ABCG2 Transports and Transfers Heme to Albumin through Its Large Extracellular Loop*1

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ABCG2 is a membrane protein belonging to the human ABC transporter family (1). Also called breast cancer resistance protein (BCRP), it was discovered for conferring resistance to the anticancer drug mitoxantrone in breast cancer cell lines (2, 3). As other members of the family, it is involved in cell protection against xenobiotics, such as the anticancer drugs mitoxantrone, 7-ethyl-10-hydroxycamptothecin (SN-38), topotecan, and daunorubicin (4), as well as antiviral agents such as HIV-1 nucleoside reverse transcriptase inhibitors (5). As expressed in the brain-blood barrier, intestine, and placenta (6, 7), it constitutes a critical target for both reversing multidrug resistance (4, 8–12) and increasing drug bioavailability (13, 14).

In mammals, heme (ferrous PPIX; see structure in supplemental Fig. S1) and hemin (ferric PPIX) play a central role in cell metabolism. Heme is the prosthetic group of several heme proteins involved in oxygen sensing, cell respiration and metabolism, and cell growth (for a review, see Ref. 15), whereas hemin is a potent erythroid cell differentiation inducer (16–18). Free heme is toxic, leading to hemolysis, proteinolysis, lipid peroxidation, reactive oxygen species generation, and vascular inflammatory disorders (19). Poorly soluble, heme and hemin require extracellular and intracellular carriers, such as albumin and hemopexin, which bind heme with a Kd of 10−6 and 10−12 M, respectively (20–22). Several reports have pointed out the role of ABCG2 in porphyrin detoxification, initiated by a study of Jonker and colleagues who described in Bcrp1−/− knock-out mice a marked sensitivity to the dietary chlorophyll-catabolite phophorbide a and an accumulation of PPIX, the heme precursor in erythrocytes and plasma, leading to acute photosensitivity (23). ABCG2 is a hematopoietic stem cell marker (24), expressed at relatively high levels by immature human hematopoietic progenitors SP, 34+/38− or 34+/KDR+ populations (25). Levels of porphyrins are normally regulated by metabolic control mechanisms of their biosynthesis; however, ABCG2 was found to prevent PPIX accumulation in erythroid progenitor K562 cells submitted to hypoxic conditions of growth (26) and to regulate PPIX levels during erythroid differentiation (27). These data point toward a role of BCRP in porphyrin homeostasis and protection; however, the molecular mechanism by which porphyrins are transported and handled for controlling their toxicity remains unknown. In addition, although ABCG2 is recognized to export PPIX (26), its capacity to export either heme or heme has not yet been demonstrated.

ABCG2 consists of a cytosolic nucleotide-binding domain fused to a transmembrane (TM) domain of six α-helices, giving an inverted domain organization by comparison to most other human ABC transporters (4). The minimal functional unit of ABCG2 is a homodimer (28–30). ABCG2 displays several topological and structural specificities, such as the presence of a large external loop, ECL3, located between TM5 and TM6. Approximately 70–80 residues long, ECL3 is large enough to constitute a full domain, although no specific role has been assigned to it. It contains three cysteine residues reported to be involved in both intermolecular (Cys592-Cys603) and intramolecular (Cys592-Cys603) disulfide bridges (31–34). ECL3 also

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2 The abbreviations used are: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; FC12, foscholine 12; Ni-NTA, nickel-nitrilotriacetic acid; HSA, human serum albumin; NATA, N-acetyltryptophanamide; PPIX, protoporphyrin IX; TEV, tobacco etch virus; TM, transmembrane; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ZnPPIX, zinc mesoporphyrin.
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| Primer | 5′–3′ Sequence | Res. sites |
|--------|----------------|-----------|
| Pfor   | AAAAAAAACATAGAGCTATTTGTTGTTCTGGGATCTGCCGCTTCTGGC | Ndel, BamHI |
| Prev  | OAAGGCTTCTGAGGAAGATCCTCTAGAGGAATCCATTATATA | |
| H583Arev | GAGTTACCTGCTGAGCCTAATATTTGGAACAAATCTTCTGCCG | |
| H583rev | CGAAGATTTTTTCTCCAAATCTATGCGCTGCGACACCCCTAACC | |
| C603Arev | GCCACAGGCAAACCTTCCGCAACTATGCGAAGATCTGCGGCA | |
| C603rev | TGGCACAAGTATGTGCGATAGTGGCGATTGCCGCG | |
| Y605Arev | GCCAGATTAAATTGCCTGCGTTACAGGATGGTTGCCTTGC | |
| Y605rev | GCCAGATCCATGGGCGGCTTACAGGATGGTTGCCTTGC | |

contains a conformational epitope recognized by the 5D3 monoclonal antibody (24), the interaction of which is prevented upon reduction of the intramolecular disulfide bridge Cys<sup>603</sup>-Cys<sup>607</sup> (35), making 5D3 a powerful conformation-sensing tool.

In the present report, we hypothesized that ABCG2 should transport hemin out of the cell, which could be particularly critical in the early stages of hematopoiesis when it is overexpressed, and hemin plays a role in differentiation. We found that ABCG2 transports zinc mesoporphyrin (ZnMP), a validated fluorescent heme analog (36, 37). We also hypothesized that hemin, once exported by ABCG2, should be transferred to an extracellular protein through interaction with an accessible domain of ABCG2 such as ECL3, which could transitorily interact with hemin after membrane translocation. We expressed the ECL3 domain in *Escherichia coli* and purified it in a folded state, as checked by biophysical methods. It was especially found to be able to interact with the 5D3 mAb only when it was oxidized. ECL3 displayed a high affinity for some, but not all, porphyrins, especially for heme and hemin, whereas it does not bind any of the known nonporphyrin substrates and inhibitors of ABCG2. We also found that hemin, when bound onto ECL3, can be rapidly transferred to human serum albumin (HSA).

