A functional study on polymorphism of the ATP-binding cassette transporter ABCG2: critical role of arginine-482 in methotrexate transport

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Overexpression of the ATP-binding cassette transporter ABCG2 reportedly causes multidrug resistance, whereas altered drug-resistance profiles and substrate specificity are implicated for certain variant forms of ABCG2. At least three variant forms of ABCG2 have been hitherto documented on the basis of their amino acid moieties (i.e., arginine, glycine and threonine) at position 482. In the present study we have generated those ABCG2 variants by site-directed mutagenesis and expressed them in HEK-293 cells. Exogenous expression of the Arg^{482}, Gly^{482}, and Thr^{482} variant forms of ABCG2 conferred HEK-293 cell resistance toward mitoxantrone 15-, 47- and 54-fold, respectively, as compared with mock-transfected HEK-293 cells. The transport activity of those variants was examined by using plasma-membrane vesicles prepared from ABCG2-overexpressing HEK-293 cells. [Arg^{482}]ABCG2 transports [^{3}H]methotrexate in an ATP-dependent manner; however, no transport activity was observed with the other variants (Gly^{482} and Thr^{482}). Transport of methotrexate by [Arg^{482}]ABCG2 was significantly inhibited by mitoxantrone, doxorubicin and rhodamine 123, but not by *S*-octylglutathione. Furthermore, ABCG2 was found to exist in the plasma membrane as a homodimer bound via cysteinyl disulphide bond(s). Treatment with mercaptoethanol decreased its apparent molecular mass from 140 to 70 kDa. Nevertheless, ATP-dependent transport of methotrexate by [Arg^{482}]ABCG2 was little affected by such mercaptoethanol treatment. It is concluded that Arg^{482} is a critical amino acid moiety in the substrate specificity and transport of ABCG2 for certain drugs, such as methotrexate.

Key words: ATP-binding cassette transporter (ABC transporter), ABCG2, acquired mutation, methotrexate, multidrug resistance, single nucleotide plymorphism.

INTRODUCTION

Individual variations in response to a drug originate from different causes, such as genetic polymorphism and altered expression levels of drug target molecules (e.g. membrane receptors, nuclear receptors and enzymes), as well as those of drug-metabolizing enzymes and drug transporters [1]. To achieve the much-talked-about 'personalized medicine', it is critically important that we understand the molecular mechanisms and functions underlying such variations in drug response.

Cancer is one of the gene-associated diseases, with multiple factors involved in its cause and progression [2]. Despite enormous costs and efforts spent on the development of cancer chemotherapies, anticancer drugs are often effective only in a relatively small proportion of cancer patients. It has long been recognized that the effectiveness of anticancer drugs can vary significantly among individual patients. Indeed, acquired and intrinsic drug resistance in cancer is the major obstacle to long-term, sustained patient response to chemotherapy.

There is accumulating evidence that active export of anticancer drugs from cancer cells is one of the major mechanisms of drug resistance. Several ATP-binding cassette (ABC) transporters underlie multidrug resistance in cancer cells by actively extruding the clinically administered chemotherapeutic drugs. Two major ABC transporters, ABCB1 (P-glycoprotein or MDR1) and ABCC1 (MRP1), have been well studied in terms of their structure and function in cancer drug resistance [3–8]. In addition, a novel ABC transporter, breast-cancer-resistant protein (BCRP), has recently been discovered in doxorubicin-resistant breast-cancer cells [9]. The same transporter has also been found in human placenta [10] as well as in drug-resistant cancer cells selected by using mitoxantrone and DNA topoisomerase I inhibitors [11–18]. The newly found ABC transporter protein is now named ABCG2 and is classified in the G-subfamily of human ABC transporter genes according to the new nomenclature. ABCG is a so-called 'half-transporter' bearing six transmembrane domains and one ATP-binding cassette.

Overexpression of ABCG2 reportedly confers cancer-cell resistance to camptothecin-based anticancer drugs such as mitoxantrone, topotecan and 7-ethyl-10-hydroxycamptothecin (SN-38: active metabolite of irinotecan). SN-38-selected PC-6/SN2-5H human lung-carcinoma cells were shown to overexpress ABCG2 with the decreased intracellular accumulation of SN-38 and its glucuronide metabolite. We have recently demonstrated that plasma-membrane vesicles prepared from those cells ATP-dependently transported both SN-38 and SN-38-glucuronide, and our results strongly suggested that ABCG2 is involved in the active extrusion of SN-38 and its metabolite from cancer cells [19].

To date, at least three variant forms of ABCG2 have been documented on the basis of amino acid moieties at position 482, which is close to the third transmembrane domain. The wild-type

Abbreviations used: ABC, ATP-binding cassette; BCRP, breast-cancer-resistant protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; MTT, bromo-3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium; RT, reverse transcription; SN-38, 7-ethyl-10-hydroxy-camptothecin; SNP, single-nucleotide polymorphism.

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form of ABCG2 has an arginine at that position [10], whereas other variants cloned from cancer cell lines [9,13] have glycine and threonine at position 482. It is currently speculated that the substrate specificities of ABCG2 may differ among those variant forms [18,20–22]. To elucidate the role of amino acid moieties at position 482 in the transport function, we have expressed each variant form of ABCG2 in HEK-293 cells and examined the substrate specificity of those variants. In the present study, we demonstrate that Arg^{482} is critically involved in methotrexate transport mediated by ABCG2. Furthermore, we provide direct evidence that the ABCG2 protein functions as a homodimer bound via a cysteinyl disulphide bond(s).

