Two populations of prelysosomal structures transporting asialoglycoproteins in rat liver
(receptor-mediated endocytosis/subcellular fractionation/hepatic glycoprotein catabolism)

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ABSTRACT Analyses by differential centrifugation of liver homogenates from rats that had received $^{131}$I-labeled asialoorosomucoid showed that, 1 min after injection, most of the intracellular ligand was associated with a particle that did not sediment at $2.5 \times 10^7$ g-min. However, by 10 min, undigested ligand became associated with a particle that did sediment at this speed. On analytical ultracentrifugation in sucrose gradients, both kinds of particles exhibited low densities (1.11–1.13 g/ml$^{-1}$). In contrast to asialoorosomucoid, $^{131}$I-labeled asialotransferrin type 3, under noncatabolic conditions, remained largely confined to the nonsedimenting particle regardless of the duration of the study. Induction of catabolism of asialotransferrin was accompanied by the appearance of the ligand in the sedimentable particle. The nonsedimentable particle was separated by immunoadsorption from other subcellular particles contained in the low-density subcellular fraction. The adsorbant, prepared by immobilizing purified antibodies to the Gal/GalN-specific lectin from rat liver on coated acrylamide beads, removed 75–80% of the asialoorosomucoid and transferrin binding capacities present, together with a similar portion of the radioligands tested (asialoorosomucoid, asialotransferrin type 3, and human diferric transferrin). Significantly, the sialyltransferase activity remained unadsorbed. From these findings, the nonsedimentable particle appears to be involved in the transport of ligands destined to such diverse fates as exocytosis or lysosomal degradation. The sedimentable particle, on the other hand, seems to represent a link between the first particle and the lysosome.

Much interest has been focused recently on the subcellular processing of proteinaceous ligands (1, 2) that are interiorized by cells through receptor-mediated endocytosis (3, 4). In mammalian hepatocytes, asialoglycoproteins are transported from the plasma membrane to the lysosome (5), and details of this transport process have been extensively studied by electron microscopy (6–10), immunoelectron microscopy (11), ultrastructural cytochemistry (12), and subcellular fractionation (12–14). These investigations showed that intracellular asialoglycoprotein is first present in smooth-surfaced vesicles and tubular structures near the sinusoidal aspect of the cell, followed by its accumulation in larger, nonsoluble structures in the GERL region (Golgi-associated endoplasmic reticulum from which lysosomes form); the disociation of the endocytosed receptor–ligand complex is effected by a change in pH before the ligand reaches the lysosome.

At present, little is known about the biochemical characteristics of this transport system. Several groups of investigators found the ligand-containing subcellular particle to be of low buoyant density (12, 14–16). However, analysis of its composition has been made difficult by the lack of suitable isolating techniques; in particular, possible contamination with Golgi-derived vesicles had to be considered in several studies (12, 15, 17).

In this report we present data which suggest that asialoorosomucoid (AsOR), during intracellular transport, is associated sequentially with two particles that have different sedimentation properties. The first particle, which also transports human asialotransferrin type 3 (HAsTF-3) and diferric human transferrin of the biantennary glycan type (2Fe-HTf), can be freed from other vesicles by adsorption to polyacrylamide beads coated with antibodies to the Gal/GalN-specific lectin.

MATERIALS AND METHODS

Materials. All radioactive compounds were purchased from New England Nuclear. Substrates for the determination of marker enzymes, 3,3'-diaminobenzidine, horseradish peroxidase (type VI), and sucrose (grade I) were from Sigma. Ovalbumin was from Worthington, and polyacrylamide beads coated with sheep anti-rabbit IgG were from Bio-Rad Laboratories. Human transferrin (HTf), HAsTF-3 (18), and AsOR (19) were prepared as before. They were iodinated by using chloroglycoluril (20) under conditions given elsewhere for transferrin (21).

Animal Experiments. Unstarved, adult Sprague–Dawley rats received the radioligands in a tail vein, and their livers were retrieved after preselected intervals as described elsewhere (15).

Homogenization. This was done with 10 strokes of a Dounce homogenizer (0.127 mm clearance) using 3 vol of ice-chilled 0.3 M sucrose containing 5 mM Tris-HCl (pH 7.4) and 1 mM edetic acid (the latter was omitted in binding studies with 2Fe-HTf). The homogenate was filtered through nylon gauze (50 mesh) and homogenized further with 6 strokes in a Dounce homogenizer of 0.076 mm clearance.

