Membrane microdomains in hepatocytes: potential target areas for proteins involved in canalicular bile secretion

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Abstract The formation of hepatic bile requires that water be transported across liver epithelia. Rat hepatocytes express three aquaporins (AQPs): AQP8, AQP9, and AQP0. Recognizing that cholesterol and sphingolipids are thought to promote the assembly of proteins into specialized membrane microdomains, we hypothesized that canalicular bile secretion involves the trafficking of vesicles to and from localized lipid-enriched microdomains in the canalicular plasma membrane. Hepatocyte plasma membranes were sonicated in Triton and centrifuged overnight on a sucrose gradient to yield a Triton-soluble pellet and a Triton-insoluble, sphingolipid-enriched microdomain fraction at the 5%/30% sucrose interface. The detergent-insoluble portion of the hepatocyte plasma membrane was enriched in alkaline phosphatase (a microdomain-positive marker) and devoid of amino-peptidase N (a microdomain-negative marker), enriched in caveolin, both AQP8 and AQP9, but negative for clathrin. The microdomain fractions contained chloride-bicarbonate anion exchanger isoform 2 and multidrug resistance-associated protein 2. Exposure of isolated hepatocytes to glucagon increased the expression of AQP8 but not AQP9 in the microdomain fractions. Sphingolipid analysis of the insoluble fraction showed the predominant species to be sphingomyelin. These data support the presence of sphingolipid-enriched microdomains of the hepatocyte membrane that represent potential localized target areas for the clustering of AQPs and functionally related proteins involved in canalicular bile secretion.—Tietz, P., J. Jefferson, R. Pagano, and N. F. LaRusso. **Membrane microdomains in hepatocytes: potential target areas for proteins involved in canalicular bile secretion. *J. Lipid Res.* 2005.** 46: **1426–1432.**

Supplementary key words aquaporins • transport • ions • solutes

Canalicular bile is formed by the osmotic filtration of water in response to osmotic gradients generated by active transport systems located at the apical and basolateral membranes of the hepatocyte. In recent years, considerable progress has been made in clarifying the molecular mechanisms of canalicular bile secretion because many of the transporters, channels, and exchangers involved have been cloned and functionally characterized (1). Impaired transport of key proteins into bile and the consequent loss of osmotic driving forces for bile secretion can result in cholestasis (1).

Work from our laboratory has described an important role for the water channel proteins, aquaporins (AQPs), in the transcellular transport of water during primary bile secretion by hepatocytes. Hepatocytes express three water channels (AQP8, AQP9, and AQP0) that are differentially localized and trafficked. In the basal state, AQP8 is present in intracellular vesicles; however, in response to a choleretic agonist, AQP8 is redistributed to the canalicular plasma membrane, where it facilitates the transport of water across the hepatocyte epithelial barrier in concert with AQP9, which is constitutively expressed on the basolateral plasma membrane (2). More recently, we reported that translocation of AQP8-containing vesicles to the apical membrane of hepatocytes is triggered by glucagon via a cAMP-dependent mechanism involving microtubules, data suggesting that this process plays a key role in hormone-induced canalicular bile secretion (3, 4).

The usefulness of detergent solubilization for isolating and characterizing integral membrane proteins was first described more than 30 years ago (5, 6). Detergents can also differentially solubilize membrane domains with different lipid and protein compositions (7–9). Differences in detergent solubility may arise for proteins with single as opposed to multiple membrane-spanning domains. The former are expected to fit better in tightly packed domains than are the latter, which require more flexibility for proper membrane accommodation. Triton-insoluble membrane domains are composed of sphingolipid-cholesterol-enriched regions arranged in a tightly packed, liquid-ordered state. The significance of this arrangement of packed lipids within a membrane, and the affinity of certain proteins and lipids for specific membrane regions or

Manuscript received 19 October 2004 and in revised form 15 February 2005 and in re-revised form 17 March 2005.

Published, JLR Papers in Press, April 16, 2005.
DOI 10.1194/jlr.M400412-JLR200

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domains, have been proposed to play important physiological roles in processes as diverse as cell surface signaling, cell adhesion and motility, and intracellular sorting (7, 10, 11). Detergent insolubility, specifically with Triton X-100, is accepted as one of several useful tools for studying cellular membranes and their characteristics.

