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The ABC transporter Abcg2/Bcrp: Role in hypoxia mediated survival

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Abstract

ABC (ATP-binding cassette) transporters have diverse roles in many cellular processes. These diverse roles require the presence of conserved membrane spanning domains and nucleotide binding domains. Bcrp (Abcg2) is a member of the ATP binding cassette family of plasma membrane transporters that was originally discovered for its ability to confer drug resistance in tumor cells. Subsequent studies showed Bcrp expression in normal tissues and high expression in primitive stem cells. Bcrp expression is induced under low oxygen conditions consistent with its high expression in tissues exposed to low oxygen environments. Moreover, Bcrp interacts with heme and other porphyrins. This finding and its regulation by hypoxia suggests it may play a role in protecting cells/tissue from protoporphyrin accumulation under hypoxia. These observations are strengthened by the fact that porphyrins accumulate in tissues of the Bcrp knockout mouse. It is possible that humans with loss of function Bcrp alleles may be more susceptible to porphyrin-induced phototoxicity. We propose that Bcrp plays a role in porphyrin homoeostasis and regulates survival under low oxygen conditions.

Abbreviations: ABC – ATP binding cassette; BSEP – Bile salt export pump; Bcrp – Breast cancer resistance protein; CFTR – Cystic fibrosis transmembrane conductance regulator; DFO – Desferoxamine; HIF – Hypoxia inducible factor; NBD – Nucleotide-binding domain; PFIC – Progressive familial intrahepatic cholestasis; PPIX – Protoporphyrin IX; SNP – Single nucleotide polymorphism; TMD – Transmembrane domain.

ABC transporters have conserved domains

ABC (ATP-binding cassette) transporters are a family of proteins present in all known living species, with approximately 1100 different proteins described in the literature and public databases. ABC proteins contain two highly conserved domains as a combination of a conserved cytoplasmic ATP-binding (ABC) domain and divergent hydrophobic transmembrane domains (TMDs) . The ABC domain consists of three highly conserved sequence motifs, the Walker A and B motifs and the ABC signature, or the so called Walker C motif. The ABC signature motif is unique to ABC proteins and distinguishes them from other ATP-binding proteins. In mammals the functionally active ABC proteins consist of at least four 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.

The TMDs of ABC proteins consist of between 4 and 8 transmembrane α-helices in each TMD. The TMD are the substrate binding sites, based upon studies with affinity probes and TMD mutants (Hafkemeyer *et al.* 1998; Loo & Clarke 1999; Zhang *et al.* 2003). Moreover, the conformational changes within the TMD are believed to be responsible for the transport of molecules by these transporters. The ABC regions are the sites that bind and/or hydrolyze cytoplasmic ATP and, ATP hydrolysis provides the energy for the uphill transport of a substrate in one direction against a concentration gradient.

In humans 49 ABC genes have been described (Gottesman & Ambudkar 2001) (see http://nutrigene.4t.com/humanabc.htm). Biological functions of some of these transporters has been facilitated by both genetic defects, so called 'accidents of nature' and the adventitious discovery of their role in causing chemotherapeutic drug resistance. An example of the former is the bile salt export pump (BSEP aka SPGP) which plays a major role in the hepatic canalicular transport of bile acids into the gut and a defect in this gene causes progressive familial intrahepatic cholestasis type II, PFIC II (Thompson & Strautnieks 2001; Chen *et al.* 2002b). Despite the conserved domains these ABC transporter proteins are functionally diverse. For instance, the cystic fibrosis transmembrane conductance regulator (CFTR) functions as a cAMP regulated membrane chloride channel (Riordan *et al.* 1989), while BSEP as mentioned above functions as a bile salt export pump. The multidrug transporter ABC proteins that cause chemotherapeutic drug resistance use cellular ATP to extrude a structurally diverse array of therapeutic compounds from cancer cells. Undoubtedly, they have important biologic roles. The development of mice that lack some of these multidrug transporters has revealed their role in protecting cells and tissues from the accumulation of toxic chemotherapeutic substances (Wijnholds *et al.* 1997; Allen *et al.* 2000; Johnson *et al.* 2001). These same studies have also resulted in identifying the normal physiological function of some of these transporters. For instance, the Mrp1 knockout mice are phenotypcially normal until challenged with an inflammatory mediator. The impaired inflammatory response of the Mrp1 knockout is a result of its inability to efflux its natural substrate, leukotriene C_4 (Wijnholds *et al.* 1997).