**EXPERIMENTAL PROCEDURES**

**Materials**—The pET15b plasmid was from Novagen. The QuikChange site-directed mutagenesis kit was from Stratagene. Luria Bertani broth, isopropyl-1-thio-β-D-galactopyranoside, and ampicillin were from Euromedex. Foscholine 12 (FC12) was from Anatrace. Nickel-nitrilotriacetic acid (Ni-NTA) and gel filtration resins and materials for chromatography were from GE Healthcare. An Amicon ultrafiltration device was from Millipore. 5D3-phycocerythrin was purchased from SERVENTIO and used as described in Ref. 27. K562 cells were kindly provided by Drs. Sheng Zhou and Albert Sorentino and used as described in Ref. 26, using K562 cells either expressing or not ABCG2 (27). Cells were lysed as described, and 50 μg of proteins were incubated at room temperature for 15 min in the presence of 0–2 ng/μl 5D3 antibody and 500 nM hemin-agarose. After incubation, the resin was pelleted by centrifugation, and unbound material was withdrawn. Pellets were washed with 1 ml of lysis buffer (150 mM NaCl, 10 mg/ml Triton X-100, and 50 mM Tris-Cl pH 8.0), suspended in 20 μl of SDS sample loading buffer (38), and centrifuged at 10,000 × g for 2 min at 4 °C. Bound and unbound materials were analyzed on a 10% SDS-PAGE and transferred to a PVDF membrane, and ABCG2 was then revealed with the BXP12 monoclonal antibody (1/500, Millipore).

**Expression Constructs**—The bcrp1 gene encoding ABCG2, referenced as QUNOQ at the UniProtKB/Swiss-Prot database, was used for constructs. The *E. coli* protein expression plasmid for producing ECL3, as displayed in Fig. 2, was constructed from alanine 562 to histidine 630 of the ABCG2 sequence by PCR using the plasmid pFastBac Dual-NinaA-BCRP (39), with the couple of forward and reverse primers Pfor and Prev displayed in Table 1. Pfor included a Ndel restriction site and encoded a tobacco etch virus (TEV) site (40); Pprev included a BamHI restriction site. The PCR product was digested by Ndel and BamHI and inserted into the corresponding sites of the pET15b plasmid. The resulting plasmid pET15b-H<sub>10</sub>ThTe-ECL3 expressed the ECL3 (A562-H630) domain fused at its N terminus to a His<sub>6</sub> tag through a thrombin site and a TEV site: MGSSHHHHHHSSGLVPRGGSHMNLYFQ/GA<sup>562</sup>-ABCG2-H<sub>10</sub><sup>60</sup>, where both cleavage sites are underlined, the cleavage position is indicated by “/,” and the ECL3 sequence is indicated in bold and italic characters. The single-point mutations H598A, C603A, and Y608A were introduced by site-directed mutagenesis using the corresponding primers displayed in Table 1.

The plasmid used for expressing full-length ABCG2 in insect cells was described previously (39); here, we extended from 6 to 10 the length of the N-terminal histidine tag and added a thrombin cleavage site. The resulting sequence H<sup>10</sup>Th-ABCG2 was MAHHHHHHHHHHHLPRGSGGMDSPPSSLVPRGS-S<sup>1</sup>–ABC<sub>2</sub>-S<sup>565</sup>.

**Expression and Purification of ECL3**—The BL21(DE3) *E. coli* bacteria strain was transformed with the expression plasmid pET15b-H<sub>10</sub>ThTe-ECL3 including wild-type or mutated sequences. A single colony of freshly transformed cells was grown overnight at 37 °C in 5 ml of Luria Bertani broth contain-
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Membrane suspension was thawed, diluted to 10 mg/ml in 50 mM HEPES, pH 8.0, 2 mM NaCl, 15% glycerol, 2 mM DTT, protease inhibitors, and incubated for 1 h before centrifugation at 15,000 × g for 1 h. The resulting pellet was suspended in 50 mM HEPES, pH 8.0, 250 mM NaCl, 15% glycerol, 40 mM dodecyl β-D-maltoside, 0.2 mM DTT, 20 mM imidazole, protease inhibitors, then incubated for 2 h and centrifuged at 100,000 × g for 1 h. The supernatant was applied to HiTrap chelating HP 1-ml column loaded with Ni2+ and equilibrated in 50 mM HEPES, pH 8.0, 250 mM NaCl, 15% glycerol, 10 mM dodecyl β-D-maltoside, 20 mM imidazole, protease inhibitors. The resin was washed with 16 bed volumes with the same buffer, and ABCG2 was eluted in the same buffer with a 0.02–1 M imidazole gradient. The protein was concentrated using an Amicon Ultra-15 concentrator device 50-kDa cutoff. The His6 tag was removed by thrombin cleavage as described above.

Absorbance Spectroscopy—A saturated solution of hemin was prepared by solubilizing about 10 mg in 100 μl of fresh 0.1 M KOH, followed by a dilution in 50 mM KH2PO4, pH 7.2, 100 mM NaCl. The solution was clarified by centrifugation at 11,000 × g for 3 min, and hemin concentration was determined at 385 nm using an extinction coefficient of 58,400 liters•mol−1•cm−1 (42). For assays, samples of 10 μM protein were subjected to UV-visible absorbance spectroscopy at room temperature on a DU 640 (Beckman) spectrophotometer, using 50 mM NaH2PO4, 2.8 mM FC12 buffer as blank. Spectra were recorded between 240 and 700 nm using a 1-cm path length cuvette, in a hemin concentration range of 0–50 μM. Difference spectra were obtained by subtracting the buffer spectrum from that of the protein-hemin complex. Spectra were normalized at 280 nm.

