MRP1 transports drug conjugates including glutathione (GSH), glucuronate, and sulfate moieties[62]. These findings suggest that *MRP1* is functionally related to the GS-X (ATP-dependent GSH S-conjugate export) pump[63]. Endogenous GS-X compounds such as LTC4 are transported by MRP1. Following homozygous deletion of *mrp1*, mice exhibit accumulation of intracellular LTC4 in bone marrow-derived leukocytes[64]. These animals suffer from impairment of inflammatory response.

MRP2, which is mainly expressed in the canalicular membrane of hepatocytes, encodes canalicular multispecific organic anion transporter (cMOAT) for hepatobiliary excretion of bilirubin glucuronides and other multivalent organic anions, including GSH S-conjugates[65,66]. Like *MRP1*-transfected cells, transfection of *MRP2* into cultured cells was found to confer elevated resistance to various antitumor agents[67,68]. MRP3, which is the closest homologue of MRP1 in the MRP family, shares 58% amino acid identity with MRP1[69,70]. Although *MRP3*-transfected cells displayed resistance to antitumor agents (etoposide and methotrexate), these cells did not show increased GSH export[70]. Over-expression of MRP4 was associated with resistance to antiviral acyclic nucleoside analogues[71]. MRP5 and MRP6 share 36% and 45%, respectively, of their amino acid identities with MRP1[72]. MRP5 is expressed in many human tissues, with relatively high levels of expression in skeletal muscles and brain, whereas expression of MRP6 is relatively restricted, with elevated levels of expression found in liver and kidney[73].

The identification of these members of the MRP gene family has been performed only in the recent years. Investigations into the function of each encoded isoform has been progressing rapidly. However, several important aspects of those genes, particularly *MRP3* to *MRP6*, remain to be investigated (Table 1).

3.2 Regulation of MRP Function

It has been demonstrated that GSH levels play an important role in the regulation of MRP1 expression, since MRP1 is functionally related to the GS-X pump[74]. The γ-glutamylcysteine synthetase (γ-GCS) catalyzes the synthesis of glutamylcysteine, which is the rate-limiting step in overall GSH biosynthesis. Thus, the cellular GSH level is substantially regulated by γ-GCS[75]. In a number of cell lines, expression of MRP1 and γ-GCS heavy chain (γ-GCSh, the catalytic subunit of γ-GCS) can be co-induced by treatment with pro-oxidants, e.g., *t*-butylhydroquinone, 2,3-dimethyoxy-1,4-naphthoquinone and menadione. These observations suggest that regulation of MRP1 and γ-GCSh may be oxidative stress-sensitive. Consistent with this idea, over-expression of the physiological antioxidant GSH in γ-GCSh-transfected cells

down-regulates MRP1 and γ-GCSh expression[76]. These findings suggest that a dynamic GSH homeostasis may be associated with response to cancer chemotherapy. Since other MRP members, e.g., MRP2, MRP3 and MRP5, also exhibit GS-X pump activity, the altered GSH content may have a broad effect on the overall function of the MRP family. However, the effects of GSH homeostasis on the function of these MRP members require further demonstrations. These findings, if proven, may have clinical relevance to trials in which modulators of MRP and GSH function are considered. The cytotoxic effects of antitumor agents may induce transient expression of MRP1 and γ-GCSh. Elevated MRP1 expression may facilitate the elimination of antitumor agents at the expense of GSH consumption, resulting in depletion of the GSH pool and subsequent downregulation of MRP1-mediated drug resistance.

As in MDR1, multiple mechanisms are likely to be involved in the regulation of MRP1 expression. Zhu and Center[77] reported that the SP1 binding sites located between -29 and -12 are involved in basal MRP1 gene expression. Gomi *et al.*[78] reported that a post-transcriptional mechanism may also be involved in the regulation of MRP1 induced by antitumor alkylating agents. Wang and Beck[79] demonstrated that wild-type p53 could suppress the transcriptional expression of MRP1 gene expression by diminishing SP1 activity. Using immunohistochemical or flow cytometric analysis, MRP1 expression was correlated with mutated p53 protein expression in human non-small cell lung cancer, colorectal cancer and acute myeloid leukemia[80,81]. The suppressive effect of wild-type p53 on MRP1 was also demonstrated in a prostate cancer cell line[82].

