FEBS Letters 567 (2004) 116–120 FEBS 28447

Minireview

ABCG2 – a transporter for all seasons

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Received 9 March 2004; accepted 14 March 2004

Available online 8 May 2004

Edited by Horst Feldmann

Abstract The human ABCG2 (ABCP/MXR/BCRP) protein is a recently recognized ABC half-transporter, which forms homodimers in the plasma membrane and actively extrudes a wide variety of chemically unrelated compounds from the cells. This protein protects our cells and tissues against various xenobiotics, with a crucial role in the intestine, liver, placenta, and the blood–brain barrier. Moreover, ABCG2 seems to have a key function in stem cell protection/regulation, and also in hypoxic defense mechanisms. Widely occurring single nucleotide polymorphisms in ABCG2 may affect absorption and distribution, altering the effectiveness and toxicity of drugs in large populations. At the clinics, overexpression of ABCG2 in tumor cells confers cancer multidrug resistance to a variety of newly developed anticancer agents. On the other hand, specific substrate mutants of ABCG2 are advocated for use as selectable markers in stem-cell based gene therapy.

Keywords: ABCG2; Multidrug transporter; Xenobiotic transport; Cancer drug resistance; Stem cell regulation; Polymorphic variant; Gene therapy; Hypoxic defense

1. Introduction – "multidrug resistance" ABC transporters

ATP-binding cassette (ABC) proteins are present in all known living species, with a relatively conserved structure which contains a combination of conserved ABC and transmembrane (TM) domains (TMDs). In mammals, the functionally active ABC proteins consist of at least four of such domains, two TMDs and two ABCs. These domains may be present within one polypeptide chain (full transporters), or within two separate proteins (half-transporters). In this latter case, functional ABC transporters need the dimerization of specific half-transporters.

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Abbreviations: ABC transporters, ATP-binding cassette transporters; ABCP, placenta-specific ABC transporter; ADME-TOX, absorption, distribution, metabolism, and toxicity; BCRP, breast cancer resistance protein; DOX, doxorubicin; MDR1, human multidrug resistance protein (P-glycoprotein, ABCB1); MRP, human multidrug resistance associated protein, ABCC1; MXR, mitoxantrone resistance protein; Sf9 cells, *Spodoptera frugiperda* ovarian cells; TK, tyrosine kinase; TM, transmembrane

The TMDs contain polypeptide chains that span the membrane multiple times, typically forming six TM α-helices in each TMD (see [1] and Fig. 1). The conformational changes within the TM domains are believed to be responsible for the transport of molecules through these transporters. The ABC regions are the sites to bind and/or hydrolyze cytoplasmic ATP and, in the active transporters, ATP hydrolysis ensures the energy for the uphill transport of a substrate. The specific interaction of ABCs with TMDs provides the transmission gear connecting substrate binding and transport to the hydrolysis of ATP.

In humans, 49 ABC genes have been described and a consistent nomenclature, based on sequence homology, has been adopted. These proteins include regulated membrane channels, e.g., the cystic fibrosis TM conductance regulator (CFTR/ABCC7), receptors also involved in complex channel formation, e.g., the sulfonylurea receptors (SUR/ABCC8 and 9), and a large number of active transporters. In this latter case, ATP hydrolysis is directly connected to substrate transport through various cellular membranes (for reviews, see [2–5]).

The multidrug transporter ABC proteins are plasma membrane glycoproteins which cause chemotherapy resistance in cancer by actively extruding a large variety of therapeutic compounds from the malignant cells. However, the same ABC transporters play an important protective function against toxic compounds in a variety of cells and tissues, especially in secretory organs, at the sites of absorption, and in blood-tissue barriers (see [6,7]).

The three major multidrug resistance ABC proteins are MDR1 (P-glycoprotein, ABCB1), multidrug resistance associated protein 1 (MRP1, ABCC1) and ABCG2 (placenta-specific ABC transporter, ABCP/breast cancer resistance protein, BCRP/mitoxantrone resistance protein, MXR). MDR1 and MRP1 can transport a large variety of hydrophobic drugs, and MRP1 can also extrude anionic drugs or drug conjugates. Additional members of the MRP/ABCC family have also been indicated to be involved in cancer multidrug resistance (for details, see [8]). The transport properties of ABCG2 are overlapping both with that of MDR1 and the MRP type proteins, thus these three proteins form a special network in chemo-defense mechanisms.

