Transcellular Transport of Polymeric IgA in the Rat Hepatocyte: Biochemical and Morphological Characterization of the Transport Pathway

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ABSTRACT Polymeric IgA (plgA) is transported by liver parenchymal cells (hepatocytes) from blood to bile via a receptor-mediated process. We have studied the intracellular pathway taken by a TEPC15 mouse myeloma plgA. When from 1 µg to 1 mg 125I-plgA was injected into the saphenous vein of a rat, 36% was transported as intact protein into the bile over a 3-h period. The concentration of transported 125I-plgA was maximal in bile 30–60 min after injection, and ~80% of the total 125I-plgA ultimately transported had been secreted into bile by 90 min. A horseradish peroxidase-plgA conjugate (~25I-plgA-HRP) was transported to a similar extent and with kinetics similar to that of unconjugated 125I-plgA and was therefore used to visualize the transport pathway. Peroxidase cytochemistry of livers fixed in situ 2.5 to 10 min after ~25I-plgA-HRP injection demonstrated a progressive redistribution of labeled structures from the sinusoidal area to intermediate and bile canalicular regions of the hepatocyte cytoplasm. Although conjugate-containing structures began accumulating in the bile canalicular region at these early times, no conjugate was present in bile until 20 min. From 7.5 to 45 min after injection ~30% of the labeled structures were in regions that contained Golgi complexes and lysosomes; however, we found no evidence that either organelle contained ~25I-plgA-HRP. At least 85% of all positive structures in the hepatocyte were vesicles of 110–160-nm median diameters, with the remaining structures accounted for by tubules and multivesicular bodies. Vesicles in the bile canalicular region tended to be larger than those in the sinusoidal region. Serial sectioning showed that the 125I-plgA-HRP-containing structures were relatively simple (predominantly vesicular) and that extensive interconnections did not exist between structures in the sinusoidal and bile canalicular regions.

Receptor-mediated endocytosis is an important cellular process by which macromolecules enter cells. Liver parenchymal cells (hepatocytes) internalize a wide variety of molecules from the circulation via this mechanism; however, once endocytosed, the ligands exhibit diverse intracellular fates. Asialoglycoproteins (41) and epidermal growth factor (6) are taken up by rat hepatocytes and degraded in lysosomes, whereas polymeric IgA (plgA) is transported from blood to bile without degradation (24, 27, 30, 40). The fates of the surface receptors can also vary in hepatocytes. The asialoglycoprotein receptor is reutilized for further rounds of ligand internalization (33, 38), whereas the receptor for epidermal growth factor is degraded when cells are exposed to the hormone (6a). Secretory component (SC), the receptor for plgA, is transported across the cell and secreted with its ligand as a complex (7, 16, 17).

Most of the plgA that is transported from blood to bile in the liver is synthesized by plasma cells found in the lamina propria of the intestinal mucosa and enters the bloodstream at the thoracic duct (15). Circulating plgA is then bound at the sinusoidal front of hepatocytes and transported across the
cell, processes mediated by SC, a plgA-specific receptor synthesized in the hepatocyte (7, 17, 23, 31, 34–36). During transport of the SC-plgA complex across epithelial cells, the membrane-anchoring domain (20–36 kD) of the transmembrane receptor is cleaved (20, 32, 34). Morphological and biochemical studies have suggested that plgA is transported across the hepatocyte within small vesicles (21, 27, 37). Another view has been put forward by Geuze et al. who reported that plgA and asialofetuin, a ligand for the asialoglycoprotein receptor, are segregated from each other in a tubule network (compartment of uncoupling of receptors and ligand) that extends from the sinusoidal cell periphery to the trans-Golgi area (bile canalicular region) of the hepatocyte (9). They suggest that plgA is sorted from asialoglycoproteins in this tubule network, with packaging of plgA in vesicles for subsequent transport to the bile front. Thus, there is not yet agreement as to the pathway by which plgA traverses the hepatocyte.

In this study we have extended the findings of Takahashi et al. (37) and Geuze et al. (9). A horseradish peroxidase (HRP) conjugate of mouse 125I-plgA (125I-plgA-HRP) and electron microscopic cytochemistry were used to identify and quantify the intracellular structures involved in the uptake, accumulation, and release of plgA by rat hepatocytes. We have found that 85% of the total 125I-plgA-HRP-containing structures at all times analyzed were discrete vesicles, with tubules and multivesicular bodies composing the remaining 15%. In addition, we have found that the vesicles in the bile canalicular region of the hepatocyte tended to be larger than those in the sinusoidal region, which suggests that the ligand is repackaged during transport. Finally, serial sections showed that in most cases vesicles and tubules were not interconnected. Preliminary portions of this work have been presented elsewhere (11).

