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Functional Characterization of Human Breast Cancer Resistance Protein (BCRP, ABCG2) Expressed in the Oocytes of *Xenopus laevis*

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ABSTRACT

To evaluate the function and substrate specificity of human breast cancer resistance protein (BCRP, ABCG2) in the absence of cofactors or heterologous partner proteins, *Xenopus laevis* oocytes were injected with cRNA of wild-type or mutant (R482T) BCRP. High expression of BCRP was observed on the oocyte surface. Accumulation and efflux assays revealed that oocytes expressing R482T transported daunorubicin (DNR), mitoxantrone (MX), rhodamine 123, and flavopiridol (FLV), whereas wild-type BCRP transported only MX and FLV, in agreement with observations in mammalian and other systems. Transport activity was completely inhibited by fumitremorgin C, a known inhibitor of BCRP. Injection of oocytes with cRNA containing mutations of serine 187 in the ATP-binding cassette signature motif (S187T or S187A) resulted in strong expression of the mutant forms; however, these oocytes were devoid of transporter activity. When oocytes were coinjected with R482T and R482T/S187T, DNR transport was inhibited in a manner dependent on the amount of R482T/S187T cRNA added, consistent with the idea that the active form of BCRP is a homodimer or homomultimer. Substrate interaction studies found that no two substrates reciprocally inhibited the efflux of the other. Although FLV proved to be an effective inhibitor of both MX and DNR transport, and MX inhibited DNR transport, the other substrates tested had only weak or no inhibitory activity, indicating a complex nature of substrate interaction with the BCRP homodimer. We conclude that the *X. laevis* oocyte heterologous expression system is a valid and effective means of studying BCRP function and substrate specificity.

Cancer drug resistance remains a major obstacle to the success of curative treatment regimens for human malignancies. In experimental systems, resistance of cancer cells to multiple drugs is often associated with reduced intracellular drug accumulation and overexpression of ATP binding-cassette (ABC) transporter proteins such as Pgp (Gottesman et al., 1996) or MRP1 (Loe et al., 1996).

Recently, BCRP was isolated from multidrug resistant MCF-7/AdrVp human breast cancer cells (Doyle et al., 1998), which were selected with doxorubicin (Adriamycin) in the presence of verapamil (Chen et al., 1990). Human BCRP is the second member of the G subfamily of the ABC transporter proteins (ABCG2), and is closely related to the white gene of *Drosophila melanogaster*. In normal tissues, BCRP expression is high in placenta, venules, and stem cells (Allikmets et al., 1998; Doyle et al., 1998; Bunting, 2002), and in the apical membrane of hepatocytes and gut epithelium (Jonker et al., 2000), where it may play a role in defense from xenobiotics and drug clearance. In human cancer cells in culture, BCRP expression confers a high level of resistance to a variety of cancer chemotherapeutic drugs, including MX (Doyle et al., 1998; Miyake et al., 1999; Ross et al., 1999), camptothecin-derived topoisomerase I inhibitors (Maliepaard et al., 1999; Nakatomi et al., 2001), methotrexate (Volk et al., 2002), and FLV (Robey et al., 2001). BCRP overexpression is found in drug-selected cancer cells derived from various types of human solid tumors (Doyle et al., 1998; Maliepaard et al., 1999; Miyake et al., 1999; Ross et al., 1999), and seems to be independent of Pgp or MRP expression. BCRP

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ABBREVIATIONS: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; MX, mitoxantrone; FLV, flavopiridol; Pgp, P-glycoprotein; MRP1, multidrug resistance-associated protein 1; MDR1, multidrug resistance gene 1; DNR, daunorubicin; ADR, doxorubicin (Adriamycin); TPT, topotecan; Rho123, rhodamine 123; PCR, polymerase chain reaction; UTR, untranslated region; HPLC, high-performance liquid chromatography; FTC, fumitremorgin C.

expression in clinical cancers is currently under investigation. Some reports suggest that the expression of BCRP mRNA or protein may correlate with resistant disease or immature phenotype, respectively, in acute myeloid leukemia (van den Heuvel-Eibrink et al., 2002; van der Kolk et al., 2002; Nakanishi et al., 2003). Furthermore, high expression of BCRP has been detected immunohistochemically in a variety of human solid tumors (Diestra et al., 2002). Ironically, expression of BCRP in human breast cancer biopsies seems to be low (Faneyte et al., 2002).

BCRP, unlike Pgp or MRP1, is a half-transporter and has only one ATP-binding domain in a single molecule (Doyle et al., 1998; Ross, 2000). ABC transporters contain two ATP-binding sites and require hydrolysis of an ATP molecule at each site to transport one substrate molecule. ABC half-transporters, in contrast, characteristically form heterodimers, such as the Tap1/Tap2 transporters involved with peptide transport, and the *Drosophila* white/scarlet or white/brown heterodimers, which transport eye pigment precursors. Current evidence indicates that the homodimeric BCRP is a functional transporter (Doyle et al., 1998; Ozvegy et al., 2001, 2002; Kage et al., 2002); however, it is possible that heterodimeric forms of BCRP combined with another half transporter exist in mammalian systems.

Mutant forms of BCRP with threonine or glycine in place of arginine at codon 482 (R482T or R482G) have been described in drug-selected cell lines (Honjo et al., 2001; Komatani et al., 2001), including the original isolate of BCRP from MCF-7/AdrVp cells (Doyle et al., 1998), which express the R482T mutation. Compared with the wild-type form, the R482T or R482G mutations are able to transport anthracyclines and Rho123 (Honjo et al., 2001; Ozvegy et al., 2002) but not methotrexate (Volk et al., 2002).

To investigate the function and substrate specificity of BCRP in the absence of cofactors or heterologous partner proteins but in the presence of post translational modifications of the expressed heterologous protein such as glycosylation, we used the African clawed frog (*Xenopus laevis*) expression system (Matthews and Colman, 1991; Wagner et al., 2000). Previously, human MDR1 (Morin et al., 1995) and mouse MDR1b (Castillo et al., 1990) have been expressed functionally in *X. laevis* oocytes. Here we present the first demonstration of the functional expression of glycosylated BCRP in *X. laevis* oocytes and provide evidence that the functional form of BCRP in these oocytes is a homodimer with complex substrate interactions.

Materials and Methods

Materials. [^{3}H]MX (3.2 Ci/mmol) was purchased from Moravek (Brea, CA). Unlabeled MX, DNR, FLV, and TPT were obtained from Immunex (Seattle, WA), Bedford Laboratories (Bedford, OH), AstraZeneca (London, England), and GlaxoSmithKline (London, England), respectively. Rho123 was purchased from Sigma-Aldrich (St. Louis, MO). TPT-selected human ovarian carcinoma cell line, Igrov1/T8 cells (Maliepaard et al., 1999) were provided by Dr. Marc Maliepaard of the Netherlands Cancer Institute. Mouse monoclonal antibodies BXP-21 and BXP-34, raised against human BCRP, were kindly provided by Dr. George Scheffer of the Free University, Amsterdam (Scheffer et al., 2000; Maliepaard et al., 2001).

Cell Culture. Igrov1/T8 cells were cultivated in RPMI 1640 (Biofluids, Camarillo, CA) supplemented 10% fetal bovine serum as described previously (Maliepaard et al., 1999).

