Localization of the Human Breast Cancer Resistance Protein (BCRP/ABCG2) in Lipid Rafts/Caveolae and Modulation of Its Activity by Cholesterol in Vitro†

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ABSTRACT

Breast cancer resistance protein (BCRP/ABCG2) is an active efflux pump that belongs to the ATP-binding cassette (ABC) transporter family. It is located in various tissues involved in drug absorption, distribution, and elimination and plays an important role in multidrug resistance. For P-glycoprotein, another member of the ABC transporter family, it is well established that it is at least partly located in cholesterol and sphingolipid-enriched domains of the plasma membrane called "lipid rafts" and that the composition of the membrane lipids may modulate its efflux activity. This study addressed the compartmentalization of BCRP in the plasma membrane and the influence of membrane cholesterol on the efflux activity of BCRP. As a cell model, we used the canine kidney epithelial cell line MDCKII-BCRP transfected with the cDNA encoding human BCRP and the corresponding parental cell line MDCKII. Cholesterol depletion with methyl-β-cyclodextrin (MβCD) provoked a 40% decrease in BCRP activity (p < 0.01) assessed with flow cytometry (pheophorbide A efflux assay). Cholesterol repletion with MβCD/cholesterol-inclusion complexes restored BCRP function, and cholesterol saturation of native cells did not further enhance BCRP activity. Communoprecipitation experiments indicated a physical interaction between BCRP and caveolin-1, and Western blot analysis after density gradient ultracentrifugation demonstrated that BCRP is located in detergent-resistant membranes that also contain caveolin-1. In conclusion, our results demonstrate for the first time that BCRP is located in membrane rafts and that cholesterol has impact on its efflux activity.

ATP-binding cassette (ABC) transporters are membrane proteins that bind ATP and use the energy of ATP hydrolysis for the extrusion of a variety of structurally diverse substrates out of the cells (Schinkel and Jonker, 2003). Breast cancer resistance protein (BCRP/ABCG2) is an only recently discovered and cloned ABC transporter (Allikmets et al., 1998; Doyle et al., 1998). In addition to its role in multidrug resistance, its substantial expression at strategic barriers such as the placenta, the small intestine, the liver, and the blood-brain barrier suggests that BCRP acts as a protective efflux pump in the placenta and at the blood-brain barrier for the extrusion of a variety of structurally diverse substrates (Schinkel and Jonker, 2003). They are characterized physicochemically by a tight packing that results in reduced fluidity compared with the disordered phase. Resistance against solubilization by cold disordered phase). Resistance against solubilization by cold

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ABBRIVIATIONS: ABC, ATP-binding cassette; BCRP/ABCG2, breast cancer resistance protein; DRM, detergent-resistant membrane; P-gp, P-glycoprotein; MDR, multidrug resistance; PBS, phosphate-buffered saline; HBSS, Hank’s balanced salt solution; MβCD, methyl-β-cyclodextrin; PFA, paraformaldehyde; PhA, pheophorbide A; FTC, fumitremorgin C; GFP, green fluorescence protein; HBSS, Hank’s balanced salt solution with 1% HEPES; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; TR, transferrin receptor; MF, median fluorescence; IP, immunoprecipitation; TNE, Tris-NaCl-EDTA.
nonionic detergents such as Triton X-100 and thus isolation with the detergent-resistant membrane ( DRM ) fraction after density gradient ultracentrifugation is a methodical criterion to evaluate whether a protein or lipid associates with lipid rafts. Caveolae are specialized lipid rafts that represent flask-shaped cell surface invaginations ( Cheng et al., 2006 ) possibly formed by a clustering of lipid rafts at the cell surface ( Simons and Ehehalt, 2002 ). The invagination process is driven by polymerized structure proteins, the caveolins ( Parton and Simons, 2007 ). On the basis of their lipid composition, lipid rafts and caveolae may “ trap ” proteins that reveal higher affinity to membrane microdomains than to the surrounding plasma membrane ( Sprong et al., 2001 ; Fastenberg et al., 2003 ). The importance of membrane lipid composition for the function of membrane proteins has been demonstrated for a number of relevant proteins involved in signal transduction ( Dykstra et al., 2003 ; Holowka et al., 2005 ) and membrane trafficking ( Kirkham and Parton, 2005 ). In addition, the ABC transporters P-glycoprotein ( Pgp ) and multidrug resistance-related protein 1 are at least partly located in these membrane microdomains ( Demeule et al., 2000 ; Hinrichs et al., 2004 ). The lipid environment has a major impact on P-gp function ( Romsicki et al., 1999 ), and variation of cholesterol content of the cell membrane modulates the activity of P-gp in vitro and ex vivo ( T冒ost et al., 2004 a, b ). Moreover, caveolae and caveolar constituents are up-regulated in multidrug-resistant cancer cells ( Lavie et al., 1998 ), suggesting that microdomains themselves might be important for the development of multidrug resistance ( MDR ) in tumors ( Liscovitch and Lavie, 2000 ). In contrast, for BCRP, so far nothing has been reported about a possible localization in membrane microdomains. The molecular mechanism of BCRP-mediated drug transport is still poorly understood, and it is not known whether and how changes in the lipid composition and compartmentalization of the plasma membrane will affect the activity of this important efflux pump. The aim of this study was therefore to examine the localization of BCRP within the plasma membrane and to investigate the influence of cholesterol on the transport activity of BCRP. As a model with substantial expression of human BCRP, the canine kidney epithelial cell line MDCKII ( Madin-Darby canine kidney cells ) stably transfected with the cDNA encoding human BCRP ( MDCKII-BCRP ) ( Pavek et al., 2005 ) was used. The corresponding parental cell line MDCKII served as a control.

