Endogenous and Exogenous Domain Markers of the Rat Hepatocyte Plasma Membrane

JAMES R. BARTLES, LELITA T. BRAITERMAN, and ANN L. HUBBARD
Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT We have used a combined biochemical and morphological approach to establish the suitability of certain endogenous and exogenous domain markers for monitoring the separation of rat hepatocyte plasma membrane domains in sucrose density gradients. As endogenous domain markers, we employed two of the integral plasma membrane protein antigens, HA 4 and CE 9, localized to the bile canalicular and sinusoidal/lateral domains, respectively, of the hepatocyte plasma membrane in rat liver tissue (Hubbard, A. L., J. R. Bartles, and L. T. Braiterman, 1985, J. Cell Biol., 100:1115-1125). We used immunoelectron microscopy with a colloidal gold probe to demonstrate that HA 4 and CE 9 retained their domain-specific localizations on isolated hepatocyte plasma membrane sheets. When the plasma membrane sheets were vesiculated by sonication and the resulting vesicles were centrifuged to equilibrium in sucrose density gradients, quantitative immunoblotting revealed that the vesicles containing HA 4 and those containing CE 9 exhibited distinct density profiles. The density profile for the bile canalicular vesicles (marked by HA 4) was characterized by a single peak at a density of 1.10 g/cm³. The density profile for the sinusoidal/lateral vesicles (marked by CE 9) was bimodal, with a peak in the body of the gradient at a density of 1.14 g/cm³ and a smaller amount in the pellet (density ≥ 1.17 g/cm³). We used this sucrose gradient fractionation as a diagnostic procedure to assign domain localizations for several other hepatocyte plasma membrane antigens and enzyme activities. In addition, we used the technique to demonstrate that 125I-wheat germ agglutinin, introduced during isolated liver perfusion at 4°C, can serve as an exogenous domain marker for the sinusoidal domain of the rat hepatocyte plasma membrane.

Epithelial cells exhibit a polarized organization, characterized in part by the differentiation of the plasma membrane (PM) into morphologically and functionally distinguishable domains. An assessment of the extent to which these different plasma membrane domains exhibit compositional differences requires their isolation and characterization. However, most attempts at domain isolation have been hampered by a shortage of definitively localized endogenous molecules that could serve to mark the domains during the isolation (for a recent exception, see references 31 and 32). By virtue of our findings reported in the preceding paper by Hubbard et al. (20), we have acquired the ability to circumvent this problem in the rat hepatocyte system. In that study, we used monoclonal antibodies in a morphological approach and established that four integral PM proteins exhibited domain-specific localizations about the surface of hepatocytes in rat liver tissue. The protein HA 4 was localized to the bile canalicular (BC) or apical domain, and the proteins CE 9, HA 116, and HA 21 were localized to the sinusoidal (SF) and lateral (LS) domains, together comprising the basolateral surface of the hepatocyte (20).

In the present paper, we have confirmed our morphological results and demonstrated the suitability of HA 4 and CE 9 as endogenous domain markers for monitoring the separation of hepatocyte PM domains in sucrose density gradients. Furthermore, by direct comparison with HA 4 and CE 9, we have assigned domain localizations for other hepatocyte PM antigens and enzyme activities and have identified conditions under which the lectin wheat germ agglutinin (WGA) can...
serve as an exogenous domain marker for the SF domain of the rat hepatocyte PM.

MATERIALS AND METHODS

Materials

N-acetyl-D-glucosamine, D-mannose, digoxigenin, yeast mannann, and neuraminidase (Type X) were obtained from Sigma Chemical Co. (St. Louis, MO); tannic acid powder was obtained from Mallinckrodt Inc. (Paris, KY); protein A was from Pharmacia Fine Chemicals (Piscataway, NJ); Ricinus communis agglutinin 1 (RCA) was from Vector Laboratories, Inc. (Burlingame, CA); and mannosyl-bovine serum albumin (BSA) (Man9-Al-BSA, see references 21 and 22) was kindly provided by Dr. Y. C. Lee (Department of Biology, The Johns Hopkins University). The sources of other reagents are given in the preceding article (20) and in other recent publications from this laboratory (2, 18, 19, 30–32).

Preparation of Proteins and Antibodies

PROTEINS: WGA was purified by affinity chromatography on ovomucoid-Sepharose 4B as described previously (2). The asialoglycoprotein receptor was purified from rat liver by affinity chromatography on asialoorosomucoid-Sepharose 4B (15). gp A was purified by electrophoresis of the M, 110,000 region of preparative polyacrylamide SDS slab gels of a WGA-agarose eluate fraction that had been depleted of HA 4 (20).

ANTIBODIES: Anti-HA 4 and anti-CE 9 IgGs were prepared as previously described (20). Polyclonal antibodies directed against the asialoglycoprotein receptor, gp A, and mouse IgG were raised in rabbits using a conventional protocol of multiple-site intradermal immunizations in complete and incomplete (for boosters) Freund's adjuvants. The anti-asialoglycoprotein receptor antibody was purified by affinity chromatography on columns of receptor-Sepharose 4B, using isotonic glycine-HCl (pH 3) elution. The rabbit anti-mouse antibody was purified by affinity chromatography on mouse IgG-Sepharose 4B, using 3 M potassium thiocyanate elution.