Preparation of Human BCRP cDNA. Human BCRP cDNA (GenBank accession no. AF098951) lacks a poly(A) tail and a standard Kozak consensus sequence. These elements were therefore added to enhance the expression of BCRP in the *X. laevis* oocyte system. To generate the poly(A) tail, BCRP cDNA was subcloned into the pSP64 poly(A) vector (Promega, Madison WI), just upstream of the 30 dA:dT residues in that vector (Construct I; see Table 1). To generate a 5′ sequence containing crucial elements enumerated by Kozak (1986, 1989), the sequence around the start codon of BCRP cDNA (CAGATGT; start codon is underlined) was modified to AAGATGG by PCR using construct I as a template. PCR was performed using *Pfx* DNA polymerase I (Invitrogen, Carlsbad, CA), and the PCR primers used were: sense, 5′-TCT CAA GAT GGC TTC CAG TAA T-3′; antisense, 5′-CAG CTA TGA CAT GAT TAC GAA T-3′. This PCR product was inserted into pCR Blunt TOPO II to make construct II (Table 1). To make construct III, the BCRP coding region from construct II was released and inserted into the pSD64TR vector, which contains portions of the 5′- and 3′-UTRs of *X. laevis* β-globin cDNA, separated by a BglII site and a stretch of 23 dA:dT and 30 dC:dG residues after the 3′-UTR (Krieg and Melton, 1984). The pSD64TR vector was a gift from Dr. Paul Welling (Department of Physiology, University of Maryland School of Medicine, Baltimore, MD). Construct III− consisted of pSD64TR containing BCRP cDNA with the additional poly(A) sequences but without the Kozak-type modifications to the start codon region. Construct III− was prepared by inserting the BCRP cDNA without Kozak-type modifications into the multiple cloning site of the pSD64TR plasmid (Table 1).

Isolation of Wild-Type BCRP (R482 BCRP) cDNA. To obtain a full-length R482 BCRP cDNA, total cellular RNA was prepared from MCF-7 cells, then subjected to reverse transcription at 37°C for 1 h in the presence of random hexamer and oligo-dT primers. This was followed by PCR using *Pfx* DNA polymerase I and PCR primers, which were designed to span the open reading frame of BCRP mRNA. The PCR primers used were: sense, 5′-CTG AGC CTT TGG TTA AGA CCG A-3′; antisense, 5′-GCT GTG CAA CAG TGT GAT GG-3′. The PCR product was ligated into the pCR Blunt TOPO II

TABLE 1
Summary of cDNA constructs made as templates for producing BCRP cRNA

Construct	Plasmid	Features of the BCRP cRNA Produced	RNA Polymerase Consensus Sequence Upstream from BCRP cDNA
I	pSP64poly(A)	R482T-poly(A)	SP6
II	pCR Blunt TOPO II	Kozak-R482T-poly(A)	T7
III	pSD64TR	5′UTR-Kozak-R482T-3′UTR-poly(A) other BCRP forms: 5′UTR-Kozak-R482–3′UTR-poly(A) 5′UTR-Kozak-R482T/S187T-3′UTR-poly(A) 5′UTR-Kozak-R482T/S187A-3′UTR-poly(A)	SP6
III−	pSD64TR	5′UTR-R482T-3′UTR-poly(A)	SP6

5′UTR, 3′UTR, portions of the 5′- and 3′-UTR of the *Xenopus laevis* β-globin gene; Kozak, modified sequences proximate to the start codon, as described under *Materials and Methods*; Poly(A), addition of a poly(A) tail, as described under *Materials and Methods*.

vector, and then the sequence was confirmed by the automated dideoxynucleoside method. The sequence was then modified to include the Kozak sequence and poly(A) tail, as described above, and was then ligated into pSD64TR, as described for the preparation of construct III.

Introduction of S187T and S187A Mutations into Human BCRP cDNA. The highly conserved serine at residue 187 in the ABC signature motif was changed to threonine or alanine using a commercially available site-directed mutagenesis system (QuikChange; Stratagene, La Jolla, CA) according to the manufacturer's protocol. These mutant forms were created using construct III as a template. Two internal complementary primers pairs were used, each containing the specific mutation. The S187T primer pair was 5′-G TTT ATC CGT GGT GTG AC T GGA GGA GAA AG-3′ and 5′-CT TTC TCC TCC AGT CAC ACC ACG GAT AAA C-3′, and the S187A primer pair was 5′-G TTT ATC CGT GGT GTG GC T GGA GGA GAA AG-3′ and 5′-CT TTC TCC TCC AGC CAC ACC ACG GAT AAA C-3′.

cRNA Synthesis. Plasmid DNA containing the desired BCRP cDNA insert (Table 1) was linearized by restriction enzyme digestion, and used as a template for synthesizing cRNA using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) and the SP6 or T7 RNA polymerase.

Microinjection of cRNA into *X. laevis* Oocytes. Adult female *X. laevis* frogs were obtained from Xenopus Express Inc. (Plant City, FL). The care and use of *X. laevis* was in strict adherence to a protocol approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee. *X. laevis* oocytes were prepared and maintained as described previously (Nakanishi et al., 2001). On the same day, oocytes were injected with 50 nl of water per oocyte (hereafter referred to as control oocytes) or 50 nl of 1 μg/μl cRNA solution. The oocytes were cultivated at 18°C in Barth's medium supplemented with 50 mg/l of gentamicin (Sigma-Aldrich, St. Louis, MO) for 2 to 3 days after cRNA injection, after which they were subjected to drug accumulation or efflux experiments.

Drug Accumulation Studies in *X. laevis* Oocytes. Groups of six to eight oocytes were incubated at room temperature in 300 μl of "uptake buffer" (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES-Tris, pH 7.5) containing either DNR (10–250 μM), MX (0.5–100 μM unlabeled MX plus 2.5 μCi/ml [^{3}H]MX), FLV (25 μM), or Rho123 (5 μM). These substrate molecules were added at time 0 of uptake. Background binding of drug was assessed by exposing the oocytes to the compounds listed above at 4°C. After incubation with drug for the desired period of time, the oocytes were washed four times with ice-cold uptake buffer. To quantify accumulation of [^{3}H]MX, each oocyte was homogenized in 5% SDS, then the radioactivity of this cell lysate was measured by liquid scintillation counting. To quantify accumulation of DNR, FLV, or Rho123, one or two drug-exposed oocytes were homogenized in 100 μl of 67% acetonitrile for 5 min, and the homogenate was centrifuged at 4°C for 10 min at 18,000*g*. The resulting supernatant (~100 μl) was submitted for HPLC analysis.

Determination of DNR or [^{3}H]MX efflux in *X. laevis* Oocytes. Before efflux studies, DNR or [^{3}H]MX was preloaded into the oocytes by 90-min exposure to 25 μM DNR or 10.8 μM [^{3}H]MX (specific activity, 0.24 Ci/mmol) at room temperature in uptake buffer. For oocytes expressing BCRP, 5 μM FTC [a specific inhibitor of BCRP (Rabindran et al., 2000)] was added to the preloading uptake buffer. After preloading, the oocytes were washed with ice-cold uptake buffer and then transferred into an excess amount of uptake buffer at room temperature (time 0 of efflux studies). At the desired efflux time, aliquots of six to eight oocytes from each treatment group were removed, and intracellular drug content was determined as described above for drug accumulation. Efflux of DNR or MX by BCRP-expressing oocytes was performed in the presence or absence of 5 μM FTC.

