Molecular Pharmacology Fast Forward. Published on October 5, 2007 as DOI: 10.1124/mol.107.038299
Molecular Pharmacology Fast Forward. Published on October 9, 2007 as doi:10.1124/mol.107.038299
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A functional steroid-binding element in an ATP-binding cassette multidrug transporter

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Running title: Steroid-protein interactions in ABCG2

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Number of text pages: 28

Number of figures: 4

Number of references: 39

Number of words in *Abstract*: 113

Number of words in *Introduction*: 400

Number of words in *Results*: 1627

Number of words in *Discussion*: 895

Abbreviations: ABC, ATP-binding cassette; a.u., arbitrary unit; DSG, disuccinimidyl glutarate; ED, 17β-estradiol; PG, progesterone; TMH, transmembrane helix

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ABSTRACT

The human breast cancer resistance protein is an ATP-binding cassette (ABC) multidrug transporter that affects the bioavailability of chemotherapeutic drugs, and can confer drug resistance on cancer cells. It is the second member of the ABCG subfamily, other members of which are associated with human steroid disorders such as hypercholesterolemia, sitosterolemia, and atherosclerosis. The molecular bases of protein-steroid interactions in ABC transporters are unknown. Here, we identify a steroid-binding element in the membrane domain of ABCG2 with a similarity to steroid hormone/nuclear receptors. The element facilitates steroid hormone binding, and mediates modulation of ABCG2 activity. The identification of this element might provide an opportunity for the development of new therapeutic ligands for ABCG2.

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INTRODUCTION

ABCG proteins are composed of an N-terminal nucleotide-binding domain followed by a membrane domain with six putative transmembrane helices (TMHs). These half-size molecules dimerise to form functionally active, full-size ABC transporters (Krishnamurthy and Schuetz, 2006). ABCG2 plays an important role in the disposition and pharmacological activity of a broad range of compounds, including chemotherapeutic drugs used in the treatment of cancer (Hardwick *et al.*, 2007). The protein is expressed on the apical membrane of cells in tissues with excretory functions, such as the apical pole of trophoblast cells in the placenta, the ducts and lobules of the breast, luminal membrane of villous epithelial cells in the small and large intestines, apical membranes of capillary vessels in the blood-brain barrier, and the canalicular membrane of hepatocytes (Maliepaard *et al*., 2001). In addition to its interaction with multiple drugs, ABCG2 can interact with a variety of steroids including 17β-estradiol (ED), progesterone (PG), testosterone, sulfated estrogens, and 17β-estradiol-17β-D-glucuronide (Janvilisri *et al.*, 2003, 2005; Suzuki *et al.*, 2003; Chen *et al.*, 2003; Cooray *et al.*, 2006).

The interaction with steroids has also been observed for other members of the ABCG subfamily. ABCG1 and ABCG4 promote cholesterol efflux from cells to high-density lipoproteins (HDL) (Wang *et al.*, 2004). ABCG1 is highly expressed in macrophages, and mediates cholesterol efflux from macrophage foam cells, providing a link between HDL levels and atherosclerosis risk. ABCG5 and ABCG8 are the defective proteins in sitosterolemia, and form a heterodimeric transporter that is responsible for dietary sitosterol/cholesterol efflux from enterocytes, thus preventing sterol overaccumulation in humans (Berge *et al.*, 2000). The observation that many ABCG

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proteins can interact with steroids raises interesting questions about the nature of protein-steroid interactions in these transporters. In this paper, we describe the identification of a functional steroid-binding element in ABCG2^{R482G}. The original cDNA encoding this ABCG2 protein was derived from S1-M1-80 cells, a mitoxantrone-resistant human colon carcinoma cell line, which encodes a glycine at amino acid 482 at the cytoplasmic end of TMH 3, instead of the wildtype arginine (Honjo *et al.*, 2001). The R482G replacement does not significantly affect the interactions of ABCG2 with Hoechst 33342 and steroid hormones (Robey *et al.*, 2003; Janvilisri *et al.*, 2005; Ozvegy-Laczka *et al.*, 2005). ABCG2^{R482G} was selected for ease of study with cationic dyes such as ethidium. In addition, its wider pharmacological spectrum enables are more exhaustive characterisation of drug-protein interactions than ABCG2^{R482} (Clark *et al.*, 2006).

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MATERIALS AND METHODS

Mutagenesis. *ABCG2* mutants were generated with the QuikChange method (Stratagene) using pGEM-BCRP R482G as a template (Janvilisri *et al.*, 2003). Mutations in the *ABCG2*^{R482G} gene were introduced using the forward primer 5'-TTT TTT CAC GTC TGT TGG TCA ATC TCA C-3' and the reversed primer 5'-ATT GAC CAA CAG ACG TGA AAA AAT CAT C-3' for G553R, the forward primer 5'-GAT GAT TTT TAT GGG TCT GTT GGT CAA TCT CAC-3' and reversed primer 5'-CCA ACA GAC CCA TAA AAA TCA TCA TAA ACA C-3' for S552M, and forward primer 5'-GGT CTG GGG GTC AAT GGC ACA ACC ATT GCA TCT TGG-3' and reversed primer 5'-ATG GTT GTG CCA TTG ACC CCC AGA CCT GAA AAA ATC-3' for L555A L558A. The mutated *ABCG2*^{R482G} genes were cloned into pNZ8048 for expression in drug-hypersensitive *Lactococcus lactis* NZ9000 Δ*lmrA* Δ*lmrCD* (Lubelski *et al.*, 2006; Venter and Van Veen, unpublished). The DNA was sequenced to ensure that only the intended changes were introduced.

