lysosomotropic amines, which accumulate within intracellular acidic organelles (5) thereby neutralizing the internal pH (18, 33), alter the intracellular distribution of several receptor species. The number of surface-binding sites for LDL (2), mannose-(29) and mannose-6-phosphate-(13) terminated ligands, alpha-2 macroglobulin (15), and asialoglycoproteins (21, 28) is markedly reduced after incubation with these agents. This reduction was seen in both the presence and absence of added ligand. However, in several cases the effect was enhanced in the presence of ligand (2, 15, 21). This loss of cell surface–binding sites is reversible since after removal of the agent the cell surface ligand–binding capacity is rapidly and fully restored (21, 29). These observations suggest that the receptors accumulate intracellularly in a nonlysosomal compartment. The aim of this study is to establish the morphologic identity of this compartment after treatment of the cells with ligand, a lysosomotropic amine, or with both.

We have reported a dose-dependent and reversible loss of surface ASGPR in Hep G2 cells treated with primaquine (PMQ) or other lysosomotropic amines (21, 28). The cells were found to possess a well-developed endocytic apparatus including compartment of uncoupling receptor and ligand (CURL) (8, 11). Using immunoelectron microscopy and antibodies against PMQ, we found that PMQ accumulates in CURL, including multivesicular bodies (MVB), lysosomes, and in the Golgi complex (25).

In the present study we used quantitative immunoelectron microscopy to describe the intracellular localization of ASGPR in Hep G2 cells, and examined the effects of the ligand ASOR and of PMQ on receptor distribution. We found that ASOR induced a partial redistribution of receptors from the plasma membrane to CURL. PMQ however, caused a dramatic disappearance of receptors from the cell surface in favor of trans-Golgi reticulum (TGR).
**Materials and Methods**

**Materials**

Human orosomucoid was provided by the American Red Cross and desialylated as described earlier (24). PMQ as the biphosphatase was obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit antibody to the human ASGPR was affinity purified as described earlier (20). Rabbit anti-human albumin (Nordic Immunology, Tilburg, The Netherlands) was affinity purified on an albumin ultragel column. Rabbit anti-cathepsin D (14) was a kind gift of Drs. K. von Figura and A. Hasilik (University of Münster, Federal Republic of Germany). Uniformly-sized protein A-gold complexes of 8 and 5 nm were prepared according to the tannic acid procedure as described (27).

**Cells**

Human hepatoma Hep G2 cells (16), clone a 16, were maintained as described previously (22). Confluent cultures were used in all experiments.

**Experimental Procedures**

Hep G2 cells were washed twice in MEM and cultured in MEM containing 10% normal rabbit serum for 4 h at 37°C. All cells were washed again and preincubated for 30-60 min at 37°C in MEM supplemented with 20 mM Hepes (pH 7.4). Thereafter, control cells that received no additional supplements were maintained in MEM Hepes for 40 min at 37°C. Cells exposed to ligand only were incubated with medium containing 200 μg ASOR/ml for 40 min at 37°C. Cells exposed to PMQ only were incubated in medium containing 300 μM PMQ for 30 min at 37°C. Cells exposed to both ligand and PMQ were first incubated for 10 min at 37°C with ASOR alone, after which PMQ was added for the final 30 min at 37°C.

**Processing for Immunocytochemistry and Morphology**

After the appropriate incubations the cells were fixed in 2% glutaraldehyde in 0.05 M phosphate buffer at pH 7.4 for 4 h at room temperature. Cells were then scraped from the dish and embedded in gelatin as previously described (6). The gelatin blocks were stored at 4°C for several weeks in 0.1 M phosphate buffer containing 0.02% sodium azide. Blocks were immersed in 2.3 M sucrose and frozen in liquid nitrogen. Cytosections were prepared according to Tokuyasu (32). Immuno-double labeling of sections was performed as previously described in detail (12). Anti-human albumin antiserum was used in the first step, followed by protein A-gold. Anti-ASGPR was used as the second antibody, followed by protein A-gold of a different diameter. In double labeling of the ASGPR with cathepsin D, anti-ASGPR was used in the first step. Sections were stained with uranyl acetate and embedded in methyl cellulose according to Tokuyasu (31).