Subcellular Fractionation. A Beckman model L8-55 ultracentrifuge was used. The homogenate was centrifuged at $3 \times 10^4$ g-min (22), and the pellet was washed twice by centrifugation at $2 \times 10^4$ g-min. The supernatants were combined and centrifuged first at $2.5 \times 10^4$ g-min and then at $1.3 \times 10^4$ g-min without washings. The pellets (P) and supernatants (S) from these three centrifugation steps are referred to as P1, P2, and P3 and S1, S2, and S3, respectively.

Preparation of the S2 Fraction for Immunoadsorption. Fifteen milliliters of the supernatant from the centrifugation at $2.5 \times 10^4$ g-min was layered over a 15-mL linear sucrose gradient, established from equal volumes of 0.47 M and 1.18 M buffered sucrose (see above) over a 6-mL cushion of 1.38 M buffered sucrose, and centrifuged at $2.3 \times 10^4$ g-min in a SW 28 rotor. The tube was emptied in 1-mL portions by layering 2 M sucrose on the bottom through a needle. Fractions containing the radiolgand (between densities 1.095 and 1.120) were pooled and diluted with H2O to the density of 0.3 M

Abbreviations: HAsTF-3, human asialotransferrin type 3; AsOR, asialoorosomucoid; HTf, human transferrin; 2Fe-HTf, diferric HTf of the biantennary glycan type.

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sucrose for concentrating by pelleting onto 0.5 ml of 2 M sucrose at 2.3 × 10³ g-min. Two milliliters of the concentrated material was loaded on a column (40 × 1.2 cm) of Sepharose 2B equilibrated with 0.3 M sucrose/0.15 M NaCl/5 mM Tris-HCl, pH 7.4. The radioactive peak appearing in the void volume was used for immunoadsorption and other assays without further treatment.

Analysis of Subcellular Fractions. Protein, density, and enzyme activities were determined as before (15). Galactosyltransferase activity was assayed according to Briels et al. (23). Values for the whole liver homogenate were calculated by summing the values for P₁ and S₁. Radioligands were measured in a Packard model 5986 multichannel analyzer.

Preparation of the Immunoadsorbent. Monospecific antiserum to the purified (24) Gal/GalN-specific lectin from rat liver were raised in rabbit and sheep. The IgG fraction was isolated by DEAE chromatography, and the antibodies were isolated by affinity chromatography on Sepharose-linked hepatic lectin. Polyacrylamide beads (see Materials) were suspended in 50 ml of 0.15 M NaCl containing 0.1% bovine serum albumin and buffered to pH 7.4 with 25 mM Tris-HCl. Ten milliliters of the suspension was centrifuged and washed three times with the same solution. To the final pellet were added 0.1 ml of the suspending fluid and 0.3 ml of a rabbit antiserum to sheep IgG. After incubation for 4–16 hr at 4°C, the beads were washed six times and then incubated for 4 hr at 22°C with 450 μg of the sheep antibodies to the hepatic lectin. Finally, the preparation was washed six times with the above solution but without albumin. Control beads were prepared in the same way using the IgG fraction (450 μg) from nonimmunized sheep.

Immunoadsorption. The method of Akio and Palade (25) was followed. The medium was 0.15 M NaCl containing 0.3 M sucrose and buffered to pH 7.4 with 5 mM Tris-HCl (final volume, 1 ml). After incubation for 3 hr at 4°C, the beads were removed by centrifugation and the supernatant was collected for analysis.

NaDodSO₄/PAGE. Linear gradients (5–12%) of polyacrylamide slab gels were prepared (26) with sucrose as a stabilizer. Samples to be applied were pelleted, suspended in 0.1 ml of the stacking gel buffer containing 2-mercaptoethanol (5%) and NaDodSO₄ (3%), and boiled for 3 min. Two gels were run simultaneously, one for staining and the other for blotting.

Electroblotting. Transfer to nitrocellulose paper was effected as described by Towbin et al. (27). Transfers were washed with 0.2 M NaCl, buffered to 7.4 with 10 mM Tris-HCl, quenched with albumin (5%), and immune-overlaid first with rabbit antibody to the rat Gal/GalN-specific lectin (16 hr, 4°C) and then with horseradish peroxidase-conjugated (28) sheep antibody to rabbit IgG (30 min, 22°C). After each antibody reaction, transfers were washed with five changes (1 hr each) of 0.2 M NaCl containing 10 mM Tris-HCl (pH 7.4), 0.2% NaDodSO₄, 0.5% Triton X-100, and 0.5% albumin. The peroxidase was activated by H₂O₂, and the color from 3,3'-diaminobenzidine was developed for 30 min in the dark.

