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Membrane cholesterol selectively modulates the activity of the human ABCG2 multidrug transporter

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Abstract

The human ABCG2 multidrug transporter provides protection against numerous toxic compounds and causes multidrug resistance in cancer. Here we examined the effects of changes in membrane cholesterol on the function of this protein. Human ABCG2 was expressed in mammalian and in Sf9 insect cells, and membrane cholesterol depletion or enrichment was achieved by preincubation with beta cyclodextrin or its cholesterol-loaded form. We found that mild cholesterol depletion of intact mammalian cells inhibited ABCG2-dependent dye and drug extrusion in a reversible fashion, while the membrane localization of the transporter protein was unchanged. Cholesterol enrichment of cholesterol-poor Sf9 cell membrane vesicles greatly increased ABCG2-driven substrate uptake, substrate-stimulated ATPase activity, as well as the formation of a catalytic cycle intermediate (nucleotide trapping). Interestingly, modulation of membrane cholesterol did not significantly affect the function of the R482G or R482T substrate mutant ABCG2 variants, or that of the MDR1 transporter. The selective, major effect of membrane cholesterol on the wild-type ABCG2 suggests a regulation of the activity of this multidrug transporter during processing or in membrane micro-domain interactions. The experimental recognition of physiological and pharmacological substrates of ABCG2, as well as the fight against cancer multidrug resistance may be facilitated by demonstrating the key role of membrane cholesterol in this transport activity.

Keywords: ABC transporters; ABCG2 transport activity; Multidrug resistance; Membrane cholesterol; ATPase activity

1. Introduction

ABCG2 is a member of the ABC multidrug resistance protein family, a plasma membrane glycoprotein, present in many human tissues, including the placenta, liver, kidney, and intestine. The tissue distribution of ABCG2 suggests that its main physiological role is the regulation of intestinal absorption and biliary secretion of potentially toxic xenobiotics. ABCG2 may also be a protective element in the maternal–fetus and in the blood–brain barrier, and the protein is present at high levels in stem cells, currently with an unknown physiological function [1–5].

ABCG2 is also used by tumor cells to fight anticancer medicines, as ABCG2 overexpression provides an active protection against a number of chemotherapeutic drugs. ABCG2 expression was found in various leukemias, in the tumors of the ovary, lung, breast, colon and gastric cancer. The anticancer cytotoxic compounds extruded by ABCG2 include mitoxantrone, topotecan, camptothecin, irinotecan, flavopiridol, as well as methotrexate and its polyglutamated metabolites [1,3,6–8].

ABCG2 is an ABC "half-transporter", which requires homodimerization for its transport function [9]. Similarly to all ABC multidrug transporters, drug extrusion by ABCG2 is closely coupled to a drug-stimulated, vanadate-sensitive ATPase activity, which requires the presence of Mg^{2+} ions [9,10]. During its substrate transport and ATP hydrolytic cycle ABCG2 occludes

Abbreviations: ABC transporters, ATP binding cassette transporters; CD, cyclodextrin; C-CD, cyclodextrin loaded with cholesterol; S-CD, cyclodextrin loaded with sitosterol; EKI, EKI-785 tyrosine kinase inhibitor; ESG, estradiol 17-beta glucuronide; E3S, estrone 3-sulfate; MDR1, multidrug resistance protein 1; MRP1, multidrug resistance associated protein 1; MTX, methotrexate; PheA, Pheophorbide A; R123, Rhodamine 123

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ADP, and this catalytic intermediate can be "trapped" in the presence of vanadate. The direct, ATP-dependent transport of several substrates of ABCG2, including methotrexate, glucuronidated or sulfated estrogens and xenobiotics, has been demonstrated [11–14].

The R482G and R482T mutant variants of ABCG2, found only in drug-selected tumor cells, show a significantly altered drug resistance pattern, as compared to the wild-type protein. These mutants efficiently transport various anthracycline derivatives or Rhodamine 123, while the wild-type ABCG2 shows very little transport activity for these compounds. In contrast, the R482G and R482T variants practically do not transport methotrexate or drug conjugates [14–17]. There are several polymorphic variants of ABCG2 present in large percentage in the human population (e.g. V12M, Q141K), and the possible alterations in the transport capacity and substrate handling of these variants have been examined in numerous experimental systems [18–24].

The functional regulation of the ABCG2 transport activity is relatively unexplored as yet. There are several data for the modulation of ABCG2 transcription during cell differentiation, cytotoxic exposure, or hypoxia, as well as after exposure to hormones or hormone derivatives [25–27]. It has also been proposed that estrogen may have a post-translational regulatory effect by altering the biosynthesis and maturation of ABCG2 [28]. Membrane cholesterol has been implicated in the regulation of many membrane proteins, including several ABC transporters, and membrane micro-domains with increased cholesterol content have been suggested to play a role in such a regulation [29,30]. In the case of ABCG2 one study found a significant cholesterol stimulation of its ATPase activity, when the protein was expressed in *Lactococcus* bacteria [31]. The authors suggested that ABCG2 may be directly involved in cholesterol transport. However, no detailed studies are available as yet for the transport and ATPase modulation of ABCG2 activity by cholesterol in eukaryote model systems.

In the present experiments we have examined the effects of membrane cholesterol modulation on the function of the human ABCG2 protein. We followed the function and localization of this transporter in intact mammalian cells, depleted from, or loaded with cholesterol. We also studied the direct transport and ATPase activity, as well as the formation of a catalytic intermediate of the human ABCG2, expressed in recombinant baculovirus-infected Sf9 cells. Sf9 cell membranes contain significantly lower cholesterol than mammalian cell membranes [32] and proteins sensitive to membrane cholesterol have different activities when expressed in this system [33,34]. Thus Sf9 cell membrane preparations provide an excellent opportunity to directly explore the effects of increasing membrane cholesterol on the characteristics of a given mammalian membrane protein.

Our experiments show a major effect of membrane cholesterol on the activity of ABCG2. We found that cholesterol depletion strongly inhibited ABCG2-dependent drug/dye extrusion in intact mammalian cells in a reversible fashion, while cellular cholesterol loading increased this transport activity. There was no effect of cholesterol loading or depletion on the general membrane localization of ABCG2 in these short-term experiments. In isolated Sf9 cell membranes cholesterol loading greatly and specifically increased the maximum transport capacity and ATPase activity of ABCG2, and increased the rate of the formation of a catalytic intermediate. Interestingly, membrane cholesterol modulation under the same conditions had only a negligible effect on the activity of ABCG2–R482G and ABCG2–R482T mutant variants, or that of the MDR1 multidrug transporter. These data indicate a significant, selective, and reversible modulation of ABCG2 activity by membrane cholesterol.

2. Materials and methods

2.1. Cell and membrane preparations

Mammalian HEK and A431 cells, selectively overexpressing the human ABCG2 or its mutant variants, were prepared either by transfection and selection as described in [23], or by retroviral transduction as described in [35]. For Sf9 cell expression, cDNAs of human ABCG2 and its mutant variants were cloned into recombinant baculovirus transfer vectors, the insect cells were cultured, and infected with the baculoviruses as described in [14]. Virus-infected Sf9 cells were harvested, cell membranes were isolated, and membrane protein concentrations were determined as described previously [9,14,36]. The membrane preparation was optimized to yield high amount of inside-out vesicles in these preparations, and the constant amount of these vesicles was assured by measuring endogenous calcium transport activity, as described in refs. [14,17].

The level of ABCG2 expression was detected by immunoblotting, using the BXP-21 monoclonal antibody and the enhanced chemiluminescence technique (ECL, Amersham Biosciences). Quantitation of ABCG2 expression was achieved by densitometry of the immunoblots.

2.2. Cholesterol loading and depletion

For the modulation of membrane cholesterol content in mammalian cells, we used cyclodextrin pretreatments. In case of intact cells (see Figure legends) 10^6–10^7 suspended cells were preincubated at 37 °C for 20–30 min in 1 ml serum-free media, containing 2–4 mM of random methylated beta (RAMEB) cyclodextrin (CD, CycloLab), either without lipids or loaded with cholesterol (C-CD, cholesterol content 4.4%). The cells were washed to remove CD or free cholesterol and used for further transport experiments within 1 h. Cell viability was tested by trypan-blue exclusion or propidium-iodide staining, indicating that CD or C-CD treatments did not significantly increase the number of dead cells. Cholesterol content of the cells was measured by the enzymatic (Molecular Probes, Amplex Red) cholesterol assay kit.

