Endogenous and Exogenous Domain Markers of the Rat Hepatocyte Plasma Membrane

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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/cm^3. 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/cm^3 and a smaller amount in the pellet (density $\geq$ 1.17 g/cm^3). 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 ^{125}I-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)[1] 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

[1] *Abbreviations used in this paper:* BC, bile canalicular; EM-ARG, electron microscope autoradiography; LS, lateral; PM, plasma membrane; RCA$_I$, *Ricinus communis* agglutinin I; SF, sinusoidal; WGA, wheat germ agglutinin.

THE JOURNAL OF CELL BIOLOGY · VOLUME 100 April 1985 1126-1138

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, digitonin, yeast mannan, 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 I (RCA_I) was from Vector Laboratories, Inc. (Burlingame, CA); and mannosyl-bovine serum albumin (BSA) (Man_{38}-AI-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 electroelution of the M_r 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 $Na^{125}I$ using modifications of the chloramine-T procedure (13). When labeling antibodies, the reaction was stopped by the addition of tyrosine instead of $Na_2S_2O_5$. ^{125}I-antibodies ($4–6 \times 10^9$ cpm/mg), ^{125}I-mannosyl-BSA ($5–11 \times 10^9$ cpm/mg) and ^{125}I-protein A ($2–4 \times 10^{10}$ cpm/mg) were repurified by gel filtration on Pharmacia PD-10 columns in the presence of 1 mg/ml of BSA and stored frozen at −70°C. ^{125}I-WGA and ^{125}I-RCA_I ($2–3 \times 10^9$ cpm/mg) were repurified by affinity chromatography on columns of ovumucoid-Sepharose 4B and Sepharose 6B, respectively, in the presence of 1 mg/ml BSA and stored frozen at −20°C (2)[2].

Isolation of Hepatocyte PM Sheets

The method of Hubbard et al. (18) was used to isolate hepatocyte PM sheets from rat livers after single-pass perfusion in situ with ice-cold saline or after labeling with ^{125}I-WGA or ^{125}I-mannosyl-BSA by perfusion (see below). Phenylmethylsulfonyl fluoride was added to a final concentration of 0.5 mM just prior to homogenization. Unless quantifying the receptor for ^{125}I-mannosyl-BSA (see below), the various fractions were resuspended in 0.25 M sucrose and stored at −70°C. Alkaline phosphodiesterase I and other marker enzymes were assayed as previously described (18). Membrane protein was measured colorimetrically by the method of Bradford (5), using BSA as a standard.

Isolated Rat Liver Perfusion

LABELING WITH ^{125}I-WGA: Isolated rat livers were labeled with 500 μg of ^{125}I-WGA at 4°C by recirculating perfusion according to the procedure of Dunn et al. (10).

QUANTIFICATION OF THE RECEPTOR FOR ^{125}I-MANNOSYL-BSA: Attempts to determine the numbers of receptors for ^{125}I-mannosyl-BSA in rat liver subcellular fractions by a direct binding assay were unsatisfactory due to high levels of nonspecific binding. Therefore we prelabeled the isolated perfused liver with ^{125}I-mannosyl-BSA at 4°C and measured the amount that could be released from the various subcellular fractions by incubation with a combination of two known inhibitors of specific binding, mannose and EDTA (1, 36).

Isolated livers were perfused with 40 μg of ^{125}I-mannosyl-BSA for 60 min at 4°C. The clearance (15–30% of that administered) was judged specific based on >95% inhibition by 20 mg of yeast mannan and >95% release after addition of EDTA to 10 mM (36). The unbound ligand was washed out with ice-cold perfusate, 500 μg of unlabeled WGA was added, and the perfusion was continued for an additional 60 min at 4°C. Hepatocyte PMs were prepared from these livers as described above. Immediately after obtaining the final PM, aliquots of each of the fractions were diluted fivefold into ice-cold dissociation medium (perfusate containing 3 mM NaN_3 and 20 mM HEPES-NaOH (pH 7.4), and 0.94 mg/ml digitonin as a membrane-permeabilizing agent [9]) either in the presence or absence of binding inhibitors (0.25 M D-mannose and 12.5 mM EDTA). After 1 h of agitation, the samples were centrifuged at 150,000 *g* (Beckman 50 Ti rotor) (Beckman Instruments, Inc., Palo Alto, CA) for 60 min at 4°C. The difference in the amount of sedimentable radioactivity in the absence and presence of binding inhibitors, which represented the amount of specifically releasable ^{125}I-mannosyl-BSA, was taken to be directly proportional to the number of specific receptors for ^{125}I-mannosyl-BSA. The stepwise recovery of specifically releasable ^{125}I-mannosyl-BSA averaged 94 ± 5% over three separate isolations of hepatocyte PMs, suggesting that the extent of spontaneous release is negligible during the fractionation.

Fractionation of Hepatocyte PM Vesicles in Sucrose Density Gradients

VESICULATION AND CENTRIFUGATION: The isolated hepatocyte PM sheets were thawed and diluted to a protein concentration of 0.75 mg/ml in 0.25 M sucrose. 2-ml aliquots of membrane suspension in capped polystyrene 14-ml conical tubes were vesiculated at 0°C in a bath-type sonicator (Laboratory Supplies Co., Hicksville, NY) using 10-s bursts, interrupted by 1-min periods with no sonication. 1.3 ml of the vesiculated PM was loaded onto 10.7-ml linear sucrose gradients (1.06–1.17 g/cm^3) buffered with 10 mM Tris-HCl (pH 7.5). After centrifugation for 16–20 h at 50,000 *g* (Beckman SW 41 Ti rotor) (Beckman Instruments, Inc.), 1-ml fractions were collected from the top automatically (Auto Densi-Flow II, Buchler Instruments Inc., Fort Lee, NJ). The pellet fraction was resuspended to 1.5 ml in 1.17 g/cm^3 gradient solution. Fraction densities were determined with a refractometer, and the fractions were stored at −20°C until analyzed further. To examine the distribution of HA 16 (see below), it was necessary to scale up the centrifugation procedure 15-fold. After sonication in 2-ml aliquots, 3.9 ml of the vesiculated PM was loaded onto each of five 32.1-ml gradients (Beckman SW 28); like-numbered fractions (3 ml) from the five gradients were pooled.

