A Domain-specific Marker for the Hepatocyte Plasma Membrane. III. Isolation of Bile Canalicular Membrane by Immunoadsorption

LAURA M. ROMAN* and ANN L. HUBBARD
*Section of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510; and
Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT

Previous immunolabeling studies (Roman, L. M., and A. L. Hubbard, 1983, J. Cell Biol., 96:1548–1558; Roman, L. M., and A. L. Hubbard, 1984, J. Cell Biol., 98:1488–1496, companion paper) established leucine aminopeptidase (LAP) as a specific marker for the bile canalicular (BC) domain of the rat hepatocyte plasma membrane (PM). In this study, we have isolated membrane from a sonicated PM vesicle fraction using anti-LAP-coated Staphylococcus aureus cells as a solid-phase immunoadsorbent. The extent and specificity of the immunoadsorption were assessed by following the behavior of LAP (the BC marker) and 32P-labeled membrane phospholipids (a uniform membrane marker). The BC fraction obtained was significantly enriched in LAP (yield: >70% of PM-LAP). Alkaline phosphatase, 5′-nucleotidase, and a 110,000-dalton glycoprotein, HA-4, were enriched in the BC fraction to the same extent as LAP (enzyme or antigen/LAP = 1.0). However, alkaline phosphodiesterase I was not enriched to the same degree (enzyme/LAP = 0.5). Contamination of this BC fraction by membrane derived from the sinusoidal domain and endoplasmic reticulum, as determined from the distribution of the asialoglycoprotein receptor and NADH cytochrome c reductase, respectively, was small (<13%).

Indirect immunofluorescence has shown that the membrane glycoprotein, leucine aminopeptidase (LAP), is localized to the bile canalicular (BC) domain of the rat hepatocyte plasma membrane in situ (1). Immunolabeling studies carried out on isolated plasma membrane (PM) sheets at the ultrastructural level have confirmed that LAP is both highly concentrated in this domain and uniformly distributed within it (2). Therefore, LAP becomes a useful marker for the isolation of BC membrane.

Several groups have attempted to separate the three domains of the hepatocyte plasma membrane by procedures that relied primarily on differences in physical parameters (i.e., size, density, surface charge, etc., references 3–6). However, most of the subfractions obtained were contaminated by membranes derived from the other plasmaemmal domains as well as from intracellular organelles. In recent years, immunological methods have been successfully used to isolate particular organelles or specialized regions of an organelle from heterogeneous mixtures of components (7–9). The domain-specific location of LAP and the availability of LAP antibodies prompted us to choose an immunoadsorption approach for the isolation of BC membrane.

Using formaldehyde-fixed, heat-inactivated Staphylococcus aureus cells complexed with anti-LAP antibodies, we have successfully isolated BC membrane from a plasma membrane fraction. In addition to LAP, the vesicle fraction we obtained was highly enriched in alkaline phosphatase, 5′-nucleotidase, and a BC antigen, HA-4, suggesting that these proteins are also concentrated in the BC domain. Alkaline phosphodiesterase (APDE) was not enriched to the same extent as these other activities. Contamination by membrane derived from either the sinusoidal domain or endoplasmic reticulum, as determined from the distribution of the asialoglycoprotein receptor (ASGPR) and NADH cytochrome c reductase, respectively, was small.

Portions of this work have been presented elsewhere (10).
MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: thymidine 5'-monophosphate p-nitrophenylester, phosphatidylcholine, sodium cyanoborohydride, Sigma 104 phosphatase substrate, Sigma Chemical Co., St. Louis, MO; adenosine 5'-monophosphate (5'-AMP) Calbiochem-Behring Corp., La Jolla, CA; glutaraldehyde and osmium tetroxide (OsO4) from Electron Microscopy Sciences, Fort Washington, PA; nontumour guinea pig whole serum, Gibco Laboratories, Inc., Grand Island, NY; thin-layer chromatography plates, with or without fluorescent indicator, Arthur Thomas, Philadelphia, PA. All other chemicals were of reagent grade. Starter cultures of S. aureus were from Dr. E. Merisko, Department of Anatomy, University of Kansas; the phospholipid standards from Dr. K. Miller, Department of Physiological Chemistry, Johns Hopkins University School of Medicine; monoclonal antibodies to the hepatic antigen HA-4 from L. Braierman, Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine; and rabbit antibodies to the asialoglycoprotein receptor from Dr. P. Zeitlin, also from the Department of Cell Biology and Anatomy, Johns Hopkins.

