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Characterization of the Role of ABCG2 as a Bile Acid Transporter in Liver and Placenta[S]

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ABSTRACT

ABCG2 is involved in epithelial transport/barrier functions. Here, we have investigated its ability to transport bile acids in liver and placenta. Cholylglycylamido fluorescein (CGamF) was exported by WIF-B9/R cells, which do not express the bile salt export pump (BSEP). Sensitivity to typical inhibitors suggested that CGamF export was mainly mediated by ABCG2. In Chinese hamster ovary (CHO cells), coexpression of rat Oatp1a1 and human ABCG2 enhanced the uptake and efflux, respectively, of CGamF, cholic acid (CA), glycoCA (GCA), tauroCA, and taurolithocholic acid-3-sulfate. The ability of ABCG2 to export these bile acids was confirmed by microinjecting them together with inulin in *Xenopus laevis* oocytes expressing this pump. ABCG2-mediated bile acid transport was inhibited by estradiol 17β-D-glucuronide and fumitremorgin C. Placental barrier for bile acids accounted for <2-fold increase in fetal cholanemia despite >14-fold increased maternal cholanemia induced by obstructive cholestasis in pregnant rats. In rat placenta, the expression of Abcg2, which was much higher than that of Bsep, was not affected by short-term cholestasis. In pregnant rats, fumitremorgin C did not affect uptake/secretion of GCA by the liver but inhibited its fetal-maternal transfer. Compared with wild-type mice, obstructive cholestasis in pregnant Abcg2(−/−) knockout mice induced similar bile acid accumulation in maternal serum but higher accumulation in placenta, fetal serum, and liver. In conclusion, ABCG2 is able to transport bile acids. The importance of this function depends on the relative expression in the same epithelium of other bile acid exporters. Thus, ABCG2 may play a key role in bile acid transport in placenta, as BSEP does in liver.

Introduction

Potentially toxic endogenous compounds, such as bile acids and biliary pigments, as well as many xenobiotics, such as drugs, toxins, and food components, are biotransformed and eliminated from the body mainly by the hepatobiliary system, with collaboration from the kidneys. The vectorial transport of these substances by hepatocytes involves sequential events, including their uptake across the basolateral plasma membrane, in some cases their metabolism, and their subsequent secretion into bile across the canalicular plasma membrane. The traffic of these compounds largely depends on the polarized expression of transport proteins.

The biliary secretion of bile acids or their conjugates with glucuronate or sulfate is carried out by efflux pumps belong-

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ABBREVIATIONS: ABC, ATP-binding cassette; BSEP, bile salt export pump; MDR, multidrug-resistance protein; MRP, multidrug resistance-associated protein; $E_2$17βG, estradiol 17β-D-glucuronide; FTC, fumitremorgin C; CA, cholic acid; GCA, glycocholic acid; TCA, taurocholic acid; TLCA, taurolithocholic acid; CGamF, cholylglycylamido fluorescein; TLCS, taurolithocholic acid-3-sulfate; wt, wild-type; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; Oatp1a1, organic anion-transporting polypeptide 1a1; Uo, uptake medium for transport studies with *X. laevis* oocytes; Uc, uptake medium for transport studies with mammalian cells; MS/MS, tandem mass spectrometry; HPLC, high-performance liquid chromatography.

ing to the superfamily of ATP-binding cassette (ABC) proteins. Among these, the bile salt export pump (BSEP, gene symbol *ABCB11*) is the major mechanism accounting for bile acid pumping into bile by hepatocytes (Gerloff et al., 1998). However, BSEP does not seem to play a major role in the transfer of these compounds in other organs, such as the placenta (Serrano et al., 2007). Other ABC transporters localized in the canalicular plasma membrane of hepatocytes are multidrug-resistance protein 1 or P-glycoprotein (MDR1, gene symbol *ABCB1*), multidrug resistance-associated protein 2 (MRP2, gene symbol *ABCC2*); and ABCG2 (also known as breast cancer resistance protein or BCRP). MDR1, which mediates the biliary secretion of hydrophobic cationic and amphipathic compounds, is not believed to be involved in the transport of bile acids (Müller et al., 1994). However, this concept has been challenged (Lam et al., 2005). MRP2 plays an important role in detoxification by transporting a wide range of compounds, mainly glutathione, sulfate, and glucuronate conjugates of lipophilic substances, including bilirubin and bile acids (Jedlitschky et al., 1997; Akita et al., 2001). MRP2 is also expressed at the apical membrane of other polarized cells, such as the placental syncytiotrophoblast (Jedlitschky et al., 2006), where this pump may play a similar role.

ABCG2 is a half-transporter that acts as a homodimer or homotetramer, and it is normally expressed in a wide variety of organs. In particular, ABCG2 is highly expressed in placental syncytiotrophoblasts (Allikmets et al., 1998; Maliepaard et al., 2001). Functional studies carried out over the last decade have indicated that ABCG2 can transport a highly diverse structural and functional range of organic substrates, including hydrophobic compounds, weak bases, organic anions, and glucuronate, sulfate, glutamylate, and glutathione conjugates of many endogenous and exogenous molecules (Robey et al., 2009). Because among the substrates of ABCG2 there are many chemotherapeutic agents, this protein, together with MDR1 and MRPs, is considered to be one of the most important ABC transporters accounting for multidrug resistance in cancer cells (Doyle et al., 1998; Robey et al., 2009).

Regarding endogenous compounds, previous studies have found the ability of ABCG2 expressed in mammalian cells to transport sulfated steroids (Imai et al., 2003; Suzuki et al., 2003). Evidence for the ability of this pump to recognize bile acids as substrates was obtained from experiments of ABCG2 expression in bacteria (Janvilisri et al., 2005). Indirect evidence suggest that orthologs of ABCG2 that are expressed in liver flukes of the genus *Fasciola* are also able to transport bile acids (Kumkate et al., 2008). In contrast, studies in P388 cells transfected with ABCG2 failed to show enhanced ability to transport taurocholate and taurolithocholate sulfate, although the latter was able to inhibit ABCG2-mediated estrone-3-sulfate transport (Suzuki et al., 2003). Moreover, using plasma membrane vesicles obtained from ABCG2 transfected cells, taurolithocholate, in both sulfated and nonsulfated forms, was able to inhibit ABCG2-mediated estrone-3-sulfate transport (Imai et al., 2003). In contrast, other authors have reported no ability of tauroursodeoxycholate to affect estrone-3-sulfate transport in plasma membrane vesicles obtained from *Sf9* cells expressing ABCG2 (Vaidya and Gerk, 2006). These findings raise the question of whether this pump is actually involved in the transport of major bile acid species across epithelia expressing this protein and involved in bile acids handling, such as in the placenta during intrauterine life and in the liver. In the present study, we have used five different experimental models to further investigate some aspects of this question.

Materials and Methods

Chemicals. Estradiol 17β-D-glucuronide ($E_2$17βG), fluorescein diacetate, fluorescein isothiocyanate, fumitremorgin C (FTC), Hoechst 33342, mitoxantrone, probenecid, sodium salts of cholic acid (CA), glycocholic acid (GCA), lithocholic acid-3-sulfate, taurocholic acid (TCA), taurolithocholic acid (TLCA), and verapamil were obtained from Sigma-Aldrich Quimica (Madrid, Spain). BODIPY-prazosin was from Invitrogen (Barcelona, Spain). [^{3}H]TCA (specific activity, 10 Ci/mmol), [^{3}H]$E_2$17βG (specific activity, 45.8 Ci/mmol), [^{3}H]mitoxantrone (specific activity, 10 Ci/mmol), and [^{14}C]taurine (specific activity, 55 mCi/mmol) were purchased from American Radiolabeled Chemicals (ITISA Biomedica, Madrid, Spain). [^{14}C]GCA (specific activity, 56 mCi/mmol) was from GE Healthcare (Barcelona, Spain). [^{3}H]Inulin (specific activity, 304.8 Ci/g), [^{14}C]inulin (specific activity, 2.2 mCi/g), and [^{3}H]CA (specific activity, 20 Ci/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA).

