

Cross-talk between Caveolae and Glycosylphosphatidylinositol-rich Domains*

Received for publication, March 6, 2001, and in revised form, June 13, 2001
Published, JBC Papers in Press, June 13, 2001, DOI 10.1074/jbc.M102039200

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Most mammalian cells have in their plasma membrane at least two types of lipid microdomains, non-invaginated lipid rafts and caveolae. Glycosylphosphatidylinositol (GPI)-anchored proteins constitute a class of proteins that are enriched in rafts but not caveolae at steady state. We have analyzed the effects of abolishing GPI biosynthesis on rafts, caveolae, and cholesterol levels. GPI-deficient cells were obtained by screening for resistance to the pore-forming toxin aerolysin, which uses this class of proteins as receptors. Despite the absence of GPI-anchored proteins, mutant cells still contained lipid rafts, indicating that GPI-anchored proteins are not crucial structural elements of these domains. Interestingly, the caveolae-specific membrane proteins, caveolin-1 and 2, were up-regulated in GPI-deficient cells, in contrast to flotillin-1 and GM1, which were expressed at normal levels. Additionally, the number of surface caveolae was increased. This effect was specific since recovery of GPI biosynthesis by gene recombination restored caveolin expression and the number of surface caveolae to wild type levels. The inverse correlation between the expression of GPI-anchored proteins and caveolin-1 was confirmed by the observation that overexpression of caveolin-1 in wild type cells led to a decrease in the expression of GPI-anchored proteins. In cells lacking caveolae, the absence of GPI-anchored proteins caused an increase in cholesterol levels, suggesting a possible role of GPI-anchored proteins in cholesterol homeostasis, which in some cells, such as Chinese hamster ovary cells, can be compensated by caveolin up-regulation.

According to the current view, the plasma membrane of mammalian cells is not a homogeneous sea of lipids but is composed of different domains with different lipid and protein compositions and different physical states. In particular, increasing evidence supports the existence of cholesterol and glycosphingolipid rich domains, which have been termed lipid rafts (1, 2). Rafts are physiologically important since they have been implicated in signaling (3, 4), cell adhesion (5), and cholesterol homeostasis (4, 6, 7), as well as in pathological pro-

cesses such as proteolytic processing of the amyloid precursor protein (8, 9) or infection by microorganisms (10).

Lipid rafts are small (50–350 nm in diameter depending on the study, cell type, and analysis method) and highly dynamic entities (11). Due to the high abundance of cholesterol and lipids with long saturated acyl chains, the lipid bilayer within these domains is in the tightly packed liquid ordered state Lo (12). Some proteins preferentially partition into Lo phases, leading to a specific protein composition (2). More than 100 proteins have been suggested to be associated with lipid rafts. Even though this might be an overestimation, considering the small size of elementary raft units, a given raft can only contain a limited number of these proteins. It is not clear at present whether each raft contains a random sample of raft components or whether some rafts contain a specific set of proteins or lipids, *i.e.* whether there are different types of rafts.

A clear subclass of rafts is, however, composed by caveolae (13). These can easily be distinguished by their shape (they are flask-like invaginations) and by the presence of membrane proteins of the caveolin family (4, 7, 13). The proposed functions of caveolae are similar to those attributed to rafts. They are believed to be implicated in signal transduction, based on the observation that caveolin-1 interacts with a number of signaling molecules (14), and in cholesterol regulation (4, 7). In the present paper, for the sake of clarity, the term raft will only be used to identify all non-caveolar rafts, as opposed to caveolae themselves. In contrast to rafts, caveolae are not ubiquitous since they are not found in cells lacking caveolins such as lymphocytes (15). However, heterologous expression of caveolin-1 will induce *de novo* formation of caveolae (16–18).

A class of raft proteins that has attracted much attention over the last decade is that of GPI¹-anchored proteins (GPI-APs). These lipid-anchored polypeptides are mainly present at the plasma membrane where they are found in rafts, *i.e.* outside of caveolae, at steady state (19), even though they may enter caveolar pits upon cross-linking with antibodies (20). GPI-anchored proteins, however, also undergo endocytosis. In CHO cells, they were shown to be internalized and then recycled back to the plasma membrane via the recycling endosome (21)² in a cholesterol-dependent (21) and sphingolipid-depend-

* This work was supported by a grant from the Swiss National Science Foundation (to F. G. v. d. G. and J. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: GPI, glycosylphosphatidylinositol; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; EMS, ethylmethane sulfonate; GPI-AP, glycosylphosphatidylinositol-anchored protein; PNS, postnuclear supernatant; DRM, detergent-resistant membrane; PI, phosphatidylinositol; PBS, phosphate-buffered saline.

² M. Fivaz, F. Vilbois, S. Thurnheer, C. Pasquali, L. Abrami, P. E. Bickel, and F. G. van der Goot, submitted for publication.

ent (22) process. In COS-7 cells, although GPI-anchored proteins were found to cycle between the plasma membrane and the Golgi apparatus, trafficking was again found to be cholesterol-dependent (23), further highlighting the functional interactions between GPI-APs and cholesterol.

We here investigated whether the absence of GPI-anchored proteins would affect lipid rafts and/or caveolae. We have used GPI-deficient lymphocytes and CHO cells. The GPI-deficient CHO cells were obtained by screening for resistance toward the bacterial pore-forming toxin aerolysin, which binds specifically to GPI-APs (24–26). Interestingly, we found that GPI-deficient CHO cells express higher levels of caveolin-1 than wild type cells, due to increased transcription, and concomitantly have an increased number of caveolae at their surface. Restoration of GPI biosynthesis by gene complementation subsequently reduced caveolin-1 expression to wild type levels. These experiments indicate that cells that lack GPI-anchored proteins somehow compensate for this loss by overexpressing caveolins. Comparison of GPI-deficient cells, which naturally express caveolins (CHO) or lack caveolins (lymphocytes), suggests that caveolin overexpression might compensate for cholesterol defects.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal anti-caveolin-1 and anti-caveolin-2 antibodies were purchased from Transduction Laboratories (Lexington, KY), polyclonal anti-caveolin-1 N20 antibodies from Santa Cruz, monoclonal anti-transferrin receptor antibodies from Zymed Laboratories Inc., polyclonal anti-LAT from Upstate Biotechnology (Lake Placid, NY), and monoclonal mouse anti-FLAG antibodies from Sigma and horseradish peroxidase-conjugated secondary antibodies from Amersham Pharmacia Biotech. Anti-Rab 5, anti- β -COP, anti-p23, anti- β 1- β 2 adaptins, anti-calnexin, and anti-flotillin antibodies were gifts from R. Jahn (MPI, Tübingen), the late T. Kreis, J. Gruenberg (University of Geneva, Geneva), A. Dautry-Varsat (Pasteur Institute, Paris), A. Helenius (ETH, Zürich), and P. Bickel (Harvard, Cambridge, MA), respectively. Fluorescent secondary antibodies were from Jackson ImmunoResearch. Peroxidase-coupled cholera toxin B-subunit was purchased from Sigma. cDNAs of PIG-L and PIG-A were those previously generated (27, 28). The caveolin-1 cDNA was a gift from P. Dupree (University of Cambridge, Cambridge) (29).

Proaerolysin Purification, Iodination, and Activation—Proaerolysin was purified as described previously (30). Concentrations were determined by measuring the optical density at 280 nm, considering that a 1 mg/ml sample has an optical density of 2.5 (31). Proaerolysin was labeled with 125 I using IODOGEN reagent (Pierce) according to the manufacturer's recommendations. The radiolabeled toxin was separated from the free iodine by gel filtration on a PD10-G25 column (Amersham Pharmacia Biotech) equilibrated with 150 mM NaCl, 20 mM HEPES, pH 7.4. We consistently obtained a specific activity of about 2×10^6 cpm/ μ g of proaerolysin. Radiolabeled proaerolysin ran as a single band on a SDS gel.

