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The Product of the ABC Half-Transporter Gene *ABCG2* (BCRP/MXR/ABCP) Is Expressed in the Plasma Membrane

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The products of the ABC gene family can be generally classified as either full-transporters of half-transporters. Full-transporters are expressed in the plasma membrane, whereas half-transporters are usually found in intracellular membranes. Recently, an ABC half-transporter, the *ABCG2* gene product Breast Cancer/Mitoxantrone Resistance Protein (BCRP/MXR), has been shown to cause mitoxantrone and topotecan resistance. The purpose of this study was to determine the expression and the intracellular localization of this protein in various drug-resistant cell lines. BCRP/MXR expression was determined by Western blot and immunohistochemistry. This protein is highly overexpressed in several drug-resistant cell lines and localizes predominantly to the plasma membrane, instead of to intracellular membranes as seen with all other known half-transporters. Therefore, BCRP/MXR is unique among the ABC half-transporters by being localized to the plasma membrane.

Since the original discovery of P-glycoprotein in a multidrug-resistant tissue culture cell line (1), an increasing number of ABC transporters has been shown to be causally associated with multidrug resistance in cancer cells (2–4). The most recent addition is the Breast Cancer Resistance Protein (BCRP) (5), which is also called mitoxantrone resistance protein (MXR) (6), or placenta ABC protein (ABC-P) (7), and is the second member of the ABCG subfamily of ABC transport proteins (gene symbol *ABCG*2). Overexpression of BCRP/MXR was shown to confer resistance to mitoxantrone, doxorubicin and daunomycin (5, 8), and various camptothecin analogs including topotecan (9, 10) by reducing the intracellular drug accumulation through active drug export. Other known multidrug resistance proteins were shown to be localized to the plasma membrane (11–13) in agreement with their function as a drug export pump. In contrast, the intracellular localization of BCRP/MXR has not yet been demonstrated. In this report, we therefore analyzed BCRP/MXR protein expression and determined its intracellular localization by immunohistochemistry in several drug resistant cell lines. The results demonstrated that the BCRP/MXR protein is predominantly expressed in the plasma membrane.

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MATERIALS AND METHODS

Cell culture and cytotoxicity assays. The parental human breast carcinoma MCF7/WT cell line, its drug resistant variants MCF7/MX (14, 15), MCF7/AdVp (subclone AdVp1000) (16, 17), MCF7/TPT300 (18) and transfected MCF7/pcDNA3 and MCF7/BCRP/MXR (5), as well as S1 and S1-M1-3.2 colon carcinoma cells (19), were grown in IMEM medium (Richter's modification) supplemented with 10% fetal bovine serum and 0.01 mg/mL ciprofloxacin at 37°C in 5% CO_2 in a humidified incubator. Cytotoxicity assays were performed as described previously (20).

Western blot analysis. Cells were seeded at a density of 1 million cells/10 cm dish. After 72 h or at 70% confluence, cells were washed twice, scraped into PBS, sonicated (3 × 10 s at maximum power with a microtip) and then centrifuged for 10 min at 1000*g* at 4°C. The supernatant was diluted 1:4 into 10 mM Tris–HCl, pH 7.5, 250 mM sucrose, and the membranes were sedimented by centrifugation at 100,000*g* for 30 min. The pellet was resuspended in 62.5 mM Tris–HCl, pH 6.8, 1% Chaps, sonicated, and solubilized for 30 min on ice. An equal volume of 2× sample buffer (0.1 M Tris–HCl, pH 6.8, 0.4 M DTT, 4% SDS) was added. The protein concentration was measured by Bradford assay (21). Samples were dispensed into aliquots and stored at −80°C. Fifty micrograms of cell membrane proteins were

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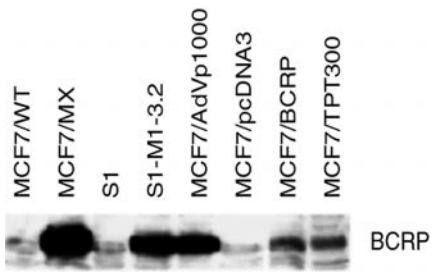

FIG. 1. Western blot for BCRP/MXR: Membrane proteins (50 μg/lane) were size fractionated by polyacrylamide gel electrophoresis, transferred to a PVDF membrane, and probed with BCRP/MXR antibody. Detection of bound antibody was by ECL.

fractionated on 10% polyacrylamide gels with a 4% stacking gel containing 8 M urea. Multimark (Novex, San Diego, CA) and/or broad range markers (Bio-Rad, Hercules, CA) were used as molecular weight standards. Proteins were transferred to a PVDF membrane (Millipore, Bedford, MA) using wet electrophoretic transfer. The membrane was washed with TBST (20 mM Tris–HCl, pH 7.5, 0.137 M NaCl, 0.01% Tween) and the nonspecific binding sites were blocked with Blotto (5% milk in TBST) overnight. The membrane was then incubated with a 1:2000 dilution of a polyclonal anti-MXR (BCRP) antibody for 2 h, followed by anti-rabbit secondary antibody for 1 h. Detection was by enhanced chemiluminescence using Super-Signal Substrate (Pierce, Rockford, IL).