Synthesis of Bile Acids Derivatives. Cholylglycylamido fluorescein (CGamF) was synthesized by binding fluorescein isothiocyanate to GCA at the carboxyl group, as described previously (Sherman and Fisher, 1986). [^{14}C]Taurolithocholic acid-3-sulfate ([^{14}C]TLCS) sodium salt was synthesized from [^{14}C]taurine and lithocholic acid 3-sulfate by an improved method of synthesis of taurine-conjugated bile acids described by Momose et al. (1997). The reaction products were separated from the excess of unreacted [^{14}C]taurine by solid-liquid extraction in octadecylsilane cartridges. The retained compounds were recovered from the cartridges with ethanol. The extract was then evaporated and dissolved in methanol for thin-layer chromatography on silica gel plates using chloroform/methanol/acetic acid/water 64:25:15:2 (v/v) as an eluent system followed by staining with 10% phosphomolybdic acid in methanol at 110°C. The [^{14}C]TLCS band was scraped off and extracted with methanol. ^{1}H-NMR analyses and deamidation reaction with cholyl-glycine hydrolase (EC 3.5.1.24; Sigma-Aldrich) were performed to confirm the formation of the desired bond.

Animals. Pregnant rats on day 21 of gestation (300–350 g) from the University of Salamanca Animal House (Salamanca, Spain), FVB wild-type (wt) mice, and homozygous constitutive Abcg2 knockout [Abcg2(−/−)] mice (FVB.129P-Abcg2^{tm1Ahs}) from Taconic Farms (Germantown, NY), were used, along with mature female frogs (*Xenopus laevis*), purchased from Regine Olig (Hamburg, Germany). The animals received humane care as outlined in the National Institutes of Health guidelines for the care and use of laboratory animals (ftp://ftp.grants.nih.gov/IACUC/GuideBook.pdf). Experimental protocols were approved by the Ethical Committee for Laboratory Animals of the University of Salamanca.

Cloning of Human ABCG2. Total RNA was isolated from JAr cells derived from human choriocarcinoma (American Type Culture Collection by way of LGC Standards, Barcelona, Spain) using RNAeasy spin columns (QIAGEN, Madrid, Spain). cDNA was synthesized from RNA by reverse transcription with oligo-dT primers using the Cloned AMV First-Strand cDNA Synthesis kit (Invitrogen). This was followed by PCR, using high-fidelity AccuPrime *Pfx* DNA polymerase (Invitrogen) and two oligonucleotide primers specific to the sequence of GenBank accession number BC021281 [forward, 5′-TTA CAG TCG ACG AGC TCT ATT AAG CTG AAA AGA TAA-3′, containing at 5′ a SalI restriction site (underlined); antisense: 5′-GAC GGT GCG GCC GCG AAT ACT TCA ATC AAA GTG CTT C-3′, containing at 5′ a NotI restriction site (underlined)]. PCR was performed using thermal conditions as follows: 1 cycle at 95°C for 2 min; 40 cycles at 95°C for 15 s, 55°C for 30 s, and 68°C for 2.5

min; and 1 cycle at 68°C for 10 min. The PCR product was then 5′-phosphorylated with T4 polynucleotide kinase (Fermentas, St. Leon-Rot, Germany) and purified. 3′ A-overhangs were added using Taq DNA polymerase (Invitrogen). The resulting product was ligated into a pGEM-T easy vector (Promega, Madrid, Spain) using T4 DNA ligase (Promega). The full-length cDNA of ABCG2 was extracted from the recombinant plasmid pGEM/ABCG2 with SalI and NotI and subcloned into the pSPORT1 vector, which includes a T7 promoter appropriate for in vitro synthesis of cRNA.

To obtain the cDNA of human ABCG2 adapted for Gateway cloning, we used the high-fidelity AccuPrime *Pfx* DNA polymerase and two specific oligonucleotide primers containing *attB* adapters (forward, 5′-CTC TAT TAA GCT GAA AAG ATA A-3′; antisense, 5′-GAA TAT TTT TTA AGA AAT AAC AAT-3′). Thermal conditions were as follows: 1 cycle at 95°C for 5 min; 40 cycles at 95°C for 15 s, 51°C for 30 s, and 68°C for 2.5 min; and 1 cycle at 68°C for 10 min. The *attB*-flanked PCR product was recombined with the *attP*-containing pDONR207 vector (Invitrogen) to generate an entry plasmid, which was recombined with the pcDNA6.2-V5 destination vector (Invitrogen), containing the cytomegalovirus promoter and a C-terminal V5-tag, to generate the expression vector. The cDNA sequence of ABCG2 was confirmed to be identical to that reported for BC021281 by automated sequencing performed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystem, Madrid, Spain).

Transport of CGamF in WIF-B9 and WIF-B9/R Cells. The polarized cell line WIF-B9, derived from the fusion of rat hepatoma Fao cells with human fibroblast WI-38 cells, and a cisplatin-resistant subline, WIF-B9/R, selected by double subcloning from cultures of WIF-B9 cells continuously exposed to increasing concentrations of cisplatin up to 10 μM (Briz et al., 2003) were cultured with appropriate media in a humidified 5% CO_2/95% air atmosphere at 37°C. Uptake experiments were carried out as described elsewhere (Briz et al., 2007). In efflux experiments, cells grown on glass coverslips were incubated with the compounds at 37°C for 1 h, washed, and then placed in a thermostatically controlled holder and examined by inverted microscopy (LSM510; Zeiss, Jena, Germany). Cells were perfused with Tyrode-HEPES medium (144 mM NaCl, 5 mM KCl, 2 mM NaH_2PO_4, 1.25 mM $CaCl_2$, 1 mM $MgSO_4$, and HEPES 10 mM, pH 7.40) containing, or not, the inhibitors to be tested at 37°C. The flow rate was 1.5 ml/min, and the volume was maintained constant at 0.5 ml. Fluorescence images were collected with a charge-coupled device camera, digitized, and integrated in real time by an image processor (Metafluor; Princeton Instruments, Trenton, NJ). In the first image of the series of photographs, a region containing a cell, but not including the canalicular zone, was drawn, delimited by boundaries. The time course of the reduction of fluorescence in these regions was monitored by acquiring images at indicated intervals.

Transport Studies in CHO Cells. Wild-type Chinese hamster ovary (CHO) cells (CHO-K1, used as control) and cells (CHO-03) stably expressing the rat organic anion-transporting polypeptide 1a1 (Oatp1a1) were used. To obtain transiently ABCG2-transfected CHO-03 cells, at approximately 70% confluence, CHO-03 cells were exposed for 6 h to serum-free Opti-MEM (Invitrogen) containing Lipofectamine LTX (Invitrogen) and the vector pcDNA6.2-V5, where the cDNA of human ABCG2 had been cloned. The transfection efficacy was 35 and 37% as measured by immunofluorescence microscopy and flow cytometry, respectively. To induce protein expression, the culture medium was replaced by a fresh one containing 5 mM sodium butyrate 24 h before carrying out transport assays. Subconfluent cell cultures (2 days after transfection) were rinsed with uptake medium used with mammalian cells (Uc) (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose, and 50 mM HEPES, pH 7.40), and the cells were subsequently incubated in the presence of 10 μM bile acids at 37°C for 15 min. Uptake was stopped by rinsing the culture dishes four times with 1.5 ml of ice-cold Uc medium. Cells were digested in 1 ml of Lowry solution (100 mM NaOH and 189 mM Na_2CO_3) to determine substrate content, which was normalized according to protein concentrations (Markwell et al., 1978). To inhibit ABCG2, the cells were treated with 5 μM FTC for 15 min before adding the substrate and for the transport period (15 min) at 37°C. The same protocol was used for inhibition in flow cytometry assays described below.

Flow Cytometry Analyses. CHO-K1 (used as control), CHO-03, and CHO-03/ABCG2 cells were collected by trypsinization and suspended in prewarmed Uc medium in the absence of substrate at 37°C. The cells were incubated with 400 nM BODIPY-prazosin, 30 μM mitoxantrone, or 10 μM CGamF at 37°C for 15 min. To stop transport processes, ice-cold Uc medium was added, and intracellular drug contents were determined with a FACSCalibur cytometer (BD Biosciences, Madrid, Spain) in approximately 10^4 cells. The fluorescence of mitoxantrone, BODIPY-prazosin, and CGamF was measured.