MATERIALS AND METHODS

Materials

Phosphorylcholine chloride (calcium salt) and o-dianisidine dihydrochloride were from Sigma Chemical Co. (St. Louis, MO); complete and incomplete Freund’s adjuvant were from Difco Laboratories Inc. (Detroit, MI); and female New Zealand white rabbits (10–12 lb) were supplied by Bunnyville (Littletown, PA). The phosphorylcholine-35S bovine serum albumin-Sepharose 4B (PC-BSA-Sepharose 4B) was a generous gift of Dr. Y. C. Lee, Johns Hopkins University. Rabbit anti-rat SC antibody was produced using soluble SC from rat bile as an immunogen. Other reagents were obtained from the same sources given in recent publications from this laboratory (1, 6, 14, 29, 41, 42) or were of the highest purity available commercially and were used without further purification.

Preparation of plgA

The plasmacytoma cell line, TECPC15, produces an IgA that binds phosphorylcholine (26) and was kindly supplied by Dr. Potter (National Cancer Institute, National Institutes of Health) and Litton Bionetics (Kensington, MD). The cells were grown in the ascites form in pristane-primed BALB/c mice where IgA concentrations reached 4–6 mg/ml ascites. Cells and clotted blood were aseptically removed from the ascites by centrifugation (1,300 g, 15 min), and sodium azide was added to a final concentration of 3 mM. The ascites could be stored in this state at 4°C for at least 8 min.

The entire purification of plgA was done at 4°C. Ascites fluid (25 ml) was clarified by centrifugation (100,000 g, 60 min) and then applied to a PC-35S-BSA-Sepharose 4B affinity column (2 x 3 cm) that had been equilibrated but not coupled to plgA. The final conjugate (three preparations) ranged from 4 to 200 gg HRP bound per 100 mg 125I-plgA, or 0.005 to 0.4 mol HRP/mol plgA (using Mr 700,000 for plgA). The 125I-plgA-HRP conjugate was characterized by native PAGE and by comparison of its transport kinetics to that of unconjugated 125I-plgA. 125I-plgA-HRP was stored at -70°C.

In Vivo Clearance and Transport into Bile

Assessment of 125I-IgA and 125I-plgA-HRP Clearance from the Blood and Transport into Bile: The in vivo clearance and transport of 125I-plgA (1–1,000 ug) and 125I-plgA-HRP (300–400 ug) were determined as previously described for other liver-specific ligands (12). The bile duct was cannulated with PE-10 Intramedic polyethylene tubing (Clay-Adams, Parsippany, NJ), and bile was collected over 10-min intervals from 0–30 min, 15-min intervals from 30–60 min, and 30-min intervals thereafter.

Characterization of 125I-plgA and 125I-plgA-HRP in Serum and Bile Samples by Affinity Chromatography: Serum and bile samples containing 125I-plgA or 125I-plgA-HRP were diluted 1/10 in TBS, 0.05% (wt/vol) BSA and applied to a small PC2S-BSA-Sepharose 4B column (0.8 x 3.0 cm), and 0.3-ml fractions were collected as described above (Preparation of plgA) with 0.05% (wt/vol) BSA in all column buffers.

Native PAGE

plgA samples were analyzed on 5–7% polyacrylamide slab gels according to the method of Maizel (19) as previously described (13), but SDS was omitted from both resolving and stacking gels to prevent dissociation of light chains from the polymers. Samples were applied in 50 mM Tris-HCl, pH 8.9, 0.4 M sucrose, 0.002% (wt/vol) bromphenol blue. Since separation of the plgA polymers required prolonged electrophoresis, horse spleen ferritin (300 zg) was used as a standard, and the gel was stopped when the fastest running band (Mr of apoferritin, 460,000) had migrated two-thirds the length of the gel. Gels were stained with Coomassie Blue, destained (19), and dried. For 125I-plgA was being analyzed, the dried gel was exposed to x-ray film (Kodak XAR-5). Bands on the developed film were quantitated using a GS300 Transmittance/Reflectance Densitometer (Hoefer Scientific Instruments, San Francisco, CA) interfaced with a Hewlett-Packard 3390A integrator (Avondale, PA).

Characterization of 125I-plgA-HRP on Nitrocellulose Transfers

The 125I-plgA-HRP polymers separated by native PAGE were electrophoretically transferred to nitrocellulose by the method of Towbin et al. (39). The nitrocellulose transfer was quantitated with 2% gelatin, 10 mM sodium phosphate, pH 7.4, 0.14 M NaCl, 3 mM KCl before incubation in 1.4 mM 3,3’-diaminobenzidine, 0.03% (wt/vol) H2O2, 50 mM Tris-HCl, pH 7.6. Bands of peroxidase reaction product appeared in ~30 s. After two 1-min washes in deionized water, the peroxidase reaction was stopped by the addition of 3% (wt/vol) trichloroacetic acid. After 15 min, the transfer was rinsed in deionized water, blotted dry, and exposed to x-ray film at -70°C with intensifying screen, and the film was developed.
Morphology

**Localization of $^{125}$I-pIgA-HRP in situ:** $^{125}$I-pIgA-HRP (0.5-1.0 mg plgA) and mannan (1.0 mg) were mixed and injected into the saphenous vein of fasted Sprague-Dawley rats under phenobarbital anesthesia, and at various times the livers were perfused in situ through the hepatic portal vein, first with 0.9% (wt/vol) NaCl for 10-20 s and then with 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 5 min (12).