QUANTITATIVE IMMUNOBLOTTING: Aliquots of gradient fractions were concentrated by precipitation, separated in 7.5% polyacrylamide SDS slab gels and transferred to nitrocellulose as described (20). Immunolabeling and autoradiography of blots were conducted as described previously (20, 30, 32). HA 4 was detected by incubation with ^{125}I-anti-HA 4 IgG, and CE 9 was detected (only under nonreducing conditions, reference 20) by incubation with anti-CE 9 ascites, followed by ^{125}I-goat anti-mouse F(ab')$_2$. The asialoglycoprotein receptor was detected by incubation with ^{125}I-anti-receptor antibody, and gp A was detected by incubation with anti-gp A antiserum followed by ^{125}I-protein A.

Labeling was quantified by scanning autoradiograms with a densitometer (Hoefer GS 300) (Hoefer Scientific Instruments, San Francisco, CA) interfaced with an integrator (Hewlett-Packard 3390A) (Hewlett-Packard Co., Palo Alto, CA). Antigen distributions were determined directly from relative peak area measurements after correction for any differences in lane width. In preliminary experiments, we observed that the response was linear for each of the antigens in the concentration range of interest. In addition, for the purpose of calculating recoveries, we included on each gel/blot three internal standard samples containing different amounts of the sonicated PM that was loaded onto the gradient. For all antigens, recoveries ranged from 75% to 125%. In the figures, the data are plotted as a percentage of the recovered antigen. The distribution of ^{125}I-WGA determined by densitometry of autoradiograms was identical to that determined by directly counting the gradient fractions.

QUANTIFICATION OF HA 16: The fraction pools obtained from a large-scale centrifugation (see above) were diluted in water and concentrated by centrifugation at 100,000 *g* (Beckman 45 Ti rotor) (Beckman Instruments, Inc.) for 90 min at 4°C. The pellets were extracted for 30 min on ice with 3 ml of Buffer A (20 mM octyl glucoside, 0.5% [vol/vol] Triton X-100, 0.3 M NaCl, 0.025 M NaP_i [pH 7.4]) (20) containing protease inhibitors (100 U/ml Trasylol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml antipain, and 1 μg/ml leupeptin). HA 16 was quantitatively immunoprecipitated from the resulting extracts (150,000 *g*, 90 min supernates) using anti-HA 16-Sepharose 4B in Buffer A containing 0.05% (wt/vol) BSA as described (20). The immunoprecipitates were directly solubilized in SDS-containing buffer under reducing conditions, electrophoresed on 7.5% polyacrylamide SDS gels, and electrophoretically transferred to nitrocellulose. HA 16 contains complex-type oligosaccharides, and it was visualized by ^{125}I-RCA_I labeling after neuraminidase treatment of the blots using a procedure that will be described in detail elsewhere.[2] Labeling was quantified by densitometric scanning of the autoradiograms.

[2] Bartles, J. R., L. T. Braiterman, and A. L. Hubbard, manuscript in preparation.

ENZYME ASSAYS: The fractions were assayed for alkaline phosphodiesterase I activity and 5'-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-gelling-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 NaP_i, 3 mM NaN_3, 0.5% (wt/vol) BSA and 0.01% (wt/vol) saponin (pH 7.4). Incubations with rabbit anti-mouse colloidal gold probe (A_{520} = 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 NaN_3, 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 × 10^7 cpm/g liver) with 500 μg of ^{125}I-WGA by perfusion at 4°C. After washing out the unbound ^{125}I-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 2,000×. The grains were assigned locations by visual inspection of photographic prints (final magnification: 5,000×). The volume densities of hepatocytes, nonhepatocytes, and intercellular spaces on these micrographs were determined stereologically (4) using a square lattice (7.5 × 7.5 mm). We analyzed three blocks, two from a single liver and a third block 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 × 10^7 cpm/g liver) with 500 μg of ^{125}I-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 3,200×. The close packing of membranes precluded analysis of the lower two-thirds of the pelleted samples. The grains were assigned a location by visual inspection of photographic prints (final magnification: 7,800×). We analyzed two blocks from the same labeled plasma membrane preparation. The data are reported as the mean ± range/2 for the 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. 1 *a*), but not on the SF domain (Fig. 1 *b*). Conversely, with the anti-CE 9 monoclonal antibody, colloidal gold was found in high density on the SF domain (Fig. 1 *d*), but not on the BC domain (Fig. 1 *c*). 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. 2 *a*). The peak of CE 9 was observed at a density of 1.14 g/cm^3, 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/cm^3. 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/cm^3. 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/cm^3 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

