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

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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 ^{32}P-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),[1] 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 plasmalemmal 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).

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[1] *Abbreviations used in this paper:* APDE, alkaline phosphodiesterase; ASGPR, asialoglycoprotein receptor; BC, bile canalicular (domain); LAP, leucine aminopeptidase; PM, plasma membrane; PSS, 0.5 ml of 20 mM Na phosphate, 0.15 M NaCl, 0.25 M sucrose, pH 7.4.

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; nonimmune guinea pig whole serum, Gibco Laboratories, Inc., Grand Island, NY; thin-layer chromatography plates, 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. Braiterman, Department of Cell Biology and Anatomy, Johns Hopkins University School of Medicine; and rabbit antibodies to the asialglycoprotein 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 mCi 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/CO₃, 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 centrifued at 100,000 g for 30 min. The supernate was removed and the pellet was resuspended to its initial volume in 0.25 M sucrose.

PREPARATION OF SONICATED PLASMA MEMBRANE VESICLES: To disrupt the plasma membrane sheets into vesicles, a suspension of plasma membranes or alkaline-extracted membranes (1 and 0.5 mg/ml, respectively, and both containing the mixture of protease inhibitors listed above) was sonicated at 0°C for a total of 120 s (eight 15-s bursts with 5-s intervals on ice) in a bath sonicator (Laboratory Supply Co., Hicksville, NY; 600 V, 80 kilocycles). The suspension was centrifuged for 10 min at 1,700 g (Beckman TJ-6, Beckman Instruments, Inc., Palo Alto, CA) and the supernate was centrifuged again under the same conditions. The final supernate was examined by phase-contrast microscopy and found to be devoid of large membrane sheets.

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 (resuspended to a concentration of 10% cell wt/vol) were centrifuged and

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 pretreat the sonicated vesicles (see below) were prepared by incubating 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.
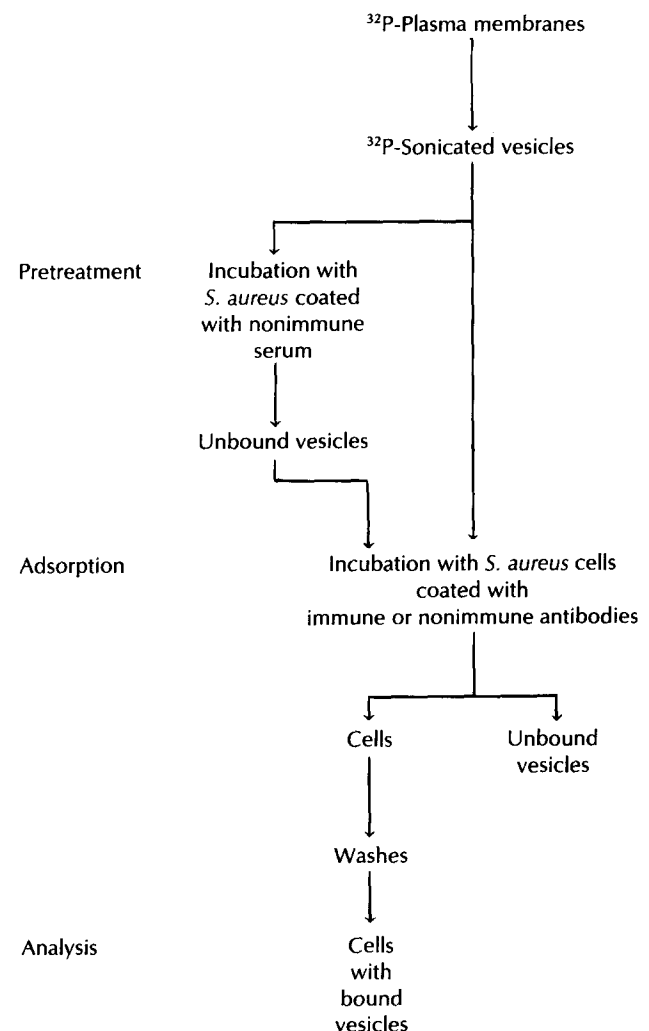

FIGURE 1   Flow sheet for the immunoadsorption of bile front membrane. The fractions underlined were analyzed for LAP activity and ³²P-radioactivity. See Materials and Methods for details.