Hypoxia signaling

Cellular adaptations to low oxygen include an increase in the expression of several key proteins such as the glucose uptake carriers (Ouiddir *et al.* 1999; Chen *et al.* 2001) and glycolytic enzymes (Semenza *et al.* 1994) that allow cells to adjust to anaerobic conditions, i.e. the 'Pasteur Effect' (Seagroves *et al.* 2001). Other cellular adaptations also occur under low oxygen. For instance, pathways involved in controlling the level of heme, a molecule whose level changes in response to cellular O_2 changes, notably the erythroid specific ALAS2 is upregulated under hypoxic conditions and this is correlated with an increase in intracellular heme levels (Hofer *et al.* 2003). A central mediator of the hypoxic response is the bHLH-PAS transcription factor complex hypoxia-inducible factor 1 (HIF-1) which is composed of the heterodimeric partners; Hif-1β and Hif-1α. Under normoxia Hif-1α is inactivated by a proteosomal degradation pathway, but when hypoxia develops Hif-1α is stabilized (Wang *et al.* 1995; Semenza 2001). The core consensus sequence for HIF-1 binding is RCGTG and multiple glycolytic enzymes (e.g., aldolase, pyruvate kinase, GAPDH, LDH) contain HIF-1 binding sites. HIF-1 also functions to regulate additional genes involved in erythropoiesis, angiogenesis, cell survival and iron metabolism. It is notable that HIF-1 deficient cells have a decreased ability to maintain cellular ATP levels (Covello & Simon 2004).

Breast cancer resistant protein (Bcrp)

Bcrp (aka ABCP, MXR, ABCG2) was almost concurrently cloned by three separate laboratories (Allikmets *et al.* 1998; Doyle *et al.* 1998; Miyake *et al.* 1999). However, the laboratory of Ross and Doyle cloned Bcrp from a cell line that had a specific resistance phenotype that was characteristic of an ABC-transporter (Doyle *et al.* 1998), i.e., an ATP-dependent reduction in the intracellular accumulation of anticancer drugs in the absence of a known multi-drug resistance transporter P-glycoprotein.

Immunohistochemical studies with monoclonal and polyclonal antibody probes confirm the predominant plasma membrane localization of the Bcrp transporter (Rocchi *et al.* 2000). Initial

studies suggested cytoplasmic staining as well, but subsequent immunofluorescence studies with confocal microscopy and careful pre-adsorption of non-specific binding antibodies demonstrated the predominance of plasma membrane immunostaining (Rocchi *et al*. 2000; Scheffer *et al*. 2000). The plasma membrane location of Bcrp differentiates this transporter from almost all other known half-transporters. Most half-transporters are predominantly localized to intracellular membranes, such as the mitochondria [M-ABC1 and ABC-7 (Shimada *et al*. 1998; Hogue *et al*. 1999)], the endoplasmic reticulum [TAP 1 and 2 (Russ *et al*. 1995; Bear *et al*. 1999)] or the peroxisomes [ALDP/ABCD1 (Corzo *et al*. 2002; Tanaka *et al*. 2002)]. The only other half-transporter with evidence suggesting partial cell surface localization is the human white homologue ABCG1 (Ewart & Howells 1998).