EXPERIMENTAL

Cloning of human [Arg^{482}]ABCG2 cDNA

Human ABCG2 cDNA was cloned from mRNA of the MCF7/BCRP clone-8 cell line [9]. Briefly, reverse transcription (RT)-PCR was carried out by using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, U.S.A.) and the following specific primers: sense 5′-CTCTCCAGATGTCTTCC-AGT-3′ and antisense 5′-ACAGTGTGATGGCAAGGGAAC-3′, where the primers were designed based on the ABCG2 cDNA sequences. The PCR reaction consisted of 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 2 min, as previously described [23]. The resulting PCR product was inserted into the pCR2.1 TOPO vector (Invitrogen) and its sequences were analysed by automated DNA sequencing (TOYOBO Gene Analysis, Tokyo, Japan). The open reading frame of the ABCG2 cDNA, thus obtained, was identical with the ABCG2 wild-type (Arg^{482}) originally named ABCP (GenBank® accession number AF103796). The [Arg^{482}]ABCG2 cDNA was removed from the pCR2.1 TOPO vector by *Eco*RI digestion. After the treatment with alkaline phosphatase, the cDNA was ligated to the *Eco*RI sites of the pcDNA3.1 Expression vector (Invitrogen) using the Rapid DNA Ligation Kit (Roche Diagnosis Co., Indianapolis, IN, U.S.A.).

Generation of variant forms by site-directed mutagenesis

The pcDNA3.1 vector carrying the [Arg^{482}]ABCG2 cDNA was used as the template, and variant forms (Gly^{482} and Thr^{482}) were created by the site-directed mutagenesis using the QuikChange Site-directed Mutagensis Kit (Stratagene, La Jolla, CA, U.S.A.) and internal complementary PCR primers as follows: 5′-CT-GATTTATTACCCATGGGGATGTTACCAAGTATT-3′ and 5′-AATACTTGGTAACATCCCCATGGGTAATAAATCAG-3′ (for the Gly^{482} variant form) or 5′-CTGATTTATTACCCATGACG-ATGTTACCAAGTATT-3′ and 5′-AATACTTGGTAACATCGT-CATGGGTAATAAATCAG-3′ (for the Thr^{482} variant form). The PCR reaction consisted 16 cycles of 95 °C for 30 s, 55 °C for 1 min and 68 °C for 15 min, and *Pfu Turbo* DNA polymerase was used for the PCR reaction. The mutations were confirmed by sequencing the inserted cDNA.

Cell culture

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) heat-inactivated fetal-calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5 % CO_2 in air. The number of viable cells was determined in a haemocytometer by Trypan Blue dye exclusion.

Table 1 Experimental conditions for quantitative PCR

			Denaturation		PCR (45 cycles)	
Gene	Amplified position	Accession no.	Temp. (°C)	Time (s)	Temp. (°C)	Time (s)
ABCG2	2062–2287	NM004827	95	30	95	15
					70	20
					82	7
ABCC1	4436–4836	NM004996	95	30	95	15
					68	20
					83	7
ABCC2	3650–4029	NM000392	95	30	95	15
					63	15
					72	15
					85	7
ABCC3	4580–4890	Y17151	95	30	95	15
					63	15
					72	15
					83	7
ABCC4	2819–3129	NM005845	95	30	95	15
					56	15
					72	15
					83	7
ABCC5	4341–4572	NM005688	95	30	95	15
					61	15
					72	15
					87	7
GAPDH	812–1130	AF261085	95	30	95	15
					68	15
					89	7

HEK-293 cells were transfected with the pcDNA3.1 vector carrying the ABCG2 cDNA (see Figure 1A below) and the LIPOFECTAMINE™ reagent (Invitrogen) according to the manufacturer's instruction. Single colonies resistant to G418 (Nacalai Tesque, Kyoto, Japan) were picked and subcultured. Selection of positive colonies was performed by immunoblotting, as described below.

Quantitative RT-PCR

Total RNA was extracted from cultured cells with the ISOGEN RNA extraction solution (WAKO Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer's protocol. cDNA was prepared from the extracted RNA in the reverse transcriptase reaction with SuperScript II RT (Invitrogen) and oligo(dT) primers according to the manufacturer's instructions. The transcriptome level of each ABC transporter was determined by quantitative PCR in a TaKaRa SmartCycler™ (TaKaRa Bio Inc., Shiga, Japan) with SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME, U.S.A.) as a fluorescence indicator and the following specific primer sets for ABCG2, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): ABCG2 (5′-GATCTCTC-ACCCTGGGGCTTGTGGA, 5′-TGTGCAACAGTGTGATGG-CAAGGGA), ABCC1 (5′-GCCCTTCCTGACAAGCTAGAC, 5′-CATATAGGCCCTGCAGTTCTGAC), ABCC2 (5′-AGGTG-GCTTGCAATTCGCCT, 5′-CCAATCTTCTCCATGCTACCG-ATGT), ABCC3 (5′-CTAGAGGCATCTTCTACGGGA, 5′-ATA-ACACTCAGTTGGGAATCGG), ABCC4 (5′-TCCCACTTGTC-ATCTTCTCTC, 5′-CAGCACTTTGTCGAACACAC), ABCC5 (5′-GTGGAGTTTGACACCCCATCGGTC, 5′-CCAATCCGG-AACTGCTGTGCGAAAG), GAPDH (5′-ACTGCCAACGTGT-CAGTGGTGGACCTGA; 5′-GGCTGGTGGTCCAGGGGTCT-TACTCCTT). PCR conditions for the detection of these transporters and GAPDH are summarized in Table 1.