Normal cells and cells treated with 300 μM PMQ for 30 min were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, embedded in 1% agar in distilled water at 70°C, and postfixed in 1% osmiumtetroxide in distilled water. Agar blocks were dehydrated in alcohol and embedded in epon. Ultrathin sections were stained with 7% uranylacetate in methanol and 0.4% leadcitrate in 0.1 M sodiumhydroxide.

**Quantitation of Receptors**

Three independent experiments were performed. Of several series of grids, sections were selected that exhibited good morphology. Approximately 1,000–2,000 gold particles labeling the ASGPR were counted for each of the three experiments. This represented ~100 cells per experiment, distributed over at least three grids. The particles were counted directly from the microscope screen. At low magnification the electron beam was placed in the top left corner of a grid mesh. After enlarging the magnification to 15,000× the grid was moved in one direction to the other side of the mesh. Thus the cells were encountered in a nonselective manner. The numbers of gold particles within each cell compartment (Table I) were expressed as the percentage of the total number of gold particles counted. The number of measurements required for a statistically reliable sample was determined by progressive mean analysis (3). 35 cell cross sections appeared to represent a reliable sample. Limits of confidence were 5% or less for at least the final three samples.

**Determination of ASOR-binding Sites**

Quantitation of specific cell surface 125I-ASOR binding was determined at 4°C as described previously (22). The total cell number of ligand-binding sites was determined by specific 125I-ASOR binding to cells at 4°C after permeabilization with saponin for 30 min at 4°C (26). In experiments in which cells were preincubated with nonradioactive ASOR, the cells were incubated for 5 min at 4°C with PBS containing no calcium but containing 5 mM EDTA at pH 5 to remove ligand from the receptor before saturation binding with 125I-ASOR (26).

**Results**

**Localization of ASGPR in Ligand-Naive Hep G2 Cells**

The normal localization of ASGPR was studied in "ligand-naive" cells, i.e., cells that had been cultured in medium without additional serum or ligand, and is shown in Figs. 1 and 2. ASGPR labeling occurred diffusely along the entire plasma membrane but was most abundant at the surface facing the culture medium. Receptors were concentrated in peripheral coated pits and vesicles. CURL was heavily labeled (Fig. 2). Double labeling with cathepsin D showed that some ASGPR-positive MVBs were poorly labeled for cathepsin D whereas others showed abundant cathepsin D labeling but did not label significantly for the ASGPR (not shown). This is in accordance with our observation that ASGPRs are present in newly formed CURL vacuoles but sort out from older ones into the CURL tubules (7). Typical secondary lysosomes showed only an occasional ASGPR label. In contrast to the situation in rat liver (9), only a little ASGPR was found in the Golgi stack (Figs. 1 and 2). TGR showed moderate ASGPR labeling (Figs. 1 and 2).

In general CURL and TGR could easily be distinguished morphologically. Although both contain anastomosing tubules and vesicles, TGR displayed a more heterogenous appearance and electron density, and showed more coated buds than did CURL (see also 8). Generally CURL occupied a peripheral position in the cells (Fig. 2) whereas TGR occurred adjacent to the Golgi complex deeper in the cell. However, some CURL elements could be detected close to a Golgi complex in the vicinity of the plasma membrane. This hampered a sharp distinction between CURL and TGR. Therefore an additional parameter was used, the presence of secretory albumin in TGR but not in CURL (8). Under the incubation conditions used, possible endocytosed albumin was not detected in CURL. Only the biosynthetic pathway including TGR was labeled for albumin. In the sections double labeled with anti-albumin and anti-ASGPR, ASGPR was distributed heterogeneously in the TGR. Some elements were enriched in ASGPR whereas others were devoid of it (Figs. 1 and 2). ASGPR-labeling of dense-coated...
buds at the TGR was low which was different from what we have previously found with the receptor for mannose-6-phosphate residues (8).

Quantitative Distribution of ASGPR in Ligand-Naive Cells

To determine the quantitative distribution of the receptors, we counted the number of gold particles confined to the various organelles as described in Materials and Methods. Particle counts were expressed as a percentage of the total number of gold particles evaluated. In ligand-naive cells (first column of Table I) the three plasma membrane domains together (lateral, bile canalicular, and those facing the culture medium and dish bottom) accounted for ~1/3 of the cells' ASGPR label. Coated pits of the plasma membrane were evaluated separately. No distinction was made between coated pits and vesicles. Together they contained ~5% of the label. CURL and TGR contained 37 and 21% of the total cellular receptor, respectively. Lysosomes contained <1% of the ASGPR labeling. The Golgi stack represented only a few percent of the label. The rough endoplasmic reticulum, the nuclear envelope, the mitochondria, and the nuclei were unlabeled. Control sections incubated with antibodies to rat amylase and rat IgA or with protein A-gold alone, gave negligible background values.

