Characterization of Proteins in Detergent-Resistant Membrane Complexes from Madin-Darby Canine Kidney Epithelial Cells†

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ABSTRACT: We previously isolated detergent-resistant membrane complexes (DRMs) that were not solubilized after extraction of Madin-Darby canine kidney cells with Triton X-100 on ice. The complexes were rich in glycosphingolipids, cholesterol, and glycosylphosphatidylinositol (GPI)-anchored proteins. In this study, we examined the protein composition of DRMs and further characterized the detergent solubility of these structures. Eight to ten cell-surface proteins, including proteins from both apical and basolateral membranes, were recovered in DRMs. Most DRM proteins, however, were not exposed to the surface of whole cells, and we did not detect the complex of cell-surface proteins described by Sargiacomo et al. in a similar study [Sargiacomo, M., et al. (1993) *J. Cell Biol. 122*, 789−807]. Almost all proteins in DRMs were solubilized by Triton X-100 at temperatures above 30 °C or by octyl glucoside on ice. In contrast, a GPI-anchored protein, placental alkaline phosphatase, was mostly solubilized by Triton X-100 after extraction at 10 °C. This protein was insoluble in ice-cold Triton X-100 when first delivered to the plasma membrane and remained so for at least 6 h after synthesis. A fraction of the lipids in DRMs remained insoluble after extraction with Triton X-100 at 37 °C. DRM lipids were not solubilized by octyl glucoside, suggesting that this detergent selectively extracts proteins from DRMs.

Although most membrane proteins and lipids are solubilized by nonionic detergents (Helenius & Simons, 1975), detergent-resistant membrane complexes (DRMs)[1] can be recovered from lysates of mammalian cells. The first of these to be described in detail were isolated from Madin-Darby canine kidney (MDCK) epithelial cells. These complexes were found to contain proteins anchored in membranes by glycosylphosphatidylinositol (GPI) and were rich in glycosphingolipids and cholesterol (Brown & Rose, 1992). Similar DRMs have now been isolated from a number of cell types (Cerneus et al., 1993; Chang et al., 1994; Cinek & Horejsí, 1992; Dráberová & Dráber, 1993; Fiedler et al., 1993; Garcia et al., 1993; Lisanti et al., 1994; Rodgers et al., 1994; Sargiacomo et al., 1993; Shenoy-Scaria et al., 1994).

DRMs probably exist in cell membranes as glycosphingolipid-rich patches or domains. These domains may play important roles both in intracellular sorting and in events at the cell surface. Glycosphingolipids can self-associate in membranes (Thompson & Tillack, 1985) and are preferentially delivered to the apical surface of epithelial cells (van Meer et al., 1987). On the basis of these observations, van Meer and Simons proposed that glycosphingolipids and apically directed proteins associate into clusters or "rafts" in the trans Golgi network (TGN), the site of sorting in many epithelial cells (Simons & van Meer, 1988; Simons & Wandinger-Ness, 1990). According to this model, the rafts are incorporated into apical transport vesicles. As we found that a GPI-anchored protein traveling along the secretory pathway first associated with DRMs in the Golgi apparatus (Brown & Rose, 1992), the rafts proposed by van Meer and Simons may be isolated as DRMs.

Glycolipid-rich domains are also present in the plasma membrane. DRMs isolated from fibroblasts and smooth muscle cells resemble non-clathrin-coated plasma membrane invaginations called *caveolae*, in that both are rounded structures of about 50 nm in diameter (Chang et al., 1994; Lisanti et al., 1994). An acidic glycosphingolipid, the ganglioside GM1, is concentrated in caveolae (Parton, 1994). In addition, DRMs from these cells are heavily labeled by antibodies directed against the 22-kDa protein caveolin, a component of the caveolar coat (Chang et al., 1994). Thus, in these cells, glycolipid-rich domains appear to be organized into caveolae.

The structure and function(s) of caveolae are not well understood. Some evidence suggests that they internalize from the plasma membrane to form endocytic vesicles, while other studies conclude that they remain at the cell surface (Rothberg et al., 1992; Severs, 1988). Recent reports clearly show that internalization can occur in at least some cell types and that internalized caveolae can fuse with organelles of the conventional endocytic pathway in fibroblasts (Parton et al., 1994). Alternatively, vesicles derived from caveolae can be transported across endothelial cell monolayers by transcytosis (Schnitzer et al., 1994). An additional process mediated by caveolae, termed potocytosis, may be involved

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[1] Abbreviations: DRM, detergent-resistant membrane; GPI, glycosylphosphatidylinositol; MDCK, Madin-Darby canine kidney; TGN, trans Golgi network, l_o, liquid-ordered; PLAP, placental alkaline phosphatase; HP-TLC, high-performance thin-layer chromatography; s-NHS-biotin, sulfo-*N*-(hydroxysuccinimido)biotin; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PI-PLC, phosphatidylinositol-specific phospholipase C; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonate; octyl glucoside, *n*-octyl β-D-glucopyranoside; FRT, Fischer rat thyroid.

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in the uptake of small molecules into the cytoplasm (Anderson et al., 1992). Both kinases (Smart et al., 1994a) and phosphatases (Parton et al., 1994) regulate the structure of caveolae, and caveolar structure is also affected by depletion (Rothberg et al., 1990) or oxidation (Smart et al., 1994b) of membrane cholesterol.

Several signaling proteins have been isolated in DRMs or localized in caveolae. A cell-surface IP3 receptor (Fujimoto et al., 1992), a plasmalemmal calcium channel (Fujimoto, 1993), and an endothelin receptor (Chun et al., 1994) are present in caveolae. The β_2-adrenergic receptor moves to these structures after ligand binding (Raposo et al., 1989) or antibody-mediated cross-linking (Dupree et al., 1993) and may be internalized in caveolae (Raposo et al., 1989). α subunits of heterotrimeric G proteins (Chang et al., 1994; Sargiacomo et al., 1993) and some nonreceptor tyrosine kinases of the Src family (Arreaza et al., 1994; Cinek & Horejsí 1992; Dráberová & Dráber, 1993; Sargiacomo et al., 1993; Shenoy-Scaria et al., 1994; Thomas & Samelson, 1992) are found in DRMs, though they have not been localized to caveolae in intact cells. In hematopoietic cells, Src-family kinases may be functionally linked to cell-surface GPI-anchored proteins [Stefanová et al. (1991); reviewed in Robinson (1991) and Brown (1993)]. These studies suggest that the association of these proteins with glycolipid-rich domains may be important in signaling.