2. Structure, transport properties and possible mechanism of action of ABCG2

The G subfamily of ABC transporters consists of five half-transporters with a domain arrangement, when the ABC

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doi:10.1016/j.febslet.2004.03.123

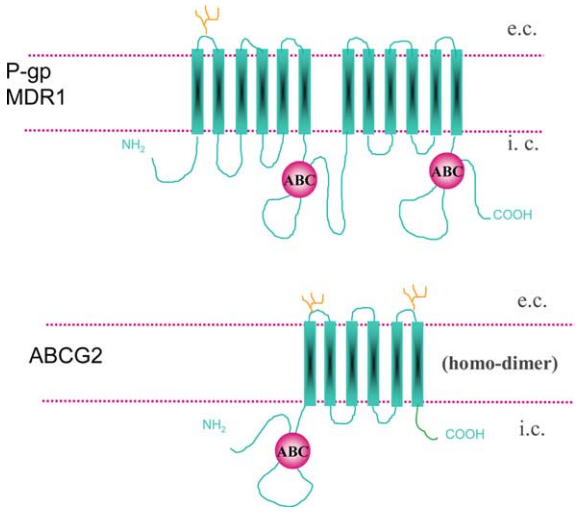

Fig. 1. Proposed membrane topology of the P-gp/MDR1 and the ABCG2 multidrug transporters.

domain is located towards the N-terminus of the polypeptide chain. The suggested membrane topology of the human ABCG2, based on various prediction models, is presented in Fig. 1. The predicted glycosylation sites are also indicated in this model. There has been no detailed study as yet for the experimental verification of this membrane topology, but the double glycosylation of the protein, when expressed in mammalian cells, has already been documented [9]. It has also been shown that the expression or transport function of ABCG2 does not require glycosylation, when the protein was expressed in insect, *Spodoptera frugiperda* ovarian cells (Sf9 cells) (see [10]). Membrane insertion and transport function most probably require the dimerization of ABCG2 (see below).

ABCG2 was first cloned from the placenta and from multidrug-resistant tumor cells, not expressing either MDR1 or MRP1 [11–13]. By now it has been established that ABCG2 functions as a high capacity drug transporter with wide substrate specificity. This protein can transport large, hydrophobic, both positively or negatively charged molecules, including cytotoxic compounds (mitoxantrone, topotecan, flavopiridol, methotrexate), fluorescent dyes (e.g., Hoechst 33342) and different toxic compounds found in normal food (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, PhIP) or pheophorbide A [14–19].

ABCG2 mediates the extrusion of the transported compounds towards the extracellular space through a process energized by ATP hydrolysis [20]. This active transport has been directly observed in inside-out membrane vesicles, when ABCG2 was shown to transport methotrexate [21].

It has been documented that ABCG2 shows a drug-stimulated, vanadate-sensitive ATPase activity, which requires the presence of Mg ions [9,10]. This ATPase activity, as in the case of other multidrug transporters, reflects the substrate recognition and is connected to substrate transport by this protein. However, when the human ABCG2 is expressed in the Sf9 insect cell system, in isolated membranes a large fraction of ABCG2-ATPase seems to be already activated by endogenous substrates, and additional substrates have only a minor effect [10]. In contrast, in certain isolated mammalian cell membranes, ABCG2-ATPase is significantly activated by the transported compounds (unpublished observations). Moreover, when human ABCG2 was expressed in a bacterial (lactobacillus) expression system, membrane cholesterol itself was found to activate this protein [22]. Thus, endogenous lipids or lipid derivatives may be natural transported substrates of ABCG2.

The active, ATP-dependent transport properties of ABCG2 have also been documented by the formation of the ATPase reaction cycle intermediate, trapped ADP, in this protein [10]. This ADP trapping reaction, which is stabilized by the ATPase inhibitor vanadate, is a general feature of all known active transporter ABC proteins [1]. Interestingly, in the case of ABCG2 only the cobalt complex of 8-azido-ATP could be used to obtain a covalent linkage of this trapped intermediate to the protein. ABCG2 has only one ABC and one TM domain, therefore, it requires dimerization to become active. There are several findings indicating that ABCG2 functions as a homodimer. In drug-selected cell lines, an exclusive upregulation of the ABCG2 mRNA and the amplification of the abcg2 gene alone was enough to circumvent drug-toxicity [13,23]. In mammalian or insect cells, transfected with the ABCG2 cDNA, ABCG2 was fully functional [9,12]. Moreover, co-expression of an inactive ABCG2 variant with the wild-type protein resulted in a loss of ABCG2 activity [24]. In this latter study, the formation of ABCG2 homodimer, bridged by a disulfide bond, was suggested.

