Sterol Transport by the Human Breast Cancer Resistance Protein (ABCG2) Expressed in Lactococcus lactis*

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The human breast cancer resistance protein (BCRP, also known as ABCG2, MXR, or ABCP) is one of the more recently discovered ATP-binding cassette (ABC) transporters that confer resistance on cancer cells by mediating multidrug efflux. In the present study, we have obtained functional expression of human BCRP in the Gram-positive bacterium Lactococcus lactis. BCRP expression conferred multidrug resistance on the lactococcal cells, which was based on ATP-dependent drug extrusion. BCRP-mediated ATPase and drug transport activities were inhibited by the BCRP-specific modulator fumitremorgin C. To our knowledge these data represent the first example of the functional expression of a mammalian ABC half-transporter in bacteria. Although members of the ABCG subfamily (such as ABCG1 and ABCG5/8) have been implicated in the transport of steroids, such a role has not yet been established for BCRP. Interestingly, the BCRP-associated ATPase activity in L. lactis was significantly stimulated by (i) steroids including cholesterol and estradiol, (ii) natural steroids such as progesterone and testosterone, and (iii) the antiestrogen anticancer drug tamoxifen. In addition, BCRP mediated the efflux of [3H]estradiol from lactococcal cells. Our findings suggest that BCRP may play a role in the transport of sterols in human, in addition to its ability to transport multiple drugs and toxins.

The emergence of multidrug resistant cancer cells is a serious problem in the chemotherapeutic treatment of human tumors. In mammals, multidrug resistance based on the active extrusion of cytotoxic drugs from the cell is mediated by several members of the ATP-binding cassette (ABC) superfamily. These include the multidrug resistance P-glycoprotein MDR1 (also termed ABCB1) and the protein MRP1 (multidrug resistance-associated protein 1, also termed ABCC1/1). The breast cancer resistance protein (BCRP, also termed MXR, ABCP, or ABCG2) is one of the more recently discovered ABC multidrug transporters in human cancer cells. BCRP confers resistance on cells to (i) toxic ions such as rhodamine 123, (ii) anticancer agents including mitoxantrone and the anthracyclines daunorubicin and doxorubicin, and (iii) the camptothecins topotecan and SN-38 (2–7). Overexpression of BCRP has been observed in several human cancer cell lines selected for drug resistance (2, 5, 8) as well as in tumor samples of cancer patients (9–11). Recently, fumitremorgin C (FTC), a novel chemosensitizing agent, was identified and shown to reverse drug resistance in human BCRP-expressing cancer cells by inhibiting BCRP-mediated drug transport (12).

BCRP is a 655-amino acid, 72.1-kDa protein and is the second member of the G subfamily of ABC transporters. Members of the G subfamily are all half-transporters and include among others (i) the Drosophila white, brown, and scarlet proteins, which are involved in the transport of eye pigment (13); (ii) ABCG1, which is thought to be involved in the transport of cholesterol and phospholipids (14); and (iii) heterodimeric ABCG5/ABCG8, which has been implicated in the transport of cholesterol and plant sterols (15). In contrast to P-glycoprotein MDR1 and MRP1, which are full size transporters, BCRP most likely functions as a homodimer (16).

In normal tissue, high expression of the BCRP is found in stem cells (17), epithelial cells of small and large intestines, ducts and lobules of the breast, endothelial cells of veins and capillaries (18), and synchtiotrophoblastic cells of the placenta (19). The localization of BCRP suggests that it could have a potential role in protection against toxins. The recent observation in BCRP knock-out mice that BCRP protects against a chlorophyll-derived dietary phototoxin and protoporphyrin is consistent with this notion (20).

