

Drug resistance-associated changes in sphingolipids and ABC transporters occur in different regions of membrane domains

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Abstract We have recently shown that two ATP binding cassette (ABC) transporters are enriched in Lubrol-resistant noncaveolar membrane domains in multidrug-resistant human cancer cells [Hinrichs, J. W. J., K. Klappe, I. Hummel, and J. W. Kok. 2004. ATP-binding cassette transporters are enriched in non-caveolar detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs) in human multidrug-resistant cancer cells. *J. Biol. Chem.* 279: 5734–5738]. Here, we show that aminophospholipids are relatively enriched in Lubrol-resistant membrane domains compared with Triton X-100-resistant membrane domains, whereas sphingolipids are relatively enriched in the latter. Moreover, Lubrol-resistant membrane domains contain more protein and lipid mass. Based on these results, we postulate a model for detergent-insoluble glycosphingolipid-enriched membrane domains consisting of a Lubrol-insoluble/Triton X-100-insoluble region and a Lubrol-insoluble/Triton X-100-soluble region. The latter region contains most of the ABC transporters as well as lipids known to be necessary for their efflux activity. Compared with drug-sensitive cells, the detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs) in drug-resistant cells differ specifically in sphingolipid content and not in protein, phospholipid, or cholesterol content. In drug-resistant cells, sphingolipids with specific fatty acids (especially C24:1) are enriched in these membrane domains. Together, these data show that multidrug resistance-associated changes in both sphingolipids and ABC transporters occur in DIGs, but in different regions of these domains.—Hinrichs, J. W. J., K. Klappe, M. van Riezen, and J. W. Kok. Drug resistance-associated changes in sphingolipids and ABC transporters occur in different regions of membrane domains. *J. Lipid Res.* 2005. 46: 2367–2376.

Supplementary key words P-glycoprotein • multidrug resistance protein 1 • glucosylceramide • C24:1 fatty acid • aminophospholipids • liquid chromatography-electrospray tandem mass spectrometry • ATP binding cassette transporters

Multidrug resistance (MDR) of cancer cells is characterized by cross-resistance toward multiple chemotherapeutic agents. Several MDR mechanisms have been described, the best characterized of which is the overexpression of ATP binding cassette (ABC) drug transporter proteins such as P-glycoprotein (Pgp) and multidrug resistance-associated protein 1 (MRP1) (1–3). These proteins function by decreasing the intracellular concentration of cytotoxic drugs. ABC proteins probably recognize these drugs in the membrane by virtue of their hydrophobic nature (4, 5). After recognition, they are either pumped out of the cell or translocated to the outer leaflet of the plasma membrane, from which they eventually diffuse in the surrounding fluid (6–8).

Most ABC transporter-overexpressing MDR cell lines display changes in lipid composition compared with drug-sensitive counterparts (9–12). Pgp and MRP1 are known to depend on the lipid environment for optimal functioning (13, 14). In membrane model systems, the ATPase activity of both proteins is dependent on the close proximity of specific phospholipids, especially phosphatidylethanolamine (PE) (15–20). Moreover, Pgp has a higher affinity for its substrates when the surrounding lipids are in the gel phase rather than in the liquid-crystalline phase (21). This gel phase occurs when lipids have a high degree of saturation, which enables them to pack tightly. This is also an important characteristic of cellular membrane microdomains (rafts) (22, 23). These domains are enriched in sphingolipids (usually containing saturated fatty acids) and cholesterol and are characterized by insolubility in

Abbreviations: ABC, ATP binding cassette; DIG, detergent-insoluble glycosphingolipid-enriched membrane domain; ESI-MS/MS, liquid chromatography-electrospray tandem mass spectrometry; GlcCer, glucosylceramide; HPTLC, high-performance thin-layer chromatography; LacCer, lactosylceramide; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Pgp, P-glycoprotein; PS, phosphatidylserine; SM, sphingomyelin; TNE, Tris-NaCl-EDTA buffer.

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cold nonionic detergents, such as Triton X-100 and Lubrol (24–26). When isolated with the use of detergent, these membrane domains are referred to as detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs).

It has been shown that caveolin-1 expression, as well as caveolae themselves, are upregulated in Pgp-overexpressing cells and that a substantial fraction of Pgp is located in caveolin-1-containing Triton X-100-insoluble membrane domains (27). On the other hand, we have recently shown that Pgp and MRP1 are not associated with caveolae and are only partly localized in Triton X-100-resistant DIGs in two human MDR cell lines. In contrast, Pgp and MRP1 are highly enriched in Lubrol-resistant membrane domains (28). In the present study, we provide evidence that Lubrol-resistant membrane domains are larger, based on higher protein, phospholipid, and cholesterol content. Moreover, they are relatively enriched in the phospholipids PE and phosphatidylserine (PS) compared with Triton X-100-resistant domains, whereas the latter are more enriched in sphingolipids. We postulate a model for ABC transporter-containing DIGs consisting of a Lubrol-insoluble/Triton X-100-insoluble region and a Lubrol-insoluble/Triton X-100-soluble region. The latter region contains most of the ABC transporters as well as lipids known to be necessary for their efflux activity.

When we compared the composition of Lubrol-resistant membrane domains between drug-sensitive cells and Pgp-overexpressing 2780AD-drug resistant cells, it became apparent that they differed only in sphingolipid content; their protein, phospholipid, and cholesterol contents were similar. In addition, differences in the fatty acid composition of sphingolipids were observed, with a prominent increase of C24:1 fatty acid in sphingolipids in Lubrol-resistant membranes of drug-resistant cells.

Therefore, MDR-associated changes in sphingolipids and ABC transporters both occur in DIGs, but in different regions of these domains.

EXPERIMENTAL PROCEDURES

Materials

The 2780AD cell line was kindly provided by Dr. E. G. E. de Vries and Dr. H. Timmer-Bosscha (Department of Medical Oncol cell lines were kindly provided by Dr. A. J. Dijkhuis (Department of Membrane Cell Biology, University of Groningen), and HepG2 cells were kindly provided by Dr. J. M. van der Mark-van der Wouden (Department of Medical Physiology, University of Groningen). The HT29^{col} cell line was obtained from HT29 G⁺ cells by selection with colchicine (10). Doxorubicin was obtained from the hospital's pharmacist. Colchicine, Triton X-100, sucrose 99+, cholesterol oxidase, peroxidase, and the monoclonal antibody anti- β -actin (clone AC-15) were purchased from Sigma (St. Louis, MO). All cell culture plasticware was obtained from Costar (Cambridge, MA). RPMI 1640 medium, DMEM, HBSS, and antibiotics were from Gibco Laboratories (Paisley, UK). Fetal calf serum was from Bodinco (Alkmaar, The Netherlands). The monoclonal antibodies anti-Pgp (C219) and anti-MRP1 (MRPr1) were from Signet Laboratories (Dedham, MA), and the mono-

clonal antibody anti-MRP2 was from Sanbio (Uden, The Netherlands). The polyclonal antibody anti-c-Src was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The monoclonal antibody anti-transferrin receptor was purchased from Zymed Laboratories, Inc. (San Francisco, CA). Trans-Blot Transfer Medium membrane was from Bio-Rad (Hercules, CA), and the ECL plus Western Blotting Detection System was from Amersham Pharmacia Biotech UK Limited (Buckinghamshire, UK). L-[U-¹⁴C]serine (specific activity > 150 mCi/mmol) and [1-¹⁴C]palmitic acid (specific activity = 60 mCi/mmol) were purchased from Amersham International (Bercks, UK). Lubrol was obtained from Serva (Heidelberg, Germany). The internal standards for liquid chromatography-electrospray tandem mass spectrometry (ESI-MS/MS) were obtained from Avanti Polar Lipids (Alabaster, AL).