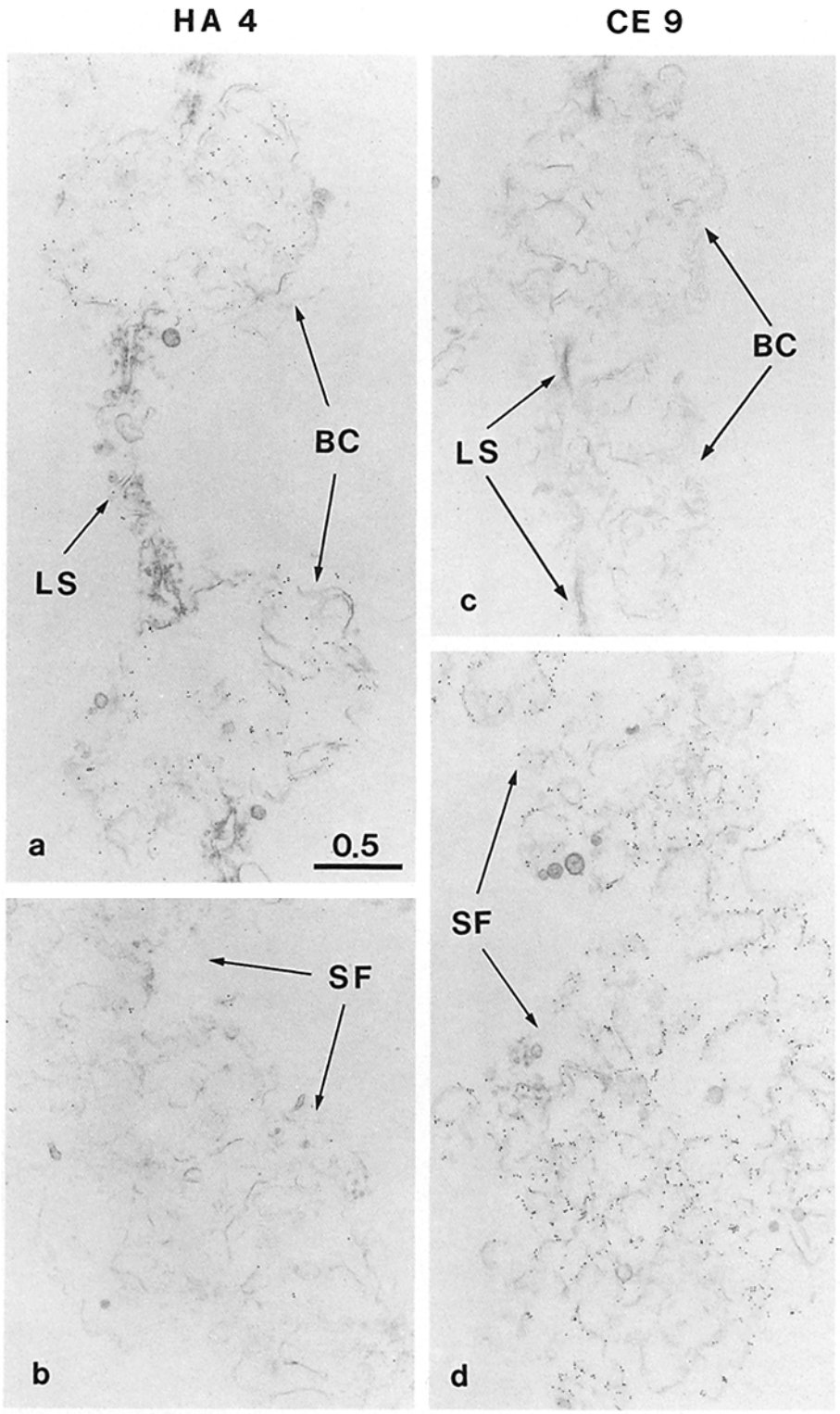
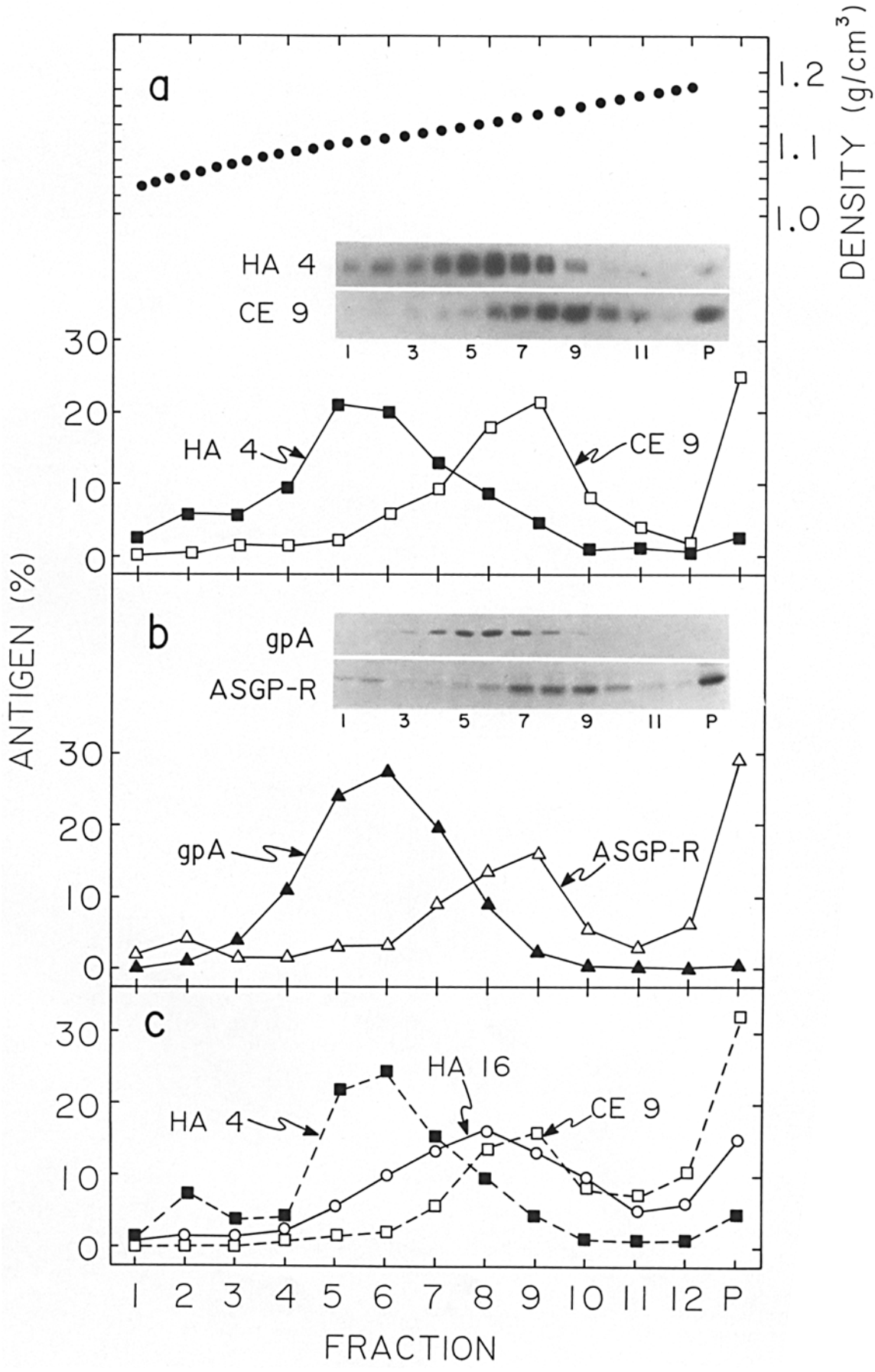


