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Biochimica et Biophysica Acta 1668 (2005) 53–63
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Single amino acid (482) variants of the ABCG2 multidrug transporter: major differences in transport capacity and substrate recognition

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Received 28 June 2004; received in revised form 19 October 2004; accepted 10 November 2004
Available online 24 November 2004

Abstract

The human ABCG2 protein is an ATP binding cassette half-transporter, which protects our cells and tissues against various xenobiotics, while overexpression of ABCG2 in tumor cells confers multidrug resistance. It has been documented that single amino acid changes at position 482 resulted in altered drug resistance and transport capacity. In this study, we have generated nine Arg-482 mutants (G, I, M, S, T, D, N, K, Y) of ABCG2, and expressed them in insect cells. All ABCG2 variants showed cell surface expression and, in isolated membranes, an ABCG2-specific ATPase activity. When methotrexate accumulation was measured in inside-out membrane vesicles, this transport was supported only by the wild-type ABCG2. In intact cells, mitoxantrone was transported by all ABCG2 variants, except by R482K. Rhodamine 123 was extruded by most of the mutants, except by R482K, Y and by wild-type ABCG2. Hoechst 33342 was pumped out from cells expressing the wild-type and all Arg-482 variants, but not from those expressing R482K and Y. Our study demonstrates that the substrate specificity of the Arg (wild-type) form is unique and that amino acid replacements at position 482 induce major alterations in both the transport activity and substrate specificity of this protein.

Keywords: Multidrug half-transporter; Single amino acid mutant; Cell surface localization; Membrane ATPase; Vesicular transport; Fluorescent dye extrusion

1. Introduction

The human ABCG2 multidrug transporter (ABCP/BCRP/MXR) is a plasma membrane glycoprotein, which belongs to the ATP binding cassette (ABC) protein family. ABCG2 is a half transporter, possessing only one ATP binding and one transmembrane domain, and most probably acts as a homodimer [1,2]. The ABCG2 protein is present in several normal tissues [3,4], and its overexpression has also been documented in drug-resistant cell lines and tumors [1].

ABCG2 transports a wide variety of compounds, including cytotoxic agents (mitoxantrone, topotecan, flavopiridol, methotrexate), fluorescent dyes (e.g., Hoechst 33342) and different toxic compounds found in normal food (e.g., 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) or pheophorbide a) [4–8]. ABCG2 mediates the extrusion of these compounds towards the extracellular space, a process energized by ATP hydrolysis [1]. Transport function and tissue distribution of ABCG2 suggest its role in protection/detoxification against xenobiotics [3,9] and, indeed, ABCG2 has been shown to influence the intestinal topotecan absorption and its secretion into the bile [10,11].

There is little information about the amino acids responsible for the substrate specificity of ABCG2. The transmembrane domain of ABC transporters is thought to be responsible for the recognition of transported substrates. In

Abbreviations: ABC, ATP binding cassette; ABCP, placenta specific ABC transporter; BCRP, breast cancer resistance protein; β-gal., β-galactosidase; Hst, Hoechst 33342; MDR1, human multidrug resistance protein (P-glycoprotein, ABCB1); MRP, human multidrug resistance associated protein, ABCC1; MXR, mitoxantrone resistance protein; MTX, methotrexate; MX, mitoxantrone; R123, rhodamine 123; Sf9 cells, *Spodoptera frugiperda* ovarian cells; SN-38, 7-ethyl-10-hydroxy-camptothecin; TM, transmembrane; wt, wild-type

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doi:10.1016/j.bbamem.2004.11.005

the case of MDR1, deletion mutants containing only the transmembrane domains still retained their substrate binding capacity [12]. Moreover, different studies have identified amino acids in TM helices 4, 5, 6, 9, 10, 11 and 12 of MDR1, responsible for drug binding and thought to form the drug-binding domain of this protein [12–15]. In colchicine selected cells, mutation of Gly 185 to Val (found in the intracellular loop between TM helices 2 and 3) occurred in the overexpressed MDR1 protein [16]. The G185V mutant conferred altered basal ATPase activity and altered interaction with substrates, as well as with the inhibitor cyclosporin A [17,18]. In case of MRP1, 2 or 3, again amino acids found in the TM region were shown to influence substrate specificity [19–21].

In some drug-selected cell lines overexpressing human ABCG2 (or its mouse ortholog), a single amino acid change at position 482 (predicted to be situated in the third TM helix) occurred [22,23]. The mutants, containing R482G, T or M (R482M or S in the mouse abcg2), showed altered substrate specificity [22–24]. Previously, we have shown that the R482G and T mutants have increased ATP hydrolytic activity, therefore they are "gain of function" mutants in this regard [25]. However, the R482G and T mutants were unable to transport methotrexate, which is a substrate of the wtABCG2 [6,26]. A recent study by Miwa et al. [27] analyzed several mutant forms of ABCG2 in conferring resistance to mitoxantrone or SN38.