Cell culture and experimental conditions

2780AD cells were cultured in RPMI 1640 containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% (v/v) heat-inactivated (56°C, 30 min) fetal calf serum. The medium of the 2780AD cells was supplemented with 1 μ g/ml doxorubicin. The cultures were passed twice per week. HT29^{col} cells were cultured in DMEM containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% (v/v) heat-inactivated (56°C, 30 min) fetal calf serum. The medium was supplemented with 50 nM colchicine 48 h after each weekly passage of the cells. SK-N-FI, SK-N-AS, and HepG2 cells were cultured in DMEM containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% (v/v) heat-inactivated (56°C, 30 min) fetal calf serum. The cultures were passed twice per week. All cells were maintained in a water-saturated atmosphere of 5% CO₂ and 95% air at 37°C. All experiments took place during the exponential growth phase of the cells.

Isolation of DIGs

DIGs were isolated from cells as described (29). For each isolation, confluent cells from two 75 cm² flasks were washed once with HBSS, harvested by scraping in 3 ml of ice-cold Tris-NaCl-EDTA buffer (TNE; 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA) containing 1% (w/v) Triton X-100 or 0.5% (w/v) Lubrol, and vortexed. After 30 min of incubation on ice, cells were homogenized further by passing the lysate at least 10 times through a 21 gauge needle. Of this lysate, 2 ml was transferred to a centrifuge tube and mixed with 2 ml of 80% (w/v) sucrose in TNE. On top of this, 4 ml of 35% (w/v) and 4 ml of 5% (w/v) sucrose in TNE were successively loaded, resulting in a discontinuous gradient. All solutions contained the following protease inhibitors: 100 μ M PMSE, 1 mM EDTA, and 1 μ M each of aprotinin, leupeptin, and pepstatin A. Gradients were centrifuged in a Beckman SW41 swing-out rotor at 36,000 rpm for 18–20 h at 4°C. Twelve fractions of 1 ml each were collected (from top to bottom), vortexed, and stored at –80°C. The protein content of all fractions was measured (30) using BSA as a standard. For (sphingo)lipid analysis, the DIGs containing fractions 3–6 were diluted three times with ice-cold TNE and centrifuged in a Beckman TLA 100.3 fixed rotor at 49,000 rpm for 1 h at 4°C. The pellet was resuspended in 1 ml of TNE and stored at –80°C.

Immunoblot analysis

Proteins from the gradient fractions were precipitated in TCA and resuspended in sample buffer. TCA-precipitated proteins were resolved on SDS-PAGE (10%) minigels and electrotransferred onto Trans-Blot Transfer Medium membrane. The membranes were rinsed with PBS and incubated (1–2 h, 20°C) with 10% (w/v) nonfat dry milk in PBS. Membranes were rinsed in washing buffer [PBS containing 0.3% (v/v) Tween 20] and incubated (2 h, 20°C) with a primary antibody against Pgp (1:300),

MRP1 (1:1,000), MRP2 (1:200), transferrin receptor (1:500), β -actin (1:500), or c-Src (1:500) in washing buffer containing 1% (w/v) nonfat dry milk. Membranes were rinsed in washing buffer and subsequently incubated for 2 h with the appropriate horseradish peroxidase-conjugated secondary antibody (1:7,500) in washing buffer containing 1% (w/v) nonfat dry milk (2 h, 20°C). After rinsing in washing buffer, membranes were incubated in chemi-luminescence substrate solution according to the manufacturer's instructions, and immunoreactive complexes were visualized by exposure of ECL hyperfilm (Amersham).

Equilibrium radiolabeling and analysis of cellular lipids

Sphingolipid pools were metabolically radiolabeled by growing the cells for 48 h in the presence of L-[U- 14 C]serine (1 μ Ci/ml), a precursor molecule for sphingolipid biosynthesis (31). Lipids were extracted (32) from the DIGs in sucrose density gradient fractions 3–6, and the cell lysate was kept apart after detergent incubation (see Isolation of DIGs above). Aliquots of the lipid extracts were taken for determination of the total amount of lipid-incorporated radioactivity. Acylglycerolipids were hydrolyzed during a 1 h incubation at 37°C in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1, v/v) containing NaOH (0.1 M). The remaining lipids were reextracted and applied to high-performance thin-layer chromatography (HPTLC) plates. Plates were developed in $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (14:6:1, v/v/v) in the first dimension and were then sprayed with 2.5% H_3BO_3 (w/v) in CH_3OH and developed in the second dimension using $\text{CHCl}_3/\text{CH}_3\text{OH}/25\%$ (w/v) NH_4OH (13:7:1, v/v/v) as the mobile phase. After autoradiography, glucosylceramide- (GlcCer), lactosylceramide- (LacCer), and sphingomyelin- (SM) containing spots were identified with the aid of standards and scraped from the plates. Radioactivity was measured by scintillation counting. Lipid levels were expressed as the percentage of radioactivity incorporated in a given lipid relative to total lipid-incorporated radioactivity of either the DIGs or the cell lysate (before DIG isolation) (33).

Alternatively, lipid pools were metabolically radiolabeled by growing the cells for 48 h in the presence of [1- 14 C]palmitic acid (1 μ Ci/ml). Cells were harvested by scraping and centrifuged, followed by lipid extraction from the cell pellet (32). In the case of analysis of DIG-associated lipids, the DIG-containing sucrose density gradient fractions 3–6 (see Isolation of DIGs above) were pooled, diluted with 2 vol of ice-cold TNE, and centrifuged in a Beckman TLA 100.3 fixed rotor at 49,000 rpm for 1 h at 4°C. The pellet was resuspended in 1 ml of TNE, followed by lipid extraction (32). The lipids were applied to HPTLC plates. Plates were developed in $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (14:6:1, v/v/v) in the first dimension and then sprayed with 2.5% H_3BO_3 (w/v) in CH_3OH and developed in the second dimension, this time using $\text{CHCl}_3/\text{CH}_3\text{OH}/25\%$ (w/v) NH_4OH (13:7:1, v/v/v) as the mobile phase. After autoradiography, GlcCer-, LacCer-, galactosylceramide-, SM-, phosphatidylcholine- (PC), PE-, and PS-containing spots were identified with the aid of standards and analyzed using phosphorimaging. Lipid levels were expressed as the percentage of radioactivity incorporated in a given lipid in either the DIGs or the cell lysate relative to the total lipid-incorporated radioactivity of the DIGs or the cell lysate, respectively (33).