DETERMINATION OF OPTIMUM ADSORPTION CONDITIONS: Antibody-coated *S. aureus* cells were incubated with pretreated sonicated vesicles for 15–120 min at 4°C with constant agitation. 60-min incubations gave the highest ratio of immune to nonimmune binding (10–20 × LAP, 8–17 × ^{32}P). Longer incubations resulted in higher nonspecific binding (data not shown). The maximum amount of LAP activity was bound (70–80%) when 250 μl of affinity-purified antibody-coated cells were incubated with 125 μg of preadsorbed membranes. When the amounts of cells and vesicles were doubled, the amount of LAP activity adsorbed also doubled. Approximately 50% of the antigen binding activity was lost when antibody was cross-linked to *S. aureus* cells with 0.025% glutaraldehyde, as assessed by the amount of LAP activity adsorbed relative to the amount of affinity-purified anti-LAP bound to the *S. aureus* cells (determined by radioimmunoassay).

PRETREATMENT OF MEMBRANE VESICLES: To reduce nonspecific binding, the sonicated vesicles were first incubated with *S. aureus* cells coated with nonimmune whole serum (Fig. 1). The antibody-coated cells were sedimented and washed with 0.5 ml of 20 mM Na phosphate, 0.15 M NaCl, 0.25 M sucrose, pH 7.4, supplemented with 5 mg/ml BSA (PSS-BSA). The cells were resuspended in PSS-BSA and transferred to plastic tubes that had been coated for 4–5 h at room temperature with a solution of 0.2 mg/ml phosphatidylcholine in PSS. Phospholipid coating was necessary to reduce nonspecific binding of the membrane vesicles to the tubes. After sedimentation, the cells were diluted to their initial volume with PSS-BSA and an equal volume of sonicated vesicles (250–500 μg) was added. After incubation at 4°C for 45 min to 1 h with constant agitation, the *S. aureus* cells were sedimented and the unbound membranes in the supernate were collected for subsequent immunoadsorption.

IMMUNOADSORPTION OF BILE FRONT-DERIVED MEMBRANE VESICLES: Immune and nonimmune IgG-coated *S. aureus* cells (± cross-linking) were washed three times in 0.5 ml PSS-BSA, transferred to PC-coated tubes, and sedimented. Unbound membrane vesicles from the pretreatment step (0.5 or 1 ml) were added directly to cell pellets and vortexed vigorously. After incubation for 45 min to 1 h at 4°C with constant agitation, the cells were sedimented and the supernate was saved. The cells were washed, once with PSS-BSA, once with PSS, and then resuspended with PSS to a final volume of 0.5 or 1.0 ml.

Analytical Procedures

ASSAYS: LAP was assayed as previously described (1). Alkaline phosphodiesterase (APDE) was assayed according to the protocol of Touster et al. (14) as described by Hubbard et al. (11). 5'-nucleotidase was determined by a radiochemical assay (15). Alkaline phosphatase activity was assessed by following the release of *p*-nitrophenol at 410 nm according to the procedure of Ray (16) as modified by Hubbard et al. (11). Protein was determined according to Bradford (17) using BSA as a standard.

SDS PAGE: Unbound fractions from the pretreatment and adsorption steps, as well as the initial sonicated vesicles, were sedimented at 100,000 g for 60 min. Membrane adsorbed to *S. aureus* (0.1 ml) was sedimented as described above. The pellets were solubilized by boiling in SDS (4%), dithiothreitol (16 mM), EDTA (4 mM), and Tris-HCl, pH 8.7 (30 mM). After alkylation in iodoacetamide (50 mM), samples were electrophoresed on 8% polyacrylamide slab gels (18), then either stained wtih Coomassie Blue or transferred to nitrocellulose (1).

IMMUNOBLOT: Transferred samples were incubated with either ^{125}I-labeled antibody (a mouse monoclonal IgG to the BC antigen, HA-4) or immune or preimmune whole serum followed by ^{125}I-protein A as previously described (1), except that the paper was preincubated with a filtered solution of 2% gelatin in wash buffer and the antibodies and protein A were diluted in filtered wash buffer containing 0.4% BSA.

IODINATION: Protein A was iodinated by a modification of the procedure of Greenwood et al. (19) as described in reference 2.