Immunofluorescence microscopy

Mock- and ABCG2-transfected HEK-293 cells were seeded on microscopic cover glasses and incubated under the above-mentioned culture conditions overnight. Cells were fixed with 4% (w/v) paraformaldehyde in PBS at room temperature. Thereafter, cell membranes were permeabilized by incubating them with 0.1% Triton X-100 in PBS at room temperature for 5 min. Cells were then treated with the BXP-21 antibody (1:20 dilution; Signet Laboratories Inc., Dedham, MA, U.S.A.) as the first antibody and subsequently with the fluorescent-dye-Cy3-conjugated anti-mouse IgG antibody (1:500 dilution; Jackson ImmunoResearch Laboratories, Inc., Baltimore, MA, U.S.A.). The immunofluorescence of HEK-293 cells was detected with a confocal laser-scanning fluorescence microscope IX70/Fluoview (Olympus; Tokyo, Japan).

Preparation of plasma-membrane vesicles from HEK-293 cells

HEK-293 cells (approx. 2×10^9 cells) were harvested by centrifugation and suspended in 100 ml of ice-cold PBS. After centrifugation at 500 ***g*** for 5 min, the cell pellet was diluted 40-fold with a hypotonic buffer [0.5 mM sodium phosphate (pH 7.0)/0.1 mM EGTA] and homogenized with a Potter–Elvehjem homogenizer. After centrifugation at 9100 ***g***, the resulting supernatant was centrifuged at 100 000 ***g*** for 30 min, and the resulting pellet was suspended in 250 mM sucrose containing 10 mM Tris/HCl, pH 7.4. The crude membrane fraction was layered over 38% (w/v) sucrose solution and centrifuged at 100 000 ***g*** for 30 min. The turbid layer at the interface was collected, suspended in 250 mM sucrose containing 10 mM Tris/HCl, pH 7.4, and centrifuged at 100 000 ***g*** for 30 min. The membrane fraction was collected and resuspended in a small volume (150–250 μl) of 250 mM sucrose containing 10 mM Tris/HCl, pH 7.4. After the measurement of protein concentration by the BCA (bicinchoninic acid) Protein Assay Kit (Pierce, Rockford, IL, U.S.A.), the membrane solution was stored at $-$ 80 °C until used.

Detection of ATP-dependent transport of [^{3}H]methotrexate

Frozen stocked membrane was thawed quickly at 37 °C, and vesicles were formed by passing the suspension through a 27-gauge needle. The standard incubation medium contained plasma-membrane vesicles (30 or 60 μg of protein), 500 μM [3′,5′,7′-^{3}H]methotrexate (Amersham Biosciences), 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM creatine phosphate and 100 μg/ml creatine kinase in a final volume of 110 μl. The reaction was started by adding [^{3}H]methotrexate to the incubation medium. The reaction was carried out at 37 °C, and the amount of [^{3}H]methotrexate incorporated into the vesicles was measured by a rapid-filtration technique previously described [24].

Gel electrophoresis and detection of ABCG2 protein

Expression of ABCG2 in HEK-293-cell membranes was determined by immunoblotting with BXP-21 (Signet), a specific antibody to human ABCG2 where membrane proteins were pretreated with or without mercaptoethanol (more details are given in the Results section). Briefly, proteins of the isolated plasma membrane were separated by electrophoresis on SDS/7.5%-(w/v)-polyacrylamide slab gels, and the proteins were electroblotted on to Hy-bond ECL® (enhanced chemiluminescence) nitrocellulose membranes (Amersham Biosciences). Immunoblotting was performed by using BXP-21 (1:250 dilution) as the first antibody and an anti-mouse IgG–horseradish peroxidase (HRP) conjugate (Cell Signaling Technology, Beverly, MA, U.S.A.) (1:3000 dilution) as the secondary antibody. HRP-dependent luminescence was developed by using Western Lighting Chemiluminescent Reagent Plus (PerkinElmer Life Sciences, Boston, MA, U.S.A.) and detected with a Lumino Imaging Analyzer FAS-1000 (Toyobo, Osaka, Japan).

Profiling of drug resistance of ABCG2-overexpressing HEK-293 cells

A growth inhibition (IC_{50}) assay was performed by seeding HEK-293 cells at a density of 1000–2000 cells/well in 96-well plates containing the culture medium. After 24 h, mitoxantrone or methotrexate was added to the culture medium at different concentrations, and cells were further incubated with the drug in a humidified tissue-culture chamber (37 °C, 5% CO_2) for 72 h. Surviving cells were detected by the bromo-3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium (MTT) assay [25]. Briefly, 20 μl of MTT solution (5 mg/ml) was added to the culture medium, and cells were incubated for 4 h at 37 °C. Thereafter, the culture medium was removed and cells were dissolved in 200 μl of DMSO. The absorbance of formazan, a metabolite of MTT, in the resulting solution was photometrically measured at a test wavelength of 570 nm and at a reference wavelength of 630 nm in a Model 550 microplate reader (Bio-Rad, Hercules, CA, U.S.A.). IC_{50} values were calculated from dose–response curves (i.e. cell survival versus drug concentration) obtained in multi-replicated experiments.

RESULTS

Expression of ABCG2 variants in HEK-293 cells

Three variant forms (i.e., Arg^{482}, Gly^{482}, and Thr^{482}) of human ABCG2 were expressed in HEK-293 cells by transfection with the pcDNA3.1 vector carrying the cDNA (Figure 1A). mRNA and protein levels of ABCG2 were detected by quantitative RT-PCR and Western blotting respectively; the mRNA levels of Arg^{482}, Gly^{482} and Thr^{482} variants in HEK-293 cells are shown in Figure 1(B). The parental HEK-293 cell line endogenously expressed ABCC1, ABCC2, ABCC4 and ABCC5, and the mRNA levels of ABCC3 and ABCG2 were below the detection limit. Similar expression profiles were observed in mock-transfected HEK-293 cells. On the other hand, in the Arg^{482}-, Gly^{482}- or Thr^{482}-variant-transfected cells, mRNA levels of ABCG2 were significantly enhanced, corresponding to 13–20% of the mRNA level of GAPDH. The expression levels of other ABC transporters, i.e., ABCC1, ABCC2, ABCC3, ABCC4 and ABCC5, were unchanged in these cells.