Our objectives here were to isolate and characterize lipid-enriched microdomains in freshly isolated rat hepatocyte plasma membranes and to begin to explore their role in canalicular bile secretion. In the studies described, we used a variety of biochemical approaches and provide evidence that Triton-insoluble microdomains exist in hepatocyte plasma membranes, are enriched in caveolin, cholesterol, and sphingomyelin, and also contain AQP8 and AQP9, multidrug resistance-associated protein 2 (MRP2), and anion exchanger isoform 2 (AE2). In addition, glucagon increases the expression of AQP8 in the microdomain fraction. These data are consistent with the notion that specific microdomains present in the hepatocyte plasma membrane may be involved in canalicular bile secretion.

EXPERIMENTAL PROCEDURES

Isolation of hepatocytes and hepatocyte plasma membranes

Highly purified (>98%) rat hepatocytes were isolated as described previously (12). Briefly, livers were perfused with oxygenated HEPES buffer containing 0.02% EGTA (Sigma) to remove blood cells and then transferred to a temperature-controlled chamber at 37°C and perfused with HEPES buffer solution containing 0.45 mg/ml collagenase D (Roche Molecular Biochemicals). After perfusion, the hepatocytes were gently removed from the biliary tree by mechanical disruption and filtered twice through 40 μm nylon mesh. The hepatocytes were purified further by isopycnic centrifugation through a discontinuous Percoll gradient (Amersham Biosciences) and washed three times in Liebovitz's L-15 medium (Invitrogen). As confirmed previously, cell viability was greater than 90% as assessed by trypan blue exclusion, and polarity was maintained during the time of the experiments (2, 13, 14).

Plasma membranes were prepared from freshly isolated hepatocytes as described previously by us (13, 14). Briefly, hepatocytes were washed and sonicated in 0.3 M sucrose containing 0.01% soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM leupeptin (Sigma). The mixed plasma membrane fraction was obtained by centrifugation at 200,000 *g* for 60 min on a discontinuous 1.3 M sucrose gradient. The plasma membrane preparations for hepatocytes have been characterized extensively for marker and organelle studies by us (2).

Isolation of hepatocyte plasma membrane microdomains

Isolation of hepatocyte plasma membrane microdomains was performed by solubilization in nonionic detergent followed by separation by buoyant density centrifugation using a modification of methods applied in other epithelia (15). Hepatocyte mixed plasma membranes were lysed and solubilized by sonication in MES-buffered saline (25 mM MES and 150 mM NaCl, pH 6.5) containing 0.5% nonionic detergent (Triton X-100) at 4°C and adjusted to 40% (w/v) sucrose by the addition of 80% (w/v) sucrose. Aliquots (3 ml) of mixed plasma membrane were then layered under a 0–30% (w/v) sucrose gradient and centrifuged for 20 h at 140,000 *g*. This preparation yielded a Triton-soluble pellet in the 40% sucrose region and a low-density, Triton-insoluble band located at the 5%/30% sucrose interface, consistent with findings generated in other epithelia (15). The gradient was fractionated into 10 aliquots (1 ml each), and each fraction was assayed for protein content, opacity, and the activity of positive and negative microdomain marker enzymes.

Characterization of gradient fractionated hepatocyte plasma membranes

Each of the 10 fractions from the 0–30% sucrose gradient was assayed for total protein (Bio-Rad) and spectrophotometric absorbance at 620 nm (a measurement of microdomain-positive opacity). In addition, each fraction was assayed for alkaline phosphatase, an accepted microdomain-positive marker, using a commercially prepared kit (Sigma), and amino-peptidase N, an accepted negative marker (15), using a colorimetric method described previously by Goldbarg and Rutenburg (16).

Membrane lipid analysis

Total membrane cholesterol and phospholipids were measured spectrophotometrically on each of 10 fractions from the 0–30% sucrose gradient using commercially available kits (Wako Chemicals, Inc., Richmond, VA) according to the protocol supplied by the manufacturer.