Detection and Quantification of DNR, FLV, and Rho123 by HPLC. HPLC for DNR, Rho123, and FLV was performed with a Beckman Coulter liquid chromatograph (Beckman Coulter, Fullerton, CA) equipped with an HP 1050 autosampler (Agilent Technologies, Wilmington, DE). For DNR or Rho123, separation was achieved with a Genesis C_{18} column (25 cm × 4.6 mm; Jones Chromatography Inc., Lakewood, CO) and a Brownlee guard column (15 cm × 3.2 mm, PerkinElmer Life and Analytical Sciences, Boston, MA). Both compounds were detected by an RF-10A_{XL} fluorescence detector (Shimadzu, Kyoto, Japan), using an excitation wavelength of 488 nm and an emission wavelength of 550 nm. The mobile phase consisted of acetonitrile, methanol, and 50 mM potassium phosphate buffer, pH 4.5 (35:10:55; v/v), and the flow rate was 1 ml/min (Pizzorno et al., 1985; Reid et al., 1990). For FLV, reverse-phase gradient HPLC was performed using a Nova-pak analytical C_{18} column (15 cm × 3.9 mm, inside diameter), with detection by a Beckman Coulter model 406 variable wavelength UV absorbance detector set at 268 nm. Mobile phase A consisted of 50 mM ammonium acetate: acetic acid (100:0.72; v/v; pH 4.15), and mobile phase B was acetonitrile, pumped in a 13-min linear gradient from 20% to 50% B in A at a flow rate of 1 ml/min. The average retention times ± S.D. for DNR, Rho123, and FLV were 9.6 ± 0.02, 8.8 ± 0.04, and 12.9 ± 0.1 min, respectively. The standard curves for DNR, Rho123, and FLV were linear over concentration ranges of 5 to 100 nM, 0.5 to 50 nM, and 0.5 to 25 μM, respectively. All of the experimental determinations of DNR, Rho123, and FLV fell within these respective ranges.

Western Blot Analysis. Oocytes were homogenized in 20 to 50 μl of homogenization buffer (150 mM NaCl; 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, and 0.5% sodium deoxycholate) per oocyte in the presence of 4 mM of a protease inhibitor (Pefabloc SC; Roche Biochemicals, Indianapolis, IN) by sonication followed by vortexing. The homogenate was subsequently centrifuged at 15,000*g* at 4°C for 15 min; then the supernatant was centrifuged again. The protein concentration of the supernatant was determined by the method of Bradford (1976). Supernatant containing 20 μg of extracted protein was subjected to 10% SDS-polyacrylamide gel electrophoresis; then, the gel was blotted by electrotransfer onto a polyvinylidene difluoride membrane. The blots were blocked with 5% nonfat dry milk and probed at room temperature for 1 h or 4°C overnight with a 1:750 dilution of the BXP-21 monoclonal antibody to BCRP (Maliepaard et al., 2001). The blots were then incubated at room temperature for 1 h with a horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (Amersham Biosciences, Piscatway, NJ), followed by enhanced chemiluminescence detection (Amersham Biosciences) using Kodak X-OMAT AR film.

Immunofluorescence and Confocal Laser Microscopy. Oocytes injected with water or BCRP cRNA (wild type, R482T) were fixed in 4% paraformaldehyde in PBS, then immersed overnight in 30% sucrose in PBS. Groups of four to six oocytes were frozen in OCT compound (Tissue-Tek; Sakura Finetek Europe, Zoeterwoude, the Netherlands) and 25-μm sections were collected on stubbed coverslips. Sections on coverslips were inverted onto 50 μl of primary antibody, BXP-34, diluted 1:50 in PBS/1% bovine serum albumin (buffer A) for 1 to 2 h at room temperature in a moisture chamber, then rinsed three times with buffer A for 10 min. The sections were probed subsequently under similar conditions with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:100 in buffer A, then rinsed twice with buffer A for 10 min. Sections were mounted in a 9:1 mixture of glycerol and Tris-HCl, pH 8, supplemented with 1 mg/ml *p*-phenylenediamine to reduce photo bleaching (Johnson et al., 1982). Immunofluorescence was observed with a Zeiss LSM confocal microscope (Zeiss, Welwyn Garden City, UK) and 63× magnification. Using an initial section from an oocyte injected with BCRP cRNA, the background fluorescence was set to zero, and the brightest fluorescence was set to saturation. These parameter settings were used for all subsequent sections.

Results

Optimization of Expression of BCRP in *X. laevis* Oocytes. In our initial attempt to express an ABC transporter in *X. laevis* oocytes, we successfully expressed Pgp as determined by Western blotting (data not shown). This was accomplished by injecting cRNA that was directly transcribed from the PI-4 plasmid, which consists of a full-length Pgp cDNA in the pSP64(polyA) vector (Hoof et al., 1991). However, injection of oocytes with BCRP cRNA directly transcribed from BCRP (R482T) cDNA (Doyle et al., 1998) failed to produce BCRP protein, as detected by Western blot examination or by functional assays. Modification of the BCRP construct by the addition of a 5′ Kozak sequence and a 3′ poly(A) tail (Table 1, constructs I and II) did not enhance BCRP expression in the oocytes (Fig. 1, A and B). Accordingly, we cloned the modified BCRP cDNA into a *X. laevis* expression plasmid (pSD64TR) in which the BCRP cDNA is flanked by the 5′ and 3′ UTRs of *X. laevis* β-globin cDNA, respectively (Table 1, construct III). The pSD64TR expression plasmid also contains an SP6 RNA polymerase recognition site upstream from the *X. laevis* β-globin 5′-UTR, and a poly(A) sequence downstream from the *X. laevis* β-globin 3′-UTR. Transcription of construct III by SP6 RNA polymerase resulted in a cRNA that produced high expression of functional transporter activity when injected in *X. laevis* oocytes (Fig. 1A). Moreover, Western blot confirmed a strong signal of the same molecular size (70 kDa) as BCRP expressed in Igrov1/T8 cells (Fig. 1B), indicating that the degree of glycosylation in the *X. laevis* oocytes approximated that achieved in the mammalian cells.

The Kozak modification of BCRP in construct III resulted in a change from serine to alanine at amino acid 2. To examine the effect of this mutation on BCRP expression, we inserted the coding region of BCRP without the Kozak modification into pSD64TR (construct III−), and then injected the oocytes with cRNA transcribed from this construct. DNR transport activity in oocytes injected with cRNA transcribed from construct III was higher and had less variability than that in oocytes injected with BCRP cRNA transcribed from construct III− (Fig. 1C). Furthermore, the mutation had no apparent effect on the substrate specificity of BCRP expressed in the oocytes, compared with that reported for BCRP in mammalian systems. Therefore, in further experiments, BCRP cRNA injected into oocytes was transcribed from Kozak-modified BCRP cDNA inserted into the pSD64TR plasmid, analogous to construct III (Table 1).