Immunostaining. To determine the subcellular localization of BCRP/MXR, cells were plated at a density of 50,000 cells/35 mm dish on coverslips, grown for 48 h to 72 h to 70% confluence, then rinsed in PBS buffer followed by fixation for 5 min in PBS containing 3.8% paraformaldehyde. The fixed cells were permeabilized by incubation for 5 min in cold methanol (−18°C). Coverslips were incubated for 45 min at 37°C with the polyclonal anti-MXR antibody (dilution 1:800). Unbound antibodies were removed by rinsing in PBS, followed by incubation for 45 min at 37°C with FITC-conjugated anti-rabbit antibody as secondary antibody (Sigma Chemical Co., St. Louis, MO). The DNA was stained using Hoechst 33342. Fluorescence images were collected using a wide-field fluorescence microscope. To analyze different focal planes, selected fluorescence images were collected as z-series in 200 nm steps on the same microscope workstation used for the imaging of the immunostaining. These data sets were then deconvolved using Delta Vision version 2.1 deconvolution software (Applied Precision Inc., Issaquah, WA) and presented as maximal intensity projections as previously described (22).

RESULTS

BCRP/MXR Expression and Drug Resistance

Breast cancer/mitoxantrone resistance protein expression was analyzed by immunoblot in various drug selected cell lines and their corresponding parental cells (Fig. 1). A large amount of BCRP/MXR protein was detected in the three cell lines MCF7/MX, MCF7/AdVp and S1-M1-3.2 which all are highly resistant to mitoxantrone and topotecan (Table I). In contrast, substantially less BCRP/MXR was detected in the MCF7/TPT300 and the transfected MCF7/BCRP cells, which were less than 5% as resistant to the two drugs tested. Of particular interest is the very low level of resistance to topotecan in MCF7/BCRP cells, despite protein levels similar to those in the MCF7/TPT300 cells. Though various reports indicated that BCRP/MXR overexpression was associated with topotecan resistance (9, 10, 23), these data suggest that possibly other factors are involved in cells that were drug selected as opposed to cells transfected with BCRP/MXR. Little, if any protein was detected in the respective parental cells.

Intracellular BCRP/MXR Localization

Several lines of evidence suggested that BCRP/MXR acts as an efflux pump reducing the intracellular drug concentration (5). Therefore, it was of interest to determine if this protein was localized in the plasma membrane, in agreement with its function as a drug efflux pump, or if it was localized in intracellular membranes, as expected from it being an ABC half-transporter (24). As shown in Fig. 2, BCRP/MXR is clearly and almost exclusively detected in the plasma membrane of MCF7/MX cells (panel B), whereas the localization in both the MCF7/AdVp (panel C) and S1-M1-3.2 (panel H) cells is less clear. Despite a clear signal by Western blot, BCRP/MXR was essentially undetectable with this method in the MCF7/TPT300 (panel D) and MCF7/BCRP (panel F) cells. Also, no evidence for BCRP/MXR expression was seen in the respective parental cells (panels A, E, and G). Since it was possible that the absence of detectable protein in some of the cells was because BCRP/MXR was only localized to certain areas of the plasma membrane that were outside of the focal plane of the pictures in panels A-H, some of the cells were also scanned in the z-axis and a series of pictures acquired that was then deconvolved by computer and presented as maximal intensity projections (Figs. 2I–2L). These images clearly show that BCRP/MXR is localized to the plasma membrane in all four cell lines analyzed in this way (MCF7/BCRP, MCF7/AdVp,

TABLE I

Relative Resistances in BCRP/MXR-Expressing Cell Lines

	IC_{50} (Fold Resistance)	
Cell Line	Mitoxantrone (nM)	Topotecan (nM)
MCF7/WT	0.42 ± 0.09 (1)	4.6 ± 1.0 (1)
MCF7/MX	796 ± 161 (1875)	6751 ± 925 (1462)
MCF7/AdVp1000	2300 ± 112 (5443)	3867 ± 763 (837)
MCF7/TPT300	26.0 ± 5.1 (61)	210 ± 54 (45)
MCF7/pcDNA3.1	2.79 ± 0.58 (1)	16.6 ± 4.6 (1.0)
MCF7/BCRP	53.6 ± 10.6 (19.2)	37.1 ± 8.5 (2.2) $p > 0.05$
S1	0.48 ± 0.15 (1)	N.D.
S1-M1-3.2	1880 ± 247 (3900)	N.D.