ATPase and Transport. The ATPase activity in inside-out membrane vesicles was determined from the rate of liberation of Pi from ATP in a colorimetric assay (Janvilisri *et al.*, 2003). For pure ABCG2^{R482G} proteins, the ATPase activity was determined using the NADH oxidation assay (Ravaud *et al.*, 2006). Hoechst 33342 and ethidium transport were measured by fluorimetry (Janvilisri *et al.*, 2003, 2005; Venter *et al.*, 2003). The transport of [1,2,6,7-^{3}H]PG (3.48 TBq/mmol) and [2,4,6,7-^{3}H]ED (3.22 TBq/mmol) (Amersham) was determined by rapid filtration (Janvilisri *et al.*, 2003, 2005).

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Drug Binding. ABCG2^{R482G} was solubilised from inside-out membrane vesicles in the presence of n-dodecyl-β-D-maltoside and purified by Ni^{2+}-NTA affinity chromatography as described for LmrA (Venter *et al.*, 2003), using elution buffer containing 250 mM imidazole. Equilibrium binding of [^{3}H]PG or [^{3}H]ED to purified ABCG2 in detergent solution, and removal of unbound steroid with dextran-coated charcoal were performed in accordance with published methods for nuclear steroid hormone receptors (Makishima *et al.*, 2002). Equilibrium binding of Hoechst 33342 to purified ABCG2^{R482G} (15 µg/ml) was measured from the fluorescence increase at excitation and emission wavelengths of 355 nm and 457 nm, respectively, and slit widths of 10 nm and 5 nm, respectively. Non-specific binding of steroid and Hoechst 33342 to hydrophobic, membrane-exposed regions in ABCG2^{R482G} was determined using half-molar quantities of the purified 12 TMH-containing galactose transporter (GalP) from *Escherichia coli* as a control, and was less than 30% of total binding obtained for ABCG2^{R482G}. GalP protein was overexpressed in *E. coli* JM1100 (pPER3), and purified by affinity chromatography according to established protocols (Ward *et al.*, 2000).

Chemical Cross-Linking and Immunoblotting. For chemical cross-linking, 50 µg of purified ABCG2^{R482G} in 50 µl of 100 mM KPi (pH 7.0) was incubated for 30 min at 20 ºC in the presence of 0.5 mM DSG and/or PG as indicated in Fig. 4. Proteins were subsequently washed and delipidated as described (Wessel and Flugge, 1984). Briefly, 100 µl methanol and 100 µl chloroform were mixed with the samples. The proteins were precipitated at the interphase by centrifugation, collected, and further washed with 300 µl methanol. After centrifugation, the pellet was allowed to dry with the tube inverted for 2-5 min. The pellet was then dissolved in 25 µl KPi (pH 7.0). Protein was subjected to 8%

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(w/v) SDS-PAGE and analysed on immunoblot using anti-His antibody (Janvilisri *et al.*, 2003; Venter *et al.*, 2003).

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RESULTS

The molecular determinants for interactions of ABCG proteins with steroids are unknown, but such interactions have been studied in great detail in nuclear steroid hormone receptors. For example, the crystal structure of the complex formed by PG and the ligand-binding domain (LBD) of the human progesterone receptor-β (hPRβ) at 1.8-Å resolution reveals that steroid hormone binding is based on a network of mutually supported hydrophobic, Van der Waals, and hydrogen-bonded interactions (Williams and Sigler, 1998) (**Fig. 1*A***). Among the regions that contribute to PG binding is Helix 3, in which L721, L718 and L715 form Van der Waals contacts with the A-ring, C-ring and the COCH3 moiety on the D-ring of the steroid, respectively (**Fig. 1 *A, B***). Residues at these positions in the LBD of other members of the steroid hormone/nuclear receptor family, including the human androgen receptor-β (hARβ) (Sack *et al.*, 2001), human glucocorticoid receptor (hGRα) (Bledsoe *et al.*, 2002), and human estrogen receptor-α (hERα) (Tanenbaum *et al.*, 1998) have established roles in ligand-binding, and form a conserved (L/M)xxLxxL motif in which x can be any residue (**Fig. 1*C***). An LxxL motif implicated in steroid binding is also conserved in the large family of human and yeast oxysterol-binding-protein-related proteins; the recent crystal structure of the closed conformation of Osh4 at 2.5-Å resolution shows the highly conserved L24 and L27 in a direct Van der Waals interaction with the sterol ligand (Im *et al.*, 2005).

