Traffic, Polarity, and Detergent Solubility of a Glycosylphosphatidylinositol–anchored Protein after LDL-Deprivation of MDCK Cells

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Abstract. Glycosylphosphatidylinositol-anchored proteins, GPI-proteins, are selectively delivered to the apical surfaces of many types of morphologically polarized epithelial cells. It has been proposed that the unit for targeting GPI-proteins to the apical surface is a membrane lipid domain. This sorting domain or molecular cluster has been equated to detergent (Triton X-100)–insoluble membrane fractions that are enriched in enriched in GPI-proteins, glycosphingolipids, and cholesterol. To determine the role of cholesterol in the formation of sorting domains and to examine its importance in the intracellular traffic and membrane polarity of GPI-proteins, we studied the behavior of a model GPI-protein, gD1-DAF, in MDCK cells cultured for 3 or 14 d without their principal source of cholesterol, serum LDL. LDL deprivation affects the intracellular traffic of gD1-DAF. Surface expression of gD1-DAF is reduced in LDL-deprived cells; this reduction is most marked after 3 d of LDL deprivation. We also find a great reduction in the fraction of gD1-DAF that is detergent-insoluble in these cells and a change in its membrane milieu defined by susceptibility to cleavage with PI-specific phospholipase C. Despite these changes, the surface polarity of gD1-DAF is no different in LDL-deprived cells than in control cells.

G LYCOSYLPHOSPHATIDYLINOSITOL (GPI)[1]-anchored proteins, are selectively delivered to the apical surfaces of many types of morphologically polarized epithelial cells, including MDCK cells (Lisanti et al., 1988; 1989; Brown et al., 1989). Models for this selective delivery must accommodate the fact that GPI-proteins are anchored to the lumenal leaflet of intracellular membranes and cannot interact directly with the sorting machinery of the cell cytoplasm. The simplest explanation for the apical targeting of GPI-proteins is that delivery to this surface is a default pathway for proteins lacking targeting signals (Matter and Mellman, 1994). However, another influential model has been developed for signal-directed targeting of GPI-proteins (Lisanti and Rodriguez-Boulan, 1990; Simons and Wandinger-Ness, 1990). This model proposes that the targeted unit is a cluster of molecules, a membrane domain enriched in GPI-proteins, glycosphingolipids (GSL), and cholesterol. Polarized delivery of this domain to the cell surface may be due to the recognition of the whole cluster by cellular sorting machinery, or to the recognition of the sorting signals of transmembrane or other proteins also associated with the cluster.

Fluorescence photobleaching recovery and fluorescence resonance energy transfer measurements indicate that molecules of one GPI-protein, gD1-DAF, are indeed clustered when they arrive at the surface of MDCK cells (Hannan et al., 1993). Clusters of other GPI-proteins have been defined as high molecular weight membrane fractions that are insoluble in cold Triton X-100 detergent (Zurzolo et al., 1994; Fiedler et al., 1993; Garcia et al., 1993; Cerneus et al., 1993; Brown and Rose, 1992; Cinek and Horejsi, 1992; Stefanova et al., 1991a, b; Stefanova and Horejsi, 1991). The detergent-insoluble fractions are enriched in both GSLs and cholesterol (Zurzolo et al., 1994; Fiedler et al., 1993; Brown and Rose, 1992). The fractions also contain protein tyrosine kinases (Arreaza et al., 1994; Sargiacomo et al., 1993; Cinek and Horejsi, 1993; Shenoy-Scaria et al., 1992; Stefanova et al., 1991) type II membrane proteins (Danielsen, 1995; Garcia et al., 1993) and caveolin (Fiedler et al., 1993; Sargiacomo et al., 1993). Though it has recently been suggested that the molecules of detergent-insoluble fractions may not be associated in membranes before detergent extraction (Mayor and Maxfield, 1995a), experiments with synthetic lipid mixtures show that the detergent insolubility depends upon membrane lipid composition, particularly content of cholesterol and sphingomyelin (Schroeder et al., 1994).

If detergent-insoluble membrane fractions do represent

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1. *Abbreviations used in this paper.* GSL, glycosphingolipid; GPI, glycosylphosphatidylinositol; LDL, low density lipoprotein; PIPLC, phosphatidylinositol-specific phospholipase C; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene.

the units for intracellular traffic and sorting of GPI-proteins, then inhibiting the synthesis of or sequestering cholesterol ought to affect both the detergent solubility and the traffic of GPI-proteins. There is some evidence that this is indeed the case. Blocking the synthesis of cholesterol, or sequestering membrane cholesterol reduces the fraction of GPI-proteins that is detergent insoluble (Hanada et al., 1995; Cerneus et al., 1993), (though not all sequestering agents have this effect on detergent insolubility (Garcia et al., 1993). Inhibiting cholesterol synthesis also reduces the amount of a GPI-protein expressed at the cell surface of neutrophils (Esfahani et al., 1993a, b) and interferes with the internalization of folate receptors in caveolae (Chang et al., 1992), and with the traffic and maturation of the GPI-anchored prion protein, PrP^{Sc} in scrapie-infected neuroblastoma cells (Taraboulos et al., 1995). Cholesterol deprivation or sequestration also alters the normal structure and function of caveolar membranes (Rothberg et al., 1990a; Chang et al., 1992; Smart et al., 1994; Schnitzer et al., 1994); these structures and associated membranes are normally detergent insoluble and enriched in GPI-proteins (Lisanti et al., 1993; Schnitzer et al., 1994; Rothberg et al., 1990b. For another view see Mayor et al., 1994).

In this paper we examine the role of cholesterol in the intracellular traffic and membrane residence of a model GPI-protein gD1-DAF. We have deprived MDCK cells of their principal source of cholesterol, serum low density lipoprotein (LDL) (Li et al., 1991), for a relatively short period, 3 d, and for an extended period, 14 d. Growing cells without LDL for 3 d reduces cell cholesterol content and increases their endogenous cholesterol synthesis (Goldstein and Brown, 1990). Hence, this regimen of LDL-deprivation is expected to have general effects on membrane composition and function as specific effects on traffic of GPI-proteins. Longer term growth of cells without LDL may allow them to compensate for reduced cholesterol levels; results in intact animals (Muriana et al., 1992; Lutton, 1991; Leikin and Brenner, 1988) indicate that cells adapt to lowered cholesterol levels with changes in lipid desaturase activity and in membrane lipid composition.

We find that the fraction of gD1-DAF on the cell surface is lower in LDL-deprived cell than in control cells. This seems to be due to the effects of LDL deprivation on the intracellular traffic of the model GPI-protein. We also find a great reduction in the fraction of gD1-DAF that is detergent-insoluble in LDL-deprived cells, and a change in its membrane milieu defined by susceptibility to cleavage with PI-specific phospholipase C. Despite these changes, the surface polarity of gD1-DAF is no different in LDL-deprived cells than in control cells. Thus, detergent-insoluble forms of gD1-DAF are not necessarily required for correct sorting of this GPI-protein to the apical surface.

Materials and Methods

Materials and Cell Culture

All chemicals were purchased from Sigma Chem. Co. (St. Louis, MO) unless otherwise indicated. MDCK cells were cultured at 37°C in an atmosphere of 5% CO_2, in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS (Intergen, Purchase, NY) and nonessential amino acids (Life Technologies, Gaithersburg, MD). LDL-deprived cells were grown

for 3–14 d in medium lacking LDL: McCoys 5A medium (Life Technologies) supplemented with 5% lipoprotein-deficient FCS (Organon-BRI, Bethesda, MD) (Martin et al., 1993). MDCK/gD1-DAF cells were the generous gift of Dr. E. Rodriguez-Boulan (Cornell University Medical School, New York, NY) (Lisanti et al., 1989). MDCK/DPP IV cells, as well as monoclonal antibodies to DPP IV, were the generous gift of Dr. A.L. Hubbard (Johns Hopkins Medical School) (Weisz et al., 1992). MDCK/PLAP-HA cells were the generous gift of Dr. D. Brown (SUNY, Stony Brook) (Arreaza et al., 1994). The expression of each protein was induced 12–24 h before an experiment by the addition of 10 mM sodium butyrate to the cultures. Cells were grown on nitric acid–cleaned glass coverslips or filters (Transwells™, Costar Corp., Cambridge, MA). 20% of the cells formed "domes" on coverslips, indicating that even on glass the cells were polarized at the time of an experiment. We were unable to detect any changes in the trans-epithelial electrical resistance of cells grown on Transwells™ in medium lacking LDL. LDL (Organon-BRI) was used at 5 μg/ml, and imipramine at 20 μM.