Radioiodinations: Proteins were iodinated with Na125I using modifications of the chloramine-T procedure (13). When labeling antibodies, the reaction was stopped by the addition of tyrosine instead of Na2SO4. 125I-antibodies (4–6 x 104 cpm/mg) and 125I-mannosyl-BSA (5.1 x 105 cpm/mg) and 125I-protein A (2–4 x 109 cpm/mg) were repurified by gel filtration on Pharmacia P-10 columns in the presence of 1 mg/ml of BSA and stored frozen at –70°C. 125I-WGA and 125I-RCA (2–3 x 109 cpm/mg) were repurified by affinity chromatography on columns of ovomucoid-Sepharose 4B and Sepharose 6B, respectively, in the presence of 1 mg/ml BSA and stored frozen at –20°C (2).
ENZYME ASSAYS: The fractions were assayed for alkaline phosphodiesterase I activity and S'-nucleotidase activity as described previously (18). In the figures, the data were plotted as a percentage of the recovered enzyme activity, typically 85-90% of that applied to the gradient.

Immunogold Localization of HA 4 and CE 9 on Isolated Hepatocyte PM Sheets

The antigens HA 4 and CE 9 were localized on agarose-embedded hepatocyte PM sheets using a modification of the procedures described by Roman and Hubbard (31). 5-nm diameter colloidal gold was prepared by reduction with tannic acid and citrate as described by Mühlpfordt (26). Affinity-purified rabbit anti-mouse antibody was adsorbed to the colloidal gold as described (7, 8). Isolated hepatocyte PM sheets in 0.25 M sucrose were embedded at a protein concentration of ~5 mg/ml in 1.5% (wt/vol) low-temperature agarose without any prior fixation. Antibody incubations were conducted for 16 h at 4°C with 50 μg/ml monoclonal anti-HA 4 or anti-CE 9 IgG in a buffer containing 0.15 M NaCl, 20 mM NaPi, 3 mM NaN3, 0.5% (wt/vol) BSA and 0.01% (wt/vol) saponin (pH 7.4). Incubations with rabbit anti-mouse colloidal gold probe (DAB = 0.4) were conducted for 3.5 h at 4°C in a buffer containing 0.15 M NaCl, 20 mM Tris-HCl, 20 mM NaN3, 1% (wt/vol) BSA and 0.01% (wt/vol) saponin (pH 8.2). Nonspecific labeling was eliminated by adsorption of the colloidal gold probe against an excess of agarose blocks containing unfixed hepatocyte PM and rat liver microsomes in the absence of saponin. Saponin was included in these experiments as a membrane-permeabilizing agent. Roman and Hubbard (31) demonstrated that saponin was required to permit access of a 5-nm colloidal gold probe to the bile canalicular lumina of isolated hepatocyte PM sheets, yet it did not cause significant extraction of the integral PM protein leucine aminopeptidase.

Electron Microscopy

ELECTRON MICROSCOPE AUTORADIOGRAPHY (EM-ARG) OF LIVER TISSUE: Isolated rat livers were labeled to high specific activity (~5 x 10^6 cpm/g liver) with 500 μCi of 125I-WGA by perfusion at 4°C. After washing out the unbound 125I-WGA with fresh ice-cold perfusate, the livers were fixed with 125 ml of ice-cold 2% (wt/vol) glutaraldehyde in 0.1 M Na-cacodylate-HCl (pH 7.4) by isolated perfusion at 30 ml/min. Blocks cut from the three largest lobes were processed for EM-ARG as described (17). For quantification of grain distribution, we photographed five randomly selected nonoverlapping fields of a single section from each block at 2,000X. The grains were assigned locations by visual inspection of photographic prints (final magnification: 50,000X). The volume densities of hepatocytes, nonhepatocytes, and intercellular spaces on these micrographs were determined stereologically (4) using a square lattice (7.5 x 7.5 mm). We analyzed three blocks, two from a single liver and one from a different liver. The data are reported as the mean ± SD for these three blocks.

EM-ARG OF ISOLATED PM SHEETS: Hepatocyte PM sheets were prepared from isolated rat livers labeled to high specific activity (~2 x 10^6 cpm/g liver) with 500 μCi of 125I-WGA by perfusion at 4°C. The PM sheets were fixed and processed for EM-ARG as described (17, 18). For quantification of grain distribution, we photographed 10 nonoverlapping fields from the top third of the membrane pellet from one section of each block at 3,200X. The close packing of membranes precluded analysis of the lower two-thirds of the pellet samples. The grains were assigned a location by visual inspection of photographic prints (final magnification: 7,800X). We analyzed two blocks from the same labeled plasma membrane preparation. The data are reported as the mean ± range/2 for these two blocks.

ANALYSIS OF GRADIENT FRACTIONS: Gradient fractions were fixed and processed for electron microscopy as described by Hubbard et al. (18).