Functional configuration of Bcrp

Bcrp has one TMD and one NBD (Figure 1); therefore, based upon structural studies indicating NBD form dimer interfaces (Higgins & Linton 2004), it is inferred that this transporter requires dimerization to become active. While Bcrp could function as either a heterodimer or homodimer there are several findings indicating that Bcrp functions as a homodimer. The initial demonstration that Bcrp transfection conferred selective resistance to its substrates suggested that Bcrp functioned by homodimerization (Doyle *et al*. 1998). This hypothesis was extended by Litman *et al*. (2002) who showed that BCRP migrated as an approximately 180 kDa complex under non-reducing conditions and as a 72 kDa band under reducing conditions. The former studies could not rule out the possibility that residues in Bcrp were simply forming disulfide linkages. Nevertheless, confirmation came from Kage *et al*. (2002) who by using BCRP transfectants tagged with either Myc or HA and performing immunoprecipitation assays which demonstrated that BCRP does indeed form a homodimer. The functional activity of Bcrp in transfected insect cells, using a baculovirus-Sf9 cell system, also argues strongly for the activity of the protein as a homodimer (Ozvegy *et al*. 2001, 2002). In this heterologous system, it is unlikely that a heterodimeric partner of Bcrp is expressed at a level sufficient to form a functionally active Bcrp heterodimer. Further support for Bcrp functioning as a homodimer was the finding that a non-functional Bcrp served as an effective dominant-negative inhibitor of Bcrp function when co-transfected with a functional Bcrp (Ozvegy *et al*. 2002).

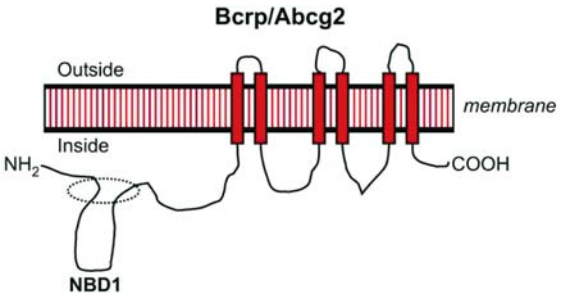

Figure 1. Proposed topology of the ABCG2 multidrug transporter – NBD = ATP (nucleotide binding domain). The solid rectangles represent the transmembrane domains.

Expression and function of Bcrp in stem cells

Evidence has evolved to reveal that ABC transporters play a crucial role in protecting hematopoietic stem cells. Stem cells from many sources are recognized by the absence of lineage markers and flow cytometric analysis with a fluorescent dye reveals a dye excluding 'side population' of cells (SP) that is attributed to the ability of these stem cells to efflux the dye Hoechst 33342. Initial attention was drawn to the ABC transporter, P-glycoprotein (encoded by the Mdr1 gene) as the transporter responsible for Hoechst 33342 dye efflux in the SP cells; however, studies in Mdr1 knockout mice found no difference in the numbers of Hoechst 33342 effluxing SP cells in the Mdr1 deficient and Mdr1 proficient animals (Zhou *et al*. 2001, 2002; Uchida *et al*. 2002). Further studies demonstrated that in mouse hematopoietic progenitor cells ($CD34^-$), Bcrp mRNA was highly expressed and when these cells differentiated, the expression of Bcrp mRNA decreased (Zhou *et al*. 2001). Paradoxically, the enforced expression of Bcrp in bone marrow cells caused a general reduction in all mature hematopoietic cells but expanded the SP population. This effect was postulated as Bcrp effluxing an endogenous molecule essential for the differentiation of stem cells. An