Previously, we have characterized the molecular basis of the drug specificity of LmrA, a half-transporter homologue of human P-glycoprotein MDR1, in the Gram-positive bacterium Lactococcus lactis (21, 22). To allow a detailed comparison of BCRP and LmrA, human BCRP was functionally expressed in L. lactis using the nisin A-induced expression system that is used for the expression of LmrA. BCRP was active as an ATP-dependent multidrug transporter in L. lactis and was able to interact with sterols. We conclude that the substrate specificity of BCRP partly overlaps with that proposed for ABCG1 and ABCG5/ABCG8. Our observations may suggest a physiological role for BCRP in sterol metabolism in human in addition to its role in mediating resistance to xenobiotics and toxins arising from dietary intake and cellular metabolism.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

L. lactis strains NZ9000 and NZ9700 (23) were grown at 30 °C in M17 medium (Difco) supplemented with 0.5% glucose. Chloramphenicol (5 μg/ml) was added where appropriate.

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¶ The abbreviations used are: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; FTC, fumitremorgin C; IC50, drug concentration required for half-maximal inhibition of the cellular growth rate; MDR, multidrug resistance; AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate.
The cells were washed three times in 50 mM KPi (pH 7.0) containing 5 mM MgSO4. To deprive cells of metabolic energy, the cell suspensions were preincubated at 30 °C for 30 min and washed three times in 50 mM KPi or Tris buffer supplemented with 1 mg bovine serum albumin/ml to an A660 of 0.5, and kept on ice until use. Cell suspensions (100 μl aliquots) were preincubated at 30 °C for 30 min in KPi buffer supplemented with 1 mM orthovanadate were obtained in parallel and subtracted from the readings.

**RESULTS**

**Expression of Human BCRP in L. lactis** — For expression of human BCRP in *L. lactis* NZ9000, the human BCRP gene present in the mammalian expression vector pcDNA3-BCRP was cloned into the lacticoccal expression vector pNZ8048 under the control of the nisin A-inducible nisA promoter. This expression system had previously been used for the expression in *L. lactis* of LmrA, the lacticoccal homologue of the human multidrug resistance P-glycoprotein MDR1 (24). The addition of 40 pg/ml nisin A to exponentially growing *L. lactis* cells harboring pNZ-BCRP resulted in the expression of a 70-kDa polypeptide that could be detected on an immunoblot by using the specific anti-BCRP antibody BXP-21 (Fig. 1). The BXP-21 antibody also showed cross-reactivity with certain constitutively expressed, endogenous membrane proteins in *L. lactis*. The 70-kDa polypeptide was expressed at a level between 0.5 and 1% of the total radioactivity.

**ATPase Assays**
The ATPase assay in inside-out membrane vesicles of *L. lactis* was based on a colorimetric ascorbic acid/ammonium molybdate assay to measure the liberation of P from ATP. Inside-out membrane vesicles prepared from BCRP-expressing and control *L. lactis* cells were diluted to a protein concentration of ~1 mg/ml in a buffer containing 20 mM K-HEPES (pH 7.0), 5 mM MgSO4, and 5 mM ATP. ATPase assays were performed at 30 °C in a 96-well plate in a reaction volume of 10 μl/well. Lipids and water-insoluble drugs were added as solutions in ethanol to a final solvent concentration below 1% (v/v). Following incubation for 10 min, the ATPase reactions were terminated by the addition of 40 μl of a freshly prepared acidic solution consisting of 0.48% (w/w) ammonium heptamolybdate tetrahydrate, 6.6% (w/v) concentrated sulfuric acid, 0.01% (w/v) potassium antimonyl tartrate, and 0.42% (w/w) ascorbic acid. Following the addition of 150 μl of H2O and 30 min of incubation at 30 °C in the dark, the absorbance of the phosphomolybdate complex formed was measured at 690 nm. ATPase activity measurements in the presence of 1 mM orthovdanate were obtained in parallel and subtracted from the readings.

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and 1% of total membrane protein, as determined by densitometric analysis of a Coomassie Brilliant Blue-stained SDS-PAGE gel. In the absence of nisin A, an approximately 10-fold lower expression level of the 70-kDa polypeptide was observed in \textit{L. lactis} cells containing pNZ-BCRP. The 70-kDa polypeptide was undetectable in control cells harboring the pNZ8048 control vector when incubated in the presence of nisin A.