HRP activity in the fixed liver was detected as described previously (41). After the diaminobenzidine reaction, sections were rinsed in Tris buffer and treated with 1% OsO$_4$, 1% K$_4$Fe(CN)$_6$·3H$_2$O, 0.1 M sodium cacodylate, pH 7.4 (filtered through a Millipore type GS filter, 0.22 μm, Millipore Corp., New Bedford, MA) for 45 min at 4°C (32). After they were rinsed in deionized water at 23°C for 30 s, the sections were dehydrated and embedded in Epon for electron microscopy.

**Quantitation of $^{125}$I-pIgA-HRP-containing structures in hepatocytes:** The numbers and distributions of intracellular structures containing $^{125}$I-pIgA-HRP were determined on thin sections from livers fixed at various times after in vivo injection of ligand. Structures were counted with the aid of a magnifying glass from random micrographs printed at a final magnification of 8,000. The hepatocyte cytoplasm was divided into three regions (sinusoidal, bile canalicular, and intermediate) and the number of $^{125}$I-pIgA-HRP-containing structures quantified in each of the regions. A structure was considered to be sinusoidal when found within 1.6 μm of the base of sinusoidal plasma membrane microvilli, bile canalicular when found within 2.2 μm of the base of bile canalicular plasma membrane, and intermediate if a structure did not fall into either of the other two regions.

Golgi-lysosome regions of hepatocytes were defined morphologically as areas in which Golgi stacks and lysosomes could be identified, and in most cases rough endoplasmic reticulum and mitochondria were absent. About 50% of such areas were near bile canaliculi (within ~2.2 μm), and the remainder were in intermediate regions as defined above. $^{125}$I-pIgA-HRP-containing vesicles, tubules, and multivesicular bodies were quantified in the Golgi-lysosome regions of the hepatocyte. Vesicles and tubes tended to have a homogeneous intensity of internal HRP reaction product, whereas multivesicular bodies were larger structures in most cases and contained distinguishable internal vesicles or other inclusions. The diameters of $^{125}$I-pIgA-HRP-positive vesicles in the sinusoidal, bile canalicular, and intermediate regions were measured on micrographs of cells from livers fixed at 7.5, 15, and 30 min after injection of the conjugate.

**Analysis of $^{125}$I-pIgA-HRP-containing structures using serial sections:** The size, shape, and possible interrelationships of conjugate-containing structures were examined on serial 70-nm sections of tissue from livers fixed 15 and 30 min after $^{125}$I-pIgA-HRP injection. Section thickness was calculated as one-half the width of section folds measured on micrographs of known (and calibrated) magnification. 13 series of 6-14 sections each (average of 8/series) were examined.

**Other Methods**

**Acid precipitation of $^{125}$I-pIgA:** Samples of bile, plasma and liver homogenate were incubated at 4°C for 30 min in 10% (wt/vol) trichloroacetic acid, 0.5% (wt/vol) phosphotungstic acid, 1 mM KI, and then sedimented (1,300 g, 15 min) at 4°C. The pellet was washed once with the trichloroacetic acid/phosphotungstic acid solution before the radioactivity associated with it was measured. The precipitated radioactivity was a measure of $^{125}$I covalently attached to pIgA.

**Radioiodination of proteins:** The plgA was radioiodinated as described previously (14) to 3-5 x 10$^6$ cpm/mg, stored in aliquots at ~70°C, and used within 6 wk after radioiodination. Protein A was radioiodinated by a chloramine-T procedure to a specific activity of 2-4 x 10$^6$ cpm/mg (29).

**RESULTS**

**Biochemical Characterization of $^{125}$I-plgA Transport**

**$^{125}$I-plgA Clearance and Transport to Bile:** The concentration of $^{125}$I-plgA in blood reached a maximum between 2.5 and 10 min after intravenous injection of the ligand and then decreased steadily out to 180 min (Fig. 1). Radioactivity was first detected in bile 20 min after ligand injection and reached its highest concentration there between 30 and 60 min (Fig. 1). Routinely, by 3 h 30-40% of the injected radioactivity had been transported to bile, a value that was independent of the $^{125}$I-plgA levels administered, which ranged from 1 μg to 1 mg (inset, Fig. 1).

In seven separate experiments, the distribution of radioactivity 180 min after injection was as follows (mean ± SD): 36 ± 4% was transported to bile, 11 ± 3% was in liver, and 34 ± 7% remained in the blood. An average of 81 ± 9% of the injected radiolabel could be accounted for in bile, blood, and liver, with the remaining 15-20% in the stomach, intestine, thyroid, spleen, kidney, lung, colon, and heart. 180 min after injection of $^{125}$I-plgA, <20% of the radioactivity in liver homogenate and >80% of the radioactivity in stomach and intestine contents were acid soluble. Therefore, ~12% of the injected radioactivity was recovered in a degraded form (iodide or small peptides) from the stomach, intestine, and liver.