Note. IC_{50} values were determined using a 7-day sulforhodamine B cytotoxicity assay. Values in parentheses represent the fold resistance relative to respective parental cells. All data are the means from at least three independent experiments, except for topotecan in MCF7/AdVp1000 cells where only two experiments were performed. N.D., not done, but S1-M1-80 cells, a derivative of S1-M1-3.2, were shown to be 640-fold resistant to topotecan (10).

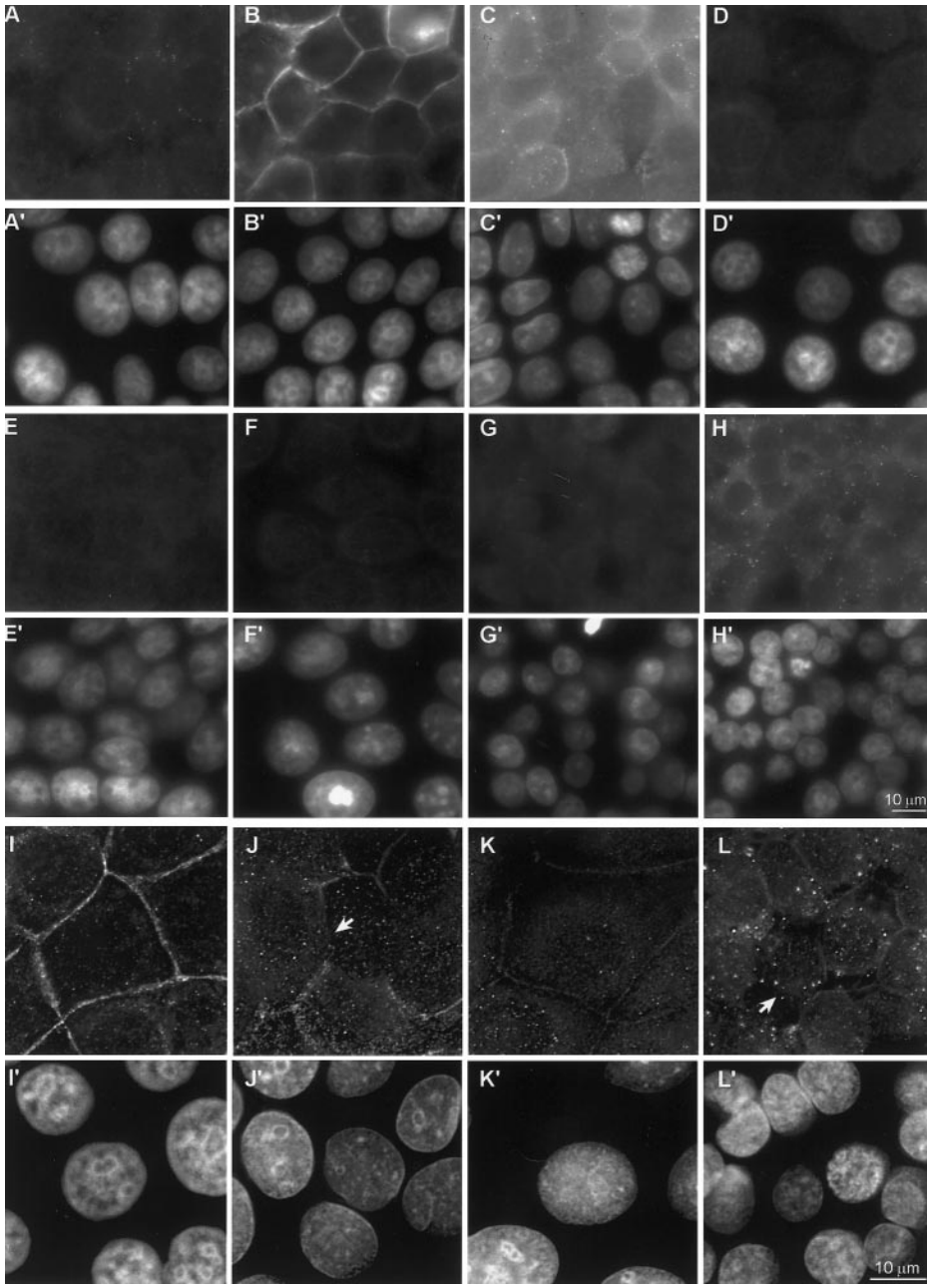

FIG. 2. Subcellular localization of BCRP/MXR: BCRP/MXR was visualized by fluorescence microscopy (A–H) with the polyclonal antibody anti-MXR (1:800), followed by FITC-conjugated anti-rabbit-secondary antibody. The DNA was stained using Hoechst 33342 (A′–L′). In I–L, multiple pictures were collected as z-series in 200 nm steps, then the data were deconvolved using Delta Vision version 2.1 deconvolution software (Applied Precision Inc., Issaquah, WA) and presented as maximal intensity projections as described under Materials and Methods. A, A′: MCF7/WT; B, B′, I, I′: MCF7/MX; C, C′, J, J′: MCF7/AdVp; D, D′: MCF7/TPT300; E, E′: MCF7/pcDNA3 (control transfectant); F, F′, K, K′: MCF7/BCRP (BCRP transfected); G, G′: S1; H, H′, L L′: S1-M1-3.2.

MCF7/BCRP, and S1-M1-3.2, panels I–L, respectively). However, the membrane staining seemed discontinuous (see e.g., arrows in panels J and L) in some of the cells, suggesting that BCRP/MXR protein distribution may not be uniform around the whole cells. Together, these results indicate that BCRP/MXR is predominantly expressed in the plasma membrane of cells, though the possibility can not be excluded that a minor fraction is also present in intracellular membranes.

DISCUSSION

Multidrug resistance in cancer cells has generally been associated with overexpression of transport proteins in

the plasma membrane of the resistant cells. Examples include the *MDR1* gene product P-glycoprotein and the multidrug resistance-associated protein MRP1 (11–13). Both of these proteins belong to the family of ATP-binding cassette (ABC) transporters that is found in all living species. In humans, so far over 40 members have been identified and grouped into eight subfamilies, and many more are predicted (24). ABC transport proteins can be classified as either full- or half-transporters. Full transporters contain two sets each of a trans-membrane and an ATP binding region, whereas half-transporters only consist of one domain each. Generally, full-transporters are found in the plasma membrane, whereas half-transporters are usually found in intracellular membranes such as the mitochondria (M-ABC1 and ABC7), the endoplasmatic reticulum (TAP1, 2) and the peroxisomes (ALD subfamily) (reviewed in 24, 25). Recently, a new half-transporter has been isolated and described and variably named breast cancer resistance protein (BCRP) (5, 8), mitoxantrone resistance protein (MXR1) (6), or placenta ABC protein (ABC-P) (7). Overexpression upon stable transfection of this gene conferred mitoxantrone and anthracycline resistance and reduced intracellular drug accumulation (5). Furthermore, Northern blot analysis demonstrated that BCRP/MXR was overexpressed in a variety of drug-selected and resistant cell lines (8). However, no protein data have so far been reported.

In the present report we for the first time present evidence of BCRP/MXR overexpression at the protein level in several drug-resistant cell lines. Very high levels of BCRP/MXR protein were observed in the three cell lines with high (>1000-fold) resistance. In contrast, substantially lower levels of BCRP/MXR (estimated at <10%) were seen in cells with comparatively moderate levels of resistance (<100-fold). Thus, even though not absolute, there appeared to be a positive correlation between BCRP/MXR protein levels and drug resistance. We also show the intracellular localization of the Breast Cancer Resistance Protein (BCRP/MXR) in the various drug resistant tissue culture cell lines. The data clearly show that BCRP/MXR is predominantly localized to the plasma membrane. This is somewhat surprising in light of the generally intracellular localization of the other known half-transporters (24), but commensurate with BCRP/MXR's function as a drug exporter. Though the exact mechanisms for targeting of full- and half-transporters to their respective membrane localization are not known, it may be noteworthy that BCRP/MXR has a domain arrangement in which the ABC-fold precedes the transmembrane region, an arrangement it shares with only the human white homologue ABC8. In contrast, all other human ABC proteins containing both domains have an arrangement in which the ABC binding fold is carboxy-terminal to the transmembrane region. Models for the *Drosophila* white protein predict it to be localized in the plasma membrane of the eye pigment cells (26). Recent evidence, however, suggests that the human ABC8 protein is expressed both on the cell surface and in intracellular compartments (27). Thus, it appears that BCRP/MXR is the first ABC half-transporter with a predominantly plasma membrane localization.

In summary, we have shown that the BCRP/MXR protein is highly expressed in various drug-resistant cell lines, and that it appears predominantly localized to the plasma membrane.

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