Cell Culture and Isolation of Proaerolysin-resistant Mutants—CHO-K1 cells were routinely maintained in 100-mm diameter Nunc dishes containing 10 ml of F-12 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine under standard tissue culture conditions in a 5% CO₂ atmosphere at 100% humidity at 37 °C. CHO-K1 cells were mutagenized with ethyl methanesulfonate (400 μ g/ml) at 37 °C for 24 h, as described (32). After growing in the complete culture medium for 4 days, the mutagenized cells were harvested, re-seeded at 400 colonies/dish, and grown in the presence of 1 nM proaerolysin for another 2 days. After incubation in toxin-free culture medium for 7 days, surviving colonies were trypsinized with a filter paper and transferred to 24-well plates. The cells were cultured in normal medium for several days, re-seeded, and then subjected to two other cycles of proaerolysin selection as above. A total of 314 separated colonies of surviving cells were formed and purified by limited dilution. After confirming toxin resistance, clones were frozen for further analysis.

Proaerolysin Binding—Confluent monolayers of CHO cells were washed three times with ice-cold PBS containing 1 mM CaCl₂ and 1 mM MgCl₂ (PBS²⁺). Cells were then incubated at 4 °C with 0.4 or 0.96 nM 125 I-proaerolysin in incubation medium containing Glasgow minimal essential medium buffered with 10 mM HEPES, pH 7.4, for 1 h. Cells were washed three times for 5 min with PBS²⁺ at 4 °C. Monolayers

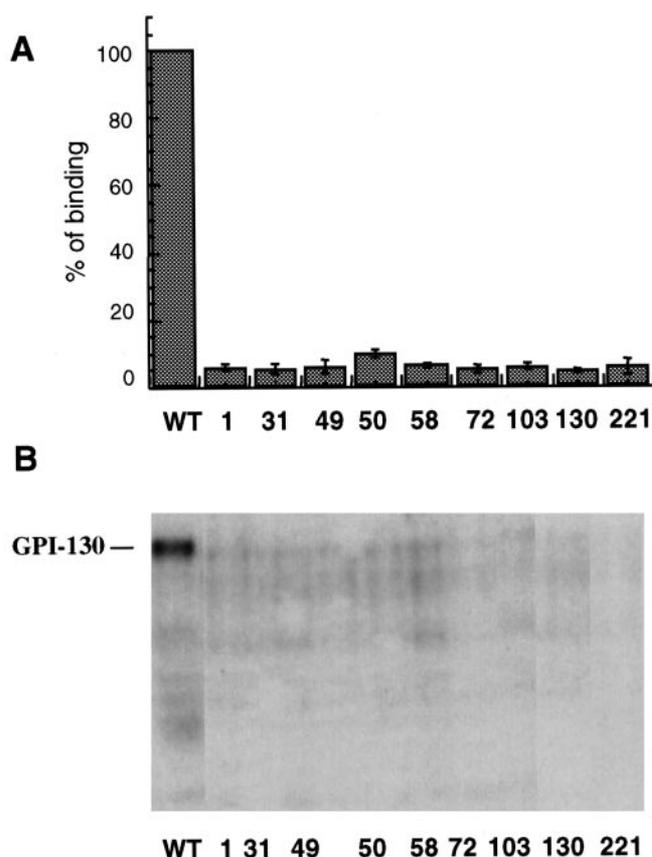


FIG. 1. Proaerolysin-resistant CHO mutant cell lines can no longer bind the toxin due to the loss of the cell surface GPI-anchored receptors. *A*, histogram of 125 I-proaerolysin binding. Monolayers of CHO wild type and mutant cells were incubated with 125 I-proaerolysin (1 nM) for 1 h at 4 °C, extensively washed, and incubated for an additional 20 min at 37 °C. The amount of radioactivity associated with the cells was counted and expressed as a percentage of the CHO wild type associated radioactivity ($n = 4$). The numbers refer to the name of mutant cell lines obtained after screening for aerolysin resistance (see text). *B*, absence of GPI-130 in proaerolysin binding-deficient CHO mutants revealed by proaerolysin overlay. 40 μ g of postnuclear supernatant of each cell type were submitted to a proaerolysin overlay assay.

were then further incubated at 37 °C in incubation medium for various times. Cells were subsequently washed with ice-cold PBS²⁺, scraped from the dish, and collected by centrifugation at $300 \times g$ for 5 min. The presence of cell-bound 125 I-proaerolysin was analyzed either by counting or by autoradiography of 10% SDS gels.

Proaerolysin Overlays—Proaerolysin overlays, to detect proaerolysin receptors, were performed as described previously (25). Briefly, membrane fractions were run on a 10% SDS gel (33). The gel was incubated in 50 mM Tris-HCl, pH 7.4, and 20% glycerol for 10 min. Proteins were then blotted onto a nitrocellulose membrane for 16 h at 100 mA in the cold using a Bio-Rad wet transfer chamber with a transfer buffer containing 10 mM NaHCO₃ and 3 mM Na₂CO₃ (pH 9.8). The nitrocellulose membrane was incubated in a binding buffer containing 50 mM NaH₂PO₄, pH 7.5, and 0.3% Tween 20 for 20 min, followed by a 2-h incubation in the presence of 2.8 nM proaerolysin (labeled or not with 125 I) diluted in the same buffer. The membrane was then washed six times for 5 min with toxin free binding buffer. Binding of proaerolysin was revealed by autoradiography using BioMax films (Eastman Kodak Co.) or using anti-aerolysin antibodies.

Plasmids, Transfections, and Generation of Stable Cell Lines—PIG-L and PIG-A genes were expressed in a pMEori expression vector (pME-EBO-FLAG) driven by Sr- α promoter composed of human T-cell lymphotropic virus-1 enhancer and simian virus 40 early promoter (27). Caveolin-1 gene was introduced in the pcDNA3 vector driven by the human cytomegalovirus promoter. Plasmids were purified using columns (Quiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. Transfection experiments in CHO cells were performed by the CaPO₄-DNA precipitation procedure described by Graham and

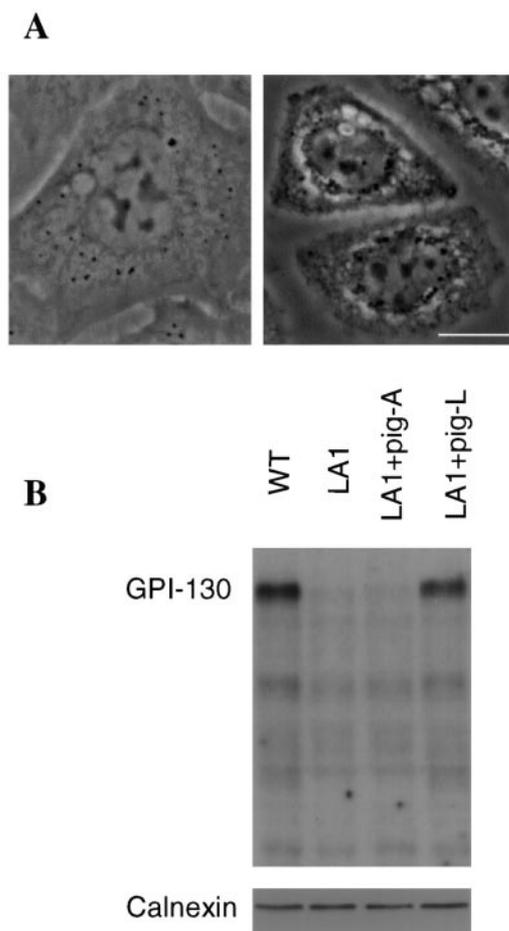


FIG. 2. Recovery of proaerolysin sensitivity upon transfection of CHO-LA1 cells with the PIG-L gene. *A*, effect of proaerolysin on the morphology of CHO-LA1 in the absence (*left*) or presence (*right*) of the PIG-L gene. Cells were incubated with 0.4 nM proaerolysin at 37 °C for 1 h. *Bar* = 10 μ m. *B*, recovery of GPI biosynthesis upon transfection of CHO-LA1 cells with the PIG-L gene. GPI-anchored proteins were detected by aerolysin overlay on PNS (40 μ g) of wild type cells, CHO-LA1, and CHO-LA1 recomplemented with the PIG-L gene. Calnexin was used as an equal loading marker.