Surprisingly, a sequence element 552-**S**GL**L**VN**L**-558 at the extracellular side of predicted TMH 5 of ABCG2 shares a significant identity with the estrogen-binding element 343-**M**GL**L**TN**L**-349 in Helix 3 of hERα (**Fig. 1*C***). This sequence element is also present in TMH 5 of ABCG8 (577-**M**IN**L**SS**L**-583). A G575R substitution at the start of this element in ABCG8 is associated with a dysfunction of ABCG5/8, causing

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sitosterolemia (Berge *et al.*, 2000). In ABCG1 and ABCG4, (S/M)xxLxxL in TMH 5 is replaced by the bulkier SxxFxxF, which might allow packing of aromatic and sterol rings as suggested for the human cholesterol-binding protein NPC2 (Friedland *et al.*, 2003) (**Fig. 1*C***).

The functional role of the SxxLxxL motif in human ABCG2^{R482G} was tested in *L. lactis*, a bacterial model which is devoid of human steroids (Janvilisri *et al.*, 2003, 2005; Venter *et al.*, 2003). A mutant form of ABCG2^{R482G} was generated in which L555 and L558 were both substituted by A (LALA mutant). These residues are equivalent to L718 and L721 in hPRβ, and L346 and L349 in hERα, respectively (**Fig. 1*C***). By analogy to the sitosterolemia-associated G574R substitution in ABCG8, G553 in the SxxLxxL motif was replaced by R (GR mutant). The LALA and GR mutations do not significantly alter the predicted topology of TMH 5 (residues 539-553 in ABCG^{R482G}, 542-556 in LALA, and 539-552 in GR), or the topology of other sections in ABCG2^{R482G} when analysed by the computer-assisted transmembrane topology prediction method MEMSAT (Jones *et al.*, 1994; Jones, 2007) on the Protein Structure Prediction Server (http://bioinf.cs.ucl.ac.uk/psipred/). This analysis is in agreement with a previously proposed topology model for ABCG2, and with the identification of intramolecular disulfide bridges in the protein (Henriksen *et al.*, 2005).

The mutant proteins were equally well expressed as ABCG2^{R482G} in the cytoplasmic membrane (**Fig. 2*A***). While steroid hormones such as ED and PG stimulated the ATPase activity of ABCG2^{R482G} up to 2.5-fold, no stimulation was obtained for the LALA and GR mutants (**Fig. 2*B***). Consistent with this observation, the mutant proteins were unable to mediate the export of [^{3}H]PG in intact cells which were preloaded with the substrate (**Fig. 2*C***). Similar results were obtained for [^{3}H]ED (data not shown). As a

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result of this inability, both steroid hormones continued to accumulate in cells expressing the LALA or GR mutant protein under conditions where active steroid efflux was observed for ABCG2^{R482G}. It is interesting to note that the observations on steroid hormone transport by ABCG2^{R482G} in our lactococcal model are supported by previous observations on [^{3}H]dihydrotestosterone transport by murine Abcg2 in prostate progenitor cell lines (Huss *et al.*, 2005).

The interaction of ABCG2^{R482G} and mutant proteins with [^{3}H]PG was further analysed in equilibrium binding assays using detergent-solubilized and affinity-purified proteins. The LALA and GR mutations were associated with a significant loss of [^{3}H]PG binding with an apparent dissociation constant (K_d) of 3.7 ± 0.5 μM and maximum binding (B_{max}) of 103.1 ± 5.2 nmol/mg protein (**Fig. 2*D***). Comparable results were obtained for the binding of [^{3}H]ED (data not shown). The first residue in the (M/L)xxLxxL motif of nuclear hormone receptors is in proximity of the substituent on the D-ring of the steroid substrate (**Figs. 1*B*, *C***). Therefore, we tested the effect of the replacement of S552 in the SxxLxxL motif in ABCG2^{R482G} on steroid selectivity. The S552 to M substitution (as observed in hERα) did not alter protein expression (**Fig. 2*A***, SM mutant) or the interaction of ABCG2^{R482G} with PG (**Fig. 2*D***), but decreased the apparent K_d for ED by 6-fold (from 4.7 ± 0.3 μM for ABCG2^{R482G} to 0.8 ± 0.2 μM for SM mutant) while the B_{max} was unaffected (106.6 ± 7.4 nmol/mg protein for ABCG2^{R482G} versus 104.2 ± 5.5 nmol/mg of protein for SM mutant) (**Fig. 2*E***). Taken together, these results suggest that the substituted residues in the steroid-binding element contribute to the interaction of ABCG2^{R482G} with steroids.