3. ABCG2 in cancer drug resistance

The overexpression of ABCG2 was observed in certain drug-resistant cell lines and tumors, providing a special multidrug resistant phenotype in these cancer cells. Human ABCG2 was shown to confer resistance against various, clinically relevant compounds, e.g., mitoxantrone, methotrexate, topotecan, SN38, and flavopiridol [13–15,25–27]. The first cloning of ABCG2 from either expressed sequence tags or heavily drug selected cell lines yielded sequences coding for different amino acids at position 482 [3–5]. As discussed below, this single amino acid change results in major differences in substrate recognition and transport by ABCG2 (see Section 5, and Fig. 2). Therefore, for some time, different experimental expressions of ABCG2 yielded different results regarding substrate handling by this protein. Based on overwhelming evidence, in the current review we refer to R482-ABCG2 as the wild-type protein. Therefore, clinical anthracyclin resistance probably cannot be caused by ABCG2, while in the case of many new anticancer agents ABCG2 may be a candidate transporter for cancer drug resistance (see Fig. 2). In fact, polymorphic variants may further modulate substrate recognition or transport activity of ABCG2, as discussed in Section 5.

We have shown recently that the human ABCG2 multidrug transporter interacts with two recently developed specific tyrosine kinase inhibitors (TKIs), Gleevec/Imatinib (STI-571) and Iressa (ZD 1839), with a high affinity [53]. In current antitumor drug research, a large variety of TKIs with increasing specificity and selectivity have been developed. These are highly promising agents for specific inhibition of malignant cell growth and metastasis formation. However, their therapeutic potential also depends on access to their intracellular targets, which may be significantly modulated by ABC membrane

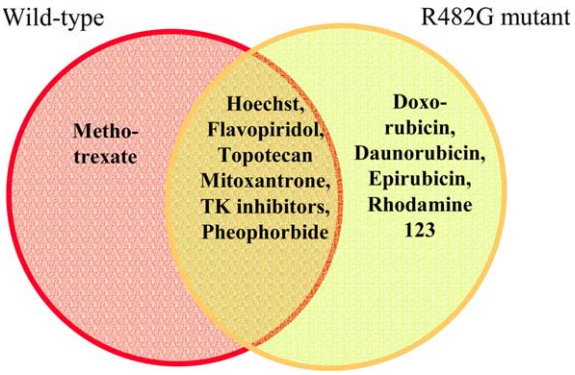

Fig. 2. Transported substrates of the wild-type and R482G ABCG2 protein.

transporters. Our results indicate that ABCG2 modulation by TKIs may be an important factor in the treatment of cancer patients, moreover, an extrusion of TKIs by ABCG2 may result in tumor cell TKI resistance. Based on the above described role of ABCG2 in tumor resistance, the selective and sensitive detection of the ABCG2 protein has a major importance in cancer diagnostics and treatment. The best known experimental approach in this regard is the analysis of the active extrusion of the fluorescent dye, Hoechst 33342 by ABCG2, by using fluorescence detection, e.g., flow cytometry (see [16]). Several other fluorescent substrates of ABCG2 (e.g., topotecan, flavopiridol, BODIPY-prazosin or mitoxantrone) have also been applied in this regard. However, this dye transport assay is usually not specific for ABCG2, and the combined use of ABCG2-specific inhibitors is suggested in these methods. A recent report suggests that the chlorophyll derivative, pheophorbide A, is an ABCG2 selective transported substrate that can be used for flow cytometry analysis of this protein [19]. Still, a highly sensitive and selective dye transport method, which could be used for in vivo sorting of ABCG2 expressing cells, is yet to be introduced.

The recent development of a monoclonal antibody, specifically reacting with the human ABCG2 protein on the cell surface, has been a major breakthrough in diagnostic applications [16]. This antibody was prepared by immunizing mice with intact mouse fibroblasts, expressing the human ABCG2. The antibody, named 5D3, was reported to inhibit the Hoechst 33342 dye transport function of ABCG2 in intact cells [16]. Recently, we have found that detection and inhibition by 5D3 strongly depends on the actual conformation of ABCG2 (Várady et al., unpublished). This conformation-sensitive interaction may be applied for the investigation of the molecular mechanism and the detection of drug interactions of ABCG2.

4. ABCG2 and tissue defense – ADME-TOX, stem cells and hypoxia

It has been increasingly recognized that transporter-mediated processes significantly modulate drug absorption, distribution, metabolism and excretion. By now it is well established that active efflux by ABC transporters in the small intestine is a major contributor to poor absorption and low bioavailability. To be effective, absorbed drugs must also enter various body compartments, e.g., cross the blood–brain barrier, and this entry is also modified by the efflux function of various ABC transporters. Transporters are common sites for drug–drug interactions, as well as interactions of drugs with endogenous substrates. These effects may lead to unexpected drug toxicity and various adverse effects (for recent review, see [7]).