Cell Cholesterol Content

Cells grown on 35-mm plates were extracted once with 3:2 isopropanol: hexane, and twice with isopropanol, and these extracts dried under a stream of N_2, and then vacuum. Free cholesterol and total cholesterol (free+ cholesteryl ester) concentrations were determined by the method of Gamble et al. (1978), an enzymatic assay which evolves a fluorescent product. Fluorescence intensities were measured on a Hitachi F-3010 fluorimeter. The protein content of the material remaining after lipid extraction was determined by the BCA assay (Pierce, Rockford, IL).

Estimates of Errors

When normalizing other values to protein content, errors were carried through calculations as coefficient of variation, c.v. (standard error as a percentage of the mean), and converted to standard errors at the last step of the calculation.

Cholesterol Biosynthesis

MDCK cells were grown to confluence in complete medium or in medium lacking LDL, and labeled with 15 μCi ^{14}C-sodium acetate (American Radiochemicals, St. Louis, MO) in the presence 100 μM sodium acetate for 20 h (Shakarjian et al., 1993). The cells were harvested and suspended in PBS at 5 × 10^6 cells/ml. 3 ml of 2:1 methanol:chloroform were used to extract 0.8 ml of cells in PBS for 1 h at 4°C. The extracts were centrifuged, and an equal volume of chloroform was added (Sleight and Pagano, 1984). The lower phase was dried under a stream of N_2. The dried sample was resuspended in chloroform and analyzed on high performance thin layer chromatography, HPTLC, plates (Alltec Associates, Birmingham, AL) in 80% hexane, 20% diethyl ether 2% acetic acid , using appropriate standards (Mangold, 1969). The cholesterol in each sample was determined by laser scanning densitometry of autoradiographic images of the HPTLC plates.

Fluorescence Polarization Microscopy

The physical state of MDCK cell apical membranes was characterized in terms of the apparent anisotropy, r, of the fluorescent probe TMA-DPH (Khury et al., 1983). Cells grown on coverslips were labeled by addition of 10 μl of a 1-mM stock of TMA-DPH (Molecular Probes, Eugene, OR) in DMF to 1 ml of DMEM with 10% FCS and incubation for 5 min at 37°C. The cells were then washed with three changes of Hepes-buffered HBSS, without phenol red, HH, and fixed in 2% formaldehyde in PBS. To measure r, labeled cells were imaged in a Zeiss Axioplan microscope using dark-field illumination. TMA-DPH was excited by 360 nm light whose intensity was reduced with neutral density filters such that TMA-DPH fluorescence faded < 5% during a measurement series. The exciting light was plane-polarized by a series MUVP-20 UV-transmitting linear film polarizer (Marks Polarized Corp., Hauppauge, NY) placed between the light source and excitation filter and the dark-field condenser. Emitted fluorescence was passed through 435–485-nm bandpass filter and an analyzer film. The polarization of the analyzer was kept constant. This arrangement, a rotatable polarizer, a dark-field condenser, and a fixed analyzer, was dictated by the construction of the microscope and the fact that the dichroic mirrors of standard epifluorescence filter cubes preferentially transmit light of one polarization.

Polarized fluorescence images were acquired using a cooled CCD camera (Photometrics, Tucson, AZ) and output to a Sun SparcStation running ISee image analysis software (Inovision, Durham, NC). To quantitate fluorescence intensity the software was used to determine the average intensity of a large area containing 10–20 cells. Fluorescence anisotropy, r, was calculated from intensities, $I_{\parallel}$ and $I_{\perp}$ as:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

GPI Anchorage of gD1-DAF Determined in Cell Extracts

MDCK/gD1-DAF cells were grown in complete medium or medium lacking LDL, metabolically labeled with ^{35}S-Translabel, washed thoroughly with PBS, and extracted with 1 ml 1% Triton X-114 in HN buffer for 10 min at 37°C. Triton X-114 was precondensed according to the method of Bordier (1981). Extracts of equal numbers of cells were incubated for 10 min at 4°C and centrifuged for 10 min at 15,000 g at 4°C. The supernatant was incubated at 37°C for 10 min, and the separated detergent phase was treated with 10 U/ml PIPLC for 15 min at 37°C. 1 ml of fresh ice cold 1% Triton X-114 solution was then added to the sample, and the sample was cycled through two additional phase separations. gD1-DAF was immunoprecipitated from each phase as described previously, and quantitated by SDS-PAGE followed by autoradiography and laser scanning densitometry of autoradiographic images.

Treatment of Live Cells with Phosphatidyl-Inositol–specific Phospholipase C

PIPLC was prepared from bacteria overexpressing *Bacillus thuringiensis* PIPLC (the generous gift of Dr. J.J. Volwerk, University of Oregon) by the method of Koke et al. (1991). We did not detect any protease activity in our preparation using the Azocol protease assay system (Calbiochem, San Diego, CA). 10 U/ml of PIPLC was applied to 100-mm plates or to coverslips of confluent MDCK/gD1-DAF cells, and incubated for 1 h at 20°C. The cells were rinsed at least three times with 10 mM Hepes, pH 7.35, with Hanks balanced salts. For determining the amount of gD1-DAF remaining after PIPLC treatment, cells treated in plates were labeled with anti-gD1 IgG and ^{125}I-protein A as described above. In one experiment cells were treated with PIPLC in suspension, washed, and labeled with Fl-Fab anti-gD1-DAF. The intensities of the labeled cells were compared by flow cytometry (Epics 752, Coulter Corp., Hialeah, FL).

Fluorescence Microscopy

Cells were fixed before labeling in 2% paraformaldehyde in PBS for 20 min at room temperature, washed with PBS, and incubated with IMF blocking buffer (1% BSA, 5 mg/ml lysine in PBS). When cells were permeabilized, 0.1% saponin was included in all subsequent incubations (Wassler et al., 1987). The cells were incubated with 1 μM rabbit polyclonal antibodies against gD1 (Showalter et al., 1981) or human alkaline phosphatase (Dako, Carpinteria, CA) or with the same concentration of monoclonal antibodies against rat DPP IV in IMF blocking buffer. Primary antibody was detected with fluorescein-conjugated goat anti–rabbit IgG (Zymed, S. San Francisco, CA), or Texas red–conjugated goat anti–rabbit IgG Fab2 (Jackson Immunoresearch), 15 μg/ml in IMF blocking solution. There was no staining apparent in cells labeled with the secondary antibody alone. The Golgi apparatus was labeled with C$_6$-NBD-ceramide (6-((n-(7-nitrobez-2-oxa-1,3-diazol-4-yl)amino)-caproyl)sphingosine) by the method of Lipsky and Pagano (1985). DiOC$_6$ (3-3'-dihexyloxacarbocyanine iodide) (Molecular Probes) was used as a marker for the endoplasmic reticulum at a concentration of 25 ng/ml (Terasaki et al., 1986). Endosomes were labeled with a fluid-phase marker by incubating cells in 10 mg/ml lysine-fixable fluorescein dextran (mol/wt 10,000, Molecular Probes) for 20 min at 37°C (Apodaca et al., 1994). The subapical vesicular compartment was also labeled with fluorescein-transferrin (Molecular Probes). Cells were serum-starved for 4 h before adding 50 μg/ml transferrin to each culture and incubation for 90 min at 37°C. After labeling, the samples were mounted in Vectashield (Vector Labs, Burlington, CA). Confocal images were obtained on a Nikon Optiphot microscope with a BioRad MRC600 confocal attachment. Digital images were taken on a Zeiss Axiovert microscope coupled to a slow-scan CCD camera.

Quantitation of Surface gD1-DAF by Digital Microscopy

MDCK/gD1-DAF cells were grown on glass coverslips, fixed, and labeled with Fl-Fab anti-gD1 as described above. Digital images of random fields (selected with phase contrast illumination, not fluorescence) were made using the CCD camera and software described above. The fluorescence intensity of each cell in a field was quantitated using ISee software.

Quantitation of Surface gD1-DAF, DPP IV, or PLAP-HA by ^{125}I- Protein A Binding

MDCK cells, grown on 24-well format Transwells™ (for MDCK/gD1-DAF or MDCK/DPP IV) or on 35-mm plates (for MDCK/PLAP-HA), were washed with PBS and fixed with 2% paraformaldehyde as described above. After incubation with IMF blocking solution, the cells were labeled with 1 μM anti-gD1 or anti-DPP IV antibody from either the apical or the basolateral surface. In experiments to determine the amount of intracellular gD1-DAF, 0.1% saponin was added to the IMF blocking buffer. MDCK/PLAP-HA cells were labeled only from the apical surface with anti-PLAP antibody (Dako, Carpinteria, CA). Labeled cells were washed with several changes of PBS over 30 min, and 1 μCi/ml ^{125}I protein A (ICN-Biomedicals, Costa Mesa, CA, specific activity >30 μCi/μg) was added to each sample from either the apical or basolateral surface as appropriate. The cells were washed with several changes of PBS over 30 min, and allowed to air dry. The filter was removed from each well, and the radioactivity measured on a gamma counter. Total protein content was determined with BCA.