Ni-NTA-Agarose Pulldown Assays—Fifty microliters of Ni-NTA magnetic beads were equilibrated with 3 × 1 ml of 50 mM NaH2PO4, pH 7.0, 150 mM NaCl, 0.1% FC12 and then incubated with 200 μg of H6ECL3/100 μl (20 nmol) for 30 min 4 °C and mild agitation. The beads were washed three times with 600 μl of the same buffer, and the amount of unbound protein was quantified by the BCA method. H6ECL3 beads were loaded on a BioSprint 15 robot (Qiagen) to carry out the binding of hemin by incubating beads in 500 μl of 50 μM hemin in the same buffer for 20 min at room temperature. The incubation was followed by three washing steps with 500 μl of the same buffer containing 300 mM NaCl. The protein was eluted in 50 μl of the same buffer containing 300 mM NaCl and 300 mM imidazole. The amount of eluted protein and hemin were quantified by the BCA method and by absorbance, respectively. Bound and unbound materials were analyzed on a 16% SDS-PAGE and revealed by Coomassie Blue staining.

Circular Dichroism (CD)—Far-UV CD of purified ECL3 was performed on a Chirascan (Applied Photophysics). Spectra were recorded at room temperature using a 1-mm path length cuvette from 260 to 185 nm, with a step size of 1 nm. Spectra were recorded in the 190–250-nm wavelength range with a 0.2-nm increment, a bandwidth of 0.5 nm, and a 1-s integration time. Spectra were processed, corrected for baseline, and smoothed by Chirascan software on the basis of the Savitzky–Golay algorithm. The percent secondary structure was calculated by deconvoluting data via the DichroWeb site (43) using methods developed by Provencencher and Glockner (44), K2d (45), CDSTSTR and a SELCON (46).
Fluorescence Spectroscopy—Experiments were performed at room temperature using a Photon Technology International Quanta Master I spectrofluorometer. All measurements were recorded in 500-μL quartz cuvettes (Hellma) of 0.5-cm path length. Data were corrected for buffer and the contribution to fluorescence and inner-filter effects of tested ligands by collecting the same spectra with N-acetyltryptophan (NATA). ECL3 (3 mM) in 50 mM NaH₂PO₄, pH 7.0, 2.8 mM FC₁₂, 20% glycerol, or ABCG2 (0.1 mM) in 50 mM HEPES, pH 8.0, 250 mM NaCl, 15% glycerol, 10 mM dodecyl-D-maltoside, 1.6 mM CHAPS, was excited at 295 nm, and the fluorescence emission was scanned from 310 to 370 nm. The concentration-dependent quenching studies for the different ligands (0–15 mM) were performed at least three times. Peak areas of corrected fluorescence spectra were fitted with the SigmaPlot software (version 11; Systat Software). Percentages of fluorescence quenching were calculated as a ratio of protein to NATA. Data were fitted assuming one binding site with different possible equations, as indicated,

\[ f = \frac{B_{\text{max}} \cdot \text{abs}(x)}{(K_d + \text{abs}(x))} \]  

(Eq. 1)

\[ f = F_{\text{min}} + ((F_{\text{max}} - F_{\text{min}}) \cdot (E + x + K_d) - \sqrt{E + x + K_d}) \]  

(Eq. 2)

which introduces a correction factor for unspecific binding.

Primary Structure Alignment—Primary structure alignments were performed with Clustal (48), carried out via the NPS@bioinformatic server.

RESULTS

ZnMP Transport by ABCG2 in K562 Cells and Modulation by the 5D3 mAb of in Vitro ABCG2 Binding to Hemin-Agarose—Because ABCG2 was shown to transport PPIX out of stem cells submitted to hypoxia (26), we hypothesized that the transporter could also be able to transport heme or hemin, which correspond to ferrous and ferric PPIX (see the corresponding chemical structure in supplemental Fig. S1). The experiment was carried out with erythroid K562 cells either transfected or
not with the cDNA encoding ABCG2 (27), following the transport of the fluorescent ZnMP. Note that although ZnMP is a validated hemin analog (36, 37), its central zinc atom cannot coordinate with protein residues such as histidine in the same way as the iron atom in heme (49).

As shown in Fig. 1A, ZnMP accumulated in K562 control cells (green), whereas they remained at a lower level in cells expressing ABCG2 (orange). However, the latter showed the same level of accumulation as observed in control cells when incubated with 1 μM ABCG2 inhibitor Ko143 (50). Hemin and heme being rather cytotoxic, especially heme as due to the ferrous state of bound iron (15), we hypothesized that their efflux could require an intermediate transfer to a carrier via an extracellular domain of ABCG2, fully accessible to this carrier. ABCG2 indeed displays such a domain, which has been shown to interact with the 5D3 mAb (35). As a first experiment to test whether or not ECL3 could play such a role in heme/hemin transfer, we took advantage of the known capacity of the protein to bind in vitro onto hemin-agarose (26) to check whether 5D3 binding could alter domain interaction with the affinity column. Results are displayed in Fig. 1B. As shown on the left, ABCG2 expressed in K562 cells (Resin/0.0/H11001) could fully bind to the hemin-agarose resin, compared with control cells (Resin/0.0/H11002), no protein remaining in the flow-through fraction as shown in the right (Flow through/0.0/+). Addition of increasing amounts of 5D3 before incubation with the resin indeed prevented the binding of ABCG2 onto hemin-agarose; the protein accumulated in the flow-through fraction proportionally to the amount of added mAb. By revealing an antagonist effect between 5D3 binding onto ECL3 and ABCG2 binding onto hemin-agarose, this experiment suggested a heme-binding capacity of ECL3.