Western blotting (Figure 1C) revealed that the three variant forms of ABCG2 were also highly expressed at the protein level. In this experiment, membrane proteins were treated with mercaptoethanol prior to SDS/PAGE, so that one single band was detected with the BXP-21 antibody at an apparent molecular mass of 70 kDa. Mock-transfected HEK-293 cells, as the negative control, did not show any immunological reaction.

Cellular localization of ABCG2 overexpressed in HEK-293 cells

Figure 2 depicts the differential interference (A and C) and immuno-fluorescence images (B and D) of mock- and [Arg^{482}]ABCG2-transfected HEK-293 cells. ABCG2 proteins

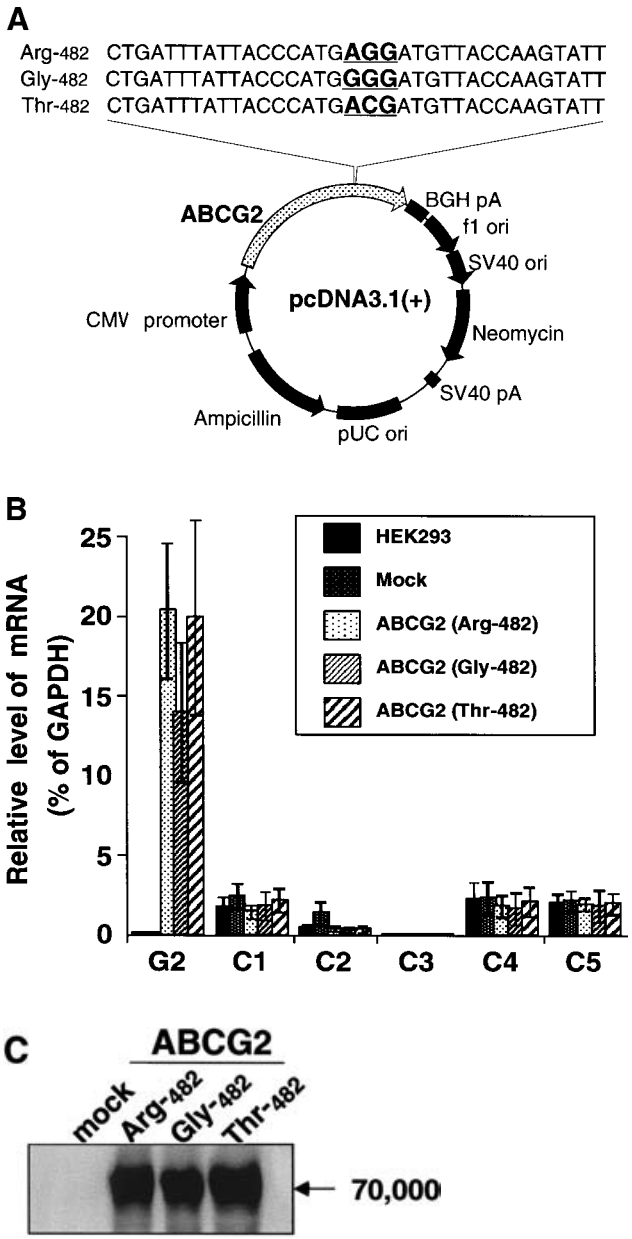

Figure 1 Expression of ABCG2 variant forms in HEK-293 cells

(**A**) Schematic illustration of the ABCG2 expression pcDNA3.1 vector. The partial cDNA sequences of three variants [Arg^{482}, Gly^{482} and Thr^{482} ('Arg-482' etc.)] are indicated. Abbreviations: BGH, bovine growth hormone; CMV, cytomegalovirus; f1ori, f1 origin of replication from the f1 filamentous phage; pA, polyadenylation sequence; pUC ori, pUC vector origin of replication; SV40, simian virus 40. (**B**) mRNA levels of ABCC1, ABCC2, ABCC3, ABCC4, ABCC5 and ABCG2 in mock- and [Arg^{482}]-, [Gly^{482}]- and [Thr^{482}]-ABCG2-transfected HEK-293 cells. Total RNA was extracted separately from those cells and cDNA was subsequently prepared by reverse transcriptase reaction. The transcriptome level of each ABC transporter was determined by quantitative PCR (for details, see the Experimental section). Data are expressed as means $\pm$ S.E.M. ($n = 4$). (**C**) Immunological detection of ABCG2 expressed in the plasma-membrane preparation from mock- and [Arg^{482}]-, [Gly^{482}]- or [Thr^{482}]-ABCG2-transfected HEK-293 cells. Membrane proteins (10 μg for each lane) were treated with mercaptoethanol prior to SDS/PAGE. Western blotting was performed as described in the Experimental section.

were probed with specific antibodies as described in the Experimental section. Strong immunofluorescence was detected at the plasma membrane of [Arg^{482}]ABCG2-transfected HEK-293 cells (Figure 2D). Similar results were obtained with HEK-293 cells expressing Gly^{482} and Thr^{482} variants as well