Preparation and Treatment of a Plasma Membrane Fraction

PLASMA MEMBRANES: Plasma membranes were prepared from the livers of male Sprague-Dawley rats by the procedure of Hubbard et al. (11). Protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 U/ml Trasylol, 1 mM benzamidine, and 1 μg/ml leupeptin and antipain) were added to the homogenization solution (0.25 M sucrose, 5 mM Tris-HCl, 1 mM MgCl₂, pH 7.4). Phenylmethylsulfonyl fluoride was added again to the final membrane preparation. The plasma membrane fraction was diluted with 0.25 M sucrose to give a final protein concentration of 1 mg/ml aliquoted into 1-ml samples, and frozen at -70°C.

32P-Labeled Plasma Membranes: To prepare 32P-labeled membranes, 5 nCi of 32P (Amersham Corp., Arlington Heights, IL; orthophosphate, carrier free in 0.5 ml PBS) was injected into the saphenous vein of an anesthetized rat, the animal (~200 g) was sacrificed by decapitation 5 h later, and PM sheets were isolated.

Alkaline-Extracted Plasma Membranes: The alkaline extraction procedure of Hubbard and Ma (12) was followed with minor modifications. Aliquots of PM (2 ml, at 1 mg/ml) were mixed with an equal volume of 0.1 M NaHCO₃, CO₂, pH 10.5, by three strokes in a Dounce-type glass homogenizer with a loose-fitting pestle. After 5 min on ice, the suspension was centrifuged at 10,000 g for 30 min at 4°C. The supernate was collected, and the pellet was resuspended in 20 mM Na phosphate, pH 7.4. The pellets were resuspended in one third the initial volume of SNET. Affinity-purified anti-LAP or nonimmune IgG (0.15-0.25 mg in PBS) was added to the S. aureus cells and the volume was adjusted to the initial 10% (wt/vol) concentration with SNET. The cells used to prepare the sonicated vesicles were washed by incubation 125 or 250 μl of S. aureus cells (at 20%) with an equal volume of nonimmune whole guinea pig serum. All antibody-cell adsorptions were incubated for 1 h at 4°C with constant agitation, after which excess antibody was removed by two cycles of sedimentation and resuspension in SNET (0.5-0.75 ml). The efficiency of IgG binding was monitored by radioimmunoassay on the antibody solution before and after exposure to the S. aureus cells. The radioimmunoassay was carried out as described (2).

Cross-linking of Antibodies to Cells: In some experiments antibodies were cross-linked to the S. aureus cells to reduce the amount of IgG eluted during the preparation of samples for SDS PAGE. The washed, IgG-coated cells were rinsed three more times in 20 mM Na phosphate, pH 7.4 (0.5 ml), and resuspended in this buffer to their initial volume, and an equal volume of 0.05% glutaraldehyde in 20 mM Na phosphate was added. After 1 h at 4°C, the cells were sedimented, washed twice with phosphate buffer, resuspended in 0.5 ml 10 mM Na cyanoborohydride (NaCNBH₄), and incubated in this solution for 30 min at room temperature to reduce the Schiff bases formed. The cross-linked cells were used for preliminary immune overlay experiments with the guinea pig anti-LAP antibodies; however, native cells were used as immunoadsorbents for several of the studies described below.

Immunoadsorption of Sonicated Vesicles with Antibody-coated S. aureus Cells

A flow sheet for the immunoadsorption protocol followed is presented in Fig. 1. Aliquots from the indicated steps were saved for 32P and enzyme analyses.

Preparation of Antibody-coated S. aureus

S. aureus Cells: Formaldehyde-fixed, heat-inactivated S. aureus cells were prepared by the procedure of Kessler (13) and stored in 2-ml aliquots at -70°C as 10% suspensions (wt/vol) in PBS containing 0.05% sodium azide. Before use, the thawed cells were passed through 30-μm nitex screening (Tetko, Elmsford, NY), divided into 0.25-0.6 ml aliquots, and washed five times by sedimentation and resuspension in SNET buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.25% Triton X-100, and 5 mg/ml BSA) as outlined by Merisko et al. (8). All centrifugations were for 1 min at 8,000 g in an Eppendorf centrifuge (Brinkman Instruments, Westbury, NY).