We also examined the effect of the LALA, GR and SM mutations on the interaction with cytotoxic drugs. In fluorimetric binding assays, the mutations did not

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affect the equilibrium binding of the ABCG2 substrate Hoechst 33342 to purified protein with a K_d of 0.47 ± 0.06 μM and B_{max} of 57.8 ± 2.2 a.u. (**Fig. 3*A***). In addition, the rates of active Hoechst 33342 extrusion in intact cells, and transport in inside-out membrane vesicles were comparable between ABCG2^{R482G} and mutant forms (**Fig. 3 *B, C***). Similar data were obtained for ethidium transport in intact cells (data not shown). The ATPase activities of ABCG2^{R482G} and mutant proteins were stimulated equally in the presence of 50 μM daunomycin (**Fig. 2*B***). Hence, the LALA, GR and SM mutations did not affect the ability of ABCG2^{R482G} to interact with multiple drugs. Together with the similar basal ATPase activities of ABCG2^{R482G}, and LALA and GR mutants (**Fig. 2*B***) and SM mutant (about 33 ± 4 nmol Pi/nmol/mg protein), these results suggest that no gross conformational changes were introduced in ABCG2^{R482G} by the LALA, GR and SM mutations.

Interestingly, PG (50 μM) stimulated the transport of Hoechst 33342 by ABCG2^{R482G} and SM, but not by the LALA and GR mutants (**Fig. 3*C***). Previous work on wildtype ABCG2^{R482} indicated that cholesterol can potentiate its ATPase and transport activities in a heterologous expression system based on *Spodoptera frugiperda* Sf9 insect cells (Pal *et al.*, 2007). Likewise, in an earlier study on ABCG2^{R482G} expressed in *L. lactis*, we observed a maximal (4-fold) stimulation of the ATPase activity of the protein in the presence of 10 μM cholesterol (Janvilisri *et al.*, 2003). However, this cholesterol-stimulated ATPase activity was not reduced by the LALA mutation (**Fig. 2*B***). In addition, cholesterol did not affect Hoechst 33342 transport by ABCG2^{R482G} and LALA mutant, or the PG-dependent stimulation of Hoechst 33342 transport by ABCG2^{R482G} at concentrations of up to 20 μM cholesterol (**Fig. 3*D***). Similar results were obtained for

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Hoechst 33342 transport by wildtype ABCG2^{R482} (**Fig. 3*E***). These findings imply that the binding of cholesterol and PG by ABCG2 occur by different mechanisms.

The PG-dependent stimulation of Hoechst 33342 transport by ABCG2^{R482G} was further analysed in kinetic experiments, and was due to a 3.3-fold enhancement of the maximum transport rate (V_{max} = 0.20 ± 0.04 a.u./s in absence of PG versus 0.67 ± 0.6 a.u./s in presence of PG) without a change in the apparent affinity for Hoechst 33342 (Michaelis constant, K_m = 2.48 ± 0.62 μM) (**Fig. 3*F***). Consistent with this observation, the equilibrium binding of Hoechst 33342 to purified ABCG2^{R482G} and mutant proteins was not affected by the presence of 50 μM PG (**Fig. 3*A***). Similar to the observations in inside-out membrane vesicles (**Fig. 2*B***), 50 μM PG stimulated the ATPase activity of purified ABCG2^{R482G} (by 2.5-fold), whereas the ATPase activities of LALA and GR mutants remained unaffected (data not shown). These experiments point to the presence of independent sites for PG and Hoechst 33342 in ABCG2^{R482G}.

In mammalian cells (Polgar *et al.*, 2006; Xu *et al.*, 2004) and insect cells (McDevitt *et al.*, 2006), monomeric ABCG2^{R482G} has been reported to oligomerise into homodimeric, homotetrameric and homooctameric forms, but the factors that influence oligomerisation are not known in detail. Subsequent to SDS-PAGE, ABCG2^{R482G} and GR mutant purified from steroid-less *L. lactis* were predominantly detected on an immunoblot as monomeric 74 kDa proteins. In addition, a weak signal was observed for the homodimeric 150 kDa form, which is stabilized due to the formation of intermolecular disulphide bonds (Xu *et al.*, 2004; Henriksen *et al.*, 2005). This signal was enhanced after chemical cross-linking of the protein with disuccinimidyl glutarate (DSG) (**Fig. 4**, *upper panel*). Interestingly, PG strongly stimulated the oligomerisation of ABCG2^{R482G} into homodimeric and homotetrameric (290 kDa) forms, respectively (**Fig.**

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4, *lower panel*). PG did not affect the oligomerisation of the GR mutant protein (**Fig. 4**, *lower panel*), consistent with the lack of PG binding by this mutant (**Fig. 2*D***). In control experiments, the presence of Hoechst 33342 (up to 5 μM) or cholesterol (up to 20 μM) failed to stimulate the oligomerisation of ABCG2^{R482G} pointing to a specific role of PG in the oligomerisation reaction (data not shown). With the notion that the ABCG2 half-transporter needs to oligomerise to form the functionally-active, full-size pump (Krishnamurthy and Schuetz, 2006; Hardwick *et al.*, 2007), the observation on the PG-dependent oligomerisation of ABCG2^{R482G} is consistent with the PG-dependent stimulation of Hoechst 33342 transport by this protein (**Figs. 3 *C, F***).