RESULTS

Localization of HA 4 and CE 9 on Isolated Hepatocyte PM Sheets

As a prelude to any actual fractionation, we wanted to determine whether HA 4 and CE 9 retained their domain-specific distributions during the isolation of hepatocyte PM from rat liver. Unfixed hepatocyte PM was embedded in agarose and incubated sequentially with anti-HA 4 or anti-CE 9 monoclonal antibody and rabbit anti-mouse–colloidal gold probe in the presence of 0.01% (wt/vol) saponin (31). With this procedure, the PM domains remain recognizable despite the poorer morphological preservation associated with the saponin-permeabilization procedure, which is required to permit access of the colloidal gold probe to the luminal aspect of the bile canaliculi (31). These membranes display a characteristic sheet-like morphology (18). The BC and LS domains retain the appearance they display in sections of intact liver tissue, and the SF domain can be recognized as membrane strands and associated vesicular profiles (SF microvilli cut in cross-section) that emanate from the LS domain at opposing ends of the sheets (see Fig. 6 and reference 18). With the anti-HA 4 monoclonal antibody, colloidal gold was found in high density on the BC domain (Fig. 1a), but not on the SF domain (Fig. 1b). Conversely, with the anti-CE 9 monoclonal antibody, colloidal gold was found in high density on the SF domain (Fig. 1d), but not on the BC domain (Fig. 1c). Labeling of the LS domain was not observed with either monoclonal antibody, even though in situ labeling indicated the presence of CE 9 on the LS domain (20). We believe that this difference is the result of limited access of the colloidal gold probe to the LS domain in the isolated PM sheets. We have observed specific labeling of the LS domain using anti-CE 9 antibody in those rare cases where there appears to have been a partial separation of the two bilayers (data not shown). Barring this difference, HA 4 and CE 9 appeared to retain their mutually exclusive domain localizations after the isolation of hepatocyte PM sheets.

Fractionation of Hepatocyte Plasma Membrane Vesicles in Sucrose Density Gradients: Behavior of HA 4 and CE 9

Isolated PM sheets were sonicated for only three or four 10-s bursts, corresponding to the time when the near complete disappearance of sheet-like structures was observed by phase-contrast microscopy. When examined by electron microscopy, this procedure was found to yield a heterogenous population of closed, spherical vesicles (50–500 nm in diameter) and a much smaller proportion of what appeared to be filament-bearing linear segments of LS membrane (data not shown).

When centrifuged to equilibrium in sucrose density gradients, the PM vesicles containing the BC marker HA 4 exhibited a density profile that was distinct from those containing the SF/LS marker CE 9 (Fig. 2a). The peak of CE 9 was observed at a density of 1.14 g/cm³, whereas the peak of HA 4 was displaced at least two fractions toward the top of the gradient at a density of 1.10 g/cm³. In addition, a much greater proportion of CE 9 than HA 4 was found in the pellet fraction, which contained membranes with densities ≥1.17 g/cm³. Thus, it would appear that we have achieved a partial separation of BC and SF/LS domains. Furthermore, the finding that HA 4 and CE 9 were on membrane vesicles of different densities served as biochemical confirmation of our morphological observations that suggested distinct localizations for these two proteins (Fig. 1 and reference 20). We observed this same general pattern of equilibrium density profiles for HA 4 and CE 9 in nine independent experiments. However, the ratio of CE 9 recovered in the pellet fraction to that recovered in the 1.14-g/cm³ peak in the body of the gradient varied over a nearly fourfold range in these nine experiments (compare CE 9 profiles in Fig. 7, a and b). We attribute this variability to differences in the efficiency of
FIGURE 1 Immunogold localization of HA 4 and CE 9 on saponin-permeabilized hepatocyte PM sheets. Unfixed hepatocyte PM sheets were embedded in agarose, incubated sequentially with monoclonal IgG and 5-nm rabbit anti-mouse colloidal gold probe in the presence of 0.01% (wt/vol) saponin, fixed and then processed for electron microscopy. The PM domains could be recognized by the following criteria: BC, collections of small vesicular profiles enclosed within an encircling membrane; LS, filament-bearing straight segments of parallel membranes emanating from both sides of the BC domain; SF, membrane strands and vesicular profiles projecting from the LS domain at opposing ends of the sheets (18). (a and b) With anti-HA 4 IgG, the colloidal gold is found in high density on the BC domain, but not the SF or LS domains. (c and d) With anti-CE 9 IgG, the colloidal gold is found in high density on the SF domain, but not on the BC or LS domains. These are unstained sections. Bar, 0.5 μm. × 25,300.
vesiculation of SF/LS membrane during sonication. The intact hepatocyte PM sheets exhibit a peak equilibrium density of 1.16-1.17 g/cm³ (data not shown).

Electron microscopy of the gradient fractions revealed no major qualitative differences in morphology between vesicles derived from BC (Fig. 3a) and SF/LS membranes (Fig. 3b). The pellet fraction was comprised primarily of filament-bearing linear segments of double membrane recognizable as LS domain, some partially vesiculated PM sheets and a smaller proportion of vesicular structures (Fig. 3, c and d).

**Figure 2** Centrifugation of hepatocyte PM vesicles in sucrose density gradients—comparison of HA 4, CE 9, and other PM antigens. Hepatocyte PM sheets were vesiculated by sonication, and the resulting vesicles were centrifuged to equilibrium in sucrose density gradients. The distributions of the antigens were determined by quantitative immunoblotting or by 125I-RCA blotting after immunoprecipitation (for HA 16); distributions are plotted as a percentage of the recovered antigen. (a) Comparison of HA 4 and CE 9. The density profiles for HA 4 (☐) and CE 9 (□) are distinct. The profile for HA 4 exhibits a single peak at a density of 1.10 g/cm³. The profile for CE 9 is bimodal, exhibiting a peak in the body of the gradient at a density of 1.14 g/cm³ and in the pellet fraction (P) at a density of ≥1.17 g/cm³. (b) Comparison of gp A and the asialoglycoprotein receptor (ASGP-R) from the same gradient as in a. The density profile for gp A (△) is coincident with that for HA 4 (☐ in a) and the density profile for the ASGP-R (▲) is coincident with that for CE 9 (□ in a). The insets show relevant portions of the autoradiograms that were used for quantification. (c) Comparison of HA 16, CE 9, and HA 4 in a large-scale density gradient experiment (see under Materials and Methods). The density profile for HA 16 (○) overlaps both those for CE 9 (□) and HA 4 (☐). □, Density.
Sucrose Gradient Fractionation using HA 4 and CE 9 as Endogenous Domain Markers: Comparison with Other PM Antigens

While the extent of separation of BC and SF/LS membranes in our sucrose gradients was admittedly incomplete, their density profiles were sufficiently different to permit us to predict domain localizations for other PM molecules. We applied this analysis to three additional integral proteins of the hepatocyte PM, the asialoglycoprotein receptor, a protein called gp A, and the antigen HA 16 (20).