ELECTRON MICROSCOPY: After immunoadsorption, 1 vol of a cell sample was diluted with 5 vol PSS, mixed with 6 vol of 2% glutaraldehyde in 0.1 M Na cacodylate, pH 7.4, and incubated for 30 min on ice. The initial sonicated vesicles were fixed in a similar manner. After sedimentation at 100,000 g for 20 min (*S. aureus* cells) or 60 min (vesicles), the pellets were processed for electron microscopy as described (20).

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

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 ^{32}P into liver phospholipids and then isolated ^{32}P-labeled PM sheets. The ^{32}P-labeled material followed the same distribution as protein in our PM isolation. The final yield of ^{32}P-labeled membrane averaged 1.1% of the homogenate radioactivity (range 0.52–1.6%). The nature of the ^{32}P-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 ^{32}P to some extent during the 5-h labeling period. Phosphatidylethanolamine and phosphatidylcholine 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 ^{32}P 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 ^{32}P radioactivity were adsorbed (Table I, experiment *A*). However, the amounts of ^{32}P-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 ^{32}P 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 ^{32}P 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%).
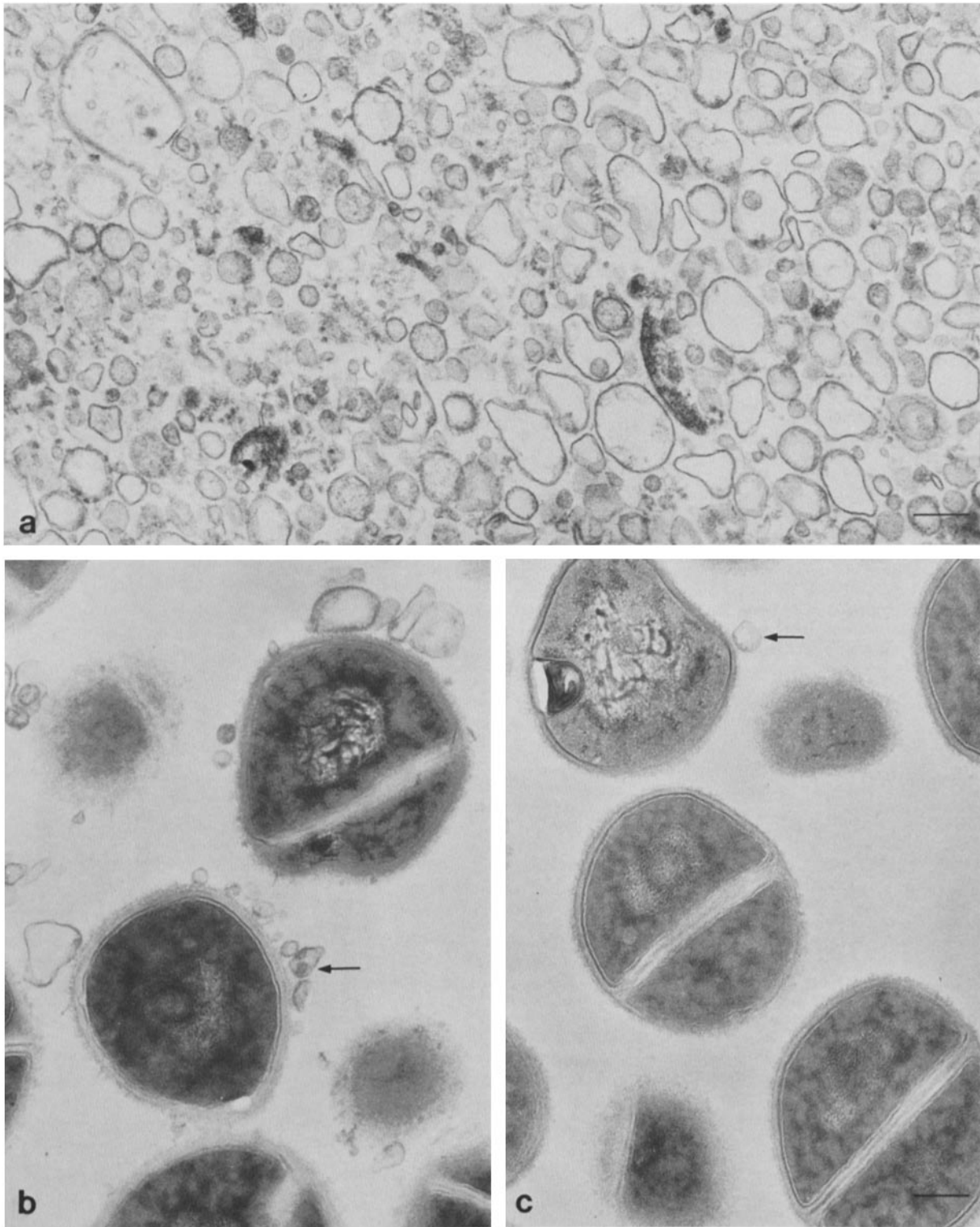
FIGURE 2 Electron micrograph of the sonicated vesicles and adsorbed membrane fraction. (a) An electron micrograph of the middle region of the initial sonicated vesicle fraction (~50% of the pellet). The size of the vesicles range from 100–1,000 nm. Junctional elements, dense fibrillar material, as well as a few mitochondria were also present. Bar, 0.2 μm. × 42,800. (b) Anti-LAP–coated S. aureus cells that had been incubated with pretreated sonicated vesicles. 90% of the cells were covered to various degrees by attached closed vesicles (arrow). (c) Nonimmune-IgG–coated S. aureus cells were nearly devoid of associated vesicles. Bar, 0.2 μm. × 42,800.