**Characterization of $^{125}$I-plgA Transported to Bile:** The nature of the radioactivity in the bile was investigated to ascertain that $^{125}$I-plgA was being transported intact across the hepatocyte. More than 90% of the radioactivity collected in bile samples up to 90 min was acid insoluble. Thereafter, the acid-insoluble radioactivity fell but was never less than 70% of the total up to 180 min.

Native PAGE followed by autoradiography was used to compare transported radioactivity with the injected $^{125}$I-plgA (Fig. 2). Three $^{125}$I-plgA bands were present in the injected sample. We have designated the three $^{125}$I bands as the dimer (band 3), tetramer (band 2), and hexamer (band 1) forms of plgA, based on their elution behavior from the Sephadex G-200 column used to purify plgA. These assignments are consistent with data on plgA from three other mouse plas-
FIGURE 2 Autoradiogram of $^{125}$I-plgA in blood and bile. Bile (25 µl) and plasma (12 µl) samples from the experiment shown in Fig. 1 were analyzed by native PAGE (see Materials and Methods). Bands 3, 2, and 1 represent the polymeric forms of plgA in our preparation which we believe to be dimer, tetramer, and hexamer, respectively. When this experiment was repeated, the distributions of radioactivity in bands 1-3 of all bile and blood samples were similar to that of the injected material.

macytomas, where tetramer and hexamer accounted for 42–76% (by weight) of the plgA (10). Bile samples collected at 30, 60, 90, and 120 min had radioactive bands that co-electrophoresed with the three $^{125}$I-plgA bands present in the injected sample. No radiolabeled ligand was detected in bile before 15 min, consistent with the radioactivity measurements. Although the experimental results presented in Fig. 2 suggest that higher polymer forms of plgA were transported to bile faster than was the dimer, the experiment was repeated using a new plgA preparation, and no apparent rate differences were observed. Thus, it is not at present clear to what extent the various polymeric species produced by the mouse TEPC plasmacytoma are differentially transported from blood to bile. However, when rat bile from uninjected animals was analyzed by native PAGE and immunoblotting (14) with an anti-alpha chain antibody, endogenous dimer, tetramer, and hexamers of IgA were all present, which indicates that the rat system can transport these polymers, whether endogenous or exogenous in origin.

$^{125}$I-plgA collected in bile samples up to 90 min, and the injected $^{125}$I-plgA showed the same extent of binding and specific hapten elution from PC25-BSA-Sepharose 4B columns, which was 70–80% of that applied. This indicated that the intact $^{125}$I-plgA in bile was immunologically active after its transport across the hepatocyte and secretion. The immunoprecipitation of SC (using rabbit anti-SC) from bile samples collected 30–60 min after $^{125}$I-plgA injection indicated that at least 40% of the transported $^{125}$I-plgA was bound to SC (data not shown).

CHARACTERIZATION OF CIRCULATING $^{125}$I-plgA: More than 80% of the radioactivity in all plasma samples collected from 0–180 min was acid precipitable, and results of native PAGE revealed the presence of three $^{125}$I-plgA bands that co-electrophoresed with those present in injected $^{125}$I-plgA samples (Fig. 2). Since by these criteria the $^{125}$I-plgA remaining in the circulation at 120 min was intact, it was puzzling why all of the molecules initially injected were not cleared. Comparable amounts (70–80%) of ligand from both injected and 120-min plasma samples bound to and were specifically eluted from PC25-BSA-Sepharose columns, which indicates that antigen binding was retained in the uncleared plgA. To examine the possibility that liver transport was progressively impaired during the experiment, a rat that had been injected 2 h earlier with a dose of $^{125}$I-plgA was challenged with another dose of $^{125}$I-plgA at 10 times greater specific activity. The result was unequivocal. The second $^{125}$I-plgA dose was transported into bile with kinetics identical to that of the first. Another possibility, that the $^{125}$I-plgA remaining in the plasma at 120 min could not be cleared, was addressed by a study of its behavior after administration to a second rat. Serum prepared from the blood of a rat that had been injected 2 h earlier with high specific activity $^{125}$I-plgA was injected into a second rat. Calculated on the basis of the $^{125}$I-plgA dose given to the first rat, only 4% more was cleared in the second animal, which indicates the near-complete inability of this $^{125}$I-plgA to be transported into the bile. We concluded that either a population of $^{125}$I-plgA less active in transport was produced after injection into rats, or $^{125}$I-plgA existed as a heterogeneous population of molecules before injection.