Synthesis of gD1-DAF

MDCK/gD1-DAF cells were labeled at 37°C with 0.1 mCi/ml ^{35}S-Translabel™ (ICN-Biomedicals) in methionine-free DME (GIBCO-BRL) for 5–45 minutes, washed with PBS, and extracted in 1 ml 60 mM octylglucoside (Calbiochem) in HN buffer (10 mm Hepes pH 7.5, 0.15 M NaCl containing 1 μg/ml each of antipain, pepstatin, and leupeptin) for 15 min at 4°C. These extracts were collected and centrifuged for 10 min at 15,000 g at 4°C. The pellets were discarded, the extracts were "precleared" with killed *Staphylococcus aureus* cells. gD1-DAF was immunoprecipitated with premixed anti-gD1 antibodies and protein–A Sepharose beads. The immunoprecipitates were washed 4–6 times in HN. The radioactivity in each immunoprecipitate was determined by liquid scintillation counting, and normalized to the total protein content of the detergent extract.

Degradation of gD1-DAF

MDCK/gD1-DAF cells, grown on 60-mm plates, were labeled with ^{35}S-Translabel™ for 30 min as described above. The cells were washed and chased for 0–4 h with the appropriate "cold" medium. The cells were washed and extracted with 60 mM octylglucoside in HN buffer, and gD1-DAF immunoprecipitated as described above. The radioactivity in the immunoprecipitates was counted on a scintillation counter, and normalized for protein content in the detergent extracts.

Detergent Solubility and Endoglycosidase H Resistance of gD1-DAF

MDCK/gD1-DAF cells grown in complete medium, in medium lacking LDL, or in this medium supplemented with LDL for the last 20 h of culture, were labeled with ^{35}S Translabel™ for 30 min as described previously; and chased for 0–120 min at 37°C with the appropriate medium. The confluent cells grown on 60-mm tissue culture plates were extracted in 1 ml 1% Triton X-100 in HN buffer for 15 min at 4°C (Skibbens et al., 1991; Brown and Rose, 1992). The extracts were centrifuged at 15,000 g for 10 min at 4°C. The pellets from each step were solubilized in 1 ml 60 mM octylglucoside in HN buffer, and the solutions extruded 5–10 times through a 22-gauge needle. gD1-DAF was immunoprecipitated from each sample as described above. Each washed immunoprecipitate was boiled for 2 min in 30 μl 1% SDS, 10 mM Tris, pH 6.8, 0.15 M NaCl, and divided into two aliquots. 10 ml of 50 mM citrate buffer, pH 5.5, with protease inhibitors was added to one aliquot, 0.1 U Endoglycosidase H (Boehringer Mannheim Indianapolis, IN) was added to the second aliquot, and both were incubated at 37°C overnight (Rosenwald et al., 1992). The reaction was stopped by the addition of Laemmli sample buffer and proteins were analyzed by SDS-PAGE and autoradiography.

In some experiments, the cell extracts were centrifuged at 25,000 g for 30 min at 4°C; the supernatant of this step was centrifuged again at 50,000 g for 30 min. The supernatant from this step was centrifuged at 100,000 g for 30 minutes. The pellets of each centrifugation were solubilized in octylglucoside, and gD1-DAF immunoprecipitated from each sample as described above.

Endocytosis of gD1-DAF

Endocytosis of Fab-labeled gD1-DAF was measured in terms of protection of Fl-Fab anti-gD1-DAF from digestion by trypsin. Replicate confluent cultures of gD1-DAF MDCK were labeled with Fl-Fab anti-gD1 on ice. The cultures were then warmed to 37°C and incubated for 15–180 min. At intervals, pairs of cultures were cooled to room temperatures and washed 3× in PBS. Cells in one culture were suspended by incubation with trypsin/EDTA solution (7 min at room T). This destroyed all fluorescence on the cell surface. Cells of the second culture were dispersed using only EDTA; cells dispersed in EDTA alone still bore surface fluorescence when examined immediately after dispersion. The dispersed cells were fixed in 2% paraformaldehyde, permeabilized with 0.1% saponin, 1% BSA, 10 mM NH_4Cl in PBS, and labeled with 1:50 Fl-goat anti–mouse Ig. The labeled cells were washed 6× in PBS, until controls not treated with primary antibody were negative, and their intensities measured by flow cytometry.

Results

Though Cholesterol Synthesis Is Increased, Cell Cholesterol Levels Are Reduced by LDL Deprivation of MDCK Cells

When fibroblasts and other types of cells are deprived of their exogenous source of cholesterol, serum LDL, they increase their cholesterol synthesis, upregulating the key enzyme of this synthesis, HMG-coA reductase (for review see Goldstein and Brown, 1990). MDCK cells grown in medium lacking LDL respond in the same way. After 1 d without LDL, incorporation of [^{14}C]acetate into cholesterol was nearly sixfold higher than in cells grown in complete medium. It increased slightly, to almost sevenfold higher than controls, after 3 d of cell growth without LDL.

MDCK/gD1-DAF cells grown in complete medium contained an average of 198 nmol cholesterol and 97 nmol cholesterol ester/mg protein. Despite increased cholesterol synthesis, cell cholesterol levels were reduced by LDL-deprivation. After 3 d without LDL cell cholesterol content (free and esterified) was 75 ± 2% of controls; after 10–14 d without LDL free cholesterol and total cell cholesterol content were 60 ± 4% and 52 ± 3% of control levels. Overnight addition of LDL to cells grown without LDL for 14 d restored the cholesterol levels to control levels (93 ± 6%).

LDL Deprivation Alters the Physical Properties of MDCK Cell Membranes

Cholesterol modifies the packing of membrane phospholipids so that ordered regions of membrane lipids are disordered and expanded in area while disordered regions are condensed (for reviews see Gennis, 1989; Shinitzky, 1984). These effects, often expressed as effects on membrane fluidity, may be tracked in terms of changes in the polarization of fluorescence of hydrophobic dyes, particularly DPH (Lentz, 1989; Shinitzky and Barenholz, 1978). We imaged polarized fluorescence of TMA-DPH, a derivative of DPH that localizes well to cell plasma membranes (Khury et al., 1983) in MDCK cells grown in complete or

LDL-deficient medium. Fluorescence anistropy, r, was calculated from the average intensities of images made with excitation and emission polarizers parallel or perpendicular to one another. r for cells grown in complete medium was 0.33 ± 0.02 (SEM), a value almost identical to that measured in MDCK cells by Giocondi et al. (1990). r was significantly lower, 0.26 ± 0.01, after 3 d of LDL-deprivation, indicating that cell membrane lipids were more disordered, or more fluid than in control cells. After 14 d of LDL-deprivation, r returned to control levels, 0.36 ± 0.02. Thus, it appears that the cells compensated for the reduction in their membrane cholesterol.

gD1-DAF Is GPI-anchored in Control and LDL-deprived Cells, but Surface gD1-DAF Is Resistant to PIPLC in LDL-deprived Cells

The changes in membrane lipids reported by our measurements of cell cholesterol content and membrane fluidity did not alter the amount of GPI-anchored gD1-DAF in LDL-deprived cells. We showed that the protein was GPI-anchored in both control and LDL-deprived cells by measuring the partition of gD1-DAF between separated detergent and aqueous phases after treating Triton X-114 extracts of cells with PIPLC (Bordier, 1981). Cleavage of PI from GPI-proteins results in their transfer from detergent phase to aqueous phase. Immunoprecipitation of ^{35}S-labeled protein from these phases showed that 70 ± 24% of gD1-DAF was sensitive to PIPLC cleavage in extracts of control cells, 66 ± 9% in extracts of cells deprived of LDL for 3 d and 70 ± 6% in extracts of cells deprived of LDL for 14 d. Thus, cells produced the GPI-anchored form of gD1-DAF whether or not they were deprived of LDL.

Though detergent extracts of gD1-DAF were equally susceptible PIPLC, treatment of intact cells with PIPLC showed that the plasma membrane environment of gD1-DAF was indeed different in LDL-deprived cells than in control cells. Using ^{125}I-protein A binding, we found that 45 ± 10% of surface gD1-DAF was removed from the surface of control cells by a 1-h incubation with PIPLC. The same treatment removed only 9 ± 25% of apical surface gD1-DAF from cells deprived of LDL for 3 d.