ECL3 Constitutes an Autonomously Folded Domain—To further check the capacity of ECL3 itself to bind heme/hemin separately from the transport sites, we studied ECL3 as an isolated and purified domain, hypothesizing that it should be long enough to fold accurately when expressed alone. Using primary structure alignments, we selected the region extending from alanine 562 to histidine 630 as possible N and C termini of such a domain. These limits are close to those recently reported from various predictions, Trp567-Trp627 (51), Leu565-Asn629 (52), or Ser563-Gly618 (35). We noted the amphipathic character of both pairs of tryptophan residues 564–567, 624–627, located at each extremity of ECL3 and likely favoring the anchoring of the loop onto the membrane, rather than belonging to the TM helices 5 and 6. We generated a chimera including a His6 tag followed by thrombin and TEV (40) endoprotease cleavage sites at the N terminus of ECL3, which were inserted for later removing the nickel affinity tag after purification because histidine residues might be potential hemin ligands. The resulting fusion protein (Fig. 2A) was produced in E. coli and then purified as

chymotrypsinogen A (21 kDa), and ribonuclease (14 kDa), fitting the elution volume data with SigmaPlot. 8, CD spectra of ECL3. The spectrum was fitted for estimating the percentage of each two-dimensional structure (see “Experimental Procedures”). The same experiment was done by adding 1–5 M urea (dashed lines). C, fluorescence spectra of ECL3 (straight line) and NATA (dashed line). D, intrinsic fluorescence quenching of ECL3 (1.5 μM), either oxidized (circles) or reduced (squares), by the 5D3 mAb, as measured at 336 nm and fitted with SigmaPlot using Equation 1.
displayed in Fig. 2B. ECL3 was abundant in the insoluble fraction P1 (arrow in B) and was solubilized with FC12 (lane S2), showing that the domain, as designed, still displayed a significant hydrophobic character. The domain was then purified by nickel affinity and gel filtration chromatographies (lanes Ni-NTA, S200), reducing the detergent concentration to 3 mM, a value roughly equivalent to twice the critical micellar concentration. Reducing this concentration below the critical micellar concentration led to protein aggregation, indicating that detergent is required for maintaining the domain in solution. DTT was added during the solubilization step and then removed during the chromatographic steps for favoring the formation of the Cys592-Cys608 disulfide bridge, which occurred during overnight incubation at 4 °C. Later, thiol titration with Ellman’s reagent (53) showed that one thiol remained free after this step. Thrombin fully digested the protein (lane Thrombin/H11001) generating a protein that migrated at a position compatible with the predicted molecular mass of 8,945 Da. TEV could never cut, which might be due to the presence of detergent as reported before (54). ECL3 was finally purified by repeating both chromatographic steps and adding one for removing thrombin.

We next examined the folding of ECL3. As shown, the protein was eluted from a S200 10/300 gel filtration at 16.7 ml (Fig. 3A), corresponding to an apparent molecular mass of 23 kDa. Taking into account the presence of detergent micelles, this value is compatible with monomeric ECL3 (~9 kDa) embedded inside a FC12 micelle (350 g/mol × ~40 molecules = 14 kDa). The CD spectrum of the domain (Fig. 3B) was typical of a α + β polypeptide which, once deconvoluted, gave the average values of α-helix, β-sheet, and coil which are indicated in the inset. Increasing concentrations of urea induced a progressive loss of secondary structure. Intrinsic fluorescence of the domain (Fig. 3C), which contains four tryptophan residues (Fig. 2A), was also typical of a folded protein in which these tryptophan residues are buried, giving a maximal emission wavelength of 336 nm, much lower than the 354-nm value obtained with NATA used as a control. A further convincing result came from the capacity of the recombinant domain to interact with the 5D3 mAb (24), which has been raised against ECL3 and is sensitive to the oxidized state of the thiol bridge Cys592-Cys608 (35). As shown in Fig. 3D, the addition of increasing amounts of the 5D3 mAb quenched the tryptophan fluorescence and led to a saturation curve (circles), allowing us to estimate a $K_d$ value of 0.06 ± 0.03 ng/μl, i.e. 4 ± 2 nM (all $K_d$ values are summarized in supplemental Table S1), assuming an average IgG molecular mass of 150,000 Da. Because the binding of 5D3 was reported to be prevented upon reduction of the Cys592-Cys608 disulfide.
bridge (35), we reproduced a similar experiment with the reduced form of ECL3 (squares). As shown, the intrinsic fluorescence of reduced ECL3 was not modified by the mAb addition, suggesting no interaction. These experiments show that the ECL3 domain is self-folded and adopts a three-dimensional structure relevant to the same region within the native full-length ABCG2 transporter.