Protein Detection by Immunofluorescence and Western Blot Assays. Immunofluorescence studies were carried out using wild-type CHO cells and noninjected oocytes, Oatp1a1/ABCG2-transfected CHO cells (48 h after transfection), and oocytes coexpressing Oatp1a1 and ABCG2 (48 h after microinjection of cRNA). Oocytes were fixed in 4% paraformaldehyde in phosphate-buffered saline, then immersed overnight in 30% sucrose in phosphate-buffered saline before being processed for immunofluorescence studies as described previously (Nakanishi et al., 2003). The samples (5-μm oocyte sections or whole mammalian cells) were permeabilized in ice-cold methanol for 1 min, and nonspecific binding sites were blocked by incubation with 5% fetal calf serum for 30 min at room temperature. Before the addition of primary antibodies, samples were washed twice with phosphate-buffered saline. Preparations were incubated at room temperature for 1 h with primary antibodies from Alexis Biochemicals (BXP-34 against ABCG2 diluted 1:20), Invitrogen (anti-V5 against ABCG2-V5 diluted 1:200), or the rabbit-anti Oatp1a1 antibody (diluted 1:250), and then for 1 h with a 1:1000 dilution of the appropriate Alexa Fluor-conjugated secondary antibody, and 4,6-diamidino-2-phenylindole to stain nuclei. Fluorescence staining was visualized using a Leica TCS SP2 confocal microscope. Crude membrane preparations were used to carry out immunoblotting analyses in 7.5% SDS-polyacrylamide gel. Rabbit polyclonal antibody anti-Oatp1a1 (K10), which was a generous gift from Dr B. Stieger (Zürich, Switzerland), and anti-ABCG2 mouse monoclonal antibody (BXP-21; Alexis Biochemicals) were used as primary antibodies. After incubation with appropriate IgG horseradish peroxidase-linked secondary antibody, immunodetection was performed with ECL (GE Healthcare).

Uptake and Efflux Studies in *X. laevis* Oocytes. Isolation and preparation of *X. laevis* oocytes were carried out as described elsewhere (Briz et al., 2002). Synthesis of cRNAs for injection into oocytes was performed using recombinant plasmids containing the open reading frame cDNA of the desired transport protein. The injected cells were maintained in modified Barth's solution at 18°C for 2 days (Briz et al., 2002).

In uptake experiments, incubations were carried out with 100 μl of uptake medium for oocytes (Uo) (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.50) containing the desired amount of substrate with or without inhibitors at 25°C for 1 h. In efflux studies, the oocytes were loaded directly by microinjection with 50 nl of Uo medium containing the desired amount of radiolabeled substrate, and its efflux was determined after transferring the oocytes to 1 ml of substrate-free Uo medium, where they were incubated at 25°C for 1 h. Transport processes were stopped by the addition of 4 ml of ice-cold Uo medium, and the oocytes were treated as described elsewhere (Briz et al., 2002). To measure the amount of Hoechst 33342, each oocyte was digested individually with 200 μl of lysis/extraction solution (10% SDS and 10 mM Tris, pH 7.4) containing 0.2 μmol of 5-carboxy-X-rhodamine as an internal standard of fluorescence and sonicated for 2 min. Then, cell lysates were mixed with 50 μl of isopropanol and centrifuged before measuring the fluorescence in the supernatant. When bile acids were microinjected, the magnitude of the leakage was evaluated by measuring the

efflux of radiolabeled inulin, which was coadministered by microinjection. Two different sets of experiments were carried out to investigate the inhibitory effect of $E_2 17\beta G$ on the ABCG2-mediated efflux of [^{14}C]TLCS from oocytes. 1) The *cis*-effect was investigated by directly microinjecting 50 nl of Uo medium containing the desired amount of [^{14}C]TLCS, either alone or together with $E_2 17\beta G$, before being transferred to 1 ml of substrate-free Uo medium. 2) In experiments designed to investigate the *trans*-effect, the oocytes were also directly microinjected with 50 nl of Uo medium containing the desired amount of [^{14}C]TLCS before being transferred to 1 ml of Uo medium with or without $E_2 17\beta G$. In these and other experiments performed in the present study, radioactivity was measured using the Ready Safe Scintillation Cocktail (Beckman Coulter España, Madrid, Spain) as scintillant.

TLCS contents in CHO cells and oocytes were determined by liquid chromatography-MS/MS (6410 Triple Quad LC/MS; Agilent Technologies, Santa Clara, CA). To accomplish this, samples were lysed in 50% or 90% methanol, respectively, containing 5 μM TCA (internal standard). Recovery from the lysis/extraction procedure, as calculated from TCA measurements, was 84 ± 2%.

In Vivo Experiments. Pregnant rats on day 21 of gestation were anesthetized with sodium pentobarbital (Nembutal N.R.; Abbott Laboratories, Madrid, Spain) (50 mg/kg body weight i.p.). When needed, polyethylene catheters were inserted into the maternal left jugular vein (for infusion) and the left carotid artery (for sampling). Bile flowing through a polyethylene catheter implanted into the common bile duct was collected in preweighed vials. In situ single-pass perfusion of rat placenta was carried out as described previously in detail (Macias et al., 2000). In brief, one of the placentas was exposed and perfused through the umbilical artery using a heparinized (0.05%) Tyrode-HEPES at 37°C at a constant inflow of 500 μl/min with a peristaltic pump. An incision in the umbilical vein was performed to permit free outflow. Perfusion pressure was measured as an indicator of placental perfusion resistance and was considered to be appropriate when it remained relatively constant and lower than 20 cm H_2O throughout the experimental period. To investigate the effect of ABCG2 inhibition by FTC on GCA transfer across the placenta-maternal liver tandem, two separate set of experiments were carried out in which GCA was administered as a bolus through the umbilical artery of one in situ perfused rat placenta (20 nmol) or the maternal jugular vein (2 nmol), alone or with FTC (50 or 5 nmol, respectively), as described elsewhere (Macias et al., 2000). To investigate the effect of blocking the maternal biliary secretion of bile acids on bile acid accumulation in maternal serum and fetal serum as well as on the placental expression of Bsep and Abgc2, complete obstruction of the maternal common bile duct was imposed on day 14 of gestation, and samples were collected on day 21, as described elsewhere (Serrano et al., 2003). Determination of rat Abcg2 and Bsep expression (Briz et al., 2007) and serum bile acid concentrations (Serrano et al., 2003) have been carried out as described previously.

To evaluate the importance of Abcg2 in bile acid transport across the placenta, wt and Abcg2(−/−) mice were used. At day 17 of pregnancy, immediately after bile duct and gallbladder ligation, 40 nmol of [^{14}C]GCA was administered through the inferior vena cava. Preliminary time-course study of radioactivity in serum and tissues revealed steady-state levels from at least min 30 to min 120 (data not shown). Accordingly, samples were collected 2 h after GCA administration. Total bile acids in maternal serum were determined enzymatically to calculate corrected specific radioactivity of [^{14}C]GCA, which was used to determine bile acid levels in maternal and fetal serum and tissues by radioactivity measurement as described previously (Vicens et al., 2007).

Statistical Methods. To calculate the statistical significance of differences among groups, the paired or unpaired *t* test or the Bonferroni method of multiple-range testing was used, as appropriate.

Results

Transport of Bile Acid Derivatives by ABCG2 in WIF-B9/R Cells. To investigate the ability of ABCG2 to transport bile acids we first used WIF-B9/R cells. As the parent WIF-B9 cells, WIF-B9/R cells display hepatocyte-like polarity and lack BSEP expression, but have the interesting characteristic, for the present study, of enhanced expression of ABCG2 (Briz et al., 2007). The fluorescent bile acid derivative CGamF was used as a potential ABCG2 substrate. Compared with WIF-B9 cells, the net uptake of fluorescein (Fig. 1A), a non–bile acid organic anion used here as a control, and CGamF (Fig. 1B) by WIF-B9/R cells was reduced. Moreover, the efflux from cells previously loaded with CGamF was significantly faster in WIF-B9/R than in WIF-B9 cells (Fig. 1C). When cells were incubated with fluorescein in the presence of verapamil (an inhibitor of MDR1), probenecid (an inhibitor of MRPs), Hoechst 33342 (a substrate of MDR1 and ABCG2), or TCA (a major bile acid), only probenecid enhanced the cellular accumulation of fluorescein (Fig. 1, D and G). However, the amount of CGamF in the cells was increased only in the presence of Hoechst 33342 (Fig. 1, E and H). Moreover, the CGamF content was decreased when cells were coincubated with TCA (Fig. 1, E and H). In cells preloaded with CGamF, both TCA and Hoechst 33342 were able to reduce CGamF efflux (Fig. 1, F and I). The effect of Hoechst 33342 was stronger than that of TCA in both cell lines (Fig. 1, F and I).

Bile Acid Transport in CHO Cells Coexpressing Oatp1a1 and ABCG2. To further investigate whether ABCG2 was able to export bile acids, we used as experimental model CHO cells transfected with ABCG2 cDNA. Moreover, to enhance bile acid load of CHO cells to better evaluate pump-mediated efflux, CHO cells permanently transfected with Oatp1a1 were used. Immunofluorescence analysis confirmed the presence of both Oatp1a1 and V5-tagged ABCG2 at the plasma membrane of CHO cotransfected cells (Supplemental Fig. 1, A–C). Western blot analyses revealed similar levels of Oatp1a1 with and without transfection with ABCG2 (Supplemental Fig. 1C), at least at 48 h, when the transport experiments were carried out. Decreased levels of Oatp1a1 mRNA detected by RT-PCR in ABCG2 transfected cells at 48 h (data not shown) suggest that the situation might not be the same at longer times.