For cholesterol loading of Sf9 cell membranes, we used two different techniques. In experiments shown in Fig. 3A, isolated membranes were prepared as described in [17], and then preincubated for 10 min at 4 °C with the indicated concentrations of CD–lipid complexes, including cholesterol-CD (cholesterol content 4.4%), sitosterol-CD (sitosterol content 1.9%), ergosterol-CD (ergosterol content 2%), or hydrocortisone–CD (hydrocortisone content 10.2%), prepared by CycloLab technology. The following transport or ATPase experiments were then performed by directly using these membrane preparations. In most experiments, during the course of the membrane preparation, before the final centrifugation step, the isolated membranes were incubated for 20 min at 4 °C with 2–4 mM of various RAMEB cyclodextrin preparations, and then cyclodextrin complexes were eliminated by a 20× dilution and a following high speed (100,000×*g*) centrifugation. Membrane preparations were stored at −80 °C in aliquots and cholesterol content was estimated by the Amplex red kit, described above.

2.3. ABCG2 activity measurements

In intact mammalian cells ABCG2 activity was measured in various assay systems. Hoechst 33342 dye uptake in a fluorescence spectrophotometer was

followed as described in [17]. Dye transport activity factor was calculated as described [17,37], that is the initial uptake rate (without inhibitor) was subtracted from the rate measured in the presence of a specific inhibitor, and divided by the uptake rate measured with the inhibitor. Mitoxantrone (MX) or Pheophorbide A (PheA) uptake was measured by flow cytometry, essentially as described in [38]. PheA uptake was followed for 12 min after the addition of 0.5 μM PheA, either in the absence or in the presence of 1 μM Ko143. Cellular PheA fluorescence was determined at excitation and emission wavelengths of 635 and 661 nm, respectively, in a FACSCalibur cytometer. Dead cells were excluded on the basis of propidium iodide staining.

In isolated Sf9 cell membranes *vanadate-sensitive ATPase activity* was measured as described in [39], using 20 min incubation times at 37 °C. MgATP-dependent uptake of ^{3}H-labeled methotrexate (MTX) and ^{3}H-labeled estradiol-17-beta glucuronide (ESG) in inside-out membrane vesicles was measured by a rapid filtration method as described [14,40], using 5-min incubations at 37 °C. We ensured that during this time period the rate of vesicular substrate uptake was linear, allowing to estimate the kinetic values. ATP-dependent transport activity of ABCG2 was determined by subtracting the uptake measured in the presence of MgAMP. MgATP-dependent transport by ABCG2 was fully inhibited by Ko143 (see Figures). MgATP-dependent Rhodamine 123 uptake by Sf9 membrane vesicles was measured by flow cytometry, by using the parameters described for Rhodamine fluorescence [14], and selecting the vesicle population based on FSC/SSC parameters. R123 uptake was followed in the presence of 1 μM R123, by taking 30 second time points for 5 min either at 37 °C or 22 °C. Again, the addition of MgAMP and Ko143 served as negative controls.

Formation of the *catalytic intermediate* ("nucleotide trapping") by the ABCG2 protein was measured as described in [17]. In brief, control or cholesterol-loaded Sf9 membranes (150 μg/assay) were incubated with 2.5 μM Co-8-azido-ATP (containing alpha-^{32}P-8-azido-ATP) for 2 or 5 min, then washed with ATP-containing media and UV-irradiated on ice. After gel-electrophoresis and electroblotting the labeled bands were visualized by Phospho-Imager and identified by immunoblotting, using the BXP-21 anti-ABCG2 antibody, as well as by Coomassie staining.

2.4. Confocal microscopy

HEK cells stably transfected with ABCG2 (wt) or ABCG2–R482G were seeded onto eight-well Nunc Lab-Tek II Chambered Coverglass (Nalge Nunc International, Rochester, NY) at 3×10^4 per well cell density, and grown for 48 h in D-MEM containing 10% FCS. Prior to the microscopy studies, the cultures were incubated with serum-free D-MEM containing 2.5 mM CD or 2.5 mM cholesterol-CD, for 20 min at 37 °C. For cholesterol repletion, the cells were first subjected to 2.5 mM CD for 20 min, then to 2.5 mM C-CD for an additional 20 min at 37 °C. Following the preincubation, the cells were gently washed, placed into serum free D-MEM, and studied by an Olympus FV500-IX confocal laser scanning microscope using an Olympus PLAPO 60× (1.4) oil immersion objective (Olympus Europa GmbH, Hamburg, Germany) at room temperature. The blue fluorescence was acquired at 405 nm excitation. The samples were subjected to 2 μM Hoechst dye, and after a 120 sec incubation time, 1 μM Ko143 was added to the medium. The activity factor was determined from the steady state fluorescence accumulation rates before and after Ko143 addition (see Fig. 1B).

For *immunofluorescence* studies the cells were seeded, cultured and preincubated with CD or C-CD as described above. For cell surface labeling, the cells were gently washed with Dulbecco's modified PBS (DPBS), and fixed with 1% paraformaldehyde in DPBS for 15 min at room temperature, and then blocked for 1 hr at room temperature in DPBS containing 0.5% bovine serum albumin. The samples were then incubated for 1 h at room temperature with the monoclonal anti-ABCG2 antibody, 5D3, conjugated with allophycocyanin (APC), diluted 5× in DPBS containing 0.5% BSA, finally washed with DPBS. For immunostaining of permeabilized cells, the CD- or C-CD pretreated samples were gently washed, and fixed with 4% paraformaldehyde in DPBS for 15 min at room temperature. After few washes with DPBS, the cells were further fixed and permeabilized in pre-chilled methanol for 5 min at −20 °C. Following further washing steps, the cells were blocked for 1 hr at room temperature in DPBS containing 2% bovine serum albumin, 1% fish gelatin, 0.1% Triton-X 100, and 5% goat serum (blocking buffer). The samples were then incubated for 1 hr at room temperature with the monoclonal anti-ABCG2, BXP-21 diluted 100× in blocking buffer. After washing with DPBS, the cells were incubated for 1 hr at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) diluted 250× in blocking buffer. As isotype controls, APC-conjugated mouse IgG2b (5 μg/ml) and mouse IgG2a (2.5 μg/ml) plus Alexa Fluor 488-conjugated goat anti-mouse IgG (1:250) were used. The APC-conjugated 5D3, the BXP-21, and the anti-mouse secondary antibodies were obtained from R&D