Effect of Ligand Administration on ASGPR Distribution

We compared ligand-naive cells with cells exposed to ligand. For this purpose Hep G2 cells were washed and preincubated for 30–60 min in medium without additional ligand or serum. Thereafter cells were incubated for 40 min at 37°C in the continuous presence of receptor-saturating concentrations of ASOR.

Cell morphology was not affected by treatment with ASOR. However receptor distribution was altered. The second column of Table I shows that receptor labeling of the plasma membrane declined from 34 to 20%. Labeling of peripheral coated pits and vesicles was not affected. The receptors lost from the surface appeared to accumulate exclusively on the endocytotic pathway, i.e., 13% in CURL, of which 5% was in MVBs. Receptor labeling of the Golgi complex and lysosomes remained unaltered.

Table I. Relative Distribution of ASGPR in Hep G2 Cell Compartments as Determined Morphologically

|                     | -PMQ | -ASOR | +PMQ | +ASOR |
|---------------------|------|-------|------|-------|
| Plasma membrane     | 34   | 20    | 4    | 4     |
| Coated pits/vesicles| 5    | 5     | 1    | 1     |
| CURL                | 37   | 50    | 47   | 46    |
| TGR                 | 21   | 22    | 42   | 46    |
| Golgi stack         | 2    | 2     | 3    | 1     |
| Lysosomes           | 1    | 1     | 3    | 2     |

Cells were washed and preincubated in serum-free medium for 30–60 min at 37°C. ASOR was added to -+ and ++ while the cells were kept at 37°C, 10 min later PMQ was added to -+ and ++. Incubation continued for 30 min. Cells were fixed and treated for immunoelectron microscopy. The numbers of gold particles present in the various cellular compartments were counted and expressed as percentages of the total number of gold particles. The values are the mean of three experiments. The limits of confidence are 5% as determined by progressive mean analysis.

Effect of PMQ Treatment on ASGPR Distribution

We have previously demonstrated (21) that incubation of Hep G2 cells with PMQ results in a considerable and reversible loss of cell surface ASOR-binding sites. In the present study we incubated cells with 300 μM PMQ for 30 min as described in Materials and Methods and quantitated the receptor redistribution.

Morphologically PMQ treatment was manifested in a disappearance of most of the microvilli on the cell surface (Figs. 5 and 6). The resulting change in cell shape, probably from the loss of surface membrane, was accompanied by an impressive increase in the number of intracellular tubules and vesicles, especially in the Golgi region. The Golgi complexes were more irregularly shaped and less contigous. Golgi cisternae and TGR were often swollen (Figs. 3 and 4). MVBs and lysosomes were enlarged and more numerous. The MVBs showed a more electron-lucent appearance and were negative for cathepsin D. As a result of the increase in the number of tubules and vesicles, the morphological distinction between the tubules of CURL and TGR was less clear than in controls. More frequently than in controls, albumin-negative but receptor-containing CURL tubules occurred in the Golgi region close to the albumin-positive TGR elements.

After PMQ incubation the distribution of the ASGPR over the cell organelles changed dramatically as shown in the third column of Table I. ASGPR surface label decreased to <5% of the control value and a significant decrease of the labeling of peripheral coated pits and vesicles was found. This was accompanied by a considerable increase of ASGPR in TGR from 21% in control cells to 42%. As in control cells, the distribution of ASGPR in TGR was not homogeneous (Fig. 5) and coated buds of TGR were not labeled. Labeling of CURL increased by 10%. Double labeling with cathepsin D showed that lysosomes were not involved in this increase. Labeling of the Golgi stack had not changed significantly.

The combined effects of 300 μM PMQ and saturating amounts of ligand on ASGPR distribution are shown in the fourth column of Table I. There was no significant difference in relative distribution of ASGPR between cells exposed to PMQ alone and cells exposed to both PMQ and ligand.