FIGURE 1 Immunogold localization of HA 4 and CE 9 on saponin-permeabilized hepatocyte PM sheets. Unfixed hepatocyte PM sheets were embedded in agarose, incubated sequentially with monoclonal IgG and 5-nm rabbit anti-mouse colloidal gold probe in the presence of 0.01% (wt/vol) saponin, fixed and then 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.


a
b
c
DENSITY (g/cm³)
1.2
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HA 4
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1 3 5 7 9 11 P
gpA
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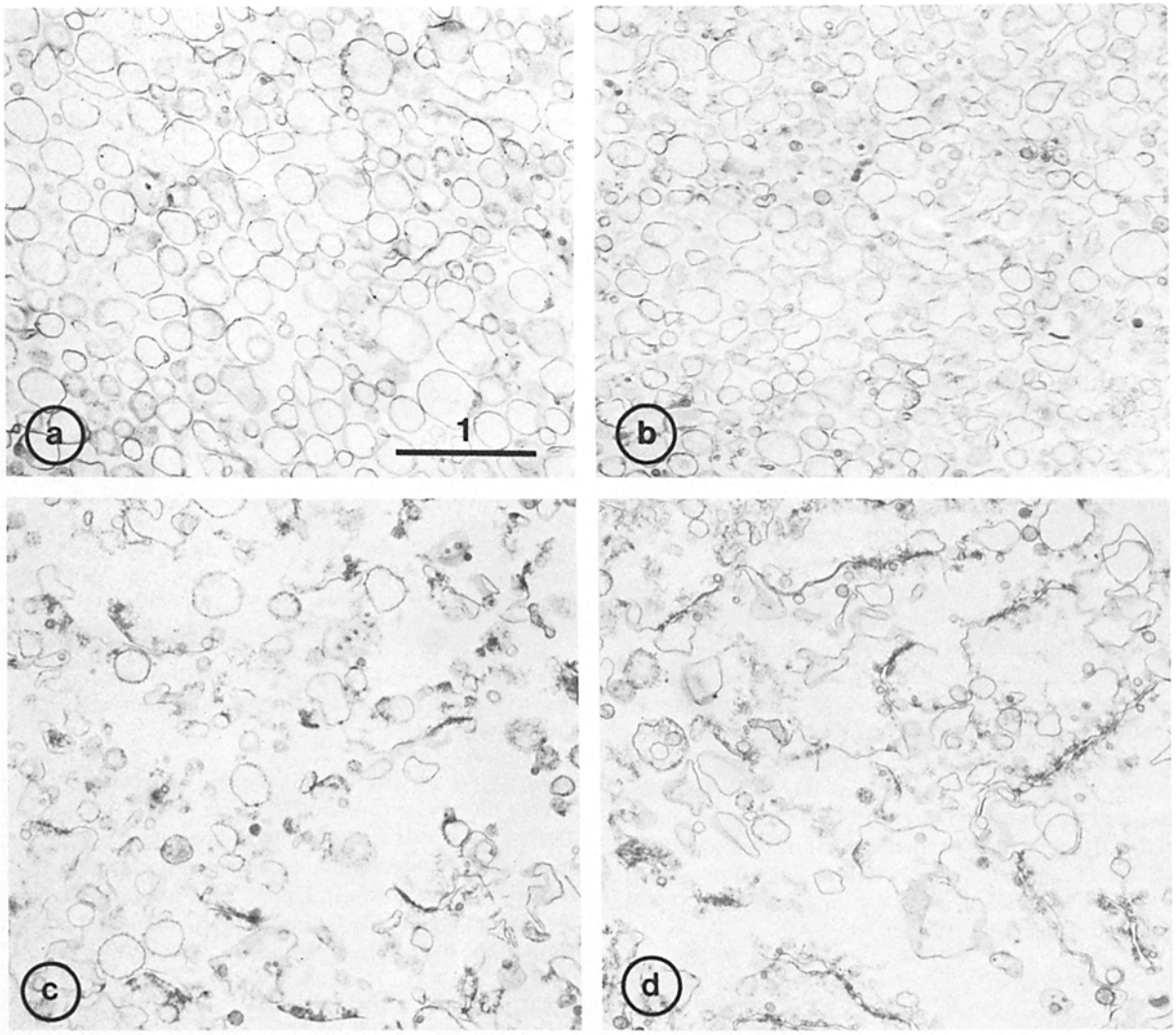