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DISCUSSION

Although steroids can be potent modulators of ion channels, for example, by acting on discrete activation and potentiation sites in the transmembrane domains of $GABA_A$ receptor isoforms (Hosie *et al.*, 2006), little information is available about the potential role of steroids in the modulation of the activity of membrane transporters. We now have identified a functional steroid-binding element in the membrane domain of $ABCG2^{R482G}$, which shares sequence similarity with Helix 3 in hERα (**Fig. 1**). By analogy to the positioning of Helix 3 in the steroid-binding site of this receptor, the steroid-binding element in $ABCG2^{R482G}$ is likely to be part of a larger steroid-binding pocket. The predicted location of the binding element at the external side of TMH 5 raises the possibility that the relatively large outer loop region connecting TMH 5 and TMH 6 contributes to the binding pocket.

The steroid interactions detected in our study appear to be steroid specific. Whereas cholesterol and PG both stimulated the $ABCG2^{R482G}$-ATPase, the LALA/GR mutations prevented this activation for PG but not for cholesterol (**Fig. 2*B***). In addition, $ABCG2^{R482G}$-mediated Hoechst 33342 transport was stimulated by PG (**Fig. 3*C***), but not by cholesterol (**Fig. 3*D***) at concentrations at which these compounds both stimulated the $ABCG2^{R482G}$-ATPase activity (**Fig. 2*B***). In agreement with the position of the steroid-binding element at the external face of the membrane, the LALA and GR mutations in the element affected interactions of $ABCG2^{R482G}$ with relatively hydrophilic PG, but not with hydrophobic, membrane-associated cholesterol. Our results on $ABCG2^{R482G}$ are consistent with recent observations by Telbisz *et al.* (2007) showing that cholesterol loading affects the ATPase activity but not Hoechst 33342 transport activity of

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ABCG2^{R482G} in the plasma membrane of human embryonic kidney (HEK) 293 cells, human skin-derived epidermoid carcinoma A341 cells, and Sf9 insect cells. In case of compulsory coupling between ATPase and substrate transport by ABCG2, these observations could be explained if cholesterol and Hoechst 33342 were both transported in an ATP-dependent fashion. Alternatively, cholesterol might enhance the basal ATPase activity of ABCG2^{R482G} without affecting transport. Although in our lactococcal system, wildtype ABCG2^{R482} behaved essentially similar to ABCG2^{R482G} with respect to the effects of cholesterol on ATPase and Hoechst 33342 transport activity (**Fig. 3*E***) (Janvilisri *et al.*, 2005), Telbisz *et al.* (2007), Pal *et al.* (2007) and Storch *et al.* (2007) found that membrane cholesterol increased substrate transport by wildtype ABCG2^{R482} in eukaryotic expression systems. The dissimilarity between these observations is possibly related to differences in the lipid composition of the eukaryotic membranes versus lactococcal membranes, and/or to differences in experimental procedures. For example, cyclodextrin was required to deplete cholesterol from the eukaryotic membranes but was not used with lactococcal membranes as the human sterols are replaced by hopanoids in these membranes (Bird *et al.*, 1971).

Our observations raise questions about the potential link between drug-binding sites and steroid-binding sites in ABCG2^{R482G}. The LALA and GR mutations strongly inhibited the equilibrium binding of PG to purified ABCG2^{R482G} (**Fig. 2*D***) and the transport of PG by the protein (**Fig. 2*C***). In contrast, the LALA and GR mutations did not affect the ABCG2^{R482G}-mediated transport of ethidium or Hoechst 33342 (**Fig. 3*B***), or the equilibrium binding of Hoechst 33342 to the purified protein (**Fig. 3*A***). As PG stimulated the transport of Hoechst 33342 by ABCG2^{R482G} (**Fig. 3*C***), these data point to independent, but interacting sites for PG and Hoechst 33342. Interacting substrate-

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binding sites in ABCG2^{R482G} were also reported in a previous study by Clark *et al.* (2006). Interestingly, the binding sites for PG and Hoechst 33342 might be associated with different levels of oligomerisation of ABCG2^{R482G}. The chemical cross-linking experiments demonstrated the PG-dependent, but not Hoechst 33342 or cholesterol-dependent formation of dimeric and tetrameric forms of ABCG2^{R482G}. In the absence of PG, no oligomerisation of ABCG2^{R482G} was observed beyond the dimer (**Fig. 4**). Taken together, these data suggest that Hoechst 33342 binding sites are present in the minimal functional (homodimeric) ABCG2^{R482G} unit and higher oligomeric assemblies derived thereof, whereas PG binding sites might be associated with higher oligomeric assemblies.

The oligomerisation of ABCG2 is also thought to be important for the trafficking of the protein from ER to plasma membrane. Previous studies on the expression of G553 mutants of ABCG2^{R482G} in HEK 293 cells resulted in impaired trafficking and enhanced degradation of these mutants in the endoplasmic reticulum compared to Wt (Polgar *et al.*, 2006). As the TMH 5-loop-TMH 6 region of ABCG2 (containing the proposed steroid-binding element) forms an oligomerisation domain when expressed alone in HEK 293 cells (Xu *et al.*, 2007), the impaired trafficking of the G553 mutants might point to a dependence of the oligomerisation of ABCG2 on endogenous steroids in these cells. Our ability to functionally express the trafficking-impaired G553 mutants in *L. lactis*, in which the insertion of membrane proteins in the plasma membrane occurs by a co-translational mechanism rather than the eukaryotic, trafficking-dependent post-translational mechanism (Mitra *et al.*, 2006), demonstrates the potential value of bacterial expression systems in the functional characterisation of mutated mammalian transport proteins.