Antibody-coating of Cells: 250- or 500-μl aliquots of washed cells were suspended to a concentration of 10% (wt/vol) were centrifuged and

Antibodies

The preparation and characterization of anti-LAP antibodies have already been described (1). Control sera were obtained from nonimmunized guinea pigs (preimmune) or purchased from Gibco Laboratories (nonimmune) and an IgG fraction was prepared as previously outlined (1).

Preparation of Antibody-coated S. aureus

S. aureus Cells: Formaldehyde-fixed, heat-inactivated S. aureus cells were prepared by the procedure of Kessler (13) and stored in 2-ml aliquots at -70°C as 10% suspensions (wt/vol) in PBS containing 0.05% sodium azide. Before use, the thawed cells were passed through 30-μm nitex screening (Tetko, Elmsford, NY), divided into 0.25-0.6 ml aliquots, and washed five times by sedimentation and resuspension in SNET buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 0.25% Triton X-100, and 5 mg/ml BSA) as outlined by Merisko et al. (8). All centrifugations were for 1 min at 8,000 g in an Eppendorf centrifuge (Brinkman Instruments, Westbury, NY).

Antibody-coating of Cells: 250- or 500-μl aliquots of washed cells were suspended to a concentration of 10% (wt/vol) were centrifuged and
We followed the distributions and enrichments of two PM marker enzymes, 5'-nucleotidase and APDE, throughout the fractionation scheme and found them to be similar to those we reported earlier (11). The relative specific activity and yield of LAP (13 ± 6-fold [range 8–26] and 9 ± 4% [range 8–17]) were lower than those of the other two PM markers, in agreement with the reports of Toda et al. (21) and Wisher and Evans (22).

For the immunoprecipitation experiments described below, it was necessary to uniformly label the plasma membrane so that nonspecific binding (i.e., non–bile front membrane) could be determined. Since no protein marker was available, we incorporated 3P into liver phospholipids and then isolated 3P-labeled PM sheets. The 3P-labeled material followed the same distribution as protein in our PM isolation. The final yield of 3P-labeled membrane averaged 1.1% of the homogenate radioactivity (range 0.52–1.6%). The nature of the 3P-labeled material (lipid vs. protein) and the identification of the radioactive species in the plasma membrane fraction were determined by lipid extraction and thin-layer chromatography as described by Kale (23). 95% of the initial radioactivity was extracted by methanol/chloroform (2:1) and 90% of the extracted label was recovered in the lower chloroform phase (85.5% of the initial counts). Analysis of the chloroform phase by thin-layer chromatography revealed that the four major classes of phospholipids were present in the extracted plasma membranes and that each had incorporated 3P to some extent during the 5-h labeling period. Phosphatidylethanolamine and phosphatidylincholine were the most abundant chemical and radiolabeled species present, in agreement with the results of Skipski et al. (24) and Takeuchi and Terayama (25).

When isolated PM sheets were sonicated and the resulting preparation was examined morphologically, the predominant components were smooth-surfaced vesicles ranging from 100 to 1,000 nm diam (Fig. 2a). Rough microsomes, mitochondria, and filaments were also found.

Immunoadsorption of Bile Front Membranes

**DISTRIBUTION OF LAP ACTIVITY AND 3P RADIOACTIVITY:** We followed the distribution of LAP activity to monitor the isolation of bile front membrane. The activity of the enzyme was not affected by incubation of the vesicles with S. aureus cells and >90% of the initial activity could be accounted for in the unbound (supernate), wash, and final cell fractions.