BCRP Expressed in *X. laevis* oocytes Localizes to the Oocyte Surface. Oocytes were injected with cRNA coding for the wild-type (R482) or mutant R482T form of BCRP, then fixed and sectioned as described under *Materials and Methods*. Exposure of these sections to the BXP-34 monoclonal antibody to BCRP resulted in immunoreactivity only in the plasma membranes of oocytes expressing wild-type (Fig. 2A) or the mutant R482T form of BCRP (Fig. 2B), as detected by immunofluorescence microscopic analysis. No immunostaining was observed in any of the internal structures of the oocytes, including the numerous yolk granules found in these cells, in oocytes expressing either form of BCRP. Control oocytes had no immunoreactivity whatsoever (Fig. 2C), although a relatively high background or autofluorescence was found in the cytoplasm, which was also present in unstained

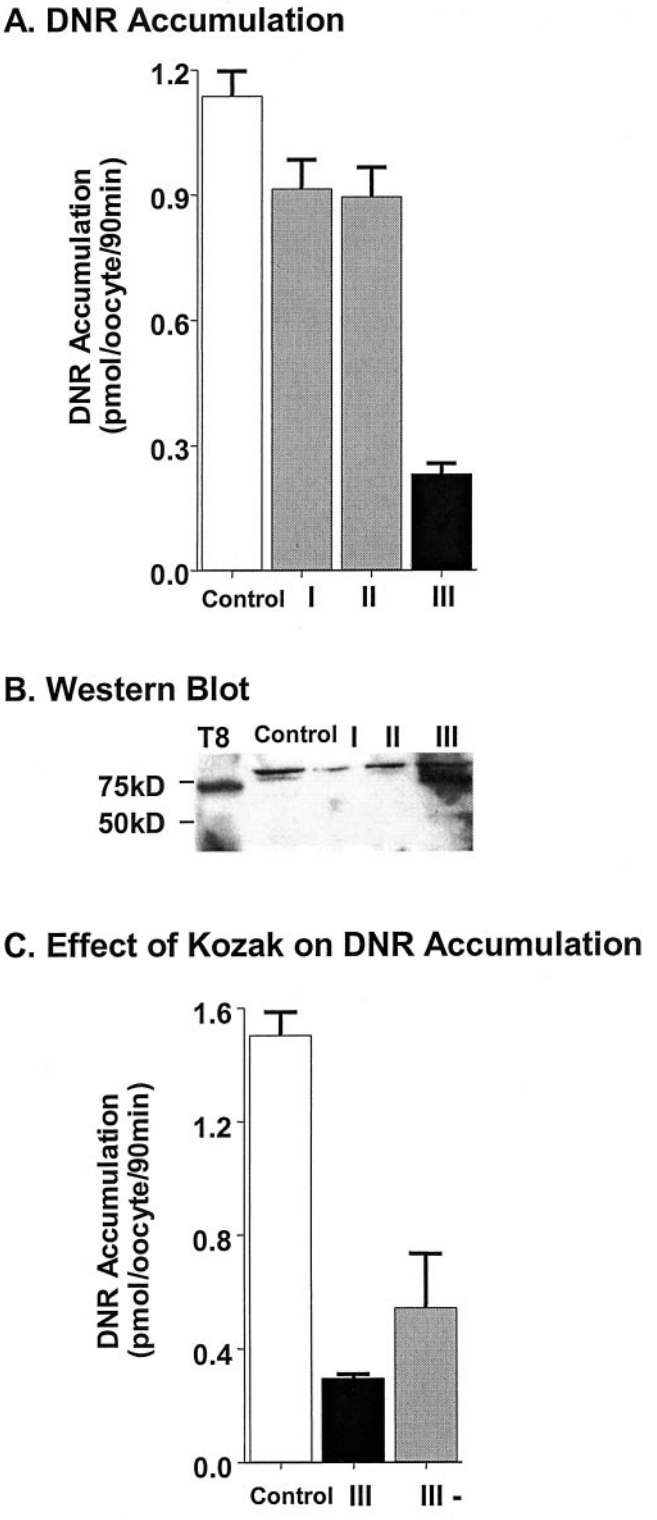

Fig. 1. Functional expression of BCRP (R482T) was examined by 10 μM DNR accumulation (A) or Western blot (B) in oocytes injected with 50 nl of water (control) or cRNA (1 μg/μl) transcribed from construct I, II, or III. For convenience, the R482T form of BCRP was used to enable monitoring of function by DNR accumulation. 25 μg of cell lysate was loaded into each lane. As control for the Western blot, cell lysate from Igrov1/T8 cells (T8) was also probed with the BXP-21 antibody. The effect of modification of BCRP sequence was examined by accumulation of 10 μM DNR (C) in oocytes injected with water (control) or with cRNA transcribed from construct III or III−. Accumulation studies were carried out for 90 min at room temperature. Each bar represents mean value ± S.E.M. of four groups of two oocytes.

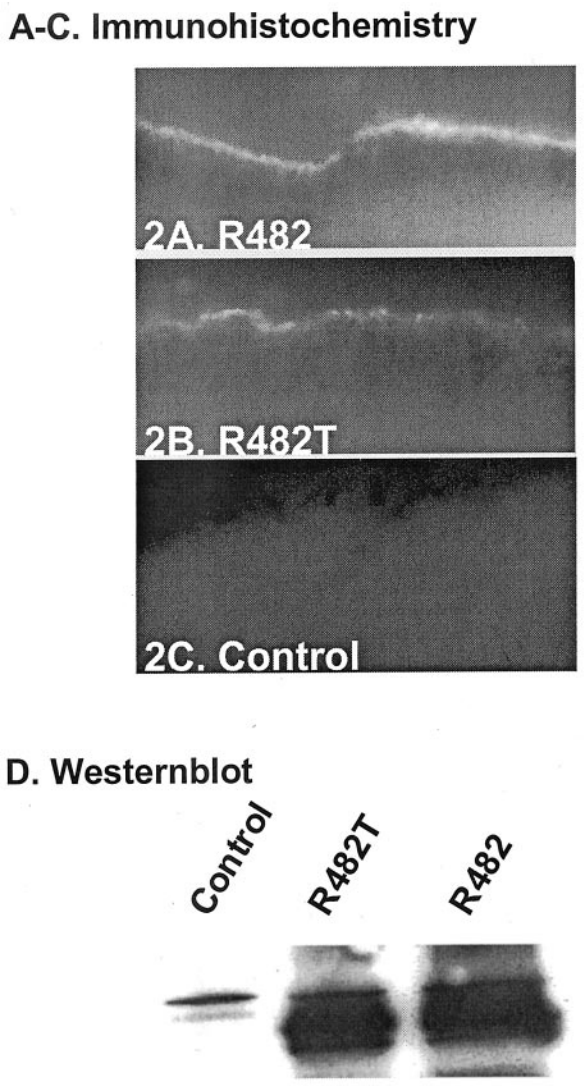

Fig. 2. Confocal immunofluorescence microscopic analysis was performed in oocytes injected with 50 nl of cRNA of BCRP (wild-type, R482) (A), BCRP (R482T) (B), or water as control (C), using the BXP-34 antibody. A and B show the distribution of BCRP (R482 or R482T) at the oocyte surface (C). Western blot shows that both BCRP forms were expressed at similar levels (D). Cell lysate (25 μg) from the oocytes injected with water (control), BCRP (R482), or BCRP (R482T) cRNA was loaded per lane of the gel. After electrophoresis and *trans*-blotting, the blots were probed with the BXP-21 antibody.

oocytes (data not shown). Western blot examination of oocytes injected with wild-type or BCRP (R482T) cRNA at the time of the immunofluorescence experiments confirmed robust expression of BCRP, with a molecular mass of 70 kDa (Fig. 2D).