Sphingolipid analysis

Lipid standards were obtained from Matrya, Inc. (Pleasant Gap, PA), and Calibochem. Silica gel G60 plates for thin-layer chromatography were purchased from Merck. Samples were prepared for lipid analysis by extraction as described previously (17–19). Fractions from the sucrose gradient were sonicated (5 min, room temperature), and an aliquot was removed for protein determination using Bradford analysis. The samples were then extracted with $CHCl_3/CH_3OH$/water/pyridine (60:30:6:1) for 48 h

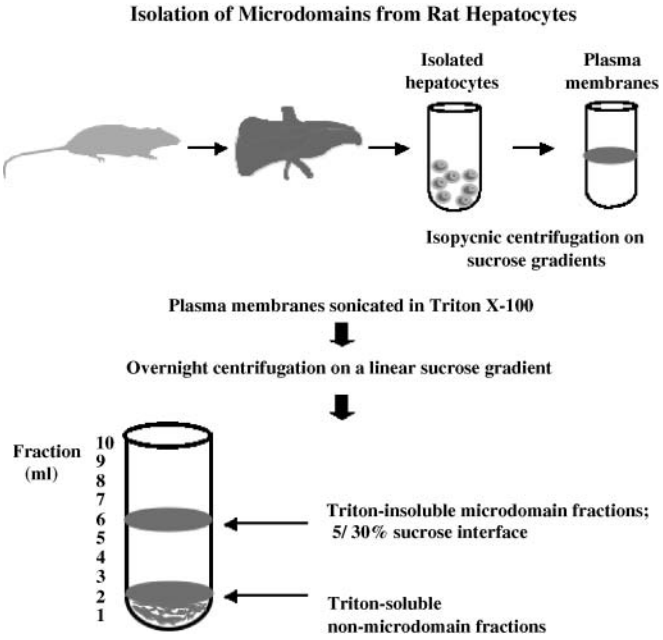

Fig. 1. Schematic methodology for the isolation of microdomains from rat hepatocytes. Rat hepatocytes were isolated and plasma membrane fractions were prepared using sucrose gradients. Plasma membranes were sonicated in Triton X-100 and centrifuged overnight to yield a Triton-soluble fraction and a Triton-insoluble microdomain fraction. Gradient fractions (1 ml each) from the 5–30% linear sucrose gradient were collected for characterization.

at 48°C in screw-top culture tubes. Insoluble particulates were removed by filtration through cotton with rinsing, and the pooled extracts were evaporated to dryness with N_2. The glycerolipids were saponified by dissolving the residue in CH_3OH (2.5 ml) with sonication (bath, 5 min, room temperature) followed by NaOH treatment (100 mM from 4 M stock for 2 h at 37°C) and neutralization with concentrated acetic acid (17 μl). The solvent was evaporated with N_2, and the lipids were dissolved in synthetic upper phase CH_3OH/water/$CHCl_3$ (94:96:6) and desalted by passage through a Waters Sep-Pak C18 column (Milford, MA). Columns were preequilibrated with 3 ml each of $CHCl_3$/CH_3OH (1:1), CH_3OH, and synthetic upper phase. After the sample was loaded, the columns were washed twice with water (2 ml) and eluted with CH_3OH (2 × 2 ml) and $CHCl_3$/CH_3OH (1:1) (2 × 2 ml). The eluting fractions were pooled, and the sphingolipids obtained were dried with N_2 and pumped under vacuum for 2 h. The residue was dissolved in a minimum volume of $CHCl_3$/CH_3OH (1:1) and chromatographed along with lipid standards on silica (G60) and developed in $CHCl_3$/CH_3OH/15 mM (aqueous) $CaCl_2$ (65:35:8). Primulin was used as a detection reagent (20), and lipids were quantified by scanning densitometry.

Immunoblotting of gradient fractionated hepatocyte plasma membranes

We performed immunoblotting using specific antibodies to caveolin-1 (a cholesterol binding protein associated with membrane microdomains of many polarized cells) and clathrin (a non-microdomain-associated protein); both antibodies were provided by Dr. Mark McNiven. In addition, we performed immunoblot analysis of each fraction for the water channel proteins AQP8, AQP9, and AQP0 using commercially available antibodies from Alpha Diagnostic International as described previously (2) and for AE2 (Santa Cruz) and MRP2 (Alexis Biochemicals). Fractions were exposed to electrophoresis on a 10% SDS-polyacrylamide gel and transferred overnight to nitrocellulose. After exposure to specific primary and secondary antibodies, protein bands were detected using an enhanced chemiluminescence detection system (ECL-Plus; Amersham Biosciences). In a separate set of experiments, freshly isolated hepatocytes were exposed to