FIGURE 3 Electron microscopy of selected gradient fractions. Fractions from the sucrose density gradient analyzed in Fig. 2, *a* and *b*, were fixed and sedimented in ultracentrifuge tubes, and the pellets were processed for electron microscopy. (*a*) Pool of gradient fractions 4–6, enriched in the BC marker HA 4. (*b*) Pool of gradient fractions 8–10, enriched in the SF/LS marker CE 9. Closed membrane vesicles comprise the predominant structures seen in both of these regions of the gradient. Different levels within each pelleted specimen (not shown) were very similar in appearance to the micrographs shown, which represent the middle level. (*c* and *d*) Pellet fraction enriched in the SF/LS marker CE 9. The top of the pelleted specimen (*c*) contains membrane vesicles and short segments of filament-bearing double membrane recognizable as LS domain. The remainder of the specimen (*d*) is comprised of LS domain and partially vesiculated hepatocyte PM sheets. Bar, 1 μm. × 19,000.

vesiculation of SF/LS membrane during sonication. The intact hepatocyte PM sheets exhibit a peak equilibrium density of 1.16–1.17 g/cm^3 (data not shown).

Electron microscopy of the gradient fractions revealed no major qualitative differences in morphology between vesicles derived from BC (Fig. 3*a*) and SF/LS membranes (Fig. 3*b*). 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 ^{125}I-RCA$_I$ 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/cm^3. The profile for CE 9 is bimodal, exhibiting a peak in the body of the gradient at a density of 1.14 g/cm^3 and in the pellet fraction (P) at a density of ≥1.17 g/cm^3. (*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. 2*a*. The results are shown in Fig. 2*b*. The density profile for the asialoglycoprotein receptor was coincident 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,[2] we have determined that HA 16 is a sialoglycoprotein with complex-type oligosaccharides that can be readily identified and quantified on nitrocellulose blots by ^{125}I-RCA$_I$ 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. 2*c*). 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.

^{125}I-WGA as a Possible Exogenous Domain Marker: Clearance of ^{125}I-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 ^{125}I-WGA can serve as an exogenous marker for the SF domain of the rat hepatocyte PM.

^{125}I-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 ^{125}I-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 × 10^6 WGA molecules bound/liver cell.[3] We routinely perfused ^{125}I-WGA through the isolated liver for a total of 60 min before preparing hepatocyte PM (see below).

The binding of ^{125}I-WGA was apparently irreversible in the absence of hapten 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 ^{125}I-WGA was specifically bound to glycoconjugates on the surfaces of the liver cells.

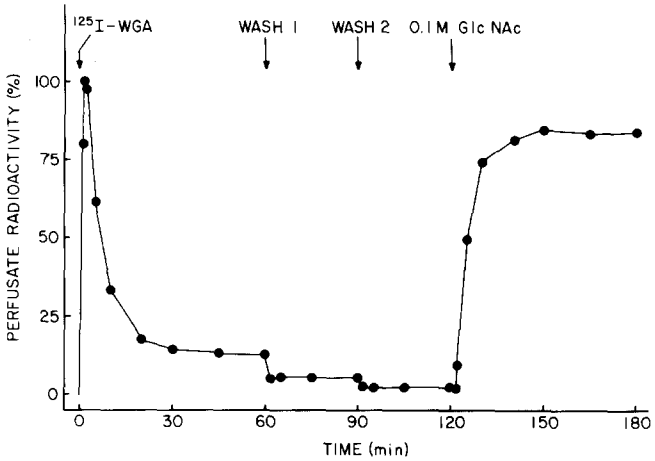

FIGURE 4 Clearance of ^{125}I-WGA in the isolated perfused liver at 4°C. ^{125}I-WGA (500 μg) was introduced into the circulation of an isolated perfused liver maintained at 4°C, and the disappearance of the lectin from the perfusate was monitored as a function of time. 90% of the lectin is cleared within 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.

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

By EM-ARG, the ^{125}I-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 canalicular lumina and the perisinusoidal space of Disse, 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 ^{125}I-WGA. By incorporating the morphometric data of Weibel (42) and Blouin et al. (4) (see ‡ in 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 SF PM than of nonhepatocyte PM in the perfused-fixed liver parenchyma (4), the bound ^{125}I-WGA was almost equally distributed between these two membranes.

Localization of ^{125}I-WGA on Isolated Hepatocyte PM Sheets

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

[3] This calculation is based on the study of Blouin et al. (4), in which it was reported that there are 1.7 × 10^8 idealized mononuclear hepatocytes and 9 × 10^7 nonhepatocytes per gram of rat liver tissue. However, since as many as 50% of the hepatocytes can be binucleate in this age of rat (14), this calculated value likely represents an underestimate.