The functionality of ABCG2 in these double-transfected CHO cells was investigated by flow cytometry analysis (Fig. 2A). Oatp1a1-expressing cells had a greater ability to take up CGamF than the wild-type cells (Fig. 2, A and B). The coexpression of ABCG2 with Oatp1a1 induced a significant reduction in the fluorescence detected in these cells. The expression of Oatp1a1 had no effect on the net uptake of BODIPY-prazosin and mitoxantrone, used here as typical ABCG2 substrates. However, expression of ABCG2 was able to significantly reduce the cell load of both compounds. When FTC, a specific ABCG2 inhibitor, was added, the ability of ABCG2 to reduce BODIPY-prazosin, mitoxantrone, and CGamF net uptake by these cells was almost abolished (Fig. 2, A–D).

Using this experimental model, we could not elucidate whether CGamF was exported itself or after being intracellularly biotransformed. Thus, to investigate whether major bile acids need to be sulfated or glucuronated to become

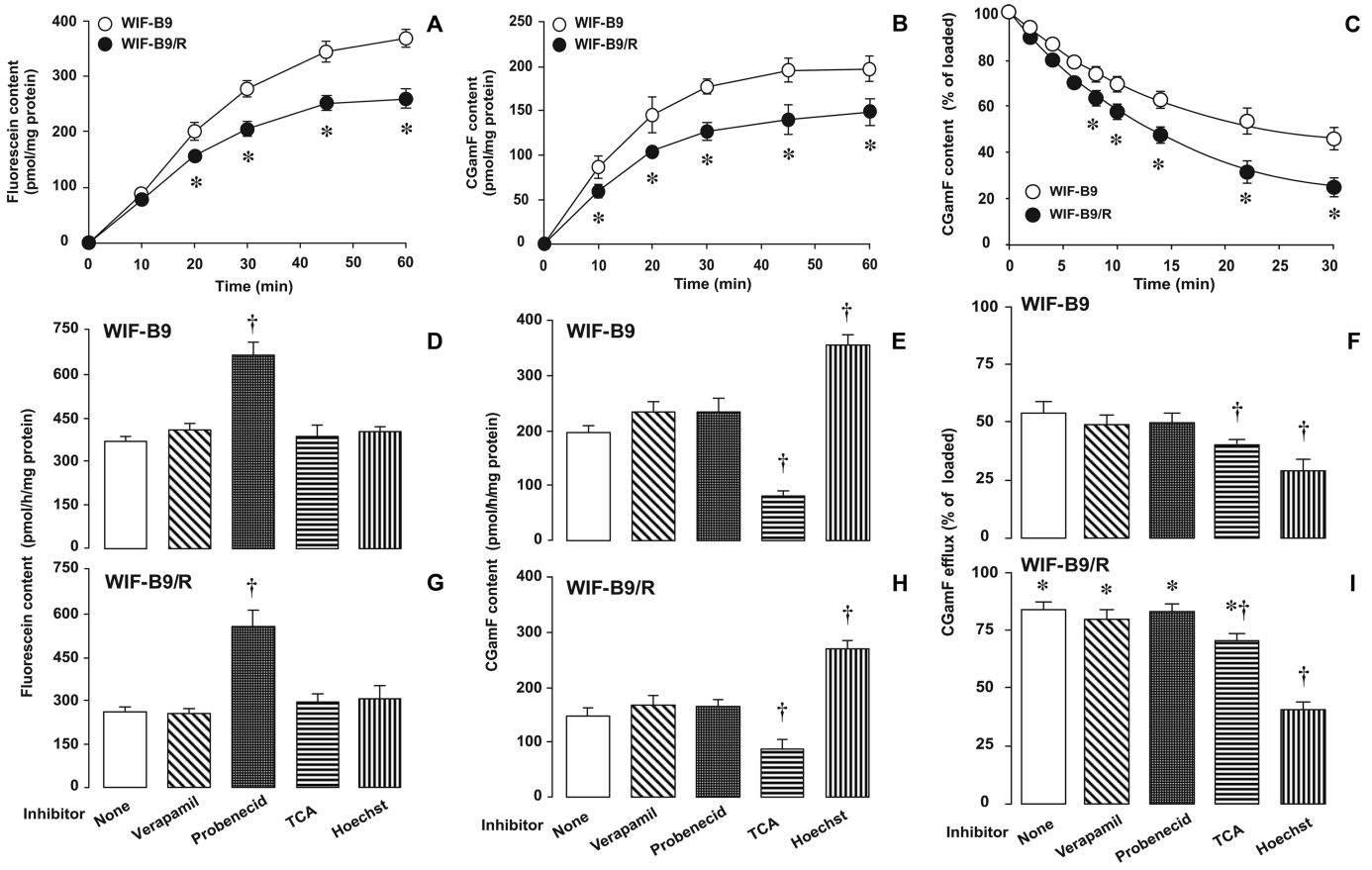

Fig. 1. Time course of fluorescein (A) and CGamF uptake (B) and CGamF efflux from preloaded cells (C) studied in wild-type WIF-B9 and cisplatin-resistant WIF-B9/R cells. Effect of 10 μM verapamil, 500 μM probenecid, 50 μM TCA, or 50 μM Hoechst 33342 on fluorescein (D and G) and CGamF (E and H) uptake and CGamF efflux (F and I). Cells were incubated with 10 μM fluorescein diacetate or 5 μM CGamF in the absence (none) or presence of the potential inhibitor at 37°C for 1 h. In efflux studies, preloaded cells in the absence of inhibitors were then placed in a thermostatically controlled holder on the stage of an inverted microscope and perfused with Tyrode-HEPES medium at 37°C containing the inhibitors to be tested. Values are means ± S.D. from three different cultures carried out in triplicate. *, $p < 0.05$ compared with WIF-B9 cells; †, $p < 0.05$ compared with results in the absence of inhibitor.

substrates of ABCG2, CHO cells, which are not expected to metabolize bile acids, were incubated with radiolabeled CA, TCA, and GCA. The uptake of these bile acid species was markedly enhanced when CHO cells expressed Oatp1a1 (Fig. 3, A–C), whereas it was significantly reduced by coexpression with ABCG2. In all cases, the presence of FTC did not affect Oatp1a1-mediated uptake but induced a significant inhibition of ABCG2-mediated efflux (Fig. 3, A–C).

Using a similar experimental design and TLCS as a model compound, we investigated the ability of ABCG2 to export sulfated bile acids. However, to differentiate between export and desulfation + export processes, instead of using a radioactivity-based method that would measure sulfated and desulfated forms together, unlabeled TLCS was used, and intracellular contents were measured by HPLC-MS/MS analysis. The coexpression of ABCG2 in CHO cells expressing Oatp1a1 induced a significant decrease in net uptake of TLCS, which was inhibited in the presence of FTC (Fig. 3D). In these cells, generation of TLCA from desulfation of TLCS was not found in any experimental group (data not shown).

Bile Acid Efflux by ABCG2 Expressed in *X. laevis* Oocytes. To further investigate the ability of ABCG2 to transport bile acids in a different experimental model, additional experiments were carried out in *X. laevis* oocytes expressing ABCG2. The transport activity of ABCG2 expressed in oocytes was confirmed upon using Hoechst 33342 and mitoxantrone, which enter the cells by simple diffusion. The expression of ABCG2 significantly reduced the net contents of both compounds (Figs. 4, A and B). The presence of FTC markedly inhibited this ability (Fig. 4, A and B).

To carry out similar experiments with bile acids Oatp1a1 was coexpressed to enhance the uptake of these compounds by the oocytes. Immunofluorescence analyses revealed a good coexpression of Oatp1a1 and ABCG2 at the plasma membrane of oocytes microinjected with cRNA of both transporters 48 h before carrying out the experiments (Supplemental Fig. 1, D–F). However, Western blot analyses revealed that the amount of proteins was affected by coexpression (Supplemental Fig. 1F). The net uptake of TLCS (Fig. 4C), as well as CA, GCA, and TCA (data not shown), reduced in oocytes expressing Oatp1a1 together with ABCG2 (Fig. 4C). This could, therefore, be due to a lower expression of Oatp1a1. However, incubation with FTC partly restored bile acid net uptake, which suggested that ABCG2 was also involved in the reduction of bile acid content. The results were consistent with findings in CHO cells described above and with the existence of either a direct efflux of the added compounds or the export of sulfated derivatives of the bile acids formed in these cells. To elucidate this question, we investigated the ability of *X. laevis* oocytes to carry out bile acid sulfation.