When sonicated vesicles were incubated with anti-LAP serum–coated S. aureus cells, 63% of the initial activity and 24% of the 3P radioactivity were adsorbed (Table I, experiment A). However, the amounts of 3P-radioactivity in both immune and nonimmune samples were higher than those predicted on the basis of the LAP activity bound in the same samples (Table I, experiment A, compare last two columns). The levels of 3P bound were substantially reduced when the sonicated vesicle preparation was pretreated with nonimmune serum–coated cells (Table I, experiment B). The amount of LAP bound was not significantly altered. In addition, the 3P ratios of material bound in the immune vs. nonimmune samples increased from two to eight using such a pretreatment. As seen in Table I (experiment C), both the amount of LAP activity bound to the immune antibody–coated S. aureus cells as well as the specificity of binding were further increased when affinity-purified anti-LAP antibodies and nonimmune IgG were used to coat the cells. That is, we could adsorb as much as 73% of the LAP activity using affinity-purified anti-LAP whereas as nonimmune IgG gave 15-fold lower adsorption (5%).

**RESULTS**

**Analysis of the Plasma Membrane and Sonicated Vesicle Preparation**

We followed the distributions and enrichments of two PM marker enzymes, 5'-nucleotidase and APDE, throughout the fractionation scheme and found them to be similar to those
OTHER APPROACHES TO REDUCE NONSPECIFIC BINDING: We were concerned that the pretreatment step removed too much of the initial LAP and 32P (~50% of each). Therefore, we examined other protocols that might reduce nonspecific binding. Removal of the cytoplasmic filaments by alkaline extraction of the PM before sonication was not effective in reducing the level of nonspecific binding (Table I, experiments C and D). These findings suggested that the filaments were not contributing to nonspecific binding.

When we examined the morphology of those membranes...
adsorbed during the pretreatment step, we observed many vesicle aggregates not in direct contact with the S. aureus cells. Experiments carried out to determine the origin of these aggregates revealed that incubation of the membrane vesicles in PSS-BSA without S. aureus cells resulted in sedimentation of 50-60% of LAP and APDE activities, as compared with incubation in 0.25 M sucrose, which resulted in the sedimentation of only 10-15% of these activities. The aggregation was not domain specific, because 5’-nucleotidase, the BC-antigen HA-4, and a sinusoidal marker, ASGPR, followed the same distribution as LAP and APDE during the preadsorption step (data not shown). Thus, the pretreatment step was necessary to remove vesicle aggregates that were generated in the immunoadsorption buffer and nonspecifically sedimented with the S. aureus cells.

**Morphology of the Immunoadsorbed Vesicle Fraction:** When S. aureus cells that had been incubated with affinity-purified anti-LAP and preadsorbed vesicles were examined by electron microscopy, they were found to be covered to various degrees with attached vesicles (Fig. 2b). The binding of closed vesicles (100-400 nm) in the adsorbed samples suggested that plasma membrane vesicles and not LAP-containing membrane fragments were recognized by the anti-LAP antibodies. S. aureus cells complexed with nonimmune IgG were essentially free of adsorbed vesicles (Fig. 2c).

The unbound vesicle fraction after immunoadsorption was morphologically similar to the initial sonicated vesicle as well as the pretreated vesicle preparations (data not shown). No obvious enrichment or depletion of a particular size or shape vesicle class was noted. This result indicated that BC vesicles were not morphologically distinguishable from those derived from the sinusoidal domain.

**Biochemical Characterization of the Adsorbed Vesicle Population**

**Table I**

**Distribution of LAP during Immunoadsorption Preadsorption of the Sonicated Vesicle Preparation**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>% Removed*</th>
<th>LAP</th>
<th>32P</th>
<th>Antibody*</th>
<th>% LAP bound</th>
<th>% 32P bound</th>
<th>% 32P predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. None</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Serum-I</td>
<td>63.4 ± 8.0</td>
<td>24.4 ± 13.0</td>
<td>15.0 ± 2.3</td>
</tr>
<tr>
<td>B. Adsorb</td>
<td>49.0 ± 6.0</td>
<td>55.0 ± 8.0</td>
<td>7.0 ± 4.0</td>
<td>Serum-NI</td>
<td>12.4 ± 3.4</td>
<td>2.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>C. Adsorb</td>
<td>47.0 ± 13.0</td>
<td>51.0 ± 11.0</td>
<td>6.2 ± 6.6</td>
<td>Serum-NI</td>
<td>17.8 ± 6.2</td>
<td>15.0 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>D. Alkaline extract</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>NI IgG</td>
<td>6.3 ± 2.0</td>
<td>2.3 ± 0.7</td>
<td>1.2 ± 0.5</td>
</tr>
</tbody>
</table>

* The amount of LAP and 32P activity removed from the initial PM vesicle preparation is expressed as percent of recovered activity associated with S. aureus cells. Recoveries were 85-95%.