Materials and Methods

Materials. Culture media, fetal calf serum, medium supplements, antibiotics, glutamine, phosphate-buffered saline ( PBS ), HEPES, and Hank s’ balanced salt solution ( HBSS ) were purchased from Invitrogen ( Karlsruhe, Germany ). Aprotinin, cholesterol, dimethyl sulfoxide, methyl- β -cyclodextrin ( MβCD ), poly-d-lysine, gelatin, and bovine serum albumin were obtained from Sigma-Aldrich ( Taufkirchen, Germany ). EDTA, NaCl, Tris, Triton X-100, SDS, glyc erol, Tween 20, β -mercaptoethanol, CaCl 2 , MgCl 2 , and parafomaldehyde ( PFA ) were obtained from AppliChem ( Darmstadt, Germany ); cell-culturing bottles were obtained from Nunc GmbH & Co. KG ( Wiesbaden, Germany ); phenoethaborid A ( PhA; 3S-(3a,4,8,21β)-14-ethyl-21-(methoxycarbonyl)-4,8,12,18-tetramethyl-20-oxo-9-vinylphorbine-5-propionic acid ) was obtained from Frontier Scientific ( Wiesbaden, Germany ); and leupeptin and pepstatin were obtained from Biomol ( Hamburg, Germany ). Nitrocellulose membranes ( Optitran BA-S 85 ) were obtained from Schleicher & Schuell ( Bassel, Germany ). Glass coverslips were purchased from Paul Marienfeld ( Lauda-Königshofen, Germany ). Anti-BCRP antibody ( BXP-21 ) was obtained from Alexis Biochemicals ( San Diego, CA ); anti-caveolin-1 antibody ( C 37120 ) was obtained from BD Biosciences ( Heidelberg, Germany ); anti-caveolin-1 antibody ( sc-894 ) was obtained from Santa Cruz Biotechnology, Inc. ( Heidelberg, Germany ); anti-transferrin receptor antibody ( clone H68.4 ) was obtained from Zymed Laboratories ( South San Francisco, CA ); and purified mouse IgG1, immunoglobulin type control was obtained from BD Biosciences. Cy5-conjugated AffiniPure donkey anti-mouse IgG ( H + L ) was obtained from Jackson ImmunoResearch Laboratories ( West Grove, PA ); ordered via Dianova, Hamburg, Germany ), and Permaflour was obtained from Immunotech ( Marseille, France ). Pumiflumorgin C ( FTC; Rabindran et al., 1998 ) was kindly provided by the National Cancer Institute ( Rockville, MD ).