TABLE 1. Hepatocyte membrane lipid composition

Gradient Fraction	Cholesterol		Phospholipid		Cholesterol-Phospholipid Ratio
	μmol/mg protein	%	*μmol/mg protein*	%	
1	0.38 ± 0.011	12.0	0.88 ± 0.06	13.6	0.43
2	0.22 ± 0.013	6.7	0.76 ± 0.01	11.8	0.28
3	0.13 ± 0.009	4.1	0.41 ± 0.03	6.4	0.31
4	0.09 ± 0.006	2.8	0.28 ± 0.02	4.3	0.32
5	0.69 ± 0.07	21.8	0.71 ± 0.07	11.0	0.97
6	0.83 ± 0.11	26.3	0.98 ± 0.11	15.2	0.84
7	0.41 ± 0.09	13.0	0.80 ± 0.09	12.4	0.50
8	0.15 ± 0.01	4.7	0.63 ± 0.08	9.7	0.23
9	0.09 ± 0.01	2.8	0.51 ± 0.06	7.9	0.17
10	0.17 ± 0.03	5.4	0.49 ± 0.03	7.6	0.34

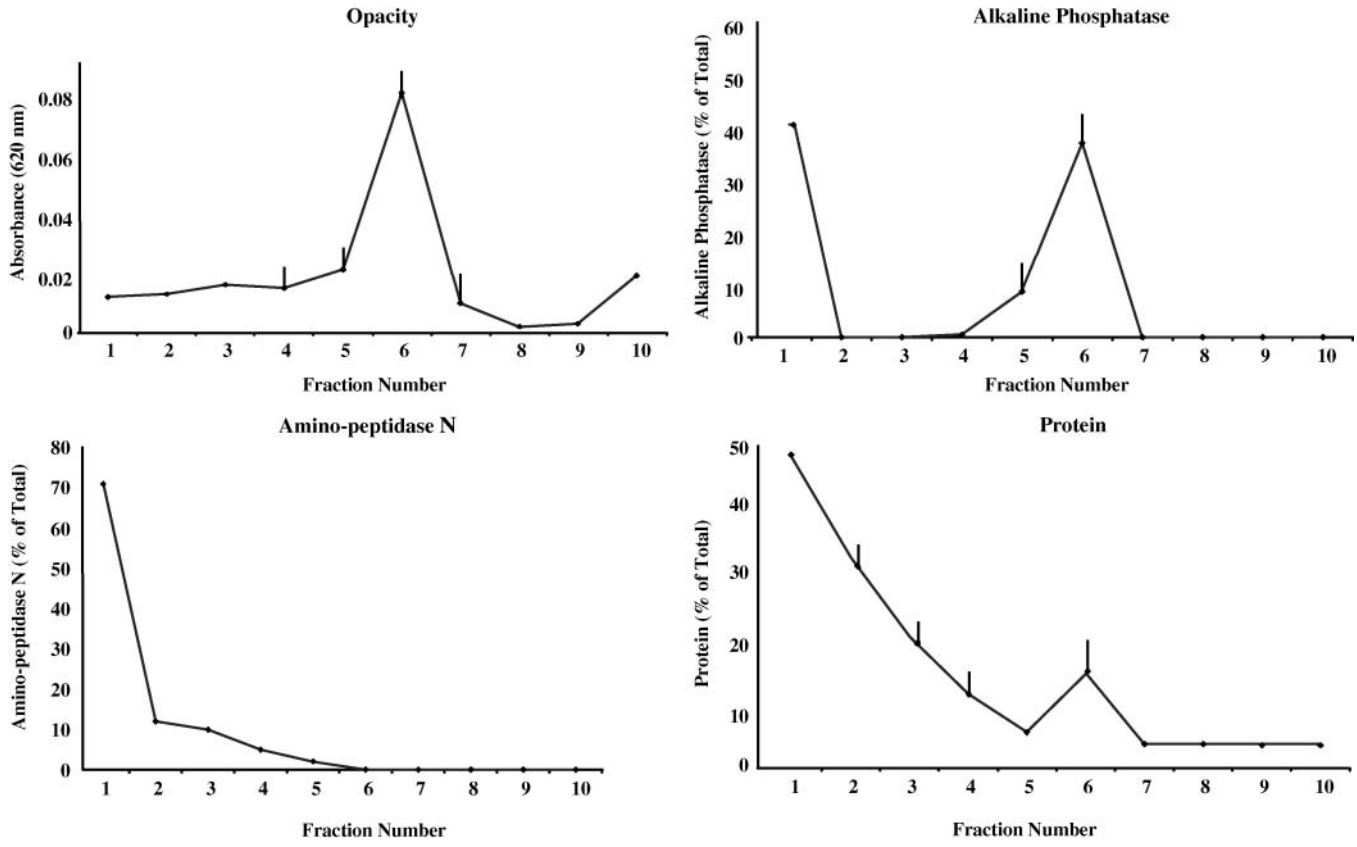

Fig. 2. Characterization of membrane microdomain fractions. Fractions of the 5–30% linear sucrose gradient (1 ml each) were collected and characterized by measuring the protein content, the opacity, and the activity of positive and negative marker enzymes. Fractions 5 and 6 represented 15 ± 3% of the total plasma membrane protein and showed an absorbance peak of 620 nm, indicating microdomain-positive opacity. Fractions 5 and 6 were enriched in alkaline phosphatase (a microdomain-positive marker) and devoid of amino-peptidase N (a microdomain-negative marker). Data are expressed as enzyme activity as a percentage of total activity (means ± SEM; n = 3).

TABLE 2. Quantitative analysis of sphingolipids

Lipid Class	Raft	Nonraft
Ceramide	21.8 ± 10	9.5 ± 3.7
Monohexoside	24.2 ± 2.4	34.0 ± 15
Dihexoside	23.5 ± 9.6	7.3 ± 4.6
Globoside	6.4 ± 1.4	4.0 ± 3.1
Sphingomyelin	18.3 ± 4.9	2.9 ± 1.4[a]
Ganglioside	10.0 ± 0.7	2.08 ± 1.8[a]

Values shown are μg lipid/mg protein (means ± SEM; n = 3).
[a] $P < 0.05$, raft versus nonraft.

1 μmol/l glucagon (Eli Lilly, Indianapolis, IN) for 10 min at 37°C before subcellular fractionation and isolation of microdomains. Immunoblot analysis was performed for AQP8 and AQP9 on the gradient fractions.

RESULTS