Accumulation and Efflux of MX or DNR in Oocytes Expressing BCRP (R482T). To determine whether functional BCRP was expressed in *X. laevis* oocytes, we monitored the accumulation of DNR and [^{3}H]MX in control or BCRP(R482T) injected oocytes. The accumulation of DNR was not saturated in control oocytes even after a 120-min incubation period, whereas the accumulation of MX reached a plateau in control oocytes after 90 min. Oocytes expressing BCRP (R482T) showed a remarkable reduction in the accumulation of DNR or MX over the 120-min period. In the experiment shown, after subtraction of background, DNR or [^{3}H]MX accumulation was only 12 or 7% of control, respectively, at 90 min (Fig. 3, A and B). This reduced accumulation of either compound was not sensitive to changes in concentration in the range of 10 to 50 μM for DNR (Fig. 3C) or 0.5 to 10.8 μM for MX (data not shown).

To examine whether the diminished accumulation of DNR or MX in the BCRP (R482T)-expressing oocytes was caused by enhanced drug efflux, the efflux of these compounds in the presence or absence of 5 μM FTC was compared with that of control oocytes. Before the efflux studies, the drug of interest was preloaded into the control or BCRP (R482T)-expressing oocytes by incubating with drug for 90 min. In the case of BCRP (R482T)-expressing oocytes, 5 μM FTC was added, which resulted in intracellular drug accumulation comparable with levels attained in the control (water injected) oocytes; after preloading and subtraction of background, the accumulation of DNR in BCRP (R482T)-injected oocytes was 3.79 $\pm$ 0.15 pmol/oocyte, whereas that in control was 4.55 $\pm$ 0.31 pmol/oocyte. [^{3}H]MX accumulation in control and preloaded R482T-expressing oocytes was 0.87 $\pm$ 0.16 and 0.96 $\pm$ 0.91 pmol/oocyte, respectively. Both DNR and MX were effluxed more rapidly from the BCRP (R482T)-expressing oocytes, compared with control (Fig. 3, D and E). This rapid efflux was not observed in R482T-expressing oocytes in the presence of FTC. BCRP-dependent efflux was most apparent during the initial 5 to 30 min for MX and DNR, respectively. Beyond this period, further efflux of these drugs did not occur, with the oocytes retaining approximately 70 or 40% of their peak DNR or MX content, respectively. This suggests that once these drugs permeate the oocytes, large proportions of the drugs are bound to intracellular sites that prevent them from effluxing from the cells.

Substrate Specificity of Wild-Type and Mutant (R482T) BCRP Expressed in *X. laevis* Oocytes. Accumulation assays using DNR, MX, FLV, and Rho123 were performed to study the effect of the Arg-to-Thr mutation at codon 482 on the substrate specificity of BCRP in the *X. laevis* oocyte system. Figure 4 shows the intracellular accumulation of the four compounds in oocytes injected with R482 or R482T BCRP in the absence or presence of FTC. Accumulation of all four compounds in the BCRP (R482T)-expressing oocytes was markedly reduced. In contrast, oocytes expressing the wild-type R482 form of BCRP displayed reduced accumulation of MX and FLV but not of DNR or Rho123, consistent with observations in mammalian systems. Addition of 5 μM of FTC to the uptake media abolished the transport activity of each form of BCRP completely.

A Mutation in the ABC Signature Motif Inactivates BCRP (R482T) Transport of DNR; BCRP Expressed in *X. laevis* Oocytes Functions as a Homodimer or Homomultimer. To determine whether dimerization is required for BCRP activity, a mutant construct was created by substituting the highly conserved serine at residue 187 in the ABC signature motif with threonine (R482T/S187T) or alanine (R482T/S187A). These mutants were expressed well in the *X. laevis* oocytes. As shown in Fig. 5A, no difference was observed in expression levels of BCRP protein among oocytes injected with cRNA encoding BCRP (R482T) or these codon 187 mutant BCRP constructs. However, when DNR accumulation experiments were carried out in the oocytes expressing the codon 187 mutant BCRP forms, they were devoid of DNR transport activity (Fig. 5B), indicating that they may serve in

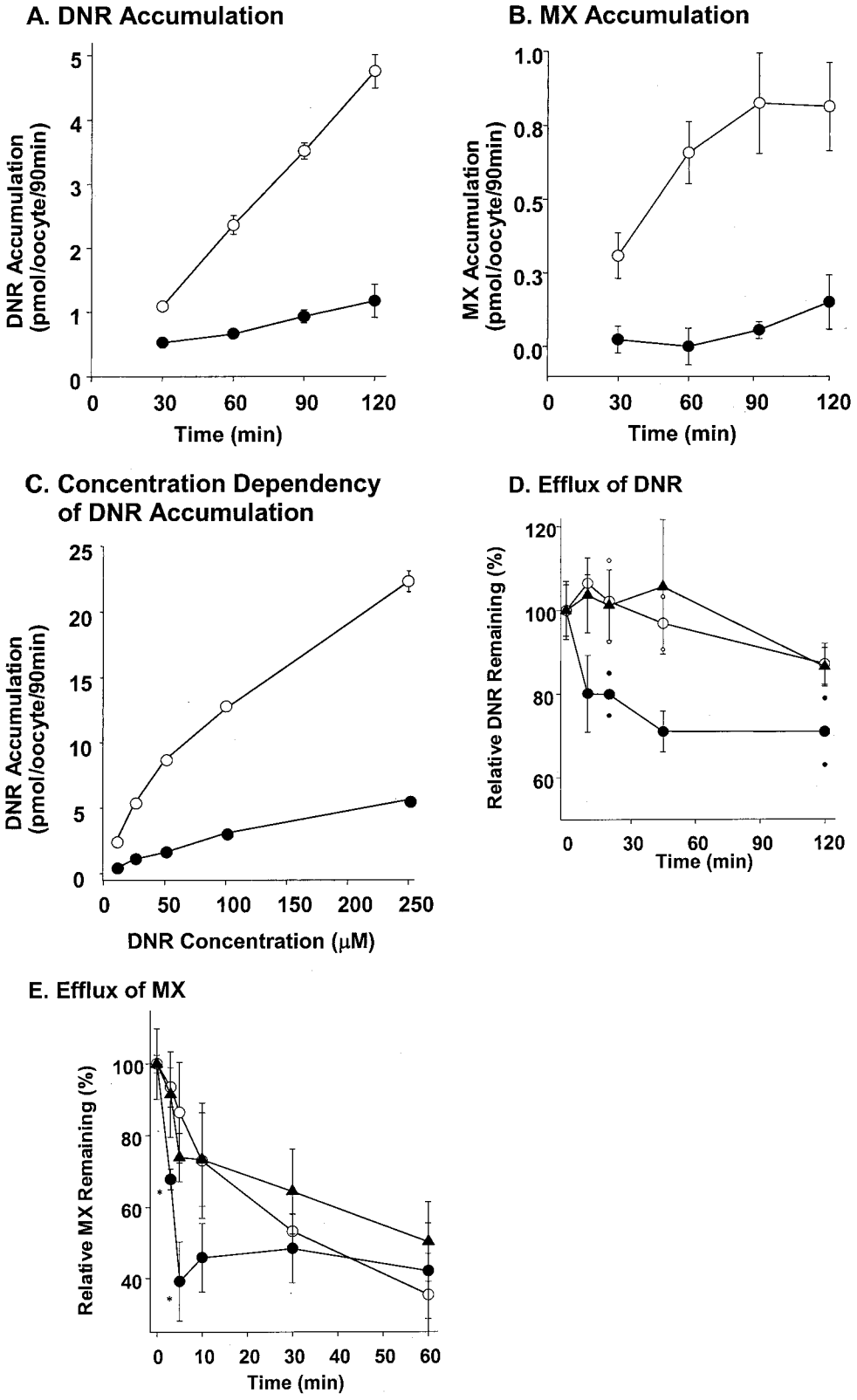

Fig. 3. Accumulation of 25 μM DNR (A), 10.8 μM MX (B) or varying concentrations of DNR (C, DNR: 10 to 250 μM) in control (○) or BCRP (R482T)-expressing oocytes (●). Efflux of DNR (D) or MX (E) from control (○) or BCRP (R482T)-expressing oocytes (●) afterpreloading with DNR or MX in the presence of 5 μM FTC was monitored at room temperature, as described under *Materials and Methods*. ▲, drug efflux in BCRP (R482T)-expressing oocytes in the presence of 5 μM FTC. Each point represents the mean value ± S.E.M. of three to four of groups of two oocytes in the DNR accumulation studies or the mean value of two to four groups of two oocytes in the DNR efflux experiments. In case of the MX assays, each value represents the mean of three to ten oocytes. *, statistically significant difference ($p < 0.05$, student's t test) between control and R482T-expressing oocytes. In D, small ○ and ● represent the values for individual points in the four instances in which only two oocytes were used for that time point.