MDCKII and MDCKII-BCRP Cells. MDCKII-BCRP cells were generated by transfection of the canine kidney epithelial cell line MDCKII with the human full-length wild-type BCRP cDNA inserted into the LZRS-IRES-GFP expression vector that also codes for green fluorescence protein ( GFP ) ( Pavek et al., 2005 ) and were kindly provided by Dr. A. H. Schinkel ( Amsterdam, The Netherlands ). The parental cell line MDCKII ( available at American Type Culture Collection, Manassas, VA ) was used as a control. Both cell lines were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

MβCD and MβCD/Cholesterol-Inclusion Complexes. Cholesterol depletion and repletion were carried out as described previously ( T冒ost et al., 2004 a,b ) with one modification: the incubation medium for cholesterol depletion consisted of a 5 mM solution of MβCD in Hank s’ balanced salt solution with 1% HEPES ( HHBSS ) instead of a 10 mM solution. Cells were incubated in suspension with this solution at 37°C for 30 min. Cholesterol repletion was also carried out at 37°C. The kinetics of cholesterol repletion in the two cell lines was recorded at 0.5, 1, 2, 5, 10, 15, 20, 25, and 30 min ( with depleted cells at t = 0 min ) in three independent experiments.

Isolation of DRMs. Isolation of DRMs was carried out as described previously ( Pohl et al., 2004 ) with the minor modification of using Triton X-100 as detergent instead of CHAPS. Briefly, the cell pellet of four cell culture flasks ( 175 cm 2 ; 80% confluence ) was dispersed in 10 mM solution. Cells were incubated in suspension with this solution at ASPET Journals on April 8, 2017 jpet.aspetjournals.org Downloaded from

Protein Assay. Total protein concentrations in each fraction after sucrose density gradient centrifugation were determined with BCA Protein Assay Kit from Pierce ( Rockford, IL ) according to the manufacturer’s instructions.
Cell Counting. For the determination of cholesterol content per cell, cells (sum of living and dead cells) were counted in a Neubauer hemocytometer. The evaluation of the viability of the cells was performed with trypan blue.

Western Blot Analysis of the Fractions. All fractions were analyzed by SDS-PAGE and Western blotting. Three proteins, namely BCRP, caveolin-1, and transferrin receptor (TR), a known nonraft marker protein (Schuck et al., 2003), were detected in immunoblot analysis; 92 μl of each fraction were mixed with 8 μl of 5× sample buffer (containing Tris-HCl, SDS, β-mercaptoethanol, bromophenol blue, and glycerol) and then subjected to a 12% SDS-PAGE and electrotransferred to nitrocellulose nitrate membranes. Blots were blocked by incubation for 1 h with 5% skim milk (w/v) in phosphate-buffered saline containing 0.1% Tween 20. Western blot analysis was carried out with murine monoclonal antibodies raised against BCRP (BXP-21), caveolin-1 (C37120), or TR (clone H68.6) diluted 1:250 (anti-BCRP) and 1:500 (anti-caveolin-1 and anti-TR) in Tris-buffered saline containing 0.1% Tween 20. The blots were then washed extensively and incubated with horseradish peroxidase-linked secondary anti-mouse antibody (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Bands were visualized by enhanced chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce).

BCRP Inhibition Assay (Flow Cytometry Efflux Assay). The BCRP inhibition assay was performed as described and validated previously (Gupta et al., 2006; Weiss et al., 2007) with minor alterations. Cells (10⁶) were suspended in 500 μl of incubation medium (Dulbecco's modified Eagle's medium without any supplements) containing 1 μM Pha and incubated at 37°C for 30 min on a rotary shaker (450 rpm). Cells were then washed once with 1 ml of ice-cold incubation medium and resuspended in 500 μl of incubation medium containing 10 μM of the specific BCRP inhibitor FTC (Rabindran et al., 1998). After incubation for 60 min at 37°C on a rotary shaker, cells were washed with 1 ml of ice-cold PBS, resuspended in ice-cold PBS, and kept on ice until flow cytometry. Intracellular fluorescence was analyzed in a BD Biosciences LSR II flow cytometer with a solid state-coherent sapphire blue laser and 530-bandpass filter for GFP and a 633 nm helium/neon laser and 660-bandpass filter for Pha.