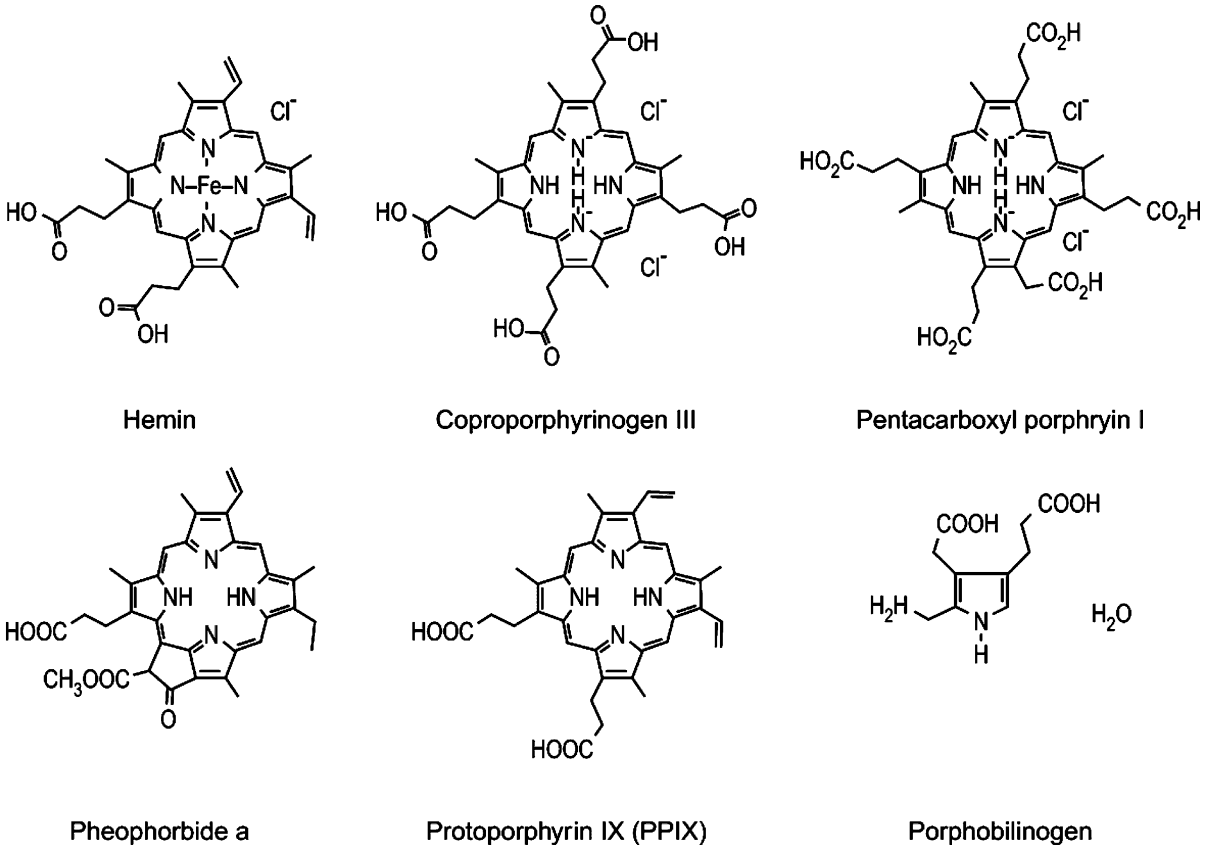

Figure 2. Schematic representation of structurally different protoporphyrin molecules. The basic structure of porphyrin consists of four pyrrole units linked by four methane bridges. Structural differences in different porphyrins arise as a result of side chain substitutions involving either methyl, vinyl or propionate groups.

alternate possibility, not discussed, was that Bcrp might extrude a molecule important for cell growth. Thus, enforced overexpression of Bcrp might be deleterious to progenitor cells. In addition to their ability to extrude Hoechst 33342, stem cells also thrive under conditions of low oxygen whereas more differentiated cells lose viability, thus revealing that stem cells are adapted to proliferate under low oxygen conditions (Cipolleschi *et al.* 1993, 2000). Notably, hematopoietic stem cells are concentrated in areas of low oxygen which was revealed by the capability of these cells to re-populate the bone marrow of lethally irradiated mice (Cipolleschi *et al.* 1993, 2000).

Bcrp and porphyrins

On the basis of its high expression in multiple stem cells (Zhou *et al.* 2001) deletion of *Bcrp* was predicted to produce a nonviable animal, however these animals were without observable defects, thus Bcrp did not appear essential for development (Zhou *et al.* 2002). As anticipated, however the hematopoietic cells of these $Bcrp^{-/-}$ animals were exquisitely sensitive to chemotherapeutic agents that are Bcrp substrates (Zhou *et al.* 2002). The laboratory of Schinkel and colleagues also developed a Bcrp-null animal and discovered that these animals also appeared normal. This changed when an unintended dietary modification revealed a susceptibility phenotype (Jonker *et al.* 2002). These animals developed a skin phototoxicity that was associated with an accidental increase in dietary chlorophyll that produced an accumulation of a chlorophyll degradation product, pheophorbide *a* that was abundant in their food. It is notable that pheophorbide *a* resembles the cellular molecule, protoporphyrin IX (Figure 2). These authors demonstrated that pheophorbide *a* accumulates at