OTHER APPROACHES TO REDUCE NONSPECIFIC BINDING: We were concerned that the pretreatment step removed too much of the initial LAP and ^{32}P (~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

TABLE I

Distribution of LAP during Immunoadsorption Preadsorption of the Sonicated Vesicle Preparation

Pretreatment	% Removed*		Antibody[‡]	% LAP[§] bound	% [32]P[§] bound	% [32]P[‖] predicted
	LAP	[32]P				
A. None	—	—	Serum-I Serum-NI	63.4 ± 8.0 ⎰9[¶] 7.0 ± 4.0 ⎱	24.4 ± 13.0 ⎰2 12.4 ± 3.4 ⎱	15.0 ± 2.3 2.1 ± 0.6
B. Adsorb	49.0 ± 6.0	55.0 ± 8.0	Serum-I Serum-NI	62.0 ± 6.6 ⎰10 6.3 ± 2.0 ⎱	17.8 ± 6.2 ⎰8 2.3 ± 0.7 ⎱	15.0 ± 1.5 1.2 ± 0.5
C. Adsorb	47.0 ± 13.0	51.0 ± 11.0	APAb NI IgG	73.0 ± 4.0 ⎰15 5.0 ± 1.0 ⎱	24.0 ± 4.0 ⎰12 2.0 ± 0.9 ⎱	17.0 ± 1.0 0.8 ± 0.4
D. Alkaline extract	—	—	APAb NI IgG	73.0 ⎰6 12.1 ⎱	38.2 ⎰6 6.0 ⎱	16.8 2.3

* The amount of LAP and ^{32}P activity removed from the initial PM vesicle preparation is expressed as percent of recovered activity associated with S. aureus cells. Recoveries were 85–95%.
[‡] I, immune; NI, nonimmune; APAb, affinity-purified anti-LAP; NI IgG, nonimmune IgG.
[§] The amount of LAP and ^{32}P bound to the S. aureus cells was determined by direct assay and expressed as a percent of initial (± preadsorption) without correction for recoveries (90–100%).
[‖] The amount of ^{32}P predicted to be associated with the adsorbed vesicles was determined from the following assumptions: (a) the membranes were uniformly labeled with ^{32}P; (b) LAP was only in the bile front membrane; and (c) the bile front membrane represented 23% of the membrane in the PM fraction.
[¶] Ratios of LAP or ^{32}P bound to the S. aureus cells in immune vs. nonimmune samples.

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. 2 b). 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. 2 c).