van der Eb (34), using caveolin-1, PIG-L, or PIG-A expression constructs. CHO cells from a confluent 60-cm² tissue culture dish were diluted 1:15 and seeded on glass coverslips in a six-well tissue culture plate in view of immunofluorescence experiments. Per well, 3 μ g of plasmid DNA were used. The expressing cells were analyzed by immunofluorescence after 48 h of incubation at 37 °C. To establish stable cell lines expressing FLAG-PIGL or FLAG-PIGA, transfectants were selected with culture medium containing 200 μ g/ml hygromycin. Stable cell lines expressing caveolin-1 were selected for resistance toward 600 μ g/ml G418. After three cycles of trypsinization and antibiotic treatment, colonies were isolated by limited dilution.

Purification of Detergent-resistant Membranes (DRMs)—Approximately 2×10^7 cells were resuspended in 0.5 ml of cold buffer (TNE) containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100 (Ultrapure, Pierce) with a tablet of Complete (Roche Molecular Biochemicals), a mixture of protease inhibitors. Membranes were solubilized by rotary shaking at 4 °C for 30 min. The DRMs were purified on either a step sucrose gradient (25) or using an Optiprep gradient (35). For purification on sucrose gradients, the cell lysate was adjusted to 40.7% sucrose (in 10 mM Tris-HCl, pH 7.4), loaded at the bottom of a SW 40 Beckman tube, overlaid with 8 ml of 35% sucrose, topped up with 15% sucrose, and centrifuged for 18 h at 35,000 rpm at 4 °C. After centrifugation, 12 fractions of 1 ml were collected. For each fraction the protein content was determined. The same protein amount of each fraction was precipitated and analyzed by SDS-PAGE, followed by toxin overlay and/or Western blot analysis. For purification on Optiprep gradients, the cell lysate was adjusted to 40% Optiprep, loaded at the bottom of a TLS.55 Beckman tube, overlaid with 600 μ l of 30% Optiprep and 600 μ l of TNE, and centrifuged for 1.5 h at 55,000 rpm at

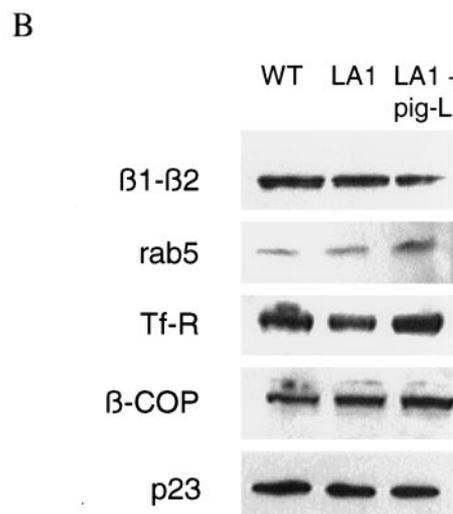
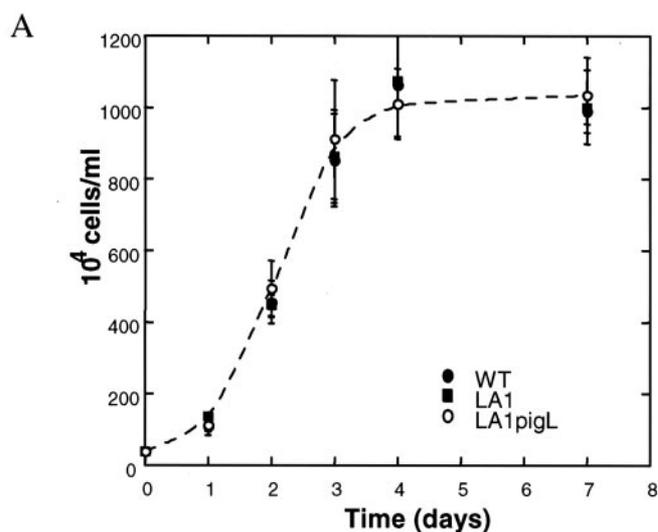


FIG. 3. Characterization of CHO-LA1 cells. *A*, growth of GPI-deficient CHO-LA1 cells was found to be indistinguishable from that of wild type and PIG-L recomplemented cells. Values represent the mean of three experiments. *B*, PNS were prepared from wild type cells, CHO-LA1, and CHO-LA1 recomplemented with the PIG-L gene. Western blot analysis (40 μ g of protein loaded/lane) shows that cellular levels of β 1- β 2 adaptins, the small GTPase Rab5, the transferrin receptor (*Tf-R*), β -COP, and p23, one of the p24 family members, were similar in wild type, mutant, and recomplemented cells.

4 °C. Six fractions of 250 μ l were collected from top to bottom. DRMs were found in fraction 3, which were subsequently precipitated with chloroform methanol and analyzed as above.

Two-dimensional Gel Analysis—DRMs were prepared from wild type and mutant CHO cells as described (36) and precipitated with 6% trichloroacetic acid in the presence of 375 μ g of sodium deoxycholate as a carrier. Two-dimensional gel electrophoresis was performed with non-linear immobilized pH gradient strips, pH range 3–10 (Amersham Pharmacia Biotech, Uppsala, Sweden) as described (36, 37).

Northern Blotting of Caveolin mRNA—Total RNA was purified from CHO wild type or LA1 cell monolayers with Nucleospin RNA II kits (Macherey-Nagel). Confluent cells in one 10-cm dish yielded ~ 10 μ g of total RNA. For Northern blot analysis, 1.25 μ g of RNA were separated on 1% agarose/formaldehyde denaturing gel and transferred on to a nylon membrane. The membrane was stained with methylene blue (presence of 28 and 18 S ribosomal RNA bands indicating intact RNA) and was hybridized to full-length random-primed ³²P-labeled (random-primed DNA labeling kit, Roche Molecular Biochemicals) caveolin cDNA (*EcoRI-XhoI* insert of 890 base pairs). The caveolin blot was