Rat hepatocyte plasma membrane microdomains were fractionated after exposure to Triton flotation on sucrose gradients and characterized using biochemical marker assays and immunoblotting according to published protocols for plasma membrane (2, 13, 14) and microdomain (15) isolation. The scheme is displayed in (**Fig. 1**). Ten fractions (1 ml each) were collected from the bottom of the gradient (fraction 1) to the top of the gradient (fraction 10). The gradient revealed a Triton-soluble pellet that we term the "nonmicrodomain" component in the 40% sucrose region and a low-density, Triton-insoluble, lipid-enriched microdomain band (fractions 5 and 6) located at the 5%/30% sucrose interface. Each fraction of the gradient (from 1 to 10) was assayed for protein content, opacity, and the activity of positive (alkaline phosphatase) and negative (amino-peptidase N) microdomain marker enzymes. As shown in **Fig. 2**, fractions 5/6 showed an absorbance peak of 620 nm, indicating positive opacity, were enriched in alkaline phosphatase (a positive marker), and were devoid of amino-peptidase N (a negative marker). The Triton-insoluble, lipid-enriched microdomain fractions represented ~15 ± 3% of the total plasma membrane protein.

Table 1 shows the results of membrane lipid analysis for each of the 10 gradient fractions. The cholesterol content and cholesterol-phospholipid ratio were highest in fractions 5/6 (representing the putative microdomains). The cholesterol-phospholipid ratios of 0.97 and 0.84, in fractions 5 and 6, respectively, are characteristic of a very stiff membrane and one of the important parameters affecting membrane fluidity. **Table 2** shows a quantitative sphingolipid class analysis of the raft versus nonraft fractions. One of the predominant sphingolipid subspecies and the hallmark of microdomains, sphingomyelin, was enriched 6-fold in the raft versus nonraft fractions. To date, no similar quantitation exists in hepatocytes or other epithelial cells.

Figure 3 shows a densitometric analysis of thin layer-chromatography of combined fractions 5/6 with a predominant peak identified as sphingomyelin by a comparative relative mobility of lipid standards.