The functional characteristics and the distribution of ABCG2 suggest a major role of this protein in the tissue protection against xenobiotics (see [28,29]). As mentioned above, ABCG2 transports a large variety of hydrophobic or partially hydrophobic molecules, and this protein was found to be physiologically expressed in the canalicular membrane of the liver, in the epithelia of small intestine, colon, lung, kidney, adrenal and sweat glands, as well as in the endothelia of veins and capillaries.

The development of abcg2 knock-out mice, achieved independently in two different laboratories [17,30], was a major step in searching for the physiological functions of this transporter. Although these knock-out animals are alive and show no major pathology, several alterations have been observed which can be connected to the lack of functional abcg2. In combination with ABCG2 inhibitor studies, carried out in normal subjects, these investigations suggested that ABCG2 has a major role in the modulation of the absorption of toxic materials from food, as well as of that of several orally applied pharmacological agents.

The Dutch group which developed an abcg2 knock-out mice found that in these animals the combination of exposure to light and an alphalpha diet, rich in chlorophyll, led to phototoxic skin lesions [17]. These lesions were demonstrated to be caused by the accumulation of a chlorophyll degradation product, pheophorbide A. As described below, in the knock-out mice studies carried out in the USA, the authors reported a role of ABCG2 in heme or related metabolite transport [54].

In addition to the role of ABCG2 in absorption, an increased biliary secretion of the transported compounds by ABCG2 may also significantly contribute to a decreased bioavailability of various agents. Indeed, ABCG2 expression was clearly shown to influence both the absorption and secretion of the anticancer agent topotecan [26,31]. Although ABCG2 expression was reported in the blood–brain barrier [32], we have no convincing data as yet about the role of this protein in this barrier function. Interestingly, a high level expression of the ABCG2 protein and its fluorescent dye extrusion function has been suggested to identify hemopoietic stem cells [16,33,34]. Moreover, this so called "side population" of progenitor cells, actively extruding the fluorescent Hoechst 33342 dye, seems to represent pluripotent stem cells in a variety of tissue sources [16,33–36]. Whether ABCG2 is required for a metabolic protection of stem cells, or may directly influence stem cell functions, e.g., differentiation, is unclear at present [16,30,37].

Recent results of Krishnamurthy et al. [54] indicate that ABCG2 expression provides an important cell survival advantage under hypoxic conditions. Progenitor cells obtained from abcg2 knock-out mice showed a reduced ability to form colonies under hypoxia. Moreover, blocking of abcg2 function in normal progenitor cells reduced survival under hypoxic conditions. In both cases, blockade of heme/porphyrin synthesis reversed this hypoxic susceptibility. Cells overexpressing ABCG2 accumulated lower amounts of porphyrin, heme was specifically bound to ABCG2, and drug transport by ABCG2 was significantly modulated by heme. ABCG2 expression was

upregulated by hypoxia through interaction with the hypoxia response elements in the ABCG2 promoter region. These studies suggest that ABCG2 permits enhanced cell survival in oxygen poor environments by reducing the accumulation of toxic heme metabolites. These findings have important implications for the chemotherapeutic treatment of solid tumors with stimulated ABCG2 expression under hypoxia and for the survival of stem cells in transplantation.

5. Mutant and polymorphic variants of ABCG2

There is little information about the structural elements responsible for the substrate recognition and transport by ABCG2. The TMDs of ABC transporters are thought to be responsible for the recognition of transported substrates. In the case of MDR1, deletion mutants containing only the TMDs still retained their substrate binding capacity [38]. Moreover, different studies identified a number of amino acids in various TM helices of MDR1 and MRPs, thought to form the drug-binding domain in these proteins [38–41].

In the case of ABCG2, in drug-selected cell lines, overexpressing human or mouse ABCG2, a single amino acid change in aa. position 482 occurred [42,43]. The mutants having R482G or R482T (R482M or R482S in the mouse abcg2) showed altered substrate specificity as compared to the wild-type protein, i.e., they conferred increased mitoxantrone or doxorubicin (DOX) resistance and rhodamine 123 transport capacity (see Fig. 2, [42,43]). In fact, these R482 mutants have increased transport and ATP hydrolytic activity, therefore they are considered as "gain of function" mutants [10]. However, the R482G and R482T mutants were not able to transport methotrexate, which is a transported substrate of the wild-type ABCG2 [15,44].