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Further studies will be required to compare steroid interactions and their effect on oligomerisation for wildtype ABCG2^{R482}; our methods and data will be useful in the detailed characterisation of steroid-binding sites in this transporter. In view of the conservation of the steroid-binding element among ABCG proteins, our findings might have a more general relevance for other members of the ABCG subfamily.

ACKNOWLEDGMENTS

We would like to thank Susan Bates and Orsolya Polgar for their comments on the manuscript and for sharing data prior to publication. In addition, we thank Peter Henderson for the kind gift of GalP-containing membrane vesicles.

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FOOTNOTES

This study was supported by the Medical Research Council and Association for International Cancer Research.

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LEGENDS FOR FIGURES

Fig. 1. Putative steroid-binding element in ABCG subfamily. (*A*) Crystal structure of hPRβ LBD (PDB 1a28). Helix 3 (segment S712 to L726 in purple) directly interacts with PG (blue). (*B*) Snapshot of the interactions between PG and segment S712 to L726 of hPRβ in which L715, L718 and L721 make Van der Waals contacts with the bound ligand, and Q725 makes a hydrogen bond. (*C*) Alignment of hPRβ, hARβ, hGRα, hERα, and ABCG proteins. Residues are highlighted by function: red (established role in ligand binding), light blue (conserved motif), and yellow (mutated in this work).

Fig. 2. Functional analyses on steroid-binding element in ABCG2^{R482G}. (*A*) Immunoblot showing no expression (Control), and equal expression of ABCG2^{R482G} (G2) and LALA, GR and SM mutant proteins in the plasma membrane. (*B*) ABCG2 ^{R482G}-ATPase activity in inside-out membrane vesicles without substrate (basal), or with 25 μM ED, 10 μM PG, 10 μM daunomycin or 10 μM cholesterol. Solid, light grey and dark grey bars refer to ABCG2^{R482G}, and LALA and GR mutants, respectively. (*C*) Transport of 50 μM [^{3}H]PG in intact cells. (*D, E*) Equilibrium binding of [^{3}H]PG (*D*) or [^{3}H]ED (*E*) to purified ABCG2 ^{R482G} proteins.

Fig. 3. Interaction of ABCG2 with Hoechst 33342. (*A*) Equilibrium binding of Hoechst 33342 to purified ABCG2^{R482G} proteins in detergent solution. ABCG2^{R482G} (●), LALA (△), GR (□), SM (▼); Hoechst 33342 binding to ABCG2^{R482G} in the presence of 25 μM PG (○). (*B*) Transport of 0.75 μM Hoechst 33342 (H) in non-expressing control cells and in cells expressing ABCG2^{R482G} (G2) or LALA, GR, or SM mutant. (*C, D*) Transport

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of 0.25 μM Hoechst 33342 (H) in inside-out control membrane vesicles or membrane vesicles containing ABCG2^{R482G} (G2) or LALA or GR mutant in the presence or absence of (*C*) 50 μM PG (± PG), or (*D)* 20 μM cholesterol (± Chol). 50 μM PG was added in (*D*) where indicated. ABCG2^{R482G}-mediated transport was initiated by the addition of ATP. Traces obtained for SM mutant were similar to those obtained for ABCG2^{R482G}. (*E*) Effects of PG and cholesterol on Hoechst 33342 transport in inside-out membrane vesicles containing wildtype ABCG2^{R482} (Wt). (*F*) Kinetic analysis of PG activation of Hoechst 33342 transport in (*C*); 0 (●), 25 (■), 50 (▼) μM PG.

Fig. 4. Effect of PG on the oligomeric state of ABCG2^{R482G}. *Lower panel*, immunodetection of purified ABCG2 ^{R482G} (G2) or GR mutant before and after chemical cross-linking in the presence of DSG, with or without PG (μM). *Upper panel*, overexposed immunoblot shows low levels of dimeric ABCG2 ^{R482G} in lanes without PG, and low levels of dimeric GR in all lanes.

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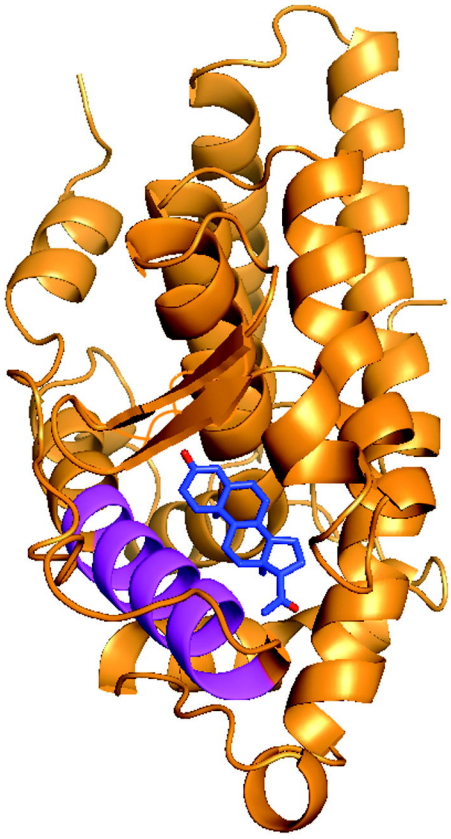
Figure 1

