ATP-binding Cassette Transporters Are Enriched in Non-caveolar Detergent-insoluble Glycosphingolipid-enriched Membrane Domains (DIGs) in Human Multidrug-resistant Cancer Cells*

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In this study we show that P-glycoprotein in multidrug-resistant 2780AD human ovarian carcinoma cells and multidrug resistance-associated protein 1 in multidrug-resistant HT29™™ human colon carcinoma cells are predominantly located in Lubrol-based detergent-insoluble glycosphingolipid-enriched membrane domains. This localization is independent of caveolae, since 2780AD cells do not express caveolin-1. Although HT29™™ cells do express caveolin-1, the ATP-binding cassette transporter and caveolin-1 were dissociated on the basis of differential solubility in Triton X-100 and absence of microscopical colocalization. While both the multidrug resistance-associated protein 1 and caveolin-1 are located in Lubrol-based membrane domains, they occupy different regions of these domains.

Treatment failure in cancer therapy is largely due to multidrug resistance (MDR), by which tumor cells are typically cross-resistant to multiple chemotherapeutic agents. Different cellular mechanisms underlying this phenomenon have been described. The overexpression of ATP-binding cassette (ABC) drug transporter proteins like P-glycoprotein (Pgp) and multidrug resistance-associated protein 1 (MRP1) are the most extensively studied examples (1–4). These proteins prevent cellular accumulation of cytotoxic drugs either by acting as an efflux pump, increasing outward transport of drugs, or by translocating the drugs to the external leaflet of the membrane, from which they eventually diffuse (5–7). Pgp, being an integral plasma membrane protein, is known to be dependent on its lipid environment for optimal functioning (8, 9).

Recent studies have indicated that virtually all MDR cells exhibit a deviating sphingolipid composition, most typically, increased levels of glucosylceramide (GlcCer) (10–12). Detergent-insoluble glycosphingolipid-enriched membrane domains (DIGs) are among other lipids such as sphingomyelin and cholesterol highly enriched in GlcCer (13, 14). DIGs share their lipid composition and insolubility in cold non-ionic detergents with caveolae (15). Caveolae are non-clathrin-coated plasma membrane vesicular invaginations of which caveolin-1 (Cav-1) is the major coat protein. Cav-1 is a 21-kDa integral membrane protein that is necessary for the formation of caveolae (16). Recently it was shown that Cav-1 expression, as well as caveolae themselves are up-regulated in Pgp-overexpressing cells and that a substantial fraction of Pgp was located in Cav-1-containing DIGs (17). Furthermore, immunoprecipitation studies suggested that Pgp and Cav-1 physically interact (18). In the present study we employed a human ovarian MDR tumor cell line, which overexpresses Pgp and GlcCer and turns out to be devoid of Cav-1. In addition we used a human colon MDR tumor cell line, which overexpresses MRP1 and GlcCer and does express Cav-1. We show that both ABC transporters, Pgp and MRP1, are predominantly located in (Lubrol-based) DIGs independently of caveolae.

**EXPERIMENTAL PROCEDURES**

**Materials—** A2780 and 2780AD cell lines were kindly provided by Dr. E. G. E. de Vries and Dr. H. Timmer-Bosscha (Dept. of Medical Oncology, University Hospital Groningen, The Netherlands). The HT29™™ cell line was obtained from HT29™™ cells by selection with colchicine (11). Doxorubicin was obtained from the hospital’s pharmacist. Colchicine, Triton X-100, sucrose (99+%), and the monoclonal antibody anti-p-actin (clone AC-15) were purchased from Sigma. All cell culture plastic ware was obtained from Costar (Cambridge, MA). Dulbecco’s modified Eagle medium, Hank’s balanced salt solution (HBSS), and antibiotics were from Invitrogen Life Technologies (Paisley, UK). Fetal calf serum was from Bovico (Alkmaar, the Netherlands). The monoclonal antibodies anti-Pgp (C219) and anti-MRP1 (MRP1) were from Signet Laboratories (Bedham, MA). The polyclonal antibody anti-Cav-1 was from Transduction Laboratories (Lexington, KY). The polyclonal antibody anti-c-Src and protein A-Sepharose CL-4B beads were from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Trans-Blet Transfer Medium membrane was from Bio-Rad and ECL plus Western blotting detection system was from Amersham Biosciences. TRITC anti-mouse and anti-rat IgG (Fab‘2) fragments were obtained from Roche Applied Science. Alexa Fluor 594 anti-rabbit, 488 anti-mouse, and 488 anti-rat were from Molecular Probes (Eugene, OR). 1-[3H]/choline from Serva (Heidelberg, Germany). All PCR primers were from Invitrogen Life Technologies and the High Fidelity PCR Master kit was from Roche Applied Science.