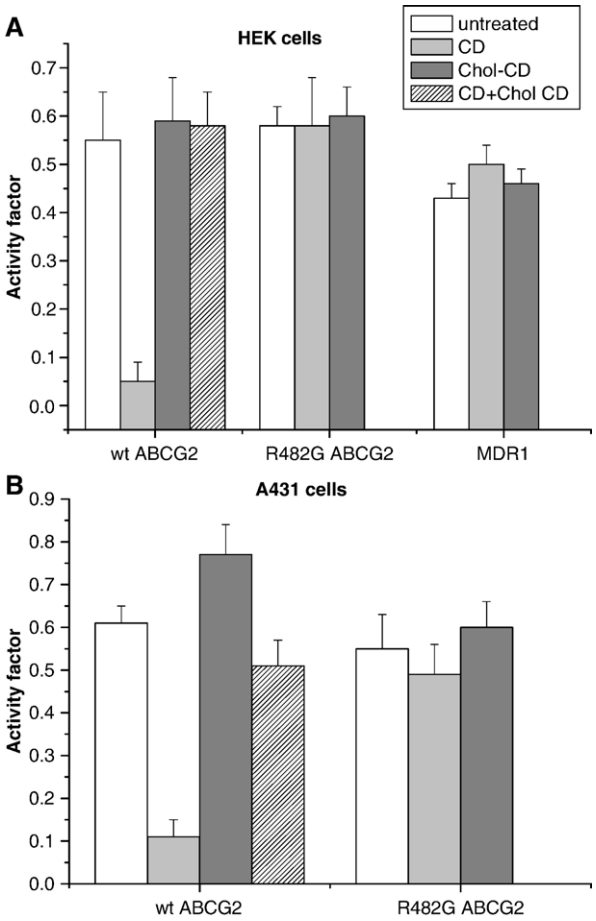


Fig. 1. Effect of cholesterol depletion or cholesterol loading on the activity of ABCG2 in intact HEK and A431 cells. Panel A. Effect of cyclodextrin (CD) or cholesterol-cyclodextrin (Chol-CD) pre-treatment on the Hoechst dye uptake activity factor in HEK cells, as measured in a fluorescence spectrophotometry assay. HEK cells, expressing the human wild-type ABCG2, the ABCG2–R482G variant, or the MDR1 protein, were pretreated in HPMI medium by 4 mM CD or 4 mM C-CD for 30 min at 37 °C, then washed twice to eliminate cyclodextrin. Hoechst dye (2 μM) accumulation was continuously measured at 37 °C in HPMI medium in a fluorescence spectrophotometer for 8 min, then 1 μM Ko143 (to the ABCG2 expressing cells) or 30 μM verapamil (to the MDR1 expressing cells) was added, and Hoechst fluorescence was measured for another 8 min. Dye extrusion activity factor was calculated based on the difference between the control uptake rate and that in the presence of inhibitor, as described in Materials and methods. The mean values±SD are presented. Empty columns: untreated cells, light gray columns: cyclodextrin (CD) pretreated cells, dark gray columns: cholesterol-cyclodextrin (C-CD) pretreated cells, striped columns: cyclodextrin (CD) pretreated, and then cholesterol repleated cells by cholesterol-cyclodextrin (C-CD) treatment. Panel B. Effect of cyclodextrin (CD) or cholesterol-cyclodextrin (Chol-CD) pre-treatment on the Hoechst dye uptake activity factor in A431 cells, as measured in a fluorescence spectrophotometry assay. The A431 cells were treated and dye accumulation measured as described for panel A. Empty columns: untreated cells, light gray columns: cyclodextrin (CD) pretreated cells, dark gray columns: cholesterol-cyclodextrin (C-CD) pretreated cells, striped columns: cyclodextrin (CD) pretreated, and then cholesterol repleated cells by cholesterol-cyclodextrin (C-CD) treatment.

Systems (Minneapolis, USA), Axxora/Alexis (Lausen, Switzerland), and Molecular Probes (Eugene, USA), respectively. The APC-conjugated mouse IgG2b, and the mouse IgG2a isotype controls were purchased from eBioscience (San Diego, USA) and DAKO Cytomation (Glostrup, Denmark), respectively. The stained samples were studied with confocal microscope specified above. The green and deep red fluorescence were acquired above 505 nm and 650 nm, using 488 nm and 633 nm excitations, respectively.

All documented measurements were carried out in at least two independent membrane preparations, at least in triplicates.

3. Results

3.1. Experiments with intact mammalian cells

For modulation of membrane cholesterol in intact mammalian cells, we applied mild treatments with beta cyclodextrin (CD), or with its cholesterol-loaded form (C-CD). Cyclodextrin complexes have been shown to be rapidly and effectively transferring cholesterol to and from cells and biological membranes [41,42].

In the experiments documented in Fig. 1A, we examined the effect of cholesterol depletion or cholesterol loading, respectively, on fluorescent dye extrusion from ABCG2-expressing intact HEK cells. The Hoechst 33342 dye is a good substrate of both the wild-type and the R482G or R482T mutant variants of ABCG2 [1,3], as well as of the MDR1 transporter [4]. This dye becomes fluorescent only inside the cells, when bound to DNA, thus increase in fluorescence directly reflects dye accumulation. In control cells this uptake is fast and not influenced by the addition of transporter inhibitors. The expression of ABCG2, its R482G mutant form, as well as MDR1 strongly decrease Hoechst dye accumulation. In the case of the addition of a specific inhibitor there is a major increase in rate of dye accumulation, reaching the level seen without transporter overexpression. As specific inhibitors, in the case of ABCG2 we used 1 μM Ko143, while in the case of MDR1 we applied 30 μM verapamil. The activity factor calculations (see Methods and 17) provide a quantitative estimate of the transporter activity in intact cells. As shown below in the isolated membrane experiments (see Fig. 3), the applied Ko143 concentration resulted in a full inhibition of ABCG2, independent of the membrane cholesterol concentrations.

Fig. 1 shows the effect of cholesterol depletion or loading on the activity factors calculated for Hoechst 33342 dye extrusion at 37 °C in ABCG2-expressing HEK (Panel A) and A431 cells (Panel B), respectively, measured in a fluorescence spectrophotometer. For demonstrating the effect of cholesterol we chose here cells expressing medium levels of the transporters, thus having a Hoechst transport activity factor between 0.4 and 0.7.

As documented in Fig. 1A, cholesterol depletion of HEK cells by 4 mM CD for 30 min at 37 °C practically eliminated Hoechst dye extrusion by the wild-type ABCG2 (the activity factor decreased from 0.54 to 0.05), while this treatment had no effect on dye extrusion in ABCG2–R482G expressing, or MDR1 expressing HEK cells. The effect of additional cholesterol loading by C-CD was not significant in these experiments in any of the above cell types.

It is also important to note, that the applied cholesterol depletion or cholesterol loading did not significantly affect the rate of Hoechst 33342 dye accumulation in the control HEK or A431 (see below) cells. Also, as measured by trypan-blue or propidium iodide exclusion, this mild pre-treatment did not increase the number of dead cells in the preparation (cell viability was over 85% in each experiment), and preserved the function of the ABCG2 mutant variants or other ABC-multidrug transporters (see also below).

Based on the enzymatic cholesterol measurements (see Methods), the original total cholesterol content in the HEK cells used in this study was 7.71 ± 1.1 μg/mg total protein, and this cholesterol content could be decreased by about 25%, to 5.71 ± 0.9 μg/mg total protein by 4 mM CD preincubation, or increased to 12 ± 1.2 μg/mg total protein, that is to about 150%, by 4 mM C-CD incubation.

When examining the possible reversibility of cholesterol depletion, we depleted HEK-ABCG2 cells from cholesterol for 30 min, as described above, and then after one washing step further incubated these cells for 20 min in a serum-free medium, or in a medium containing 4 mM Cholesterol-CD. When measuring Hoechst dye uptake in these cells we found that the original activity factor of 0.54 ± 0.06 decreased to 0.05 ± 0.04 during CD treatment, and the activity factor was increased to 0.58 ± 0.07 after cholesterol reloading. Thus, the modulation of ABCG2 transport activity by cholesterol was fully reversible.

As shown in Fig 1B, we have also performed Hoechst dye uptake experiments in ABCG2-expressing A431 tumor cells, overexpressing ABCG2 after a retroviral transduction [35]. In these cells the results were essentially similar to those obtained in HEK cells. The activity factor in the ABCG2 expressing A431 cells decreased to very low levels after 30 min cholesterol depletion, and increased to slightly above the control values after cholesterol loading. Cholesterol repletion of cholesterol-depleted cells restored the activity factor to the level of the control cells. Thus cholesterol modulation of ABCG2 transport activity in both HEK and A431 cells was also fully reversible and selective for the wild-type protein.

In experiments not documented here in detail, we have repeated these cellular transport experiments by measuring the uptake of mitoxantrone (MX) and Pheophorbide A (PheA), both transported compounds for both ABCG2 and its R482G variant, by using flow cytometry. We found that after cholesterol depletion the HEK cells expressing the wild-type ABCG2 had a significantly lower extrusion capacity, while cholesterol depletion had no effect on MX or PheA extrusion in HEK cells expressing the R482 mutant variant.

In order to demonstrate the major effect of membrane cholesterol on the ABCG2-dependent Hoechst dye transport in intact cells, we performed similar experiments in a real-time microscopic transport assay system, by using confocal microscopy. Cellular Hoechst dye uptake was directly, *in situ* followed under the microscope, and the changes in the rate of dye uptake were analyzed by determining the mean fluorescence in a selected number [10–12] of cell nuclei.

As shown in Fig. 2A, left panel, after the Hoechst dye addition, ABCG2-expressing HEK cells showed a low level of

nuclear staining, which was greatly increased upon the addition of the ABCG2 inhibitor, Ko143. CD-pretreatment of the attached HEK cells significantly increased Hoechst dye uptake, while C-CD pretreatment rather decreased this accumulation. The actual dye accumulation rate was determined in a 60- to 120-s steady state uptake period, before and after the addition of Ko143 (see Fig. 2A, upper right panel), and the activity factors were calculated. The lower right panel shows that the activity factor was significantly decreased in the CD-treated, while increased in the C-CD treated cells. Moreover, the decrease in activity factor caused by CD treatment was fully reversible upon a following C-CD incubation. Again, dye extrusion activity by the ABCG2–R482G variant was practically unaffected by CD or C-CD treatment. These experiments confirm that Hoechst 33342 dye extrusion in HEK cells, expressing the wild-type ABCG2, is strongly modulated by membrane cholesterol, while Hoechst transport by the ABCG2–R482G variant is not affected by this membrane lipid. Cell viability was unchanged in these studies—when measured by propidium iodide exclusion, viability was above 85% in all samples examined.