DRMs can also be isolated from cells such as lymphocytes (Fra et al., 1994) and neuroblastoma cells (Gorodinsky et al., 1994) that do not contain morphologically recognizable caveolae. In addition, electron microscopic studies suggest that caveolae are rare or nonexistent in the apical membrane of MDCK cells (R. Parton, personal communication), although DRMs derived from this membrane can be isolated. Thus, glycolipid-rich domains that are not present in caveolae can still be isolated from cell lysates as DRMs. This conclusion is also supported by the observation that MDCK cell DRMs are generally much larger than caveolae and can reach almost 1 μm in diameter (Brown & Rose, 1992; Sargiacomo et al., 1993).

We previously extracted liposomes with Triton X-100 to determine how detergent-resistant proteins and lipids associate with each other, and what causes their surprising insolubility. Lipids with saturated chains and high melting temperatures were the most detergent-resistant (Schroeder et al., 1994). A condensed or liquid-ordered (l_o) phase can exist in equilibrium with the liquid-crystalline phase in artificial liposomes that are rich in cholesterol (Sankaram & Thompson, 1991). This suggested that DRMs might be present in the l_o phase. We found that liposomes with a lipid composition similar to that in DRMs from cells had the same "fluidity" as bona fide l_o phase liposomes. A GPI-anchored protein was not solubilized from the DRM-like liposomes, even in the absence of other proteins (Schroeder et al., 1994). On the basis of these findings, we proposed that sphingolipid-rich l_o phase domains exist in the membranes of eukaryotic cells and that acyl chain interactions underlie the clustering of these molecules and their detergent resistance (Schroeder et al., 1994). GPI-anchored proteins generally have saturated acyl chains (McConville & Ferguson, 1993) and might be expected to partition into a detergent-resistant l_o phase.

Glycosphingolipid-rich membrane domains may thus play a role in processes as diverse as intracellular sorting and cell-surface signaling. Understanding how the organization of proteins and lipids into specialized domains facilitates these processes requires a thorough characterization of these molecules. In this study, we examined the proteins and lipids in DRMs from MDCK cells and compared the solubilization behavior of the proteins and lipids in the membranes.

EXPERIMENTAL PROCEDURES

Materials. MDCK strain II cells (Arreaza et al., 1994; Brown & Rose, 1992) and cloned lines of these cells stably expressing human placental alkaline phosphatase (PLAP) (Brown et al., 1989) were maintained as previously described (Arreaza et al., 1994). Antibodies to $p62^{yes}$ (Yes) (Sudol & Hanafusa, 1986) and PLAP were as previously described (Arreaza et al., 1994; Brown & Rose, 1992). Rabbit antiserum against the heterotrimeric G protein $G_{i\alpha}$ was the gift of Dr. Craig Malbon (SUNY, Stony Brook) (Watkins et al., 1987). Mouse monoclonal anti-caveolin antibodies were obtained from Transduction Laboratories (Lexington, KY). High-performance thin-layer chromatography (HP-TLC) plates were from Merck. Transwell filters (Brown et al., 1989) were from Costar (Cambridge, MA). Streptavidin bound to agarose beads and sulfo-*N*-(sulfosuccinimido)biotin (s-NHS-biotin) were from Pierce (Rockford, IL). The enhanced chemiluminescence (ECL) reagent and horseradish peroxidase-conjugated streptavidin (HRP-streptavidin) were from Amersham (Arlington Heights, IL). EXPRE^{35}S^{35}S protein labeling mix (>1000 Ci/mmol; referred to as "[^{35}S]-methioinine") was from New England Nuclear (Boston, MA). Recombinant *Bacillus cereus* PI-PLC was purified from overexpressing *Escherichia coli* (the gift of J. Volwerk) according to Koke et al. (1991) as described (Arreaza & Brown, 1995).

Preparation of Detergent-Resistant Membranes. The procedure was as previously reported (Arreaza et al., 1994) except that the lysis buffer and all sucrose gradient solutions contained 0.1 M sodium carbonate at pH 11 unless otherwise indicated.

Re-extraction of DRMs. In some experiments, pelleted membranes were resuspended in 1 mL of TNE with a 25-gauge needle and syringe and re-extracted for 30 min under various conditions. Lysates were then subjected to centrifugation for 15 min at top speed in a microfuge (for analysis of proteins in the soluble and insoluble fractions) or were diluted to 35 mL and subjected to centrifugation at 100000*g* for 1 h (for analysis of lipids in the insoluble fraction).

Separation of Detergent-Soluble and -Insoluble PLAP. Cells expressing PLAP were labeled using a pulse−chase protocol and then lysed on ice for 20 min at pH 6.2 in Mes−buffered saline [MBS (Sargiacomo et al., 1993)] with 1% Triton X-100 or buffer D (50 mM Tris-HCl, pH 8, 62.5 mM EDTA, 0.4% deoxycholate, 1% Triton X-100). PLAP was recovered by immunoprecipitation from detergent-soluble and -insoluble fractions (Brown & Rose, 1992).

Protein Labeling. Metabolic labeling of cells to steady-state or by a pulse−chase protocol was as previously described (Brown & Rose, 1992). To biotinylate all DRM proteins, 0.02 mg/mL s-NHS-biotin in 0.5 mL of TEA buffer (10 mM triethanolamine, pH 9; 2 mM $CaCl_2$; 125 mM NaCl) or phosphate-buffered saline (PBS) (Brown & Rose, 1992) was added to the detergent phase after Triton X-114 phase partitioning (see below, Analysis of DRM Proteins) and

incubated for 30 min on ice. Biotinylation was quenched by the addition of ammonium chloride to 8.3 mM before precipitation of proteins with trichloroacetic acid. Biotinylation of cell-surface proteins on cells grown on plastic (Lisanti et al., 1988) or filters (Lisanti et al., 1988) was performed twice in TEA buffer or PBS for 15 min each time.

To determine whether PLAP was detergent-insoluble when first delivered to the plasma membrane, we subjected cells expressing PLAP to a published procedure (le Bivic et al., 1989) involving pulse labeling of proteins, surface biotinylation at various times of chase, and sequential immunoprecipitation and absorption of PLAP on streptavidin−agarose beads. Triton-soluble and -insoluble fractions were separated before immunoprecipitation.

Analysis of DRM Proteins. Except in the re-extraction experiments described above, DRMs were solubilized in buffer containing 1% Triton X-114 and subjected to phase partitioning (Bordier, 1981). Proteins in the detergent phase were then mixed with 1 mL of TNE and precipitated with 15% trichloroacetic acid. Precipitates were washed with acetone and then diethyl ether to solubilize all lipids, solubilized in gel loading buffer, and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (Laemmli, 1970) on gels containing 11% acrylamide. Precipitates appeared to dissolve completely in the gel loading buffer. Metabolically labeled proteins were detected by fluorography (Bonner & Laskey, 1974). Blots containing proteins transferred to nitrocellulose were incubated with HRP−streptavidin or with specific antibodies and then HRP-conjugated secondary antibodies as appropriate. Proteins were detected by ECL. Blots to be reprobed were stripped as described (Arreaza et al., 1994).

