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ABCG2 membrane transporter in mature human erythrocytes is exclusively homodimer

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Abstract

The human ABCG2 protein, a member of ABC transporter family, was shown to transport anti-cancer drugs and normal cell metabolites. Earlier studies have demonstrated the expression of ABCG2 in hematopoietic stem cells and erythroid cells; however little is known about the expression and activity of ABCG2 in mature erythrocytes. In this report, we show that ABCG2 in mature human erythrocytes migrates with an apparent molecular mass of 140 kDa, under reducing conditions, on Fairbanks SDS gel system. In contrast, tumor cells expressing higher levels of ABCG2 show no detectable homodimers, when resolved under identical reducing conditions. Analysis of the same membrane extracts from tumor cells and human erythrocytes on Laemmli SDS gel system, where samples are boiled in the presence of increasing concentrations of disulfide reducing conditions and then analyzed, migrate with an apparent molecular mass of 70 kDa or a monomer. Drug transport studies using Pheophorbide A, a substrate of ABCG2, show the protein to be active in erythrocytes. Furthermore, Fumitremorgin C, a specific inhibitor of ABCG2 increases the accumulation of Pheophorbide A in erythrocytes and drug-resistant cells but not in the parental drug-sensitive cells. Given the ability of ABCG2 to transport protoprophyrin IX or heme, these findings may have implications on the normal function of erythrocytes.

Keywords: Erythrocytes; ABCG2; Drug resistance; ATP-binding cassette; Homodimer; Membrane protein

The breast cancer-resistant protein (or ABCG2, also known as the mitoxantrone-resistant protein, and ABC placenta) is a member of the ATP-dependent binding cassette (ABC) family of transporters. Similar to other well-characterized ABC transporters, namely P-glycoprotein (P-gp1 or ABCB1) and multi-drug resistance protein 1 (MRP1 or ABCC1), ABCG2 was initially discovered in a multidrug-resistant cell line, MCF7/AdrVp [1]. ABCG2 has been shown to confer drug resistance in tumor cells, and to mediate the transport of anti-cancer drugs, including mitoxantrone, methotrexate, camptothecans (SN-38, topotecan), and flavopiridol (for recent reviews see [2,3]). In normal tissue, ABCG2 is found in the canalicular membrane of the liver, in the epithelia of the small intestine, colon, kidney, placenta, and sweat glands. More recently, ABCG2 expression has been found in hematopoietic stem cells. This latter "side population" of progenitor cells is representative of pluripotent stem cells. It has been suggested that the expression of ABCG2 protects this "side population" from cytotoxic substrates [4,5]. Furthermore, increased expression of ABCG2 has been demonstrated in erythroid maturation and was shown to decrease intracellular protoporphyrin IX, a natural substrate of ABCG2 [6]. Consistent with these findings, erythrocytes from ABCG2 knocked-out mice showed significant increase in intracellular protoprophyrin IX and a decrease in survival further confirming the protective role of ABCG2 in normal tissue [7]. Recently, ABCG2 has been implicated in the transport of heme, and was shown to enhance hypoxic cell survival through interactions with heme [8].

The human ABCG2, with a molecular mass of 72 kDa, encodes one transmembrane domain (TMD, with six transmembrane α-helices) and one nucleotide binding domain

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(NBD) all within a 655 amino acids primary sequence. This is in contrast with other ABC transporters (e.g., ABCB1 and ABCC1), which has led some to label ABCG2 "a half-transporter". Consistent with the latter, two monomers are necessary to form a fully active ABCG2 transporter. Several reports have described ABCG2 as a functional homodimer, possibly as a homotetramer [9–14]. These latter studies have suggested that homodimerization through inter-disulfide bonds between two ABCG2 monomers. Mutational analysis of all 12 cysteine residues in human ABCG2, including the three extracellular cysteines, Cys592, 603, and 608, suggested that inter-disulfide bridge at Cys-603 in the 3rd extracellular domain between two monomers is important, but not essential, for oligomerization of ABCG2 [15,16].

The expression of ABCG2 in hematopoietic stem cells and erythroid cells can protect normal tissue from toxic agents, similar to other ABC transporters. Moreover, the enhanced expression of ABCG2 in hematopoietic stem cells has led some to speculate a role for ABCG2 in preventing the accumulation of a differentiating factor in stem cells [4]. While others have observed the expression of ABCG2 in Ter119+ erythroid precursors and natural killer lymphocytes [4]; however little is known about the expression and activity of ABCG2 in mature erythrocytes. In this report we demonstrate that ABCG2 is expressed in mature human erythrocytes isolated from at least eight different adults. In these cells, ABCG2 migrated almost exclusively as 140-kDa protein on Fairbanks SDS gel system under reducing conditions. By contrast, ABCG2 from mitoxantrone-resistant tumor cells (MCF7/Mitox) migrated exclusively as a 72 kDa under identical Fairbanks gel system. Interesting, analysis of ABCG2 from mature erythrocytes and MCF7/Mitox tumor cells using the Laemmli gel system, in the presence of reducing agent, migrates as a monomer (or 72-kDa protein). The significance of these finding in the normal transport functions of ABCG2 in erythrocytes is discussed.