We used quantitative immunoblotting to determine the distributions of membrane vesicles containing the asialoglycoprotein receptor and gp A in the same gradient that had been analyzed above for HA 4 and CE 9 in Fig. 2a. The results are shown in Fig. 2b. The density profile for the asialoglycoprotein receptor was identical with that of CE 9, suggesting a SF/LS localization for the receptor. In contrast, the density profile for gp A was coincident with that for HA 4, suggesting a BC localization for this protein.

Since our anti-HA 16 monoclonal antibody does not react with denatured HA 16 on blots (20), it was necessary to develop an alternate method to examine the distribution of HA 16 in the sucrose gradients. In work to be reported elsewhere, we have determined that HA 16 is a sialoglycoprotein with complex-type oligosaccharides that can be readily identified and quantified on nitrocellulose blots by 125I-RCA labeling after neuraminidase treatment. We used this technique to monitor the distribution of HA 16 after immunoprecipitation from the fractions of a scaled-up density gradient (Fig. 2c). The density profile for HA 16 was found to overlap both those for CE 9 and HA 4, suggesting a BC and SF/LS localization for this antigen.

125I-WGA as a Possible Exogenous Domain Marker: Clearance of 125I-WGA by the Isolated Perfused Rat Liver at 4°C

To make our method of domain separation more generally accessible, we wanted to find an exogenous molecule that could serve as a domain marker in fractionation experiments of this type. In this and the following sections, we present evidence that 125I-WGA can serve as an exogenous marker for the SF domain of the rat hepatocyte PM.

125I-WGA was rapidly cleared after being introduced into the circulation of the isolated perfused rat liver at 4°C (Fig. 4). 50% of the standard administered dose of 500 μg of 125I-WGA was cleared within 10 min, and 90% was cleared within 45 min. Maximum clearance represented the binding of 60–120 μg of lectin/g of liver (wet weight) or 3–6 × 109 WGA molecules bound/liver cell. We routinely perfused 125I-WGA through the isolated liver for a total of 60 min before preparing hepatocyte PM (see below).

The binding of 125I-WGA was apparently irreversible in the absence of hepatic sugar inhibitors. When the unbound lectin was removed by dilution of the perfusate (WASH 1 and WASH 2, arrows in Fig. 4), a negligible amount of the bound lectin (<1% of that initially cleared) dissociated and reappeared in the perfusate. However, >95% of the initially cleared lectin was released into the perfusate by the addition of N-acetyl-D-glucosamine to a final concentration of 0.1 M (0.1 M GlcNAc, arrow in Fig. 4). This suggested that the 125I-WGA was specifically bound to glycoconjugates on the surfaces of the liver cells.

Localization of the Bound 125I-WGA in Rat Liver Tissue

By EM-ARG, the 125I-WGA could be found at the surface of both hepatocytes and nonhepatocytes (predominantly Kupffer and endothelial cells) (Fig. 5 and Table I). Those grains found over cytoplasm or over the intercellular spaces, including sinusoidal and bile canaliculi, amounted to only 10% of the total number of grains and could be completely ascribed to background. At the hepatocyte surface, >98% of the grains were confined to the SF domain, with <2% on the BC and LS domains. The surfaces of the nonhepatocyte cell types were also effectively labeled with 125I-WGA. By incorporating the morphometric data of Weibel (42) and Blouin et al. (4) (see Table I), we can estimate that the nonhepatocyte PM was labeled 2.6 ± 0.7 times more heavily than the SF domain of the hepatocyte PM, on the basis of PM surface area. However, since there is three times more surface area of hepatocyte PM than of nonhepatocyte PM in the perfused-fixed liver parenchyma (4), the bound 125I-WGA was almost equally distributed between these two membranes.

Localization of 125I-WGA on Isolated Hepatocyte PM Sheets

EM-ARG was used to examine the distribution of 125I-WGA on hepatocyte PM sheets isolated from a rat liver labeled with 125I-WGA (Fig. 6 and Table II). The analysis