GPI-anchored proteins were identified by partitioning into the Triton X-114 aqueous phase after treatment for 1 h with 10 units/mL PI-PLC (Lisanti et al., 1988). Proteins in both phases were precipitated with trichloroacetic acid before SDS−PAGE analysis.

Lipid Analysis. DRMs were re-extracted in detergent, and insoluble material was collected by centrifugation, as described above under *Re-extraction of DRMs*. Pelleted lipids were extracted and analyzed by quantitative thin-layer chromatography (Brown & Rose, 1992), except that only neutral lipids were analyzed. Charred lipids were quantitated by scanning densitometry and comparison to standards on the same plate (not shown), using a Bio-Rad GS-670 imaging densitometer. Several aliquots of each sample were analyzed to ensure that all values fell on the standard curve.

RESULTS

Proteins in DRMs. We used two different techniques to determine the total profile of integral membrane proteins in DRMs. The first method was to label cells metabolically to steady state with [^{35}S]methionine prior to formation of the complexes. Alternatively, proteins in DRMs were labeled by biotinylation after solubilization at 37 °C. Both techniques would be expected to label all DRM proteins. DRMs were isolated as described in Experimental Procedures, and proteins were separated by SDS−PAGE. [^{35}S]methionine-labeled proteins were visualized by fluorography (Figure 1, lane 1), whereas biotinylated proteins were transferred to nitrocellulose and visualized with HRP−streptavidin (Figure 1, lane 2). About 20−25 major proteins were detected by each method. As expected, the two patterns were very similar.

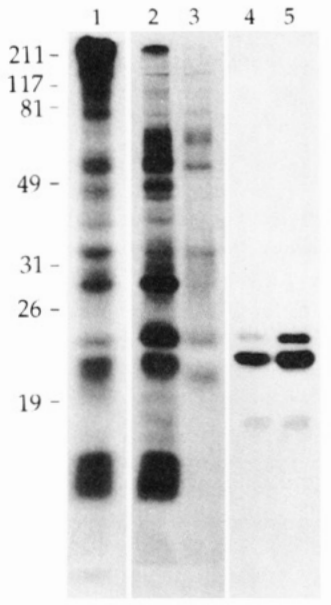

FIGURE 1: Detection of proteins in DRMs. Lane 1: DRMs were prepared from the lysate of MDCK cells in one 100-mm dish after metabolic labeling with 50 μCi/mL of [^{35}S]methionine for 18 h, pooled with the lysate of one 100-mm dish of unlabeled cells as a carrier. Lane 2: DRMs from cells in two 100-mm dishes were subjected to biotinylation after solubilization. Lane 3: DRMs were prepared from cells in two 100-mm dishes after surface biotinylation. All lanes were from the same gel. Positions of molecular weight standards are indicated. Lane 1 was removed and subjected to fluorography. Lanes 2 and 3 were transferred to nitrocellulose, and biotinylated proteins were detected with HRP−streptavidin. Lanes 4 and 5: The blot was then stripped and reprobed with antibodies to caveolin.

To determine how many of these proteins were present on the surface of intact cells, surface-exposed proteins were labeled with the membrane-impermeant biotinylation reagent s-NHS-biotin before preparation of DRMs. Blotted proteins were detected with HRP−streptavidin, as shown in Figure 1, lane 3. (High molecular weight proteins in this profile and that of total biotinylated proteins are seen more clearly in Figure 4, lanes 3 and 4.) Comparison of lanes 2 and 3 in Figure 1 revealed that many major DRM proteins could not be labeled by biotinylation from the surface. These proteins might be localized to intracellular membranes or might be present on the cytoplasmic face of the plasma membrane. The two patterns cannot be compared quantitatively, as the conditions used to biotinylate total and cell-surface proteins were different. Major surface-exposed proteins detected reproducibly in this and other experiments have molecular masses of about 20, 25, 32, 58, 74, 83, 100, and 200 kDa.

Previously, we found that the yield of DRMs varied between preparations. We speculated that this resulted from nonspecific adherence of DRMs to high-density detergent-insoluble material, causing them to pellet in the sucrose gradient (Brown & Rose, 1992). In an attempt to minimize this effect, extractions were routinely performed in the presence of alkaline carbonate (pH 11) to disrupt protein−protein interactions. Although the yield of membranes was generally greater under these conditions than when the extraction was performed under mildly acidic conditions, there was no qualitative difference in the profile of either

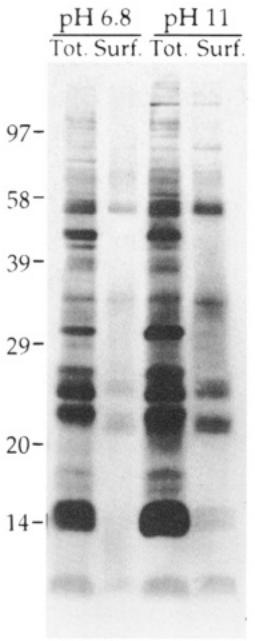

FIGURE 2: Effect of extraction pH on recovery of proteins in DRMs. DRMs were prepared by extraction at pH 6.8 in MBS containing 1% Triton X-100 (lanes 1 and 2) or at pH 11 as in Experimental Procedures (lanes 3 and 4). s-NHS-biotin was added to intact cells (lanes 2 and 4) or to DRMs after solubilization (lanes 1 and 3). Samples from cells in two 100-mm dishes were applied to each lane. Proteins were separated by SDS−PAGE and transferred to nitrocellulose. Biotinylated proteins were detected with HRP−streptavidin.

surface-exposed or total proteins in the DRMs (Figure 2, compare lanes 1 and 2 with 3 and 4).

PLAP Is a Major Cell-Surface Protein of DRMs. DRMs from MDCK cells stably expressing the GPI-anchored PLAP contained a major 67-kDa protein that was not present in complexes isolated from untransfected cells (Figure 3A). This protein could be depleted from solubilized DRMs with specific antibodies, showing that it was PLAP (Figure 3B). These data show that PLAP was one of the most abundant cell-surface proteins in DRMs prepared from cells expressing the protein.

Membrane Polarity of Cell-Surface DRM Proteins. Most plasma membrane proteins in epithelial cells are restricted to either the apical or basolateral surface (Simons & Fuller, 1985). We used apical or basolateral domain-specific biotinylation of cells grown on polycarbonate filters (Lisanti et al., 1988) to determine the polarity of cell-surface DRM proteins. DRMs were prepared, blotted, and analyzed as in Figure 1. Results are shown in Figure 4. For comparison, DRMs from surface-biotinylated plastic-grown cells (Surf.) or DRMs labeled by biotinylation after solubilization to detect the total protein profile (Tot.) were also examined. Proteins of 20, 25, and 74 kDa were primarily apical (A), while a doublet of about 100 kDa and a protein of about 200 kDa were primarily basolateral (B). Proteins of 32, 58, and 83 kDa appeared to be present on both membranes.