Electrospray tandem mass spectrometry

Sphingolipids were extracted and analyzed by ESI-MS/MS on a PE-Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray source as described previously (34). Dry N_2 was used as the nebulizing gas at a flow rate of 6 l/min. The ion spray needle was held at 5,500 V, and the orifice and ring voltages were kept low (40 and 220 V, respectively) to prevent collisional decomposition of molecular ions before entry into the first quadrupole; the orifice temperature was set to 500°C. N_2

was used to collisionally induce dissociations in Q2, which was offset from Q1 by 40–50 V. Q3 was then set to pass molecularly distinctive product ions (N ions) of m/z 264.4. Multiple reaction monitoring scans were acquired by setting Q1 and Q3 to pass the precursor and product ions of the most abundant sphingolipid molecular species. For example, for the ceramides, these transitions occur at m/z 538.7/264.4, 566.5/264.4, 622.7/264.4, 648.7/264.4, 650.7/264.4, 676.5/264.4, and 678.5/264.4, which correspond to ceramides with a d18:1 sphingoid base (sphingosine) and C16:0, C18:0, C22:0, C24:1, C24:0, C26:1, and C26:0 fatty acids, respectively. Quantitation was achieved by spiking the samples before extraction with the C12-fatty acid homologues of Cer, SM, and GlcCer.

Cholesterol and phosphate determination on cell lysate and DIGs

Triton X-100- and Lubrol-based cell lysates were prepared, and part of this lysate was used to isolate DIGs (see Isolation of DIGs above). The DIG-containing sucrose density gradient fractions 3–6 were pooled. After protein determination (30) on both the lysate and the pooled DIG-containing fractions, lipids were extracted (32). In the extract, the cholesterol concentration was determined spectrophotometrically by a cholesterol oxidase/peroxidase assay (35). The phosphorus content, as a measure of the phospholipid content in the lysate and the pooled DIG-containing fractions, was determined by a phosphate assay (36).

RESULTS

Lubrol-based DIGs contain twice the amount of proteins and phospholipids compared with Triton X-100-based DIGs

We have recently shown that Pgp and MRP1 are predominantly located in Lubrol-based DIGs of the human MDR tumor cell lines 2780AD and HT29^{col}, respectively (28). When Triton X-100 was used to isolate DIGs, the ABC transporters were much less enriched in detergent-insoluble fractions. In this study, we analyzed the lipid composition of Lubrol-based DIGs to assess the specific characteristics of these domains that may render them a more suitable environment for ABC transporter localization compared with Triton X-100-based DIGs.

To accurately quantify the difference in protein and lipid levels between both types of DIGs, Triton X-100-based and Lubrol-based lysates were prepared from 2780AD and HT29^{col} cells and fractionated in a discontinuous sucrose density gradient. The detergent-insoluble membrane domains in floating fractions 3–6 were pooled. Their protein content was measured and expressed as micrograms of DIG-associated protein relative to milligrams of protein of the starting material (cell lysate) used for the DIG isolation (Fig. 1A, prot). Lubrol DIGs of HT29^{col} cells contained twice the amount of protein of Triton X-100 DIGs, whereas Lubrol DIGs of 2780AD cells contained ~2.5 times the amount of protein. Phospholipid levels, as determined by phosphate content, in DIGs from both cell lines were in agreement with the protein levels. Phospholipid content was expressed as nanomoles of DIG-associated lipid relative to milligrams of protein of the starting material (cell lysate) used for the DIG isolation (Fig. 1A, phos). Although phospholipid levels in Lubrol-based DIGs

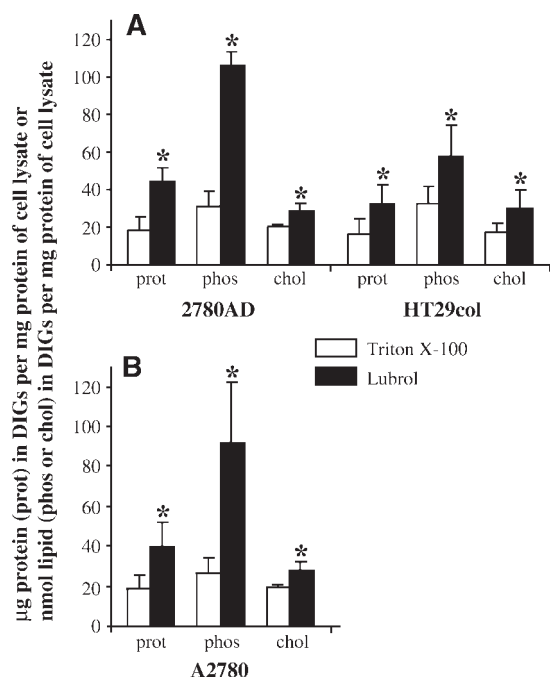


Fig. 1. Protein, phospholipid, and cholesterol levels of low-density, detergent-insoluble membrane domains. Triton X-100 and Lubrol lysates prepared from 2780AD (A), HT29^{col} (A), and A2780 (B) cells were fractionated by flotation in a discontinuous sucrose density gradient. Detergent-insoluble floating fractions 3–6 were combined, and their protein content was determined. Protein (prot) content was expressed as micrograms of detergent-insoluble glycosphingolipid-enriched membrane domain (DIG) protein per milligram of cell lysate protein. Phospholipid and cholesterol contents were determined after total lipid extraction. Phospholipid content was assessed by measuring lipid-phosphate. Phospholipid (phos) and cholesterol (chol) contents were expressed as nanomoles of DIG lipid per milligram of cell lysate protein. Open bars, Triton X-100-based DIGs; closed bars, Lubrol-based DIGs. Data represent means \pm SD of three independent experiments. * These values are significantly ($P < 0.05$) different from those of the Triton X-100-based DIGs as determined by two-tailed, unpaired t -test.

of HT29^{col} cells were almost twice as high as those in Triton X-100-based DIGs, the levels in Lubrol-based DIGs of 2780AD cells were three times those of Triton X-100-based DIGs. Cholesterol levels, expressed as nanomoles of DIG-associated cholesterol relative to milligrams of protein of the starting material (cell lysate), were also significantly higher in Lubrol-based DIGs compared with Triton X-100-based DIGs in both cell lines (Fig. 1A, chol). These data indicate that a given amount of cell lysate yields more membrane domain material (protein, phospholipids, and cholesterol) in the case of Lubrol-based DIGs compared with Triton X-100-based DIGs. Next, we compared the total cholesterol content of total cell lysate (e.g., 43.9 ± 8.9 nmol/mg cell lysate protein in HT29^{col} cells) with DIG-associated cholesterol. The latter was now expressed relative to DIG-associated protein. Cholesterol was highly enriched relative to protein in both Triton X-100-based DIGs ($1,012.7 \pm 237.2$ nmol/mg DIG-associated protein in HT29^{col} cells) and Lubrol-based DIGs (902.8 ± 167.6 nmol/mg DIG-associated protein in HT29^{col} cells). Given

the double amount of protein associated with Lubrol-based DIGs, these DIGs had approximately double the amount of cholesterol compared with Triton X-100-based DIGs in HT29^{col} cells. Thus, Lubrol-based DIGs contained at least twice the amount of protein and phospholipid and significantly increased cholesterol compared with Triton X-100-based DIGs.