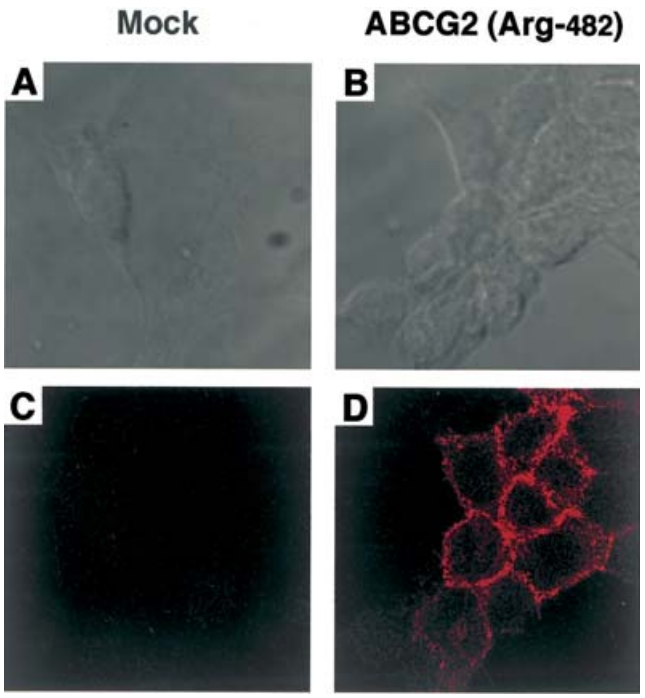

Figure 2 Cellular localization of ABCG2 in HEK-293 cells

Differential interference (**A**) and immunofluorescence (**B**) images of mock-transfected HEK-293 cells; differential interference (**C**) and immuno-fluorescence (**D**) images of [Arg^{482}]ABCG2-transfected HEK-293 cells. The ABCG2 protein was immunologically linked with the fluorescent Cy3 probe, as described in the Experimental section.

(results not shown). On the other hand, no immunofluorescence was detected in mock-transfected HEK-293 cells as the negative control (Figure 2B). Thus it is proven that those three ABCG2 variants were expressed predominantly in the plasma membrane of HEK-293 cells.

Drug-resistant profile of ABCG2-transfected HEK-293 cells

Figure 3 shows the cellular resistance profiles of ABCG2-expressing HEK-293 cells to mitoxantrone and methotrexate. In this experiment, HEK-293 cells were incubated with mitoxantrone or methotrexate at different concentrations as indicated at 37 °C for 72 h. Overexpression of Arg^{482}, Gly^{482} and Thr^{482} variants conferred HEK-293 cells resistance to mitoxantrone by 15-, 47- and 54-fold respectively as compared with the mock-transfected HEK-293 cells (Figure 3A). The cellular resistance of Gly^{482}- or Thr^{482}-variant-expressing cells was even higher than that of Arg^{482}-variant-expressing cells. On the other hand, as shown in Figure 3(B), HEK-293 cells expressing Arg^{482}, Gly^{482}, and Thr^{482} variants did not exhibit any significant resistance to methotrexate as compared with mock-transfected cells.

ATP-dependent transport of methotrexate by [Arg^{482}]ABCG2

The function of Arg^{482}, Gly^{482} and Thr^{482} variants was examined by using plasma-membrane vesicles prepared from HEK-293 cells overexpressing those variants. Figure 4 depicts the time courses of methotrexate transport into plasma-membrane vesicles in the presence or absence of ATP. As previously described [24], the ATP concentration was maintained at constant levels for a period sufficient for measurement (at least 20 min) with the creatine

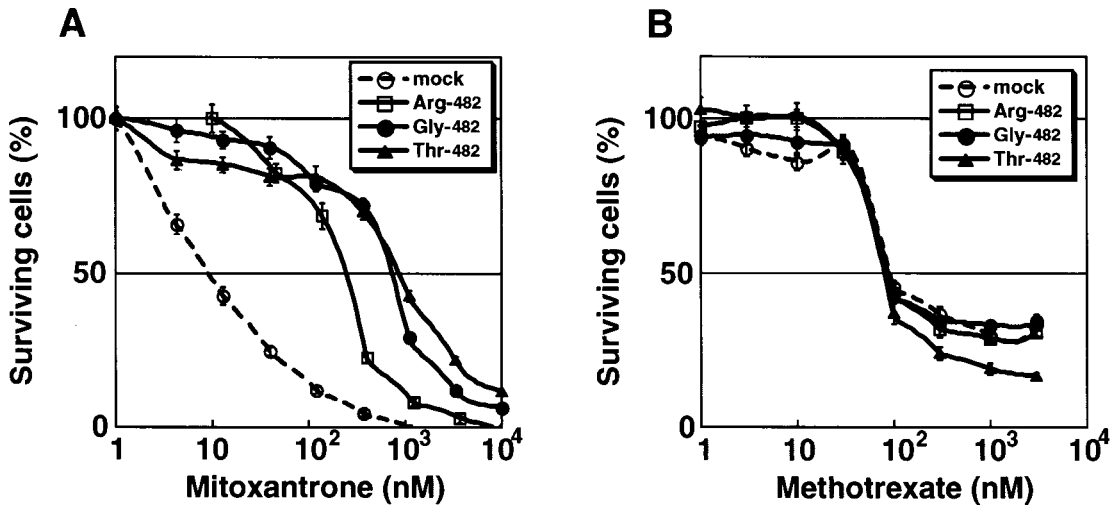

Figure 3 Cellular resistance of ABCG2-expressing HEK-293 cells to mitoxantrone and methotrexate

HEK-293 cells transfected with the mock vector or [Arg^{482}]-, [Gly^{482}]- and [Thr^{482}]-ABCG2 expression vectors were incubated in 200 μl of the culture medium containing mitoxantrone or methotrexate at different concentrations in 96-well plates in a humidified tissue-culture chamber (37 °C, 5 % CO_2). After 72 h, surviving cells were detected by the MTT assay (see the Experimental section). Data are expressed as means ± S.E.M. for multi-replicated ($n = 8$–12) experiments.