In a recent study [45], several amino acids in the TM helices of ABCG2 have been mutated and the protein variants were analyzed by measuring drug resistance in intact cells. The mutants showed significantly different drug resistance patterns, indicating the importance of these residues in the substrate specificity of ABCG2. In a recent, detailed study we have expressed nine different ABCG2 position 482 variants in Sf9 cells [Özvegy et al, unpublished], and demonstrated that an exchange of this single amino acid was not detrimental for the correct folding or basic transport activity of ABCG2, but induced major alterations in both the transport activity and substrate specificity of this protein. These variants may be applied to express this multidrug transporter with the desired transport profile for various cell protection applications (see below).

Recent studies have identified two frequently occurring polymorphisms in ABCG2, which are non-synonymous SNPs, resulting in amino acid substitution in the coding region of the protein. These SNPs yield ABCG2 variants containing V12M and Q141K. When these variants were expressed in mammalian or insect cells, a different pattern of drug resistance, and a significantly altered transport capacity and substrate handling has been observed [46,47] [Morisaki et al., unpublished]. Population data for the actual frequency of these variants, the possible effects of the wild-type/variant heterodimerization (probably present in heterozygous individuals) and the detailed functional characterization of the ABCG2 variants are challenging tasks in this research area.

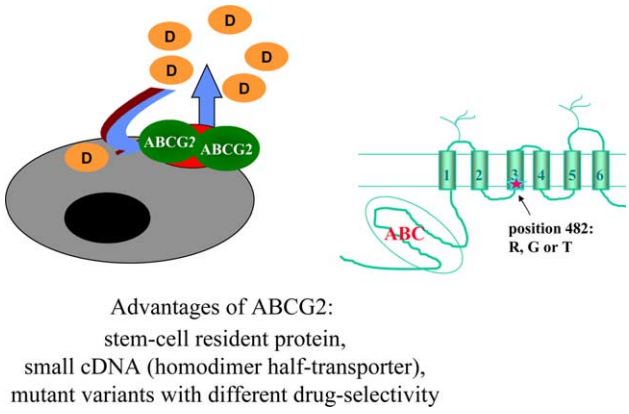

Fig. 3. Gene-therapy application of the mutant ABCG2 as a selectable marker protein.

6. ABCG2 in stem cells – possible gene therapy applications

The co-expression of a human drug-resistance protein with a therapeutic gene product should allow both an enrichment of the corrected cells and an in vivo drug selection during clinical gene therapy. The use of the MDR1-P-glycoprotein as such a selectable marker has been widely investigated and advocated, while other studies reported major problems with this approach (see [48–51]).

Recent studies in our laboratory suggest that a mutant ABCG2 protein is an ideal candidate for human stem cell protection and for use as a selectable marker in gene therapy (Fig. 3). The cDNA encoding this protein is relatively small (about 2 kbase) and the active dimer is spontaneously formed in the overexpressing cells [52]. Since the R482G variant of ABCG2 has different substrate specificity than the wild-type protein (Fig. 2), this mutant has a special advantage in gene therapy applications.

We have documented that when the mutant ABCG2 was co-expressed with a therapeutic gene, the expression of the therapeutic gene in hematopoietic progenitor cells corrected the loss-of-function mutation responsible for human chronic granulomatous disease. At the same time, the mutant ABCG2 protein selectively protected the transduced cells against clinically applicable cytotoxic agents. Overexpression of ABCG2 did not affect in vitro hematopoietic cell maturation or the restoration of granulocyte function by the therapeutic gene. The in vivo applicability of such a tailor-made drug resistance ABC transporter as selectable marker still awaits further studies.

7. Conclusions and perspectives

The human ABCG2 protein is clearly a multi-task transporter, having important roles in a variety of physiological and pathological functions. Protection of our tissues, including the stem cells, against xenobiotics and hypoxia, modulation of drug absorption and distribution, are the positive sides of ABCG2 expression. In contrast, multidrug resistance caused by ABCG2 is a major challenge at the cancer clinics. However, even this drug resistance function may be put to good use in medical therapy, since the application of ABCG2 may promote stem-cell based gene therapy.

Acknowledgements: This work has been supported by research grants from OTKA, ETT and OM, Hungary (T-35126, T-31952, T35926, T38337, D 45957, ETT, and NKFP). Balázs Sarkadi is a recipient of a Howard Hughes International Scholarship. We appreciate the partly unpublished information provided for this review by Susan E. Bates and John D. Schuetz.

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