PE/PS versus sphingolipid enrichment in Lubrol- versus Triton X-100-based DIGs

We next examined whether the lipid class composition of both types of DIGs was also different. We compared the lipid class composition between Lubrol- and Triton X-100-based DIGs and in comparison with the total membranes in both 2780AD and HT29^{col} cells. Total lipid pools were metabolically radiolabeled to equilibrium with [$1\text{-}^{14}\text{C}$]palmitic acid. Lubrol- and Triton X-100-based cell lysates were prepared and fractionated on discontinuous gradients. The low-density membranes of detergent-insoluble floating fractions 3–6 were isolated, and their lipid composition as well as that of total cell lysate were analyzed by thin-layer chromatography. Overall, the lipid patterns were highly comparable between both cell lines with regard to both the total membranes and the two types of DIGs (Fig. 2A, B). For the sphingolipids, Triton X-100-based DIGs were highly enriched in these lipids compared with the total cell membranes in both the 2780AD and HT29^{col} cell lines, with SM representing the largest share (Fig. 2A, B). Although the Lubrol-based DIGs were also enriched in sphingolipids compared with the total membranes, their levels were only approximately half those of the Triton X-100-based DIGs (Fig. 2A, B). Concerning phospholipids, PC represented the largest share in both Triton X-100- and Lubrol-based DIGs. The levels of the main phospholipids PC, PE, and PS in Triton X-100-based DIGs did not differ significantly from those of the total cell membranes. However, in Lubrol-based DIGs, PS and PE levels were significantly higher compared with those of total membranes (and compared with Triton X-100-based DIGs; Fig. 2A, B). Especially in HT29^{col} cells, the PE level was close to the PC level in Lubrol-based DIGs (Fig. 2A). Thus, Lubrol-based DIGs have relatively low sphingolipid and relatively high PE and PS levels compared with Triton X-100-based DIGs.

Sphingolipid alterations in drug-resistant 2780AD cells relative to drug-sensitive A2780 cells occur to a large extent in Triton X-100-based DIGs

Pgp-overexpressing drug-resistant 2780AD cells have been shown to display an altered sphingolipid composition compared with drug-sensitive A2780 cells (11). Here, we analyzed the sphingolipid class compositions of both Triton X-100- and Lubrol-based DIGs in both drug-resistant 2780AD and drug-sensitive A2780 cells. Total lipid pools were metabolically equilibrium-labeled with L-[$U\text{-}^{14}\text{C}$]serine. Lubrol- and Triton X-100-based cell lysates were prepared and fractionated on discontinuous gradients. The low-density membranes of detergent-insoluble floating fractions 3–6 were isolated, and their sphingolipid class com-

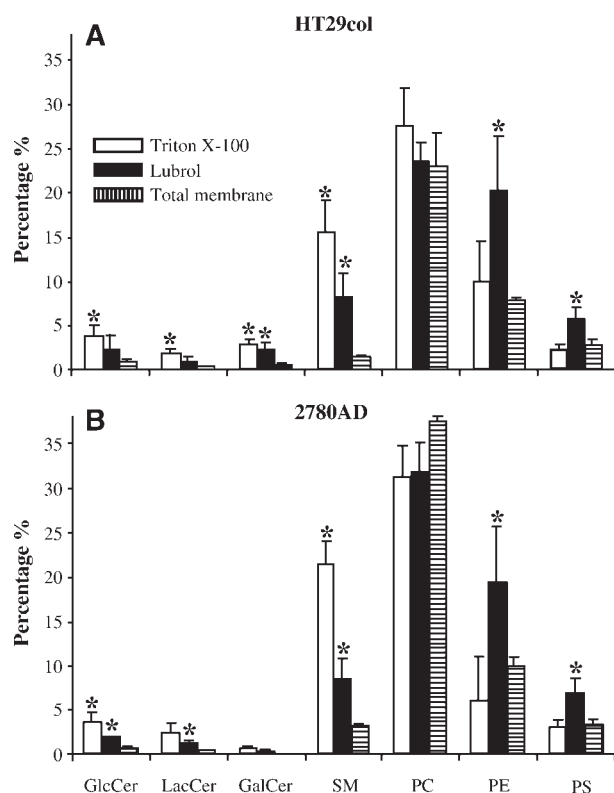


Fig. 2. Lipid composition of detergent-insoluble membrane domains. Total lipid pools of HT29^{col} (A) and 2780AD (B) cells were metabolically radiolabeled by culturing the cells in the presence of 1 μ Ci/ml [14 C]palmitic acid for 48 h. Cell lysate, Triton X-100-based DIGs, and Lubrol-based DIGs were obtained as described in Experimental Procedures. Lipids were extracted from the DIGs in sucrose density gradient fractions 3–6 and subjected to two-dimensional TLC, followed by autoradiography and phosphorimaging analysis. Open bars, Triton X-100-based DIGs; closed bars, Lubrol-based DIGs; striped bars, cell lysate. GlcCer, glucosylceramide; LacCer, lactosylceramide; GalCer, galactosylceramide; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. Data (means \pm SD, $n = 3$) are expressed as percentages of the radioactivity incorporated in a given lipid in DIGs or cell lysate relative to total lipid-incorporated radioactivity of the DIGs or the cell lysate, respectively. * These values are significantly ($P < 0.05$) different from those of the cell lysate as determined by two-tailed, unpaired t -test.

positions were analyzed. The sphingolipid class compositions of both Lubrol- and Triton X-100-based DIGs of the drug-resistant 2780AD cells were different from those of the drug-sensitive A2780 cells (Table 1). Although SM levels and especially GlcCer levels were higher in DIGs from drug-resistant cells, LacCer levels were lower. The amounts of sphingolipids relative to total (radiolabeled) lipids were three times higher in Triton X-100-based DIGs compared with Lubrol-based DIGs. For example, in drug-resistant 2780AD cells, the sum of all measured sphingolipids in Triton X-100-based DIGs represented 62% of total (radiolabeled) lipids in the DIGs, against 23% in Lubrol-based DIGs. Therefore, both Triton X-100- and Lubrol-based DIGs display large differences in sphingolipid class composition between drug-resistant and drug-sensitive cells,