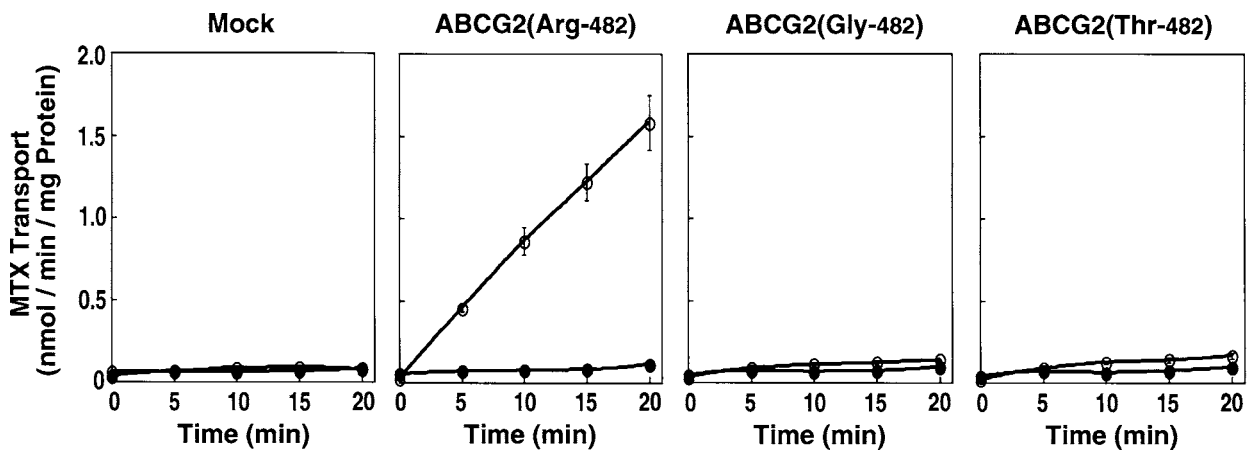

Figure 4 ATP-dependent transport of methotrexate in plasma-membrane vesicles prepared from ABCG2-transfected HEK-293 cells

Plasma-membrane vesicles were prepared from mock- and [Arg^{482}]-, [Gly^{482}]- or [Thr^{482}]-ABCG2-transfected HEK-293 cells as described in the Experimental section. Plasma-membrane vesicles (60 μg of protein) were incubated with 20 μM [^{3}H]methotrexate (MTX) in the absence (●) or presence (○) of 1 mM ATP in the medium containing 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM creatine phosphate and 100 μg/ml creatine kinase. The incubation was carried out at 37 °C and then stopped at different time points as indicated in the Figure. The amount of [^{3}H]methotrexate incorporated into the membrane vesicles was measured by a rapid-filtration technique. Results are expressed as means ± S.E.M. for triplicate experiments.

phosphate and creatine kinase reaction system. It is important to note that ATP-dependent methotrexate transport was observed only in plasma-membrane vesicles prepared from HEK-293 cells overexpressing the Arg^{482} variant. No significant transport activity was detected from the Gly^{482} or Thr^{482} variant-expressing HEK-293 cells.

ATP-dependent transport of methotrexate by [Arg^{482}]ABCG2 was further characterized with the plasma-membrane-vesicle system. Figure 5(A) demonstrates a relationship between the methotrexate concentration and the rate of ATP-dependent transport. On the basis of this result, the apparent K_m value for methotrexate was calculated to be 5.7 mM. In addition, as shown in Figure 5(B), ATP-dependent methotrexate transport mediated by [Arg^{482}]ABCG2 was inhibited by doxorubicin, mitoxantrone and rhodamine 123 at a concentration of 500 μM. In contrast, *S*-octylglutathione, which is a strong and competitive inhibitor for the GS-X (ATP-dependent glutathione-S-conjugate export) pump, e.g., ABCC1 and ABCC2 [24], had a minimal inhibitory effect (Figure 5B).

Existence of ABCG2 as a homodimer in the plasma membrane

As shown in Figure 1(C), ABCG2 was detected as one single band at a molecular mass of 70 kDa under reducing conditions. On the other hand, without mercaptoethanol treatment, ABCG2 exhibited a molecular mass of 140 kDa. Figure 6(A) shows the effect of increasing concentrations of mercaptoethanol on the apparent molecular mass of [Arg^{482}]ABCG2. After a 20 min incubation with 100 mM mercaptoethanol at room temperature, the molecular mass of [Arg^{482}]ABCG2 completely

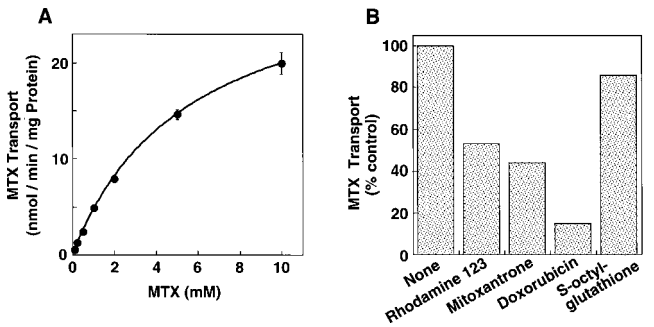

Figure 5 Kinetic properties of ATP-dependent methotrexate transport by [Arg^{482}]ABCG2