In order to examine the possible changes in ABCG2 localization during cholesterol depletion, we have performed immunostaining of the HEK cells by using confocal microscopy. We used both a cell-surface reactive anti-ABCG2 monoclonal antibody (5D3) [43], and another monoclonal antibody (BXP21) [44], recognizing an intracellular epitope of ABCG2. As documented in Fig. 2B, both antibodies detected exclusive plasma membrane staining, both in the control, and in the CD-treated, cholesterol-depleted cells. A similar picture was observed in the cholesterol repleted HEK cells as well (not shown).

These experiments strongly suggested that in intact cells membrane cholesterol has a major effect on the wild-type ABCG2 protein, while the morphology or the basic functions of the cells were unaltered under the mild cholesterol depletion applied. Moreover, the ATP-dependent transport by ABCG2-R482G or MDR1 was also not significantly affected under these conditions.

3.2. Experiments with isolated Sf9 cell membranes

In order to explore the molecular details of the cholesterol effects observed in intact cells, ABCG2 and its R482G, R482T mutant variants were expressed in Sf9 cells. Sf9 cells, as compared to mammalian cells, contain low level of endogenous membrane cholesterol, thus in this system a direct effect of cholesterol loading can be better examined [32–34]. Sf9 cells were engineered to express high amounts of the human ABCG2 variants, at about equal transporter protein levels [14]. Moreover, in isolated membranes of Sf9 cells both direct vesicular transport, drug-stimulated ATPase activity and catalytic intermediate formation (nucleotide trapping) could be examined [17].

For cholesterol loading of Sf9 cell membranes we applied a preincubation of the membranes at 4 °C with sterol-containing beta cyclodextrin, followed by a removal of this agent during the further centrifugation steps. The original membrane cholesterol content in our Sf9 cell membrane preparations was between 5 and 8 μg/mg membrane protein, and with 1–5 mM cholesterol-cyclodextrin (C-CD) preincubation this cholesterol content could be gradually increased up to 60–80 μg cholesterol/mg membrane protein. As a comparison, we found that the cholesterol content of the HEK or MCF7 human cell membrane preparations was between 30 and 40 μg/mg membrane protein, irrespective of the presence or absence of ABCG2 expression, while isolated red cell membranes contain up to 100 μg cholesterol/mg protein [45].

3.3. Vesicular transport studies

We carried out direct, ATP-dependent substrate transport measurements by using isolated inside-out membrane vesicles of ABCG2-expressing Sf9 cells [14,17]. In the first set of experiments we studied the effects of various sterol–CD complexes directly added to the membrane vesicles in a short preincubation period at 4 °C (see Materials and methods). Methotrexate (MTX) transport activity of the wild-type ABCG2 was measured at 100 μM MTX concentration, by the addition of 5 mM ATP in a 5 min incubation period at 37 °C. To correct for any endogenous MTX transport activity, ABCG2-dependent active MTX uptake was calculated by subtracting MTX uptake measured in the presence of 1 μM Ko143, fully inhibiting ABCG2.

As documented in Fig. 3A, the addition of 1–4 mM CD did not have any significant effect of MTX transport activity. However, Cholesterol-CD greatly increased ABCG2-dependent MTX uptake in a concentration-dependent manner (we found a maximum effect at 4 mM C-CD, data not shown in detail). There was no such transport increase in the presence of 1-4 mM sitosterol-CD or hydrocortisone-CD, while ergosterol-CD actually slightly decreased ABCG2-dependent MTX uptake. It has been shown earlier that all these sterol-CD complexes allow a rapid exchange of sterol molecules with hydrophobic binding materials [41].

In order to further investigate the effect of cholesterol loading of Sf9 cell membranes, the following experiments were performed by C-CD pretreated, cholesterol preloaded isolated membrane preparations. In these experiments the preloaded membranes, with stable membrane sterol content, could be characterized in detail. As shown in Fig. 3B, preloading of the membranes with cholesterol (C-CD) greatly increased the MTX transport activity of the wild-type ABCG2, to yield a 15–20 fold increase, when the initial membrane cholesterol was elevated to 65 μg cholesterol/mg membrane protein. We found a similar large increase in the ABCG2-dependent uptake of estradiol beta-17-glucuronide (ESG—see below), and of estrone 3-sulfate (E3S—not shown) in the Sf9 membrane vesicles after cholesterol loading. We found that pretreatment with free cyclodextrin (CD) did not significantly affect either the MTX (see Fig. 3), ESG or E3G transport activity of the human ABCG2 protein. Also, when we applied sitosterol (S) loaded CD (Fig. 3B—part 4) we found no effect on the ABCG2-dependent transport activity. Ko143, a specific inhibitor of ABCG2, abolished substrate transport both in the control and cholesterol-loaded membrane vesicles.

The R482G or R482T variants of ABCG2 have significantly different substrate handling properties than the wild-type protein. These mutant variants transport certain negatively charged compounds, e.g. MTX, ESG or E3S only with a very low activity [17–21]. As shown in Fig. 3B, MTX transport by the ABCG2–R482G variant was very low both in the control