**Table II**

**Enrichment of LAP Activity during Immunoadsorption**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Relative specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>98 ± 4.0</td>
<td>0.19 ± 0.02</td>
<td>1</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>94.5 ± 4.0</td>
<td>5.0 ± 0.2</td>
<td>26</td>
</tr>
<tr>
<td>Initial pretreated sonicated vesicles</td>
<td>0.36 ± 0.06</td>
<td>7.2 ± 0.2</td>
<td>38</td>
</tr>
<tr>
<td>Adsorbed membranes</td>
<td>0.28 ± 0.06</td>
<td>29.0 ± 2.9</td>
<td>153</td>
</tr>
</tbody>
</table>

* The homogenate and plasma membrane values were determined immediately after isolation of the PM fraction and separately from the last two fractions. Approximately 1% of the total PM was used in the pretreatment and immunoadsorption experiment (~250 μg protein).

**Table III**

**Distribution of Other Activities Present in the PM Fraction**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Relative specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>4,414 ± 3.4</td>
<td>0.050 ± 0.005</td>
<td>1</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>17.0 ± 1.0</td>
<td>0.5 ± 0.02</td>
<td>26</td>
</tr>
<tr>
<td>Initial pretreated sonicated vesicles</td>
<td>0.28 ± 0.06</td>
<td>7.2 ± 0.2</td>
<td>38</td>
</tr>
<tr>
<td>Adsorbed membranes</td>
<td>0.28 ± 0.06</td>
<td>29.0 ± 2.9</td>
<td>153</td>
</tr>
</tbody>
</table>

* The homogenate and plasma membrane values were determined immediately after isolation of the PM fraction and separately from the last two fractions. Approximately 1% of the total PM was used in the pretreatment and immunoadsorption experiment (~250 μg protein).

**Table IV**

**Distribution of Other Activities Present in the PM Fraction**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Relative specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>4,414 ± 3.4</td>
<td>0.050 ± 0.005</td>
<td>1</td>
</tr>
<tr>
<td>Plasma membranes</td>
<td>17.0 ± 1.0</td>
<td>0.5 ± 0.02</td>
<td>26</td>
</tr>
<tr>
<td>Initial pretreated sonicated vesicles</td>
<td>0.28 ± 0.06</td>
<td>7.2 ± 0.2</td>
<td>38</td>
</tr>
<tr>
<td>Adsorbed membranes</td>
<td>0.28 ± 0.06</td>
<td>29.0 ± 2.9</td>
<td>153</td>
</tr>
</tbody>
</table>
nucleotidase and alkaline phosphatase, two plasma membrane enzymes reported to be concentrated in the bile front domain (26, 27), were adsorbed to nearly the same extent as was LAP (i.e., enzyme/LAP = 1.15 and 1.11, respectively). APDE, however, demonstrated a different distribution, with ~25% of the activity adsorbed under conditions that yielded ~50% LAP binding. This result was unexpected, since Siera-kowska et al. (28) and Smith and Peters (29) have suggested that APDE was concentrated in the bile front domain. The lower amount of LAP activity bound to the S. aureus cells seen in these experiments, relative to those reported in Table I (~45 vs. 70%), was attributed to the use of cross-linked cells. However, the distribution of the three enzymes was the same using native (uncross-linked) antibody-coated cells in two experiments (data not shown). The distribution of NADH cytochrome c reductase, an endoplasmic reticulum marker, was also examined (Table III), since endoplasmic reticulum represents ~20% of the membrane in our PM fraction. This activity was substantially depleted in the adsorbed preparation as compared with that in the initial PM vesicle preparation and there was no specific binding.