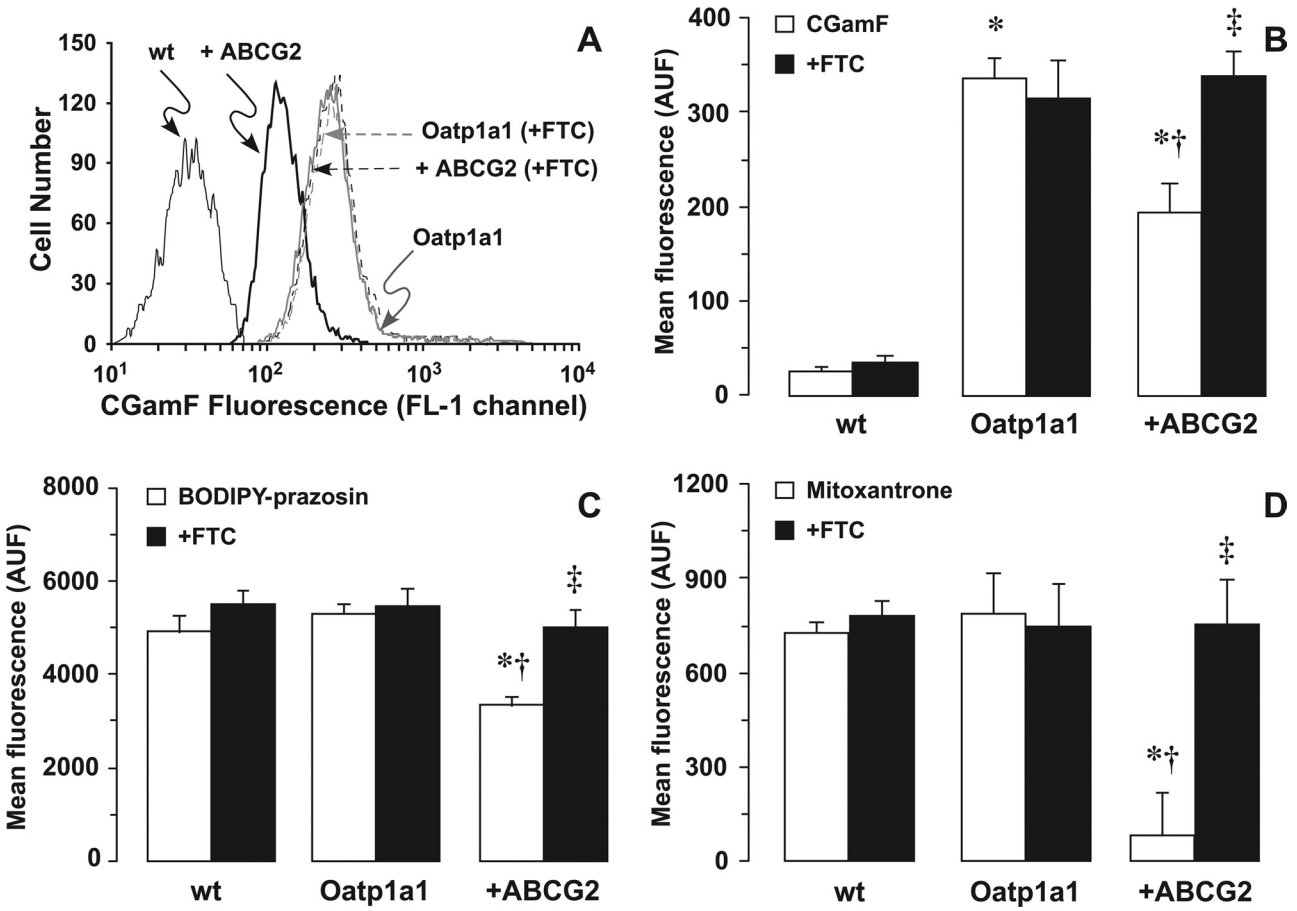

Fig. 2. Transport of typical ABCG2 fluorescent substrates and CGamF by wt CHO-K1 cells and cells expressing Oatp1a1 alone (Oatp1a1) or together with ABCG2 (+ABCG2). These cells were incubated with 10 μM CGamF (A and B), 400 nM BODIPY-prazosin (C), or 30 μM mitoxantrone (D) in the presence or the absence of 5 μM FTC at 37°C for 15 min. A representative frequency histogram corresponding to CGamF fluorescence after 15-min incubation of the cells at 37°C with this compound is depicted in A. Values shown in B to D are means ± S.D. of the mean fluorescence values (AUF, arbitrary units of fluorescence) obtained by flow cytometry analysis from at least three determinations per data point using cells from three different cultures. *, $p < 0.05$ compared with wt cells; †, $p < 0.05$ compared with Oatp1a1-expressing cells; ‡, $p < 0.05$ comparing cells incubated with and without FTC.

Oocytes expressing Oatp1a1 alone were incubated with TLCA, and both the medium and the lysate of oocytes was analyzed by HPLC-MS/MS. The results indicated that only 1.7% of the TLCA loaded had been transformed into TLCS (0.8 pmol · h^{-1} · $oocyte^{-1}$) (data not shown). Therefore, the efflux of bile acids observed in ABCG2-expressing oocytes (>5 pmol · h^{-1} · $oocyte^{-1}$ for CA, GCA, and TCA) (data not shown) cannot be accounted for by the export of sulfated derivatives but was mainly due to the transport of their nonsulfated forms.

To directly observe bile acid efflux in the absence of coexpression interferences, the amount of bile acid was determined in oocytes at different times after microinjection of [^{3}H]CA, [^{14}C]GCA, [^{3}H]TCA, or [^{14}C]TLCS together with radiolabeled inulin in the presence or the absence of FTC. A significantly faster progressive decrease of [^{14}C]TLCS content in cells expressing Bsep or ABCG2 was observed (Fig. 5A), whereas [^{3}H]inulin content (used here as a negative control) was only slightly modified in all groups along the experimental period (Fig. 5A). When the net efflux (content at min 0 min versus min 60) after incubation in bile acid-free medium for 1 h of TCA, GCA, and CA was measured, an endogenous component of bile acid export was found that was markedly higher than that found for TLCS. However, the expression of ABCG2 was able to further enhance the export of these bile acids, which was abolished in the presence of FTC (Fig. 5B).

Finally, we analyzed the effect of $E_2$17βG on the TLCS efflux mediated by Bsep- or ABCG2-expressing oocytes preloaded with the substrate (with or without inhibitor) by microinjection (Fig. 6). An inhibitory effect of $E_2$17βG on Bsep- and ABCG2-mediated TLCS efflux when $E_2$17βG was placed either extracellularly (*trans*-effect) or in the intracellular medium (*cis*-effect) was found.

In Vivo Experiments. To investigate the relative importance of Abcg2 inhibition in the transport of bile acids by the liver and placenta, we administered [^{14}C]GCA with and without FTC through the jugular vein of anesthetized pregnant rats or the umbilical artery of a single-pass perfused placenta. In these experiments, when drug administration was carried out through the umbilical artery, the same proportions of substrate and inhibitor were used, but concentrations were 10-fold higher. The reason was that in previous studies, we have found that in this experimental model, approximately only 10% of perfused bile acid crosses the placenta during the single pass. When administered through the jugular vein, [^{14}C]GCA secretion into bile was not significantly lower than that observed in absence of FTC (Fig. 7A).