Assessment of $^{125}$I-plgA-HRP Conjugate

We next determined the clearance and transport characteristics of the HRP conjugate of $^{125}$I-plgA. The low endogenous HRP activity in bile allowed a direct comparison of the radioactivity and peroxidase activity transported to bile in our initial characterization of the conjugate. The kinetics of $^{125}$I-plgA-HRP transport into bile measured both ways was similar.
to that of $^{125}$I-plgA (Figs. 1 and 3). Both radioactivity and peroxidase activity first appeared in bile at 20 min and reached a maximum concentration at 60 min, within the 30-60-min period in which bile $^{125}$I-plgA usually reached a maximum. However, more of the injected peroxidase activity (56%) than of the injected radioactivity (38%) was transported to bile (inset, Fig. 3). The $^{125}$I-plgA-HRP radioactivity present in the liver (23% of injected) after the 120-min experiment was somewhat higher than the 6-15% range seen for the 180-min experiments. This probably reflects the greater percentage of injected $^{125}$I-plgA-HRP in transit through the hepatocyte at 120 min.

We next characterized the injected and transported conjugates by native PAGE, the results of which are shown in Fig. 4. The three bands observed with $^{125}$I-plgA alone were also present in the injected preparation of $^{125}$I-plgA-HRP and contained both radiolabel and HRP activity. A comparison of the relative amounts of radioactivity and peroxidase activity in each band indicated that HRP was preferentially coupled to higher polymeric forms. There was also radioactivity at the top of the separating gel that had relatively less HRP activity (Fig. 4, A vs. B). This radiolabeled material may represent species that have more than one plgA molecule bound per HRP molecule, with possible inactivation of HRP in such conjugates. When the $^{125}$I-plgA-HRP transported to bile was analyzed on native gels, both radioactivity and HRP activity were detected in the three plgA bands that corresponded to the major plgA's injected (Fig. 4). A discrete band containing HRP activity was also present above band 1, but its content of $^{125}$I was not detectable above the background in that region of the gel (Fig. 4B).

Finally, we found that >70% of both peroxidase activity and radioactivity transported to bile between 45 and 60 min after $^{125}$I-plgA-HRP injection was bound and specifically eluted from PC$_{25}$-BSA-Sepharose 4B. Thus, by several criteria, the HRP in bile was coupled to $^{125}$I-plgA, which indicates that the conjugate had crossed the hepatocyte intact.

**Ultrastructural Examination of $^{125}$I-plgA-HRP Transport Across the Hepatocyte**

To characterize the transport of $^{125}$I-plgA-HRP across the hepatocyte, the types and cellular distribution of structures that contained this electron microscopic tracer were determined at various times after in vivo administration of the conjugate. Fig. 5 illustrates qualitatively the types of $^{125}$I-plgA-HRP-containing structures present in the sinusoidal, intermediate, and bile canalicular regions of hepatocytes 30 min
FIGURE 5 Intracellular localization of plgA-HRP in hepatocytes after its in vivo administration. Livers were fixed by perfusion 30 min after in vivo injection of the conjugate (0.55 mg) and processed for HRP cytochemistry. (a and b) Sinusoidal region. HRP reaction product is present in small vesicles (small arrowheads) and tubules. SL, sinusoidal lumen; SF, sinusoidal front. (c) Intermediate region. Reaction product is found near nuclei (N), and in vesicles similar in size to those at the periphery, in multivesicular bodies, and also in larger vesicles (large arrowheads), but not in lysosomes (Ly) or stacks of Golgi (Go), which are also in this area. (d) Bile canalicular (BC) region. Conjugate is present in larger vesicles (large arrowheads) and in multivesicular bodies (MVB) as well as in the bile canalculus itself. Bar, 0.5 μm. × 24,000.

after injection of the conjugate, when the distribution of labeled structures was approximately equal in these regions. Vesicles were the predominant labeled structures seen in every region of the cell at all times after injection. They accounted for ~85% of all 125I-plgA-HRP-structures, with tubules and multivesicular bodies composing the rest. However, the sizes of labeled vesicles found in the sinusoidal region (Fig. 5, a and b) tended to be smaller than those near the bile canaliculus (Fig. 5d). Our quantitation of these qualitative observations is presented below.

CELLULAR DISTRIBUTION OF 125I-plgA-HRP-CONTAINING STRUCTURES: To obtain an indication of the pathway and kinetics of ligand transport, the cellular distribution of 125I-plgA-HRP structures was determined at various times after intravenous injection of conjugate. The results presented in Table I show a progressive redistribution of 125I-plgA-HRP structures from the sinusoidal area to intermediate and bile canicular regions of the hepatocyte cytoplasm between 2.5 and 10 min after administration of 125I-plgA-HRP. The percentage of structures in the sinusoidal region decreased from 94 to 47 in this period, approaching a steady state at ~40% of total structures. The conjugate appeared in structures in the intermediate region before the bile canicular region, which suggests that 125I-plgA-HRP was delivered
to the intermediate region immediately after the sinusoidal region (see 2.5 min in Table I). The percentage of structures in the intermediate region reached a maximum at 10–12.5 min (at 34 and 36%, respectively) and decreased steadily thereafter. The rate at which the percentage of total structures in the bile canicular region increased for the first 15 min (from 0 to 30%) was faster than the increase between 15 and 45 min (from 30 to 39%). As illustrated qualitatively in Fig. 5, most labeled structures were vesicles. When the diameters of 125I-plgA-HRP vesicles in the three cellular regions of livers fixed 7.5, 15, and 30 min after conjugate injection were measured, vesicles in the bile canicular region were found to be larger than those in the sinusoidal area of the hepatocyte. The results are presented in Fig. 6. The sizes of vesicles in the intermediate compartment fell between those in the other two regions (data not shown).