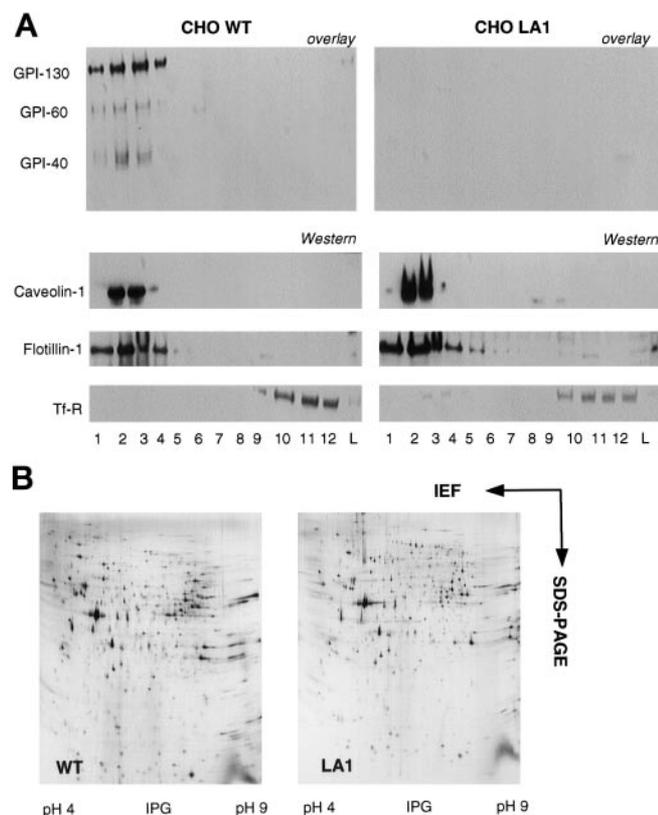


FIG. 4. Isolation of detergent-resistant membranes from wild type and CHO-LA1 cells. DRMs were purified from wild type and CHO-LA1 cells using sucrose flotation gradients. **A**, fractions were collected from the top to the bottom of the gradient and equal amounts of proteins (20 μ g) from each fraction were separated by SDS-PAGE and blotted onto nitrocellulose. The distribution of GPI-anchored proteins was analyzed by aerolysin overlay and that of caveolin-1, flotillin-1, and the transferrin receptor (*Tf-R*) by Western blotting. **B**, the two-dimensional protein map of DRMs from wild type and LA1 CHO cells were obtained by two-dimensional gel electrophoresis followed by high sensitivity silver staining. The repeated two-dimensional gel analysis of DRMs from the two cell types as well as the comparison of the gels using the MELANIE two-dimensional gel analysis software indicated that the protein patterns were essentially identical. GPI-anchored proteins are not detectable on the gel of CHO wild type cells, because the DRMs were not treated with PI-phospholipase C (36) prior to two-dimensional SDS-PAGE and, moreover, CHO GPI-anchored proteins are not abundant enough to be seen on such a gel. IEF, isoelectric focusing; IPG, immobilized pH gradient.

prehybridized for 24 h at 65 °C and then hybridized overnight at 65 °C to the probe at 1×10.6 cpm/ml hybridization buffer. Washed blots were visualized on Kodak X-Omat film, exposed to a phosphorimager plate, and analyzed using Quantity One software (Bio-Rad) under conditions where the signals were linear with respect to the amount of sample applied.

Immunofluorescence—Fixation and permeabilization of CHO cells were performed by incubation 4 min at -20 °C in methanol. After three washes in PBS plus 0.05% bovine serum albumin, fixed cells were reacted for 30 min at room temperature with polyclonal anti-caveolin 1 antibodies (1:100). Cells were then washed three times with PBS, 0.5% bovine serum albumin and incubated for 30 min at room temperature with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:50). Cells were analyzed with a Zeiss Axiophot fluorescence microscope.

Cholesterol Quantification by Thin Layer Chromatography—Lipids were chloroform/methanol-extracted from cell extract (equivalent of 40 μ g of protein). Lipids were separated on Kiesel gel 60 (Merck, Germany) with a solvent system of heptane:ethyl ether:acetic acid (18:6:2). Cholesterol was visualized after incubating the sheet in a solution of 3% copper acetate with 8% H_3PO_4 and drying 5 min at 160 °C. TLC plates were then analyzed by densitometry and cholesterol amount quantified using Scan-Analysis software.

Other Methods—To quantify cell growth, wild type and mutant CHO cells were seeded in complete medium at the density of 4.10^5

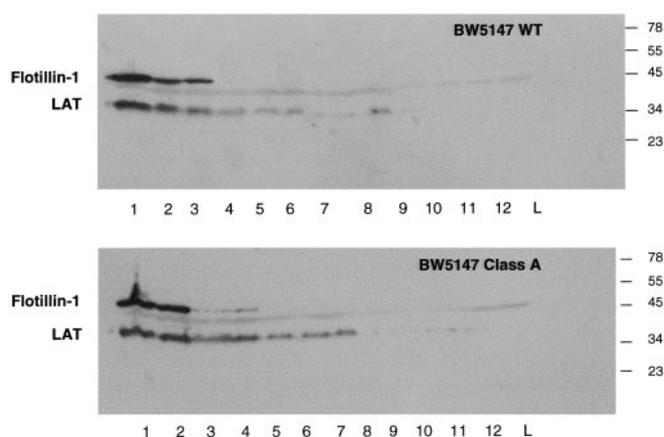


FIG. 5. Isolation of DRMs from lymphocytes expressing or not expressing GPI-anchored proteins. DRMs were purified from wild type and class A BW5147 cells using sucrose flotation gradients. Fractions were collected from the top to the bottom of the gradient, and equal amounts of proteins (20 μ g) from each fraction were analyzed by SDS-PAGE and Western blotting using anti-flotillin and LAT antibodies.

cells/ml at day 0 and counted every 24 h. Cell surface caveolae were quantitated by electron microscopy exactly as described previously (38). SDS-PAGE was performed as described by Laemmli (33). Western blot analysis was carried out using peroxidase-conjugated sheep anti-mouse or anti-rabbit IgG as a secondary antibody, which was detected by chemiluminescence using Super Signal reagents (Pierce). Protein concentrations of cellular fractions were determined with bicinchoninic acid (BCA, Pierce).

RESULTS AND DISCUSSION

Generation of CHO Mutant Cell Lines Deficient in GPI Biosynthesis—In order to generate cell deficient in GPI biosynthesis, we have made use of the bacterial pore-forming toxin aerolysin (26). This toxin specifically binds to GPI-anchored proteins on mammalian cells, and the binding capacity is retained after SDS-PAGE of cellular extracts and blotting onto a nitrocellulose membrane (toxin overlay assay) (24, 25). The main determinant for aerolysin binding is not the protein moiety but the glycan core common to all GPI-anchored proteins (39). Therefore, aerolysin does not generally discriminate between different GPI-anchored proteins, although some exceptions exist (40). Thus, all cells expressing GPI-anchored proteins are likely to be sensitive to aerolysin and will ultimately die when exposed to the toxin (25). Conversely, cells lacking GPI-anchored proteins should be insensitive to aerolysin, as suggested by several examples (40, 41) and should therefore multiply even in the presence of the toxin. Aerolysin therefore potentially constitutes a very powerful tool to generate GPI-deficient cells. We found that this is indeed the case by screening for aerolysin-resistant CHO cells. We have recently identified and characterized the GPI-anchored protein composition of CHO cells by two-dimensional gel analysis² and shown that a protein with apparent molecular mass of 130 kDa is by far the most abundant GPI-anchored protein in this cell type. This protein, called here GPI-130, can readily be detected by performing an aerolysin overlay assay on a postnuclear supernatant after one-dimensional SDS-PAGE (can be seen in Fig. 1B).