3.3.1 Clinical relevance of MRP in cancer chemotherapy

The clinical relevance of MRP in resistance to cancer chemotherapy has not been thoroughly investigated. The investigations are likely to be more complex than that of MDR1 because: [i] Co-expression of multiple MRP isoforms is often observed in MDR cell lines selected with a single antitumor agent; therefore, reliable methodologies to differentiate the contribution from each member are desirable. To this end, isoform-specific probes for individual members have to be used. Given the fact that many MRP transporters have similar substrate spectra, although affinities toward the same substrate differ among the isoforms, modulators of individual MRP isoforms may not be readily available. Monoclonal antibodies that recognize specific MRP isoforms have been produced[83,84]. Alternatively, it may be possible to explore neutralizing antisense oligonucleotides to specific *MRP* mRNAs without cross-reacting with other isoforms[85]. [ii] In tumor cells, cellular locations of expressed MRP may not be membrane-located or properly spanned into the membrane lipid bilayer. Thus, measurement of MRP expression by biochemical means needs to be coupled to immunohistochemical determination. [iii] MRP-mediated transport of antitumor agents requires

GSH or other organo-anionic constituents. Thus, measurable levels of MRP expression may not actually reflect the transport activities.

Despite these difficulties, some progress has been noted. Expression of *MRP1* mRNA and MRP1 was detected in a wide spectrum of human cancers[86]. Sullivan *et al.*[87] showed that MRP1 expression was more frequent in prostate cancer than in benign glandular elements, and Fukushima *et al.*[81] showed that the frequency of MRP1 expression in colorectal carcinoma was significantly higher than in adenoma. Previously we demonstrated that the expression level of *MRP1* mRNA was higher in human colorectal carcinoma than in the matched non-tumor specimens[88]. These findings suggest that MRP1 expression is up-regulated during carcinogenesis.

Several studies have found expression levels of MRP1 to be of prognostic significance. In non-small cell lung cancer patients who underwent postoperative chemotherapy, Oshika *et al.*[80] showed that the prognosis of patients with MRP1-positive tumors was significantly worse than that of patients with MRP1-negative lesions. Studying breast cancer patients with small tumors (<2 cm) and negative lymph node metastasis, Nooter *et al.*[89] showed that the prognosis of patients with MRP1-positive tumors was significantly poorer than that of patients with MRP1-negative tumors.

Endo *et al.*[90] investigated the relationship between MRP1 expression and chemosensitivity to cisplatin, doxorubicin, etoposide, and mitomycin C in 75 patients with gastric cancer. The MTT assays showed that MRP1 positive gastric cancer tissue was less sensitive to cisplatin, doxorubicin, and mitomycin C compared with MRP1 negative tissue. A similar tendency was noted with etoposide. Campling *et al.*[33] demonstrated a significant correlation between doxorubicin resistance and MRP1 expression levels, but found no correlation between MRP1 expression levels and sensitivity to cisplatin, etoposide or vincristine.

Young *et al.*[91] studied the relationship between *MRP2-5* mRNA expressions and chemosensitivity in 23 human lung cancer cell lines. They noted a significant correlation between *MRP3* expression levels and drug resistance to doxorubicin, etoposide, vincristine and cisplatin. In addition, there was a significant correlation between *MRP3* and *MRP1* mRNA expression levels. Like MRP1, MRP3 may contribute to the drug-resistance phenotype of human lung cancer cells.

Several compounds have been reported to modulate drug resistance in MRP1 over-expressing cell lines. These include the calcium channel blockers verapamil and nicardipine, the tiapanmil analogue, the cyclosporin analogue PSC-833, tyrosine kinase inhibitors, and others[74]. The specificity of these modulators have not been conclusively demonstrated. Moreover, their clinical utilities have not been explored.