Materials and methods

Materials. ABCG2-specific antibody BXP-21 was purchased from Kamiya Biomedical Co. (Seattle, WA). Na^+/K^+-ATPase-specific monoclonal antibody was purchased from Sigma. All other chemicals were of the highest grade available.

Cell culture and plasma membrane preparation. Human breast carcinoma cells MCF7 and their drug-resistant clone, MCF7/Mitox, were grown in α-MEM containing 10% fetal calf serum (Bio Media). Plasma membranes were prepared as described previously [17]. Protein concentrations were determined by the Lowry method [18]. Erythrocytes ghosts were prepared as described previously [19]. Briefly, freshly drawn erythrocytes in the presence of sodium-citrate were first washed with PBS three times, and then run on a Ficoll gradient to remove leukocytes. Erythrocytes were washed three more times with ice-cold PBS prior to lysis in hypotonic buffer 5P8 (5 mM sodium phosphate, pH 8.0). Hemolysis was initiated by rapid mixing in the presence of protease inhibitors (2 mM PMSF). Lysed erythrocytes were centrifuged at 4 °C for 10 min at 14,000 rpm (16,000*g*) and the supernatant removed by aspiration. The latter wash was repeated four more times and the final pellet suspended in PBS with protease inhibitors.

Western blots. Plasma membranes from tumor cells (MCF7 and MCF7/Mitox cells) or erythrocytes (10–100 μg) were resolved by SDS–PAGE using the Fairbanks gel system [20] and transferred to nitrocellulose membranes using wet electroblotting as outlined by Towbin et al. [21]. The nitrocellulose membrane was blocked in 5% fetal bovine serum, 5% skim milk, and 7.5 mM NaN_3 in PBS, and incubated with various ABCG2-specific antibodies at varying dilutions (1:1000–1:3000 v/v) overnight at 4 °C. Membranes were washed, and incubated with varying dilutions (1:3000–1:6000 v/v) of goat anti-rabbit or mouse antibody conjugated to horseradish peroxidase. Immuno-reactive proteins were visualized by chemiluminescence using Pico or Femto SuperSignal Substrate (Pierce).

Flow cytometry analysis. ABCG2 activity in MCF7 mammalian tumor cells was assessed by FACS analysis as previously described [22] with some modifications. Briefly, cells were washed twice with incubation buffer (Hepes 10 mM, NaCl 150 mM, KCl 5 mM, $CaCl_2 \times 2H_2O$ 1.8 mM, $MgCl_2 \times 6H_2O$ 1 mM, glucose 10 mM, pH 7.4). Freshly drawn human erythrocytes were collected by finger prick in EDTA-filled 0.5 mL Eppendorf tubes and re-suspended in PBS and run on an equal volume Ficoll gradient (to remove leukocytes), and subsequently washed twice with incubation buffer. Cells MCF7 (0.5×10^6–1.0×10^6 cells/ml) and whole erythrocytes (0.5% hematocrit) were incubated with 2 μl PhA with or without ABCG2-specific or non-specific inhibitors: 1–10 μM FTC, 50 μM verapamil, 50 μM *cis*-platin, and incubated in 37 °C hot water bath for 30 min. Cells were then washed once with ice-cold incubation buffer and then incubated for 1 h at 37 °C in PhA-free medium with ABCG2-specific or non-specific inhibitors. Following the efflux phase cells are washed once again with ice-cold incubation buffer, resuspended in 1 mL of incubation buffer and kept on ice in the dark and analyzed immediately (within 30 min), using the FACS Aria flow cytometer (Becton–Dickinson, CA). PhA fluorescence was measured with a 488-nm argon laser and a 670-nm filter. At least 10,000 events were collected for all of the flow cytometry studies, and by gating forward versus side scatter from a dot blot we were able to determine cellular debris and dead cells from our target population. Results are representative of at least two separate experiments done in triplicate, and calculated as % increase of mean channel fluorescence (MCF).

Results and discussion

Several reports have now shown that ABCG2 mediates the transport of normal cell metabolites, including protoprophyrin IX and heme [6,7]. Given these latter findings, it was of interest to examine the expression of ABCG2 in normal tissue that contains high levels of heme, such as mature human erythrocytes. Fig. 1A shows a Western blot of plasma membrane extracts from drug-sensitive (MCF7) or -resistant (MCF7/Mitox) tumor cells and human erythrocytes probed with ABCG2-specific monoclonal antibody, the BXP-21. The results in Fig. 1A show a BXP-21 reactive polypeptide with an apparent molecular mass of 72 kDa in drug-resistant tumor cells (MCF7/Mitox, lane 2) using a Fairbanks gel system. The parental drug-sensitive cells (MCF7, lane 1) showed a much weaker signal for this 72-kDa polypeptide; while membrane extracts from several human erythrocytes (lanes 3 and 4) showed a significant expression of this 72-kDa protein. These observations are consistent with the expression of ABCG2 protein in breast tumor cells that have been selected for mitoxantrone resistance [23] from the drug-sensitive breast tumor cell lines MCF7. In addition, lanes 3 and 4 revealed a 140-kDa BXP-21 reactive protein that was not observed in membrane extracts from tumor cells (lanes 1 and 2). The 140-