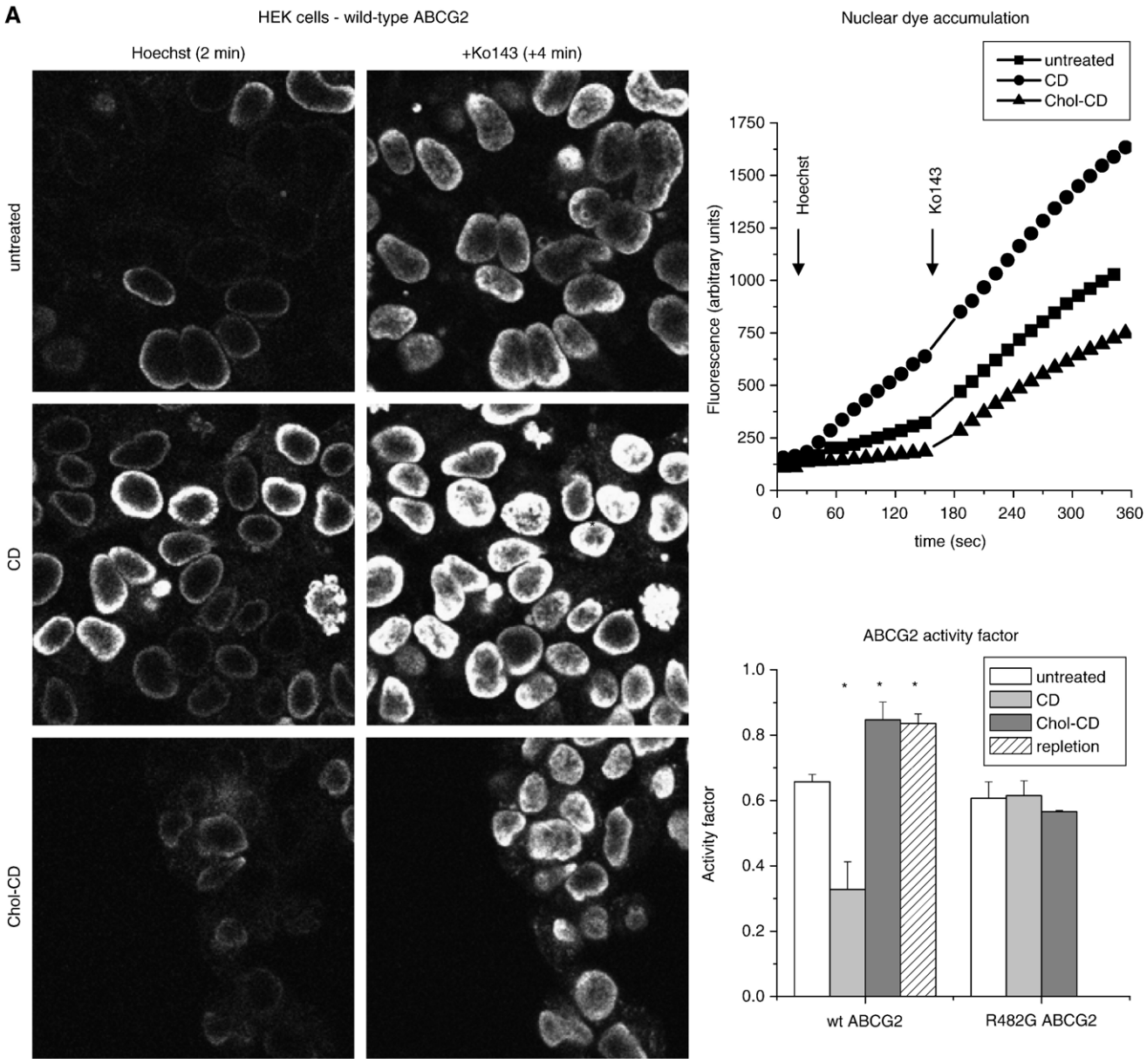


Fig. 2. Confocal microscopy studies on the effect of membrane cholesterol on Hoechst dye uptake and ABCG2 localization in ABCG2-expressing HEK cells. (A) Effect of cyclodextrin (CD) or cholesterol-cyclodextrin (Chol-CD) pre-treatment on the Hoechst dye uptake in HEK cells, as followed in a confocal microscopy assay. Cholesterol depletion or repletion by CD or C-CD was carried out in the surface attached HEK cells by 2.5 mM CD or 2.5 mM C-CD for 20 min at 37 °C, in serum free D-MEM medium, as described in Materials and methods. Hoechst dye accumulation was measured in cells expressing the human wild-type ABCG2 or the ABCG2–R482G variant at 37 °C, and cellular dye content was estimated based on the fluorescence in selected regions of interests. Activity factor, based on the difference between the control uptake rate and that in the presence of 1 μM Ko143, was calculated as described in Materials and methods. Left panel: confocal microscopy picture of HEK cells after 2 min of the Hoechst dye addition, and after an additional 4 min, following the addition of the ABCG2 inhibitor, Ko143. Upper right panel: nuclear Hoechst dye accumulation averaged in 12 cells during the experiment described above. ■—Hoechst dye uptake in the control, untreated HEK cells, expressing the wild-type ABCG2 transporter, ●—Hoechst dye uptake in cyclodextrin (CD) pretreated HEK cells, expressing the wild-type ABCG2 transporter, ▲—Hoechst dye uptake in cholesterol-cyclodextrin (C-CD) pretreated HEK cells, expressing the wild-type ABCG2 transporter. Lower right panel: the calculated ABCG2 activity factors based on the average values obtained in at least 3 independent experiments. Empty columns: untreated HEK cells, light gray columns: cyclodextrin (CD) pretreated HEK cells, dark gray columns: cholesterol-cyclodextrin (C-CD) pretreated HEK cells, striped columns: cyclodextrin (CD) pretreated HEK cells, after a second treatment with cholesterol-cyclodextrin (C-CD—see Materials and methods). The mean values ± SD are presented, the stars indicate significant differences. (B) Effect of cyclodextrin (CD) pre-treatment on the immunolocalization of ABCG2 protein in HEK cells, followed by confocal microscopy. Cholesterol depletion by CD was carried out as described for panel A. Untreated control, or cholesterol-depleted (CD) ABCG2-expressing HEK cells were fixed and immunostained as described in Materials and methods, by the 5D3 monoclonal antibody, reacting with an extracellular epitope, or by BXP21, reacting with an intracellular epitope of ABCG2.

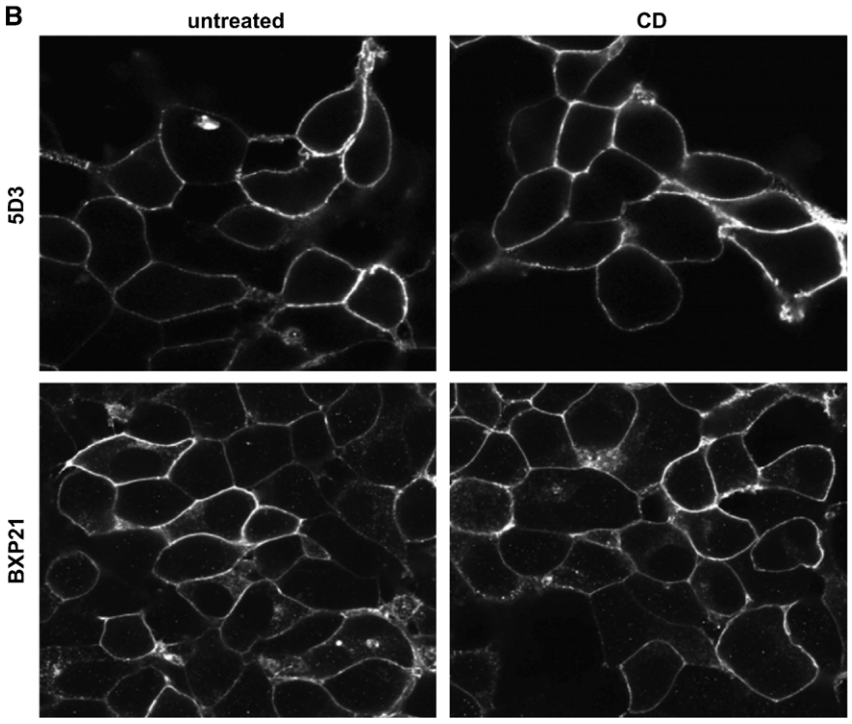


Fig. 2 (*continued*).

and in the cholesterol-loaded Sf9 membrane vesicles. Similarly, the MTX transport rate by the ABCG2–R482T variant was below 50 pmol/mg membrane protein/min, irrespective of the membrane cholesterol content. We found a similar lack of significant ESG and E3S transport by the R482G and R482T variants, irrespective of the cholesterol content of the membrane vesicles (not shown).

It is important to note that we found little effect of sterol loading or CD treatment on the endogenous ATP-dependent calcium transport activity, used to estimate the vesicle content of the Sf9 membrane preparations [17,40]. In the transport experiments documented in this manuscript, CD or sterol-CD treatment of the vesicles before the actual transport experiment did not affect, while preloading with CD-cholesterol during membrane preparation decreased the relative membrane vesicular content by 20–30%, as measured by endogenous calcium transport activity. Thus in these experiments the increase in the ABCG2 transport activity is probably underestimated, as we did not perform a correction of the data based on the calcium transport activity.

All these experiments suggest that the cholesterol effect was specific for the wild-type ABCG2, and depletion of other lipid constituents of the Sf9 cell membrane (e.g. by unloaded CD), an increase in other related membrane sterols, or non-specific permeability changes caused by various CD complexes could not be accounted for the observed effects.

In the following experiments we examined how cholesterol loading affected the kinetic parameters of MTX and ESG transport by ABCG2. Fig. 4A shows the MTX concentration dependence of the ABCG2-dependent MTX uptake in control, and cholesterol-loaded Sf9 membrane vesicles, respectively. In the original, untreated Sf9 cell membrane vesicles (containing 6–8 μg cholesterol/mg membrane protein) the rate of MTX uptake was low, with an apparent Vmax of about 0.5 nmol MTX/mg membrane protein/min. In contrast, in the C-CD pretreated vesicles (in this experiment containing 55 μg cholesterol/mg membrane protein), MTX uptake had an estimated Vmax of about 10 nmol MTX/mg membrane protein/min. The apparent Km of MTX uptake was about 0.5 mM in both cases, but the proper determination of the Km and Vmax values in these experiments was hindered by the low solubility of MTX at higher than 3 mM concentrations. Fig. 4A also documents that the R482G variant of ABCG2 had a very low MTX transport activity, irrespective of the MTX concentrations examined.

When we examined the effect of membrane cholesterol on the ATP concentration dependence of the MTX uptake in the ABCG2-containing Sf9 membrane vesicles we found that, irrespective of the membrane cholesterol content, MTX transport had a saturable ATP-dependence, with an apparent Km of 0.6–0.8 mM ATP and a maximum transport rate at about 5 mM ATP. These values are in agreement with the data in the literature for the ATP-dependence of vesicular transport by ABCG2 [17,21–23]. Again, neither the R482G, nor the R482T variants showed any MTX transport activity, irrespective of the ATP concentration or the cholesterol content of the Sf9 cell membrane vesicles.