The results with ^{125}I-protein A were confirmed and extended using flow cytometry to measure the amount of Fl-Fab anti-gD1-DAF bound to cells before and after treatment with PIPLC. Within error no gD1-DAF was removed by PIPLC treatment, even at 37°C from the surface of cells deprived of LDL for 3 d. PIPLC treatment at 20°C removed < 15% of surface gD1-DAF from cells deprived of LDL for 14 d. PIPLC treatment at 37°C removed about half the surface gD1-DAF from these cells. The changes in PIPLC susceptibility parallel those in membrane fluidity and detergent solubility of gD1-DAF (see below).

The Amount of gD1-DAF at the Cell Surface Is Reduced by LDL-Deprivation

Unexpectedly, there was less gD1-DAF at the cell surface of LDL-deprived cells than at the surface of control cells. This was apparent even in images of MDCK gD1-DAF cells, labeled with appropriate antibodies for immunofluorescence. Control cells showed a pattern typical of apical staining for a membrane protein (Fig. 1 a). Fluorescence
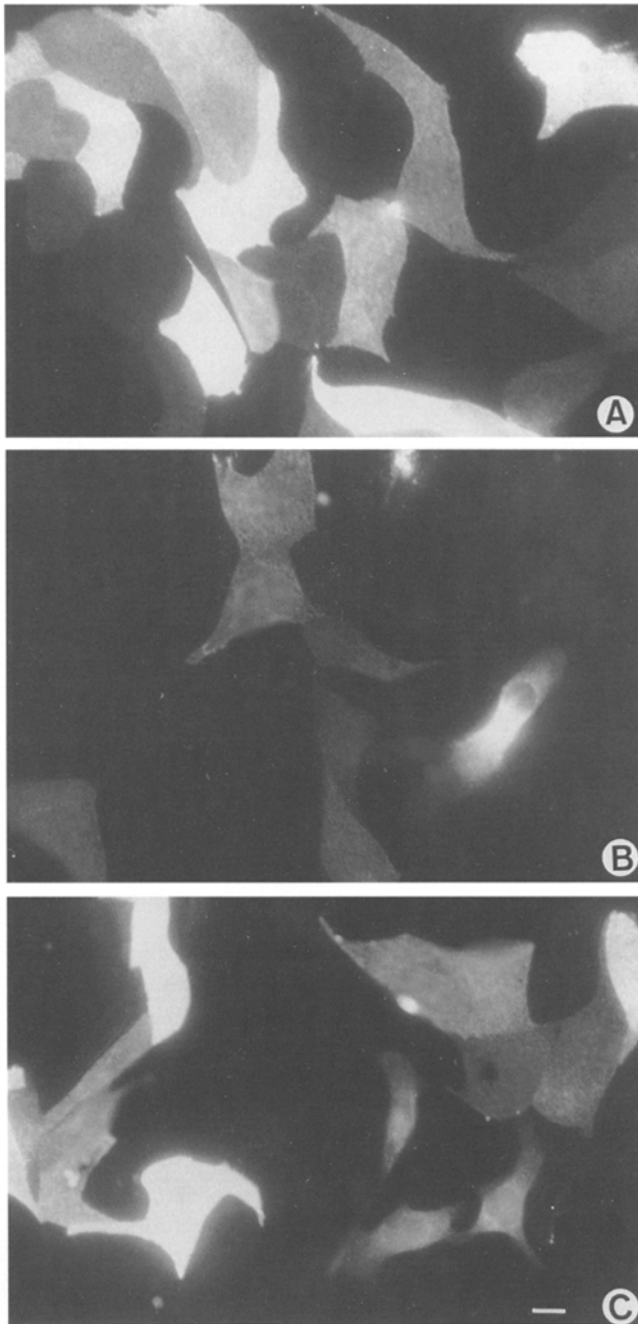
Figure 1. Immunofluorescence images of gD1-DAF at the apical surface of MDCK cells grown in complete medium or in medium lacking LDL. MDCK cells were grown to confluence on glass coverslips in complete medium (*A*), in medium lacking LDL for 3 d (*B*), or in medium lacking LDL supplemented with added LDL for the last 20 h of culture (*C*). All cells were treated with 10 mM sodium butyrate for 20 h before fixation to induce expression of gD1-DAF. The cells were then labeled with rabbit anti–gD1-DAF and fluorescein-conjugated goat anti–rabbit IgG antibodies. All images were obtained and processed under identical conditions. Bar, 10 μm.

intensity varied from cell to cell. This variation was seen at a range of times, 12–20 h, after adding sodium butyrate to the cells to induce the expression of gD1-DAF. The intensity of labeling of gD1-DAF was significantly reduced in cells grown without LDL for 3 d (Fig. 1 *b*); it was somewhat higher, but still lower than controls, in cells grown without LDL for 14 d (data not shown). Addition of LDL to the LDL-deficient medium for the entire time of deprivation (3 d) restored the labeling for gD1-DAF to control levels (Fig. 1 *c*).

We used digital microscopy to quantitate the binding of fluorescent Fab anti-gD1 to the apical surface of cells growing on glass. 3 d without LDL reduced the level of surface gD1-DAF to 53 ± 2% of controls; it was 75 ± 1% of controls after 14 d without LDL. Cells growing on Transwells™ showed the same changes in fluorescence intensity as those growing on glass.

The amount of gD1-DAF on the apical and basal surfaces of MDCK cells growing on Transwells™ was also quantitated using anti-gD1 IgG, followed by ^{125}I-protein A. The errors of this method are higher than those of our digital images, mainly because of errors in estimating cell protein. However, our values for antibody binding at the apical surface were. in good agreement with values obtained by digital microscopy. After 3 d without LDL, binding of anti-gD1 to the apical surface averaged 60% (range 40–65% in three experiments). After 14 d without LDL, binding of anti-gD1 to the apical surface averaged 72% (range, 60–85% in three experiments) that of control cells grown in complete medium. Basolateral levels of gD1-DAF were less affected by LDL-deprivation than were apical levels of the protein. After 3 d without LDL, the basolateral surface bound an average of 85% (45–100%) of control levels of anti-gD1. Binding was 91% of controls (range 60–110%) after 14 d without LDL.

The Polarized Distribution of gD1-DAF Is Unaffected by LDL-Deprivation

Though the fraction of gD1-DAF at the cell surface was decreased by LDL-deprivation, the distribution of this surface gD1-DAF between apical and basolateral surfaces was unaffected. Confocal microscopy of cells labeled at both surfaces showed that the most gD1-DAF was at the apical surface of control cells (Fig. 2, *a* and *b*); and of cells deprived of LDL for 3 d (Fig. 2, *c* and *d*).

We quantitated the polarity of gD1-DAF by measuring binding of anti–gD1-DAF IgG with[125]I-protein A. While observed polarity, the ratio of IgG bound to the apical and basal surfaces, varied from experiment to experiment, in no case did LDL-deprivation significantly reduce surface polarity of gD1-DAF (Fig. 3).

## Surface Expression of Transmembrane Proteins Is Not Reduced in LDL-deprived Cells

Although LDL-deprivation reduced the amount of gD1-DAF on the cell surface, it did not reduce the surface amount of two transmembrane proteins, the type II protein DPP IV and the type I protein PLAP-HA. After 3 d of cell growth without LDL the amount of DPP IV at the cell surface was more than twice that in cells grown in complete medium. 93% of surface molecules were at the apical surface of control and LDL-deprived cells. LDL-deprivation of cells for 14 d restored the amount of apical DPP IV to control levels (93% of controls); but the amount at the basolateral surface remained high (201% of
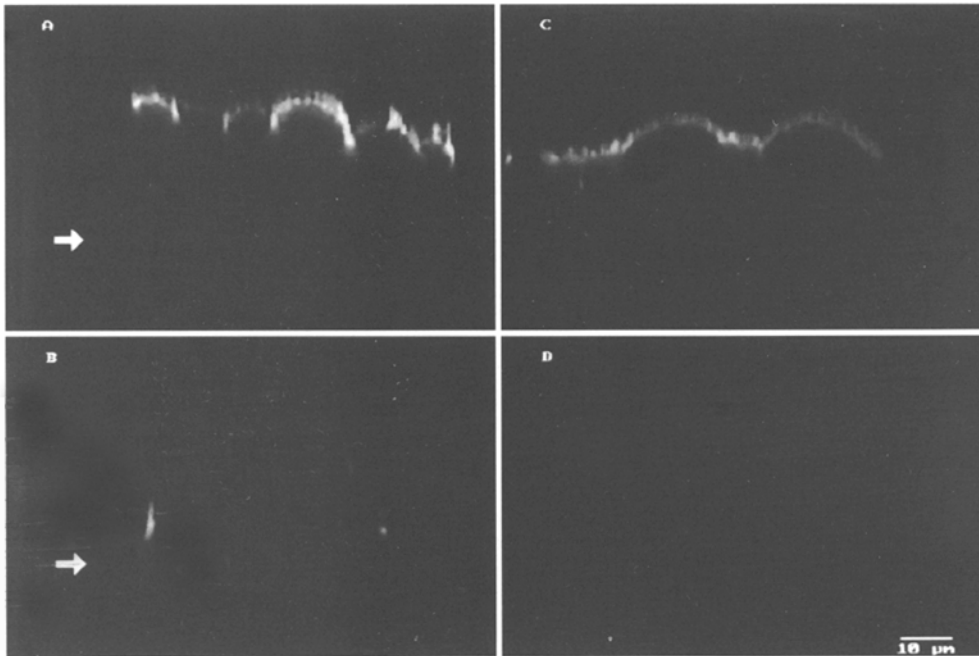
Figure 2. gD1-DAF is concentrated at the apical surface of cells grown in complete medium or in medium without LDL. MDCK cells were grown to confluence (3 d) on Transwells™ in complete medium (*A* and *C*) or in medium lacking LDL (*B* and *D*), treated for 20 h with 10 mM sodium butyrate, fixed, and labeled with rabbit anti–gD1-DAF from both the apical and the basolateral surface. The apical surface was labeled with rhodamine-conjugated goat anti–rabbit Ig antibodies, and the basolateral surface was labeled with fluorescein-conjugated goat anti–rabbit Ig antibodies. Confocal images, perpendicular to the plane of the cells, were obtained from the same field of cells detecting the apical, Texas red, (*A* and *B*), and the basolateral fluorescein (*C* and *D*) signals independently. The arrow indicates the position of the Transwell™ surface. Bar, 10 μm.