TABLE 1. DIG lipids in MDR 2780AD cells versus drug-sensitive A2780 cells

Detergent and Lipid	A2780 Relative to		2780AD Relative to	
	DIGs	Cell Lysate	DIGs	Cell Lysate
Triton				
GlcCer	3.1 \pm 0.40	0.39 \pm 0.09	6.1 \pm 0.46 ^a	0.86 \pm 0.15 ^a
LacCer	6.8 \pm 1.2	0.86 \pm 0.22	3.4 \pm 0.18 ^a	0.53 \pm 0.17
SM	44.4 \pm 7.3	5.5 \pm 0.35	52.9 \pm 9.2	8.0 \pm 1.1 ^a
PC	29.6 \pm 3.1		31.2 \pm 3.5	
PE	7.7 \pm 4.6		6.0 \pm 4.9	
PS	3.1 \pm 0.92		3.1 \pm 0.81	
Lubrol				
GlcCer	0.95 \pm 0.13	0.24 \pm 0.07	2.2 \pm 0.22 ^a	0.60 \pm 0.18 ^a
LacCer	2.1 \pm 0.61	0.54 \pm 0.23	1.2 \pm 0.24	0.31 \pm 0.03
SM	13.5 \pm 1.5	3.3 \pm 0.47	19.3 \pm 3.6 ^a	5.2 \pm 0.89 ^a
PC	29.9 \pm 1.8		31.9 \pm 3.2	
PE	20.8 \pm 7.0		19.3 \pm 6.3	
PS	6.6 \pm 2.0		6.9 \pm 1.5	

DIG, detergent-insoluble glycosphingolipid-enriched membrane domain; GlcCer, glucosylceramide; LacCer, lactosylceramide; MDR, multidrug resistance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin. Sphingolipid pools of A2780 cells and 2780AD cells were metabolically radiolabeled by culturing the cells in the presence of 1 μ Ci/ml L-[14 C]serine for 48 h. Phospholipid pools were metabolically radiolabeled by culturing the cells in the presence of 1 μ Ci/ml [14 C]palmitic acid for 48 h. Triton X-100- and Lubrol-based DIGs were isolated as described in Experimental Procedures. The lipids were extracted from the DIGs in sucrose density gradient fractions 3–6 and, in the case of sphingolipids, subjected to an alkaline hydrolysis procedure to remove glycerol-based lipids. The remaining lipids were subjected to two-dimensional TLC followed by autoradiography and scintillation counting or phosphorimaging analysis. Data (means \pm SD, $n = 3$) are expressed as percentages of the radioactivity incorporated in a given lipid in the DIGs relative to total lipid-incorporated radioactivity of either the DIGs or the cell lysate (before DIG isolation).

^a These values are significantly ($P < 0.05$) different from those of A2780 cells, as determined by two-tailed, unpaired t -test.

but Triton X-100-based DIGs are more enriched in sphingolipids than Lubrol-based DIGs.

C24:1 sphingolipids are highly enriched in Triton X-100-based DIGs of drug-resistant 2780AD cells

DIGs are believed to owe their insolubility, in cold non-ionic detergents such as Triton X-100 and Lubrol, to the strong bonds between the long, mainly saturated fatty acid chains of sphingolipids (37, 38). If sphingolipid alterations in 2780AD cells play a structural role in DIGs, those with long fatty acid chains and minor unsaturations would be expected to be involved. Because GlcCer and SM were increased in drug-resistant 2780AD cells, we compared their fatty acid distribution and that of their common precursor Cer between DIGs of drug-sensitive 2780AD cells and those of drug-resistant A2780 cells. Triton X-100-based cell lysates from both cell lines were prepared and fractionated on discontinuous gradients. The low-density membranes of detergent-insoluble floating fractions 3–6 were isolated, and their sphingolipid species compositions were analyzed by ESI-MS/MS. The fatty acid distributions were comparable between GlcCer, SM, and Cer in one cell type (Table 2), with a slight preference for shorter chain fatty acids in SM compared with Cer and GlcCer. However, fatty acid distributions were highly different in DIGs

TABLE 2. Triton X-100-based DIG sphingolipid species in MDR 2780AD cells versus drug-sensitive A2780 cells

Lipid	Cell Type	C16	C18	C20	C22	C24:1	C24	C26:1	C26
Cer	A	51.3 ± 3.7	9.0 ± 1.1	2.6 ± 1.1	8.5 ± 2.3	14.9 ± 1.2	13.0 ± 0.8	0.4 ± 0.0	0.2 ± 0.0
	AD	60.9 ± 3.1 ^a	4.8 ± 0.7 ^a	1.3 ± 0.6	4.1 ± 1.2 ^a	21.0 ± 1.9 ^a	7.7 ± 2.3 ^a	0.3 ± 0.0 ^a	0.1 ± 0.0 ^a
GlcCer	A	47.2 ± 5.0	7.1 ± 0.3	1.8 ± 0.0	12.2 ± 3.1	14.5 ± 2.0	21.3 ± 3.4	0.5 ± 0.1	0.4 ± 0.1
	AD	54.3 ± 8.1	3.7 ± 0.0 ^a	1.1 ± 0.1 ^a	7.7 ± 0.9 ^a	22.6 ± 3.8 ^a	11.9 ± 3.5 ^a	0.4 ± 0.1	0.2 ± 0.0 ^a
SM	A	68.6 ± 2.1	9.7 ± 1.1	1.8 ± 0.6	4.2 ± 0.6	9.8 ± 1.1	5.6 ± 0.6	0.4 ± 0.2	0.1 ± 0.0
	AD	71.0 ± 2.5	4.3 ± 0.3 ^a	0.7 ± 0.1 ^a	2.1 ± 0.3 ^a	16.9 ± 2.6 ^a	4.7 ± 0.3 ^a	0.2 ± 0.0	0.1 ± 0.0

From A2780 (A) and 2780AD (AD) cells, Triton X-100-based DIGs were obtained as described in Experimental Procedures. Lipids were extracted from the DIGs in sucrose density gradient fractions 3–6 and subjected to alkaline hydrolysis to remove glycerol-based lipids. Sphingolipid species were analyzed by liquid chromatography-electrospray tandem mass spectrometry. Each sphingolipid with a specific fatty acid is expressed as the percentage of the total species of that sphingolipid class (means ± SD, *n* = 3).