Immunological Characterization of the Adsorbed Vesicle Preparation

We next examined the distribution of two domain-specific markers throughout the immunoadsorption procedure using an immunological approach. ASGPR is a marker for the sinusoidal surface and the antigen termed HA-4 is concentrated in the bile canalicular domain. Both antigens have been localized to their respective domains by indirect immunofluorescence (P. Zeitlin, L. Braiterman, and A. Hubbard, unpublished data). Aliquots from each step in the adsorption protocol were prepared for SDS PAGE, electrophoresed, transferred to nitrocellulose, and then incubated with the appropriate antibodies as described in Materials and Methods. The results of this analysis are presented in Fig. 3. Quantitation of autoradiograms by densitometry revealed that the HA-4 antigen was adsorbed to anti-LAP-coated S. aureus cells to the same extent as was LAP, while the ASGPR was not. That is, under conditions where 77% of the LAP activity was adsorbed onto the S. aureus cells, 70% of the HA-4 antigen was bound to the immunosorbent. However, ~4% of the ASGPR present in the preadsorbed sonicated vesicles was adsorbed onto the anti-LAP-coated S. aureus. This latter data suggests that the BC vesicle fraction was not significantly contaminated with membrane derived from the sinusoidal domain.

DISCUSSION

Kawajiri et al. (30) first described a solid-phase affinity adsorption protocol for the subfractionation of rat liver microsomes. We have adopted this procedure for the isolation of bile front membrane. Using LAP as a specific probe for this domain and formaldehyde-fixed, heat-inactivated S. aureus cells complexed with anti-LAP antibodies, we have obtained a membrane fraction significantly enriched in LAP activity.

Biochemical and Immunological Characterization of the Adsorbed Vesicle Population

Enzyme analyses carried out on the adsorbed vesicle population revealed that LAP was significantly enriched over the homogenate value (153-fold). This enrichment is about three times higher than that reported by Inoue et al. (6) for a BC-enriched fraction obtained by differential centrifugation following Ca"'-mediated precipitation of lateral, sinusoidal, and intracellular membranes. However, our value may be an overestimate owing to the uncertainty in determining the protein concentration of initial vesicle fraction and the adsorbed BC membranes. Nonetheless, the substantial amount of PM-LAP adsorbed onto the S. aureus cells, and the low amount of non-BC contamination, indicates that a significant purification of the BC membrane has been achieved.

The maximum adsorption of LAP-positive vesicles we obtained was 80%. Since we have not attempted a second immunoadsorption with the remaining 20%, we do not know at present if these vesicles contain LAP in an accessible orientation (e.g., right-side-out) or an orientation that would not be recognized by our antibody (e.g., inside-out). In the preceding paper (2), we have shown that our anti-LAP preparation does not bind to the cytoplasmic side of the BC membrane.

We found that 5'-nucleotidase, alkaline phosphatase, and the antigen HA-4 were adsorbed to the same extent as was
FIGURE 3  Distribution of two domain-specific antigens during immunoadsorption of BC membranes (immunoblots). Aliquots from each step of the immunoadsorption protocol were prepared for SDS PAGE, electrophoresed, transferred to nitrocellulose, and then incubated with specific antibodies to the BC antigen, HA-4 (top panel), or the sinusoidal front antigen, ASGPR (bottom panel). The lanes from left to right, with the fraction of each applied to the gel in parentheses, are SV, sonicated vesicles (0.1); PI-SV, pretreated sonicated vesicles (0.1); PI-Cell, cells from pretreatment (0.1); C-Cell, antibody-coated S. aureus control (0.2); UB-1, unbound fraction after incubation with immune anti-LAP-S. aureus (0.5); B-I, bound fraction-immune (0.2); B-Nl, bound-nonimmune (0.2); UB-Nl, unbound-nonimmune (0.5). Arrowheads designate the position of HA-4 (110 kdalton) and ASGPR (85 and 43 kdalton). The reactive components at ~55 and 26 kdalton in the top panel, lanes PI-Cell, C-Cell, B-I, and B-NI, have been tentatively identified as the heavy and light chains of immunoglobulin (by co-migration with standards). The band migrating at ~68 kdalton in the bottom lanes PI-Cell, UB-I, and UB-Nl appears to be albumin.