controls). Total cellular levels of DPP IV were unaffected by cholesterol deprivation (data not shown).

PLAP-HA is a chimeric protein, with the extracellular domain of PLAP fused to the transmembrane domain of influenza hemagglutinin A (Arreaza and Brown, 1995).
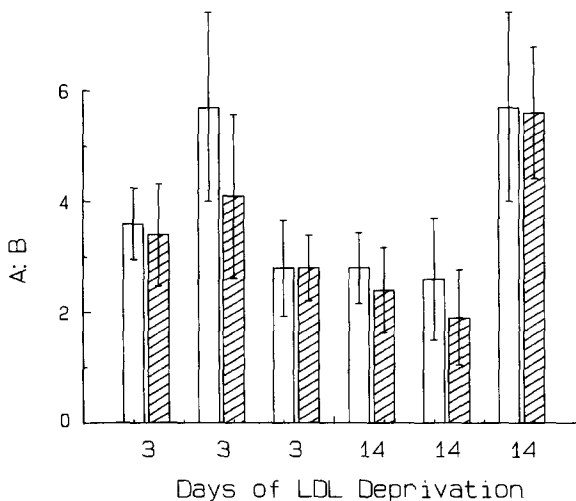
Figure 3. Surface polarity of gD1-DAF in control cells and in cells grown without LDL for 3 or 14 d. Surface polarity of gD1-DAF (*A:B*) was determined using anti–gD1-DAF and ^{125}I protein A for cells grown to confluence on Transwells™ in complete or LDL-deficient medium. Control cells (*open bars*); cells grown without LDL for 3 or 14 d as indicated on the figure (*hatched bars*). The vertical bars show standard errors for each set of determinations.

The amount of PLAP-HA at the surface of cells grown without LDL for 3 d (on plastic) was not significantly different from that on the apical surface of control cells, 110 ± 3% of controls.

The Biosynthesis, Maturation, and Degradation of gD1-DAF Are Unaffected by LDL-Deprivation

The reduction in amount of gD1-DAF at the cell surface after LDL-deprivation could be due to changes in the metabolism or in the intracellular traffic of maturing gD1-DAF. Therefore, we compared the biosynthesis and degradation of gD1-DAF and its rate of progress through the Golgi complex (measured in terms of maturation N-linked oligosaccharides) in control and in LDL-deprived cells.

The synthetic rate of gD1-DAF was determined by pulse labeling with ^{35}S-Translabel™ and immunoprecipitation of gD1-DAF from cells grown in either complete medium or in medium without LDL. The synthetic rate of gD1-DAF was the same in cells grown without LDL for 3 d (Fig. 4 *a*) or 14 d (data not shown) as it was in controls. Pulse-chase experiments showed that there was no effect of LDL-deprivation on the degradation rate of gD1-DAF (Fig. 4 *b*). No gD1-DAF was immunoprecipitated from the culture medium following overnight ^{35}S-labeling of cells grown in complete medium or in medium lacking LDL (data not shown).

When cells were pulse labeled during the time of treatment with 10 mM sodium butyrate to induce expression of gD1-DAF–labeled molecules could first be detected after 8 h. The kinetics of their synthesis were the same in con-
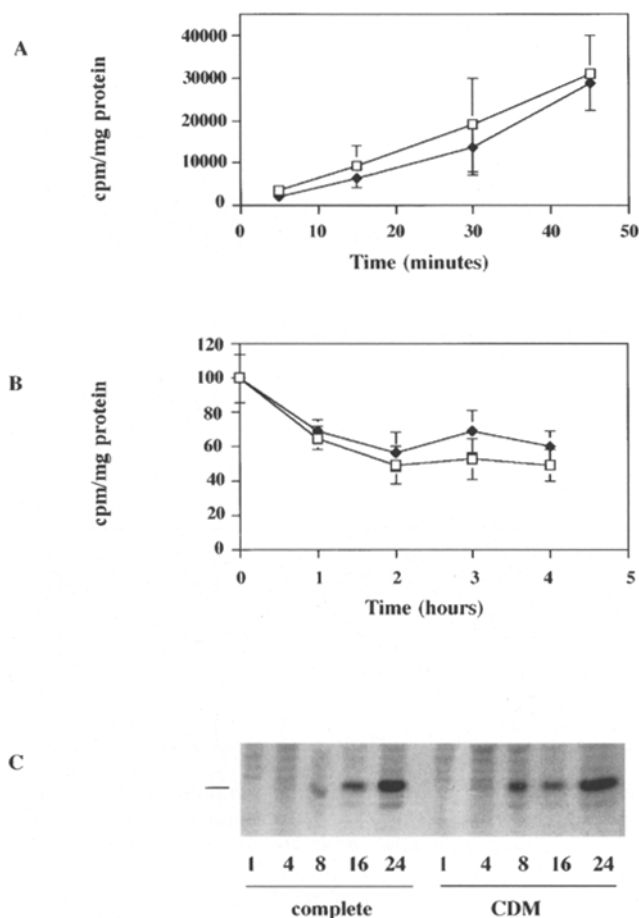
Figure 4. The rates of synthesis and degradation of gD1-DAF, and its synthetic rate during the time of induction for cells grown in complete medium or in medium lacking LDL. The biosynthetic rate of gD1-DAF in MDCK cells (*A*) grown in complete medium (◆) or in medium lacking LDL for 3 d (□) was determined by pulse labeling for 5–45 min with 0.1 mCi/ml ^{35}S-Translabel™; gD1-DAF was immunoprecipitated from octylglucoside extracts of the labeled cells. The amount of radioactivity in each sample was determined by scintillation counting and normalized for the total amount of protein in each sample. The degradation rate of gD1-DAF (*B*) was determined by pulse labeling for 30 min, and chase in cold medium for 0–4 h. gD1-DAF was immunoprecipitated and quantitated as described for the synthetic rate. The synthetic rate of gD1-DAF throughout the time of induction with 10 mM sodium butyrate (*C*) was determined in cells grown in complete medium or in medium lacking LDL for 3 d by pulse labeling for 30 min after induction for 1, 4, 8, 16, or 24 h.

trol and LDL-deprived cells for measurements made 8, 16, or 20 h after adding sodium butyrate to the cultures (Fig. 4 *c*).

The newly synthesized protein moved through the Golgi complex of LDL-deprived cells at the same rate as it moved through the Golgi complex of control cells. With time after synthesis there was an increase in the apparent molecular weight of the protein, as well as increasing resistance to cleavage by endo H (Fig. 5). The mature protein was the same apparent molecular mass (50–55 kD) and was resistant to endo H whether or not cells were grown with LDL. The rate of maturation of the oligosaccharides of the nascent gD1-DAF, measured by endo H resistance,

was also the same in control and in LDL-deprived cells (Fig. 6).