**Cell Culture and Experimental Conditions—** A2780 and 2780AD cells were cultured in RPMI 1640 containing 100 units/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 mg/ml doxorubicin. The cultures were passed twice a week. HT29™™ and HT29™™ cells were cultured in Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, 100 mg/ml streptomycin and 10% (v/v) heat-inactivated (56 °C, 30 min) fetal calf serum. The cultures were passed once a week. The medium of the HT29™™ was supplemented with 50 μM colchicine, 48 h after the weekly passage of the cells. All cells were maintained in a water-saturated atmosphere of 5%
CO₂/5% air at 37 °C. All experiments took place during the exponential growth phase of the cells.

**Reverse Transcriptase Polymerase Chain Reaction**—Total RNA was isolated from cells using RNeasy Mini Kit according to manufacturer's instructions. Single-stranded cDNA was synthesized from RNA (1 µg) using oligo(dT)12-18 primer (0.5 µg), Superscript RT (200 units), 5× First Strand Buffer (4 µl), dithiothreitol (10 mM), and 0.5 mM of each dNTP in a total volume of 20 µl. The RNA sample – oligo(dT)12-18 primer were denatured at 65 °C for 15 min and placed on ice for 5 min, before adding them to the reaction mixture. Reverse transcription was performed for 1 h at 37 °C and the samples were subsequently heated for 5 min at 99 °C to terminate the reverse transcription reaction. PCR was performed with High Fidelity PCR Master, using 2.5 µl of the CD135, 0.5 µl of sense and antisense, 5'-ATGTCACGCACGATTTCC-3' and 5'-CTTGAAATTGGCACCAGGAA-3' (608-bp amplified product); β-actin: sense, 5'-AACACCCCAAGCCTGATAC-3'; and antisense, 5'-GTGTCCACGCACCATTC-3' (254-bp amplified product). In each experiment, β-actin was used as an internal control. Water as well as RNA were used as negative controls, to check for potential contamination. Ten microliters of PCR product was loaded on a 2.5% agarose gel stained with ethidium bromide.

**Isolation of DIGs**—DIGs were isolated from cells as described (18). 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-NCI-EDTA buffer (TNE) (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA) containing 1% (w/v) Triton X-100 or 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. Two ml of TNE were then added 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 5% (w/v) sucore in TNE were successively loaded, resulting in a discontinuous gradient. All solutions contained the following protease inhibitors: 100 µM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1 µg each of aprotinin, leupeptin, and pepstatin A. Gradients were centrifuged at 15,000 rpm for 1 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 using bovine serum albumin as standard.

**Immunoprecipitation**—The DIG containing fractions were adjusted to 3 ml with ice-cold TNE and centrifuged in a Beckman TL100A 1.5 fixed rotor at 49,000 rpm for 45 min at 4 °C. The pellet was resuspended in 200 µl of TNE, containing Triton X-100 or Lubrol and protease inhibitors (see “Isolation of DIGs”). After protein determination, 5 µg of the DIGs was immunoprecipitated by overnight incubation at 4 °C with anti-Cav-1 antibody, conjugated to protein A-Sepharose CL-4B beads prepared with dimethyl pimelimidate. beads were spun down by centrifugation at 10,000 rpm, for 1 min, at 4 °C and washed five times with TNE, containing Triton X-100 or Lubrol and protease inhibitors. Immunoprecipitated proteins were eluted with sample buffer (Tris-HCl (0.25 M), pH 6.5, containing 5% (w/v) sodium dodecyl sulfate, 5% (v/v) 2-mercaptoethanol, 40% glycerol, and 0.1% (w/v) bromophenol blue) by boiling (3 min). the boiling step was omitted with MRP1.