FIGURE 4 Clearance of 125I-WGA in the isolated perfused liver at 4°C. 125I-WGA (500 μg) was introduced into the circulation of an isolated perfused liver maintained at 4°C, and the disappearance of the lectin from the perfusate was monitored as a function of time. 90% of the lectin is cleared within 45 min. When unbound lectin is removed by dilution of the perfusate at 60 and 90 min (WASH 1 and WASH 2, arrows), a negligible amount of the bound lectin dissociates and reappears in the perfusate. However, at 120 min, when N-acetyl-d-glucosamine is added to a final concentration of 0.1 M GlcNAc (arrow), >95% of the bound lectin is released into the perfusate.
Figure 5  Localization of $^{125}$I-WGA in liver tissue by EM-ARG. An isolated rat liver was labeled to high specific activity ($\sim 5 \times 10^7$ cpm/g liver) with 500 ng of $^{125}$I-WGA by perfusion at 4°C, fixed, and then processed for EM-ARG. The autoradiographic grains, representing the bound $^{125}$I-WGA, appear to circumscribe the sinusoidal lumina (SL) and are largely excluded from areas over cytoplasm or intercellular space. A closer examination (see inset) reveals the grains to be localized over the surfaces of both hepatocytes and nonhepatocytes, including the Kupffer cells (not shown) and the highly attenuated endothelial cells (EN) at the periphery of the sinusoidal lumina. At the hepatocyte surface, the grains are localized almost exclusively over the sinusoidal surface (SF), whose microvilli can be seen to protrude into the perisinusoidal space of Disse (DS) between the hepatocytes and the overlying endothelial cells. For the purposes of quantifying grain distribution (see Table I), we could readily distinguish the hepatocytes from endothelial cells by virtue of a difference in the apparent electron density of their cytoplasms. This difference is often partially obscured by photoreproduction of the original micrographs. BC, bile canaliculi. Bar, 5 $\mu$m. \( \times 4,800. \) (Inset: bar, 2 $\mu$m. \( \times 9,000. \))
indicated that a minimum of 90% and a maximum of 95% of the membrane-associated autoradiographic grains were localized over the SF domain of these isolated hepatocyte PM sheets. A relatively small percentage of the grains (1.5%) was found over unidentifiable membrane. This value could be thought of as an upper estimate for the extent of contamination with $^{125}$I-WGA-labeled nonhepatocyte PM.

The $^{125}$I-WGA remained both membrane-associated and accessible during the isolation of hepatocyte PM, because (a) 90-95% of the lectin could be sedimented from the filtered homogenate and all other fractions by centrifugation at 100,000 g for 60 min, and (b) 80-90% of the lectin could be released from the fractions into the 100,000 g supernate by 0.1 M N-acetyl-D-glucosamine in perfusate. Table III shows the distribution of $^{125}$I-WGA and other markers during the PM isolation procedure. 16 ± 2% of the $^{125}$I-WGA in the filtered homogenate was obtained in the final PM fraction, with an enrichment of 29 ± 3-fold. The distribution of the hepatocyte PM marker enzyme alkaline phosphodiesterase I (Table III) and those of other organelle marker enzymes (data not shown) were similar to those observed previously without a prior $^{125}$I-WGA perfusion (18). This indicates that the lectin did not significantly alter the fractionation. The

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**TABLE I**

| Location     | Grains* | Relative grains/Surface area* |
|--------------|---------|-----------------------------|
| Hepatocytes  | 40 ± 3  | -                           |
| Surface      | 35 ± 3  | 0.76 ± 0.04                 |
| SF           | 34 ± 3  | 1.0 ± 0.06                  |
| LS           | 0.4 ± 0.6| 0.07 ± 0.09                 |
| BC           | 0.2 ± 0.2| 0.03 ± 0.03                 |
| Cytoplasm    | 5 ± 1   | -                           |
| Nonhepatocytes | 56 ± 3 | -                           |
| Surface      | 55 ± 3  | 2.6 ± 0.7                   |
| Cytoplasm    | 0.7 ± 0.4| -                          |
| Intercellular Space | 4.3 ± 0.7| -                          |

* Reported as the mean ± SD for three randomly selected blocks, two from the same liver and the third from a different liver. We localized 500 ± 40 grains/block, corresponding to 2,300 ± 700, 340 ± 30 and 1,400 ± 100 grid points/block overlying hepatocytes, nonhepatocytes and intercellular space, respectively, in the accompanying stereological analysis.

* The close packing of the membrane sheets in these pelleted specimens made some domain assignments ambiguous and, therefore, necessitated the creation of these overlap categories.

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**TABLE II**

| Location     | Grains* | % |
|--------------|---------|---|
| SF           | 84 ± 2  |   |
| LS           | 1.3 ± 0.5|   |
| BC           | 0.6 ± 0.1|   |
| SF or LS†    | 4 ± 1   |   |
| LS or BC     | 0.7 ± 0.1|   |
| BC or SF     | 1.4 ± 0.8|   |
| Not identified| 1.5 ± 0.7|   |
| Not over membrane | 6.3 ± 0.5|   |

† Hepatocyte PM sheets were isolated from a rat liver that had been labeled to high specific activity (~2 × 10⁷ cpm/g liver) with 500 μg of $^{125}$I-WGA by isolated perfusion at 4°C. The membranes were fixed, sedimented in an ultracentrifuge and the membrane pellets were analyzed by EM-ARG as described under Materials and Methods.

* Reported as the mean ± SD for two randomly selected blocks, both from the same membrane preparation. We localized 455 ± 57 grains/block corresponding to 71 ± 10 BC domain/block and 100 ± 10 SF "displays"/block. (One SF "display" refers to the collection of membrane strands and associated vesicular profiles emanating from one end of an intact hepatocyte PM sheet.)

* The close packing of the membrane sheets in these pelleted specimens made some domain assignments ambiguous and, therefore, necessitated the creation of these overlap categories.