CONCLUSION

P-gp was the first human ABC transporter protein cloned. The discovery of MRP1 expands our understanding of the molecular basis of multidrug resistance. MDR and MRP are among the most intensively studied ABC transporter proteins because multidrug resistance is a major cause of cancer chemotherapy failure. We have learned a great deal about the biology of these two drug transporters, particularly in cultured cell systems. Major efforts have also been devoted to the investigation of whether expression of MDR and MRP plays a role in clinical drug resistance in cancer chemotherapy, and from these studies we hope to develop strategies that may circumvent multidrug resistance by modulating MDR and MRP expression.

In light of the MDR and MRP gene families' function as transporters of many antitumor agents, their frequent up-regulation in human neoplasms, and the association between their expression and treatment efficacies in certain human malignancies, make it likely that MDR and MRP play an important role in clinical drug resistance, at least in certain forms of cancer. Likewise, the expression of these transporters may be a prognostic predicator of the treatment outcomes. However, the challenge remains the development of effective strategies to circumvent MDR- and MRP-mediated drug resistance in clinical settings.

Finally, more than 40 ABC transporter sequences have been identified in the human genome, and perhaps as many remain to be explored. These yet-to-be identified ABC transporters may also contribute to resistance to cancer chemotherapy. By exploiting the entire spectrum of drug resistance mechanisms, we will learn the overall complexity of the MDR phenotype. These studies may eventually enable us to design better strategies to combat drug resistance in cancer treatment.

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Methods in Enzymolgy

CONJUGATION ENZYMES
(Edited by Helmut Sies and Lester Packer)

Chapter: Transporters

High-speed screening of human ABC transporter function and genetic polymorphisms: New strategies in pharmacogenomics

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Running Title: High-speed screening of human ABC transporters

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ABSTRACT

The drug transporters represent an important mechanism in cellular uptake and efflux of drugs and their metabolites. Hitherto a variety of drug transporter genes have been cloned and classified into either solute carriers (SLC) or ATP-binding cassette (ABC) transporters. Such drug transporters are expressed in various tissues such as the intestine, brain, liver, kidney, and importantly cancer cells, to play critical roles in the absorption, distribution and excretion of drugs. We recently developed high-speed functional screening and quantitative structure-activity relationship (SAR) analysis methods to study the substrate specificity of ABC transporters and to evaluate the effect of genetic polymorphisms on the function. These methods would provide powerful and practical tools to screen synthetic and natural compounds, and deduced data can be applied to the molecular design of new drugs. Furthermore, we here demonstrate a new "SNP array" method to detect genetic polymorphisms of ABC transporters in human samples.

I. Introduction

ATP-binding cassette (ABC) proteins form one of the largest protein families encoded in human genome (Dean *et al.*, 2001; Holland *et al.*, 2003). Hitherto more than 48 human ABC protein genes have been identified and sequenced (Klein *et al*, 1999). It has been reported that mutations of ABC protein genes are causative in several genetic disorders in humans (Dean *et al.*, 2001). Many of human ABC proteins are involved in membrane transport of drugs, xenobiotics, endogenous substances or ions, thereby exhibiting a wide spectrum of biological functions (Schinkel and Jonker, 2003). Based on the arrangement of molecular structure components, i.e. nucleotide binding domains and topologies of transmembrane domains, hitherto reported human ABC proteins are classified into 7 different sub-families (A to G) (Klein *et al.*, 1999; Borst and Oude Elferink, 2003; Ishikawa, 2003). The HUGO Human Gene Nomenclature Committee developed a new system of nomenclature for the human ABC-transporter family. The new nomenclature scheme was implemented in 1999, and detailed information is available on the web site at URL: <http://www.gene.ucl.ac.uk /nomenclature/genefamily/abc.html>.