**RESULTS**

Observations from the subcellular fractions are summarized in Fig. 1. The salient point is the time-dependent partition of 131I-labeled AsOR (131I-AsOR) between particles sedimentable (P₂) and nonsedimentable (P₃) at 2.5 × 10⁵ g-min. *

*Non sedimentable particles after the second centrifugation step are in the supernatant designated S₂. However, any free radioligand from plasma membrane and hepatic residual blood is also in S₂. To distinguish between free and entrapped ligand, the third centrifugation is performed that pellets the nonsedimentable particles from the second centrifugation in P₃.

![Fig. 1. Distribution of marker enzymes and radioligands following differential centrifugation of homogenized livers from rats injected with 125I-labeled HAsTF-3 (125I-HAsTF-3) or 131I-AsOR. In each quardrate, the histograms from left to right depict the P₁, P₂, and S₂ fractions (see Subcellular Fractionation in Materials and Methods). The relative specific activity (% activity/% protein) of the fractions is plotted against their relative protein content (29). The values are means from three to six experiments, the bars denoting standard errors. (A) Sialytransferase; (B) glucose-6-phosphatase; (C) alkaline phosphodiesterase I; (D) acid phosphatase; (E) 131I-AsOR (2 μg/100 g of body weight) injected 1 min beforehand; (F) 131I-AsOR (2 μg/100 g) injected 10 min beforehand; (G) 125I-HAsTF-3 (2 μg/100 g) injected 10 min beforehand; (H) 125I-HAsTF-3 (2 μg/100 g), followed 5 min later by unlabeled HAsTF (0.5 mg/100 g), and liver removed 12 min after the second injection. The total recoveries were: 87.0% ± 0.2% (A); 93.5% ± 0.5% (D); 96.2% ± 1.6% (C); 79.6% ± 1.5% (D); 101.3% ± 0.5% (E); 97.5% ± 0.9% (F); 88.1% ± 1.8% (G); 88.2% ± 1.5% (H); and 97.3% ± 0.5% (protein).
hepatic lectin as described in Materials and Methods. To facilitate particle identification, the donors were prepared by injecting 125I-HAS-TF-3, diferric 125I-labeled HTF (125I-HTF), or 121I-AsOR 10–20 min before the removal of their livers. Preliminary studies showed that the fraction of radioligands removed from the solution by the immunoabsorbant was proportional to the reactants (coated beads and subcellular fraction). Results obtained under optimal reaction conditions are summarized in Table 1, and several features should be noted. First, immunoadsorption with antibodies to the lectin not only depleted 76% of the lectin present but also a comparable fraction of the tracer asialoglycoproteins. Furthermore, tracer transferrin and transferrin binding capacity were depleted to similar extents. Second, sialyltransferase levels remained unaffected by this treatment. It should be pointed out in connection with the changes seen for acid phosphatase and phosphodiesterase I that the preparations used for adsorption contained <1% of the former and <10% of the latter present in the homogenates.

The effects of immunoadsorption were also assessed by slab gel electrophoresis and electroblotting (Fig. 3). The polypeptide composition of the subcellular preparation used for adsorption differed from that of carbonate-treated rat liver plasma membranes, although numerous components appeared to be common. After adsorption with antibodies to the Gal/GalN-specific hepatic lectin, there was a marked reduction in one of the polypeptide bands that migrated close to the Mr 45,000 standard. The rat lectin subunit has been reported to have a Mr of 43,000 (34); therefore, the results of the electrophoresis and the blot and the fact that the antibody preparation in the free (uncoupled) state inhibited 121I-AsOR binding by purified plasma membranes from rat liver permit us to conclude that the band under consideration was the lectin. Densitometric gel scans were indicative of partial losses affecting a limited number of additional components on immunoadsorption, mainly in the higher molecular weight region (Mr >93,000). However, this requires further confirmation with gels of different compositions and having less noisy base lines.

Preliminary studies similar to the ones described above with the sedimentable particle in fraction S2 indicated that it contains undegraded 121I-AsOR (Fig. 4) and that degradation of the ligand in these particles is not induced by incubation at pH 5.0 and 37°C for 1 hr.

DISCUSSION

The present method of preparing the nonsedimentable, ligand-containing particles from fraction S2 was derived from our earlier publication. As was shown there (17), these particles are uncoated vesicles with an average diameter of ~150 nm. Thus, they are smaller than isolated receptosomes (35). The modifications introduced are aimed at the better preservation of these delicate structures.