Cells were submitted to chemical mutagenesis with EMS and then selected for the ability to grow in the presence of 1 nM proaerolysin, the aerolysin precursor (for review see Ref. 26). Among 314 mutants, most were unable to bind aerolysin (Fig. 1A) and 9 were selected for further analysis. In agreement with the lack of proaerolysin binding, we could not detect GPI-130 by toxin overlay in any of the mutants (identified by numbers in Fig. 1B). The absence of GPI-130 suggested that the cells might

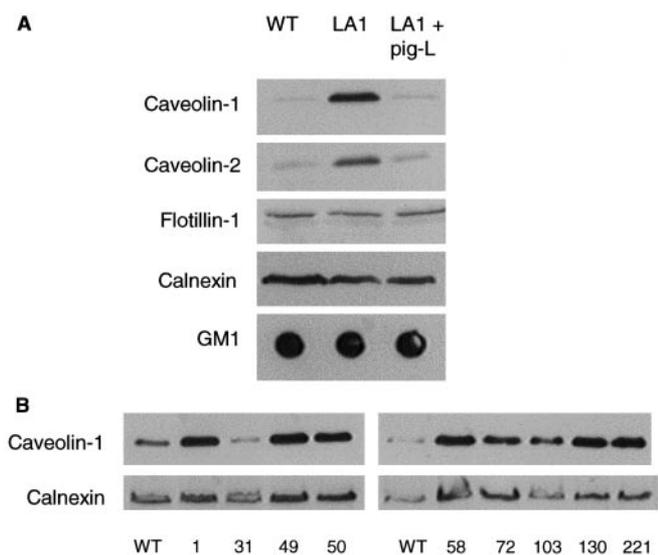


FIG. 6. Effects of GPI deficiency on the levels of raft/caveolae markers. *A*, PNS were prepared from wild type cells, CHO-LA1, and CHO-LA1 recomplemented with the PIG-L gene. The amounts of caveolin-1, caveolin-2 and flotillin were assayed by Western blot analysis (40 μ g of total protein/lane). Calnexin was used as an equal loading marker. The amounts of GM1 were analyzed by dot blot using peroxidase-coupled cholera toxin B-subunit. *B*, PNSs were prepared from wild type and various GPI-deficient cell lines (the numbers refer to the mutant cell lines, obtained after screening for aerolysin resistance, which no longer express GPI-130 or alkaline phosphatase (see text and Fig. 1)). The amounts of caveolin-1 was assayed by Western blot analysis (30 μ g of total protein/lane). Calnexin was used as an equal loading marker.

indeed be deficient in the biosynthesis of GPI-anchored proteins in general. This was confirmed by the observation that none of the mutants were able to heterologously express the GPI-anchored protein human alkaline phosphatase (data not shown).

CHO mutants deficient in GPI biosynthesis have previously been generated by EMS mutagenesis (for reviews see Refs. 42 and 43). Most of the obtained mutants were deficient in the enzyme responsible for the *N*-deacetylation of *N*-acetylglucosamine phosphatidylinositol, which is encoded by the PIG-L gene (27, 44). We therefore tested whether our mutants were also PIG-L-deficient, by transfecting cells with a PIG-L construct. Expression of alkaline phosphatase and sensitivity toward proaerolysin were assayed. Among the nine selected mutants, only the CHO-LA1 mutant cell line (labeled 1 in Fig. 1B) was sensitive toward proaerolysin after PIG-L transfection, as witnessed by a recovery of ER vacuolation, a characteristic effect of aerolysin on fibroblast-like cells (25, 45) (Fig. 2A). A second frequent target gene of EMS mutagenesis is the DMP2 gene, encoding an ER protein implicated in the synthesis of dolichol-phosphate mannose, a mannose donor for GPI as well as for *N*-glycans and C-mannosylation (46). By gene recomplementation, we found that mutant CHO-LA49 (labeled 49 in Fig. 1B) was affected in the DMP2 gene. It remains to be determined what step of GPI biosynthesis is affected in the other mutant cells.

Characterization of the CHO-LA1 GPI-deficient Cell Line—The CHO-LA1 GPI-deficient cell line was chosen for further analysis, and a stable CHO-LA1 cell line expressing the PIG-L gene was generated. In agreement with their sensitivity toward aerolysin (Fig. 2A), CHO-LA1 cells recomplemented with the PIG-L gene were able to express GPI-130 (Fig. 2B).

We first checked that the absence of GPI-anchored proteins did not drastically affect cellular physiology. As shown in Fig. 3A, the growth curve of CHO-LA1 was similar to that of wild type and PIG-L recomplemented cells. We next verified that a variety of

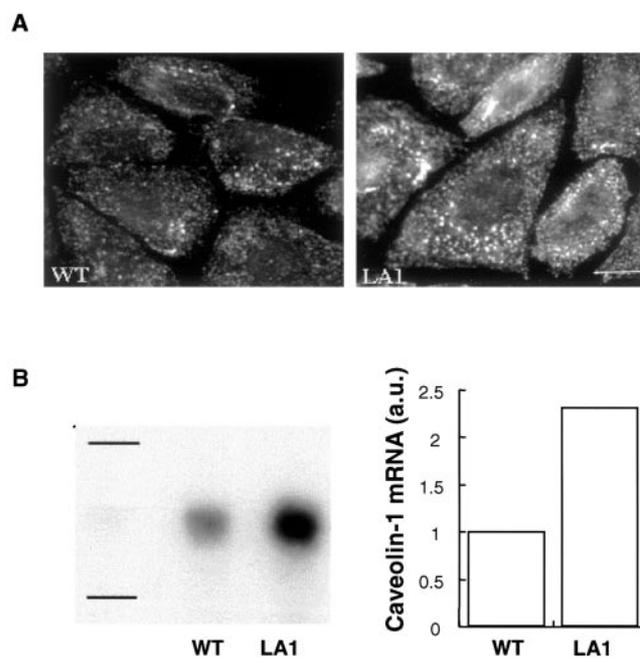


FIG. 7. Up-regulation of caveolin-1 in GPI-deficient cells. *A*, the distribution of caveolin-1 was analyzed by immunofluorescence on wild type and CHO-LA1 cells. Cells were fixed and permeabilized with methanol and incubated for 30 min at room temperature with polyclonal anti-caveolin-1 antibodies, followed by 30 min with fluorescein isothiocyanate anti-rabbit IgGs. Bars = 10 μ m. *B*, detection of caveolin mRNA by Northern blot analysis using the full-length caveolin-1 DNA as probe on 1.25 μ g of total RNA of wild type or LA1 CHO cells. Quantification using a phosphorimager revealed a 2.3-fold increase in caveolin-1 mRNA ($n = 2$).

TABLE I
The number of cell surface caveolae is increased in GPI-deficient CHO cells

	Wild type	LA1	LA1 + PIG-L
No. of caveolae	65	108	61
No. of unit cells counted	60	60	60
No. of caveolae/unit cell	1.1	1.8	1.0

proteins involved in key cellular functions were expressed to normal levels. The β 1- β 2 adaptins, implicated in clathrin-mediated vesicular transport (47), and the small GTPase Rab5, involved in early steps of endocytosis (48), were expressed to similar levels in wild type and GPI-deficient LA1 cells (Fig. 3B). Also levels of transferrin receptor and of β -COP, one of the components of the COPI coat involved biosynthetic and endocytic transport (49, 50), were similar in wild type and mutant cells (Fig. 3B). Finally, we analyzed the levels of p23, a type I membrane protein that has been implicated in organizing the early secretory pathway (51) as well as in transport of GPI-anchored proteins in yeast (52, 53). Levels of p23 were also similar in wild type and mutant cells (Fig. 3B).

The above observations suggest that the absence of GPI-anchored protein does not grossly alter the physiology of CHO cells in culture. This contrasts with the importance of GPI-anchored proteins in mouse embryonic development (54).