Identification of GPI-Anchored Proteins in DRMs. GPI-anchored proteins partition into the detergent phase during Triton X-114 phase separation (Bordier, 1981; Brown & Rose, 1992; Lisanti et al., 1988). After removal of the hydrophobic anchor with phosphatidylinositol-specific phospholipase C (PI-PLC), however, these proteins partition into the aqueous phase. DRMs were isolated from apically or

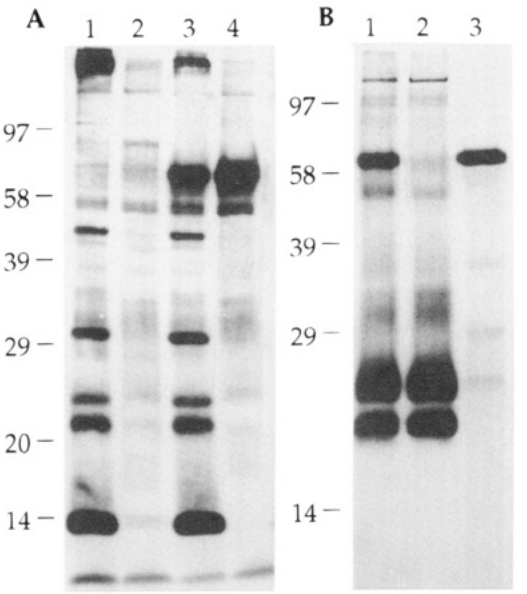

FIGURE 3: Detection of PLAP in DRMs. (A) DRMs were prepared from two 100-mm dishes of MDCK cells (lanes 1 and 2) or MDCK cells expressing PLAP (lanes 3 and 4) after surface biotinylation of cells (lanes 2 and 4) or biotinylation after solubilization of DRMs (lanes 1 and 3). (B) Lane 1: DRMs were prepared from cells expressing PLAP in two 100-mm dishes after cell-surface biotinylation. Lane 2: As in lane 1, except that PLAP was removed by immunoprecipitation. Shown are TCA-precipitated proteins remaining in the supernatant after immunoprecipitation. Lane 3: PLAP immunoprecipitated from the sample in lane 2 is shown. In both A and B, all proteins were separated by SDS−PAGE and transferred to nitrocellulose. Biotinylated proteins were detected with HRP−streptavidin.

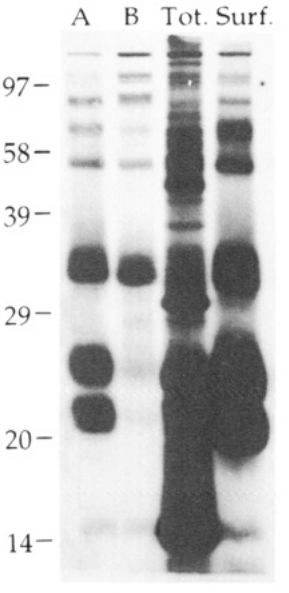

FIGURE 4: Surface polarity of DRM proteins. Proteins in cells grown on two 24-mm Transwell filters were biotinylated from the apical (A) or basolateral (B) surface. DRMs prepared from each set of filters were pooled with DRMs from two unlabeled 100-mm dishes as carrier. DRMs were also prepared from two 100-mm dishes of MDCK cells after surface biotinylation (Surf.) or biotinylation after solubilization (Tot.). Proteins were separated by SDS−PAGE and transferred to nitrocellulose. Biotinylated proteins were detected with HRP−streptavidin.

basolaterally biotinylated cells. After solubilization of the membranes in Triton X-114 and phase partitioning, detergent phases were incubated with or without PI-PLC, and the phase separation was repeated. Biotinylated proteins in both

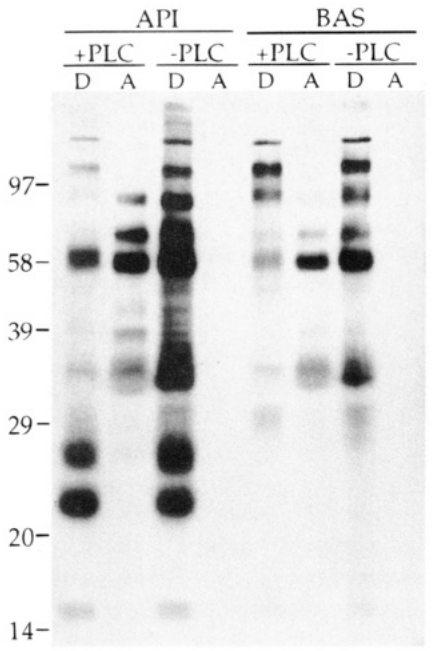

FIGURE 5: GPI-anchored proteins in DRMs. Proteins in cells grown on three 24-mm Transwell filters were biotinylated from the apical (API) or basolateral (BAS) surface. DRMS prepared from each set of filters were pooled with DRMs from one unlabeled 100-mm dish of cells as carrier. DRMs were solubilized in Triton X-114 and subjected to phase separation. Proteins in the detergent phase were incubated with (+PLC) or without (−PLC) PI-PLC. Phase separation was repeated, and proteins in the aqueous (A) and detergent (D) phases were separated by SDS−PAGE, transferred to nitrocellulose, and detected with HRP−streptavidin.

detergent and aqueous phases were subjected to blotting and detected with HRP−streptavidin as shown in Figure 5. As previously reported (Brown & Rose, 1992; Lisanti et al., 1988), five or six proteins were present in the aqueous phase only after PI-PLC treatment and were thus anchored by GPI. As shown earlier (Lisanti et al., 1988, 1990), all of these were more abundant on the apical than the basolateral membrane. Several of these (especially proteins of 58, 74, and 83 kDa) were among the most abundant cell-surface proteins in DRMs. Other major cell-surface proteins, of about 20, 25, 100, and 200 kDa, were not cleaved by PI-PLC and were thus probably not GPI-anchored. Apically labeled bands of about 58 kDa were present in both detergent and aqueous phases after PI-PLC treatment. It is possible that a GPI-anchored protein was only partially released from its anchor by PI-PLC. Alternatively, two different 58 kDa proteins, only one of which is GPI-anchored, may be present on the apical surface of the cells.

In the polarity study shown in Figure 4, a protein of 83 kDa appeared to be present in comparable amounts on both apical and basolateral membranes. The data shown in Figure 5 demonstrated that the apical protein of this size was GPI-anchored, whereas the basolateral protein was not. This result suggested that two well-polarized 83-kDa proteins comigrated in the experiment shown in Figure 4. Two other proteins that appeared to be present on both surfaces in Figure 4 were 58 and 32 kDa in size. The basolateral pools of both of these proteins were largely cleaved by PI-PLC (Figure 5, lanes 2 and 6) and thus appear to be missorted GPI-anchored proteins.