**Immunoblot Analysis**—Cells were washed twice with HBSS and harvested with a rubber policeman. The total amount of cellular protein was determined, using bovine serum albumin as standard. Subsequently, a known amount of cells was dissolved in sample buffer at the ratio 1:1 (w/v) and incubated for 5 min at 95 °C. In the case of MRP1 immunoblotting, the sample was incubated for 5 min at 95 °C step was omitted. Protein from each fraction was trichloroacetic acid-precipitated and resuspended in sample buffer. Cells, immunoprecipitated proteins, or trichloroacetic acid-precipitated proteins (DIGs) were resolved on SDS-PAGE (10%) gels. Proteins were electrotransferred onto Trans-Blot Transfer Medium membrane. The membranes were rinsed with PBS and incubated with 5% (w/v) nonfat dry milk in PBS. Membranes were rinsed in washing buffer (PBS containing 0.3% (w/v) Tween 20) and incubated (2 h, 20 °C) with a primary antibody against Pgp (1:300), MRP1 (1:1000), Cav-1 (1:500), TR (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 (1:7500) (ECL) in washing buffer containing 1% (w/v) nonfat dry milk (2 h, 20 °C). Membranes were incubated in chemiluminescence substrate solution, according to the manufacturer's instructions, and immunoreactive complexes were visualized by exposure of Kodak Bio-max film.

**Microscopy**—After treatment with detergent, cells were fixed and permeabilized in methanol (−20 °C) over 5 min. Thereafter, cells were washed four times with PBS, followed by a 30-min incubation with PBS/1% (w/v) bovine serum albumin. Fixed cells were incubated for 1.5 h at room temperature with a primary antibody against MRP1 (1:300), Cav-1 (1:150), TR (1:150), or Pgp (1:100) in PBS/1% (w/v) bovine serum albumin, followed by two washes with PBS/1% (w/v) bovine serum albumin. Thereafter, fixed cells were incubated for 1.5 h at room temperature with the appropriate TRITC-conjugated antibody (1:30) or Alexa Fluor-conjugated antibody (1:1000) in PBS/1% (w/v) bovine serum albumin, followed by two washes with PBS/1% (w/v) bovine serum albumin. After mounting, images from the cells were taken with a confocal scanning laser microscope (True Confocal Scanner SP2; Leica, Heidelberg, Germany) equipped with argon-krypton and neon lasers and coupled to a Leica DM RXE microscope. Alexa Fluor images were taken at λem = 488 nm and λex = 505–530 nm, and Alexa Fluor 594 images were taken at λem = 543 nm and λex = 580–620 nm. TRITC images were taken at λem = 543 nm and λex = 570–620 nm, in the case of single staining, and at λem = 580–690 nm, in the case of double staining.