^aThese values are significantly (*P* < 0.05) different from those of A2780 cells, as determined by two-tailed, unpaired *t*-test.

from drug-resistant versus drug-sensitive cells (Table 2, Fig. 3). Most notably, all measured sphingolipids (i.e., Cer, GlcCer, and SM) with C24:1 were three times more abundant in DIGs from drug-resistant 2780AD cells compared with those from drug-sensitive cells, as indicated by a value of 3 for the ratio 2780AD/A2780 (Fig. 3). C16 was the major species in all measured sphingolipids in the DIGs (Table 2). In the cases of Cer and GlcCer, the C16 species were relatively abundant in DIGs of drug-resistant cells compared with the total enrichment factor (Fig. 3). Apart from C24:1 and C16, all other fatty acid species were relatively more abundant in DIGs of drug-sensitive cells compared with the total enrichment factor. Overall, the levels of GlcCer, SM, and Cer were higher in DIGs of 2780AD cells, as indicated by the sum of all fatty acid species (Fig. 3, Total). The increases of GlcCer and SM were comparable to those observed using [¹⁴C]serine equilibrium labeling. Thus, Triton X-100-based DIGs differ significantly in sphingolipid species composition between drug-resistant 2780AD cells and drug-sensitive A2780 cells, with drug-resistant cells displaying higher levels of C24:1 and to a lesser extent C16 fatty acid-containing sphingolipids.

Drug-resistant 2780AD cells do not have altered amounts of DIGs or an altered phospholipid composition

Given the changes in sphingolipid levels in drug-resistant 2780AD cells (11) and the finding that they represent an important share of DIG lipids, we investigated whether 2780AD cells have altered amounts of DIGs compared with A2780 cells. From both cell lines, equal amounts (based on protein content) of Lubrol- and Triton X-100-treated cell lysates were fractionated on discontinuous gradients. The low-density membranes of detergent-insoluble floating fractions 3–6 were isolated, and their protein, phospholipid, and cholesterol contents were measured. Between DIGs of A2780 and 2780AD, no significant differences were observed in any of the three parameters (Fig. 1B; compare with Fig. 1A). Therefore, we conclude that the amounts of DIGs present in drug-resistant 2780AD cells are no different from those in drug-sensitive A2780 cells.

To investigate whether drug-resistant versus drug-sensitive cells differed in the levels of individual phospholipid classes in DIGs, total lipid pools of both A2780 and 2780AD cells were metabolically radiolabeled to equilibrium with

[1-¹⁴C]palmitic acid. Lubrol- and Triton X-100-based cell lysates were prepared and fractionated on discontinuous gradients. The low-density membranes of detergent-insoluble floating fractions 3–6 were isolated, and the phospholipid classes were analyzed. Between DIGs of A2780 and 2780AD, no significant differences were observed in PC, PE, or PS levels (Table 1).

Finally, it is notable that the differences between Lubrol- and Triton X-100-based DIGs found in 2780AD cells (Fig. 1A) were also observed in A2780 cells (Fig. 1B). Lubrol-based DIGs contained two times more protein and three times more phospholipid, as well as increased levels of cholesterol, compared with Triton X-100-based DIGs. Also, the phospholipid composition differed significantly between Lubrol- and Triton X-100-based DIGs in A2780 cells (Table 1). Although levels of PC were comparable between both types of DIGs, Lubrol-based DIGs contained higher levels of PE and PS.

ABC transporters are also predominantly located in Lubrol-based DIGs of non-MDR tumor cells

In previous studies, we have shown in two MDR cell lines that ABC transporters are predominantly localized in Lubrol-based DIGs compared with Triton X-100-based DIGs (10, 11). Both MDR cell lines were derived from their parental counterparts by culturing with increasing concentrations of amphipathic substrates (cytostatics) and are under continuous selective pressure from the cytostatic. The two cell lines highly overexpress the ABC transporters Pgp and MRP1, respectively, compared with their parental counterparts (Fig. 4C). To examine whether this specific Lubrol-based DIG localization is a general characteristic of ABC transporters, we analyzed the localization of ABC transporters in cell lines that were not exposed to cytostatics and consequently do not overexpress the transporters. Triton X-100- and Lubrol-based DIGs were prepared from HepG2 human liver tumor cells as well as SK-N-FI and SK-N-AS human neuroblastoma cells. The cell lysates were fractionated in a discontinuous sucrose density gradient, and the fractions were analyzed for Pgp, MRP1, or MRP2 immunoreactivity (Fig. 4A). Pgp, MRP1, and MRP2 were all substantially enriched in the Lubrol-insoluble fractions 3–6 compared with the Lubrol-soluble fractions 8–12 (Fig. 4A). In the case of Triton X-100, enrichment of ABC trans-

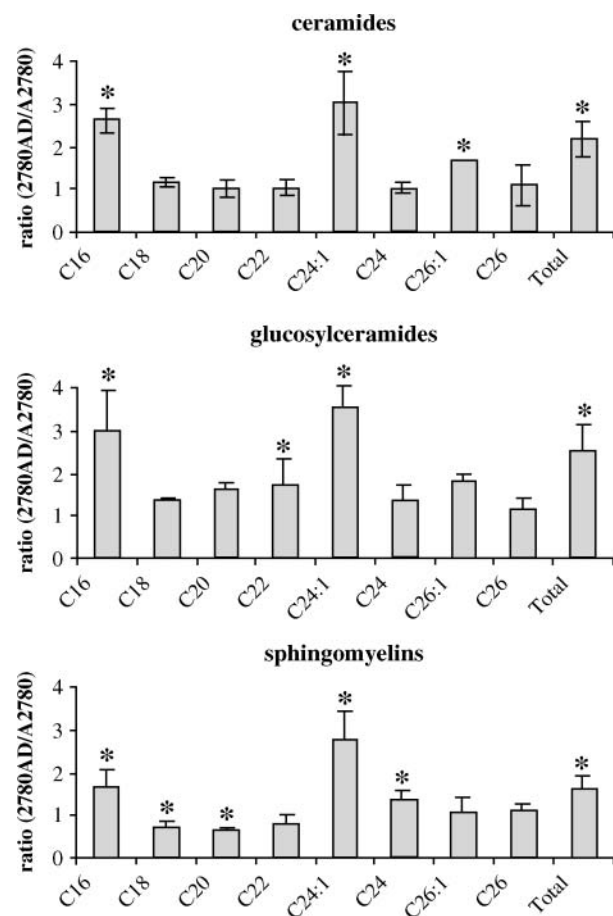


Fig. 3. Sphingolipid fatty acid distribution of Triton X-100-based DIGs from 2780AD versus A2780 cells. From A2780 and 2780AD cells, Triton X-100-based detergent-insoluble membrane domains were obtained as described in Experimental Procedures. Lipids were extracted from the DIGs in sucrose density gradient fractions 3–6 and subjected to alkaline hydrolysis to remove glycerol-based lipids. Sphingolipids were analyzed by liquid chromatography-electrospray tandem mass spectrometry. The ratio (2780AD/A2780) was calculated by dividing the amount of a given sphingolipid with a specific fatty acid in 2780AD-derived DIGs by the amount in A2780-derived DIGs (means \pm SD, $n = 3$). * These values are significantly ($P < 0.05$) different as determined by the one-sample t -test comparing ratios of three independent experiments to a ratio of 1.

porters in DIG fractions was less pronounced (Fig. 4A). This pattern was consistent with ABC transporter localization in 2780AD and HT29^{col} cells. As controls, c-Src as well as transferrin receptor were localized in Lubrol-insoluble fractions 3–6, whereas β -actin was localized in Lubrol-soluble fractions 8–12 (data not shown) (28). Lubrol-insoluble fractions contained roughly twice the amount of protein compared with the Triton X-100-insoluble fractions in all cell lines tested (Fig. 4B). The sucrose gradient density profile (Fig. 4D) indicates that DIGs in fractions 3–6 may well be a mixture of membrane domains with different densities. Therefore, in human tumor cell lines, ABC transporters are predominantly located in Lubrol-based DIGs, irrespective of whether these cell lines are exposed to cytostatics or not.