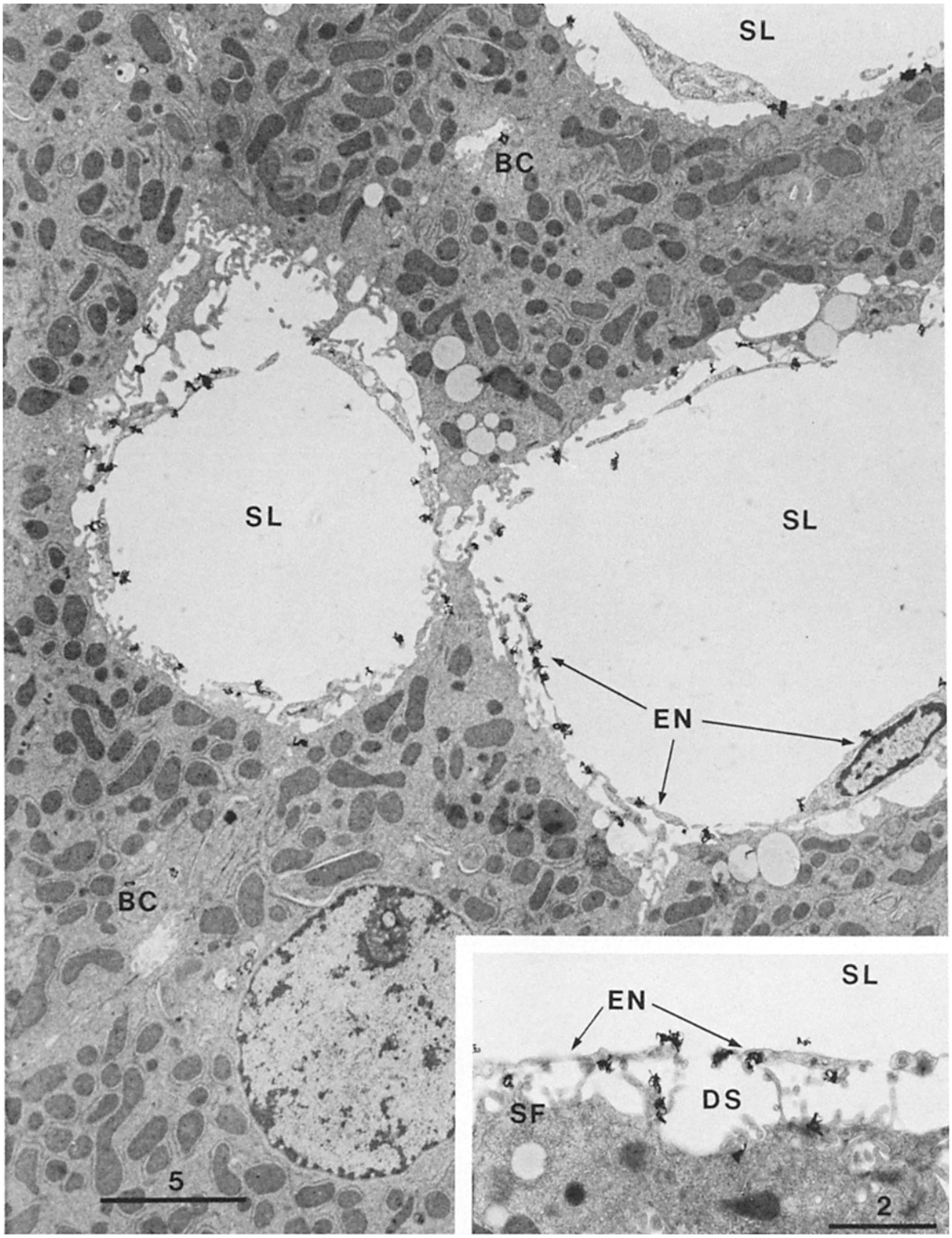

FIGURE 5 Localization of ^{125}I-WGA in liver tissue by EM-ARG. An isolated rat liver was labeled to high specific activity (~5 × 10^7 cpm/g liver) with 500 μg of ^{125}I-WGA by perfusion at 4°C, fixed, and then processed for EM-ARG. The autoradiographic grains, representing the bound ^{125}I-WGA, appear 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 μm. × 4,800. (*Inset*: bar, 2 μm. × 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 organellar 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

TABLE I

Localization of ^{125}I-WGA in Liver Tissue by EM-ARG

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	—

Isolated perfused rat livers were labeled to high specific activity (~5 × 10^7 cpm/g liver) with 500 μg of ^{125}I-WGA at 4°C, fixed, and analyzed by EM-ARG as described under Materials and Methods.

* 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.

‡ Reported as the mean ± SD for the three blocks. For each block, this value was calculated by dividing the number of grains/grid point overlying the relevant cell type by the surface area-to-cell volume ratio for the relevant membrane or membrane domain. (This latter quantity was calculated from the combined morphometric data of Weibel [42] for the hepatocyte and Blouin et al. [4] for the nonhepatocyte. In units of $\mu m^2/\mu m^3$, its value is 0.77, 0.15, and 0.13 for the SF, LS and BC domains of the hepatocyte. The weighted average of its value over the nonhepatocyte cell types is 3.2 $\mu m^2/\mu m^3$.) These values were normalized to the same number of grains/grid point overlyling cells, averaged and the means were normalized to a value of 1.0 for the SF domain of the hepatocyte.

TABLE II

Localization of ^{125}I-WGA on Isolated Hepatocyte PM Sheets by EM-ARG

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 × 10^7 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 ± range/2 for two randomly selected blocks, both from the same membrane preparation. We localized 455 ± 57 grains/block corresponding to 71 ± 10 BC domains/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.