Existence of Rafts in GPI-deficient Cells—We next investigated whether the absence of GPI-anchored proteins affected raft integrity. Since rafts have the biochemical property of being resistant to certain non-ionic detergents at 4 $^{\circ}$ C, we have analyzed whether DRMs could be purified by flotation gradients from CHO-LA1 as from wild type CHO cells. As shown in Fig. 4A, DRMs could readily be obtained from mutant cells and were enriched in caveolin-1 as well as flotillin-1, a protein

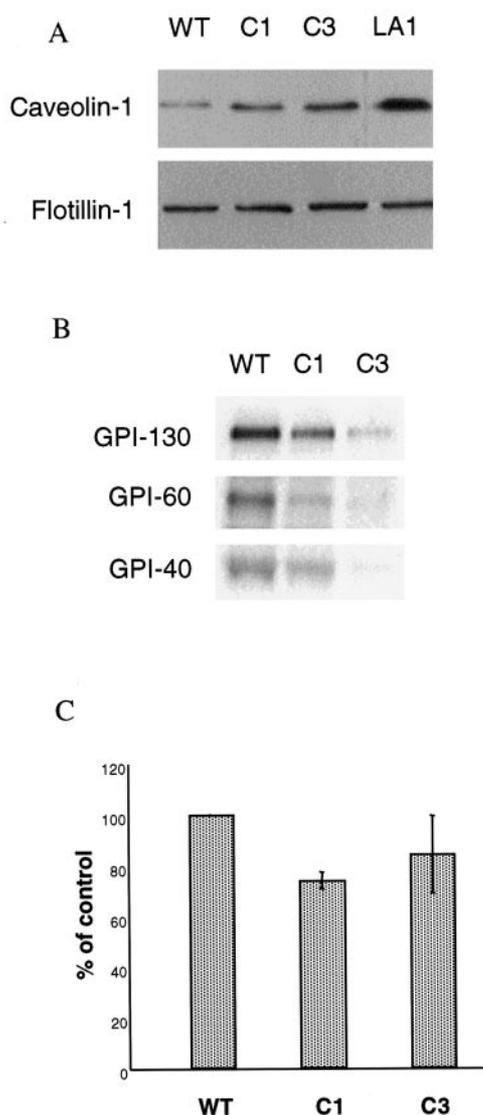


FIG. 8. Down-regulation of GPI-anchored proteins upon over-expression of caveolin-1. *A*, CHO cell lines overexpressing caveolin-1 (called CHO C1 and C3) to various extents were generated. The amounts of caveolin-1 in the various cell types were analyzed by Western blotting (40 μ g of protein loaded/lane). *B*, the levels of GPI-anchored proteins in caveolin-1-overexpressing cells was measured by aerolysin overlay on DRMs prepared using Optiprep gradients. *C*, histogram of 125 I-proaerolysin binding. Monolayers CHO wild type and caveolin-1-overexpressing cells were incubated with 125 I-proaerolysin (0.4 nM) for 1 h at 4 $^{\circ}$ C and extensively washed. The amount of radioactivity associated with the cells was counted, normalized to the amount of protein, and expressed as a percentage of the CHO wild type-associated radioactivity ($n = 4$).

reported to be associated with caveolae and other subdomains of the plasma membrane (55–57). Note that after purification of DRMs, two additional GPI-anchored proteins could be detected by toxin overlay in wild type cells, GPI-40, and GPI-60. The protein composition of DRMs from wild type and GPI-deficient cells was very similar as witnessed by two-dimensional gel analysis (Fig. 4B).

The only detectable difference between DRMs from wild type and GPI-deficient cells as analyzed in Fig. 4A was that the latter contained more caveolin-1. This raised the possibility that CHO-LA1 cells might have fewer or no rafts and compensate for this loss by forming a greater number of caveolae. Since both rafts and caveolae are found in DRMs, the analysis of DRMs of CHO cells does not allow to address this issue.

We therefore analyzed lymphocytes since they lack caveolae

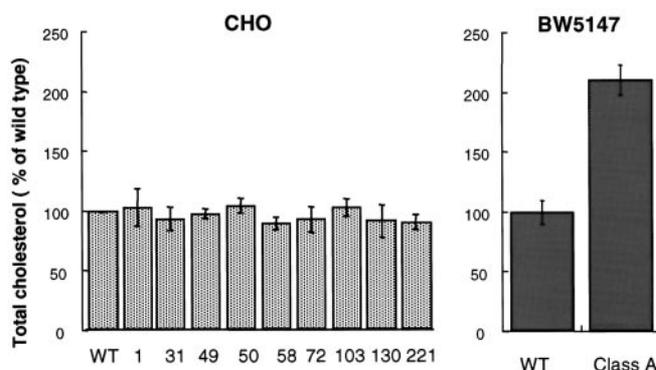


FIG. 9. Cholesterol levels in GPI-deficient cells. The total cholesterol content of wild and GPI-deficient CHO cells as well as wild type and class A BW5147 lymphocytes was measured by TLC analysis on cell extracts (equivalent of 40 μ g of protein). TLC plates were then analyzed by densitometry ($n = 3$).

(15). DRMs were prepared from control lymphocytes (BW5147) and mutants, which are deficient in the first step of GPI biosynthesis, *i.e.* the transfer of *N*-acetylglucosamine to phosphatidylinositol (58) (BW5147/A; Ref. 59). As raft markers, we have used flotillin-1 and the transmembrane protein linker for activation of T cells (LAT) (60). As shown in Fig. 5, DRMs, enriched in flotillin-1 and LAT, could be obtained from both wild type and GPI-deficient lymphocytes, indicating that GPI-anchored proteins are not necessary for the structural integrity of rafts. Since BW5147/A cells are mutated in the first step in GPI biosynthesis (42, 58), these cells also lack non-protein-linked or so-called free GPIs. These free glycolipids are abundant in many mammalian cells (10^5 to 10^7 molecules/cell; Ref. 61), and are found at the plasma membrane as well as in intracellular organelles (62). The physiological role of free GPIs is not known. They are, however, likely to concentrate in rafts since they share the same lipid moiety as GPI-anchored proteins. The present observations demonstrate that not only GPI-anchored proteins but also free GPIs are not required for raft integrity.

Increased Expression of Caveolin-1 in GPI-deficient Cell Lines—The observation that caveolin-1 was more enriched in DRMs of CHO-LA1 cells than in those of wild type cells (Fig. 4A) could be due to a stronger association of caveolin-1 with DRMs in the mutant cells or to higher expression levels of this protein. We therefore compared the amounts of caveolin-1 in postnuclear supernatants of wild type and CHO-LA1 cells. Care was taken to perform experiments on wild type and mutant cells that were at similar confluence since cell confluence can affect the level of caveolin-1 expression (63). As shown in Fig. 6A, the total amount of caveolin-1 was higher in CHO-LA1 cells but was restored to normal levels upon expression of the PIG-L gene, but not of the PIG-A gene (data not shown). The increase in caveolin-1 in LA1 cells was accompanied by an up-regulation of caveolin-2 (Fig. 6A). The levels of other markers of DRMs such as flotillin-1 and GM1 were, however, not affected (Fig. 6A), suggesting that the absence of GPI-anchored protein specifically affects caveolin levels.

According to this hypothesis, all mutant cell lines deficient in GPI biosynthesis should express higher levels of caveolin-1 than wild type cells, independently of the gene in the GPI biosynthesis that is mutated. As shown in Fig. 6B, this is indeed the case: all our previously mentioned GPI-deficient cell lines (which no longer express GPI-130, as shown in Fig. 1, or exogenous alkaline phosphatase) showed increased levels of caveolin-1. One possibility could have been that the increase of caveolin-1 is not due to the absence of GPI-anchored proteins but rather to the accumulation of GPI intermediates. This

seems unlikely for the following reasons. First, CHO-LA1 and CHO-LA49 are mutated in two proteins involved in the early steps of GPI biosynthesis and therefore accumulate GlcNAc-PI but not mannosylated intermediates. Second, although CHO-LA49 cells are likely to accumulate less GlcNAc-PI than CHO-LA1 (see reason below), they express similarly high levels of caveolin-1 as CHO-LA1 cells. As mentioned above, CHO-LA49 cells were affected in DPM2, a protein that has been shown to regulate the GPI-N-acetyl glucosaminyltransferase (64). In the absence of DPM2, the enzyme activity of GPI-N-acetyl glucosaminyltransferase was found to be reduced by 3-fold leading to a decreased production of the GPI intermediate GlcNAc-PI.