Effects of PMQ on Distribution of ASOR-binding Sites

We next determined biochemically the number of ASOR-binding sites at the cell surface relative to the total cellular ASOR-binding sites in control cells or cells exposed to either ligand, or PMQ, or both (Table II). In eight independent experiments the surface ASOR-binding sites were found to be 41 ± 11% (SD) (range 26–64%) of total cell ASOR-binding sites. As seen in Table II, the total cell receptor content was unaltered by incubation with either 300 μM PMQ, or 200 μg/ml ASOR, or both. However, the amount of cell surface receptor was reduced significantly in all three cases. In the presence of ASOR alone, surface receptor was reduced by 50 to 22% of total cell receptor. In the presence of PMQ alone, surface receptor was reduced by 65 to 15% of total cell receptor.

Discussion

We have recently described the localization of the ASGPR in rat liver hepatocytes and quantified the receptor distribu-
Figures 3 and 4. (Fig. 3) Golgi stack (G) and TGR (T) of Hep G2 cell incubated in the presence of 300 μM PMQ and saturating concentration of ASOR for 30 min. Label of both ASGPR and albumin are present in the swollen TGR profiles (T). G, Golgi cisternae. Bar, 0.25 μm. (Fig. 4) Like Fig. 3 but single labeled with 10 μm gold for the presence of ASGPR to show the intense labeling of TGR (T) as compared with the Golgi cisternae (G). As in many cells, the Golgi cisternae are swollen as a result of PMQ treatment. Bar, 0.25 μm.

There is a close similarity in distribution between hepatocytes and Hep G2 cells: in hepatocytes 36% of total cellular receptors is present at the cell surface and 39% in Hep G2. CURL contains 31 and 37%, respectively, and the total Golgi complex, including TGR, contains 23% in both cases. However the amount of receptors present in the Golgi cisternae differ remarkably. In Hep G2 cells, the stacks of Golgi cisternae contain only a small fraction (2%) of the cell's receptors, whereas the Golgi stacks in hepatocytes contain a major pool of receptors (14). A corresponding difference between hepatocytes and Hep G2 cells was found for the mannose-6-phosphate receptor (8, 10). The significance of this difference is presently unknown.

We were previously unable to detect an effect of added ligand on ASGPR distribution in rat liver parenchymal cells after infusion with asialofetuin in vivo for 60 min (9). However, as the physiological concentration of ASGPs present in the serum is unknown, it is possible that the rate of ligand administration in vivo was far below that necessary for saturation. This problem has been overcome in the present cell culture system. Using ASOR, which is a higher affinity ligand than asialofetuin (19), we found that the continuous administration of a saturating amount of ligand for 40 min resulted in a 14% decrease of cell surface receptors and a corresponding increase of receptors in CURL. Strikingly, the receptor content of TGR remained unchanged, as was the case for all other organelles.

Previous biochemical studies on the ASGPR in Hep G2 cells (4) have shown that the receptors are able to internalize ligand and recycle back to the cell surface every 7 min. In the present study we exposed Hep G2 cells to ASOR for 40 min. Thus most of the receptors on the cell surface had ample time to complete several cycles of ligand internalization. A passage of the 14% surface receptors through TGR would have lead to an increase in TGR receptors during this time period. Since the receptor content of TGR had not significantly increased, in contrast to CURL, these results suggest that a major fraction of the surface ASGPRs recycle directly from CURL, bypassing the TGR. That a minority of recycling surface receptors travel through TGR may well be possible since a small increase in TGR receptors may have been overlooked.

A direct CURL to plasma membrane recycling pathway raises the question as to the possible function of the relatively large fraction of one-fifth of total cellular receptors present in TGR under normal and ligand stimulated conditions. It is unlikely that the TGR receptors only represent newly synthesized molecules in transit to the cell surface. On the basis of life time (τt = 30 h) (20) and transit time (45–60 min) (20) one can estimate that only 2.5–3.5% of the cell's receptors...
Cells were washed and preincubated for 30 min at 37°C with 200 μg/ml unlabeled ASOR, or 300 μM PMQ, or both. Thereafter at 4°C, cells were washed and preincubated for 30 min at 37°C with 200 μg/ml unlabeled ASOR or 300 μM PMQ, or both. Total specific 125I-ASOR binding for all 12 samples was set to 100%. Whether such a connection between the endocytotic and biosynthetic pathways exists under normal conditions is currently under study in our laboratory. It remains to be established whether the PMQ effects were the result of neutralization of acidic compartments or whether additional factors were involved.

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