(**A**) Relationship between the methotrexate (MTX) concentration and the ATP-dependent MTX transport rate. Plasma-membrane vesicles (30 μg of protein) from [Arg^{482}]ABCG2-expressing HEK-293 cells were incubated with [^{3}H]methotrexate (MTX) at different concentrations (0–10 mM) in the incubation medium containing 1 mM ATP, 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM creatine phosphate and 100 μg/ml creatine kinase at 37 °C for 20 min. The amount of [^{3}H]methotrexate incorporated into the membrane vesicles was measured as shown in Figure 3. Data are expressed as means ± S.E.M ($n = 3$ for 0–0.5 mM; $n = 4$ for 1–10 mM). (**B**) Inhibition of ATP-dependent methotrexate transport by rhodamine 123, mitoxantrone, doxorubicin and *S*-octylglutathione. Plasma-membrane vesicles (30 μg of protein) were incubated with [^{3}H]methotrexate (500 μM) in the presence of those inhibitors (500 μM for each) in the incubation medium containing 1 mM ATP, 250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM creatine phosphate and 100 μg/ml creatine kinase at 37 °C for 20 min. The amount of [^{3}H]methotrexate incorporated into the membrane vesicles was measured in the same manner. Data are normalized for the amount of [^{3}H]methotrexate incorporated during the incubation in the absence of inhibitors.

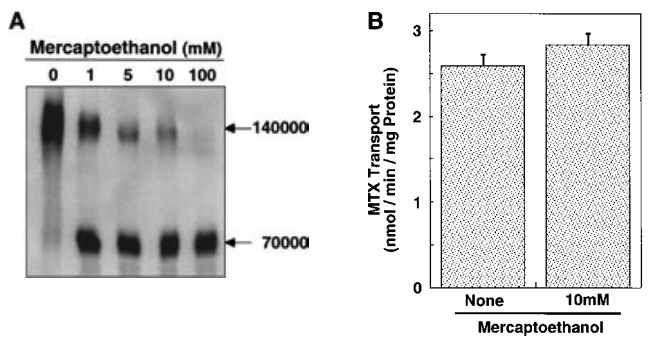

Figure 6 Effect of mercaptoethanol on dimerization of ABCG2 (A) and ATP-dependent methotrexate transport (B)

(**A**) The plasma membrane from [Arg^{482}]ABCG2-transfected HEK-293 cells was incubated with mercaptoethanol at final concentrations of 0, 1, 5, 10 and 100 mM in the medium containing 250 mM sucrose and 10 mM Tris/HCl, pH 7.4, at room temperature for 20 min. Thereafter, membrane proteins (2 μg for each lane) were separated by SDS/PAGE, and ABCG2 was immunologically detected by Western blotting, as shown in Figure 1(C). (**B**) The plasma membrane from [Arg^{482}]ABCG2-transfected HEK-293 cells was pre-incubated in the absence or presence of 10 mM mercaptoethanol at room temperature for 20 min, as described above. ATP-dependent methotrexate (MTX) transport was detected in the plasma-membrane vesicles as described in the Experimental section; the transport reaction was performed at 37 °C for 20 min. Data are expressed as means ± S.E.M. for triplicate experiments.

shifted to 70 kDa on Western-blotting analysis (Figure 6A). This phenomenon was not unique for the Arg^{482} variant, since similar molecular-mass shifts were observed for the Gly^{482} and Thr^{482} variants as well (results not shown). These results suggest that ABCG2 exists in the plasma membrane as a homodimer bound through cysteinyl disulphide bond(s). It is noteworthy that ATP-dependent methotrexate transport was little affected by mercaptoethanol treatment, in spite of the breakage of the inter-peptide disulphide bonds (Figure 6B).

DISCUSSION

ATP-dependent methotrexate transport by [Arg^{482}]ABCG2

In the present study, we have established HEK-293 cell lines that overexpress three variant forms of human ABCG2 (Figures 1 and 2). Using the plasma-membrane vesicles prepared from those cells, we clearly demonstrate that [Arg^{482}]ABCG2 transports methotrexate; however, the other variants, Gly^{482} and Thr^{482}, do not (Figure 4). The present study is the first report providing direct evidence that ABCG2 is able to transport methotrexate. Our finding strongly suggests that the Arg^{482} close to the third transmembrane domain is a critical amino acid residue involved in the substrate specificity of ABCG2. Since the arginine residue is positively charged under physiological conditions, it is likely that its positive charge is a prerequisite for interactions between the active site of ABCG2 and anionic substrates such as methotrexate. However, as indicated by the weak inhibition by *S*-octylglutathione (Figure 5B), structural factors may also be important for the substrate recognition besides the negative charge. In fact, methotrexate transport was much more strongly inhibited by the glutathione conjugate of mitomycin C than by S-octylglutathione (H. Mitomo and T. Ishikawa, unpublished work).

Previous studies [26,27] have predicted the existence of energy-dependent transport systems for the methotrexate efflux from cells. Molecular cloning and functional expression of ABC transporters led to the identification of human ABCC1, ABCC2, ABCC3 and ABCC4 as methotrexate transporters [28–30]. These transporters belong to the C-subfamily of human ABC transporters and exhibit broad spectra of substrate specificity towards organic anions, including glutathione, glucuronide and sulphate conjugates, as well as nucleotide analogues [7,8]. The K_m values for these ABC transporters in respect of methotrexate were reportedly in the range 200–500 μM [28–30]. Whereas HEK-293 cells endogenously express ABCC1, ABCC2, ABCC4 and ABCC5 (Figure 1B), ABCG2 expression was predominantly enhanced by transfection with the exogenous cDNA (Figures 1B and 1C). ABCG2 proteins were localized at the plasma membrane of HEK-293 cells (Figure 2). The K_m value of ATP-dependent transport by [Arg^{482}]ABCG2 was estimated to be 5.7 mM for methotrexate (Figure 5A), approximately one order of magnitude greater than those reported for the above-mentioned ABCC-subfamily transporters.