**ECL3 Specifically Binds Porphyrins**—We first monitored the binding of hemin (see the structure of each tested compound in supplemental Fig. S1) by absorbance, following the evolution of the Soret peak at 412 nm which appears in the presence of ECL3 (Fig. 4A). The interaction led to an increase in absorbance at this wavelength when increasing the hemin concentration. The absorbance values, plotted in Fig. 4B, gave a saturation curve from which a $K_d$ of $1.33 \pm 0.72 \mu M$ was estimated ($K_d$ values are summarized in supplemental Table S1), assuming one hemin bound per domain. This binding ratio was confirmed experimentally by pulldown experiments as displayed in Fig. 4C using Ni-NTA magnetic beads (see “Experimental Procedures”). Results show that 20 nmol of ECL3 bound to the beads binds 16 nmol of hemin, whereas after washing, elution leads to the removal of 16 nmol of ECL3 and 12 nmol of hemin. Both ratios suggest that up to one hemin binds to the domain. The ECL3-hemin interaction was also monitored by intrinsic fluorescence, leading to similar results (Fig. 4D, circles) with a $K_d$ of $0.88 \pm 0.18 \mu M$. As shown, the maximal quenching of intrinsic fluorescence was approximately 90%. When hemin iron, present as a ferric state Fe$^{3+}$, was reduced by dithionite to ferrous state, Fe$^{2+}$, as in heme, a higher maximal quenching of intrinsic fluorescence was observed (Fig. 4D, diamonds), and the $K_d$ of $0.47 \pm 0.40 \mu M$ indicated a 2-fold higher affinity than for the oxidized form. We also monitored hemin binding to the reduced form of ECL3 (Fig. 4D, squares) which led to a similar saturation curve, although despite a lower maximal quenching (60%), the $K_d$ of $1.03 \pm 0.69 \mu M$ was close to that obtained with oxidized species. This suggested that reduction of the Cys$^{592-608}$ disulfide bridge does not dramatically alter hemin binding.

We then examined the interaction of other porphyrins, such as PPIX, ZnPPIX, CoPPIX, phaeophorbide a, and vitamin B12, with ECL3. As shown in Fig. 5A, binding of PPIX when monitored by quenching of intrinsic fluorescence led to a saturation curve (circles) reaching about 80% and giving a $K_d$ of $1.21 \pm 0.71 \mu M$. The same experiment carried out after reduction of ECL3 also led to a saturation curve of fluorescence quenching (squares), giving a $K_d$ of $3.40 \pm 0.01 \mu M$. ZnPPIX, which usually reaches a high level in ferropenic anemia, as well as CoPPIX, were found to bind efficiently to ECL3, as shown in Fig. 5B (circles and squares, respectively), with respective $K_d$ values of $0.80 \pm 0.01 \mu M$ and $0.71 \pm 0.38 \mu M$. By contrast to these porphyrins, we observed a much reduced interaction of phaeophorbide a (Fig. 5B, triangles), producing only 30% of maximal quenching, with a high $K_d$ value of $3.42 \pm 0.50 \mu M$. No change at all of ECL3 intrinsic fluorescence was observed upon addition of either another porphyrin-type molecule, vitamin B12 (supplemental Fig S2A), which is not an ABCG2 substrate (55), or bilirubin, a product of heme catabolism (supplemental Fig S2B).

Finally, we measured the binding of nonporphyrin substrates of ABCG2, such as mitoxantrone (3) and riboflavin (56), and other drugs that bind to ABCG2 but are not transported, such as doxorubicin and rhodamine 123 (12). Results are displayed in Fig. 5C; as shown, all these compounds did not modulate the intrinsic fluorescence.

**FIGURE 5. Binding of porphyrin and nonporphyrin substrates to ECL3, as monitored by quenching of intrinsic fluorescence.** A, binding of PPIX to ECL3, either oxidized (circles) or reduced (squares). B, binding of ZnPPIX (circles), CoPPIX (squares), and phaeophorbide a (triangles) to oxidized ECL3. C, Interaction of mitoxantrone (circles), doxorubicin (squares), rhodamine 123 (diamonds), and riboflavin (triangles) with ECL3. Experiments were carried out and analyzed as described in the legend of Fig. 4D.
fluorescence of ECL3, suggesting no interaction with the domain. We observed that the dual inhibitor of ABCG2 and ABCB1, GF120918, was able to bind but with very low affinity ($K_D$ of 8 ± 6 μM) and unspecific contribution (supplemental Fig. S2C).

**Single-point Mutations H583A and C603A of ECL3 Alter Hemin Binding**—We noticed that ECL3 displays a significant sequence similarity with the heme-binding domain of cytochrome $b_6$, with 14.3% identity and 15.6% similarity, as shown in Fig. 6A. The alignment showed, in addition, that residues His$^{583}$, Cys$^{603}$, and Tyr$^{605}$ of ECL3 are close to the histidine residues of cytochrome $b_6$ (boxed in Fig. 6A) which are involved in the strong chelation of hemin iron. We mutagenized these residues into alanine and monitored by intrinsic fluorescence the binding capacity of the mutant proteins toward hemin and PPIX. The quenching curves are displayed in supplemental Fig. S3 and summarized in supplemental Table S1.

**ABCG2 First Saturated with Mitoxantrone or Doxorubicin Binds Hemin but Not Pheophorbide $a$**—We observed that the dual inhibitor of ABCG2 and ABCB1, GF120918, was able to bind but with very low affinity ($K_D$ of 8 ± 6 μM) and unspecific contribution (supplemental Fig. S2C).
expected from Fig. 4D. In addition, the fluorescence signal remained stable with time after the addition of hemin (see the dark trace e.g. between 100 and 150 s), indicating that the hemin is stably bound to the domain. Further addition of 3 μM apoHSA fully reverted ECL3 fluorescence to its initial level, as quickly as for hemin binding, indicating that hemin was released from the domain. Note that the intrinsic fluorescence signal of ECL3 was corrected from the contribution of HSA as detailed in supplemental Fig. S6. The addition of a preformed HSA-hemin complex at the same concentration did not modify the fluorescence of the ECL3-hemin complex (dashed gray trace in Fig. 8A), indicating no change in hemin interaction with ECL3. The same type of experiment was carried out using the whole ABCG2 preloaded with mitoxantrone and doxorubicin, as described above. As displayed in Fig. 8B, comparable results were obtained, with, however, a larger increase of ABCG2 fluorescence signal upon addition of HSA. These experiments suggested that hemin is released from ECL3 upon addition of HSA. We then monitored the absorbance of hemin bound to ECL3 in the presence of increasing concentrations of HSA. As shown in Fig. 8C, addition of 5 μM hemin to 10 μM apo-ECL3 (dashed black trace) generated the expected Soret peak at 412 nm of the ECL3-hemin complex (dark trace), with the addition of two shoulders at 360 and 385 nm corresponding to 1 μM unbound hemin. Despite this, upon addition of HAS, the peak at 412 nm decreased, even at low (0.5–1 μM) concentrations of HSA, with a new Soret peak at 402 nm of the HSA-hemin complex (57). This result is in agreement with a transfer of hemin from ECL3 to HSA which also supposed an interaction between HSA and ABCG2. We probed such an interaction by monitoring the intrinsic fluorescence signal of ABCG2 in the presence of increasing HSA concentrations. This led to a typical saturation curve displayed in Fig. 8D, reaching to approximately 60% quenching and a rather high Kd of 3.3 ± 1.1 μM, suggesting that such an interaction occurs between both proteins.