LAP, suggesting that these membrane markers are also concentrated in the BC domain of hepatocytes. However, APDE distributed differently, indicating that ~50% of this activity is associated with noncanalicular membranes in our PM preparation. This last observation was unexpected, since several groups have reported a canalicular distribution for APDE by both subcellular fractionation (29) and enzyme cytochemistry (28). In addition, APDE is enriched in our plasma membrane fraction to the same extent as 5’-nucleotidase. However, there are several possible explanations for the apparent discrepancy. First, enzyme cytochemistry localizes activities not antigens, thus there are uncertainties regarding the specificity of the substrate for only one enzyme. Secondly, subfraction of the hepatocyte PM has to date yielded heterogeneous fractions containing membrane derived from all three plasmalemmal domains. Thus, caution must be used in assigning an enzyme activity to a particular domain based on its sedimentation characteristics. Since the BC membrane fraction we have obtained represents one of the purest such preparations, our finding that APDE is relatively depleted from it suggests to us that several enzymes in different PM locations may be hydrolyzing the same substrate.

The absence of ASGPR in our adsorbed BC vesicle fraction suggests that it is not significantly contaminated by sinusoidal membrane and confirms data obtained by others in our laboratory (11) that the ASGPR is not present in the BC domain but is present in the other two domains.

This receptor appears to be concentrated in coated pits along the sinusoidal surface of hepatocytes in situ (31). Thus, we were concerned that such regions might form coated vesicles during sonication, resulting in a distribution of the receptor during immunoadsorption that did not represent that of the whole sinusoidal domain. Two observations argue against such a concern: (a) the receptor is also present outside of coated pits (31); and (b) the sonicated vesicle preparation contains a number of vesicle profiles that contain coated regions. This latter observation indicates that all coated pits do not form coated vesicles during sonication.

The low percent of the initial PM NADH cytochrome c reductase (2.3%) adsorbed onto the S. aureus cells indicates that the BC fraction is not significantly contaminated by membrane derived from the endoplasmic reticulum. If we assume that only membrane derived from the BC, sinusoidal front, and endoplasmic reticulum were adsorbed to the S. aureus cells (a reasonable assumption, considering they account for ~93% of all the membrane in the initial PM preparation [11]), then 20% of the membranes initially present were adsorbed, and 87% of these were derived from the BC. The remaining 13% (at most) were derived from the endoplasmic reticulum (<5%) and sinusoidal front (~10%).

Other Methods for the Isolation of Hepatocyte Plasma Membrane Domains

A number of groups (3–6, 22, 32) have attempted to isolate a particular membrane domain by procedures that have relied primarily on physical parameters. While enrichment of do-

2 Taking the example of the BC, ~22% of the membrane in the PM fraction is derived from BC (11). The amount of BC membrane in the final adsorbed fraction is estimated from PM-BC (22%) times the percent of PM-LAP activity present in the adsorbed fraction (78%) which equals 17.2%, normalized to the total amount of membranes (of all types) adsorbed (20%).
main-specific markers has been achieved by these schemes, the fractions still showed contamination by membrane derived from the other PM domains and intracellular organelles. Elements of the endoplasmic reticulum were the major contaminants in all of these studies even when the domain fractions were derived from isolated PM (5).

The Use of Immunoadsorption to Isolate Membrane Subfractions

Immunoadsorption has been demonstrated to be an efficient method to isolate various membrane subfractions. Ito and Palade (7) used polyacrylamide beads coated with rabbit anti--NADPH cytochrome c reductase to subfractionate vesicles derived from the Golgi apparatus. Merisko et al. (8) employed S. aureus cells complexed with antiallactan antibodies to isolate coated vesicles from porcine brain. More recently, Miljanich et al. (9) used polyacrylamide beads coated with antisera directed against electric organ synaptic vesicles derived from the Gogli apparatus. Merisko et al. (8) demonstrated here. In addition, immunoadsorption in conjunction with immunoprecipitation could be used for biosynthesis and transport studies, where the movement of components into or out of a particular membrane domain could be assessed.

We would like to thank Ms. A. Daniel for preparation of the manuscript, Mr. T. Urquhart for his photographic work, and Dr. J. Bartles for helpful discussions.

This project was supported by a National Institutes of Health grant to Ann Hubbard (GM29185).

Received for publication 12 September 1983, and in revised form 20 December 1983.

REFERENCES