Variant forms of ABCG2 and drug-resistance phenotypes

Recently, Volk et al. [22] suggested that overexpression of the wild-type ([Arg482]ABCG2) mediates methotrexate resistance. Since the mitoxantrone-selected MCF7/MX cell line was used in their study, actual molecular mechanisms underlying the cross-resistance to mitoxantrone and methotrexate were not known. Our present study on ATP-dependent methotrexate transport mediated by [Arg^{482}]ABCG2 in part supports their hypothesis. Indeed, HEK-293 cells transfected with [Arg^{482}]ABCG2 were resistant to mitoxantrone (Figure 3A). However, [Arg^{482}]ABCG2-transfected cells did not exhibit significant resistance to methotrexate (Figure 3B). This may be explained by the high K_m value (5.7 mM) of [Arg^{482}]ABCG2 in respect of methotrexate (Figure 5A). In addition, it is also true that the Arg^{482}-variant-transfected cells grew approx. 20% more slowly than the Gly^{482}- and Thr^{482}-variant-transfected cells, suggesting that overexpression of ABCG2 could result in an enhanced efflux of unknown, but physiologically important, substances from cells.

Until now, several acquired mutations were documented for ABCG2 cloned from drug-resistant cell lines [20,31]. Drug-resistant phenotypes vary among different cell lines expressing

variant types of ABCG2. In fact, transfectants with the wild-type (Arg^{482}) were not resistant to topotecan [18], whereas overexpression of the Gly^{482} and Thr^{482} variants conferred resistance to mitoxantrone, doxorubicin, daunorubicin and various camptothecin analogues, including topotecan [9,14–16]. Furthermore, Gly^{482} and Thr^{482} variants mediated the efflux of rhodamine 123 and doxorubicin from cells; however, Arg^{482} did not [20,21]. These findings strongly suggest that arginine at position 482 has a critical role in the substrate specificity of ABCG2. The identification of mutations at 482 in ABCG2 may explain some discrepancies observed in the cross-resistance profiles of human cancer-cell lines. Likewise, one mutation 'hot spot' was identified at the same amino acid position 482 in mouse Abcg2 [32]. In the case of mouse Abcg2, variants of arginine, serine and methionine significantly affect the drug-resistance profile in cancer-cell lines [32].

To examine the function of ABCG2 as a drug transporter, we have previously established ABCG2-overexpressing Sf9 [fall armyworm (*Spodoptera frugiperda*)] insect cells and tried to measure drug-induced ATPase activity [23]. However, because of a high activity of endogenous ATPase present in the plasma membrane, we were not able to accurately measure the drug-induced ATPase activity of ABCG2 [23]. Özvegy et al. [21] had a similar difficulty in the case of the [Arg^{482}]ABCG2. In this context, the vesicle transport system we used in the present study may offer a simple and practical tool to elucidate the substrate specificity and transport activity of human ABCG2 and its variants.

Homodimer formation of ABCG2

Since ABCG2 is an ABC half-transporter, it has been suspected that ABCG2 functions as a homodimer or heterodimer [15]. Most recently, using cross-linking reagents and specific antibodies, Litman et al. [33] provided evidence for the formation of an ABCG2 homodimer. In addition, Kage et al. [34] have recently established PA317 transfectants expressing c-Myc oncoprotein (Myc)- and haemagglutinin (HA)-epitope-tagged ABCG2 proteins, and they demonstrated that those hybrid proteins formed S–S homodimers. In the present study, by expressing native ABCG2 (without tag) in HEK-293 cells, we could provide more direct evidence that human ABCG2 exists in the plasma membrane as a homodimer bound through cysteinyl disulphide bond(s) (Figure 6). Treatment with mercaptoethanol decreased the apparent molecular mass of ABCG2 from 140 to 70 kDa (Figure 6A). It is noteworthy that ATP-dependent transport of methotrexate by [Arg^{482}]ABCG2 was little affected by mercaptoethanol treatment (Figure 6B). Thus disulphide-bond formation does not appear to be required for the transport function of ABCG2.

On the basis of the cDNA sequence, a total of 12 cysteine residues exist in the ABCG2 peptide. From a biochemical point of view, it may be interesting to study which cysteine residues participate in the disulphide-bond formation and how inter-peptide disulphide bonds are formed in the cell. Furthermore, ABCG1, ABCG4, ABCG5 and ABCG8 have been hitherto identified as ABC half-transporters [35], and it would be of interest to know whether those half-transporters also form homodimers or heterodimers via disulphide bonds as does ABCG2.

Concluding remarks

ABCG2 is expressed in human normal tissues such as placental syncytiotrophoblasts, the epithelium of the small intestine and colon, the liver canalicular membrane, and ducts and lobules of the breast [36]. In addition, expression of ABCG2 is detected in venous and capillary endothelium [36]. Furthermore, relatively high expression of ABCG2 is observed in approx. 30% of acute-myeloid-leukaemia patients [37] and is correlated with an immature immunophenotype as determined by expression of the surface marker CD34 [38]. Not only expression levels, but also intrinsic genetic polymorphisms and acquired mutations of ABCG2, are suggested to affect the pharmacokinetic profile of drugs and thereby lead to individual variations in the drug response. Recently, two single-nucleotide polymorphisms (SNPs) leading to amino acid substitutions (i.e., $Val^{12} \rightarrow$ Met and $Gln^{141} \rightarrow$ Lys) for ABCG2 have been found in the Japanese population [39]. It is critically important to validate such SNPs and mutations by examining the actual function of the resulting gene products.

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