125I-plgA-HRP Structures in the Golgi-Lysosome Area: Previous work from this laboratory established that epidermal growth factor (6) and asialoglycoproteins fixed 7.5, 15, and 30 rain after conjugate injection were accumulated that plgA-HRP vesicles in the three cellular regions of livers measured, vesicles in the bile canicular region were found to be larger than those in the sinusoidal area of the hepatocyte. The results are presented in Fig. 6. The sizes of vesicles in the intermediate compartment fell between those in the other two regions (data not shown).

### Table I. Distribution of plgA-HRP-Containing Structures in Hepatocytes After In Vivo Injection

| Time after administration (min) | Conjugate preparation used | Intracellular region (% of total structures) |
|---------------------------------|----------------------------|---------------------------------------------|
|                                 |                           | Sinusoidal* | Intermediate | Bile canaliculal* |
| 2.5 (65)                        |                           | 94          | 6            | 0                |
| 5 (351)                         | 3                         | 86          | 10           | 4                |
| 7.5 (570)                       | 4                         | 57          | 24           | 19               |
| 10 (1,038)                      | 3, 4                      | 47          | 34           | 19               |
| 12.5 (553)                      | 4                         | 37          | 36           | 27               |
| 15 (1,338)                      | 2, 3, 4                   | 45          | 25           | 30               |
| 30 (1,286)                      | 3                         | 35          | 29           | 36               |
| 45 (125)                        | 3                         | 40          | 21           | 39               |

Livers were fixed in situ at various times after administration of 125I-plgA-HRP (0.5–1.0 mg plgA) and mannan (1.0 mg) and then processed for peroxidase cytochemistry as described in Materials and Methods. Each discrete structure was scored as one, regardless of size and shape. Since three separate preparations of conjugate have been used in the above experiments, the conjugate(s) used for each administration time is indicated. The standard deviation was <10% for the combined data at 5 and 10 min and in the intermediate region at 15 min. However, the combined data for the sinusoidal and bile canaliculal regions at 15 min had standard deviations of 10 and 16%, respectively. The data for each region are presented as a percentage of total structures scored at a given time.

Structures with 1.6 μm of the base of the sinusoidal plasma membrane microvilli. Structures within 2.2 μm of the base of the bile canaliculal membrane.

Figure 6. The distribution of vesicle sizes in the sinusoidal and bile canaliculal regions. The diameters of 125I-plgA-HRP-positive vesicles in the sinusoidal (A, C, and E) and bile canaliculal (B, D, and F) regions were measured on micrographs of cells from livers fixed at 7.5 (A and B), 15 (C and D), and 30 (E and F) min after injection of the conjugate. 50 vesicles were measured at each time point. Medians are indicated by the arrowheads.

### Table II. Classification of plgA-HRP-Containing Structures in Golgi-Lysosomal Regions

| Time after administration (min) | Structures in Golgi-Ly- | Multivesicular bodies* |
|---------------------------------|------------------------|------------------------|
|                                 | sorosome region (% of total structures) | Tubules* | Vesicles* |
| 2.5                             | 0                      | 1                      |
| 5                               | 7                      | 0                      | 62        | 38        |
| 7.5                             | 19                     | 4                      | 83        | 13        |
| 10                              | 29                     | 5                      | 80        | 15        |
| 12.5                            | 35                     | 4                      | 75        | 21        |
| 15                              | 31                     | 9                      | 79        | 12        |
| 30                              | 21                     | 8                      | 87        | 5         |
| 45                              | 26                     | 12                     | 78        | 10        |

* The experiment was the same as that described in Table I. Examples of vesicles, tubules, and multivesicular bodies are shown in the micrographs of Fig. 5.
* Tubular structures ≤700 nm long.
* Multivesicular bodies are vesicles ≥200 nm in diameter that contain distinguishable vesicles or other inclusions in their lumen.
* No structures in Golgi-lysosomal region of cell.

(41), two ligands destined for lysosomal degradation, accumulated in endosomal structures within the Golgi-lysosome regions of hepatocytes before their entry into bona fide lysosomes. Therefore, we identified and quantified the 125I-plgA-HRP-containing structures in such regions to determine if a similar accumulation was occurring before secretion of plgA (Table II). Ligand did enter Golgi-lysosome regions early and reached significant proportions there (~30% of total structures) before biochemical evidence of release into bile (>15 min). However, approximately half of these regions were very near bile canaliculal (~2.2 μm). More important, most labeled structures were neither stacks of Golgi complexes, lysosomes, nor endosomes typical of those in which lysosomal ligands accumulate. Rather, plgA-HRP was found predominantly in vesicles, as it was in all other regions of the cell. However, the combined percentage of tubules and multivesicular bodies in Golgi-lysosome areas was somewhat greater than that found over all cellular regions (~13–25 vs. 15%). Nonetheless, there was remarkably little change in the distribution of 125I-plgA-HRP among vesicles, tubules, and multivesicular bodies in Golgi-lysosomal areas for times after 5 min.