DISCUSSION

MDR cancer cells are often characterized by the overexpression of ABC transporters as well as an altered membrane lipid composition (11, 39). Concerning sphingolipids, a typical increase in GlcCer level is observed in many MDR tumor cell types (9, 40, 41). Sphingolipids, with usually saturated fatty acyl chains, strongly interact with cholesterol in cell membrane domains named DIGs because of their insolubility in cold nonionic detergents (22–26). DIGs have been shown to accommodate ABC transporters, such as Pgp. Caveolae, a specific subclass of DIGs coated with caveolin-1, have been reported as the site of Pgp localization and enrichment (27, 42). We have recently shown that Pgp and MRP1 are localized in noncaveolar membrane domains in the human tumor cell lines 2780AD and HT29^{col}, respectively. These membrane domains were defined by their insolubility in the detergent Lubrol. Furthermore, in HT29^{col} cells, the increase in GlcCer level occurred concomitant with MRP1 upregulation during resistance acquisition, whereas both were enriched in Lubrol-based DIGs (43).

In this study, we set out to investigate 1) whether the lipid composition of Lubrol-based DIGs has specific characteristics that could favor the localization of ABC transporters; 2) whether MDR-associated sphingolipid alterations occur in DIGs of 2780AD cells, and if so, whether they occur in a specific type of DIG (i.e., Triton X-100- or Lubrol-based); and 3) whether additional changes occur in other components of DIGs (i.e., phospholipids, cholesterol, and protein content) in drug-resistant cells.

1) An interesting characteristic of the Lubrol-insoluble membrane domains is their relative enrichment in the aminophospholipids PE and PS compared with Triton X-100-insoluble membrane domains. The latter were relatively more enriched in sphingolipids. Both types of DIGs were highly enriched in cholesterol, relative to total cell lysate. This indicates that the main building units of rafts (i.e., cholesterol and sphingolipids) are present in both types of DIGs, but part of the sphingolipids appears to be replaced by PE and PS in Lubrol-based DIGs. It is conceivable that PE has partly substituted for SM concerning interactions with cholesterol in Lubrol-based DIGs (44). Localization of ABC transporters in aminophospholipid-enriched membrane domains is compatible with the well-known dependence of ABC transporters on PE and PS for their ATPase activity. It has been shown that for the isolation of active Pgp, the protein needs to retain tightly bound lipids, whereas total delipidation results in the complete loss of ATP hydrolysis activity. These tightly bound lipids have been identified as mainly PE and PS (15, 16). Furthermore, it has been shown that PE stimulates the ATPase activity of both Pgp and MRP1, whereas PS additionally stimulates MRP1 ATPase activity (17–20).

Lubrol-based DIGs contain twice the amount of protein and phospholipid compared with Triton X-100-based DIGs. This observation is consistent with the model that Triton X-100-based DIGs are part of larger Lubrol-based DIGs, which is supported by other observations (45, 46). Immu-