Metabolism of xenobiotics including drugs is widely referred to phase I and II systems, where phase I includes oxidation of xenobiotics and phase II deals with the conjugation of phase I products (Fig. 1). The oxidative metaboism in the phase I system is mediated by cytochrome P-450 (CYP) or flavin mixed-function oxidases. Some of activated xenobiotics can interact with DNA and/or proteins in cells to cause toxic effects. In the phase II system, on the other hand, activated hydrophobic xenobiotics are converted into hydrophilic forms via conjugation reactions with glutathione, sulfate or glucuronide. This phase II metabolism is regarded as the detoxification process of xenobiotics. However, in some cases, the phase II system is a critical step in the formation of genotoxic electrophiles. Furthurmore, accumulation of the resulting metabolites in cells can lead to a decrease in the detoxification activity of the phase II system. Therefore, the phase III system must take a task to eliminate Phase II metabolites from cells (Ishikawa, 1992). Several ABC transporters, including ABCB1, ABCB11, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, and ABCG2, are considered to be major players in the

considered to be major players in the phase III detoxification system (Borst and Oude Elferink, 2002; Schinkel and Jonker. 2003.)

II. Expression of human ABC transporter ABCG2 in insect cells

II-1 Human ABC transporter ABCG2

The breast cancer resistant protein (BCRP) has been discovered in doxorubicin-resistant breast cancer cells (Doyle *et al.*, 1998). Since the same transporter has also been found in the human placenta (Allikmets *et al.*, 1998) as well as in drug-resistant cancer cells selected in mitoxantrone (Miyake *et al.*, 1999), the transporter was also called ABCP or MXR1. This ABC transporter protein is now named ABCG2 and has been classified in the G subfamily of human ABC transporter genes according to the new nomenclature.

The *ABCG2* gene is located on chromosome 4q22 and spans over 66 kb, consisting of 16 exons and 15 introns (Bailey-Dell *et al.*, 1999). ABCG2 is expressed endogenously in placental trophoblast cells, the epithelium of the small intestine and liver canalicular membrane, as well as in ducts and lobules of the breast. In addition, expression of ABCG2 is detected in venous and capillary endothelium. In particular, the high expression of ABCG2 in trophoblast cells suggests that the pump is responsible either for transporting compounds into the fetal blood supply, or removing toxic metabolites. The apical localization in the epithelium of the small intestine and colon indicates a possible role of ABCG2 in the regulation of the uptake of *p.o.* administered drugs as well as the protection against toxic xenobiotics (Maliepaard et al. 2001; Zhou et al., 2001; Jonker *et al.*, 2002). In addition, we have recently provided direct evidence that ABCG2 transports SN-38, an active metabolite of irinotecan (CPT-11), and its glucuronide conjugate (Nakatomi *et al.*, 2001; Yoshikawa *et al.*, 2003).

II-2 Construction of expression vector

Human ABCG2 cDNA was cloned from cDNA of the MCF7/BCRP clone-8 cell line by PCR, as described previously (Mitomo *et al.*, 2003). The PCR product is first inserted into the

pCR2.1 TOPO vector, and its sequence is analyzed by automated DNA sequencing. The cDNA is then inserted into the pNNK vector (Fig. 2) that was modified from pPSC8 (Protein Sciences Co.) by Nosan corporation (Yokohama, Japan). Briefly, ABCG2 cDNA-containing pCR2.1 TOPO vector is digested by *Eco*RI, and ABCG2 cDNA is removed. After the treatment of alkaline phosphatase, ABCG2 cDNA is ligated to the *Eco*RI site of the pNNK vector using the Rapid DNA ligation kit (Roche Diagnosis Co., Indianapolis, IN). Recombinant virus is constructed according to the methods described by Summers and Smith (1987) and O'Reilly *et al.* (1994).

II-3 Transfection to insect cells

Sf9 insect cells are diluted with Sf-900II serum free medium at a cell density of 0.2 x 10^6 cells/ml. 5 ml (total of 1.0 x 10^6 cells) of the cell suspension is kept in a 25-cm^2 culture dish. Allow cells to attach the dish for 1 hour at room temperature, and the following reagents are added: 5 μl (358 ng/μl) of the ABCG2/pNNK vector, 5 μl (17 ng/μl) of liner AcNPV DNA (Protein Sciences Co.), 20 μl of Cellfectin Reagent (Invitrogen Co., Cat. No. 10362-010) and 180 μl of Sf-900II serum free medium (Invitrogen Co., Cat. No.10902-088). Following the incubation at 28℃ for 7 days, the cell suspension is centrifuged at 3,000 x *g* for 15 min at 4℃. The resulting supernatant is collected and defined as the primary viral solution.