Shown in **Fig. 4** are comparative immunoblots of the gradient fractions 1–10 for caveolin, clathrin, AQP0, AQP8, and AQP9, AE2, and MRP2. Bands for caveolin, a cholesterol binding protein associated with membrane microdomains, were observed at the appropriate molecular mass of ~22 kDa in fractions 2 and 5/6, indicating its presence in the plasma membrane and its enrichment in the microdomain fractions. The immunoblot for clathrin

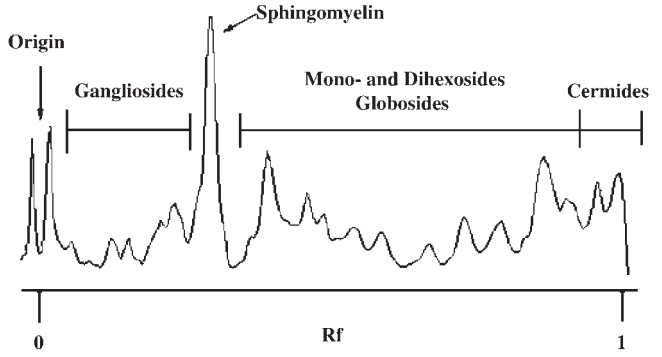

Fig. 3. Sphingomyelin is the predominant sphingolipid in hepatocyte microdomains. Sphingolipids were extracted from isolated microdomain fractions and chromatographed along with lipid standards on thin-layer silica gel plates as described in Experimental Procedures using $CHCl_3/CH_3OH/15$ mM $CaCl_2$ (65:35:8) as the developing solvent. Bands were visualized with primulin, and the thin-layer chromatograph was quantified by scanning densitometry under ultraviolet irradiation. Quantitative data in Table 2 are expressed as means ± SEM (n = 3). R_f, relative mobility.

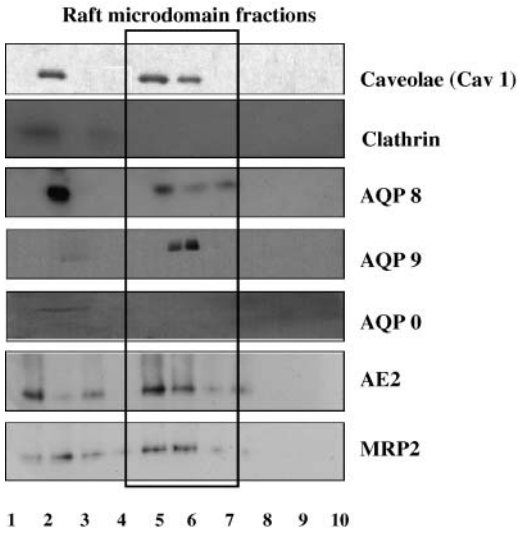

Fig. 4. Hepatocyte microdomains are enriched in caveolin, aquaporins 8 and 9 (AQP8 and AQP9), anion exchanger isoform 2 (AE2), and multidrug resistance-associated protein 2 (MRP2). Immunoblots were performed on each of the 1 ml fractions collected from the sucrose gradient to isolate microdomains. Each fraction was run on a 10% SDS-PAGE gel, exposed to specific primary and secondary antibodies, and visualized using ECL. Corresponding to fractions 5 and 6, which displayed the microdomain-positive markers, we did not observe a band for AQP0 or clathrin.

(a non-microdomain-associated protein) was negative in all fractions of the gradient. In the blots for both AQP8 (34 kDa) and AQP9 (28 kDa), bands were observed corresponding to the microdomain fractions. AQP0 was present in the gradient pellet but was not observed in the microdomain fractions. Bands for AE2 and MRP2 were also observed at their appropriate molecular masses and enriched in the microdomain fractions. These data support the concept that water channel proteins (AQPs) associate with or cluster in a sphingolipid-enriched microdomain portion of the membrane and may also cluster with other functionally associated proteins that, together, provide the solute and ions to facilitate the production of bile.

Figure 5 shows comparative immunoblots for the distribution and expression of both AQP8 and AQP9 in the microdomain fractions isolated in the basal state and after pretreatment of hepatocytes with glucagon. The immunoblot fractions were analyzed by densitometric scanning, and the relative densitometry units of fractions 5 and 6 (representing the microdomain-associated fractions) were pooled, expressing the basal condition as 100%. After treatment of hepatocytes with glucagon, AQP8 (an agonist-regulated AQP) was increased in the microdomain fractions (5 and 6) by 3.7-fold compared with the same fractions in the basal condition, whereas AQP9 (nonregulated) was unchanged.