In each sample, 30,000 cells were counted. Cell debris was eliminated by gating the living cells in the forward versus side scatter. BCRP-positive MDCKII-BCRP cells were additionally gated using their GFP signal. To quantify the activity of BCRP, the ratio between the median fluorescence (MF) with inhibitor (FTC) and without inhibitor during the efflux period was calculated and normalized to the effect observed in the corresponding parental cell line according to the following equation:

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\text{Inhibition ratio} = \frac{\text{MF with FTC in MDCKII BCRP}}{\text{MF without FTC in MDCKII BCRP}} \times \frac{\text{MF with FTC in MDCKII parental}}{\text{MF without FTC in MDCKII parental}}
\]

Flow cytometry experiments were performed at least three times in untreated, depleted, repleted (0.5, 1, and 30 min), and saturated cells on different days.

Comimmunoprecipitation Experiments. Comimmunoprecipitation experiments were carried out with Protein G Immunoprecipitation Kit (Sigma-Aldrich) according to the manufacturer's instructions. In brief, the pellet of one cell culture flask (100 cm²) was washed with PBS and resuspended in 500 μl of ice-cold 1× immunoprecipitation (IP) buffer provided by the kit. A mixture of protease inhibitors (1 mg/ml Pefabloc, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml apronin) was added, and the suspension was lysed by ice on 30 min. The total cell lysate was centrifuged at 3000 rpm for 5 min at 1°C. The supernatant was transferred into a filter column (provided by the kit), and 5 μg of antibody (anti-BCRP (BXP-21), anti-caveolin-1 (C37120), or anti-caveolin-1 (sc-894)) were added. The samples were incubated at 4°C for 6 h on an inversion mixer; 30 μl of protein G agarose beads were then added to the cell lysate, and the samples were incubated overnight at 4°C on an inversion mixer. After incubation, samples were washed five times with ice-cold 1× IP buffer and once with 0.1× IP buffer. The pellet was resuspended in 40 μl of 1× sample buffer and prepared for Western blotting as described above. The communoprecipitation experiment was performed three times with untreated cells and depleted cells (5 mM solution of MβCD in HHBSS for 30 min at 37°C). As a negative control and for the exclusion of unspecific precipitations, the experiment was also conducted with the parental cell line MDCKII lacking human BCRP. Furthermore, control experiments without the addition of any antibody, experiments with nonspecific mouse IgG (purified mouse IgG1, immunoglobulin isotype control), experiments with protein G agarose beads alone, and experiments without cell lysis but the addition of anti-BCRP antibody or anti-caveolin-1 antibody, respectively, were performed. The latter experiment was carried out to exclude the possibility that the light chains of the BCRP antibody constitute a signal that appears at a molecular weight of approximately 20 to 25 kDa and thus seems to be a signal of caveolin-1 (molecular weight, 21 kDa).

Immunofluorescence Procedure. To investigate whether cholesterol depletion leads to an internalization of BCRP from the cell membrane to intracellular compartments, immunofluorescence experiments with untreated and depleted MDCKII-BCRP cells were performed. Cells were seeded in six-well plates on glass coverslips (diameter, 1 cm) coated with poly-D-lysine and collagen R. Confluent monolayers were washed once with PBS containing 0.1 mM CaCl₂ and 0.1 mM MgCl₂ [PBS(−)] to preserve tight junctions and then incubated for 30 min at 37°C with HHBSS (untreated cells) or HHBSS containing 5 mM MβCD (depleted cells). Coverslips were washed twice with PBS(+) and fixed with 4% PFA in PBS(+) for 15 min. PFA was quenched by incubating the cells with 0.2% bovine serum albumin in PBS(+) three times for 10 min. Cells were permeabilized with 0.1% Triton X-100 in PBS(+) for 3 min and washed again three times for 5 min with PBS(+). Permeabilization with Triton X-100 is required because the antibody used for BCRP staining (BXP-21) recognizes an intracellular domain of BCRP. After blocking for 30 min with 0.2% gelatin in PBS(+), the cell monolayer was incubated with 30 μl of primary antibody [1:10 dilution of BXP-21 in 0.2% gelatin/PBS (+)] or 30 μl of 0.2% gelatin/PBS (+) for negative control in a moisture box at room temperature. Coverslips were washed three times for 5 min with 0.2% gelatin/PBS(+) and then incubated with 30 μl of secondary antibody [1:100 dilution of Cy5-conjugated AffiniPure donkey anti-mouse IgG (H + L) in 0.2% gelatin/PBS (+)] in a moisture box. Coverslips were washed again three times with PBS(+), dipped once in deionized water to remove salts, and then embedded with aqueous mounting medium. BCRP staining was analyzed with a DM IRE 2 TCS SP II confocal laser-scanning microscope from Leica (Bensheim, Germany).