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**FIGURE 6** Localization of $^{125}$I-WGA on isolated hepatocyte PM sheets by EM-ARG. An isolated rat liver was labeled to high specific activity (~2 × 10⁷ cpm/g liver) with 500 μg of $^{125}$I-WGA by perfusion at 4°C. Hepatocyte PM sheets were isolated, fixed, and sedimented in ultracentrifuge tubes, and the pellets were processed for EM-ARG. Autoradiographic grains, representing the bound $^{125}$I-WGA, are present over the membrane strands and vesicular profiles comprising the SF domain, but not over the BC or LS domains (see Table II). Bar, 1 μm. x 12,500.
TABLE III
Distribution of 125I-WGA and Other Markers During the Isolation of Hepatocyte PM

| Fraction                  | 125I-WGA (n = 5) | Alkaline Phosphodiesterase I (n = 3) | 125I-mannosyl-BSA receptor (n = 3) | SF Membrane (Calculated) |
|---------------------------|------------------|-------------------------------------|------------------------------------|--------------------------|
|                           | %                | %                                   | %                                  | %                        |
| Filtered homogenate       | 100              | 100                                 | 100                                | 100                      |
| 280 g, 5 min              |                  |                                     |                                    |                          |
| Supernate                 | 89 ± 2           | 84 ± 8                              | 80 ± 10                            | 97                       |
| Pellet                    | 8 ± 2            | 19 ± 4                              | 5 ± 1                              | 11                       |
| 1500 g, 10 min            |                  |                                     |                                    |                          |
| Supernate                 | 52 ± 7           | 34 ± 6                              | 70 ± 10                            | 37                       |
| Pellet                    | 33 ± 3           | 49 ± 1                              | 10 ± 2                             | 53                       |
| Floatation 1.18 g/cm³ layer | 20 ± 3         | 45 ± 5                              | 4 ± 1                              | 34                       |
| Pellet                    | 4 ± 3            | 0.8 ± 0.2                           | 2 ± 1                              | 6                        |
| 1500 g, 10 min            |                  |                                     |                                    |                          |
| Supernate                 | 4 ± 2            | 6 ± 1                               | 2 ± 1                              | 6                        |
| PM                        | 16 ± 2           | 35 ± 3                              | 3.1 ± 0.3                          | 27                       |
| Enrichment (-fold)        | 29 ± 3           | 68 ± 7                              | 5.8 ± 0.5                          | 51                       |

*Reported as the mean ± SD for indicated number of isolations (n).

For each fraction, this value was calculated from the equation (% recovery of SF membrane) = (1 + a/β) (% recovery of 125I-WGA) - (a/β) (% recovery of 125I-mannosyl-BSA receptor), where α = 2.6, the ratio of the relative grains/surface area values for nonhepatocyte PM and hepatocyte SF PM as determined in Table I, and β = 0.33, the ratio of the surface area contributions from these two membranes per g of liver parenchyma as determined from the combined morphometric data of Weibel (42) and Blouin et al. (4).

*Ratio of the specific activity (cpm or U/mg of membrane protein) in the final PM preparation to that in the filtered homogenate.

The difference between the distributions of 125I-WGA and alkaline phosphodiesterase I (Table III) reflected the contribution of 125I-WGA-labeled nonhepatocyte PM to the overall distribution of 125I-WGA. We have monitored the distribution of nonhepatocyte PM independently using 125I-mannosyl-BSA, a neoglycoprotein ligand for the mannose/N-acetylglucosamine receptor in the plasma membranes of the Kupffer and endothelial cells of rat liver (1, 34). When corrected for the contribution of the 125I-WGA-labeled nonhepatocyte PM (see § in Table III), the distribution of 125I-WGA-labeled SF membrane was quite similar to that observed for alkaline phosphodiesterase I during the isolation procedure.

Fractionation of the 125I-WGA-Labeled Hepatocyte PM Vesicles in Sucrose Density Gradients: Comparison of 125I-WGA, Endogenous Domain Markers, and Conventional Marker Enzymes

When the 125I-WGA-labeled hepatocyte PM sheets were vesiculated by sonication and centrifuged to equilibrium in sucrose density gradients, 125I-WGA, HA 4, and CE 9 displayed the density profiles shown in Fig. 7a. First, the BC domain marker HA 4 and the SF/LS domain marker CE 9 exhibited the same distinct density profiles as were observed in the absence of 125I-WGA (compare Fig. 7a with 2a). Second, the profile for 125I-WGA was coincident with that for the SF/LS domain marker CE 9 (Fig. 7a), suggesting that the 125I-WGA remained associated with the SF domain during both vesiculation and fractionation in the sucrose density gradients.

We have ruled out trivial explanations for the coincidence of the 125I-WGA and CE 9 distributions shown in Fig. 7a. For example, we demonstrated that BC vesicles could bind 125I-WGA simply by adding the 125I-WGA after vesiculation. In this case, a much larger proportion of the 125I-WGA was found to co-fractionate with the BC marker HA 4 (Fig. 7b) than when the 125I-WGA was added during isolated perfusion (Fig. 7a). This observation was consistent with the detection of a number of 125I-WGA-binding glycoproteins in these BC...
jugates on the external surface of the vesicles. Thus, it appears approach to establish the suitability of certain endogenous

duced into the isolated perfused rat liver at 4°C can serve as that, under the conditions employed here, ~25I-WGA intro-

duced by prolonging sonication or by prior alkaline extraction of PM markers relative to the filtered homogenate (Table III). An estimate that the vesicles in the SF/LS and BC peaks are enriched 90- to 150-fold in their corresponding PM markers. 4 We believe that the difference in density between BC and SF/LS domains can be explained by a difference in the number of vesicles. On isolated PM sheets, only the SF and LS domains display a relatively dense submembranous network of protein filaments (18, 19). In preliminary experiments, we have observed that vesicle densities can be progressively reduced by prolonging sonication or by prior alkaline extraction of filaments from the PM sheets (unpublished observations).