**DISCUSSION**

We show that ABCG2 exports heme, formally its fluorescent analog ZnMP (36, 37), and binds porphyrins, preferably the metal-chelated forms, to its large extracellular domain ECL3 and can release them to HSA. Bound compounds are restricted to porphyrins because nonporphyrin molecules, such as mitoxantrone, riboflavin, or doxorubicin (the latter binds to ABCG2 (12) but is only transported by the mutated R482T/G trans- complexed to hemin (gray dashed trace). ECL3 intrinsic fluorescence was recorded at 336 nm. The contribution of HSA to the fluorescence signal was corrected as detailed in supplemental Fig. S6. B, intrinsic fluorescence of ABCG2 (0.1 μM) preincubated with 5 μM mitoxantrone and doxorubicin was recorded with time before and after addition at the indicated times of 5 μM hemin and then 6 μM HSA. The ABCG2 intrinsic fluorescence was recorded at 328 nm. The contribution of HSA to the fluorescence signal was corrected as detailed in supplemental Fig. S7. C, ECL3 to HSA hemin transfer monitored by UV absorbance. The experiment was carried out as described in Fig. 4 with 10 μM apoECL3 (dashed trace), 5 μM hemin and 0–12.5 μM HSA (black to brown traces). The Soret bands at 402 and 412 nm resulting from the interaction of hemin with HSA and ECL3, respectively, are indicated. D, interaction of HSA with ABCG2 monitored by quenching of ABCG2 intrinsic fluorescence. Fluorescence experiments were carried out and analyzed as detailed in the legend of Fig. 3C, correcting the intrinsic fluorescence of ABCG2 with that of NATA in the presence of the same concentrations of HSA.

**FIGURE 8. Hemin transfer from ECL3 to HSA.** A, intrinsic fluorescence of ECL3 (3 μM) was recorded with time before and after addition at the indicated times of 2 μM hemin, and then 3 μM HSA, either alone (black trace) or complexed to hemin (gray dashed trace). ECL3 intrinsic fluorescence was recorded at 336 nm. The contribution of HSA to the fluorescence signal was corrected as detailed in supplemental Fig. S6. B, intrinsic fluorescence of ABCG2 (0.1 μM) preincubated with 5 μM mitoxantrone and doxorubicin was recorded with time before and after addition at the indicated times of 5 μM hemin and then 6 μM HSA. The ABCG2 intrinsic fluorescence was recorded at 328 nm. The contribution of HSA to the fluorescence signal was corrected as detailed in supplemental Fig. S7. C, ECL3 to HSA hemin transfer monitored by UV absorbance. The experiment was carried out as described in Fig. 4 with 10 μM apoECL3 (dashed trace), 5 μM hemin and 0–12.5 μM HSA (black to brown traces). The Soret bands at 402 and 412 nm resulting from the interaction of hemin with HSA and ECL3, respectively, are indicated. D, interaction of HSA with ABCG2 monitored by quenching of ABCG2 intrinsic fluorescence. Fluorescence experiments were carried out and analyzed as detailed in the legend of Fig. 3C, correcting the intrinsic fluorescence of ABCG2 with that of NATA in the presence of the same concentrations of HSA.
porter (4)) do not bind to the domain. Heme and hemin appear to be the preferred ligands, because (i) they display the highest binding affinities for both the isolated ECL3 domain (0.5–1 \( \mu \)M) and the full-length ABCG2 transporter (0.2 \( \mu \)M); (ii) the H583A and C603A single-point mutations alter more markedly the binding of hemin than that of metal-free corresponding PPIX; and (iii) phophorhbia, which is transported by ABCG2 (26), displays a low binding affinity for ECL3 (3 \( \mu \)M), which is even lower for the full protein (\( >8 \) \( \mu \)M) when saturated with MTX or doxorubicin, suggesting that phosphorhbia will not bind to ECL3 under physiological conditions.

ECL3 is topologically located downstream from the membrane transport-binding sites and close to them (4, 51). It is consequently better positioned to bind heme or hemin coming from the transport sites of ABCG2 than those already present in the extracellular space. The relatively low affinity of ECL3, either isolated or within ABCG2, for heme and hemin (micro- molar to submicromolar range) reinforces that hypothesis, especially when taking into account the affinity of albumin for hemin in the same compartment, which is 100-fold higher (\( 10^{-8} \) M). The extracellular location of ECL3 likely favors a role for delivering heme, e.g. to a serum protein such as albumin (or hemopexin, not yet tested) (22), a hypothesis strengthened by our experiments showing that apoHSA releases hemin bound to ECL3 by direct interaction with ABCG2.