Statistical Analysis. Data were analyzed using GraphPad Prism version 4.0 and InStat version 3.05 (GraphPad Software Inc., San Diego, CA). Statistical analysis was performed with repeated measures analysis of variance and Bonferroni multiple comparison test to compare the protein and cholesterol distribution in the fractions of the density gradient after ultracentrifugation between MDCKII and MDCKII-BCRP, to compare the kinetics of cholesterol repletion in MDCKII and MDCKII-BCRP cells, and to compare the percentage of cells in the flow cytometry gates after cholesterol modulation experiments. Unpaired Student's t test was used for the comparison of cholesterol content in untreated cells and in cells after cholesterol depletion, repletion, and saturation as well as for the comparison of native cholesterol content in parental and the BCRP-overexpressing cell line. Mann-Whitney U test was employed to compare the inhibition ratios of untreated, depleted, repleted, and saturated cells. Results are displayed as mean ± S.E.M. A p value of ≤0.05 was considered significant.
Results

Characterization of Density Gradient Ultracentrifugation Fractions. Western blot analyses of density gradient ultracentrifugation fractions in the parental cell line MDCKII (A) and the BCRP-overexpressing cell line MDCKII-BCRP (B) are displayed in Fig. 1 (one representative experiment out of four). The DRM fraction was found at the interface between 30% sucrose and TNE buffer as a cloudy band. BCRP was predominantly located in the DRM (raft) fractions (fractions 8, 9, and 10) colocalized with caveolin-1. Only small amounts of BCRP were found in nonraft fractions (Fig. 1B). In the parental cell line, the localization of caveolin-1 was similar to that in MDCKII-BCRP, but no BCRP was detected in MDCKII cells. TR, a nonraft protein, was predominantly found in the nonraft fractions 1 through 4 (bottom of the ultracentrifugation tube; Fig. 1C).

The relative protein distribution (in percentage of total protein amount of the tube) in the 12 fractions of the density gradient after ultracentrifugation is shown in Fig. 2. The protein distribution in the parental cell line MDCKII was not different from that in the BCRP-overexpressing cell line MDCKII-BCRP ($p > 0.05$ for the comparison of protein content in each fraction of MDCKII versus the corresponding fraction of MDCKII-BCRP). This indicates that both cell lines are comparable in regard to the DRM analysis. The largest amount of protein was found in fractions 1 through 4 (bottom of the tube), and only small amounts were present in the DRM fractions 8 through 10.

Figure 3 displays the relative cholesterol distribution (in percentage of total cholesterol amount of the tube) in the 12 ultracentrifugation fractions. There was no difference between the relative cholesterol amounts in the fractions of MDCKII and MDCKII-BCRP ($p > 0.05$ for the comparison of a particular fraction of MDCKII with the corresponding fraction of MDCKII-BCRP). High amounts of cholesterol were found in fractions 1 through 5 and DRM fractions 9 through 11, indicating that cellular cholesterol partly floated to the top of the tube.