The presence of 125I-HAS-TF-3 in the S2 particle confirms our previous results (17). More importantly, the present data show that HTF and AsOR are also transported by the same particles and that the preponderance of all three ligands is adsorbed by beads coated with antibodies to the Gal/GalN-specific lectin. Thus, the lectin appears to be a prominent constituent of the particle under consideration, a view also supported by the data in Fig. 3. Gel chromatography of the preparation in the presence of edetic acid before immunoadsorption precludes the possibility that the 121I-AsOR found in this fraction could have been adherent to the outer surface of the particle.

The lectin antibody technique is a convenient approach to the separation of the S2 transport vesicles from unrelated subcellular organelles, above all from the pronounced sialyl- and galactosyltransferase activities present in such preparations (12, 15, 17). Unfortunately, the vesicles thus captured cannot be dissociated from the adsorbant intact, and therefore their properties had to be inferred from analyses of the material that failed to adsorb. Even so, it is clear that with one-third of the protein removed (Table 1), the overall change effected by the adsorption in the electrophoretogram was minor. This suggests that the particle is likely to possess a reduced complement of polypeptides by comparison to plasma membrane and the other structures present. A similar conclusion was recently reached for receptosomes (35).

In HeLa cells, subcellular transferrin and transferrin receptor are localized in a vesicle that, in sucrose density gra-

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Table 1. Partition of the subcellular components following the treatment of the partially purified S2 fraction from rat liver homogenate with polyacrylamide beads coated with different IgGs

<table>
<thead>
<tr>
<th>IgG used for coating</th>
<th>Protein</th>
<th>Gal/GalN lectin</th>
<th>AsOR</th>
<th>HAS-TF-3</th>
<th>HTF</th>
<th>TBC</th>
<th>PD-I</th>
<th>APhase</th>
<th>STase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(5)</td>
<td>(1)</td>
<td>(3)</td>
<td>(8)</td>
<td>(1)</td>
<td>(1)</td>
<td>(5)</td>
<td>(6)</td>
</tr>
<tr>
<td>Nonspecific</td>
<td>8 ± 3</td>
<td>2</td>
<td>18 ± 2</td>
<td>20 ± 4</td>
<td>9</td>
<td>8</td>
<td>10 ± 3</td>
<td>20 ± 3</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>Anti-lectin</td>
<td>33 ± 2</td>
<td>76</td>
<td>78 ± 1</td>
<td>78 ± 1</td>
<td>74</td>
<td>71</td>
<td>66 ± 3</td>
<td>73 ± 1</td>
<td>16 ± 3</td>
</tr>
</tbody>
</table>

Percentages refer to quantities present before treatment. Numbers of experiments are given in parentheses; values are means ± SEM, where appropriate. TBC, binding capacity for diferric HTF; PD-I, phosphodiesterase I; APhase, acid phosphatase; STase, sialyltransferase. The lectin (17) and transferrin binding (36) were assayed according to published methods.
AsOR appeared in the sedimentable (P$_2$) particle after passage through the nonsedimentable (S$_2$) particle. Little HASTF-3 was detected in P$_2$ under recycling conditions—the rate of hepatic degradation of this protein is low; however, after rerouting $^{125}$I-HASTF-3 to the lysosomes (30), the sedimentable particle exhibited high levels of $^{125}$I activity. This may be due to the undegraded ligand released by the lysosomes. Although with a mean density difference of 0.01, the P$_2$ and S$_2$ particles do not lend themselves to separation by gradients. The fortuitous observation regarding their differential sedimentation at 2.5 x 10$^{-3}$ g-min provided the key to the present preparative procedure. The unequal sedimentation behavior of both particles is likely due to a difference in size (29), which is in keeping with the morphological studies showing that the ligand-containing subcellular structures become larger as they move towards the lysosomes (8).

It would be difficult to equate either of the particles under consideration here with particular structures seen in the electron microscope in unfractionated cells. In vivo, galactose-terminated ligands are rapidly transferred to the GERL region (7, 10). Thick-sectioning shows the existence of an extensive (acid phosphatase-positive) tubular network in this region (37) that is likely to be destroyed during homogenization. Similarly, the rupture of multivesicular bodies could set free vesicles containing their ligands; of interest in this connection is the recent observation that, in rat reticulocytes, recycling of transferrin occurs from these structures (38). The receptors of the vesicles within multivesicular bodies are everted (39), and AsOR binding by the nonsedimentable particles is sensitive to trypsinization and exhibits no latency (17).

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