\textbf{Heterologously Expressed BCRP Is Active as a Multidrug Transporter—}The drug resistance of \textit{L. lactis} cells harboring pNZ-BCRP was compared with that of cells harboring the pNZ8048 control vector. The growth rate of the two strains in liquid culture containing 40 \(\mu\)g/ml nisin A was determined at increasing concentrations of ethidium bromide, Hoechst 33342, rhodamine 123, or tetramethylrhodamine. The concentrations of drugs necessary to reduce the growth rate of cells by 50\% (IC\(_{50}\)) are listed in Table I. BCRP expression in \textit{L. lactis} significantly increased the drug resistance of the organism.

To test whether drug extrusion from the cell is the underlying mechanism of drug resistance in \textit{L. lactis} expressing BCRP, ethidium bromide uptake in cells was measured by monitoring the fluorescence of the intracellular ethidium-poly nucleotide complex. In the presence of glucose, the uptake of ethidium bromide in cells expressing BCRP was significantly lower than that observed in control cells without BCRP (Fig. 2). This difference in ethidium accumulation between the two cell types reflected a higher ethidium efflux rate in BCRP-expressing cells. Upon the addition of the BCRP-specific inhibitor FTC, the ethidium accumulation in cells expressing BCRP was similar to that observed in control cells in the presence or absence of FTC, pointing to the inhibition of BCRP activity under these conditions.

The fluorescent lipophilic dye Hoechst 33342 is transported by BCRP expressed in mammalian cells (19, 25). To further analyze the activity of BCRP in \textit{L. lactis}, the transport of Hoechst 33342 was studied in \textit{L. lactis}-derived inside-out membrane vesicles in which the nucleotide-binding domain of BCRP was exposed on the outside surface of the membrane. The addition of Hoechst 33342 to the inside-out membrane vesicles resulted in a rapid increase in fluorescence up to a steady state level because of the partitioning of the dye in the hydrophobic environment of the phospholipid bilayer (Fig. 3). The subsequent addition of MgATP resulted in a rapid quenching of the Hoechst 33342 fluorescence in membrane vesicles containing BCRP (Fig. 3A) but not in control membrane vesicles without BCRP (Fig 3B). FTC strongly inhibited the ATP-dependent quenching of Hoechst 33342 fluorescence in membrane vesicles containing BCRP. In contrast, no significant changes in the steady state level of Hoechst 33342 fluorescence were observed in BCRP-containing and control membrane vesicles in the presence of AMP-PNP alone or AMP-PNP plus FTC (Fig. 3). These observations point to the BCRP-dependent transport of Hoechst 33342 from the phospholipid bilayer into the aqueous lumen of the membrane vesicles.

Functional studies in membrane vesicles of \textit{L. lactis} also

\begin{table}[h]
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\begin{tabular}{|c|c|c|}
\hline
\textbf{Drug} & \textbf{IC\(_{50}\)} & \textbf{Control} \\
\hline
Ethidium bromide & \(40 \pm 0 \mu\)g/ml & \(8 \pm 0 \mu\)g/ml \\
Hoechst 33342 & \(>200 \mu\)M & \(40 \pm 0 \mu\)M \\
Rhodamine 123 & \(>200 \mu\)M & \(131 \pm 6.6 \mu\)M \\
Tetramethylrhodamine & \(14.4 \pm 0.9 \mu\)M & \(8.8 \pm 0.8 \mu\)M \\
\hline
\end{tabular}
\caption{Drug survival characteristics of BCRP-expressing \textit{L. lactis} cells, compared with cells not expressing BCRP (control).}
\end{table}
aimed at the ATPase activity of BCRP. In contrast to control inside-out membrane vesicles, BCRP-containing inside-out membrane vesicles displayed a significant amount of ortho-vanadate-sensitive ATPase activity that was stimulated up to 5-fold in the presence of daunomycin (Fig. 4A), a substrate of BCRP (2). The concentration of daunomycin required for half-maximal stimulation (SC50) of the vanadate-sensitive ATPase activity was about 20 nM. FTC significantly reduced the daunomycin-stimulated vanadate-sensitive ATPase activity, with an IC50 concentration below 2.5 nM (Fig. 4B). These data strongly suggest that the drug-stimulated vanadate-sensitive ATPase activity is associated with BCRP activity. 