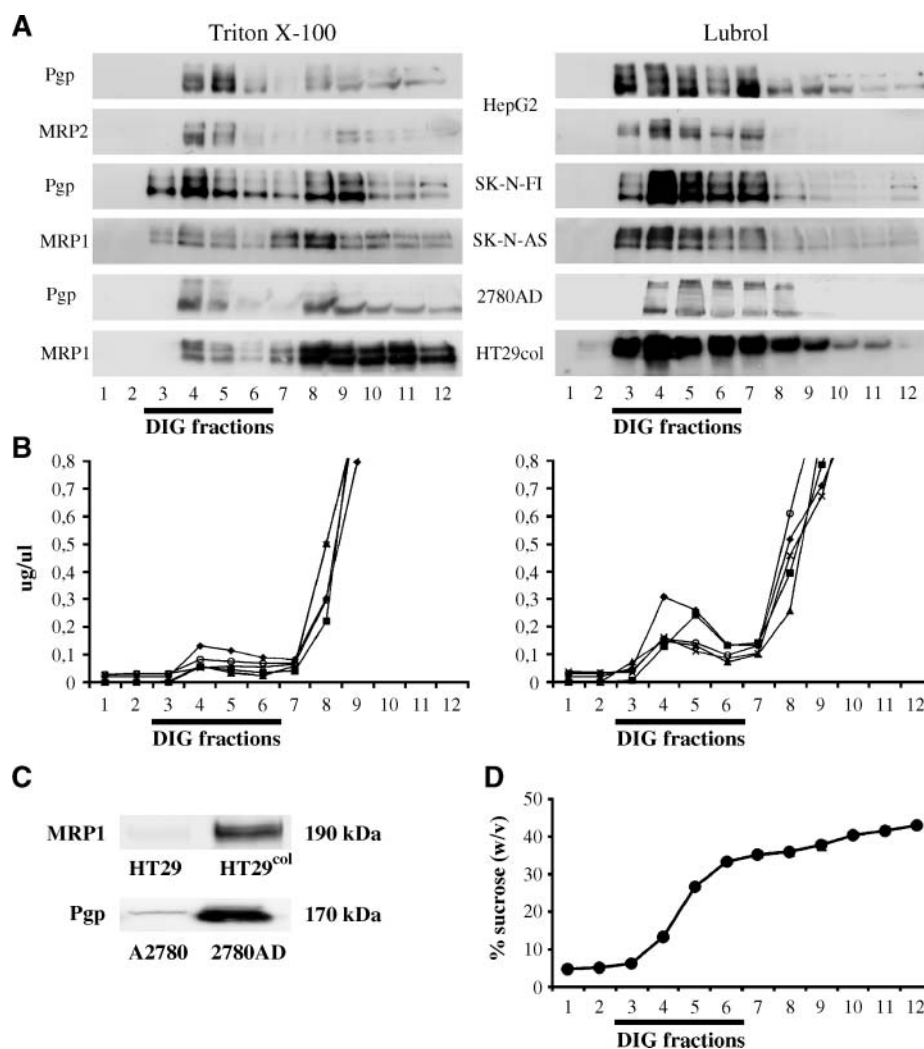


Fig. 4. Localization of ATP binding cassette transporters in low-density, detergent-insoluble membrane domains. **A:** Lubrol lysates prepared from HepG2, SK-N-FI, SK-N-AS, 2780AD, and HT29^{col} cells were fractionated by flotation in a discontinuous sucrose density gradient. Aliquots of each fraction, containing 10 μ g of protein, were subjected to SDS-PAGE and immunoblotting (see Experimental Procedures). Monoclonal antibodies against P-glycoprotein (Pgp) and multidrug resistance-associated proteins 1 and 2 (MRP1 and MRP2) were used for detection. **B:** The protein contents (μ g/ μ l of each fraction) of all obtained sucrose density gradient fractions were measured and compared between Triton X-100-based and Lubrol-based cell lysates. The protein contents of fractions 9–12 extended from 0.8 to 1.5 mg/ml. Diamonds, HepG2; squares, SK-N-FI; triangles, SK-N-AS; crosses, 2780AD; open circles, HT29^{col}. **C:** 2780AD cells highly overexpress Pgp, whereas MRP1 is highly overexpressed in HT29^{col} cells. Equal amounts of cell protein were loaded on the cells, as confirmed by actin staining (data not shown). **D:** Sucrose gradient density profile. The density of each fraction was measured optically, using a refractometer. Data represent means \pm SD of four independent measurements, including two with Triton X-100-based and two with Lubrol-based DIG isolations (which yield very similar data).

noprecipitation studies have shown that at least part of the Lubrol-based DIGs of HT29^{col} cells consisted of a Triton X-100-insoluble part and a Triton X-100-soluble part. MRP1 was localized predominantly in the Triton X-100-soluble part of the Lubrol-based DIGs (28). Although it remains to be fully proven, it is tempting to conclude that Lubrol-based DIGs consist of a Triton X-100-insoluble core, which is highly enriched in sphingolipids, and a surrounding Triton X-100-soluble region. The latter is moderately enriched in sphingolipids and relatively rich in aminophospholipids. It is this region that harbors most of the ABC transporter molecules (47).

It should be noted that this study, which is based on the use of cold detergent isolation of membrane domains, does not support conclusions concerning the actual size of ABC transporter-containing membrane domains under dynamic physiological conditions. As has been discussed extensively (48), especially Triton X-100-based DIG isolation procedures may be hampered by detergent-driven reorganization of lipids during the isolation, as has become apparent from model membrane studies with small liposomes. This may include detergent-induced raft formation and/or detergent-induced coalescence of rafts to larger membrane structures. On the other hand, a recent

article argues against detergent-induced artifacts during raft isolation, based on studies in giant unilamellar vesicles (i.e., liposomes that have cell-like dimensions). In these giant unilamellar vesicles, which allow direct visualization of membrane behavior, the detergents Triton X-100 and Brij 98 did not induce domain formation or domain coalescence (49). Under normal physiological conditions, rafts may be very small structures (48), possibly just starting out as single-protein molecules with a surrounding lipid shell (50) of specific lipids recruited by the protein of interest. In this context, our study reveals that ABC transporters are associated with specific lipids that are partly different from those associated with "traditional" raft marker proteins found in Triton X-100-based DIGs. Such very small rafts may subsequently grow into larger membrane domains (e.g., as a result of protein-protein interactions) (51). In fact, what we have defined as the DIG fractions may well be a collection of membrane domains that all have ABC transporters but that differ in size and composition (lipid/protein content). This notion is supported by the observation that DIGs are found in sucrose density gradient fractions 3–6, which display a range of different sucrose densities and thus may harbor membrane domains with different densities.

2), 3) The sphingolipid and phospholipid compositions as well as the cholesterol and protein contents of both Triton X-100- and Lubrol-based DIGs were compared between MDR 2780AD cells and their drug-sensitive counterpart (A2780 cells). Both Triton X-100- and Lubrol-based DIGs of 2780AD cells differed in sphingolipid class composition from those of A2780 cells in a manner reminiscent of the differences in total cellular sphingolipid composition observed previously (11). Moreover, the difference between DIGs of the drug-resistant and drug-sensitive cell lines was limited to sphingolipids. All other measured parameters [i.e., the levels of the main phospholipids (PC, PE, and PS), the total phospholipid content, and protein and cholesterol contents] were similar in DIGs from drug-resistant and drug-sensitive cells. Hence, DIGs from both cell lines differed specifically in sphingolipid class composition. It can be calculated [based on the data from Table 1 in combination with published data (11) on sphingolipid levels in whole cells] that the amount of each measured sphingolipid class in Triton X-100-based DIGs represented at least 69% of its total cellular pool. This means that the MDR-associated differences in cellular sphingolipid composition occur to a large extent in Triton X-100-based DIGs.

When analyzing the fatty acid distribution of DIG sphingolipids using ESI-MS/MS, the most striking observation was that sphingolipids with C24:1 were three times more abundant in DIGs of drug-resistant 2780AD cells compared with those of drug-sensitive A2780 cells. This corresponds with the finding that C24:1 SM was most typically enriched in rafts compared with total plasma membrane (52). Enrichment of C24:1 in all measured sphingolipids in DIGs from 2780AD cells suggests that the MDR-associated changes in the sphingolipid composition of drug-resistant cells at least partly fulfill a structural role in DIGs of these cells. In the cases of Cer and GlcCer, the C16 spe-

cies also were relatively more abundant in DIGs of drug-resistant cells. This is consistent with other observations indicating the relative abundance of short-chain fatty acid in raft lipids (52). C16-Cer, in contrast to C24-Cer, is associated with signal transduction in apoptosis (53). Although beyond the scope of this study, it is conceivable that the C16-GlcCer increase in DIGs of 2780AD cells is correlated with the C16-Cer increase and involved in an apoptosis escape pathway, as described previously (54, 55).

In conclusion, MDR-associated sphingolipid composition changes in drug-resistant 2780AD cells were localized in DIGs and, more specifically, in Triton X-100-based DIGs. Moreover, sphingolipid composition changes were the only compositional changes observed in the DIGs of these drug-resistant cells. In addition to changes in sphingolipid class composition, each sphingolipid class displayed an altered fatty acid composition relative to drug-sensitive cells. These changes in fatty acid distribution are compatible with a role for sphingolipids in the structural organization of DIGs. This study does not provide evidence for a functional role of DIG-associated sphingolipid changes in MDR. In fact, because Pgp was shown to be especially enriched in Lubrol-based DIGs of 2780AD cells (28), it is not likely that its function is directly facilitated by the MDR-related sphingolipid alterations, which occur largely in the Triton X-100-based DIGs. However, as discussed above, sphingolipids and Pgp may be localized in different regions of a large membrane domain, which harbors a Triton X-100-insoluble core and a Triton X-100-soluble/Lubrol-insoluble perimeter (47). As such, this large membrane domain may be involved in MDR [e.g., in providing a suitable membrane environment for optimal Pgp function and/or in ensuring close proximity of Pgp and its amphipathic substrates (cytostatics)]. Although this study shows that ABC transporter localization in Lubrol-based DIGs is not dependent on contact of the cells with cytostatics, future research in our laboratory will attempt to further dissect these options. ■

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