(A)



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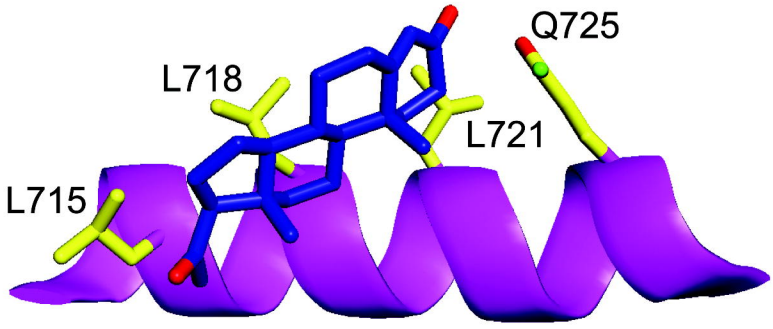

(C)

hPRβ	712-	S	S	L	**L**	T	S	**L**	N	Q	**L**	G	E	R	**Q**	L	-726
hARβ	698-	A	A	L	**L**	S	S	**L**	N	E	**L**	G	E	R	**Q**	L	-712
hGRα	557-	W	R	I	**M**	T	T	**L**	N	M	**L**	G	G	R	**Q**	V	-571
hERα	340-	A	S	M	**M**	G	L	**L**	T	N	**L**	A	D	R	**E**	L	-354
hABCG2	549-	M	I	F	**S**	G	L	**L**	V	N	**L**	T	T	I	A	S	-563
hABCG8	574-	G	G	F	**M**	I	N	**L**	S	S	**L**	W	T	V	P	A	-588
hABCG1	577-	L	L	F	**S**	G	F	**F**	V	S	**F**	D	T	I	P	T	-591
hABCG4	546-	L	L	F	**S**	G	F	**F**	V	S	**F**	K	T	I	P	T	-560