Taken together, the observations of (i) multidrug resistance and reduced ethidium bromide accumulation in cells expressing BCRP, (ii) the ATP-dependent Hoechst 33342 transport and the drug-stimulated vanadate-sensitive ATPase activity in inside-out membrane vesicles containing BCRP, and (iii) the inhibition of these drug transport and ATPase activities by the BCRP inhibitor FTC demonstrate the functional expression of human BCRP in *L. lactis*. 

Interaction of BCRP with Sterols—Although BCRP (ABCG2) was originally identified as a multidrug transporter, other members in the ABCG subfamily (e.g. ABCG1 and ABCG5/G8) have been implicated in the transport of sterols. To explore the interaction of BCRP with sterols, the effect of sterols on the BCRP-associated ATPase activity was examined. Surprisingly, the sterols estradiol and cholesterol both stimulated the BCRP-associated ATPase activity about 4-fold, with SC50 values of about 10 and 8 nM, respectively (Fig. 5A). In addition, the BCRP-associated ATPase activity was stimulated 4-fold by the natural steroid progesterone and 7-fold by testosterone at SC50 values of 5 and 15 nM, respectively, (Fig. 5B). Finally, the estrogen receptor modulator tamoxifen (26) stimulated the BCRP-associated ATPase activity almost 3-fold with an SC50 of about 50 nM (Fig. 5C). None of these sterols significantly affected the low level of vanadate-sensitive ATPase activity observed in control membrane vesicles lacking BCRP (Fig. 5).

The ability of BCRP expressed in *L. lactis* to interact with estradiol was further analyzed in Hoechst 33342 transport
assays in which estradiol was included as a competing substrate. As shown in Fig. 6A, the presence of estradiol significantly inhibited the BCRP-mediated transport of Hoechst 33342 in inside-out membrane vesicles. The degree of inhibition by estradiol was proportional to the concentration of estradiol used, suggesting that estradiol is a potential transport substrate for BCRP. Estradiol did not affect the fluorescence of Hoechst 33342 in control membrane vesicles without BCRP (data not shown). The ability of BCRP to transport estradiol was directly assessed by measuring the uptake of [3H]estradiol in L. lactis cells. In the presence of glucose, BCRP-expressing cells exhibited a 4-fold lower uptake of [3H]estradiol than the control cells or BCRP-expressing cells in the absence of glucose (Fig. 6B). These results demonstrate the BCRP-mediated transport of estradiol in L. lactis. Interestingly, the amount of cell-associated estradiol was not reduced in glucose-energized L. lactis cells expressing LmrA, suggesting the lack of a significant LmrA-mediated transport of estradiol under the experimental conditions (Fig. 6B).

DISCUSSION

ABC transporters are important contributors to cellular lipid transport and homeostasis, and their dysfunction is often associated with human disease phenotypes. For example, P-gly-
coprotein MDR3 (ABCB4) mediates the transport of phosphatidylcholine across the canalicular membrane during bile formation, and mutations in the MDR3 gene are a cause of progressive familial intrahepatic cholestasis (27, 28). ABCR (ABCA4) probably transports a complex of retinaldehyde and phosphatidylethanolamine in the retina of the eye, and malfunctioning of this transporter results in Stargardt’s macular dystrophy (29). ABCA1 is crucial for the elimination of excess body cholesterol, and mutations in the ABCA1 gene have been causatively linked to familial high density lipoprotein deficiency and Tangier disease (30). The multidrug resistance P-glycoprotein MDR1 (ABCB1) transports a wide variety of hydrophobic compounds and has been shown to catalyze the transbilayer movement of phospholipid analogues (31), sphingomyelin (32), cholesterol (33), and progesterone (34) but not sitosterol (35). Although members of the ABCG subfamily have been implicated in sterol transport, BCRP (ABCG2) has been characterized as an efflux system for multiple drugs and cellular toxins (2–7, 20). However, in view of its broad substrate specificity and its presence in tissues producing steroid hormones (18, 19), BCRP may also be involved in local lipid transport processes that may have remained undetected in BCRP knock-out mice (20).