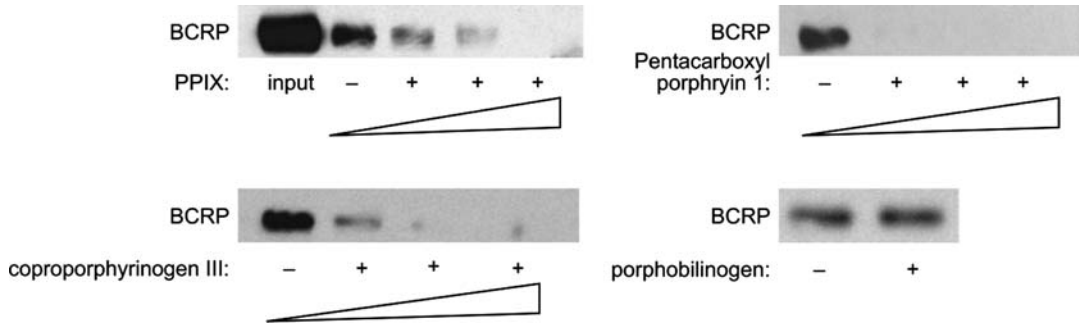

Figure 3. BCRP precipitation after association with hemin-agarose is reduced by the addition of tetrapyrroles. An aliquot of membrane lysate from cells programmed to express BCRP was incubated with hemin-agarose in the presence of increase concentration (0, 40, 80 and 120 μM) of structurally different porphyrins. Hemin-agarose precipitated samples were probed for BCRP by immunoblotting.

reduced levels in Bcrp overexpressing cells and inferred that this porphyrin directly interacted with and was a Bcrp substrate (Jonker *et al*. 2002). Subsequent studies confirmed that pheophorbide *a* was a Bcrp substrate (Robey *et al*. 2004). Because pheophorbide *a* structurally resembles protoporphyrin IX (a heme precursor) one interpretation is that pheophorbide *a* directly caused skin phototoxicity. However, an alternate possibility is that pheophorbide *a* increased the accumulation of phototoxic cellular porphyrins (e.g. heme) by altering the distribution of heme between mitochondria, cytosol and plasma membrane (Lim 1989; Bohm *et al*. 2001). These studies, coupled with the high level of Bcrp in erythroid cells (Jonker *et al*. 2002), suggest Bcrp might have a role in controlling porphyrins and/or heme levels in these cells. The possibility that Bcrp interacted with heme and protoporphyrin molecules was assessed by first determining if Bcrp interacted with heme [(Krishnamurthy *et al*. 2004) and Figure 3]. This was tested by preparing lysates from Bcrp expressing cells followed by incubation with the affinity resin, hemin-agarose. After the complex was precipitated, the bound proteins were resolved by gel electrophoresis and Bcrp was identified by anti-Bcrp antibodies. To determine the specificity of binding, various concentrations of hemin (as a competitor) were added into the mixture of hemin-agarose and Bcrp containing plasma membranes. Hemin dose dependently decreased the amount of Bcrp precipitated from the mixture, which indicates Bcrp specifically interacts with Heme. To further probe the protoporphyrin structural requirements, we performed an additional competition assay with structurally different protoporphyrin molecules (Figures 2 and 3). We challenged the Bcrp hemin-agarose complex with different protoporphyrins at various concentrations and discovered that the least effective competitor was porphobilinogen, a precursor of the tetrapyrroles. In contrast, the most potent tetrapyrrole was a pentacarboxylate porphyrin followed by coproporphyrinogen III and protoporphyrin IX. These studies indicate that the tetrapyrrole structure is an important molecular feature in the interaction with Bcrp. These results provide a strong argument in favor of these compounds being Bcrp substrates and suggest Bcrp has a protective role under conditions where defects in heme biosynthetic enzymes such as uroporphyrinogen decarboxylase or coproporphyrinogen oxidase result in the accumulation of porphyrins leading to protoporphyria and phototoxicity.