Activity of BCRP in MDCKII and MDCKII-BCRP Cells after Cholesterol Depletion, Repletion, and Saturation. The kinetics of cholesterol repletion are displayed in Fig. 4A. Maximum cholesterol levels were reached after 20 min in both cell lines. Baseline cholesterol levels in the range of those in untreated cells were achieved in less than 1 min. There were no significant differences between the relative cholesterol content in MDCKII and MDCKII-BCRP ($p > 0.05$). Results of cholesterol quantification in untreated, depleted, repleted ($t = 0.5, 1,$ and $30$ min), and saturated cells are presented in Fig. 4B (MDCKII) and 4C (MDCKII-BCRP) ($n = 3–8$). The cholesterol level did not differ significantly between untreated parental and BCRP-overexpressing cells ($p > 0.05$). Cholesterol depletion led to an approximate 50% reduction of cholesterol content from $10.2 \pm 1.0$ fmol/cell to $4.9 \pm 1.0$ fmol/cell in MDCKII and from $10.6 \pm 1.2$ fmol/cell to $5.4 \pm 1.2$ fmol/cell in MDCKII-BCRP.
to 5.4 ± 1.2 fmol/cell in MDCK-II-BCRP (p = 0.004 for MDCKII and p = 0.015 for MDCK-II-BCRP). Short-term cholesterol repletion restored baseline cholesterol levels [MDCKII, 9.7 ± 1.3 fmol/cell (0.5 min; p > 0.05 versus untreated cells and p = 0.024 in comparison to depleted cells) and 10.3 ± 1.2 fmol/cell (1 min; p > 0.05 versus untreated cells and p = 0.013 versus depleted cells); MDCK-II-BCRP, 10.6 ± 0.9 fmol/cell (0.5 min; p > 0.05 versus untreated cells and p = 0.024 versus depleted cells) and 11.0 ± 0.6 fmol/cell (1 min; p > 0.05 versus untreated cells and p = 0.015 versus depleted cells)], whereas long-term cholesterol repletion (30 min) and cholesterol saturation caused an increase in cholesterol content by a factor of 3 to 4 in relation to untreated cells: MDCK-II-repleted (30 min), 29.1 ± 4.3 fmol/cell; MDCKII-saturated, 40.1 ± 7.2 fmol/cell; MDCK-II-BCRP-repleted (30 min), 42.8 ± 9.2 fmol/cell; and MDCK-II-BCRP-saturated, 48.5 ± 9.2 fmol/cell (p < 0.001 for MDCKII, p < 0.01 for repleted MDCK-II-BCRP, and p < 0.001 for saturated MDCK-II-BCRP).

Figure 4D summarizes the effect of cholesterol depletion, short-term repletion (0.5 and 1 min), long-term repletion (30 min), and saturation on the activity of BCRP. Cholesterol depletion provoked a 40% decrease in PhA transport expressed as inhibition ratio (p < 0.01), and both short- and long-term cholesterol repletion as well as cholesterol saturation restored BCRP function (p > 0.05 for the comparison of BCRP activity in repleted or saturated cells with the baseline activity in untreated cells). The appearance of the dot plots, the gates, and the percentage of cells in the gates in the forward versus side scatter were unchanged (p > 0.05) before and after cholesterol modulation experiments in both cell lines, suggesting that the viability of the cells was not significantly influenced by the experimental procedure (Fig. 5).

There was no shift in MF by the addition of FTC in the parental cell line.

**Coimmunoprecipitation Experiments.** The Western blot analysis of one representative coimmunoprecipitation experiment in untreated MDCKII-BCRP and MDCKII cells is shown in Fig. 6A. A strong signal at 72 kDa (BCRP) and at 21 kDa (caveolin-1) was visible in MDCKII-BCRP cells, indicating that caveolin-1 was coimmunoprecipitated by the anti-BCRP antibody (BXP-21). In the parental cell line MDCKII, which served as a negative control, no BCRP was detected, and there was only a small signal at 21 kDa (caveolin-1). In contrast, coimmunoprecipitation experiments in depleted cells (Fig. 6B) showed only a faint caveolin-1 signal (21 kDa) in MDCKII-BCRP. In the parental cell line MDCKII, a small signal at 21 kDa was also present. Control experiments without antibody, with nonspecific mouse IgG, and with protein G agarose beads with or without cell lysates did not show any signal in Western blot analysis. However, the immunoprecipitation experiment with anti-caveolin-1 antibody (C37120) only caused a caveolin-1 signal (Fig. 6C). We therefore repeated the coimmunoprecipitation experiment with a different antibody against caveolin-1 (sc-894) but also failed to detect BCRP (Fig. 6D).