In the following experiments we examined the ESG concentration dependence of ATP-dependent ESG uptake in Sf9 membrane vesicles (Fig. 4B). Similarly to that seen for MTX, in the cholesterol-loaded vesicles this transport showed simple saturation kinetics, with an apparent Vmax of 700 pmol

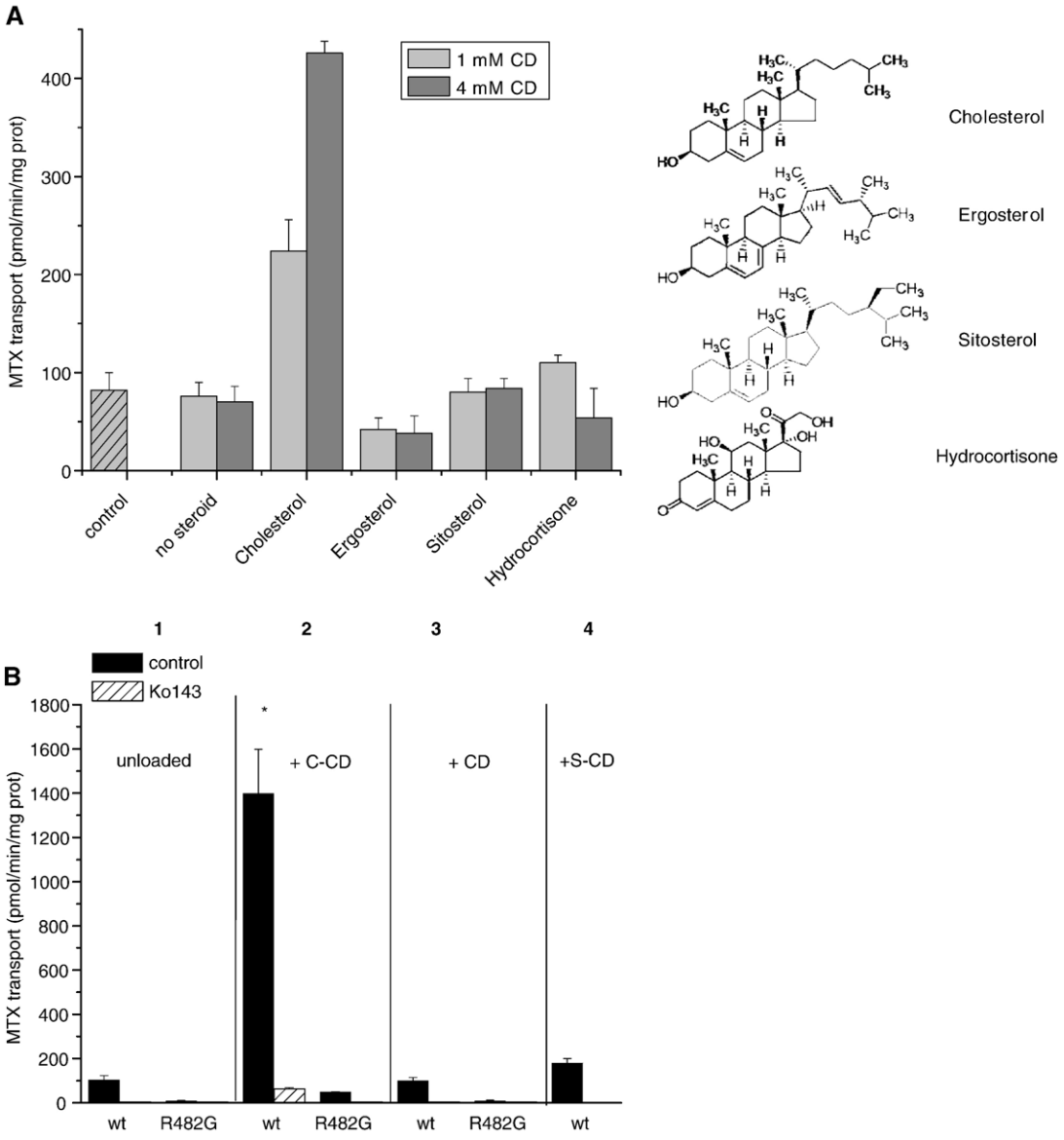
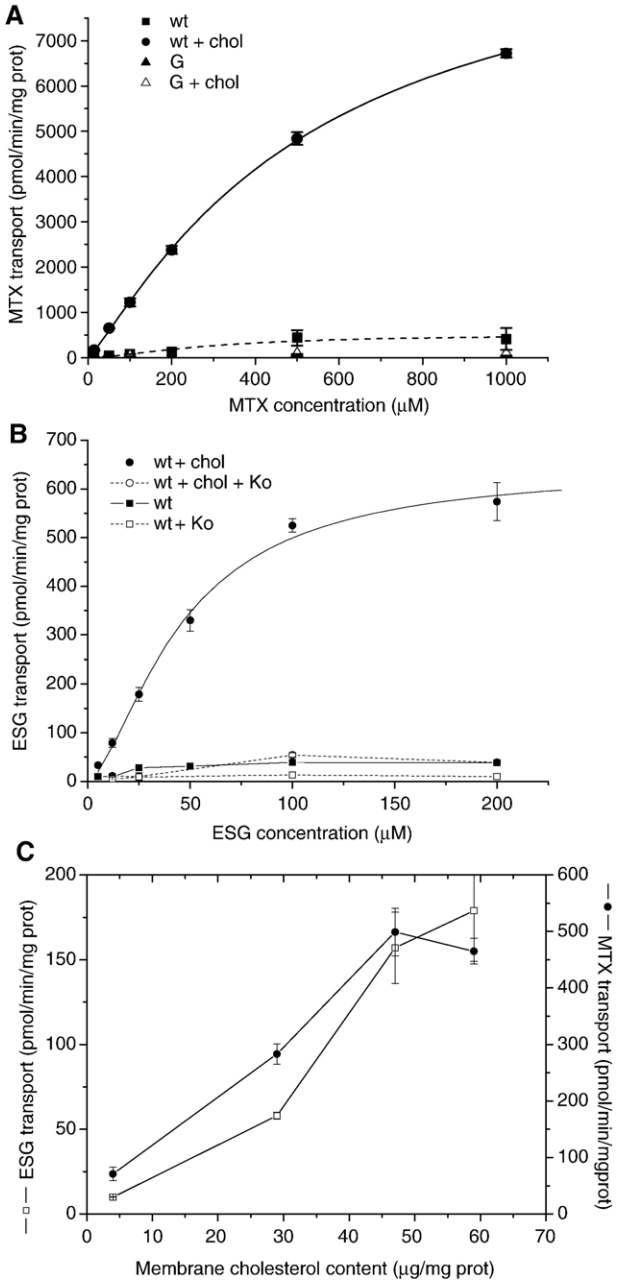


Fig. 3. (A) Effect of CD and sterol-CD complexes on the ATP-dependent transport of MTX in inside-out Sf9 membrane vesicles. Isolated membranes were preincubated for 10 min at 4 °C with CD or with the indicated concentrations of CD–lipid complexes, including cholesterol-CD, sitosterol-CD, ergosterol-CD, or hydrocortisone-CD. MTX uptake was measured at 100 μM MTX concentration for 5 min at 37 °C in membrane vesicles containing the human wild-type ABCG2 (WT), or R482G–ABCG2 (G) transporter. ABCG2-dependent transport was calculated by subtracting MTX uptake measured in the presence of 1 μM Ko143. Light gray columns: 1 mM CD or sterol-CD, dark gray columns: 4 mM CD or sterol-CD. The respective structures of the sterols applied are shown in the right panel. (B) Effect of cholesterol and sitosterol loading on ATP-dependent MTX uptake in Sf9 membrane vesicles preloaded with sterol-CD complexes. MTX uptake was measured at 100 μM MTX concentration for 5 min at 37 °C in membrane vesicles containing the human wild-type ABCG2 (WT), or R482G–ABCG2 (G) transporter. Dark columns: vesicular MTX transport without inhibitor, striped columns: MTX transport in the presence of the specific ABCG2 inhibitor, Ko143 (1 μM). Part 1 demonstrates MTX uptake in the unloaded, control vesicles (containing 8 μg cholesterol/mg membrane protein), Part 2 shows MTX uptake in cholesterol-cyclodextrin (C-CD) pre-treated vesicles (containing 56 μg cholesterol/mg membrane protein), and Part 3 shows MTX uptake in empty cyclodextrin (CD) pre-treated vesicles (containing 5 μg cholesterol/mg membrane protein). Part 4 shows the effect of sitosterol loading on ATP-dependent MTX uptake in Sf9 membrane vesicles, when the vesicles were pre-treated with sitosterol-beta-cyclodextrin (S-CD), similarly to the CD or C-CD pre-treatments. The mean values±SD are presented. Significant differences are indicated by stars.

ESG/mg membrane protein/min, while the apparent Km for ESG was about 45 μM. In the control Sf9 membrane vesicles ESG uptake was too low for a proper estimation of Km. An apparent saturation of this uptake was also achieved above 100 μM ESG, with an apparent Vmax of less than 50 pmol ESG/mg membrane protein/min.

These experiments suggest that modulation of the MTX and ESG transport by membrane cholesterol has a predominant effect on the Vmax, that is increasing the substrate transport capacity of this active transporter. Cholesterol may also slightly modulate the substrate affinity and the transporter/substrate interactions, but we need further studies in this respect.

Fig. 4C demonstrates the stimulation of the MTX and ESG transport in inside-out Sf9 cell membrane vesicles by different membrane cholesterol levels. The effect of cholesterol on the vesicular transport was measured at MTX and ESG concentrations (50 μM MTX and 25 μM ESG, respectively) below saturating values. In these experiments pooled membrane preparations, containing the same amount of human ABCG2, but loaded to different cholesterol levels, were applied. Although a slight difference in these activation curves may be observed, the effect of cholesterol on both ABCG2-dependent MTX and ESG transport was maximum above 55 μg cholesterol/mg membrane protein. Thus cholesterol modulation of ABCG2 transport activity was the most pronounced in the range of physiological cholesterol levels in various mammalian cellular membranes (see Discussion).