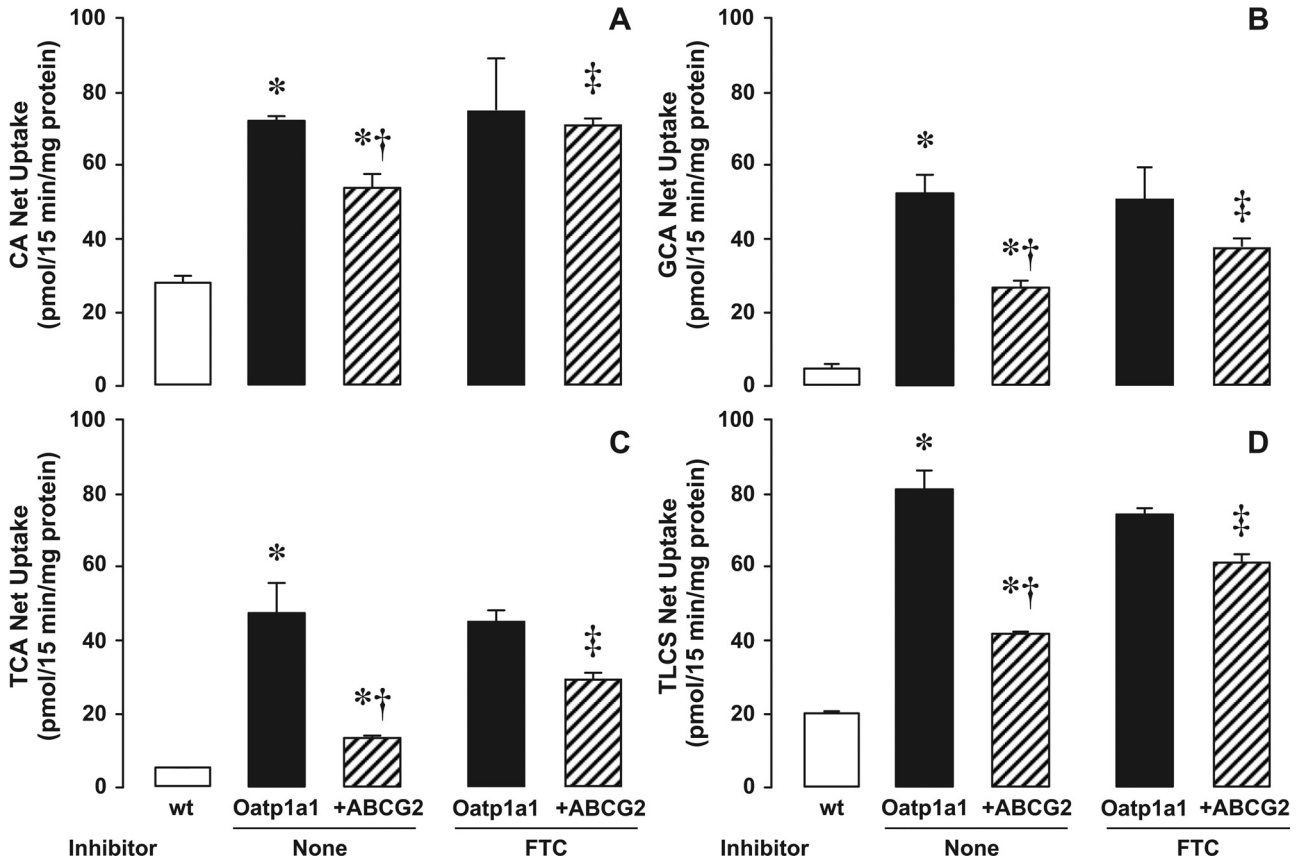

Fig. 3. Net bile acid uptake by wt CHO-K1 cells and cells expressing Oatp1a1 alone (Oatp1a1) or together with ABCG2 (+ABCG2). Cells were incubated with 10 μM [^{3}H]CA (A), [^{14}C]GCA (B), [^{3}H]TCA (C), or TLCS (D) in the presence or the absence of 5 μM FTC at 37°C for 15 min. Bile acid content was determined by radioactivity measurement except for TLCS, in which case it was determined by HPLC-MS/MS. Results are means ± S.D. from at least three determinations per data point using cells from three different cultures. *, $p < 0.05$ compared with wt; †, $p < 0.05$ compared with Oatp1a1-expressing cells; ‡, $p < 0.05$ comparing ABCG2-expressing cells incubated with and without FTC.

In contrast, when both compounds were given through the umbilical artery, a marked reduction in placental transfer of [^{14}C]GCA—and the subsequent secretion into bile—was observed (Fig. 7B).

To evaluate the relevance of Abcg2 in placental barrier for bile acids, we measured serum bile acids in mothers and fetuses after a week of obstructive cholestasis. The expression of Abcg2 in rat placenta was much higher than that of Bsep (Table 1). Moreover, the level of mRNA of Abcg2 was not impaired by obstructive cholestasis (Table 1). These findings contrast with the reported decreased expression of ABCG2 in the duodenum of patients with obstructive cholestasis (Zimmermann et al., 2006). Recent studies in Abcg2 knockout mice suggest that Abcg2 does not have a significant role in the adaptive response to cholestasis in the liver as may happen in the kidney and intestine (Mennone et al., 2010). The stability of placental Abcg2 expression during cholestasis observed here probably justifies that only a moderate increase (<2-fold) in fetal serum bile acid concentrations was found despite these serum bile acid concentrations were markedly increased (>14-fold) in the mothers (Table 1).

To further evaluate the importance of ABCG2 in the placental barrier for bile acids, we used Abcg2(−/−) mice. Bile duct ligation followed by intravenous administration of radiolabeled GCA to pregnant mice permitted the attainment of a steady state in radioactivity distribution in maternal and fetal tissues that lasted from at least min 30 to min 120 after administration (data not shown). Combination of radioactivity measurements and total bile acid determination revealed that, although a tendency toward lower levels in Abcg2(−/−) mice was observed, there was no significant difference between wild-type and Abcg2(−/−) pregnant mice regarding serum bile acid concentrations or the amount of these compounds in liver and kidney (Fig. 8). In contrast, in fetuses, markedly higher levels of bile acids in serum (3021%), liver (1091%), and placenta (528%), but not in kidney (99%), were found in Abcg2(−/−) mice (Fig. 8).

Discussion

The present study provide strong evidence for the ability of ABCG2 to transport both sulfated and nonsulfated bile acids, for the role of this pump in the placental barrier for bile acids, and for its sensitivity to the inhibition by cholestatic steroids, such as $E_2 17\beta G$, also able to inhibit BSEP-mediated bile acid secretion into bile.

WIF-B9 cells were used as a first approach because these cells form canaliculus-like structures able to concentrate CGamF. The uptake of CGamF by rat hepatocytes and its biliary secretion closely resemble that of the parent bile acid moiety GCA. CGamF has been suggested to be a substrate of BSEP (Mita et al., 2006). However, despite an almost complete absence of BSEP expression in WIF-B9 cells (Wakabayashi et al., 2004; Briz et al., 2007) they are able to extrude

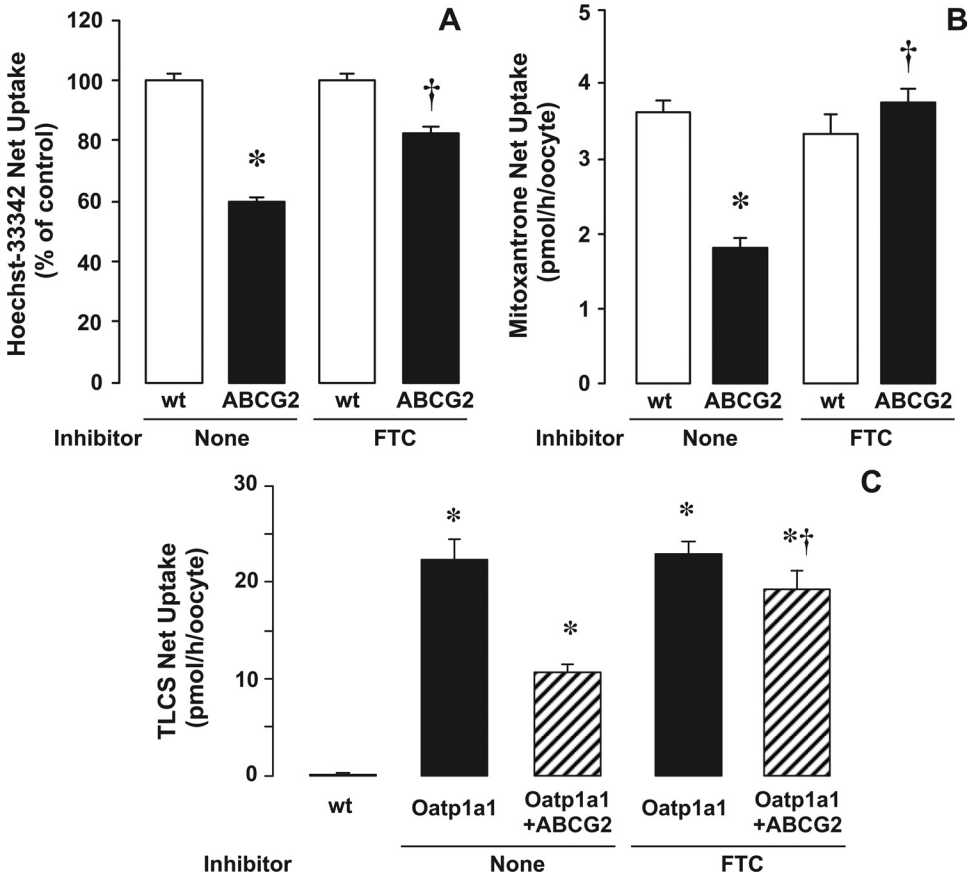

Fig. 4. Functional expression of human ABCG2 alone or coexpressed with rat Oatp1a1 in *X. laevis* oocytes. Oocytes were incubated with 200 μM Hoechst 33342 (A), 10 μM [^{3}H]mitoxantrone (B), or 50 μM TLCS (C) in the presence or the absence of 5 μM FTC at 25°C for 1 h. TLCS content in oocytes was determined by HPLC-MS/MS. Values are means ± S.D. from 20 to 30 determinations per data point using oocytes from three different frogs. *, $p < 0.05$ compared with wt cells; †, $p < 0.05$ on comparing ABCG2-expressing oocytes incubated with or without FTC by the paired t test.