**RESULTS**

Caveolin-1 Expression Is Not Altered in MDR Tumor Cells—It was previously shown that caveolae and Cav-1 expression are up-regulated in Pgp overexpressing HT29 cells (17). We compared Cav-1 expression of two human MDR tumor cell lines, which had acquired their drug resistance through selection with chemotherapeutic agents, with the drug-sensitve parental cell lines. Ovarian 2780AD tumor cells overexpress Pgp, while colon HT29 ¯c tumors overexpress MRP1 (11, 12). Lysates of HT29(ª), HT29(¯c), A2780, and 2780AD cells were subjected to SDS-PAGE/immunoblotting and analyzed for Cav-1 immunoreactivity (see “Experimental Procedures”). The levels of Cav-1 were similar in drug-resistant HT29(º) cells and their parental HT29(‡) counterpart (Fig. 1A). No clear Cav-1 expression was detectable in A2780 cells, either in the parental A2780 cells or in drug-resistant 2780AD cells (Fig. 1A). The slight staining at the molecular weight of Cav-1 observed especially in the A2780 cells is most likely unspecific, since multiple bands of similar intensity were seen throughout the blot in all four cell lines. This conclusion is strengthened by the fact that specific Cav-1 staining was absent in 2780 cells (A and AD) as judged by confocal scanning microscopy after immunostaining with anti-Cav-1 antibody (Fig. 1B). To further confirm that Cav-1 expression did not occur in 2780 ovarian carcinoma cells, we examined Cav-1 expression at the mRNA level by RT-PCR, using specific primers (see “Experimental Procedures”). No expression of Cav-1 was observed in either A2780 or 2780AD cells (Fig. 1C). In conclusion, Cav-1 is not expressed at all in 2780 cells and not up-regulated in drug-resistant HT29 cells.

**ABC Transporters Are Predominantly Located in Lubrol-based DIGs of MDR Cancer Cells**—Pgp overexpressing 2780AD cells did not express Cav-1 and therefore lack caveolae. We next examined whether Pgp is localized in non-caveolar DIGs. Triton X-100-based lysates were prepared from 2780AD cells, followed by fractionation in a discontinuous sucrose density gradient and analysis of the fractions for Pgp immunoreactivity. Pgp was enriched in the detergent-insoluble fractions 4 and 5, but a considerable amount was located in the detergent-soluble fractions 8–12 (Fig. 2A). c-Src, an established marker of Triton X-100-based DIGs (20), was indeed predominantly concentrated in the detergent-insoluble fractions 4–6, while the non-DIG markers TR and β-actin were localized in the deter-
Fig. 1. Expression of Cav-1 in 2780 and HT29 cells. A, lysates of drug-resistant 2780AD and HT29<sup>col</sup> cells and their drug-sensitive counterparts (2780 and HT29<sup>con</sup>) were subjected to SDS-PAGE and immunoblotting (see "Experimental Procedures"). A polyclonal antibody was employed for detection of Cav-1. B, after fixation, A2780, 2780AD, and HT29<sup>col</sup> cells were stained immunocytochemically, employing a TRITC-conjugated antibody (control) or a polyclonal antibody against Cav-1 followed by a TRITC-conjugated antibody and analyzed by confocal microscopy (see "Experimental Procedures"). Bars, 10 μm. C, the expression levels of Cav-1 mRNA in A2780 and 2780AD cells were determined by RT-PCR, using specific primers. The PCR products were separated on a 2.5% agarose gel. A, A2780; AD, 2780AD; G, HT29<sup>con</sup>; col, HT29<sup>col</sup>; control. Shown are typical results from three independent experiments.

Fig. 2. Localization of Pgp in low density, detergent-insoluble membrane domains. Triton X-100 (A) or Lubrol (B) lysates prepared from 2780AD cells were fractionated by flotation in a discontinuous sucrose density gradient. Aliquots of each fraction, containing 10 μg of protein, were subjected to SDS-PAGE and immunoblotting (see "Experimental Procedures"). Monoclonal antibodies against Pgp, TR, and β-actin and a polyclonal antibody against c-Src were employed for detection. Shown are typical results from three independent experiments.

Fig. 3. Localization of MRP1 in low density, detergent-insoluble membrane domains. Triton X-100 (A) or Lubrol (B) lysates prepared from HT29<sup>col</sup> cells were fractionated by flotation in a discontinuous sucrose density gradient. Aliquots of each fraction, containing 10 μg of protein, were subjected to SDS-PAGE and immunoblotting (see "Experimental Procedures"). Monoclonal antibodies against MRP1, TR, and β-actin and polyclonal antibodies against Cav-1 and c-Src were employed. Shown are typical results from three independent experiments.