Bcrp and Hypoxia

Recent results indicate that Bcrp expression provides an important cell survival advantage under hypoxic conditions (Krishnamurthy *et al*. 2004). Progenitor cells obtained from Bcrp knock-out mice had compromised survival under hypoxia. Moreover, blocking function of Bcrp in progenitor cells from wild type mice reduced survival under hypoxic conditions which indicates that interruption of Bcrp transport activity can interfere with hypoxic survival. Because Bcrp-null hematopoietic cells have greater levels of protoporphyrin IX (PPIX), and

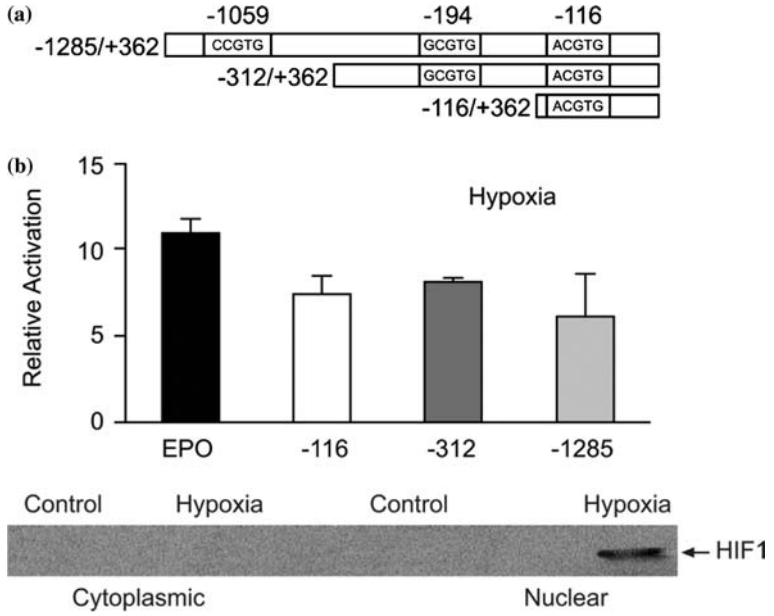

Figure 4. BCRP promoter activity under hypoxic conditions. (a) Core sequence of hypoxia response element in the Bcrp promoter and the deletion constructs (fused to the luciferase reporter gene) used in promoter activity assays is depicted. (b) NIH3T3 Cells transfected with the Bcrp promoter deletion constructs were exposed to hypoxia and the luciferase activities were determined. (c) Hypoxia stabilizes Hif-1alpha protein which localizes to the nucleus.

cells overexpressing Bcrp have decreased PPIX concentration, we tested if inhibition of heme biosynthesis increased cell survival during hypoxic exposure. Consistent with Bcrp role in extruding cytotoxic porphyrins, reduction of heme/porphyrin biosynthesis reversed this hypoxic susceptibility of Bcrp-null hematopoietic cells.

Further study of Bcrp regulation suggested its expression was regulated by hypoxia or its mimetics. Bcrp expression was upregulated by either hypoxia or the iron-chelator DFO which stabilizes Hif-1α by inhibiting a prolyl hydroxlyase involved in tagging Hif-α for proteosomal degradation (Krishnamurthy *et al*. 2004). Three hypoxia response elements are found in the Bcrp promoter (Figure 4). These studies suggest that Bcrp permits enhanced cell survival in oxygen poor environments by reducing the accumulation of toxic heme metabolites and that Bcrp is specifically activated by HIF-1. These findings have important implications for the survival of stem cells under hypoxic conditions and the chemotherapeutic treatment of solid tumors. However, two issues are important. First, the level of HIF-1 expression is not the same in all tumors (Hopfl *et al*. 2004). Second the kinetics of stabilization of Hif-1α varies between tissues. For, instance in liver Hif-alpha achieves a maximum after 1 hour of hypoxia, whereas in brain 5 hours are required. Thus, it is possible that the magnitude of HIF-1 activation will strongly influence Bcrp upregulation.