Caveolin. Sargiacomo et al. (1993) also examined the protein composition of DRMs from MDCK cells (see Discussion). These workers identified a major 22-kDa protein in the membranes as caveolin, a component of the caveolar coat. On the basis of the fact that caveolin comigrated on one-dimensional gels with a protein that could be biotinylated from the cell surface, these workers proposed that caveolin was exposed to the cell surface. In an attempt to reproduce this result, the blot shown in Figure 1, lanes 2 and 3, was stripped and reprobed with antibodies directed against caveolin. The two bands seen in Figure 1, lanes 4 and 5, probably corresponded to two different forms of caveolin that differ by an unknown post-translational modification (Dupree et al., 1993). The two forms of caveolin comigrated with two major proteins in DRMs (compare lanes 2 and 4). (It is not clear why the upper band in lane 4 was labeled less intensely than that in lane 5. Possibly, biotinylation of a site that was accessible only after solubilization interfered with the binding of anti-caveolin antibodies.) The more slowly migrating form of caveolin comigrated with a prominent band in lane 3, consistent with the possibility that caveolin might be labeled from the surface. In contrast, the more rapidly migrating form of the protein was not biotinylated from the surface (compare lanes 3 and 5). Thus, it appeared that at least one form of caveolin was not exposed to the cell surface. This is consistent with results of Dupree et al. 1993), who proposed that the protein may assume a hairpin structure in the membrane, with both amino and carboxyl termini in the cytosol.

Solubilization of DRM Proteins. PLAP can be solubilized from stably transfected MDCK cells by octyl glucoside on ice or by Triton X-100 at 37 °C (Brown & Rose, 1992). In contrast, extraction of cells with any of several other detergents did not solubilize the protein (Brown & Rose, 1992). To determine the detergent solubility of other proteins in DRMs, the membranes were prepared as usual and then subjected to a second extraction under one of the conditions described below. Soluble and insoluble material were separated, and proteins in both fractions were analyzed by SDS−PAGE followed by fluorography or blotting.

We first tested CHAPS and octyl glucoside, as the effect of these detergents on PLAP solubilization was known. We also exposed cells to a low concentration of saponin before extracting with ice-cold Triton X-100, as Cerneus et al. have shown that endogenously expressed PLAP in the BeWo trophoblast-like cell line is solubilized by this treatment (Cerneus et al., 1993). The solubilization of metabolically labeled proteins is shown in Figure 6A and that of surface-biotinylated proteins in Figure 6B. All re-extractions were performed on ice. Most proteins were solubilized to some extent by re-extractions with Triton X-100 after saponin pretreatment, although solubilization was not complete [Figures 6A and 6B, compare P (pellet) and S (soluble) fractions)]. Proteins were solubilized more efficiently by extraction with octyl glucoside. In contrast, most proteins were not solubilized by re-extraction with CHAPS.

To investigate the temperature dependence of DRM protein solubilization, DRMs prepared from [^{35}S]methionine-labeled cells were re-extracted with 1% Triton X-100 at 0, 13, 22, 30, or 37 °C, as shown in Figure 6C. Most proteins remained insoluble after a second extraction on ice, were partially solubilized when the extraction was performed at 22 °C, largely extracted at 30 °C, and almost completely solubilized at 37 °C.

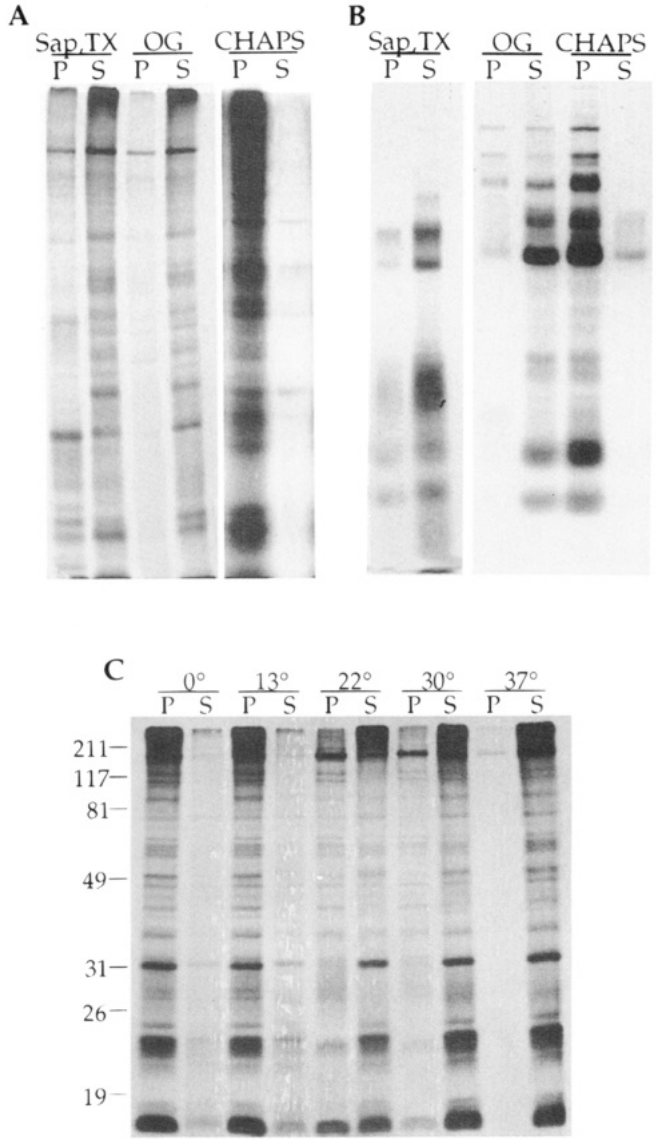

FIGURE 6: Solubilization of proteins in DRMs. (A) DRMs were prepared from methionine-labeled cells in a 100-mm dish. Pelleted DRMs were divided in half and re-extracted under one of the following conditions: TNE with 1% Triton X-100 on ice after pretreatment with 0.2% saponin for 40 min on ice (Sap, TX); or TNE containing 60 mM octyl glucoside (OG). In a separate experiment, 50% of the DRMs from one 100-mm dish were re-extracted with TNE containing 30 mM CHAPS. All samples were subjected to centrifugation for 15 min at top speed in a microfuge. The pellets were dissolved directly in gel loading buffer, whereas proteins in the supernatant were first precipitated with TCA. SDS–PAGE and fluorography were performed. (B) As in A, except that cells were subjected to surface biotinylation instead of metabolic labeling. Blotted biotinylated proteins were detected with HRP–streptavidin. The first two lanes and the last four lanes are from separate experiments. In each case, 50% of the DRMs from cells in one 100-mm dish were re-extracted under each condition. (C) DRMs were prepared and re-extracted with Triton X-100 as in A. Re-extractions were performed at the indicated temperatures (°C). Soluble (S) and pelletable (P) fractions were prepared and analyzed as in A.