a manner analogous to a dominant-negative mutation, if coinjected with the active BCRP (R482T) cRNA. Indeed, when BCRP (R482T) was coinjected with varying amounts of the "dominant-negative" construct S187T/R482T, the transport of DNR was significantly inhibited in a manner dependent on the amount of the S187T mutant construct added (Fig. 5C), strongly suggesting that homodimerization or multimerization is essential for BCRP activity. The degree of inhibition seemed to plateau after the addition of 48 ng of the "dominant-negative" construct; for technical reasons, we were unable to inject the oocytes with amounts of S187T mutant cRNA in excess of 72 ng.

Substrate Interaction Studies. To investigate the mechanism of BCRP substrate recognition, we examined the accumulation of a known BCRP substrate drug in the presence of other BCRP substrate drugs in oocytes expressing BCRP. First, DNR accumulation in BCRP (R482T)-expressing oocytes was studied (Fig. 6A). In the absence of competitor, DNR accumulation was reduced to approximately 10% of control, which was completely reversed by the addition of FTC, a known inhibitor of BCRP. Addition of 10-fold molar excess of FLV also caused complete inhibition of BCRP transport of DNR, with intracellular DNR accumulation increasing to control levels (Fig. 6A). In contrast, MX and Rho123 only partially inhibited DNR transport by BCRP (R482T), and 10-fold molar excess of TPT was not inhibitory at all. When BCRP substrate inhibition of MX accumulation was studied (Fig. 6B) using a 23-fold molar excess of Rho123, TPT, DNR, or FLV as competitors, only FLV caused partial but statistically significant reversal of BCRP R482 or R482T transport of MX. Interestingly, when the competition studies were done with respect to FLV accumulation (Fig. 6C), none of the competing BCRP substrates was able to inhibit FLV efflux by wild-type or R482T BCRP, despite their presence in a 10-fold or, in the case of DNR, a 100-fold molar excess relative to FLV. The competitive inhibition studies are summarized in Table 2.

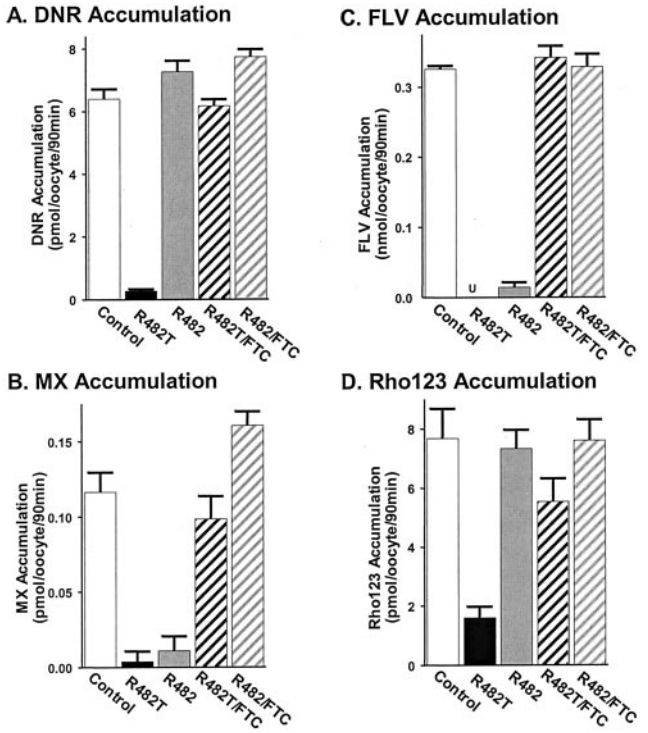

Fig. 4. Accumulation of BCRP substrates DNR (25 μM, A), MX (0.5 μM, B), FLV (25 μM, C), and Rho123 (5 μM, D) was measured in control (□), BCRP (R482T)-expressing oocytes (■) or BCRP (R482, wild-type)-expressing oocytes (▨). Hatched columns show accumulation of in the presence of 5 μM FTC. Accumulation of these four substrates in control oocytes was measured in the absence or presence of 5 μM FTC, but no significant differences were observed. Each bar represents the mean ± S.E.M. of at least four individual assays. U, intracellular levels of drug were undetectable.