Increase in the Number of Caveolae in GPI-deficient CHO-LA1 Cells—Up-regulation of caveolin-1 has been shown previously to be accompanied by an increase in the number of caveolae (38, 65, 66). Immunofluorescence analysis of wild type and CHO-LA1 suggested that indeed the number of caveolae was higher in the GPI-deficient cells (Fig. 7A) since the punctate staining pattern appeared more intense and dense for CHO-LA1 cells. This was confirmed when analyzing the cells by electron microscopy. Non-coated 50–100-nm flask-shaped invaginations at the plasma membrane, typical of caveolae, were counted for each cell type. CHO-LA1 cells had almost twice as many caveolae as wild type (Table I). Importantly, the number of caveolae returned to wild type levels when LA1 cells were complemented with the PIG-L gene.

This higher expression of caveolin-1 in CHO-LA1 is most likely due to an increase in transcription since the level of caveolin-1 mRNA was increased by 2.5-fold when compared with wild type cells, as determined by Northern blot analysis using a caveolin-1 cDNA probe (Fig. 7B). We cannot, however, exclude that mRNA stability was increased.

Inverse Correlation between the Levels of Caveolin-1 and of GPI-anchored Proteins—The above experiments show that the absence of GPI-anchored proteins leads to an up-regulation of caveolins and an increase in the number of caveolae. To confirm this apparent inverse correlation, we tested whether overexpression of caveolin-1 would lead to a reduction of the amount of GPI-anchored proteins. Two cell lines, CHO C1 and C3, were generated that overexpress caveolin-1 to different extents, which are, however, lower than CHO-LA1 GPI minus cells (Fig. 8). As shown in Fig. 8, the increase in caveolin-1 levels was accompanied by a decrease in the amounts of GPI-anchored proteins (GPI-130, GPI-60, and GPI-40) as well as an expected decrease in aerolysin binding (Fig. 8B).

The inverse correlation we have observed here between caveolin-1 and GPI-anchored protein expression is consistent with the previous observations that a reduction in caveolin-1 in ovarian carcinoma leads to an increase in the levels of GPI-anchored folate receptor (67) and that the overexpression of placental alkaline phosphatase in baby hamster kidney cells leads to a decrease in caveolin-1 levels (data not shown).

Effect of GPI Deficiency on Total Cholesterol Content—Finally, since GPI-anchored proteins are enriched in cholesterol-rich membranes and since cholesterol appears to regulate transport of GPI-APs (21, 23), we wondered whether the absence of GPI-anchored proteins would affect total cholesterol contents. As shown in Fig. 9, all our CHO GPI-deficient cell lines (described in Figs. 1 and 6) had total cholesterol contents similar to those for wild type cells. Since caveolin-1 has been shown to play a role in cholesterol homeostasis (6, 7), the maintenance of wild type cholesterol levels in GPI-deficient CHO cells could be due to the increased amount of caveolins. To test this possibility, we measured total cholesterol levels in wild type and GPI-deficient BW5147 lymphocytes. As shown in Fig. 9, class A lymphocytes had a 2.1-fold higher cholesterol

level than wild type cells. Taken together, the above observations support the hypothesis that caveolins are up-regulated in GPI-deficient CHO cells in response to cholesterol changes.

Conclusions—We here show that GPI-anchored proteins as well as free GPIs are not required for the structural integrity of lipids rafts. In agreement with our observations, it was recently shown (68) that erythrocytes from patients with paroxysmal nocturnal hemoglobinuria, which lack glycosylphosphatidylinositol-anchored proteins (mutation in PIG A gene), still contained rafts.

Interestingly, we found that in the absence of GPI-anchored proteins, lymphocytes had elevated levels of cholesterol. Similar observations were made on paroxysmal nocturnal hemoglobinuria erythrocytes, which were found to have 2.37 times more cholesterol than control erythrocytes (68). In contrast, we found that CHO cells stringently maintain their cholesterol levels in the absence of GPI-anchored proteins. One possible explanation for the differences observed between lymphocytes and erythrocytes, on one hand, and CHO cells, on the other, is that the latter cells contain caveolins, which have been implicated in cholesterol homeostasis. Strikingly, GPI-deficient CHO cells express higher levels of caveolins than wild type cells and have more caveolae at their plasma membrane. It is therefore tempting to speculate that caveolins are up-regulated in GPI-deficient cells in response to cholesterol changes. Further investigation will be required to confirm the hypothesis that GPI deficiency affects cholesterol regulation, in a manner that can be compensated for by caveolins.

Acknowledgments—We are very grateful to Robert Hyman providing us with GPI-deficient lymphocytes. We thank Filipo Passardi for contributions toward this work during short term training in the laboratory. We thank Paul Dupree for caveolin-1 cDNA; Perry Bickel for the anti-flotillin-1 antibodies; Marie-Claire Velluz, Margaret Lindsay, and Kathryn Green for technical assistance; and Jean Gruenberg and Frank Lafont for critical reading of the manuscript.

REFERENCES

1. Simons, K., and Ikonen, E. (1997) *Nature* **387**, 569–572
2. Brown, D. A., and London, E. (1998) *Annu. Rev. Cell Dev. Biol.* **14**, 111–136
3. Janes, P. W., Ley, S. C., Magee, A. I., and Kabouridis, P. S. (2000) *Semin. Immunol.* **12**, 23–34
4. Schlegel, A., Pestell, R. G., and Lisanti, M. P. (2000) *Front. Biosci.* **5**, D929–937
5. Kasahara, K., Watanabe, K., Takeuchi, K., Kaneko, H., Oohira, A., Yamamoto, T., and Sanai, Y. (2000) *J. Biol. Chem.* **275**, 34701–34709
6. Fielding, C. J., and Fielding, P. E. (1997) *J. Lipid Res.* **38**, 1503–1521
7. Ikonen, E., and Parton, R. G. (2000) *Traffic* **1**, 212–217
8. Simons, M., Keller, P., De, S. B., Beyreuther, K., Dotti, C. G., and Simons, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 6460–6464
9. Lee, S. J., Liyanage, U., Bickel, P. E., Xia, W., Lansbury, P. T., Jr., and Kosik, K. S. (1998) *Nat. Med.* **4**, 730–734
10. Fivaz, M., Abrami, L., and van der Goot, F. G. (1999) *Trends Cell Biol.* **9**, 212–213
11. van der Goot, F. G., and Harder, T. (2001) *Semin. Immunol.* **13**, 89–97
12. London, E., and Brown, D. A. (2000) *Biochim. Biophys. Acta* **1508**, 182–195
13. Parton, R. G. (1996) *Curr. Opin. Cell Biol.* **8**, 542–548
14. Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998) *J. Biol. Chem.* **273**, 5419–5422
15. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1994) *J. Biol. Chem.* **269**, 30745–30748
16. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 8655–8659
17. Lipardi, C., Mora, R., Colomer, V., Paladino, S., Nitsch, L., Rodriguez-Boulan, E., and Zurzolo, C. (1998) *J. Cell Biol.* **140**, 617–626
18. Vogel, U., Sandvig, K., and van Deurs, B. (1998) *J. Cell Sci.* **111**, 825–832
19. Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J., and Oh, P. (1995) *Science* **269**, 1435–1439
20. Maxfield, F. R., and Mayor, S. (1997) *Adv. Exp. Med. Biol.* **419**, 355–364
21. Mayor, S., Sabharanjak, S., and Maxfield, F. R. (1998) *EMBO J.* **17**, 4626–4638
22. Chatterjee, S., Smith, E. R., Hanada, K., Stevens, V. L., and Mayor, S. (2001) *EMBO J.* **20**, 1583–1592
23. Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D., and Lippincott-Schwartz, J. (2001) *J. Cell Biol.* **153**, 529–542
24. Nelson, K. L., Raja, S. M., and Buckley, J. T. (1997) *J. Biol. Chem.* **272**, 12170–12174
25. Abrami, L., Fivaz, M., Glauser, P.-E., Parton, R. G., and van der Goot, F. G. (1998) *J. Cell Biol.* **140**, 525–540
26. Abrami, L., Fivaz, M., and van Der Goot, F. G. (2000) *Trends Microbiol.* **8**, 168–172