To study sterol transport by human BCRP, we have expressed the protein in L. lactis, a bacterium that is devoid of mammalian sterols but that synthesizes haptanoids to regulate membrane fluidity (36). This property gives L. lactis an advantage over insect cells and mammalian cells where the sterol content of the plasma membranes can vary between 5 and 25%, which may hinder direct measurements of sterol transport and sterol-stimulated ATPase activities in the experimental setting. In addition, the expression of BCRP in L. lactis allows a comparison of its substrate specificity with that of the ABC half-transporter LmrA, a lactococcal homologue of P-glycoprotein MDR1. Three lines of experimental evidence suggested that BCRP was functionally expressed in L. lactis. Firstly, BCRP expression conferred multidrug resistance on cells. Secondly, BCRP expression enhanced the efflux of ethidium in cells and the transport of Hoechst 33342 in inside-out membrane vesicles. Both activities were inhibited in the presence of the BCRP-specific modulator FTC. Finally, BCRP-containing inside-out membrane vesicles displayed a vanadate and FTC-sensitive ATPase activity, which was stimulated by drugs (such as daunomycin) that are transported by BCRP. The observation of a BCRP-associated ATPase activity in inside-out membrane vesicles of L. lactis is consistent with published studies in which a drug-stimulated vanadate-sensitive ATPase activity is detected in isolated membranes of insect cells (37) and mammalian cells (38) was shown to be due to the presence of BCRP.

The BCRP-associated ATPase activity in inside-out membrane vesicles of L. lactis was also significantly stimulated in the presence of sterols, including estradiol, cholesterol, progesterone, testosterone, and tamofoxen. It has been shown for P-glycoprotein MDR1 that the lipid environment can significantly influence the characteristics of purified and functionally reconstituted protein (39). Hence, the stimulation of the BCRP-associated ATPase activity in inside-out membrane vesicles of L. lactis by mammalian sterols could reflect a requirement of BCRP for the presence of these sterols in its lipid environment. However, the observations of (i) the efflux of [3H]estradiol in BCRP-expressing L. lactis cells but not LmrA-expressing cells (Fig. 6B) and (ii) the inhibition of BCRP-mediated Hoechst 33342 transport by estradiol (Fig. 6A) at concentrations that stimulate the BCRP-associated ATPase activity (Fig. 5A) imply competition between sterols and drugs for binding to common binding sites in BCRP. These data would also argue against indirect mechanisms of coupling between the estradiol-induced stimulation of BCRP-associated ATPase activity and [3H]estradiol translocation, in which (i) [3H]estradiol transport would represent a secondary flux associated with the active translocation of an endogenous compound (e.g. lipid) by BCRP and (ii) simultaneously, estradiol would interact with an allosteric binding site, rather than a transport site, to enhance the BCRP-associated ATPase activity.

Interestingly, as the concentration of daunomycin or sterols increased beyond what stimulated the BCRP-associated ATPase maximally, the ATPase activity then decreased (Figs. 4 and 5) similar to observations for the P-glycoprotein MDR1 ATPase (34, 39). The biphasic pattern of stimulation and inhibition of the drug/sterol stimulated BCRP-associated ATPase activity may depend on the saturation state of BCRP transport sites, with enhanced binding of drug/sterol to transport sites at the inside surface of the membrane at low substrate concentrations and reduced dissociation of these substrates from release sites at the outside surface of the membrane at high substrate concentrations. Alternatively, the inhibition of the BCRP-associated ATPase activity at high drug/sterol concentrations may reflect changes in the lipid environment, i.e. in lateral pressure, that are less optimal for BCRP activity.

Altogether the data presented in this paper suggest that human BCRP is able to interact with sterols and that BCRP may play a role in the transport of sterols, steroids, and estrogen receptor antagonists used in the treatment of breast tumors, in addition to its ability to transport chemotherapeutic drugs and cellular toxins.

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