**Immunofluorescence in Untreated and Depleted Cells.** Immunofluorescence of untreated and depleted MDCKII-BCRP cells demonstrated that after cholesterol depletion there was no evidence for changes in localization of BCRP (Supplemental Data Fig. 1).

**Discussion**

MDR is a major problem in cancer chemotherapy. It is characterized by the overexpression of ABC transporters...
such as P-gp and BCRP that mediate export of cytostatic drugs. Several studies revealed changes in membrane protein and lipid composition in the multidrug-resistant phenotype. Beyond increased expression of ABC transporters, increased amounts of caveolin-1, larger numbers of caveolae (Lavie et al., 1998), and elevated membrane cholesterol content (Gayet et al., 2005) were found in multidrug-resistant cells. These changes in protein and lipid amounts in multidrug-resistant cells are associated with functional changes in proteins. Indeed, for P-gp it has been reported in various studies that this transporter at least partly localizes to lipid rafts and that the membrane lipid composition and particularly the membrane cholesterol content is essential for the function of P-gp (Troost et al., 2004a,b). So far, the location of BCRP within the membrane was not specified, and it is only known that cholesterol content may affect BCRP ATPase assay activity in isolated insect cell membranes transfected with BCRP (Pál et al., 2007). However, ATPase activity does
not necessarily coincide with efflux activity as shown for P-gp (Schwab et al., 2003). The results of our study now demonstrate that BCRP is mainly located in DRMs and that cholesterol may substantially alter transport activity of BCRP.

MβCD treatment induced a 50% reduction of cellular cholesterol content along with a significant decrease (40%) in BCRP activity, whereas the viability of the cells remained unaltered. Immunofluorescence data indicate that cholesterol depletion does not substantially alter localization of BCRP within the cell. Changes in BCRP activity after cholesterol modification are therefore most likely attributed to alterations in membrane composition and cholesterol content. The mechanism by which cholesterol influences BCRP function is not clear, and several types of interactions are conceivable. 1) Cholesterol is an essential component of lipid rafts/caveolae and contributes to the formation of these microdomains, which might be required for optimal BCRP activity (e.g., via the interaction of BCRP with caveolin-1 or by enabling the dimerization of the monomers). 2) Cholesterol may modulate the function of BCRP by interacting with the transmembrane regions of the transporter and modulating the nucleotide-binding domains as suggested for P-gp (Romsidek and Sharom, 1999). 3) Cholesterol might also influence the binding process of the often-lipophilic BCRP substrates as shown for P-gp (Romsicki and Sharom, 1999).

Interestingly, cholesterol content was reconstituted already within 1 min of repletion with longer incubation times, leading to an excess of cholesterol. Both short-term (0.5 and 1 min) and long-term cholesterol repletion (30 min) with MβCD/cholesterol-inclusion complexes restored BCRP baseline activity. In contrast to the corresponding results for P-gp in human peripheral blood mononuclear cells (Troost et al., 2004a) and the P-gp-overexpressing cell line L-MDR1 (Troost et al., 2004b), cholesterol saturation did not increase BCRP activity beyond baseline values of native cells. A possible explanation might be that BCRP in MDCKII cells is mainly located in rafts/caveolae, which is in contrast to P-gp, where a significant portion is located outside of those domains (Lavie et al., 1998; Ghetie et al., 2004; Troost et al., 2004b).

Indeed, our density gradient ultracentrifugation analysis provided clear evidence for the predominant localization of BCRP in lipid rafts. The absence of the nonraft marker protein TR in these fractions substantiates the contention that the presence of BCRP in DRM fractions is not caused by unspecific floatation.