**DISCUSSION**

We demonstrated that the HA 4 and CE 9 display mutually exclusive domain localizations on unfixed rat hepatocyte PM sheets using a particulate colloidal gold tracer and immunoelectron microscopy (Fig. 1). HA 4 is localized to the BC domain, and CE 9 is localized to the SF domain, in accordance with the results of our immunofluorescence and immunoperoxidase experiments on frozen sections of rat liver tissue (20). Our results suggest that the two integral PM proteins HA 4 and CE 9 do not randomize or redistribute within the plane of the bilayer under our conditions of homogenization and subcellular fractionation. A similar result was recently obtained by Roman and Hubbard, who localized the integral membrane protein leucine aminopeptidase to the BC domain of the hepatocyte PM in rat liver tissue (30) and on isolated hepatocyte PM sheets (31). Thus, it appears that there is a mechanism for maintaining surface domains on the isolated hepatocyte PM. These PM sheets are known to retain a full complement of junctional elements and a submembranous cytoskeletal network (18, 19).

**Domain-specific Distributions of HA 4 and CE 9 on Isolated Hepatocyte PM Sheets**

When hepatocyte PM sheets are vesiculated by sonication and centrifuged to equilibrium in sucrose density gradients, BC vesicles (marked by HA 4) and SF/LS vesicles (marked by CE 9) were found to exhibit distinct density profiles (Fig. 2). This serves as biochemical confirmation of our morphological experiments that suggested that these antigens were localized to different domains (Fig. 1 and reference 20). We estimate that the vesicles in the SF/LS and BC peaks are enriched 90- to 150-fold in their corresponding PM markers. 4 By judicious selection of fractions from the leading edge of the BC peak and from the trailing edge of the SF/LS peak, one can obtain PM vesicles that are enriched five- to 10-fold in one domain over the other. Vesicles so derived, containing predominantly HA 4 or CE 9, appear qualitatively similar when examined by electron microscopy, whereas the pellet is comprised predominantly of LS membrane segments (Fig. 3). We believe that the difference in density between BC and SF/LS vesicles can be explained by a difference in the number of peripheral membrane proteins associated with the two types of vesicles. On isolated PM sheets, only the SF and LS domains display a relatively dense submembranous network of protein filaments (18, 19). An analysis of the distribution of protein in the sucrose gradients (data not shown) reveals an additional 1.8- to 2.2-fold enrichment for the markers.

**Figure 8 Centrifugation of 125I-WGA-labeled PM vesicles in su-

crose density gradients—comparison of 125I-WGA, HA 4, and con-

ventional marker enzymes.** 125I-WGA-labeled hepatocyte PM sheets were vesiculated by sonication, and the resulting vesicles were centrifuged to equilibrium in sucrose density gradients. The distributions of 125I-WGA, HA 4, alkaline phosphodiesterase I, and 5'-nucleotidase are plotted as a percentage of the recovered radio-

activity, antigen, or enzyme activity. (a) Alkaline phosphodiesterase I (APDE). The density profile for alkaline phosphodiesterase I activity (■) overlaps those for the BC marker HA 4 (○) and the SF marker 125I-WGA (△). (b) 5'-nucleotidase (5'-NUC). The density profile for 5'-nucleotidase activity (■) is nearly coincident with that for the BC marker HA 4 and distinct from that of the SF marker 125I-WGA (both replotted from panel a using dashed lines). ○, Density.
HA 4 and CE 9 as Endogenous Domain Markers for Monitoring BC and SF/LS Domains

While the extent of separation of BC and SF/LS domains in our sucrose gradients is only partial, the profiles are sufficiently distinct to be used as a diagnostic procedure to complement morphological techniques in assigning domain localizations for PM molecules. Furthermore, since all three domains are recovered almost equally during the isolation of hepatocyte PM sheets (18) and since all of the antigens and enzymes so far examined are recovered in high yield from the sucrose density gradients, the method would appear to permit quantification of an antigen's distribution between the BC and SF/LS domains. This approach was exemplified in our experiments with additional integral PM proteins, the asialoglycoprotein receptor, gp A, and HA 16 (Fig. 2). The domain localization of the asialoglycoprotein receptor has remained equivocal, despite its being the object of a relatively large number of morphological studies (11, 12, 18, 20, 23, 40, 41). The density profile for the asialoglycoprotein receptor is coincident with that of CE 9, suggesting a SF/LS localization. This result is consistent with multiple published observations from this laboratory: (a) those of Hubbard et al. (18), who localized asialoglycoprotein binding sites to the SF and LS domains using EM-ARG; (b) those of Roman and Hubbard (32), who found the receptor to be depleted from BC vesicles isolated by immunoadsorption using anti-leucine aminopeptidase antibody; and (c) those reported in our companion paper (20), where we localized the receptor to the SF and LS domains in sections of rat liver using anti-HA 116 monoclonal antibody and an immunoperoxidase technique. The density profile for gp A was coincident with that for HA 4, suggesting a BC localization. In this case, our gradient analysis proved to be particularly valuable, because the corresponding morphological experiments are presently precluded by the fact that this anti-gp A antibody reacts only with the reduced and SDS-denatured form of gp A on immunoblots. The density profile for HA 16 was seen to overlap both those for CE 9 and HA 4, confirming the SF/LS and BC localization predicted by our morphological experiments (20).