CGamF. This interesting characteristic is maintained in WIF-B9/R cells (Briz et al., 2007). Moreover, we have observed here that WIF-B9/R cells were more efficient in reducing intracellular levels of CGamF than WIF-B9 cells. These findings imply that other pumps expressed in these cells [i.e., rat Mdr1, rat Mrp2 and human ABCG2 and rat Abcg2, which are up-regulated in WIF-B9/R (Briz et al., 2007)] must account for CGamF export from these cells. Studies carried out using typical selective inhibitors revealed that verapamil, a well known inhibitor of MDR1, had no effect on CGamF efflux. Probenecid, which is able to inhibit several MRP isoforms, also had no effect. In contrast, Hoechst 33342, which can be transported by both MDR1 and ABCG2, reduced CGamF efflux. This, together with the absence of an effect of verapamil, suggested that CGamF efflux from WIF-B9 and WIF-B9/R cells was mainly mediated by Abcg2/ABCG2. In this experimental model, TCA had a strong effect on net CGamF content in uptake studies, whereas it had a mild effect in assays of CGamF efflux from preloaded cells. To elucidate whether this was due to a dual inhibitory effect on the uptake and efflux processes involved in determining net CGamF contents, a different experimental model was required.

Thus, we used CHO cells expressing human ABCG2. The expression of this pump induced a significant reduction in the cell contents of typical ABCG2 substrates such as BODIPY-prazosin and mitoxantrone. Likewise, the ability to efficiently take up CGamF that had been induced in CHO cells by expressing rat Oatp1a1, which is known to carry out bile acid uptake efficiently (Jacquemin et al., 1994), was markedly reduced by ABCG2 coexpression. Under our experimental conditions, this was not due to a reduced abundance of Oatp1a1 protein when ABCG2 was coexpressed. ABCG2-mediated export, which was sensitive to the specific inhibitor FTC, was not restricted to bile acid derivatives (i.e., CGamF and TLCS) but was extended to major bile acids, both unconjugated and conjugated with glycine or taurine.

To further confirm the ability of ABCG2 to transport bile acids and to investigate its sensitivity to the presence of steroids that induce cholestasis, we expressed ABCG2 in *X. laevis* oocytes. In these cells, ABCG2 behaved as a functional export pump able to enhance the efflux of its typical substrates, Hoechst 33342 and mitoxantrone. Because Hoechst 33342 is taken up by simple diffusion and mitoxantrone mainly by means of a flip-flop mechanism, there was no need in these experiments to increase the uptake of these compounds before evaluating ABCG2-mediated efflux. In contrast, as discussed above for CHO cells, bile acids were poorly taken up by wild-type cells (Fig. 4C), hence the export activity of ABCG2 could not be detected (data not shown). Coinjection of Oatp1a1 and ABCG2 mRNA permitted enhanced bile acid uptake and hence detected the ability of ABCG2 to export sulfated and nonsulfated bile acids. However, two factors seemed to be involved in the reduction of bile acid content in coinjected oocytes: 1) a reduced uptake due to lower Oatp1a1 expression and 2) an enhanced ABCG2-mediated and FTC sensitive bile acid export. This was further confirmed by efflux assays after direct injection of the substrate into oocytes expressing ABCG2 alone.

Several studies have demonstrated that sulfated steroids inhibit the ABCG2-mediated transport of drugs, such as mitoxantrone and camptothecins (Imai et al., 2002), and phys-

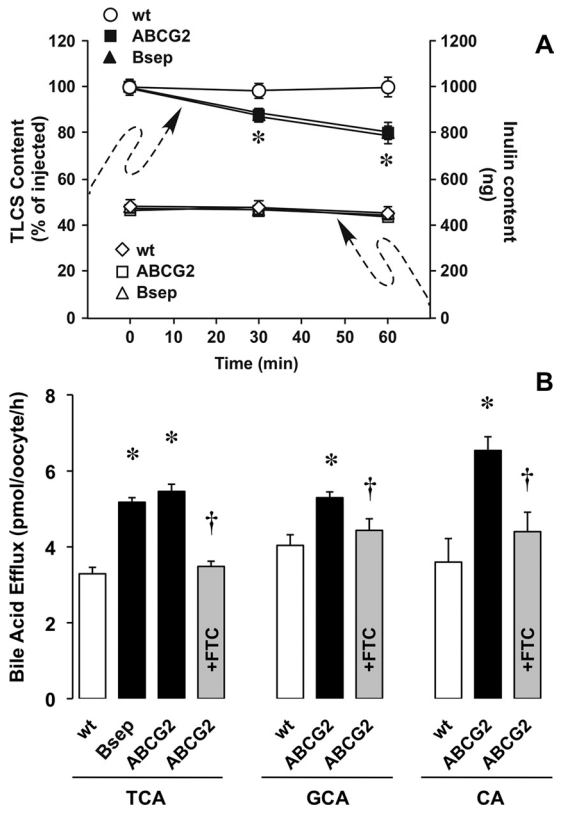

Fig. 5. Time course of [^{14}C]TLCS efflux from oocytes previously loaded by microinjection. Wild-type oocytes and oocytes expressing human ABCG2 or rat Bsep were microinjected with 50 nl of a solution containing 100 μM [^{3}H]inulin and 300 μM [^{14}C]TLCS before being incubated in substrate-free medium (A). Similar experiments were carried out injecting [^{3}H]CA, [^{14}C]GCA, or [^{3}H]TCA and measuring the efflux (content at min 0 versus min 60) after incubation in bile acid-free medium at 25°C for 1 h in the presence or the absence of 5 μM FTC (B). Values are means ± S.D. from 20 to 30 determinations per data point using oocytes from three different frogs. *, $p < 0.05$ compared with wt cells; †, $p < 0.05$ on comparing ABCG2-expressing oocytes incubated with or without FTC.

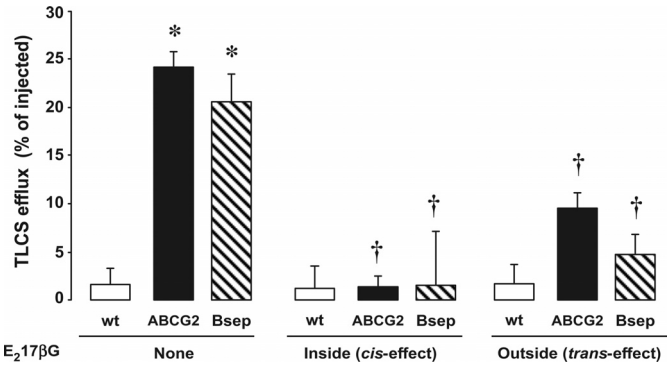

Fig. 6. Effect of $E_2$17βG on ABCG2-mediated efflux of TLCS from *X. laevis* oocytes. Oocytes expressing ABCG2 or Bsep and wt cells were microinjected with 50 nl of solution containing 150 μM [^{14}C]TLCS alone or together with 750 μM $E_2$17βG (*cis*-effect) before being incubated in the absence or the presence of 50 μM $E_2$17βG (*trans*-effect) at 25°C for 1 h. Values are means± S.D. from at least 10 determinations per data point using oocytes from two different frogs. *, $p < 0.05$ compared with wt cells; †, $p < 0.05$ on comparing with efflux in the absence of $E_2$17βG.

iological substrates, such as estrone-3-sulfate (Imai et al., 2003). Moreover, it has been described that $E_2$17βG is able to inhibit Bsep-mediated bile acid transport (Stieger et al., 2000; Vallejo et al., 2006), which may account for its choles-