II-4 Purification of baculovirus by plaque assay

The primary viral solution is diluted 10^4, 10^5, 10^6 and 10^7–folds with Sf-900II serum free medium. On the other hand, 4 ml of the Sf9 cell suspension (0.5 x 10^6 cells/ ml) is put into each 60-mm dish. After Sf9 cells become a monolayer, the culture medium is removed from the dish. 1 ml of the diluted viral solution is added to each dish containing the cell monolayer. Sf9 cells are then incubated with gentle shake at room temperature for 1 h. After the viral solution was removed, Sf-900II serum free medium with 0.5% SeaKem GTG Agarose (FMC Co., Cat. No. 50070) is added to the dish. Sf9 cells are further cultured at 28℃ for 7 days. Recombinant plaques (without polyhedrin) are collected with a Pasteur pipette. Each plaque

together with agarose should be suspended in 1 ml of serum-free Sf-900II medium. Thereby, the primary viral solution is obtained.

II-5 Amplification of recombinant virus and titer check

Dilute Sf9 cells in Sf-900II serum free medium at a cell density of 1.0 x 10^6 cells/ml, and add 2 ml (total of 2.0 x 10^6 cells) of the cell suspension to each 25-cm^2 culture dish. Allow the cells to attach the dish for 1 hour at room temperature. Thereafter, 0.5 ml of the purified primary viral solution and 3 ml of serum-free Sf-900II medium are added to the dish. The cell suspension, thus obtained, is incubated at 28℃ for 72 hour. After centrifuged at 3,000 x *g* for 15 min at 4℃, the resulting supernatant is recovered and defined as the first-generation of viral solution. 5 ml of this viral solution and 10 ml of fresh serum-free Sf-900II medium are added into a 75-cm^2 culture dish containing Sf9 cells (6 x 10^6 cells). After 72 hour post-infection, the cell suspension is centrifuged at 3,000 x g for 15 min at 4℃, and the supernatant (15 ml) is recovered and stored at 4℃ as the second-generation of viral solution.

*expres*SF+® cells (Protein Sciences Co.) are maintained to grow in serum-free Sf-900II medium. The cells in the exponential multiplication stage are withdrawn and diluted to 1.5 x 10^6 cells/ml with the same medium. Then, 1 ml of the second-generation viral solution is added to the cell suspension (100 ml) in a 250-ml Erlenmeyer flask. The cell suspension is maintained with gentle rotation (130 rpm) at 28℃, and cells are harvested at 72 hours post infection. After centrifuged at 3,000 x *g* for 15 min at 4℃, the supernatant is recovered and defined as the third-generation of viral solution.

To determine the viral titer, the third-generation of viral solution is diluted to 10^5, 10^6, 10^7 and 10^8-folods with Sf-900II serum-free medium. 4 ml of the Sf9 cell suspension (0.5 x 10^6 cells/ml) is put into each 60-mm dish. After Sf9 cells have become a monolayer, the culture medium is removed from the dish. 1 ml of the diluted viral solution is added to each dish containing the cell monolayer. Sf9 cells are then incubated with gentle shake at room temperature for 1 h. After the viral solution was removed, Sf-900II serum free medium with 0.5% SeaKem GTG Agarose (FMC Co., Cat. No. 50070) is added to the dish. Sf9 cells are

further cultured at 28℃ for 7 days. Resultant plaques are counted and the viral titer is determined.

II-6 Expression of ABCG2 in expresSF+® cells

*expres*SF+® cells in the exponential multiplication stage are diluted to a cell density of 1.5 $\times 10^6$ cells/ml with Sf-900II serum free medium, and 100 ml of the cell suspension is mixed with the third third-generation of viral solution in a 250-ml Erlenmeyer flask. MOI at this time should be set at 1.0. Cells are maintained with a gentle rotation (130 rpm) at 28℃. Three days after the infection, *expres*SF+® cells are harvested by centrifugation. Cells are subsequently washed with phosphate-buffered saline (PBS) at 4°C, collected by centrifugation, and stored at –30°C until used.