Furthermore, the results of the communoprecipitation experiments suggest a physical interaction between BCRP and caveolin-1. Caveolins are integral membrane proteins and important structural components of caveolae (Anderson, 1998). By homo-oligomerization and cholesterol-binding, caveolins assume the characteristic appearance and morphology of caveolae (Rietveld and Simons, 1998). Moreover, they can interact with other proteins and form a scaffold that promotes the assembly of many different classes of signaling molecules to generate preassembled signaling complexes (Okamoto et al., 1998). The caveolin gene family comprises three members: caveolin-1, -2, and -3. Caveolin-1 and -2 are expressed in miscellaneous cell types and most abundantly in adipocytes, endothelial cells, and fibroblastic cell types, whereas caveolin-3 is a muscle-specific protein (Okamoto et al., 1998; Razani et al., 2002). In this study, caveolin-1 was communoprecipitated by the anti-BCRP antibody BXP-21 in the overexpressing cell line MDCKII-BCRP, supporting a molecular interaction between the two proteins. The origin of the only faint signal of caveolin-1 in the parental control cell line MDCKII is unclear. However, the caveolin-1 signal in the BCRP-overexpressing cell line MDCKII-BCRP was much stronger; therefore, this signal is most likely not related to unspecific precipitation. Furthermore, the absence of unspecific precipitation was assessed by a series of control experiments that all lacked any signal in Western blot analysis. Experiments without cell lysates confirmed that the 21-kDa band indeed represents caveolin-1 and is not related to the presence of the light antibody chains of BXP-21 that would also appear at 20 to 25 kDa. In the cholesterol-depleted state, both cell lines had only small signals of caveolin-1, indicating that cholesterol withdrawal disrupts the physical interaction between BCRP and caveolin-1 in MDCKII-BCRP cells. These findings therefore emphasize the importance of an intact lipid raft structure for the molecular interaction of the two proteins and BCRP function.

Studies in G protein α-subunits demonstrated that these subunits interact with caveolin-1 and that they have consensus amino acid sequences, the so-called “caveolin-binding motifs”: ϕXϕXXXϕ and ϕXXXϕXϕ, where ϕ is an aromatic residue (Trp, Phe, or Tyr) and X is any amino acid residue (Couet et al., 1997). These caveolin-binding motifs can interact with caveolin-1 by serving as a ligand for the caveolin-scaffolding domain. The amino acid sequence of BCRP contains a region with a “reverse caveolin-binding domain” [amino acid residues 571–579 (PSIPRYGF)]. Thus, an interaction between caveolin-1 and BCRP might take place via this domain. Transport activity of BCRP requires the assembly as homodimer (Kage et al., 2002; Litman et al., 2002) or even homotetramer (Xu et al., 2004). Caveolin-1 might facilitate the formation of these dimers or tetramers by binding BCRP and thus requiring proximity between two or more BCRP molecules.

However, in Western blot analysis of independent communoprecipitation experiments with two different anti-caveolin-1 antibodies (C37120 and sc-884), we failed to detect a signal at 72 kDa, indicating that BCRP could not be coprecipitated by the anti-caveolin-1 antibody. There are three possible explanations for this observation. 1) The BCRP expression level in the MDCKII-BCRP cells is comparatively low (Dr. A. H. Schinkel, personal communication), with mRNA expression 10 times lower than caveolin-1 expression \(p < 0.0001\) (unpaired Student's \(t\) test), as measured by real-time reverse transcriptase polymerase chain reaction (J. Weiss and C. H. Storch, unpublished data). Therefore, the number of BCRP molecules that interact and thus coprecipitate with caveolin-1 might be too low to be detected in Western blot. The overall amount of BCRP molecules that are precipitated by an anti-BCRP antibody might be much higher, because presumably only a fraction of all BCRP molecules interact with caveolin-1. 2) The antibodies against caveolin-1 might recognize a sequence in the caveolin-1 molecule where the interaction with BCRP takes place (possibly caveolin-scaffolding domain), thus leading to a disruption of this interaction. 3) The size of an IgG antibody (approximately 140 kDa) is much larger than that of caveolin-1 (21 kDa). Therefore, the linkage of the antibody to caveolin-1...
could sterically interfere with the caveolin-1-BCRP interaction and thus prevent coimmunoprecipitation of BCRP. In conclusion, our in vitro results indicate that BCRP is located in rafts/caveolae and that the lipid constitution and in particular membrane cholesterol has an impact on the efflux activity of BCRP in the canine kidney epithelial cell line MDCKII. Furthermore, our study provides in vitro evidence for a physical and possibly functional interaction between BCRP and caveolin-1, which might be of importance for an optimal operation of BCRP. These findings could be relevant with regard to the known up-regulation of ABC transporters and the elevated cholesterol and caveolin-1 levels observed in the multidrug-resistant phenotype.

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