125I-WGA Can Serve as an Exogenous Domain Marker

We believe that the above examples demonstrate the utility of our sucrose gradient fractionation scheme, using endogenous domain markers, as a complement to a morphological approach in localizing components of the rat hepatocyte PM. Furthermore, we anticipate that this type of methodology will prove useful in the analysis of PM domains in other epithelial cell systems. Clearly, however, the success of such a procedure rests heavily upon prior knowledge of a domain-specific localization for at least some membrane components: in our case the endogenous domain markers, HA 4 and CE 9. We realize that the PM of few epithelial cells is as well characterized as that of the rat hepatocyte with respect to endogenous domain markers. Therefore, we wanted to find an exogenous molecule that could serve as a domain marker in fractionation experiments of this type. We have presented evidence in this paper that 125I-WGA can serve as an exogenous marker for the SF domain of the rat hepatocyte PM.

The location of 125I-WGA was monitored from its introduction in the perfused liver through the fractionation of hepatocyte PM vesicles in the sucrose density gradients. At all stages, the lectin remained associated with SF membrane, yet accessible to release by a hapten sugar. We can not presently rule out the possibility that some of the lectin might have redistributed from the SF domain to the LS domain either during or after sonication. However, we think that this would be unlikely, because there was no detectable redistribution to BC vesicles, and yet these vesicles contain a number of 125I-WGA-binding glycoproteins (unpublished data) and could bind the lectin when it is added after sonication (Fig. 7b). Taken together, these results indicate that, under controlled conditions, 125I-WGA can serve as an exogenous marker for the SF domain of the rat hepatocyte PM. As such, 125I-WGA represents an easily assayable and more generally accessible alternative to CE 9 or the asialoglycoprotein receptor for monitoring SF membrane vesicles in these sucrose density gradients.

Our success with 125I-WGA as an exogenous domain marker in the isolated perfused rat liver gives us a reason to speculate that the lectin could be of general utility as an exogenous marker for the exposed PM domains of other epithelial cells, whether in isolated organs or in tissue culture. For example, one could label the brush border of intestinal epithelia, or one could alternately tag both the apical and basolateral domains of the PM in epithelial cell lines like the Madin-Darby canine kidney line that can form a tightly sealed monolayer on porous substrata (6, 16, 29).

Comparison to Other Fractionations

Several investigators have reported the separation of BC and SF/LS domains by isopycnic centrifugation in sucrose gradients (e.g., see references 25, 37, 44). Most (but not all) investigators have found a population of so-called heavy vesicles (density 1.18-1.20 g/cm³) enriched in marker enzyme activities like Na⁺, K⁺-ATPase and glucagon-sensitive adenylate cyclase, which have been variously attributed to the SF and LS domains (3, 28), and they have found a population of so-called light vesicles (density: 1.12-1.13 g/cm³) enriched in other marker enzyme activities, such as 5'-nucleotidase, alkaline phosphodiesterase I, alkaline phosphatase and leucine aminopeptidase, which have been variously attributed to the BC domain (3, 27, 35, 38, 39, 43). There are two potential dangers in the use of these marker enzyme activities. First, there is little indication that many of the commonly followed enzymatic activities can actually be ascribed to a single enzyme or organelle. Second, most of the marker enzymes have been assigned domain localizations in sections of liver tissue using cytochemistry, with all the attendant uncertainties of differential inactivation during fixation, limited substrate access, and reaction product diffusion (for recent exceptions, see references 24, 31, and 33).

It is difficult to make a comparison of our sucrose gradient fractionation to others that have been reported for the rat hepatocyte PM, because none of the other investigators have assessed domain distributions using proven domain-specific antibody and lectin probes. Therefore, we assayed the distributions of two conventional hepatocyte PM marker enzymes in our gradients (Fig. 8). The density profile for 5'-nucleotidase is nearly coincident with that for HA 4, suggesting a predominantly BC localization for this activity. In this regard, 5'-nucleotidase activity would appear to be an easily assayable and more generally accessible alternative to HA 4 or gp A for monitoring BC vesicles in these sucrose density gradients.
These results appear to differ from those of Matsuura et al. (23), who recently found that immunoreactive 5'-nucleotidase is roughly equally distributed between BC and SF/LS domains using ferritin immunoelectron microscopy on intact hepatocytes isolated by homogenization of fixed rat livers. The density profile for alkaline phosphodiesterase I activity overlaps both those for HA 4 and II-WGA, suggesting that it is more equally distributed between BC and SF/LS domains. These results appear to differ from those of others, who have found alkaline phosphodiesterase I activity to be predominantly associated with the light, putative BC vesicles (25, 44).

Concluding Remarks

We have used monoclonal antibodies to identify and definitively localize several interesting proteins of the hepatocyte PM. We believe that our results, reported here and in the preceding article (20), illustrate the value of a combined morphological and biochemical approach to protein localization. Most of the proteins we have examined (CE 9, HA 4, HA 21, HA 116 [the asialoglycoprotein receptor], gp A, [recently identified as dipetidyl dipetidase IV], and 5'-nucleotidase activity) appear to be domain-specific. These proteins likely represent some molecular correlates of the long-prepared functional and morphological polarity of hepatocytes and of polarized epithelial cells in general. However, there are presently two exceptions to this domain-specific localization (HA 16 and alkaline phosphodiesterase I activity), which suggests that the extent of molecular restriction within the PM domains of polarized epithelial cells is not complete. Clearly, many more proteins must be localized before any generalizations can be made. Our sucrose gradient fractionation will provide a relatively simple way to tentatively localize these additional molecules.

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