DISCUSSION

The key finding reported here relates to the isolation and characterization of sphingolipid-enriched microdomains of the plasma membrane of rat hepatocytes. We found that regions of the hepatocyte plasma membrane fractionated by isopycnic centrifugation had the following characteristics: *i*) Triton insolubility; *ii*) enrichment in cholesterol and sphingomyelin; *iii*) the presence of caveolin; *iv*) contained the water channel proteins AQP8 and AQP9 as well as AE2 and MRP2; and *v*) exposure of hepatocytes to glucagon increased the expression of AQP8 in the microdomain-associated fractions. Collectively, these data support the notion that there are distinct membrane microdomains on the plasma membrane of hepatocytes, to which functionally related "flux proteins" (14) for water, ions, and solutes involved in canalicular bile secretion may be targeted and physically clustered.

Our data are in general agreement with those in model systems from several other cell types, including cultured MDCK cells, fibroblasts, cerebral cortex, and intestinal epithelia (8, 15). In each case, the low-density, detergent-insoluble membrane fraction was strongly opaque at 620 nm, with the bulk of the protein in the soluble regions of the gradient and detergent-insoluble fractions containing the putative microdomains, which constitute 6–18% of the protein yield. As in our results, amino-peptidase N (a non-

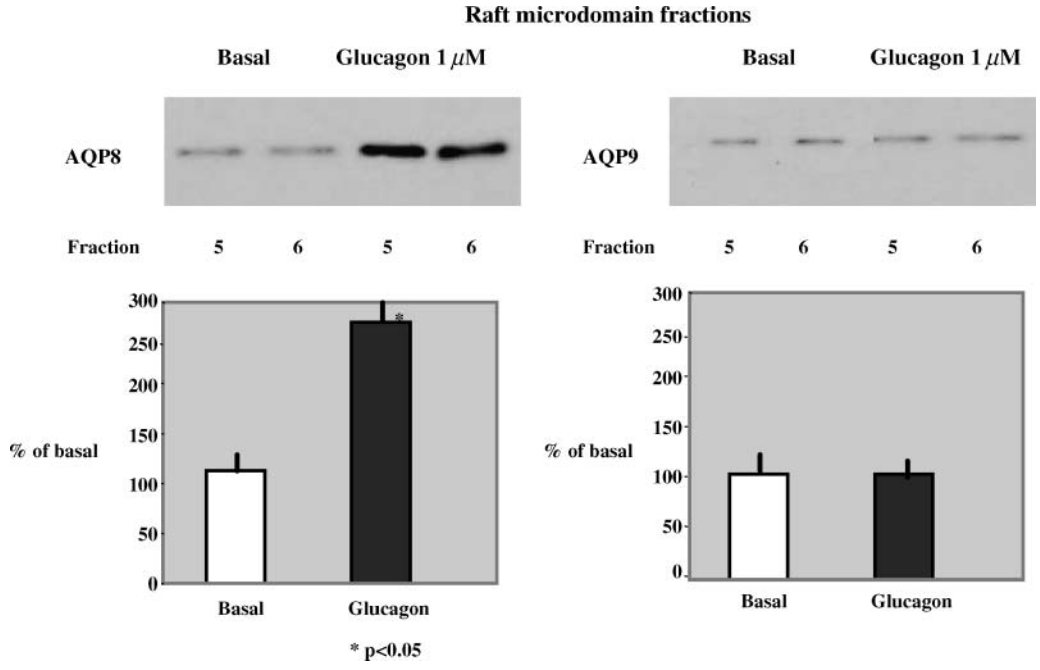

Fig. 5. Glucagon increases the localization and expression of AQP8 in plasma membranes and microdomain fractions. Rat hepatocytes were isolated, and half of the cells were exposed to 1 μmol/l glucagon and incubated at 37°C for 10 min. Hepatocyte plasma membranes and microdomain fractions were prepared as described in Experimental Procedures. Representative immunoblots for fractions of the microdomain gradient in both the basal and glucagon conditions are shown as well as relative densitometric analysis for both AQP8 and AQP9. The mean densitometry of fractions 4 and 5 (representing the microdomain fractions) of the basal condition (control, 100%) is plotted versus glucagon treatment. To obtain sufficient protein for the immunoblots, nonmicrodomain fractions from the gradient were pooled. Each lane was loaded with 30 μg of protein. Data represent three separate experiments and are expressed as means ± SEM. * $P < 0.05$ for the glucagon effect.