However, both Pgp (Fig. 4, J and L) and MRP1 (Fig. 4, A and C) were completely resistant to Lubrol treatment. TR was completely resistant to Lubrol treatment but very strongly reduced after Triton X-100 treatment in both HT29<sup>col</sup> (Fig. 4, G—I) and 2780AD cells (Fig. 4, M—O), while Cav-1 was completely resistant to both Triton X-100 and Lubrol treatment (Fig. 4, D—F).

MRP1 Is Not Localized in Caveolae—MRP1 co-fractionated with Cav-1 in Lubrol-based DIGs, but not in Triton X-100-based DIGs, suggesting that the two proteins do not co-localize in caveolae in intact cells. This was further examined by double immunostaining and confocal scanning microscopy (Fig. 5, A—C). Hardly any colocalization of MRP1 and Cav-1 was observed, as indicated by the virtual absence of yellow staining in the merged image (Fig. 5C). MRP1 was homogeneously distributed over the membrane in contrast to Cav-1, which displayed a dot-like staining pattern, indicative of local accumulation.
Also TR and Cav-1 showed hardly any colocalization (Fig. 5, D–F), while TR and MRP1 did colocalize to a large extent (Fig. 5, G–H).

Cav-1 and MRP1 Are Localized in Different Regions of Lubrol DIGs—Although MRP1 and Cav-1 did not colocalize, they were both found in the Lubrol-based DIG fractions. We therefore investigated if these two proteins might be localized in different regions of the Lubrol-based DIGs. Triton X-100-based and Lubrol-based lysates were prepared from MRP1 overexpressing HT29col cells and fractionated in a discontinuous sucrose density gradient. The detergent-insoluble floating fractions 4–6 were used to immunoprecipitate proteins with anti-Cav-1 antibodies. During the immunoprecipitation procedure the detergent-resistant fractions were treated with solubilization buffer containing either Triton X-100 or Lubrol. The anti-Cav-1 immunoprecipitated complexes that were retained during this procedure were analyzed for MRP1 or TR immunoreactivity.

Starting with Triton X-100-based DIGs neither MRP1 nor TR was co-immunoprecipitated with Cav-1 (Fig. 6). This was also the case when Lubrol-based DIGs were immunoprecipitated with anti-Cav-1, using Triton X-100 in the solubilization buffer. However, when Lubrol was used in the solubilization buffer instead of Triton X-100, both MRP1 and TR were co-immunoprecipitated with Cav-1. Fig. 6 also shows that Cav-1 was immunoprecipitated under all conditions. In fact, Cav-1 appears to be somewhat more concentrated in Triton X-100-based DIGs compared with Lubrol-based DIGs, but still does not co-immunoprecipitate MRP1 or TR.
possible to play a role in the cholesterol flux to the DIGs. As Cav-1 is thought to be an important factor in the transport of cholesterol to the plasma membrane (25), caveolae could then act as plasma membrane terminals from where free cholesterol flows to non-caveolar regions of the membrane, e.g. DIGs (26, 27). Furthermore, it has been proposed that caveolin-dependent cholesterol efflux pathways may play a role in delivering drugs from various intracellular membranes, into which they partition, to plasma membrane-localized drug efflux pumps (28).

While the role of caveolae in MDR is still not clear, this study shows that the presence of caveolae is not an absolute requirement for the development of MDR. We have shown that ABC transporters are preferably localized not in caveolae, but rather in non-caveolar Lubrol-based DIGs. The physiological significance of this localization is at present unknown. It has been shown that disturbance of DIG integrity by cholesterol depletion does not necessarily affect the functioning of Pgp, located in these DIGs (29). On the other hand, cholesterol depletion did disturb the normal apical trafficking of Pgp in HepG2 cells (23). Recently, we obtained evidence showing that MRP1 and GlcCer are coordinately up-regulated and enriched in rafts during multidrug resistance acquirement in HT29<sup>col</sup> cells. This indicates that DIGs may indeed be important determinants in MDR acquisition and DIG-associated changes in sphingolipid composition.

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