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

ESTIMATION OF THE ENRICHMENT OF BILE FRONT MEMBRANE: A summary of the enrichment of LAP activity from an initial homogenate through immunoadsorption is presented in Table II. Since BSA was included in all incubations, we were not able to measure the protein concentration of the adsorbed vesicle population. Thus, we used the distribution of ^{32}P and the specific radioactivity (^{32}P/mg protein)

TABLE II

Enrichment of LAP Activity during Immunoadsorption

Fraction	Total activity	Specific activity	Relative specific activity
	μmole/h	μmole/h per mg protein	
Homogenate	984*	0.19	1
Plasma membranes	94.5	5.0	26
Initial pretreated sonicated vesicles	0.36	7.2[‡]	38
Adsorbed membranes	0.28	29.0[‡]	153

* 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).
[‡] The protein values used to determine the specific activity of the initial and adsorbed samples were calculated from the ^{32}P values. The specific activity of ^{32}P in the PM preparation was 9.0 × 10^{4} cpm/mg PM protein. Sample calculation:

[32]P in pretreated sonicated vesicles

$$= \frac{4{,}414 \text{ cpm} + 9.0 \times 10^4 \text{ cpm/mg PM protein}}{(\text{measured}) \quad (^{32}\text{P sp act in PM})} \cong 0.050 \text{ mg protein}$$

LAP in adsorbed membranes

$$= 0.28 \text{ μmol/h} ÷ 0.0097 \text{ mg protein} = 29 \text{ μmol/h per mg protein} \\ (\text{measured})$$

of ^{32}P in the PM fraction to estimate the amount of vesicle protein adsorbed onto the antibody-coated cells. The 1.5-fold increase in the relative specific activity between the plasma membrane and initial preadsorbed vesicle fraction may have been due to removal of non–LAP-positive components (i.e., unbroken plasma membrane sheets, lateral membranes with associated filaments, mitochondria, and lysosome-like structures) or variability in LAP measurements between the PM and adsorbed vesicles. As can be seen from Table II, >70% of the initial LAP activity was bound to the cells with an enrichment of 153-fold over that in the homogenate.

DISTRIBUTION OF OTHER ACTIVITIES PRESENT IN THE PM FRACTION: Having established a procedure that gave significant enrichment of LAP activity, we next assessed the distribution of several conventional plasma membrane enzymes, 5′ nucleotidase, alkaline phosphatase, and APDE. The results of this study are presented in Table III. Both 5′-

TABLE III

Distribution of Selected Enzymes after Immunoadsorption*

	Percent initial activity bound			
	Immune	Nonimmune	I/NI	Enzyme/LAP[§]
1. Alkaline phosphatase	56.7 ± 0.7[‡]	7.3 ± 0.7	8.3 ± 0.9	1.15
LAP	48.5 ± 5.0	5.2 ± 0.9	9.2 ± 1.1	
2. 5'-Nucleotidase	48.4 ± 5.7	3.8 ± 0.7	13.0 ± 2.2	1.11
LAP	45.7 ± 3.8	5.5 ± 0.6	8.5 ± 1.7	
3. APDE	25.3 ± 2.7	2.2 ± 1.2	10.0 ± 4.4	0.53
LAP	49.4 ± 7.3	6.9 ± 1.5	7.3 ± 1.1	
4. NADH cytochrome c reductase	6.7 ± 1.2	4.3 ± 2.0	2.0 ± 1.1	0.03
LAP	73.0 ± 2.4	4.3 ± 1.2	18.0 ± 5.4	

* Experimental series No. 1–3 were carried out with cross-linked *S. aureus* cells (500 μl) and 500 μg sonicated PM vesicles. This treatment reduced the binding capacity of the cells. In series No. 4, 500 μl native *S. aureus* cells were used, accounting for the higher percent adsorption of LAP (73%). The immunoadsorption protocol and the enzyme activity determinations were modified in the following manner: for 5'-nucleotidase, the input of membrane was increased from 250 to 500 μg and the unbound fraction and the washes were first concentrated by ultracentrifugation (100,000 g, 60 min), resuspended to ~20% of their initial volume with PSS-BSA, and then assayed; for alkaline phosphatase, the bound activity was determined from the difference between the activity in the initial sample and the unbound fractions (i.e., initial-unbound [supernate + washes]), because the *S. aureus* cells had endogenous phosphatase activity. Enzyme recoveries were >85%.
‡ Average of three experiments ± SD.
§ (I − NI of enzyme) + (I − NI of LAP).