A Fraction of gD1-DAF Is Found Intracellularly in LDL-deprived Cells
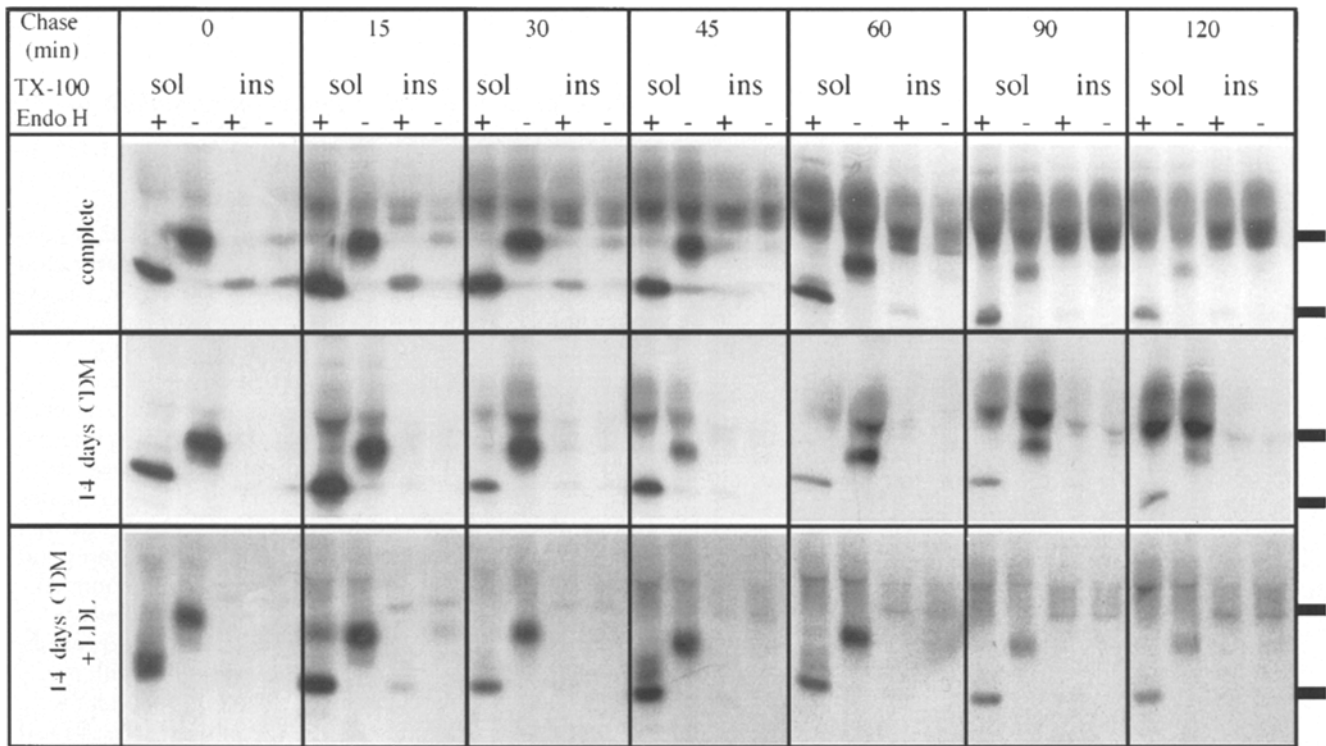
All of the experiments described in the previous section indicate that although the amount of protein at the surface of LDL-deprived cells was lower than the surface of control cells, the metabolism of gD1-DAF and its maturation in the Golgi were unaffected by LDL-deprivation. The cytoplasm of LDL-deprived cells labeled strongly for gD1-DAF, particularly after 3 d of LDL-deprivation (Fig. 7).

Comparing the binding of anti-gD1 IgG and ^{125}I protein-A to intact cells with binding to saponin-permeabilized, cells we estimated that 10% of the total gD1-DAF was internal in control cells and 35% was internal in cells grown without LDL for 3 d. Given the errors of these estimates (which depend upon measurement of cell protein as well as on measurement of bound protein A), this is in good agreement with our finding that the average amount surface gD1-DAF in these cells was 50–60% of controls.

Labeling for gD1-DAF was concentrated at the periphery of the cells (Fig. 7 *a*) and did not colocalize with labels for the endoplasmic reticulum or the Golgi complex (Figs. 7, *b* and *c*). Confocal xz-sectioning of labeled cells showed that internal label was concentrated in the apical cytoplasm (Fig. 8 *A*). While the rate of endocytosis of gD1-DAF was higher in LDL-deprived cells than in controls (Fig. 9), we could not colocalize cytoplasmic gD1-DAF with endosomes labeled by Fl-transferrin (Fig. 8 *B*) or Fl-dextran (data not shown). We also note that there was considerably more intracellular gD1-DAF in 3-d deprived cells than 14-d deprived cells even though the rate of endocytosis was the same in 3-d and 14-d LDL-deprived cells.

Little gD1-DAF Is Found in a Triton X-100–Insoluble Fraction of LDL-deprived Cells

LDL-deprivation altered membrane fluidity, and the membrane milieu and cellular distribution of gD1-DAF, but did not affect its surface polarity. Since a cholesterol-enriched detergent-insoluble fraction of gD1-DAF is thought to be the unit for its sorting and targeting (Brown and Rose, 1992), we determined the appearance of this fraction after pulse labeling control and LDL-deprived cells (Fig. 5). The detergent-insoluble fraction was defined by centrifugation of ice-cold Triton X-100 extracts for 150,000 gmin (Brown and Rose, 1992). In control cells almost all newly synthesized gD1-DAF molecules were initially soluble in ice-cold Triton X-100. The detergent-insoluble fraction increased with increasing chase time. After 2 h chase an average of 63% of gD1-DAF was detergent-insoluble in control cells (Fig. 5 *a*). Centrifugation for up to 3 × 10⁶ gmin did not increase the yield of detergent-insoluble gD1-DAF from control cells. In cells deprived of LDL for 3 d, 35% of gD1-DAF was detergent-insoluble 2 h after labeling (data not shown). The yield of detergent insoluble material increased to 50% if extracts were centrifuged for 7.5 × 10⁵ gmin and did not increase upon further centrifugation. Only 3% of gD1-DAF was pelleted by 150,000 gmin from extracts of cells grown without LDL for 14 d

Chase (min)	0				15				30				45				60				90				120			
TX-100	sol		ins		sol		ins		sol		ins		sol		ins		sol		ins		sol		ins		sol		ins	
Endo H	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−

complete

14 days CDM

14 days CDM + LDL

Figure 5. Changes in detergent solubility and in endoglycosidase H sensitivity of gD1-DAF with time after synthesis in control and LDL-deprived cells. MDCK cells were grown in complete medium (*top panel*), without LDL for 14 d (*central panel*), or without LDL for 13 d, and with LDL added for the last 20 h of culture (*bottom panel*). The cells were pulse labeled for 30 min with ^{35}S-Translabel™, followed by a chase in the appropriate cold medium for 0–120 min, as indicated at the top of the figure. Material that was soluble (*sol*) in 1% Triton X-100 was separated from material that was insoluble (*ins*) in 1% Triton X-100 at 4°C by centrifugation for 150,000 g/min. The insoluble material was solubilized in 60 mM octylglucoside, and gD1-DAF was immunoprecipitated from both fractions. Each immunoprecipitate was split in half, and one half was incubated with endoglycosidase H overnight. The samples were subjected to SDS-PAGE and autoradiography. 43-kD (*upper*) and 28-kD (*lower*) molecular mass markers are indicated. Endoglycosidase–H sensitive gD1-DAF has an apparent molecular mass of 40-kD, and 38-kD after endoglycosidase H digestion. The two fully glycosylated, endoglycosidase H–resistant forms of gD1-DAF have apparent molecular masses of 50 and 55 kD. Note that different amounts of labeled protein were loaded on each gel.

(Fig. 5 *b*). The yield of insoluble material was increased to 30% by centrifugation for 7.5×10^5 gmin and was not increased by further centrifugation. Addition of LDL for 20 h to cells LDL-deprived for 14 days increased the fraction of detergent-insoluble gD1-DAF, defined by 150,000 gmin centrifugation, from 3 to 30% (Fig. 5 *c*).

Cholesterol carried by LDL is separated from its carrier in lysosomes and must leave this compartment if it is to be used by the cell. Imipramine has been shown to trap LDL-derived cholesterol in lysosomes (Rodriguez-Lafrasse et al., 1990; Roff et al., 1993). We found that the fraction of Triton X-100–insoluble gD1-DAF pelleting at 150,000 gmin centrifugation was reduced to 1–3% in control cells grown for 20 h in complete medium containing 20 mM imipramine. Again, more gD1-DAF, 30% of the total, was pelleted from detergent extracts of these cells by 750,000 g/min centrifugation.