To date, ABCG2 has been identified to export phophorhbia (a (23) and PPIX (26, 27) for preventing intracellular accumulation of these compounds. Our data show that it also transports hemin and heme, in agreement with protein binding to a hemin-agarose resin (26). Such an efflux is also likely related to protecting from damages that such molecules may produce (19), being more toxic than metal-free porphyrins (15). In that context, ECL3 is strategically located to handle these compounds after membrane translocation and before their extracellular release. FLVCR, a membrane protein of the MFS family, has been shown to export hemin out of the cell (37, 58), making the ABCG2-mediated efflux of hemin apparently redundant. One possible explanation could lie in the expression pattern of these proteins during hematopoiesis: ABCG2, which is a hematopoietic stem cell marker (24), is particularly expressed at high levels in the early stages of hematopoiesis (25), whereas the level of FLVCR is particularly high during erythropoiesis. This suggests complementary roles of the transporters.

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REFERENCES

1. Dean, M., Hamon, Y., and Chimini, G. (2001) J. Lipid Res. 42, 1007–1017
2. Ross, D. D., Yang, W., Abruzzo, L. V., Dalton, W. S., Schneider, E., Lage, H., Dietel, M., Greenberger, L., Cole, S. P., and Doyle, L. A. (1999) J. Natl. Cancer Inst. 91, 429–433
3. Litman, T., Brangi, M., Hudson, E., Fetsch, P., Abati, A., Ross, D. D., Miyake, K., Resau, J. H., and Bates, S. E. (2000) J. Cell Sci. 113, 2011–2021
4. Sarkadi, B., Ozvég-Laczka, C., Némét, K., and Várádi, A. (2004) FEBS Lett. 567, 116–120
5. Wang, X., and Baba, M. (2005) Antivir. Chem. Chemother. 16, 213–216
6. Maliepaard, M., Scheffer, G. L., Faneyte, I. F., van Gastelen, M. A., Pijnenborg, A. C., Schinkel, A. H., van De Vijver, M. J., Schepers, R. J., and Schel- lens, J. H. (2001) Cancer Res. 61, 3458–3464
7. van Herwaarden, A. E., and Schinkel, A. H. (2006) Trends Pharmacol. Sci. 27, 10–16
8. Ahmed-Belkacem, A., Pozza, A., Muñoz-Martínez, F., Bates, S. E., Casta- nys, S., Gamarro, F., Di Pietro, A., and Pérez-Victoria, J. M. (2005) Cancer Res. 65, 4852–4860
9. Ahmed-Belkacem, A., Macalou, S., Borrelli, F., Capasso, R., Fantorresu, E., Taglialatela-Scafati, O., and Pietro, A. D. (2007) J. Med. Chem. 50, 1933–1938
10. Boumendjel, A., Macalou, S., Ahmed-Belkacem, A., Blanc, M., and Di Pietro, A. (2007) Bioorg. Med. Chem. 15, 2892–2897
11. Nicole, E., Boumendjel, A., Macalou, S., Genoux, E., Ahmed-Belkacem, A., Currup, P. A., and Di Pietro, A. (2009) Adv. Drug Deliv. Rev. 61, 34–46
12. Pozza, A., Perez-Victoria, J. M., Sardo, A., Ahmed-Belkacem, A., and Di Pietro, A. (2006) Cell Mol. Life Sci. 63, 1912–1922
13. Seamon, J. A., Rugg, C. A., Emanuel, S., Calcagno, A. M., Ambudkar, S. V., Middleton, S. A., Butler, J., Borowski, V., and Greenberger, L. M. (2006) Mol. Cancer Ther. 5, 2459–2467
14. Bihorel, S., Camenisch, G., Lemaire, M., and Scherrmann, J. M. (2007) J. Neurochem. 102, 1749–1757
15. Tsiftsoglou, A. S., Tsamadou, A. I., and Papadopoulou, L. C. (2006) Pharm- acol. Ther. 111, 327–345
16. Sassa, S. (1988) Semin. Hematol. 25, 312–320
17. Baliga, B. S., Mankad, M., Shah, A. K., and Mankad, V. N. (1993) Cell Prolif. 26, 519–529
18. Zhu, Y., Hon, T., and Zhang, L. (1999) Biochem. Biophys. Res. Commun. 258, 87–93
19. Kumar, S., and Bandyopadhyay, U. (2005) Toxicol. Lett. 157, 175–188
20. Muller-Eberhard, U., and Fraig, M. (1993) Am. J. Hematol. 42, 59–62
21. Wardell, M., Wang, Z., Ho, J. X., Robert, J., Rulker, F., Ruble, J., and Carter, D. C. (2002) Biochem. Biophys. Res. Commun. 291, 813–819
22. Taketani, S. (2005) Tohoku J. Exp. Med. 205, 297–318
23. Jonker, J. W., Buitelaar, M., Wagenaar, E., Van Der Valk, M. A., Scheffer, G. L., Scheper, R. J., Plosh, T., Kuipers, F., Ellerink, R. P., Rosing, H., Beijnen, J. H., and Schinkel, A. H. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 15649–15654
24. Zhou, S., Schuetz, J. D., Bunting, K. D., Colapietro, A. M., Sampath, J., Morris, J. J., Lagutina, I., Grosveld, G. C., Osawa, M., Nakauchi, H., and Sorrentino, B. P. (2001) Nat. Med. 7, 1028–1034
25. Scharenberg, C. W., Harkey, M. A., and Torok-Storb, B. (2002) Blood 99, 507–512
26. Krishnamurthy, P., Ross, D. D., Nakashita, T., Bailey-Dell, K., Zhou, S., Mercer, K. E., Sarkadi, B., Sorrentino, B. P., and Schuetz, J. D. (2004) J. Biol. Chem. 279, 24218–24225
27. Zhou, S., Zong, Y., Nye, P. A., Nair, G., Stewart, C. F., and Sorrentino, B. P. (2005) Blood 105, 2571–2576
28. Bhatia, A., Schäfer, H. J., and Hrycyna, C. A. (2005) Biochemistry 44, 10893–10904
29. Polgar, O., Ozvég-Laczka, C., Robey, R. W., Morisaki, K., Okada, M., Tamaki, A., Koblos, G., Elkind, N. B., Ward, Y., Dean, M., Sarkadi, B., and Bates, S. E. (2006) Biochemistry 45, 5251–5260
30. Polgar, O., Robey, R. W., Morisaki, K., Dean, M., Michejda, C., Sauna, Z. E., Ambudkar, S. V., Tarasova, N., and Bates, S. E. (2004) Biochemistry 43, 9448–9456
31. Özvegy, C., Litman, T., Szakács, G., Nagy, Z., Bates, S., Váradi, A., and
Hemin Binding to the Large Extracellular Loop of ABCG2