The aim of the present work was to analyze how different Arg-482 mutants influence the function of human ABCG2. Therefore, we have created seven additional ABCG2-R482 mutants (I, M, S, D, N, K and Y), representing various amino acid properties. We have expressed these mutants in the baculovirus-Sf9 insect cell expression system, which allows the investigation of both the transport and ATP-hydrolytic functions of ABCG2. Moreover, in this heterologous expression system there is no potential endogenous dimerization partner for the human ABCG2, thus the mutant variants function only as uniform homodimers.

The ATP hydrolytic capacity of the mutants was measured in isolated membrane vesicles, and the effect of potential substrates and inhibitors on this activity was determined. We also compared the MgATP-dependent methotrexate transport capacity of wtABCG2 and nine Arg-482 mutants using inside-out membrane vesicles. Additionally, we analyzed the transport of several fluorescent compounds (mitoxantrone, rhodamine 123 and Hoechst 33342) in intact cells expressing these mutants.

2. Materials and methods

2.1. Materials

ATP, methotrexate, mitoxantrone, Na-orthovanadate, prazosin, propidium iodide and rhodamine 123 were purchased from Sigma. Hoechst 33342 was purchased from Molecular Probes. Ko143 was a generous gift from Dr. G. Koomen (Division of Experimental Therapy, The Netherlands Cancer Institute, and Laboratory of Organic Chemistry, University of Amsterdam, Amsterdam, The Netherlands). [^{3}H]methotrexate was purchased from Moravek Biochemicals.

2.2. Generation of transfer vectors possessing different human ABCG2 cDNAs

pAcUW21-L/ABCG2 (wild-type, R482G, T or K86M/R482G) was constructed as described earlier [25]. In this study, we used the K86M-R482 single mutant, which was generated by cloning the *Not*I–*Spe*I fragment of pAcUW21-L/K86M-R482G [25] into the corresponding site of pAcUW21-L/R482. The seven additional Arg-482 variants were created using ABCG2-R482G cDNA as a template by overlap extension PCR [25,28]. The same outer primer pairs were used and the same cloning strategy was performed as described previously [25]. The two internal complementary primer pairs containing the specific mutation were: 5′-tta tta cca atg atc atg tta cc-3′ and 5-′gg taa cat gat cat tgg taa taa-3′ (R482I), 5′-tta tca gat cta tta ccc atg-3′ and 5′-gg taa cat cat cat ggg taa t-3′ (R482M), 5′-ta ccc atg tcg atg tta cca a-3′ and 5′-t tgg taa cat cga cat ggg ta-3′ (R482S), 5′-cc atg gac atg tta cca tcg att ata-3′ and 5′-tat aat cga tgg taa cat gtc cat gg-3′ (R482D), 5′-atg tta cca tcg att ata ttt acc-3′ and 5′-cc atg aat atg tta cca tcg att ata-3′ (R482N), 5′ -tta tta cct atg aag atg tta-3′ cc and 5′-gg taa cat ctt cat agg taa taa-3′ (R482K) and 5′-tta tta cct atg tac atg tta cc-3′ and 5′-gg taa cat gta cat agg taa taa-3′ (R482Y). The mutations were confirmed by sequencing the *Pst*I–*Msc*I fragments of the constructs.

2.3. Generation of recombinant baculoviruses

Recombinant baculoviruses carrying the different human ABCG2 cDNAs were generated as described [25,29]. ABCG2 protein expression was determined by immunoblotting and immunoflow cytometry (see below).

2.4. Membrane preparation and immunodetection of ABCG2

Virus-infected Sf9 cells were harvested after 72 h of infection. Membranes were isolated by differential centrifugation [30] and stored at −80 °C. The membrane protein concentrations were determined by the modified Lowry method [29]. Immunoblot detection was performed as described in Ref. [25]. The expression level of different ABCG2 mutants was determined by densitometry of the immunoblots (BioRad ChemiDoc).

Immunoflow cytometry was performed by labeling 2–5×10^5 Sf9 cells after 40 h of infection [25] at 37 °C, by using the anti-ABCG2 monoclonal antibody 5D3 (eBioscience), which recognizes a cell-surface epitope of human ABCG2 [4]. The antibody was used in a final concentration of 1 μg/ml, and binding was visualized by the addition of a