Discussion

Growing MDCK cells without LDL, their exogenous source of cholesterol, altered the global physical properties of their plasma membranes and had specific effects on the milieu and traffic of a GPI-protein, gD1-DAF, expressed in these cells. However, LDL depletion did not alter surface polarity of gD1-DAF. Under all conditions examined, the ratio of the amount of the GPI-protein at the apical surface to the basal surface was ∼3:1. The effects of LDL depletion on the traffic of gD1-DAF, on its detergent solubility and on its sensitivity to PIPLC treatment of intact membranes must be due to some combination of global changes in cell membranes and specific changes in the lipid environment of gD1-DAF (and presumably other GPI-proteins) consequent to LDL deprivation.

Studies of synthetic lipid bilayers show that cholesterol disorders relatively ordered regions of the bilayer and orders relatively disordered regions (for reviews see Gennis, 1989; Shinitzky, 1984). This helps to maintain the physical state of the bilayer at some average of lipid orientations and motions. The cholesterol content of plasma membranes of mammalian cells may be altered in vivo by diet, or in vitro by exchanging cholesterol between cell membranes and liposomes. When membranes are depleted of cholesterol by these means the apparent order of their membrane lipid increases; repletion of membrane cholesterol has the opposite effect (Bastiaanse et al., 1994; No-
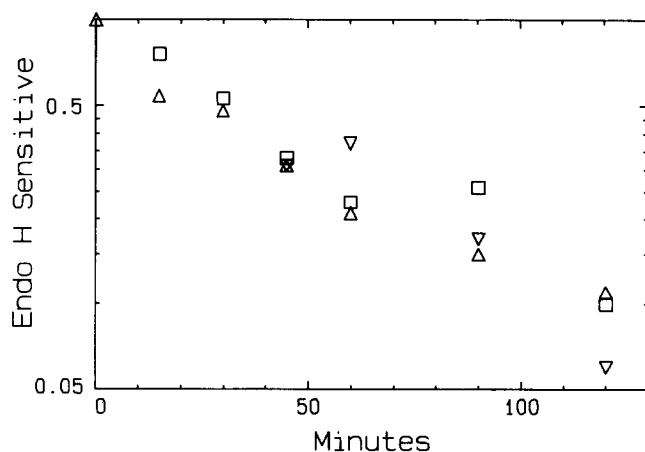
Figure 6. Maturation of oligosaccharides of gD1-DAF after synthesis, measured in terms of resistance to cleavage by endoglycosidase H. The autoradiograms of Fig. 5 and another autoradiogram of extracts of cells deprived of LDL for 3 d were digitized, and the digital images were used to determine the fraction of gD1-DAF that was sensitive to cleavage by endoglycosidase H during a 2-h chase. △, cells grown in complete (LDL-containing) medium; □, cells grown with LDL for 14 d; ▽, cells grown without LDL for 3 d.

vak et al., 1994; Muriana et al., 1992; Bonnekoh et al., 1990; Brasitus et al., 1988; Leikin and Brenner, 1988; Sinensky, 1980). As might be expected from these results, depriving MDCK cells of their principal source of cholesterol, LDL, resulted in changes in the ordering of membrane lipids. These were measured in terms of r, the anistropy of fluorescence of TMA-DPH. Unlike its parent compound DPH which partitions into all cell membranes, TMA-DPH localizes to the outer leaflet of the plasma membrane (Giocondi et al., 1990). While the fluorescence behavior of DPH and TMA-DPH is complex, and is not easily interpreted in detail, it is clear that r is a phenomenological parameter that reflects both the rate and the extent of reorientation of the probe, parameters that are sensitive to the composition and lateral packing of membrane lipids (Toptygin and Brand, 1994; Lentz, 1989). After 3 d without LDL, r was lower than that in controls, implying that the membrane lipids of cells grown without cholesterol were more disordered, more "fluid" than those of controls. After 14 d without LDL, membrane order as probed by TMA-DPH had returned to a state approximating that of control cell membranes. Since the cholesterol content of these cells remained significantly lower than that of control cells, the change in r must reflect compensatory changes in the lipid composition and organization of the plasma membrane.

Addition of the GPI tail to nascent gD1-DAF was as efficient in LDL-deprived cells as in controls. LDL-deprivation did not change the sensitivity of detergent-solubilized gD1-DAF to PIPLC. However, in intact LDL-deprived cells, membrane gD1-DAF was resistant to cleavage by PIPLC, indicating changes in the membrane environment of this protein after LDL-deprivation. In control cells, about half of surface gD1-DAF was sensitive to PIPLC. After 3 d of LDL-deprivation, surface gD1-DAF was almost completely resistant to PIPLC, even at 37°C. By 14 d
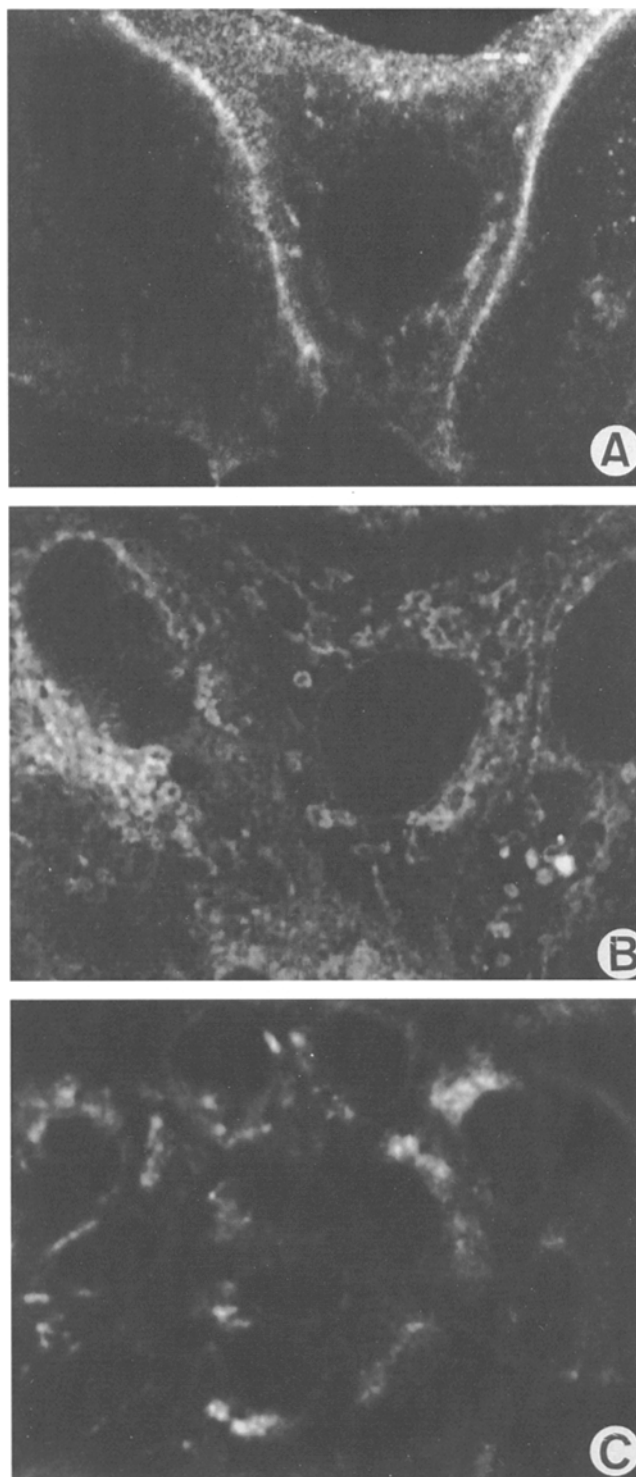
Figure 7. Intracellular gD1-DAF does not colocalize with markers for ER or Golgi complex. MDCK cells were grown to confluence (3 d) on glass coverslips in medium lacking LDL, treated with 10 mM sodium butyrate 20 h, fixed, and permeabilized with 0.1% saponin. The cells were double labeled with rabbit antibodies against gD1-DAF and Texas red–conjugated Fab2 anti–rabbit IgG (*A*), or with DiOC$_6$ to label the ER (*B*). Confocal images of the same field were obtained by gD1-DAF labeling and ER labeling. (*C*) A separate slide was labeled with C$_6$NBD-ceramide to mark the Golgi. Bar, 10 μm.
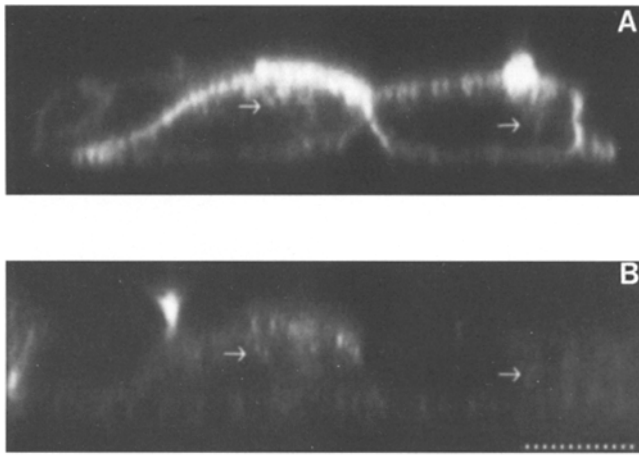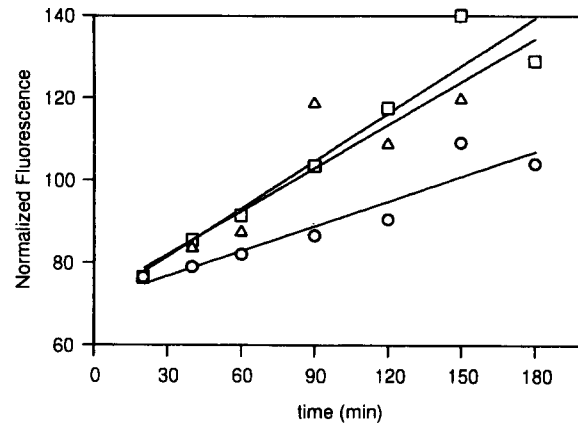
Figure 8. Subapical localization of intracellular gD1-DAF in LDL-deprived cells. Cells were labeled for gD1-DAF and with Fl-transferrin at 37°C for 90 min; then they were fixed, permeabilized, and labeled for gD1-DAF. (A) Apical and subapical localization of gD1-DAF. (B) Localization of Fl-transferrin. While the two labels are in the same regions (examples marked by arrows), there is no extensive colocalization of labels. Bar, 10 μm.