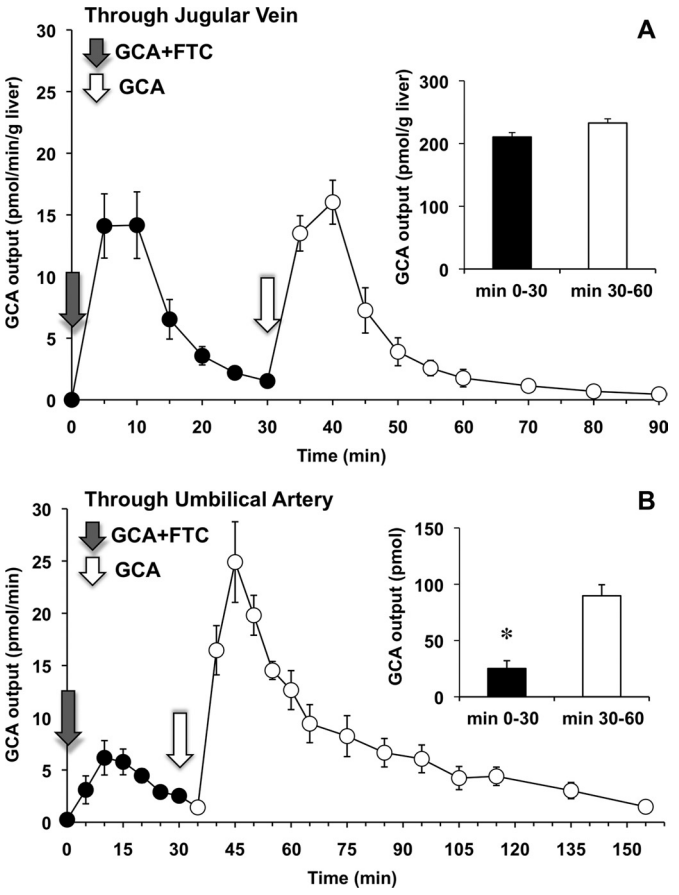

Fig. 7. Effect of FTC on [^{14}C]GCA secretion into rat bile when this bile acid was administered as a bolus alone or together FTC through the jugular vein of anesthetized pregnant rats (A) or the umbilical artery of in situ perfused placentas (B). Cumulative output over 30 min periods after bolus administration is depicted in the insets. Values are means ± S.D. from five different animals. *, $p < 0.05$ compared with and without FTC.

TABLE 1

Effect of obstructive cholestasis during pregnancy in rats on serum bile acid concentrations in mothers and fetuses and expression levels of Abcg2 and Bsep in placenta

Cholestasis was induced surgically in pregnant rats by complete obstruction and section of the common bile duct on day 14 of pregnancy. Samples were collected on day 21 of pregnancy. Steady-state mRNA levels were measured in triplicate by real-time quantitative reverse transcription-PCR and normalized by the 18S rRNA content in the same sample. Data are presented as mean ± S.D.

	Serum Bile Acids		Placental mRNA Level	
	Mothers	Fetuses	Bsep	Abcg2
	μM (n)		*% of adult male rat liver (n)*	
Control	19 ± 3 (6)	25 ± 3 (6)	1.7 ± 0.3 (12)	160 ± 29 (12)
Cholestasis	273 ± 46 (6)[a]	40 ± 6 (6)[a]	2.1 ± 0.6 (12)	131 ± 28 (12)

[a] $P < 0.05$ compared with control group by Student's *t* test.

tatic effect when administered to isolated perfused rat liver (Vallejo et al., 2006). In the present study, we observed the ability of $E_2$17βG to inhibit ABCG2 both from inside (*cis*-effect versus substrate location) and outside (*trans*-effect) the cells. The *cis*-effect could be due to the interaction of $E_2$17βG as a substrate of this transporter. Controversial results regarding the ability of ABCG2 to transport $E_2$17βG have been reported (Imai et al., 2003; Suzuki et al., 2003; Grube et al., 2007). The findings of the present study (data not shown) are consistent with ABCG2-mediated $E_2$17βG transport; hence it

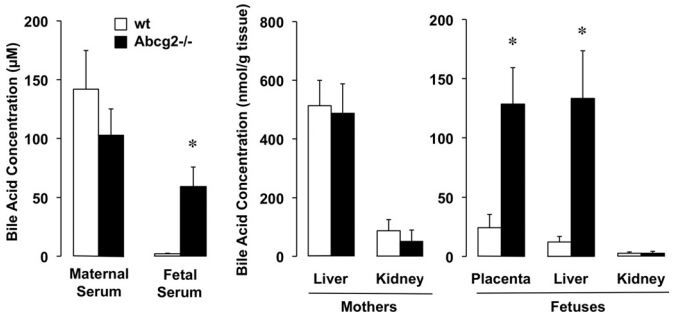

Fig. 8. Bile acid distribution in maternal and fetal serum and tissues after administering 40 nmol of [^{14}C]GCA immediately after bile duct and gallbladder ligation in wt and Abcg2(−/−) mice at day 17 of pregnancy. Samples were collected 2 h after GCA administration. Values are means ± S.D. from five different pregnant mice and 15 conceptuses per group. *, $p < 0.05$ compared with wt.

may be possible that $E_2$17βG could compete with other substrates from inside the cell. The *trans*-inhibitory effect might be similar to that of chemosensitizers able to circumvent ABC protein-dependent resistance to anticancer chemotherapy, as has been discussed elsewhere (Vallejo et al., 2006).

Considering the situation in the bile canaliculi, the present results indicate that in the absence of BSEP expression or function, a certain degree of bile acid secretion is expected to occur through alternative transporters, such as ABCG2 and perhaps MDR1 (Lam et al., 2005). This is what actually happens in Bsep knockout mice (Wang et al., 2001) and in patients suffering from progressive familial intrahepatic cholestasis type 2 as a result of inactivating mutations in the *ABCB11* gene (Strautnieks et al., 1998). In both cases, however, alternative bile acid exporting activity is probably insufficient to maintain bile acid secretion at physiological rates, and cholestasis appears (Strautnieks et al., 1998; Wang et al., 2001). This is consistent with the results obtained here using in vivo models. Inhibition of Abcg2 in rat liver by FTC induced a nonsignificant reduction in liver uptake/secretion of intravenously administered GCA. Moreover, lack of this pump does not result in enhanced levels of bile acids in the livers of Abcg2(−/−) mice (data not shown).

Kinetic studies in different models to compare K_m and hence the efficacy of transport for different transporters and bile acid species would help us to understand the role of ABCG2 versus other pumps under physiological and pathological circumstances. However, the results of the present study suggest a similar ability of ABCG2 and BSEP to transport bile acids. Accordingly, the different physiological role of these pumps in bile acid transport would probably be due to the relative expression of both genes. Regarding the absolute abundance of mRNA, the proportion of ABCG2 to BSEP in human liver has been found to be approximately 1 to 100 (Serrano et al., 2007). Thus, although the contribution of ABCG2 to bile acid secretion into bile is possible, its magnitude under physiological circumstances is probably very low. Moreover, when cholestasis is due to the inhibitory effect of steroids, such as $E_2$17βG, ABCG2 cannot compensate BSEP inhibition because ABCG2 is itself also sensitive to these compounds.

The situation may well be very different in the placenta. Since the existence of ATP-dependent mechanisms for bile acid transport across the apical membrane of human trophoblast was described previously (Bravo et al., 1995; Marin et al., 1995), the actual transporter accounting for this process has been sought (Macias et al., 2009). BSEP, in principle the main candidate, seems to be poorly expressed in human placenta (Serrano et al., 2007). The expression in human placenta of MRP2 and MRP4, also able to transport bile acids and bile acid derivatives, is also low (Serrano et al., 2007). Although MRP3 is expressed in placenta in the same order of magnitude as in the liver (Serrano et al., 2007), this is a basolateral transporter. In contrast, ABCG2 is highly expressed at the apical membrane of the trophoblast. The dramatic reduction in bile acid transfer across the rat placenta observed here when Abcg2 was inhibited by FTC and the marked reduction of the placental barrier for bile acids in Abcg2(−/−) pregnant mice provides strong evidence for an important role of this protein in the handling of bile acids by the trophoblast.

In conclusion, these results indicate that ABCG2 is able to transport sulfated and nonsulfated bile acids. The physiological relevance of this ability depends upon the relative expression in the same epithelium of ABCG2 and another bile acid transporters. Thus, ABCG2 may play a key role in bile acid transport in placenta, as BSEP does in liver.

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Authorship Contributions

Participated in research design: Blazquez, Briz, Cassio, and Marin.

Conducted experiments: Blazquez, Briz, Romero, Rosales, Monte, Vaquero, Macias, and Cassio.

Contributed new reagents or analytic tools: Blazquez, Romero and Cassio.

Performed data analysis: Blazquez, Briz, Romero, Rosales, Monte, Vaquero, Macias, Marin.

Wrote or contributed to the writing of the manuscript: Blazquez, Briz, Cassio, and Marin.

Other:

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