The experiments shown in Fig. 4 demonstrate that increased membrane cholesterol did not convert the ABCG2–R482G mutant into an efficient MTX or ESG transporter. However, in order to further explore the effect of cholesterol on the substrate specificity of the mutant and wild-type ABCG2, we have also examined Rhodamine 123 (R123) uptake by the Sf9 membrane vesicles. R123 is a transported substrate of the ABCG2–R482G variant, while this compound is practically not transported by the wild-type protein. As shown in Fig. 5, MgATP-dependent, rapid vesicular R123 uptake was well measurable by flow cytometry in Sf9 membrane vesicles, expressing the human R482G–ABCG2 protein. This transport was fully inhibited by 1 μM Ko143. Under the same conditions membrane vesicles containing the wild-type ABCG2 showed no measurable R123 uptake. Also, no such ATP-dependent Rhodamine uptake was observed in the control, beta-galactosidase expressing Sf9 membrane vesicles.

These data indicate that cholesterol loading of the vesicles (in these experiments to 55–62 μg cholesterol/mg membrane protein) did not evoke R123 uptake in the vesicles containing the wild-type ABCG2, while slightly increased (as an average by 25–30%) both the initial rate and the maximum level of the R123 uptake in the R482G–ABCG2 vesicles. This effect was negligible as compared to the cholesterol effect seen on the MTX or ESG transport activity of the wild-type ABCG2 transporter. We have performed these experiments with similar results with three independent membrane preparations, both at 22 °C and 37 °C, and in Fig. 5 we show a representative experiment, performed in triplicates, at 22 °C.

Fig. 4. Effect of cholesterol loading on ATP-dependent MTX and ESG uptake in Sf9 membrane vesicles. MTX and ESG uptake was measured for 5 min at 37 °C at 5 mM ATP in membrane vesicles containing the human wild-type ABCG2 (WT), or R482G–ABCG2 (G) transporter. During this time period drug uptake was linear. The mean values±SD are presented. Panel A MTX concentration dependence of MTX uptake. ABCG2-specific uptake at each substrate concentration was calculated by subtracting the rate obtained in the presence of 1 μM Ko143 ABCG2 inhibitor. ■—MTX uptake in the control vesicles (8 μg cholesterol/mg membrane protein), containing the human wild-type ABCG2 (WT) transporter, ●—MTX uptake in cholesterol-loaded vesicles (56 μg cholesterol/mg membrane protein), containing the human wild-type ABCG2 (WT) transporter, ▲—MTX uptake in the control vesicles (8 μg cholesterol/mg membrane protein), containing the human R482G ABCG2 (G) transporter, △—MTX uptake in cholesterol-loaded vesicles (62 μg cholesterol/mg membrane protein), containing the human R482G ABCG2 (G) transporter. Panel B ESG concentration dependence of ESG uptake. ■—ESG uptake in the control vesicles (8 μg cholesterol/mg membrane protein), containing the human wild-type ABCG2 (WT) transporter, □—ESG uptake in the control vesicles (8 μg cholesterol/mg membrane protein), containing the human wild-type ABCG2 (WT) transporter, in the presence of 1 μM Ko143, ●—ESG uptake in cholesterol-loaded vesicles (56 μg cholesterol/mg membrane protein), containing the human wild-type ABCG2 (WT) transporter, ○—ESG uptake in cholesterol-loaded vesicles (56 μg cholesterol/mg membrane protein), containing the human wild-type ABCG2 (WT) transporter, in the presence of 1 μM Ko143. Panel C Relative stimulation of MTX and ESG transport by cholesterol loading in Sf9 membrane vesicles. MTX uptake (●) was measured at 50 μM MTX concentration, while ESG transport (□) was measured at 25 μM ESG, for 5 min at 37 °C, in Sf9 membrane vesicles containing the human wild-type ABCG2. Membranes from the same cell preparation, containing identical amount of ABCG2, were pre-loaded by variable C-CD concentrations to contain different levels of cholesterol. On the figure for each data point the mean values±SD are presented.

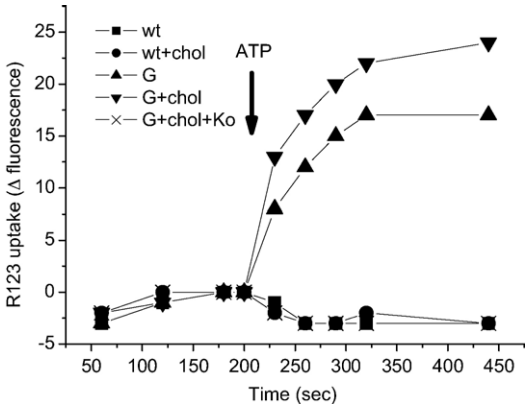


Fig. 5. Effect of cholesterol loading on ATP-dependent Rhodamine 123 (R123) uptake in Sf9 membrane vesicles. R123 uptake was measured by flow cytometry (see Materials and methods) at 1 μM R123 concentration, by taking 30-s time points for 5 min at 22 °C in membrane vesicles containing the human wild-type ABCG2 (WT), or R482G–ABCG2 (G) transporter. The figure shows a representative experiment, depicting the mean values of a triplicate measurement. The SD values for these data points were within the size of the symbols. ■—R123 uptake in the control vesicles (8 μg cholesterol/mg membrane protein), containing the human wild-type ABCG2 (WT) transporter, ●—R123 uptake in cholesterol-loaded vesicles (56 μg cholesterol/mg membrane protein), containing the human wild-type ABCG2 (WT) transporter, ▲—R123 uptake in the control vesicles (8 μg cholesterol/mg membrane protein), containing the human R482G ABCG2 (G) transporter, ▼—R123 uptake in cholesterol-loaded vesicles (62 μg cholesterol/mg membrane protein), containing the human R482G ABCG2 (G) transporter. ×—R123 uptake in cholesterol-loaded vesicles (62 μg cholesterol/mg membrane protein), containing the human R482G ABCG2 (G) transporter, in the presence of 1 μM Ko143.

As a summary, membrane cholesterol greatly and selectively increased substrate transport by the wild-type ABCG2, while we found no major changes by cholesterol in the substrate handling properties of the mutant ABCG2 protein variants.

3.4. Membrane ATPase measurements

In the following experiments we examined the effect of cholesterol loading on the membrane ATPase activity of the ABCG2 and the MDR1 proteins. Vanadate-sensitive membrane ATPase activity, selectively blocked by a specific inhibitor, reflects the transport activity of a number of the ABC multidrug transporters [1,5,39]. As reported earlier, the ABCG2-ATPase activity can be specifically inhibited by Fumitremorgin C or its analog, Ko143. This "basal" ABCG2-ATPase activity is relatively high in isolated Sf9 cell membrane vesicles, but a significant substrate-activation could not be detected in the case of the wild-type protein in the Sf9 cell membrane preparations. In contrast, many substrates caused a strong activation for the ABCG2-ATPase of the R482G or R482T variants [9,14]. The significant substrate-stimulation of the human MDR1-ATPase activity in Sf9 membranes has also been extensively documented [4,39].

Fig. 6A shows the vanadate-sensitive ATPase activity of the wild-type ABCG2 as well as the R482G and the R482T variants, both in the absence and presence of two potential transported substrates. In these studies we selected prazosin, and the EKI-785 tyrosine kinase inhibitor (EKI), as these compounds were shown to be substrates both for the wild-type, as well as the R482G or R482T variants of ABCG2 [14,35].