Figure 9. Endocytosis of gD1-DAF from the apical surface of control and LDL-deprived cells. Endocytosis of Fab-labeled gD1-DAF was measured in terms of protection of Fl-Fab anti–gD1-DAF from digestion by trypsin. Cells grown on 35-mm dishes were labeled with Fl-Fab at 0°C, washed, and warmed to 37°C. At intervals pairs of dishes were cooled to room temperature. Cells in one dish were suspended using trypsin + EDTA, while cells in the other dish were suspended with EDTA only. The suspended cells were fixed in 2% PFA. After permeabilizing and adding Fl-anti-Ig to enhance the signal, cell intensities were measured by flow cytometry. The figure plots trypsin-resistant fluorescence as a fraction of total fluorescence (measured on cells dispersed with EDTA). They are not corrected for cell autofluorescence. Values >1 are due, at least in part, to dissociation of surface Fab during suspension of cells in EDTA. O, controls; △, 3 d of LDL deprivation; □, 14 d of LDL deprivation.

of LDL-deprivation, when fluorescence polarization measurements suggest that membranes have returned to some physical state approximating that of control cell membranes, we found that while all gD1-DAF was still resistant to PIPLC at 20°C, about half was cleaved from the cells at 37°C. This change with prolonged LDL-deprivation is consistent with our results on the changes in apparent membrane fluidity with the time of LDL-deprivation.

There are other reports that membrane composition can alter the exposure of GPI proteins or glycolipids. Hanada et al. (1993) found that a GPI-protein, CD14, was more sensitive to PIPLC in membranes of sphingolipid-deficient cells than in control cells. Hamilton et al. (1994) summarize and extend observations showing that the accessibility of a GSL antigen to antibody depends upon membrane composition.

The amount of gD1-DAF on the cell surface was significantly reduced by LDL-deprivation for 3 d. This effect was specific for gD1-DAF (and other GPI-proteins) to the extent that the surface display of two transmembrane proteins, DPP IV and PLAP-HA, was not reduced by LDL-deprivation. After 14 d deprivation surface gD1-DAF levels approached but did not reach control levels. Though the amount of gD1-DAF at the surface of LDL-deprived cells was reduced compared to controls, the biosynthesis and degradation of the protein were unchanged. Its progress through the Golgi complex to the point of mature N-linked glycosylation was also unchanged.

gD1-DAF molecules in the cytoplasm of LDL-deprived cells are concentrated beneath the apical surface. Labeling for cytoplasmic gD1-DAF does not colocalize with markers for endoplasmic reticulum or Golgi or with endosomes containing Fl-transferrin, or a fluid-phase marker, fluorescein-dextran. We suggest that cytoplasmic gD1-DAF is in vesicles whose normal traffic is altered as a result of cholesterol deprivation of MDCK cells. There is more intracellular gD1-DAF in cells deprived for three than in cells

deprived for 14 d, even though endocytic rates are the same (and higher than controls) after 3 and 14 d LDL-deprivation. This, together with our failure to colocalize cytoplasmic gD1-DAF and endosomes, suggests that the rate or efficiency of intracellular vesicle traffic was reduced after 3 d of LDL deprivation and that compensatory changes in membrane lipid composition partly restored traffic to normal after 14 d LDL-deprivation.

The key change in vesicle traffic in LDL-deprived cells could be in the efficiency of vesicle fusion in both the outbound transport pathway and in vesicle recycling. The importance of cholesterol for membrane fusion has been demonstrated for fusion of enveloped viruses with animal cells (Nieva et al., 1994; Cheetham et al., 1994) and for fusion of liposomes with mycoplasma membranes (Tarshis et al., 1993).

The increase in surface gD1-DAF in cells deprived of LDL for 14 d, relative to those deprived of LDL for 3 d, would then, like the results on fluorescence polarization, reflect changes in the physical properties and organization of cell membranes.

Other workers have reported that cholesterol deprivation of nonpolarized cells affects and alters traffic of vesicles containing GPI-proteins. Mayor and Maxfield (Mayor, S., and F.R. Maxfield. 1995. Mol. Biol. Cell. 6:231a) found that cholesterol deprivation decreased the retention time for GPI-proteins in cytoplasmic vesicles; this appeared to reflect the diversion of GPI-proteins from sorting endosomes. Such diversion could be due to reduced fusion efficiency of inbound vesicles subsequent to LDL-deprivation and reduction in membrane cholesterol. Taraboulos et al.

(1995) showed that acute cholesterol deprivation of infected cells interfered with the degradation of the GPI-anchored cellular prion protein PrP^C and blocked the conversion of PrP^C to scrapie prion protein PrP^{Sc}. They suggested that in cholesterol-deprived cells, export to surface of PrP^c is slowed possibly because the protein spends more time in internal recycling compartments. Again, this delay could reflect reduced fusion efficiency for trafficking vesicles.

Despite all the changes in its intracellular traffic and surface display, LDL-deprived cells maintained the polarity of surface gD1-DAF. The average amount of gD1-DAF on apical surfaces was approximately three times that on basolateral surfaces of cells under all conditions of cell culture. Since Triton X-100–insoluble fractions of GPI-proteins are thought to represent the specialized lipid environment required for polarized delivery of these molecules to the cell surface (Brown and Rose, 1992), it might be expected that the detergent insolubility of gD1-DAF was the same in LDL-deprived as in control cells. Instead, we found that an insoluble fraction defined by centrifugation of detergent extracts of control cells did not contain gD1-DAF when isolated from LDL-deprived cells. At first sight this implies that polarized delivery of gD1-DAF was independent of the lipid (and protein) clusters isolated after detergent extraction of membranes. However, an appreciable amount of gD1-DAF was pelleted after fivefold longer centrifugation.Thus, the size of the lipid cluster that can be maintained in detergent was altered by LDL-deprivation, particularly after 14 d. We could never pellet >30% of the total cell gD1-DAF; this is about half the amount pelleted from extracts of control cells. We suggest that alterations in membrane lipid composition altered the detergent solubility of the putative sorting units but did not completely disperse them.

Though cells increase their endogenous cholesterol synthesis in response to LDL-deprivation, endogenously synthesized cholesterol apparently does not contribute significantly to the formation of detergent-insoluble units of gD1-DAF. On the other hand, blocking the egress of cholesterol from lysosomes, by culturing cells in medium containing both serum LDL and imipramine (Rodriguez-Lafrasse et al., 1990; Roff et al., 1993), changed the detergent-insoluble fraction of gD1-DAF from one pelleting at 150,000 gmin to one pelleting at 750,000 gmin. The requirement for exogenously derived, as opposed to endogenously synthesized, cholesterol may reflect the route to the surface proposed for endogenously synthesized cholesterol (Urbani and Simoni, 1990), a route that bypasses the Golgi complex.

Despite the many changes observed after LDL-deprivation of MDCK cells, they continued to maintain a polarized distribution of gD1-DAF. On the other hand, the putative sorting units, defined by detergent solubility, were altered and were not recovered as efficiently from LDL-deprived cells as from controls. It is clear that further definition of the sorting unit of GPI-proteins will be necessary to determine its composition and traffic in polarized cells.

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