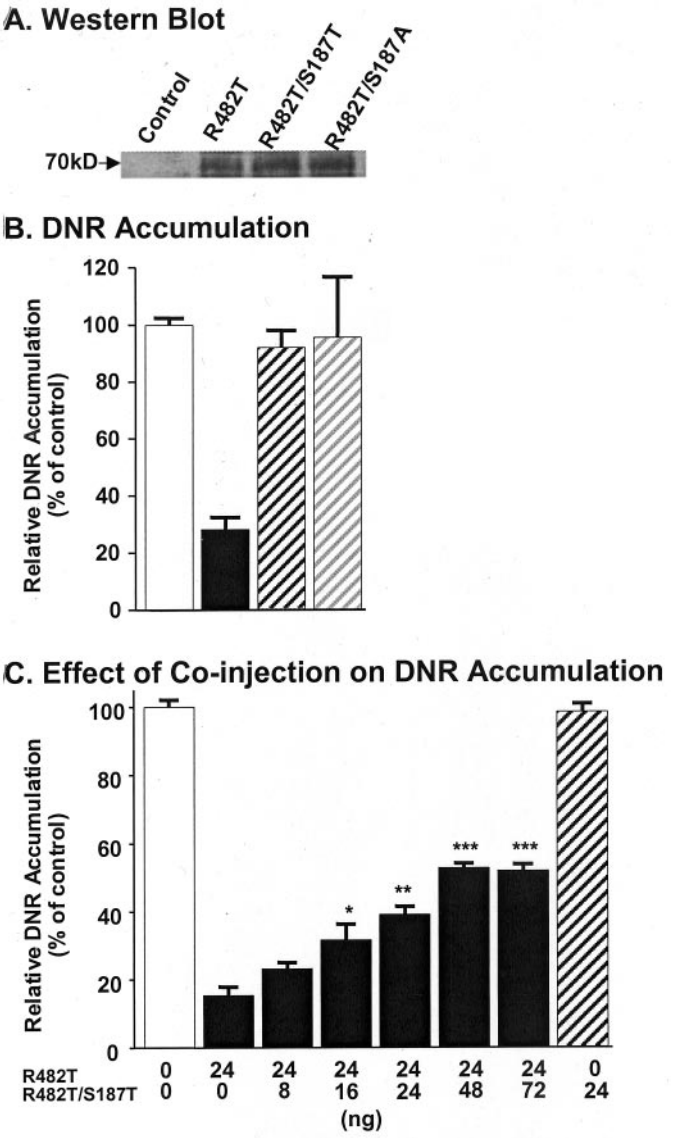

Fig. 5. Dominant-negative type studies. Expression of dominant-negative construct was confirmed by Western blot (A) and by DNR accumulation (25 μM, B) in control or R482T-expressing oocytes (■), R482T/S187T-expressing oocytes (▨), or R482T/S187A-expressing oocytes (gray ▨). Oocytes were injected with 50 μl of each cRNA (1 μg/μl). DNR accumulation (25 μM, C) was determined in oocytes coinjected with 24 ng of R482T cRNA and different amounts (0 to 72 ng) of the R482T/S187T construct cRNA (■). As control, the accumulation of 25 μM DNR was also determined in control (□) or oocytes injected with 24 ng of R482T/S187T cRNA only (▨). Each bar represents the mean value ± S.E.M. of three to four groups of two oocytes. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ represent statistically significant differences (Student's t test) in coinjected oocytes compared with accumulation in oocytes injected with R482T cRNA only.

Discussion

This work is the first description of the expression of human BCRP in *X. laevis* oocytes and demonstrates that this heterologous expression system is a valid model for examining the physical and functional characteristics of this ABC half transporter. *X. laevis* oocytes injected with BCRP cRNA synthesize a protein with the molecular mass of glycosylated BCRP and the immunological characteristics of native human BCRP protein. Our results further demonstrate that oocytes injected with mutant R482T or wild-type BCRP cRNA express BCRP in the oocyte plasma membrane, as evidenced by confocal immunofluorescence microscopic analysis. Accumulation and efflux assays using various BCRP substrate molecules indicate that the oocyte-expressed BCRP functions in a manner analogous to that observed in mammalian systems. Moreover, the *X. laevis* model indicates that a mutation of serine at codon 187 in the ABC-signature motif of BCRP is not only devoid of transporter activity but also serves in a manner analogous to a dominant-negative inhibitor of BCRP function when this mutant cRNA is coinjected with BCRP without the codon 187 mutation, suggesting that the functional form of BCRP is a homodimer or homomultimer. Finally, substrate interaction studies of BCRP expressed in *X. laevis* oocytes predict a complex interaction of BCRP substrates and inhibitors.

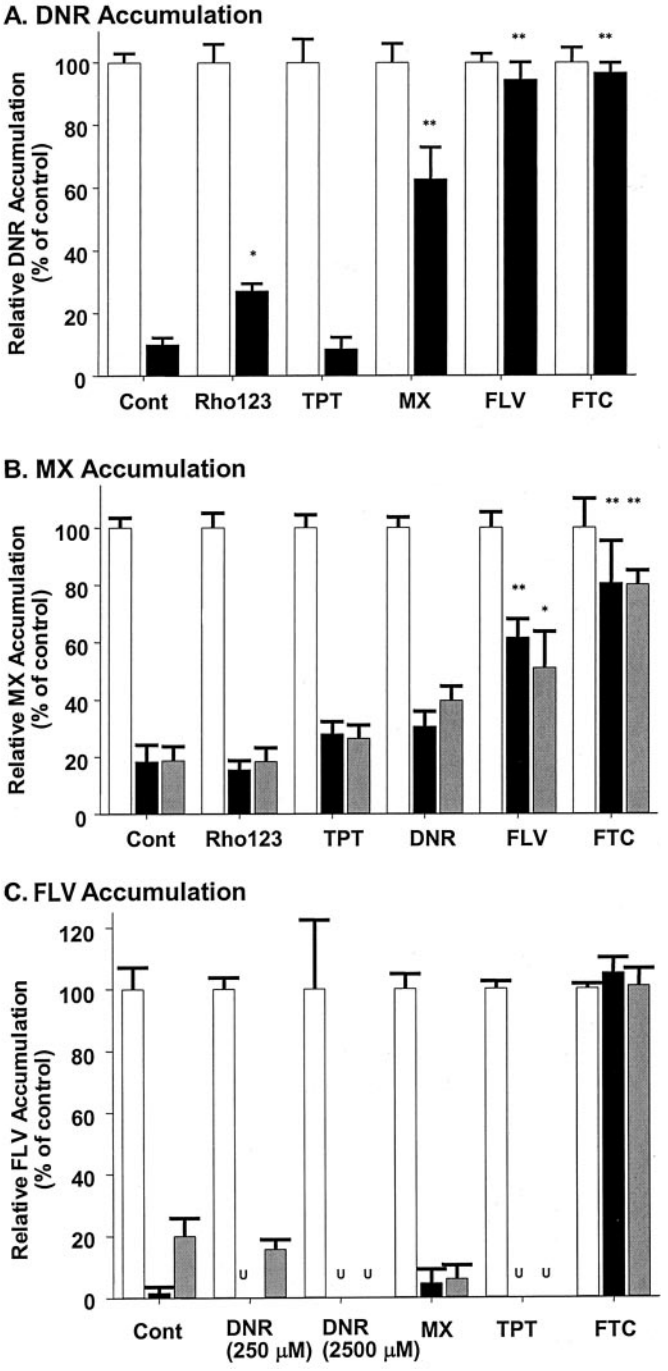

Fig. 6. Substrate interaction studies. Accumulation of 25 μM DNR (A), 10.8 μM MX (B), or 25 μM FLV (C) was determined in the presence of different BCRP substrates (Rho123, TPT, MX, or FLV, 250 μM), or FTC (5 μM). □, accumulation of these drugs in control oocytes for each condition (100%); ■, accumulation in BCRP (R482T)-expressing oocytes; ▨, accumulation in BCRP (R482, wild-type)-expressing oocytes. Each bar represents the mean ± S.E.M. of more than four individual assays. U, intracellular levels of drug were undetectable. *, $p < 0.05$; and **, $p < 0.01$ represent a statistically significant difference (Student's t test) in accumulation in BCRP-expressing oocytes in the absence and presence of inhibitors.

The present work validates the *X. laevis* oocyte expression system as a facile means of assessing BCRP function, particularly with respect to the effects of mutant sequences on transporter activity. Validation of the assay is based our observation that the function of BCRP expressed in the oocytes parallels that observed in mammalian systems (Doyle et al., 1998; Honjo et al., 2001; Robey et al., 2001; Minderman et al., 2002); the R482T mutant form of BCRP expressed in the oocytes efficiently transported DNR, Rho123, MX, and FLV, whereas the oocyte-expressed wild-type form transported only MX and FLV. Furthermore, FTC, in concentrations known to inhibit BCRP function in mammalian systems (Rabindran et al., 2000) also caused complete inhibition of both mutant R482T and wild-type BCRP forms expressed in the oocytes. In contrast to Sf9 insect cells, the other heterologous system in which BCRP has been expressed (Ozvegy et al., 2001, 2002), BCRP expression in *X. laevis* oocytes results in a glycosylated form of the protein. A potential limitation of the *X. laevis* oocyte expression system, however, lies in difficulties encountered in measuring efflux rates of DNR or MX. In our studies, the diminished accumulation of DNR or MX displayed by BCRP R482T-expressing oocytes correlated with greater initial efflux rates of these drugs compared with control oocytes. Despite this rapid initial drug efflux in the BCRP-expressing oocytes, approximately 70% of preloaded DNR or 40% of preloaded MX remained thereafter in the cells, presumably bound in a stable steady state (Fig. 3, D and E). The amount of drugs remaining in the oocytes is higher than the proportion of drug that remains in BCRP-overexpressing mammalian cells that are similarly preloaded with drug (Doyle et al., 1998; Maliepaard et al., 1999; Minderman et al., 2002). This phenomenon may be caused by nonspecific binding of these hydrophobic BCRP substrates to intracellular structures such as the abundant yolk granules, which occupy approximately 50% of the cellular volume of *X. laevis* oocytes (Gurdon and Wickens, 1983). This high background in the efflux experiments tended to make efflux measurements imprecise, and precluded our performing Michaelis-Menten kinetic studies of BCRP transport as measured by initial rates of efflux.