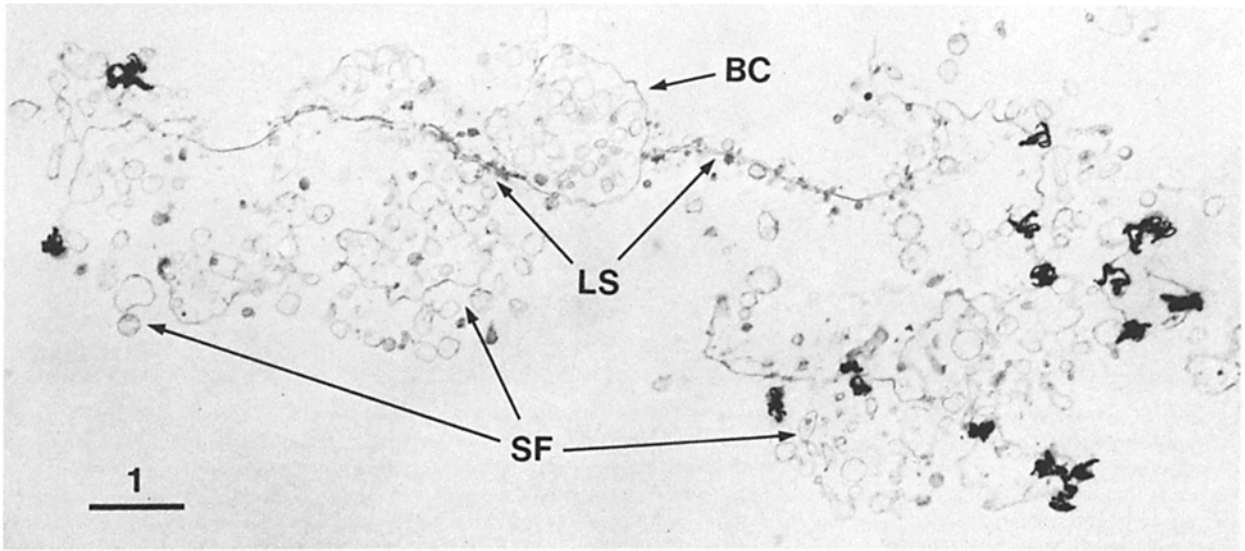

FIGURE 6 Localization of ^{125}I-WGA on isolated hepatocyte PM sheets by EM-ARG. An isolated rat liver was labeled to high specific activity (~2 × 10^7 cpm/g liver) with 500 μg of ^{125}I-WGA by perfusion at 4°C. Hepatocyte PM sheets were isolated, fixed, and sedimented in ultracentrifuge tubes, and 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. × 12,500.

TABLE III

*Distribution of ^{125}I-WGA and Other Markers During the Isolation of Hepatocyte PM**

Fraction		^{125}I-WGA (n = 5)	Alkaline Phosphodiesterase I (n = 3)	^{125}I-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
Flotation	Interface	20 ± 3	45 ± 5	4 ± 1	34
	1.18 g/cm^3 layer	6 ± 1	6 ± 1	2 ± 1	9
	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 + $\alpha\beta$) (% recovery of ^{125}I-WGA-) − ($\alpha\beta$) (% recovery of ^{125}I-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.

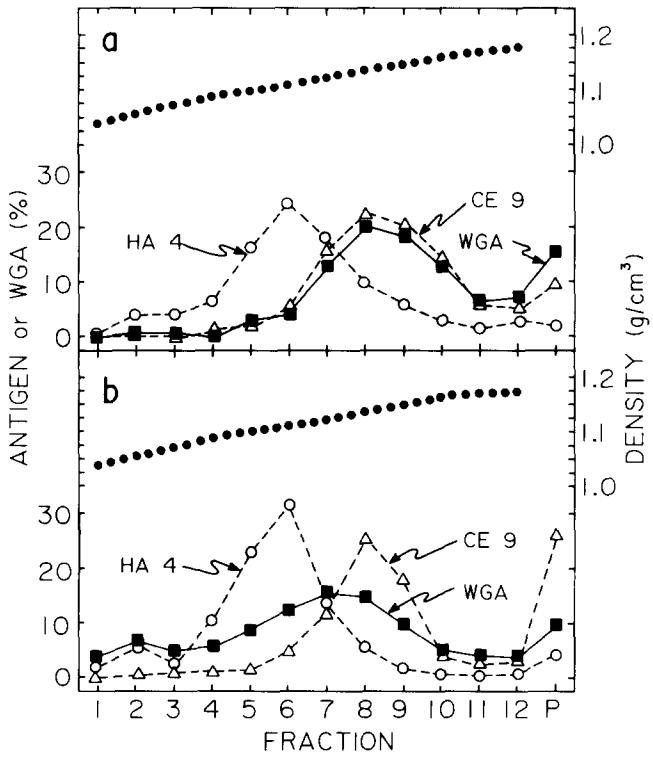

FIGURE 7 Centrifugation of ^{125}I-WGA-labeled PM vesicles in sucrose density gradients—comparison of ^{125}I-WGA, HA 4, and CE 9. Hepatocyte PM sheets were vesiculated by sonication, and the resulting vesicles were centrifuged to equilibrium in sucrose density gradients. The PM vesicles were labeled with ^{125}I-WGA (~6 μg of lectin/mg of PM protein) at 4°C, either as usual during isolated liver perfusion (*a*) or during a 30-min period immediately following sonication (*b*). The distributions of ^{125}I-WGA, HA 4, and CE 9 are plotted as a percentage of the recovered radioactivity or antigen. (*a*) Labeling with ^{125}I-WGA by isolated liver perfusion. The density profile for ^{125}I-WGA (■) is coincident with that for the SF/LS marker CE 9 (△) and distinct from that of the BC marker HA 4 (○). (*b*) Labeling with ^{125}I-WGA after sonication. The density profile for ^{125}I-WGA (■) overlaps those of the BC marker HA 4 (○) and the SF/LS marker CE 9 (△). ●, Density.

difference between the distributions of ^{125}I-WGA and alkaline phosphodiesterase I (Table III) reflected the contribution of ^{125}I-WGA-labeled nonhepatocyte PM to the overall distribution of ^{125}I-WGA. We have monitored the distribution of nonhepatocyte PM independently using ^{125}I-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 ^{125}I-WGA-labeled nonhepatocyte PM (see ‡ in Table III), the distribution of ^{125}I-WGA-labeled SF membrane was quite similar to that observed for alkaline phosphodiesterase I during the isolation procedure.