Several proteins, including Yes, caveolin, and the α subunits of heterotrimeric G proteins, have been reported to be present in MDCK cell DRMs (Arreaza et al., 1994; Sargiacomo et al., 1993). To determine whether these proteins were solubilized under the same conditions that solubilized the major DRM proteins, DRMs were re-extracted as in Figure 6. Proteins in the supernatant (S) and pellet (P) fractions were analyzed on a blot probed sequentially with antibodies directed against Yes, $G_{i\alpha}$, and caveolin (Figure 7). All three proteins were largely extracted under the three conditions, as was true for most [^{35}S]methionine-labeled proteins shown in Figure 6. Caveolin was only about 50% solubilized when re-extracted with Triton X-100 after saponin pretreatment, possibly indicating a particularly tight association of this protein with detergent-resistant membranes.

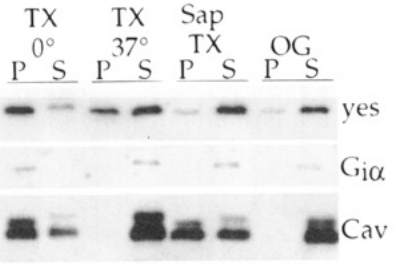

FIGURE 7: Extractability of Yes, $G_{i\alpha}$, and caveolin. DRMs were prepared and re-extracted under the conditions defined in Figure 6A, except that proteins were not labeled. In addition, some DRMs were re-extracted with TNE with 1% Triton X-100 at 0 °C or at 37 °C. Soluble (S) and pelletable (P) fractions were prepared and analyzed as in Figure 6A. The blot was probed sequentially with antibodies against Yes, $G_{i\alpha}$, and caveolin (Cav). Proteins were detected with appropriate HRP-conjugated secondary antibodies and ECL.

We next extended our characterization of the Triton X-100 solubility of PLAP. In these experiments, DRMs were not isolated from sucrose gradients. Instead, the ability of PLAP to pellet after centrifugation in a microfuge was taken to indicate its association with DRMs. This assumption was based on our earlier finding that insoluble PLAP migrated with DRMs in sucrose gradients, and could be detected in these membranes by electron microscopy (Brown & Rose, 1992). We have previously shown that PLAP is insoluble when the extraction is performed on ice, but is solubilized at 37 °C. To determine the temperature dependence of solubilization more precisely, surface-biotinylated cells were extracted at 0, 10, 20, or 37 °C (Figure 8A). PLAP was recovered by immunoprecipitation from soluble (S) and insoluble (P) fractions. The protein was largely extracted at 10 °C, and completely solubilized at 20 °C. Thus, this GPI-anchored protein was extracted at a lower temperature than most proteins in the DRMs (Figure 6). This result suggests that GPI-anchored proteins may associate with the membranes differently from other proteins. In agreement with Cerneus et al. (1993), saponin pretreatment of cells caused complete solubilization of surface-biotinylated PLAP by Triton (Figure 8B).

Sargiacomo et al. reported that extraction under slightly acidic conditions increased the insolubility of a GPI-anchored protein (Sargiacomo et al., 1993). The effect of pH on extraction of cell-surface PLAP was examined using a pulse–chase protocol. MDCK cells expressing PLAP were labeled with [^{35}S]methionine, chased for 3 h, and lysed on ice with 1% Triton X-100 under either mildly acidic conditions (MBS) or mildly alkaline conditions (buffer D). In contrast to the results of Sargiacomo et al., the pH of the extraction buffer did not affect the solubilization of PLAP (Figure 8C).

Solubility of PLAP on the Plasma Membrane. We have previously shown that PLAP remains detergent-insoluble through association with DRMs for at least 3 h after synthesis

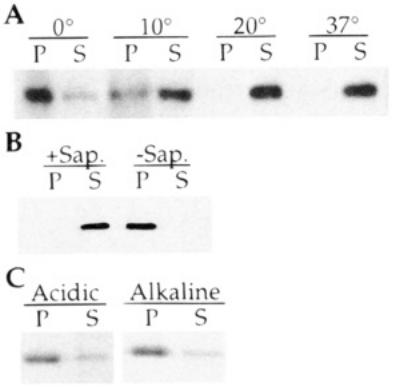

FIGURE 8: Triton X-100 solubility of PLAP under different extraction conditions. (A) Cells expressing PLAP were subjected to surface biotinylation and were then extracted for 20 min with 1 mL of TNE/TX 100 at one of the indicated temperatures. (B) Cells were subjected to surface biotinylation and then incubated with TNE with (+Sap) or without (−Sap) 0.2% saponin for 40 min on ice before extraction with TNE/TX 100 on ice as usual. (C) Cells were pulse-labeled with 0.5 mCi/mL [^{35}S]methionine for 30 min and then chased for 3 h in media containing excess unlabeled methionine. Cells were extracted on ice with 1 mL of MBS/TX100 (pH 6.8) or buffer D (pH 8). (A−C) Soluble and insoluble fractions were separated as in Figure 6A. PLAP was recovered from both fractions by immunoprecipitation and subjected to SDS−PAGE. Each sample was prepared from cells in one 35-mm dish. (A and B) Proteins were transferred to nitrocellulose. PLAP was detected with HRP−streptavidin and ECL. (C) A fluorograph is shown.

(Brown & Rose, 1992). Hannan and Edidin have shown that PLAP (L. Hannan and M. Edidin, personal communication) and another GPI-anchored protein (Hannan et al., 1993) are clustered and immobile when first delivered to the plasma membrane. The proteins then gradually uncluster and become mobile on the cell surface. To determine whether clustering and immobility reflected the association of PLAP with glycolipid-rich membrane domains, we examined the solubility of the protein in ice-cold Triton X-100 when it first reached the cell surface, and after long times of chase. First, we used a pulse−chase procedure coupled with surface biotinylation to detect PLAP when it arrived at the plasma membrane and determined the solubility of newly delivered protein in ice-cold Triton X-100 as described in Experimental Procedures. Results are shown in Figure 9A. When PLAP was first detected on the surface, after 20−30 min of chase, it was predominantly insoluble in detergent. (A faint band with a slightly higher mobility than PLAP seen in the soluble fraction after 20 min of chase was not consistently observed.)

To determine whether PLAP remained insoluble as long as it was on the cell surface, pulse-labeled PLAP was recovered from soluble and insoluble fractions by immunoprecipitation after chase times of up to 6 h. Using this protocol, PLAP in all organelles of the secretory pathway was recovered. A relatively large fraction of the protein was detergent-soluble at early times of chase (Figure 9B), as PLAP is soluble in Triton while it is in the early compartments of the secretory pathway (Brown & Rose, 1992). After 3 h of chase, however, when most PLAP should have reached the plasma membrane, it was almost completely insoluble. The protein remained insoluble even after 6 h of chase (Figure 9B). Thus, a long residency on the plasma membrane did not affect its extractability. Together, these results suggest that PLAP is always insoluble in ice-cold Triton X-100 when it is on the plasma membrane.