27. Nakamura, N., Inoue, N., Watanabe, R., Takahashi, M., Takeda, J., Stevens, V. L., and Kinoshita, T. (1997) *J. Biol. Chem.* **272**, 15834–15840
28. Tarutani, M., Itami, S., Okabe, M., Ikawa, M., Tezuka, T., Yoshikawa, K., Kinoshita, T., and Takeda, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7400–7405
29. Kurzchalia, T. V., Dupree, P., Parton, R. G., Kellner, R., Virta, H., Lehnert, M., and Simons, K. (1992) *J. Cell Biol.* **118**, 1003–1014
30. Buckley, J. T. (1990) *Biochem. Cell Biol.* **68**, 221–224
31. van der Goot, F. G., Hardie, K. R., Parker, M. W., and Buckley, J. T. (1994) *J. Cell Biol.* **269**, 30496–30501
32. Kuge, O., Nishijima, M., and Akamatsu, Y. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1926–1930
33. Laemmli, U. K. (1970) *Nature* **227**, 680–685
34. Graham, F. L., and van der Eb, A. J. (1973) *Virology* **54**, 536–539
35. Harder, T., Scheiffele, P., Verkade, P., and Simons, K. (1998) *J. Cell Biol.* **141**, 929–942
36. Fivaz, M., Vilbois, F., Pasquali, C., and van der Goot, F. G. (2000) *Electrophoresis* **21**, 3351–3356
37. Pasquali, C., Fialka, I., and Huber, L. A. (1997) *Electrophoresis* **18**, 2573–2581
38. Hailstones, D., Sleer, L. S., Parton, R. G., and Stanley, K. K. (1998) *J. Lipid Res.* **39**, 369–379
39. Diep, D. B., Nelson, K. L., Raja, S. M., McMaster, R. W., and Buckley, J. T. (1998) *J. Cell Biol.* **273**, 2355–2360
40. Gordon, V. M., Nelson, K. L., Buckley, J. T., Stevens, V. L., Tweten, R. K., Elwood, P. C., and Leppla, S. H. (1999) *J. Biol. Chem.* **274**, 27274–27280
41. Brodsky, R. A., Mukhina, G. L., Nelson, K. L., Lawrence, T. S., Jones, R. J., and Buckley, J. T. (1999) *Blood* **93**, 1749–1756
42. Kinoshita, T., Ohishi, K., and Takeda, J. (1997) *J. Biochem. (Tokyo)* **122**, 251–257
43. Stevens, V. L. (1995) *Biochem. J.* **310**, 361–370
44. Stevens, V. L., Zhang, H., and Harreman, M. (1996) *Biochem. J.* **313**, 253–258
45. Abrami, L., Fivaz, M., Decroly, E., Seidah, N. G., François, J., Thomas, G., Leppla, S., Buckley, J. T., and van der Goot, F. G. (1998) *J. Biol. Chem.* **273**, 32656–32661
46. Maeda, Y., Tomita, S., Watanabe, R., Ohishi, K., and Kinoshita, T. (1998) *EMBO J.* **17**, 4920–4929
47. Kirchhausen, T. (2000) *Nat. Rev. Mol. Cell Biol.* **1**, 187–198
48. Zerial, M., and McBride, H. (2001) *Nat. Rev. Mol. Cell Biol.* **2**, 107–117
49. Kreis, T. E., Lowe, M., and Pepperkok, R. (1995) *Annu. Rev. Cell Dev. Biol.* **11**, 677–706
50. Aniento, F., Gu, F., Parton, R. G., and Gruenberg, J. (1996) **133**, 29–41
51. Rojo, M., Pepperkok, R., Emery, G., Kellner, R., Stang, E., Parton, R. G., and Gruenberg, J. (1997) *J. Cell Biol.* **139**, 1119–1135
52. Muniz, M., Morsomme, P., and Riezman, H. (2001) *Cell* **104**, 313–320
53. Muniz, M., and Riezman, H. (2000) *EMBO J.* **19**, 10–15
54. Nozaki, M., Ohishi, K., Yamada, N., Kinoshita, T., Nagy, A., and Takeda, J. (1999) *Lab. Invest.* **79**, 293–299
55. Bickel, P. E., Scherer, P. E., Schnitzer, J. E., Oh, P., Lisanti, M. P., and Lodish, H. F. (1997) *J. Biol. Chem.* **272**, 13793–13802
56. Lang, D. M., Lommel, S., Jung, M., Ankerhold, R., Petrausch, B., Laessing, U., Wiechers, M. F., Plattner, H., and Stuermer, C. A. (1998) *J. Neurobiol.* **37**, 502–523
57. Volonte, D., Galbiati, F., Li, S., Nishiyama, K., Okamoto, T., and Lisanti, M. P. (1999) *J. Biol. Chem.* **274**, 12702–12709
58. Ferguson, M. A. J. (1999) *J. Cell Sci.* **112**, 2799–2809
59. Sugiyama, E., DeGasperi, R., Urakaze, M., Chang, H. M., Thomas, L. J., Hyman, R., Warren, C. D., and Yeh, E. T. (1991) *J. Biol. Chem.* **266**, 12119–12122
60. Harder, T., and Kuhn, M. (2000) *J. Cell Biol.* **151**, 199–208
61. van't Hof, W., Rodriguez-Boulan, E., and Menon, A. K. (1995) *J. Biol. Chem.* **270**, 24150–24155
62. Baumann, N. A., Vidugiriene, J., Machamer, C. E., and Menon, A. K. (2000) *J. Biol. Chem.* **275**, 7378–7389
63. Galbiati, F., Volonte, D., Engelman, J. A., Watanabe, G., Burk, R., Pestell, R. G., and Lisanti, M. P. (1998) *EMBO J.* **17**, 6633–6648
64. Watanabe, R., Murakami, Y., Marmor, M. D., Inoue, N., Maeda, Y., Hino, J., Kangawa, K., Julius, M., and Kinoshita, T. (2000) *EMBO J.* **19**, 4402–4411
65. Scherer, P. E., Lisanti, M. P., Baldini, G., Sargiacomo, M., Mastick, C. C., and Lodish, H. F. (1994) *J. Cell Biol.* **127**, 1233–1243
66. Yang, C. P., Galbiati, F., Volonte, D., Horwitz, S. B., and Lisanti, M. P. (1998) *FEBS Lett.* **439**, 368–372
67. Bagnoli, M., Tomassetti, A., Figini, M., Flati, S., Dolo, V., Canevari, S., and Miotti, S. (2000) *Oncogene* **19**, 4754–4763
68. Samuel, B. U., Mohandas, N., Harrison, T., McManus, H., Rosse, W., Reid, M., and Haldar, K. (2001) *J. Biol. Chem.* **276**, 29319–29329