**Electron Microscopic Serial Section Analysis of 125I-plgA-HRP-Containing Structures**: Many sets of serial sections were examined to obtain a clearer picture of the size, shape, and interrelationships of the structures containing 125I-plgA-HRP. A representative series of nine sections is presented in Fig. 7. Structures identified as vesicles on single micrographs were confirmed to be simple spherical structures in serial sections and between 50 and 295 nm in diameter. Measurements of tubules that were sectioned along their long axis indicated that they could reach at least 700 nm in length. More important, our serial section analysis indicated that the number of tubules was underestimated by only ~5–10%2 in the analysis of single micrographs presented in the analysis.
FIGURE 7 Serial section micrographs of bile canalicular and sinusoidal regions in a liver 30 min after plgA-HRP administration. Livers were fixed by perfusion and processed for HRP cytochemistry. The predominant labeled structures around the bile canaliculus (BC) are discrete vesicles that span a maximum of three 70-nm sections. Several are marked by small arrowheads. Tubules (t) span more than three successive sections, and multivesicular bodies (MVB) span more than five. Near the sinusoidal front (SF), reaction product is in an anastomosing network of tubules and several small vesicles (+ in a–c). Go, Golgi complex. × 16,000.
Table II (due to tubules sectioned transverse to their long axis, or close to transverse, appearing as vesicles as in Fig. 7, c-f). Multivesicular bodies (MVB in Fig. 7, a-e) were large, somewhat spherical structures >200 nm in diameter. These structures were occasionally seen in continuity with tubules (see Fig. 5), and in some cases vesicles appeared to be in continuity with tubules (e.g., see Fig. 7, b and c).

**DISCUSSION**

The purpose of this study was to characterize the transcellular transport of pIgA across the rat hepatocyte. Toward this goal, we biochemically characterized the transport of our 125I-plgA (TEPC15) and electron microscopic tracer (125I-plgA-HRP). Once satisfied that these molecules were being transported as expected, we undertook electron microscopic studies to characterize the plgA transport pathway.

**Biochemical Characterization of 125I-plgA and plgA-HRP Transport**

Unlike asialoglycoproteins and asialoagalactoglycoproteins, where >80% of the injected dose is cleared by the liver in 10 min (12), 125I-plgA is cleared both less effectively and more slowly (40–55% in 180 min). The results indicate that mouse 125I-plgA may exist as a mixture of transport-active and -inactive molecules before injection into animals. Perhaps SC-binding sites on some of the plgA molecules are inactivated during the preparation of plgA or its radioidnation. Alternatively, the transport-inactive molecules may lack their J chain and be unable to bind to their receptor, as Brandtzæg and Prydz have suggested (2). However, values in the literature for rat and human 125I-plgA uptake by the rat agree nicely with ours and are from 30 to 67% of injected 125I-plgA transported to bile in intact rats (17, 22, 25, 30, 40), 27% in an isolated perfused liver (7), and 53% in cultured hepatocytes (18).

When we analyzed the specific electron microscopic tracer, 125I-plgA-HRP, we found that the transport kinetics of both radioactivity and peroxidase activity were similar (Fig. 3); however, relatively more peroxidase activity was transported to bile than was radioactivity. This difference may have been caused by the nature of the plgA to which HRP was conjugated. HRP was reacted with a mixture of plgA that contained one part 125I-plgA and 100 parts unlabeled plgA. Therefore, HRP was coupled mainly to unlabeled plgA. The greater transport of HRP activity could therefore be accounted for by better binding of the unlabeled plgA-HRP conjugate to SC than to the labeled plgA and labeled conjugate. Thus, the plgA-HRP conjugate is probably a better probe of plgA transport than is the radiiodnated ligand. The substantial transport of both plgA and peroxidase activity (Fig. 3), together with our finding that 125I-plgA-HRP was transported into bile as an intact conjugate, indicated that the localization of HRP-containing structures accurately reflected the behavior of plgA itself.