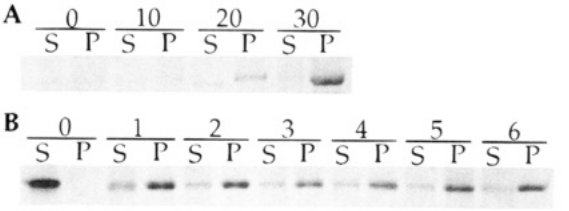

FIGURE 9: Kinetic analysis of PLAP solubility in Triton X-100. Cells expressing PLAP in one confluent 35-mm dish were used per time point. (A) Cells were labeled for 20 min with media containing 1 mCi/mL [^{35}S]methionine and then chased with media containing excess unlabeled methionine for the indicated times (in minutes). Cells were then subjected to surface biotinylation and lysed. Soluble and insoluble fractions were separated, and PLAP was recovered from each by immunoprecipitation. Immune complexes were eluted from Pansorbin and incubated with streptavidin−agarose beads. Biotinylated proteins were eluted and analyzed by SDS−PAGE and fluorography. (B) Cells were labeled for 5 min with 0.5 mCi/mL [^{35}S]methionine and then incubated for the indicated times (in hours) in media containing excess unlabeled methionine. Cells were lysed on ice with 1 mL of buffer D. Soluble and insoluble fractions were separated. PLAP was recovered from both fractions by immunoprecipitation and analyzed by SDS−PAGE and fluorography.

Table 1: DRM Lipids Remaining Insoluble after Re-Extraction under Various Conditions[a]

lipid	13 °C	22 °C	37 °C	OG	saponin
Ch	93	72	34	87	33
PC	68	71	23	94	33
PE	66	69	11	86	18
SM	96	84	30	86	32
CB	66	61	21	79	44
Forss	84	69	28	89	39
LacCer	67	70	22	76	40

[a] DRMs were re-extracted with TNE/TX on ice or at the indicated temperatures, with TNE/TX on ice after saponin pretreatment, or on ice with 60 mM octyl glucoside (OG). The amount of each lipid that remained insoluble under each condition, as a percentage of the amount that remained insoluble after re-extraction by TNE/TX on ice, was calculated. All experiments except the 37 °C extraction were performed twice; it was performed five times. Average values are shown; experiments varied by less than 20%. Ch, cholesterol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; CB, cerebrosides; Forss, Forssman antigen; LacCer, lactosyl cerebroside.

Solubility of DRM Lipids. We next examined the solubilization of lipids from DRMs, to determine whether lipids and proteins were extracted coordinately. DRMs were prepared as usual, collected by centrifugation, and then re-extracted under one of the following conditions: Triton X-100 at 0, 13, 22, or 37 °C; octyl glucoside on ice; or Triton X-100 on ice after saponin pretreatment. Membranes that remained insoluble after re-extraction were collected by centrifugation. Pelleted neutral lipids were extracted and analyzed as described in Experimental Procedures. Lipids were quantitated by scanning densitometry, as shown in Table 1. The amount of each lipid that remained insoluble under each condition is expressed as a percentage of the amount that remained insoluble after re-extraction with Triton X-100 on ice. Increasing the temperature of the extraction caused a gradual increase in the amount of lipid that was solubilized, although even at 37 °C, 20−30% of the lipids remained insoluble. Treatment with octyl glucoside at 0 °C, however, extracted DRM lipids very poorly. This was in striking contrast to the efficient solubilization of DRM proteins by this detergent.

DISCUSSION

We examined the protein profile of MDCK cell DRMs. About 25 major proteins were detected, either by metabolic labeling of cells before lysis or by biotinylation after solubilization. Only 8−10 major proteins could be labeled by a membrane-impermeant biotinylation reagent added to cells before lysis. Therefore, most DRM proteins are probably located in intracellular membranes or on the cytoplasmic surface of the plasma membrane in intact cells. DRMs contained both apical and basolateral proteins, and several major cell-surface proteins in the membranes were GPI-anchored. DRMs from cells extracted at pH 6.8 or pH 11 had very similar protein compositions.

van Meer, Simons, and colleagues proposed that apical proteins associate with glycolipid-rich membrane "rafts" in the TGN and are then packaged into specific transport vesicles for delivery to the apical surface (Simons & van Meer, 1988; Simons & Wandinger-Ness, 1990). DRMs are likely to be derived from these rafts. Thus, the presence of basolateral membrane proteins in DRMs was surprising. These proteins might be expected to associate with glycolipid-rich domains in the TGN as well as on the basolateral cell surface. For this reason, the role of these domains in apical sorting is unclear. Possibly, domains in the TGN are heterogeneous and exhibit subtle variations in lipid composition or other factors. Apical proteins might associate only with a subpopulation of the rafts, those involved in apical sorting.

Insolubility of PLAP: PLAP Is a Major DRM Protein. We showed previously that PLAP was not solubilized by Triton X-100 from liposomes with a lipid composition similar to that in DRMs, even in the absence of other proteins (Schroeder et al., 1994). This supported our model that acyl chain interactions were responsible for the presence of PLAP in DRMs. The finding that PLAP was a major component of DRMs prepared from overexpressing cells also supports this theory. As PLAP is present on the extracellular side of the membrane, a putative "PLAP binding" protein would also need to be exposed to the cell surface. PLAP is one of the most abundant surface-exposed proteins in DRMs (Figure 3). Therefore, any "binding protein" should have been saturated by the high level of PLAP in the membranes.

Insolubility of PLAP: Clustering, Immobility, and Detergent Resistance. Hannan and Edidin showed that gD1-DAF, a GPI-anchored protein, was clustered and immobile for at least 30 min after it reached the surface of MDCK cells (Hannan et al., 1993). This behavior gradually changed, and the protein was largely mobile at steady-state. PLAP expressed in MDCK cells showed similar behavior (L. Hannan and M. Edidin, personal communication). We wondered whether the same factors were responsible for immobility and detergent insolubility. However, we found that PLAP was insoluble as soon as it reached the plasma membrane, and remained so after 6 h of chase. Thus, detergent insolubility does not correlate with clustering and immobility.