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

When the ^{125}I-WGA-labeled hepatocyte PM sheets were vesiculated by sonication and centrifuged to equilibrium in sucrose density gradients, ^{125}I-WGA, HA 4, and CE 9 displayed the density profiles shown in Fig. 7*a*. 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 ^{125}I-WGA (compare Fig. 7*a* with 2*a*). Second, the profile for ^{125}I-WGA was coincident with that for the SF/LS domain marker CE 9 (Fig. 7*a*), suggesting that the ^{125}I-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 ^{125}I-WGA and CE 9 distributions shown in Fig. 7*a*. For example, we demonstrated that BC vesicles could bind ^{125}I-WGA simply by adding the ^{125}I-WGA after vesiculation. In this case, a much larger proportion of the ^{125}I-WGA was found to co-fractionate with the BC marker HA 4 (Fig. 7*b*) than when the ^{125}I-WGA was added during isolated perfusion (Fig. 7*a*). This observation was consistent with the detection of a number of ^{125}I-WGA-binding glycoproteins in these BC

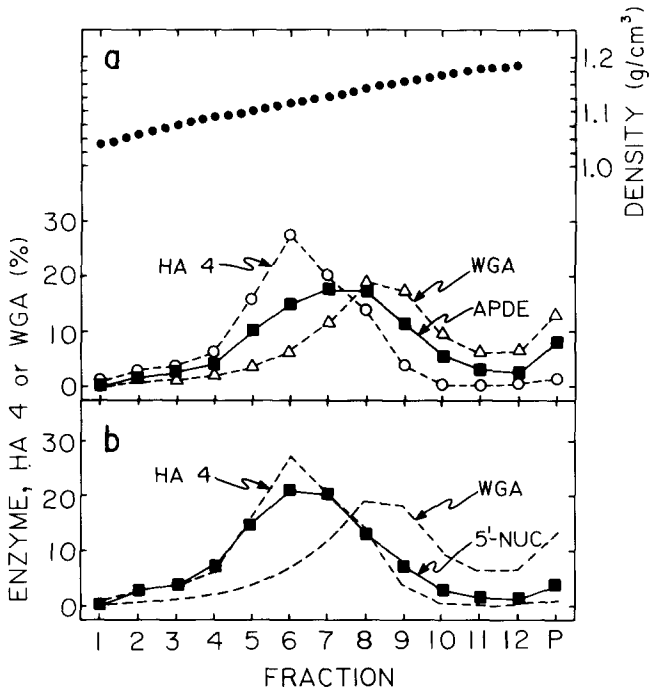

FIGURE 8 Centrifugation of ^{125}I-WGA-labeled PM vesicles in sucrose density gradients—comparison of ^{125}I-WGA, HA 4, and conventional marker enzymes. ^{125}I-WGA-labeled hepatocyte PM sheets were vesiculated by sonication, and the resulting vesicles were centrifuged to equilibrium in sucrose density gradients. The distributions of ^{125}I-WGA, HA 4, alkaline phosphodiesterase I, and 5′-nucleotidase are plotted as a percentage of the recovered radioactivity, 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 ^{125}I-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 ^{125}I-WGA (both replotted from panel *a* using dashed lines). ●, Density.

vesicles by lectin blotting (data not shown). Furthermore, the ^{125}I-WGA was not trapped within the lumina of the vesicles, because 75–90% could be released into a 100,000 *g*, 60 min supernate after incubation in perfusion medium containing 0.1 M *N*-acetyl-D-glucosamine for 60 min at 4°C. This suggested that the ^{125}I-WGA was specifically bound to glycoconjugates on the external surface of the vesicles. Thus, it appears that, under the conditions employed here, ^{125}I-WGA introduced into the isolated perfused rat liver at 4°C can serve as an exogenous marker for the SF domain of the rat hepatocyte PM.

Finally, we compared the density profiles for two conventional hepatocyte PM marker enzymes, alkaline phosphodiesterase I and 5′-nucleotidase, with those of the BC marker HA 4 and the SF marker ^{125}I-WGA (Fig. 8). The density profile for alkaline phosphodiesterase I (Fig. 8*a*) was seen to overlap those for HA 4 and ^{125}I-WGA, suggesting that this enzyme activity was almost equally distributed between the BC and SF/LS domains. In contrast, the density profile for 5′-nucleotidase (Fig. 8*b*) was nearly coincident with that for HA 4, which suggests that this enzyme activity was localized primarily to the BC domain.

DISCUSSION

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 hepatocyte PM domains in sucrose density gradients. Though we are certainly not the first to claim the resolution of BC and SF/LS domains on the basis of density (see below), we are, to our knowledge, the first to use domain-specific antibody and lectin probes to precisely monitor the location of the domains during such a fractionation.

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

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).

Distinct Density Profiles for HA 4 and CE 9 in Sucrose Gradients

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). 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).

[4] The isolated hepatocyte PM sheets show a 50- to 70-fold enrichment of PM markers relative to the filtered homogenate (Table III). 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.

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).

^{125}I-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 ^{125}I-WGA can serve as an exogenous marker for the SF domain of the rat hepatocyte PM.

The location of ^{125}I-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 ^{125}I-WGA-binding glycoproteins (unpublished data) and could bind the lectin when it is added after sonication (Fig. 7*b*). Taken together, these results indicate that, under controlled conditions, ^{125}I-WGA can serve as an exogenous marker for the SF domain of the rat hepatocyte PM. As such, ^{125}I-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 ^{125}I-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/cm^3) 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/cm^3) 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. (24), 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 ^{125}I-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 dipeptidyl dipeptidase IV], and 5′-nucleotidase activity) appear to be domain-specific. These proteins likely represent some molecular correlates of the long-appreciated 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.

We would like to thank Dr. Y. C. Lee for providing the mannosyl-BSA, Ed Shapland for technical assistance, Tim Connolly and Hans Stukenbrok (Yale University School of Medicine) for assistance with electron microscopic techniques, Tim Connolly and Tom Urquhart for photographic assistance and Arlene Daniel for help in preparing the manuscript.

This work was supported by National Institutes of Health grants to A. L. Hubbard (GM29185) and J. R. Bartles (AM06708 and AM34138).

Received for publication 5 October 1984, and in revised form 19 December 1984.

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