**Electron Microscopic Study of 125I-plgA-HRP Transport**

The distribution of 125I-plgA-HRP-containing structures in the hepatocyte during the continuous uptake of conjugate indicated that after internalization at the sinusoidal front, 125I-plgA-HRP rapidly appeared in both the intermediate and bile canalicular regions of cells (Table I). 125I-plgA-HRP-containing structures could be detected in the latter region as early as 5 min after administration of the conjugate, and their percentage continued to increase up to 45 min. Within the intermediate and bile canalicular regions, many of the labeled structures could be localized to areas in which Golgi complexes and lysosomes were also present. HRP conjugates of asialoorosomucoid (41) and epidermal growth factor (6), two polypeptides destined for lysosomal degradation, appear in Golgi-lysosome areas of hepatocytes 4–5 min after their administration, the same time at which 125I-plgA-HRP structures appeared in these areas (Table II). However, the types of structures that contain these conjugates were qualitatively different from those that contain plgA-HRP. Although none of the three ligands was present in either bona fide stacks of Golgi complexes or in lysosomes, asialoorosomucoid and epidermal growth factors were present in a more complicated array of tubulovesicular structures than those seen for 125I-plgA-HRP. plgA was found predominantly in vesicles. The different distributions of lysosome- vs. bile-destined ligands suggest either that these ligands enter the hepatocyte by internalization into different structures or that sorting of these ligands may begin soon after endocytosis and continue to near completion during transit of the molecules to Golgi-lysosomal areas. Alternatively, segregation may occur rapidly in the Golgi-lysosomal areas, with the resulting prelysosomal and presecretion compartments having relatively long residence times. Since HRP conjugates of asialoorosomucoid, epidermal growth factor, and plgA occur in similar structures in the sinusoidal region of the hepatocyte, we believe they enter cells in the same vesicles and tubules. This would require a sorting of ligands within the cell. The dual ligand experiments of Courtoy et al. and of Geuze et al. support this hypothesis (5, 9).

Several studies have suggested that plgA is present only in vesicles during its transport across the hepatocyte (4, 21, 37), and another study has implicated smooth endoplasmic reticulum in addition to vesicles in 125I-plgA transport (27). We saw no evidence for the involvement of smooth endoplasmic reticulum in serial sections of 125I-plgA-HRP-containing structures (Fig. 7). However, 85% of the total 125I-plgA-HRP-containing structures were vesicles. Nonetheless, we did find tubules and multivesicular bodies that contained conjugate (Fig. 5). Since vesicles appeared much more often than did tubules or multivesicular bodies in juxtaposition to the bile canalicular plasma membrane, vesicles are probably the structures responsible for secretion of 125I-plgA into the bile. Furthermore, they were generally larger than ligand-containing vesicles near the sinusoidal front, implying transfer of ligand during its transport, fusion of vesicular carriers, or both. Similar types of large vesicles around bile canaliculi were reported by Renston et al. to contain unconjugated HRP, a fluid-phase pinocytic marker (28). However, this tracer, which was administered at very high doses (10 mg/100 g body wt), was even more abundant in multivesicular bodies and secondary lysosomes of hepatocytes, which suggests that it non-selectively filled all those endocytic pathways that originate at the sinusoidal front (i.e., lysosomal, transcellular, and perhaps as yet unidentified routes). Our 125I-plgA-HRP conjugate exhibited much greater selectivity and at lower doses.

It is not currently clear why 125I-plgA-HRP was found in...
multivesicular bodies, structures commonly thought to be lysosomal precursors (6, 8, 41). At this time, we cannot exclude the possibility that multivesicular bodies have a role in plgA transport. We believe a more likely explanation is that some 125I-plgA-HRP molecules were directed to lysosomes because of simple missorting, or because of an alteration of injected conjugate that still allowed its specific uptake into hepatocytes but not its secretion into bile.

The 125I-plgA-HRP-containing tubules we have described may be analogous to the compartment of uncoupling of receptors and ligand described by Geuze et al., who used immunoelectron microscopy to detect endogenous plgA (9). The authors describe this compartment as an interconnected tubule network extending from the sinusoidal cell periphery to the trans-Golgi area, with the tubules participating most significantly in plgA sorting occurring at the sinusoidal-lateral cell corners of the hepatocyte. Our data showed the presence of 125I-plgA-HRP-containing tubules in both sinusoidal and bile canalicular regions of the cell with no overwhelming preference for one location over the other. In addition, our serial section analysis of 125I-plgA-HRP-containing structures strongly indicated no interconnections of tubules between the sinusoidal and bile canalicular regions (Fig. 7). Thus, tubules probably do play a role in the transport of plgA across the hepatocyte, since they are found throughout the hepatocyte, but they appear to be concentrated in the sinusoidal and bile canalicular regions. However, more work is required to resolve the apparent differences between our results and those of Geuze et al. (9), since different morphologic methods were used in the two studies.

125I-plgA-HRP did not appear in the bile until 20 min, even though significant numbers of ligand-containing structures had accumulated in the bile canalicular region by 10 min (Fig. 3 and Table I). This observation suggested that there was a lag between 125I-plgA vesicle accumulation in the bile canalicular region and fusion with the bile canalicular plasma membrane. The reason for such a lag is at present unclear but may involve the proteolytic cleavage of the membrane SC (M, 120,000 in reducing SDS PAGE) to free SC, a step that appears to be concentrated in the sinusoidal and bile canalicular regions. However, more work is required to resolve the apparent differences between our results and those of Geuze et al. (9), since different morphologic methods were used in the two studies.

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