A characteristic of ABC transporters is the highly con-

TABLE 2
Summary of substrate interaction studies

Substrate (BCRP form)	Substrate Competitor					Inhibitor
	DNR	MX	FLV	TPT	Rho123	FTC
DNR (R482T)		Partial	Yes	No	±	Yes
MX (W or R482T)	No		Partial	No	No	Yes
FLV (W or R482T)	No	No		No	N.D.	Yes

W, wild type; R482T, BCRP R482T; No, no inhibition by competitor; Yes, inhibition by competitor, $P < 0.01$; Partial, partial (approximately 50%) inhibition by competitor, $P < 0.01$; ±, slight (approximately 20%) inhibition by competitor, $P < 0.05$; N.D., no data.

served "ABC signature" motif, located between the Walker A and B motifs of the nucleotide-binding domain. Of the 15 amino acids in the ABC signature consensus pattern, the sequence LSGGQ is highly conserved (Higgins, 1992). Within the LSGGQ sequence itself, the amino acids serine and glycine are highly conserved among ABC transporters in the pattern XSXGX. The LSGGQ motif in ABC transporters is thought to interact with the Walker A site of the opposing nucleotide binding domain and is displaced when ATP is hydrolyzed; hence, LSGGQ may be involved in a crucial step in drug or substrate transport (Loo et al., 2002). The functional significance of LSGGQ is further illustrated by the finding that missense mutations in codons corresponding to the highly conserved serine or glycine of the motif in the cystic fibrosis transmembrane conductance regulator gene cause the disease cystic fibrosis (Tsui, 1992). In the half-transporter BCRP/ABCG2, the sequence corresponding to the ABC signature LSGGQ is ^{186}VSGGE^{190}. Hence, we made two mutations of serine 187 in this signature motif of BCRP R482T, one to an amino acid that, like serine, has a polar side chain (threonine, S187T) and the other to a nonpolar side chain amino acid (alanine, S187A). The R482T mutant form of BCRP was used because it allowed us to monitor BCRP function by DNR accumulation. Both mutations of S187 were expressed well in *X. laevis* oocytes but had no transporter activity by DNR accumulation assays. This illustrates how crucial serine 187 is to the function of BCRP, because substitution of another polar amino acid (threonine) causes loss of transporter activity. To our knowledge, this is the first report of an inactivating mutation in the ABC signature motif of BCRP. Inactivation of BCRP function has been reported for mutations in the Walker A motif as measured by expression in Sf9 (Ozvegy et al., 2002) or PA317 cells (Kage et al., 2002) and regions of BCRP other than the ABC signature domain (Kage et al., 2002).

When the S187T mutation was coexpressed with BCRP R482T in the oocytes, the S187T mutant protein acted as would a dominant-negative inhibitor of BCRP transport of DNR, with the degree of inhibition increasing in proportion to the amount of S187T cRNA injected. This illustrates an advantage of the oocyte expression system for "dominant-negative" type studies, because precise amounts of inhibitor and native cRNA can be injected into the oocytes. Although it is possible that the inhibition caused by the S187A/T mutants was the result of the mutant saturating a limiting and essential oocyte cellular component crucial for protein transport or maturation, it is more likely that the inhibition was analogous to a "dominant-negative" effect of the S187T mutant. The latter is in agreement with previous observations of dominant-negative BCRP constructs possessing a mutation in the Walker A motif (K86M) (Ozvegy et al., 2002) or putative 5th transmembrane domain (L553P) (Kage et al., 2002), supporting the notion that the active form of BCRP is a homodimer.

In dominant-negative type studies, if one postulates that the inactive (I) and active (A) forms have exactly equal dimerization affinities and that the I:A heterodimer is not active, then a 50:50 mixture of I and A should result in a 75% reduction in transport activity (expected dimers are 25% I:I, 25% I:A, 25% A:I, and 25% A:A). In our studies, we observed only approximately 25% inhibition at equimolar ratios of I and A (24 ng of S147T injected), and the degree of inhibition of BCRP activity seemed to reach plateau at approximately 40% after the addition of 48 ng of the "dominant-negative" construct. This may have occurred because the affinity of A-to-A and I-to-I homodimeric partners may be greater than that of the S187T mutant-to-active BCRP heterodimeric partners. Alternatively, it is possible that the active/inactive heterodimer/multimer does possess some (albeit limited) transport activity. Because of injection volume constraints and the viscosity of highly concentrated cRNA solutions, we were technically unable to inject the oocytes with amounts of the S187T mutant form in excess of 72 ng.

Recent reports suggest that the efflux of one BCRP substrate can be inhibited by the presence of another BCRP substrate (Robey et al., 2001; Ozvegy et al., 2002). In the substrate interaction studies in BCRP expressing *X. laevis* oocytes presented here (Fig. 6 and Table 2), we found a significant inhibitory effect of FLV on BCRP-mediated MX transport, in good agreement with Robey et al. (2001); furthermore, FLV and MX significantly inhibited DNR accumulation in R482T-expressing oocytes. However, of the substrates studied reciprocally for competition (DNR, MX, or FLV), no two substrates caused mutual inhibition of transport of the other. The observation that known substrates frequently do not inhibit the transport of other substrates has been made for Pgp (Barecki-Roach et al., 2003). These investigators hypothesized that of compounds transported by Pgp, those with slow rates of cellular influx are good 'substrates' for Pgp, whereas those that rapidly permeate the plasma membrane may be more effective inhibitors than substrates (Barecki-Roach et al., 2003). Among cytotoxic compounds selected from the National Cancer Institute Drug Screen repository, many compounds were found to be either substrates or inhibitors of Pgp, but few compounds were found to be both, suggesting that multiple regions of Pgp are involved in drug transport (Scala et al., 1997). Distinct substrate binding sites designated 'H' or 'R' are described for Pgp; substrates that interact with the H site are competitive inhibitors of transport of each other but are allosteric activators of the transport of substrates that interact with the R site and vice versa (Garrigos et al., 1997; Shapiro and Ling, 1997; Shapiro et al., 1999). It is possible that BCRP also has multiple substrate interaction sites; however, because we did

not observe reciprocal inhibition of transport by any pair of the substrates tested, we are unable to designate distinct substrate binding sites at this time.

This study demonstrates the functional expression of human BCRP in *X. laevis* oocytes as a drug-extrusion pump, confirms that the sole expression of BCRP in this heterologous system can confer a pure BCRP phenotype, and shows for the first time the functional requirement for serine 187 in the ABC signature motif of BCRP. Further elucidation of the transport mechanism and substrate specificity of BCRP would not only be of biological interest in terms of cellular xenobiotic protective mechanisms in normal and cancer cells but might also impact BCRP substrate drug delivery and disposition.

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