III. Preparation of plasma membrane vesicles and detection of ABCG2 protein

III-1 Plasma membrane vesicles from insect cells

Plasma membrane vesicles can be prepared from either Sf9 cells or *expres*SF+® cells in the same way. Fig. 3 demonstrates the procedure of plasma membrane preparation. The harvested and frozen cells should be quickly thawed, diluted with 30 ml of an ice-cold hypo-tonic buffer (0.5 mM Tris/Hepes, pH 7.4. and 0.1 mM EGTA) and then homogenized with a Potter-Elvehjem homogenizer. After centrifugation at 2,000 x *g* for 10 min at 4°C, the supernatant is collected, whereas the precipitate is further homogenized with a Potter-Elvehjem homogenizer in another 30 ml of the hypo-tonic buffer. After centrifugation at 2,000 x *g*, the resulting supernatant was collected and combined with the first supernatant fraction. The supernatant (total of 60 ml) is centrifuged at 100,000 x *g* for 30 min. The pellet, thus obtained, is suspended in 10 ml of 250 mM sucrose containing 10 mM Tris/Hepes, pH 7.4, and homogenized with a Potter-Elvehjem homogenizer. The crude membrane fraction is carefully layered over 40% (w/v) sucrose solution and centrifuged at 100,000 x *g* for 30 min. The turbid layer at the interface should be collected with a Pasteur pipette, suspended in 250 mM sucrose containing 10 mM Tris/Hepes, pH 7.4, and

centrifuged at 100,000 x *g* for 30 min. The membrane fraction is collected and resuspended in a small volume (250 to 500 µl) of 250 mM sucrose containing 10 mM Tris/Hepes, pH 7.4. After the measurement of protein concentration by the BCA Protein Assay Kit (PIERCE, Rockford, IL, USA), the membrane preparations should be frozen and stored either at –80°C until used.

III-2 Immunological detection of ABCG2 in plasma membrane vesicles

The amount of ABCG2 expressed in the cell membrane vesicles is determined by immunoblotting with BXP-21 (SIGNET, Dedham, MA, USA), a specific antibody to human ABCG2. Briefly, proteins of the plasma membrane are separated by electrophoresis on 10% sodium dodecyl sulfate (SDS) polyacrylamide slab gels, and the proteins are subsequently electroblotted onto Hy-bond ECL nitrocellulose membranes (Amersham, Buckinghamshire, UK). Immunoblotting is 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, USA) (1:3000 dilution) as the secondary antibody. HRP-dependent luminescence is developed by using Western Lighting Chemiluminescent Reagent Plus (PerkinElmer Life Sciences, Boston, MA, USA) and detected by Lumino Imaging Analyzer FAS-1000 (TOYOBO, Osaka, Japan). According to this immunoblotting procedure, ABCG2 monomer expressed in insect cell membranes is detected at a molecular weight of 68,000, the sample is treated with mercaptoethanol (Fig. 4A). To analyze the transport activity of ABCG2 variants, it is critically important to normalize the expression level of each variant protein. Fig. 4B clearly demonstrates a linear relationship between the signal intensity of immunoblotting and the logarithmic value of the amount of protein applied to the eloctrophoresis. Based on the linear relationship, the expression levels of ABCG2 and its variants in different plasma membrane preparations can be quantitatively estimated and normalized.

IV. High-speed screening to measure the transport activity of ABCG2 and its variants

IV-1 Development of a high-speed screening system

The original assay method to measure ATP-dependent transport of organic anions into inside-out plasma membrane vesicles was developed by Ishikawa (1989) and subsequently used by other researchers (Ishikawa *et al.*, 1990; Keppler *et al.*, 1998). Recently we have improved the method to enhance the speed of screening and to profile the substrate specificity of ABC transporters. To detect the transport activity of ABCG2, we use methotrexate (MTX) as a substrate (Mitomo *et al.*, 2003; Ishikawa *et al.*, 2003).