microdomain protein) in each study was found exclusively in the first few fractions of the sucrose gradients. In addition, we have extended these findings by making the observation that the water channel proteins AQP8 and AQP9, AE2, and MRP2 are enriched in the sphingolipid-enriched microdomain fractions of the plasma membrane, consistent with their role in regulated canalicular bile flow. Our observation that caveolin is present in the microdomain fraction is reasonable based on the known interaction of caveolae and sphingolipids. Caveolae, the flask-shaped invaginations of the plasma membrane that contain the coat protein caveolin, have been implicated in cell signaling and the uptake of small molecules, and they function as signal transduction centers. The sphingolipid-rich microdomain portion of a membrane forms an annulus around the neck of the caveolae, thereby implying a direct association between the two and substantiating a potential role in targeting at the canalicular membrane of hepatocytes.

Our data showed the highest cholesterol and phospholipid values in the detergent-insoluble fractions of the gradient, supporting the concept that these microdomains have an ordered structure. The cholesterol-phospholipid ratio of nearly 1.0 observed in these same fractions of the hepatocyte plasma membrane represents a stiff portion of the membrane, a biophysical property that should facilitate the clustering of specific molecules in these regions.

The clustering of cholesterol, glycosphingolipids, glycosylphosphatidylinositol-anchored proteins, and other proteins in distinct domains within membranes facilitates molecular interactions and provides potential target regions by bringing molecules into close association and/or increasing the local concentration of specific molecules that are required for specialized cellular functions. Sphingomyelin was the most abundant sphingolipid subspecies found in the microdomain fraction; these are the first detailed data characterizing phospholipids in hepatic epithelia and confirming the prominence of sphingomyelin, a microdomain characteristic lipid.

In two recent studies by separate investigators (21, 22), the model of cultured polarized hepatocytes is described as an unresolved model in which to study lipid microdomains and their role in the trafficking of proteins to the apical membrane. Hepatocytes are described to represent a case in which distinct lipid microdomains operate in both the direct and indirect apical transport of resident proteins (21, 22). To date, sorting principles and polarized trafficking have been largely described in MDCK cells. Although these cells are abundant and easy to work with, the extrapolation of these findings to water transport in hepatocytes should be regarded with caution.

We found that AQP8 and AQP9 were present primarily in fractions 5 and 6, those regions of the hepatocyte plasma membrane that were Triton-insoluble, that contained a very high cholesterol-phospholipid ratio and caveolin, and that were enriched in sphingomyelin. These novel observations are important because they are the first data in any cell type that show AQPs to be localized to specific regions of the plasma membrane, a finding compatible with our hypothesis that specific regions of the plasma membrane may serve as target areas for the insertion (i.e., AQP8) or constitutive expression (i.e., AQP9) of proteins involved in canalicular bile secretion. The observation that glucagon caused an increase in the expression of AQP8 in the Triton-soluble, nonmicrodomain fractions (1–3) supports and complements our previous findings related to the hormone-regulated membrane insertion of AQP8 in hepatocytes (4) and the observation that glucagon induces a redistribution of AQP8 from intracellular vesicles to the plasma membrane (3). The observation that AQP8 (but not AQP9) is significantly and specifically increased in the sphingolipid-enriched microdomain-associated fractions (5 and 6) after exposure to glucagon is novel and extends the hypothesis that canalicular bile secretion results in part from agonist-induced insertion and clustering of AQP8 into specific regions or microdomains of the plasma membrane.

To extend our findings, we observed AE2 and MRP2 in the microdomain fraction. It is reasonable to speculate that the agonist-responsive vesicles in hepatocytes that contain AQP8 likely contain other molecules, such as AE2 and MRP2, necessary to establish the osmotic gradients that drive the passive movement of water, because both proteins participate in bile secretion and their targeting can be stimulated by cAMP (23, 24, 25). In addition to genetic defects, the study of the normal and altered trafficking of hepatocellular transport systems and the role of lipid-enriched microdomains involved in bile secretion may provide a molecular correlate for the functional changes that occur in cholestasis.

This work was supported by National Institutes of Health Grant DK-24031 and by the Mayo Foundation.

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