Sarkadi, B. (2001) Biochem. Biophys. Res. Commun. 285, 111–117

32. Kage, K., Tsukahara, S., Sugiyama, T., Asada, S., Ishikawa, E., Tsuruo, T., and Sugimoto, Y. (2002) Int. J. Cancer 97, 626–630

33. Kage, K., Fujita, T., and Sugimoto, Y. (2005) Cancer Sci. 96, 866–872

34. Henriksen, U., Fog, J. U., Litman, T., and Gether, U. (2005) J. Biol. Chem. 280, 36926–36934

35. O¨zvegy-Laczka, C., Laczko´, R., Hegedus, C., Litman, T., Va ´rady,G., Goda, K., Hegedus, T., Dokholyan, N. V., Sorrentino, B. P., Váradi, A., and Sarkadi, B. (2008) J. Biol. Chem. 283, 26059–26070

36. Worthington, M. T., Cohn, S. M., Miller, S. K., Luo, R. Q., and Berg, C. L. (2001) Am. J. Physiol. Gastrointest. Liver Physiol. 280, G1172–G1177

37. Quigley, J. G., Yang, Z., Worthington, M. T., Phillips, J. D., Sabo, K. M., Sabath, D. E., Berg, C. L., Sassa, S., Wood, B. L., and Abkowitz, J. L. (2004) Cell 118, 757–766

38. Montigny, C., Penin, F., Lethias, C., and Falson, P. (2004) Biochim. Biophys. Acta 1660, 53–65

39. Trometer, C., and Falson, P. (2010) in Heterologous Expression of Membrane Proteins: Methods and Protocols (Mus-Veteau, I., ed) pp. 105–117, Humana Press, Totowa, NJ

40. Kapust, R. B., Tözse´r, J., Fox, J. D., Anderson, D. E., Cherry, S., Copeland, T. D., and Waugh, D. S. (2001) Protein Eng. 14, 993–1000

41. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76–85

42. Li, S. D., Su, Y. D., Li, M., and Zou, C. G. (2006) Acta Biochim. Biophys. Sin. 38, 63–69

43. Whitmore, L., and Wallace, B. A. (2004) Nucleic Acids Res. 32, W668–W673

44. Provencher, S. W., and Glöckner, J. (1981) Biochemistry 20, 33–37

45. Andrade, M. A., Chacón, P., Merelo, J. J., and Morán, F. (1993) Protein Eng. 6, 383–390

46. Seeraram, N., and Woody, R. W. (2000) Anal. Biochem. 287, 252–260

47. Falson, P., Penin, F., Divita, G., Lavergne, J. P., Di Pietro, A., Goody, R. S., and Gautheron, D. C. (1993) Biochemistry 32, 10387–10397

48. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680

49. Scheidt, W., and Lee, Y. (1987) in Metal Complexes with Tetrapyrole Ligands I, pp. 1–70, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany

50. Allen, J. D., van Loevezijn, A., Lakhai, J. M., van der Valk, M., van Tellingen, O., Reid, G., Schellens, J. H., Koomen, G. J., and Schinkel, A. H. (2002) Mol. Cancer Ther. 1, 417–425

51. Li, Y. F., Polgar, O., Okada, M., Esser, L., Bates, S. E., and Xia, D. (2007) J. Mol. Graph. Model 25, 837–851

52. Hazai, E., and Bikádi, Z. (2008) J. Struct. Biol. 162, 63–74

53. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70–77

54. Mohanty, A. K., Simmons, C. R., and Wiener, M. C. (2003) Protein Expr. Purif 27, 109–114

55. Jonker, J. W., Merino, G., Musters, S., van Herwaarden, A. E., Bolscher, E., Wagenaar, E., Mesman, E., Dale, T. C., and Schinkel, A. H. (2005) Nat. Med. 11, 127–129

56. van Herwaarden, A. E., Wagenaar, E., Merino, G., Jonker, J. W., Rosing, H., Beijnen, J. H., and Schinkel, A. H. (2007) Mol. Cell. Biol. 27, 1247–1253

57. Monzani, E., Bonafè, B., Fallarini, A., Redaelli, C., Casella, L., Minchiotti, L., and Galliano, M. (2001) Biochim. Biophys. Acta 1547, 302–312

58. Keel, S. B., Doty, R. T., Yang, Z., Quigley, J. G., Chen, J., Knoblaugh, S., Kingsley, P. D., De Domenico, I., Vaughn, M. B., Kaplan, J., Palis, J., and Abkowitz, J. L. (2008) Science 319, 825–828