The frozen stocked membrane should be quickly thawed, and membrane vesicles are formed by passing the membrane suspension through a 27-gauge needle. For the measurement of ABCG2-mediated MTX transport, the standard incubation medium should contain plasma membrane vesicles (10 or 50 μg of protein), 200 μM [3',5',7'-^{3}H]MTX (Amersham, Buckinghamshire, UK), 0.25 M sucrose, 10 mM Tris/Hepes, 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 100 μl. The incubation is carried out at 37°C. After a specified time (20 min for the standard condition), the reaction medium is mixed with 1 ml of the ice-cold stop solution (0.25 M sucrose, 10 mM Tris/Hepes, pH 7.4, and 2 mM EDTA) to terminate the transport reaction. Subsequently, aliquots (280 μl per well) of the resulting mixture are transferred to MultiScreen™ plates (Nihon Millippore KK, Tokyo, Japan) (Fig. 5A). Under aspiration, each well of the plate is rinsed by the 0.25 M sucrose solution containing 10 mM Tris/Hepes, pH 7.4 four times (4 x 200 μl for each well) in an EDR384S system (BioTec, Tokyo, Japan) (Fig. 5B). [^{3}H]MTX thus incorporated into the vesicles is measured by counting the radioactivity remaining on the filter of MultiScreen™ plates, where each filter is placed in 2 ml of liquid scintillation fluid (Ultima Gold, Packard BioScience).

Fig. 6 depicts the time course of MTX transport into plasma membrane vesicles. MTX transport into ABCG2-expressing plasma membrane vesicles was greatly enhanced in the presence of ATP. In plasma membrane vesicles prepared from mock-infected cells, ATP-dependent transport of MTX was not observed.

IV-2 Functional screening of genetic polymorphisms of ABCG2

There is accumulating information on the genetic polymorphism of ABCG2 (Ishikawa et al. 2004; Cevenak et al., 2005 for recent reviews). Fig. 7A shows the hitherto reported alterations of amino acid residues due to non-synonymous SNPs and acquired mutations. Quantitative studies should be carried out to precisely evaluate functional changes associated with such genetic polymorphisms. For this purpose, variant forms (V12M, G51C, Q126stop, Q141K, T153M, Q166E, I206L, E334stop, N590Y, D620N, R482G, and R482T) have been created by the site-directed mutagenesis using the QuikChange Site-directed Mutagensis Kit (Stratagene). Recombinant baculoviruses to express the above-mentioned variant forms of ABCG2 in insect cells were generated with the BAC-TO-BAC Baculovirus Expression Systems (Invitrogen), as described previously (Ishikawa et al., 2004). Insect Sf9 cells (1 x 10^6 cell/ml) were infected with the recombinant baculoviruses and cultured in the EX-CELL™ 420 Insect serum-free medium (JRH Bioscience, Levexa, KS, USA) at 26°C with gentle shaking. 48 hours after the infection, cells were harvested by centrifugation. Cell membranes were prepared as described above (Fig. 3). Fig. 7B shows our data on the MTX transport activity of those ABCG2 variants after the normalization of expression levels. No MTX-transport activity was observed in variants of Q126stop, E334stop, R482G, and R482T.

V. High-speed screening of human ABCB1 and its variants

V-1 Human ABC transporter ABCB1

Human ABCB1 (P-glycoprotein or MDR1) was identified because of its overexpression in cultured cancer cells associated with an acquired cross-resistance to multiple anticancer drugs, such as colchitine, doxorubicin, daunorubicin, vincristiene and VP16 (Ling, 1997; Ambudkar *et al.*, 1999). Besides cancer cells, ABCB1 is expressed in many normal tissues; e.g., the apical domain of the enterocytes of the gastrointestinal tract (jejunum and duodenum), the endothelial cells lining the small vessels of the human cortex, the lumenal membrane of capillary endothelial cells of the brain,