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Figure 2

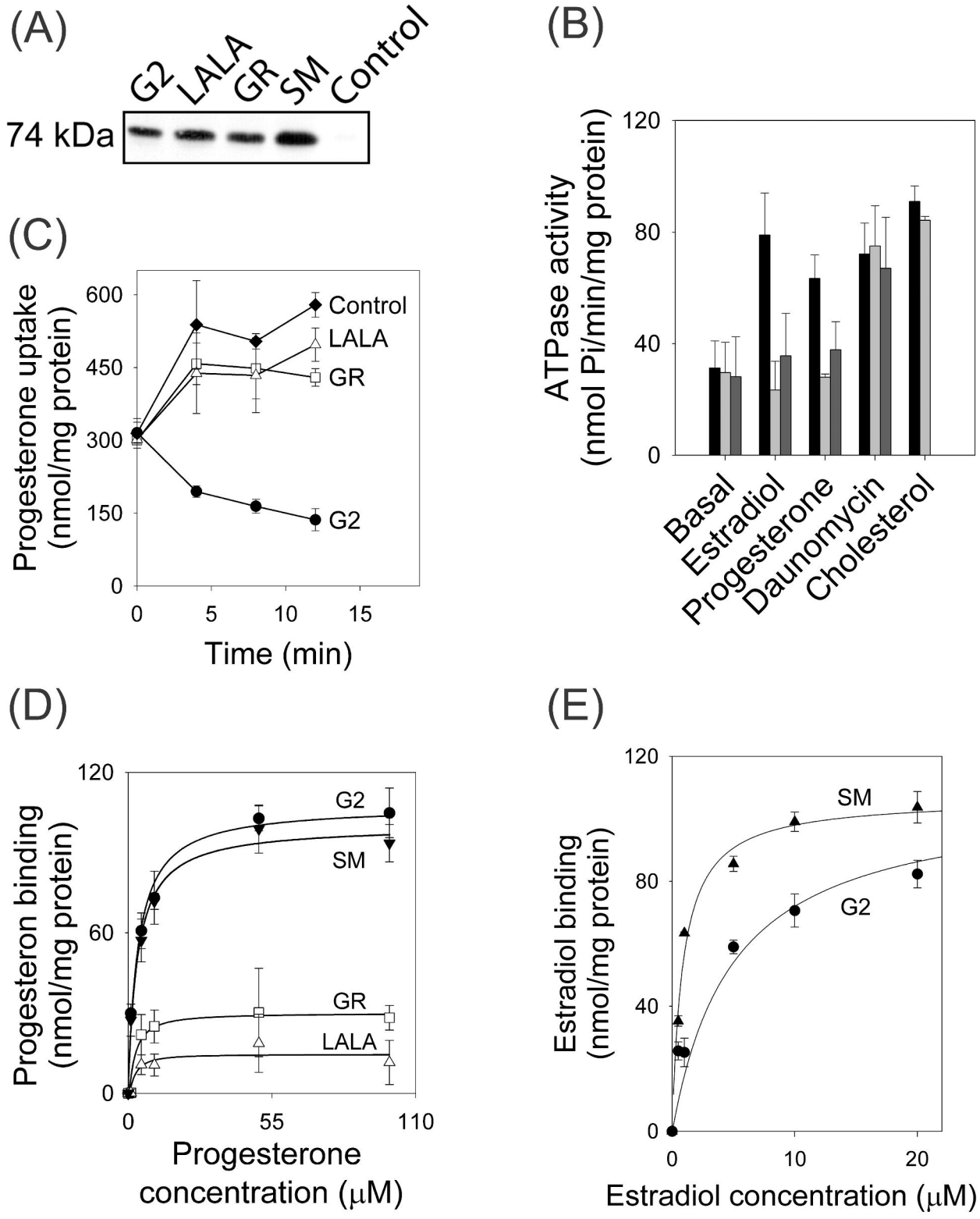

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Figure 3

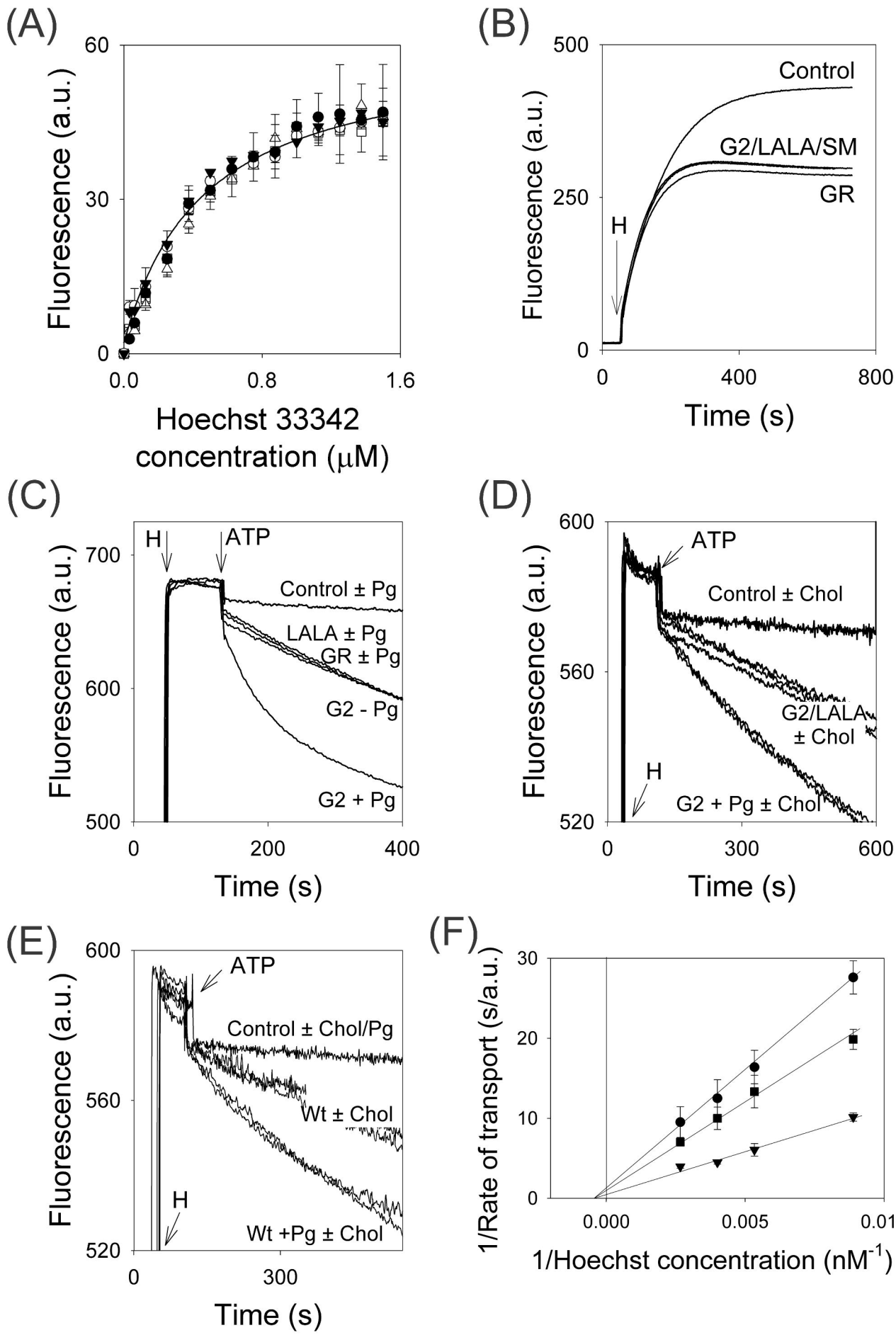

Figure 4