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 Sierakowska 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 immunoadsorbent. 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
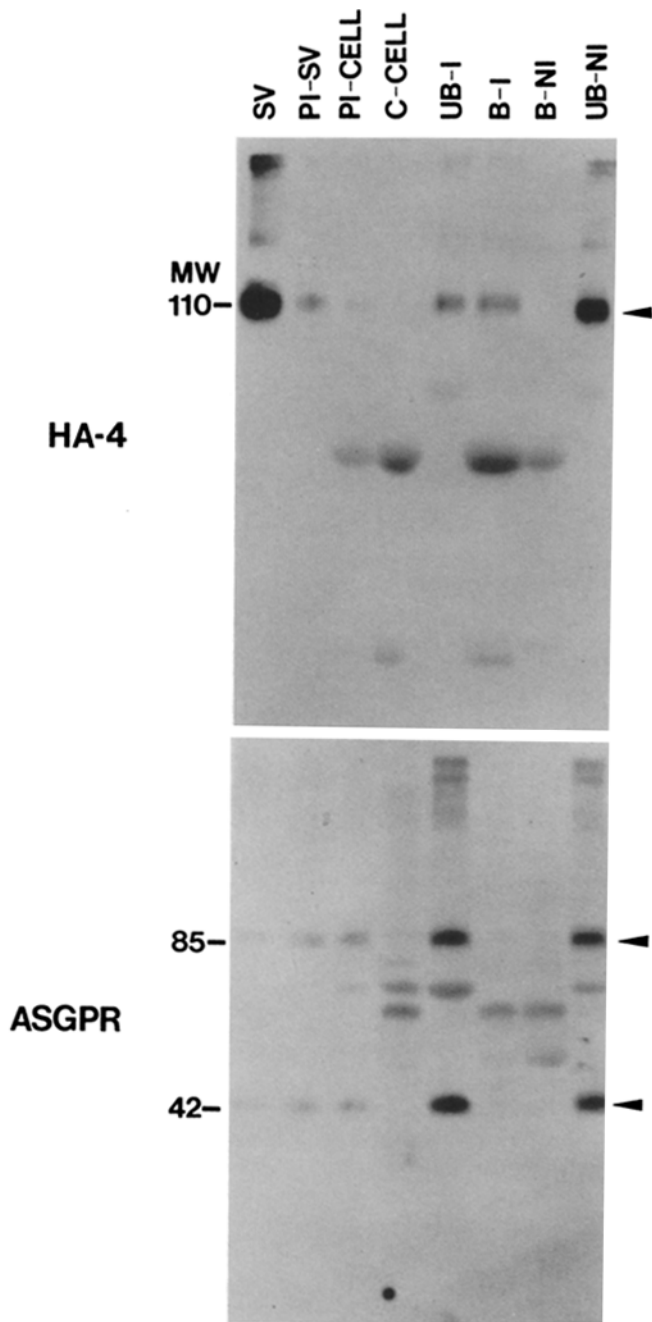
FIGURE 3 Distribution of two domain-specific antigens during immunoadsorption of BC membranes (immunoblots). Aliquots from each step of the immunoadsorption protocol were prepared for SDS PAGE, electrophoresed, transferred to nitrocellulose, and then incubated with specific antibodies to the BC antigen, 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-I, unbound fraction after incubation with immune anti-LAP-S. aureus (0.5); B-I, bound fraction-immune (0.2); B-NI, bound-nonimmune (0.2); UB-NI, 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-NI 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 (<3%) and sinusoidal front (~10%).[2]

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-

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[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 anticlathrin antibodies to isolate coated vesicles from porcine brain. More recently, Miljanich et al. (9) used polyacrylamide beads coated with antiserum directed against electric organ synaptic vesicles to isolate a membrane fraction enriched in "active zones" (the region of the nerve terminal plasma membrane where synaptic vesicles fuse).

We have used immunoadsorption to isolate BC membrane from hepatocyte PM. The technique is rapid, requiring ~4 h of total incubation time after the initial isolation of a PM fraction. The protocols followed are relatively simple, requiring only four solutions and a microcentrifuge. At present this technique is not preparative, since ~1% of the protein from a single PM preparation is used for one immunoadsorption, with ~75 μg of BC membrane recovered in the final fraction. However, it could easily be scaled up 10–100-fold. Nonetheless, in its present form, this technique is amenable to characterization of the BC membrane proteins, as we have 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.

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