Human variants of Bcrp

In cell lines selected for chemotherapeutic resistance, Bcrp acquired a single amino acid change at position 482 (Honjo *et al*. 2001; Allen *et al*. 2002). The mutants having R482G or R482T (R482M or R482S in the mouse Bcrp) showed altered transport properties as compared to the wild-type protein (Honjo *et al*. 2001; Allen *et al*. 2002). In fact, these R482 mutants have increased transport and ATP hydrolytic activity, therefore they are considered as 'gain of function' mutants (Ozvegy *et al*. 2002). However, the R482G and R482T mutants were not able to transport methotrexate, which is a substrate only transported by the wild-type Bcrp (Volk *et al*. 2002; Chen *et al*. 2003). Recent, detailed studies,

expressing a number of amino acid 482 mutant variants, have indicated that mutant Bcrp proteins have variable substrate recognition, drug resistance patterns, and transport activity. These experiments demonstrated that an exchange of a single amino acid at position 482 was not detrimental for the correct folding of Bcrp, but induced major alterations in both the transport activity and substrate specificity of this protein (Miwa *et al*. 2003). One implication of these studies is the possibility that natural variations in Bcrp (non-synonymous single-nucleotide polymorphisms) may result in individuals with different capacities to transport endogenous Bcrp substrates such as heme and porphyrin molecules.

Partial sequencing of Bcrp exons from human genomic DNA has demonstrated the presence of single nucleotide polymorphisms (SNPs). The SNPs in Bcrp that produce non-synonymous changes (i.e., amino acid substitutions) are at amino acids 12 (V12M), 141 (Q141K), 206 (I206L), and 590 (N590Y). The most frequent polymorphisms being the exon 2 SNP (G34A/V12M) and the exon 5 SNP (C421A/Q141K), which produce changes in amino acids 12 and 141 (Imai *et al*. 2002; Mizuarai *et al*. 2004). These variants produce functional alterations as shown by different patterns of drug resistance, and a significantly altered transport capacity (Imai *et al*. 2002; Mizuarai *et al*. 2004). Because the allele frequency of these variants differs among ethnic groups (Zamber *et al*. 2003) and because individuals may be heterozygotes for these non-synonymous SNPs it is possible that wild type/variant heterodimerization occurs. Thus, depending upon the functional capability of the Bcrp allele, these heterozygous and homozygotic individuals might manifest phenotypes similar to the Bcrp knockout mouse with enhanced levels of porphyrins and phototoxicity. For instance, the increases in PPIX in erythrocytes found in the Bcrp-null animal are characteristic of some of the human porphyrias. In variegate porphyria, the activity of the penultimate enzyme, protoporphyrinogen oxidase, in heme biosynthesis is reduced (Brenner & Bloomer 1980). While patients have a photosensitivity, they share an additional similarity to the $Bcrp^{-/-}$ mice; the unexplained accumulation of erythrocyte protoporphyrin. Another porphyria, erythrocytic protoporphyria (EPP) has been linked to autosomal dominant mutations in the ferrochelatase gene, which catalyzes the insertion of iron into PPIX (Blom *et al*. 1990; Chen *et al*. 2002a). The EPP phenotype exhibits photosensitivity and it is possible that Bcrp modulates the phenotype of ferrochelatase deficiency, especially if some Bcrp alleles exhibit a 'gain of function' for transport of porphyrins.

Perspective

The ABC transporters extrude a diverse array of structurally dissimilar compounds often providing resistance to multiple toxic chemotherapeutic agents. Based on this feature it has been proposed that their primary role is protection from accumulation of exogenous toxins. This view was consistent with an old perspective based upon a single-transporter capable of moving multiple substrates. Contemporary knowledge based upon genomic analysis and biochemical studies reveal multiple ABC transporters which perform specific roles under physiological conditions which permit the export of a select set of substrates and tissue distribution. This suggests their role as protectors from exogenous cytotoxins may be purely coincidental or opportunistic because of the accommodating nature of their substrate binding pockets. Further support for unique biological roles of ABC transporters is provided by findings in lower organisms. For instance, in yeast, an ABC transporter regulates mating by controlling the concentration of a-factor (Ketchum *et al*. 2001) or in plants an ABC transporter regulates the number and opening of stomatal cells (Gaedeke *et al*. 2001). Undoubtedly, the fact that Bcrp interacts and transports porphyrins fits, in part, into a role of protection. However, we propose a hypothesis that the role of Bcrp is to regulate the level of cellular heme and porphyrins as these molecules have potent effects on gene expression. Thus, it is possible that Bcrp's primary biological role is to regulate cellular porphyrins and disruption of its function leads to cytotoxicity.

Acknowledgements

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