Solubilization of Lipids and Proteins. We proposed that glycolipid-rich domains in the l_o phase may be present in cell membranes and may give rise to DRMs during detergent extraction. We also suggested that GPI-anchored proteins associate with these domains through acyl chain interactions (Schroeder et al., 1994). In apparent contrast to the prediction of our model, PLAP was solubilized when cells were extracted at temperature above 5−10 °C, and was insoluble only when extracted on ice. This might suggest that l_o phase domains do not exist in cells at physiological temperatures. However, most lipids and proteins in DRMs were not fully solubilized at temperatures as high as 22 °C, and some lipid remained insoluble even after extraction at 37 °C. Detergent resistance may not be a perfect indicator of the physical state of lipids; lipids in gel-phase membranes are solubilized at temperatures slightly below their acyl-chain transition temperatures (Ribeiro & Dennis, 1973). Thus, it is plausible that glycolipid-rich l_o phase membrane domains may exist in cells at physiological temperatures. GPI-anchored proteins may associate with these domains differently than do other proteins.

Both proteins and lipids were largely solubilized from DRMs by Triton X-100 at 37 °C and by Triton X-100 on ice after saponin pretreatment, suggesting that the two might be solubilized coordinately. However, some lipids remained insoluble after extraction with Triton at 37 °C, whereas protein solubilization was virtually complete. Thus, lipids may be more resistant to extraction from the membranes than proteins. This effect was very striking in the case of octyl glucoside. Lipids were largely resistant to extraction by this detergent, whereas proteins were completely solubilized.

We showed previously that liposomes with a lipid composition similar to that of DRMs from cells were equally resistant to Triton extraction at 37 °C and on ice (Schroeder et al., 1994). In contrast, most lipids in DRMs from cells were solubilized at 37 °C. Subtle differences in lipid composition between cell membranes and the liposomes tested, or the presence of proteins in cellular DRMs, may affect the temperature dependence of lipid solubilization.

Cellular Origin of DRMs. According to our model, l_o phase domains in cell membranes are isolated as DRMs after detergent extraction (Schroeder et al., 1994). However, our data do not address the size and distribution of these domains in membranes. These remain major unanswered questions. DRMs from MDCK cells range from about 50−100 nm to 1 μm in diameter (Brown & Rose, 1992). Such large structures may not exist as discrete domains in cells. Instead, it is possible that smaller domains exist in membranes. Small glycosphingolipid clusters have been observed in cellular membranes (Rock et al., 1990). Possibly individual GPI-anchored proteins associate with small clusters of detergent-resistant lipids. These may coalesce into the large structures that we observed after solubilization of surrounding regions of membrane. This interpretation is consistent with recent observations of another group (Mayor & Maxfield, 1994). These workers extracted cells with detergent before fixation and then observed GPI-anchored proteins in the remnant structures by immunofluorescence or immunoelectron microscopy. Surprisingly, large regions of apparently unsolubilized plasma membrane were detected in these cells. GPI-anchored proteins, which were initially uniformly distributed in the membrane, were slightly more clustered after detergent extraction. However, no large domains that were enriched in GPI-anchored proteins were observed.

We cannot exclude the possibility that proteins isolated in DRMs do not associate with specialized domains before extraction. These proteins might be inherently poorly soluble in Triton X-100, and might "hop" into DRMs in the same lysate, after solubilization of most membrane components.

However, our earlier observations concerning the solubility of PLAP make this explanation unlikely (Brown & Rose, 1992). We found that the protein is solubilized by Triton X-100 immediately after synthesis, while it is in the endoplasmic reticulum. This behavior probably reflects the fact that the endoplasmic reticulum is poor in sphingolipids and cholesterol. Domains enriched in these lipids would not be expected to form there. If PLAP were simply inherently insoluble in Triton X-100, though, and "hopped" into DRMs after detergent extraction, we would expect to recover it in these domains regardless of its membrane of origin.

Comparison with Published Data. While these experiments were in progress, a similar study by Sargiacomo et al. appeared (Sargiacomo et al., 1993). Several results reported in that paper differ from those described here. First, the authors found that most of the major proteins in DRMs could be labeled by biotinylation from the cell surface. Second, they showed that GPI-anchored proteins were present in DRMs, but were not among the major surface-exposed proteins in the membranes. Third, they reported that DRM proteins were present in roughly equal amounts on the apical and basolateral membranes. In addition, extraction of cells at a slightly acidic pH increased the yield of DRM proteins. Finally, the pattern of cell-surface proteins that they detected in DRMs was different from that described here. The major surface-exposed proteins identified in their study were proposed to form a complex that bound to a GPI-anchored protein during biosynthetic transport and was also important in the biogenesis of caveolae (Lisanti et al., 1993; Sargiacomo et al., 1993).

The finding that most of our DRM proteins were not exposed to the cell surface, while almost all of theirs were, may have a technical explanation. Sargiacomo et al. labeled all the proteins in DRMs by adding s-NHS-biotin to cells after permeabilization with a low concentration of Triton X-100 (Sargiacomo et al., 1993). The protein profile observed using this method was similar to that obtained when the reagent was added to intact cells. In separate experiments, however, they metabolically labeled DRM proteins using [^{35}S]methionine. Many more proteins were labeled this way than by biotinylation after permeabilization. These conflicting results suggest that the biotinylation reagent did not have access to all proteins in DRMs after the permeabilization. In contrast, the profiles of total DRM proteins that we observed by metabolic labeling and by biotinylation after solubilization were very similar.

The basis of the other discrepancies is not clear. The difference in pH of the extraction buffers used in the two studies did not affect the results (Figure 2). In most cases, we performed Triton X-114 phase separation on solubilized DRM proteins and examined proteins only in the detergent phase. In contrast, Sargiacomo et al. did not routinely use this detergent. However, they tested the effect of Triton X-114 on DRM proteins, and found that only a few minor proteins partitioned into the aqueous phase (Sargiacomo et al., 1993). We also found that very few DRM proteins partitioned exclusively into the aqueous phase (data not shown). Thus, our use of Triton X-114 phase separation does not explain the observed differences.

One explanation is the following: Their DRMs may be identical to ours but contain the protein complex they described in addition to other proteins. This complex may be much more abundant than the other proteins, making the latter difficult to detect. This would explain why GPI-anchored proteins were major surface-exposed proteins in our DRMs, but were minor components of their membranes. This model would also explain another difference between our results. Sargiacomo et al. found many fewer proteins in DRMs prepared from Fischer rat thyroid (FRT) cells than from MDCK cells (Sargiacomo et al., 1993). In contrast, we found comparable amounts of protein in DRMs from the two cell lines (M. J. Burns and D. A. Brown, unpublished results). The presence of the abundant protein complex in MDCK cell DRMs, but not FRT cell DRMs, would produce a striking difference in overall protein level, even if other proteins were present in similar amounts in DRMs from the two cell types. It is not clear, however, why this complex of abundant proteins was not observed in our MDCK DRMs. It is possible that these proteins were spurious contaminants in the preparation of Sargiacomo et al., rather than bona fide components of DRMs.

Conclusions. Together, these studies support our proposal that glycolipid-rich domains exist in cell membranes, and that a limited number of cellular proteins may associate with them. Further characterization of the function(s) of these structures remains a challenge for the future.

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