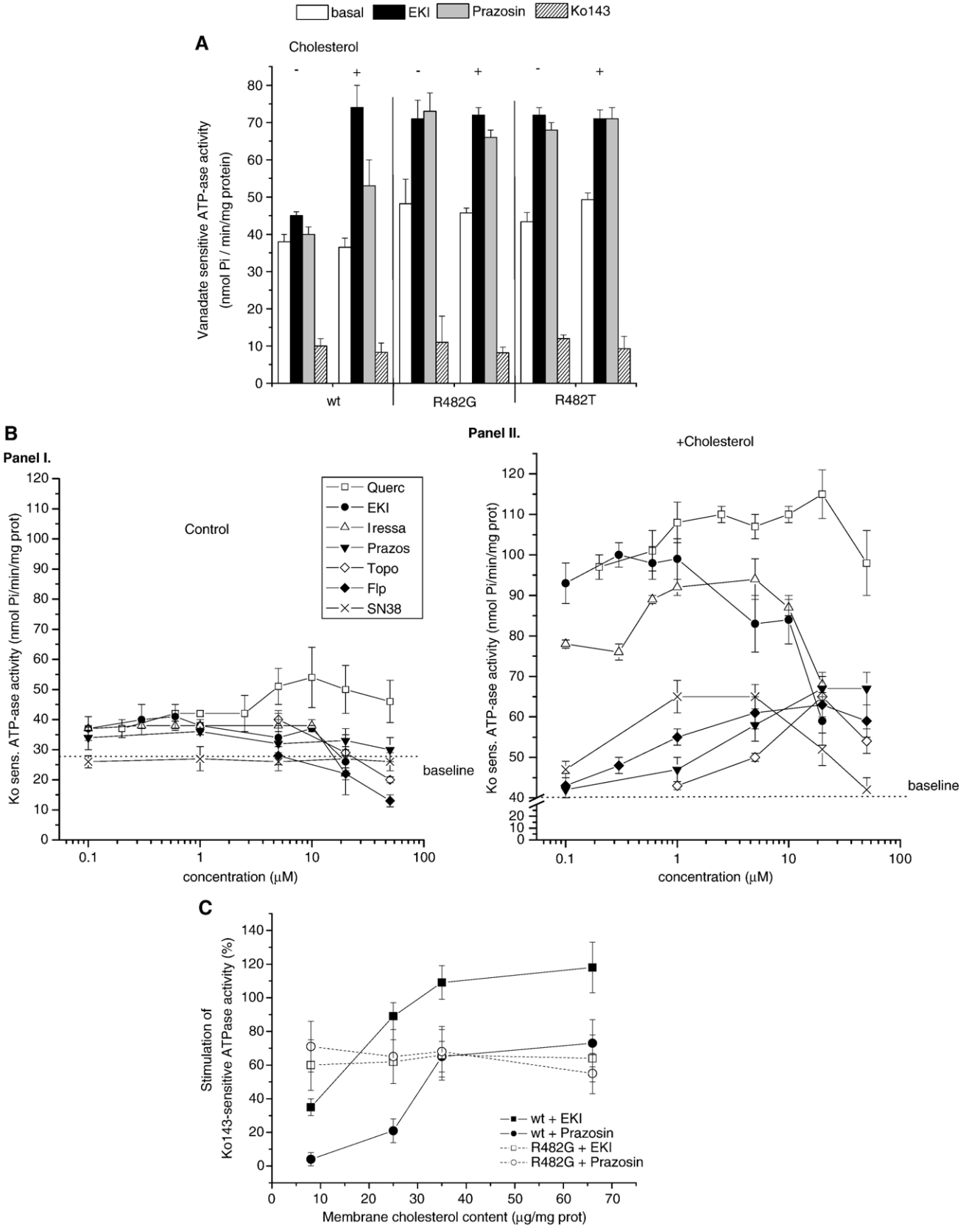
In Fig. 6A we document the respective ATPase activities measured at two different membrane cholesterol levels, that is in the control (6–8 μg cholesterol/mg membrane protein) and cholesterol-loaded (50–65 μg cholesterol/mg membrane protein) Sf9 cell membranes, respectively. Ko143 was shown to fully and selectively inhibit the transport activities in all these ABCG2 variants [14], and the level of the endogenous vanadate-sensitive ATPase activity in the control Sf9 cell membranes was in the range of that measured in the presence of Ko143 in the ABCG2 expressing membranes (about 8–10 nmol/mg membrane protein/min). These data indicate that the Ko143 sensitive fraction of the membrane ATPase activity closely correlates with the activity of the ABCG2 protein.

As documented in Fig. 6A, cholesterol loading of the Sf9 cell membranes did not significantly affect, or only slightly increased the basal ATPase activity of all the three ABCG2 variants, and did not affect the low background ATPase activity measured in the presence of Ko143. However, cholesterol loading greatly increased the drug-stimulated ATPase activity of the wild-type ABCG2 in the presence of both substrates, while it had no such effect in the case of the R482G or R482T mutant variants. Thus, the effect of cholesterol on the drug-stimulated ATPase activity is in close correlation with the observed alterations in the direct vesicular substrate transport, while the basal ATPase activity may not be directly related to this transport.

In the following experiments we have examined the effects of several transported substrates on the ABCG2-ATPase activity (the Ko143-sensitive fraction) in isolated Sf9 cell membranes in a concentration range of 0.1–50 micromolar. According to our earlier studies [14,35], in this system we did not observe, or obtained only a minor stimulation of the ABCG2-ATPase activity by the compounds examined. As shown in Fig. 6B, the compounds already indicated to be transported substrates of ABCG2, that is the tyrosine kinase inhibitor Iressa (Gefitinib), the widely applied topotecan, the irinotecan metabolite SN38, the experimental anticancer agent flavopiridol, and the flavonoid compound quercetin, all produced a major stimulation of the ABCG2-ATPase activity in the cholesterol-loaded Sf9 cell membranes (Panel II), while there was only a small stimulation in the control membranes (Panel I). In the case of quercetin and some tyrosine kinase inhibitors this stimulation was observed already in sub-micromolar concentrations and produced very high maximum ATPase activity levels. These data show that in cholesterol-loaded Sf9 membranes ABCG2 substrate screening, based on measuring Ko143-sensitive ATPase activity, can be efficiently and reliably performed. This is an important practical point in drug development studies, as the Sf9 membrane system is a powerful method for measuring substrate modulation or inhibition of ABC transporter ATPase activity, due to the much higher and well-controlled expression level of membrane proteins than achievable in mammalian cell membranes.

Fig. 6C shows the membrane cholesterol content dependence of the percent drug-stimulation of the ABCG2-ATPase activity by prazosin and EKI. In the case of the wild-type ABCG2, drug-

stimulation was greatly increased by increasing membrane cholesterol levels. Although the cholesterol dependence of this modulation was somewhat different for the two substrate molecules applied, a maximum effect of membrane cholesterol above 40 μg cholesterol/mg membrane protein was observed in both cases. Fig. 6C also depicts the effect of membrane



cholesterol on the prazosin and EKI stimulation of the ATPase activity of the ABCG2–R482G variant. In the case of this mutant transporter, increasing membrane cholesterol levels caused only a slight decrease in the relative substrate stimulation.

In experiments not documented here in detail, we have repeated these experiments with human MDR1-containing Sf9 membrane vesicles, and analyzed the verapamil-stimulation of the MDR1-ATPase under various cholesterol loading conditions. We found that the basal activity of the MDR1-ATPase was slightly increased, while the verapamil-stimulated maximum ATPase activity, measured at 50 μM verapamil, was practically unchanged in cholesterol-loaded Sf9 membranes. A detailed study on the cholesterol modulation of MDR1 and MRP1 activities is underway in our laboratory.

3.5. Nucleotide trapping measurements

In order to explore the molecular mechanism of the cholesterol effect on human ABCG2, we examined the vanadate-sensitive nucleotide trapping in the control and the cholesterol-loaded isolated Sf9 cell membranes, respectively. Most active ABC transporters form a catalytic intermediate, stabilized by the presence of vanadate, which can be visualized through UV-dependent photo-cross-linking and covalent labeling by alpha ^{32}P-8-azido-ATP [1,3,4]. As we documented earlier [17], in the case of ABCG2 this experiment requires the use of Co-alpha ^{32}P-8-azido-ATP, and nucleotide trapping is entirely vanadate-dependent.

In our earlier nucleotide trapping experiments, carried out in human ABCG2-containing Sf9 cell membranes, we found that in the case of wild-type ABCG2 the addition of drugs, e.g. prazosin, did not increase, but rather slightly decreased the formation of this intermediate (see 17). In contrast, nucleotide trapping in the ABCG2–R482G variant was significantly increased by transported substrates.

In the current study we repeated these experiments in Sf9 cell membranes, expressing ABCG2, either without or with cholesterol preloading. As shown in Fig. 7, in the control Sf9 cell membranes vanadate-dependent nucleotide trapping was well measurable, but the addition of prazosin or EKI only slightly decreased the formation of this intermediate. When calculating the average values in three independent experiments, corrected by the immunoblot loading control of ABCG2, the relative ABCG2 labeling was decreased to 45% by prazosin and to 55% by EKI. Cholesterol loading did not alter the basic level of ABCG2-nucleotide trapping (the mean relative value of ABCG2 labeling was 105%). However, the addition of prazosin (a relative increase to 125%), or even more significantly, of EKI (an increase to 180%), stimulated nucleotide trapping in the cholesterol-loaded membranes, in contrast to the strong

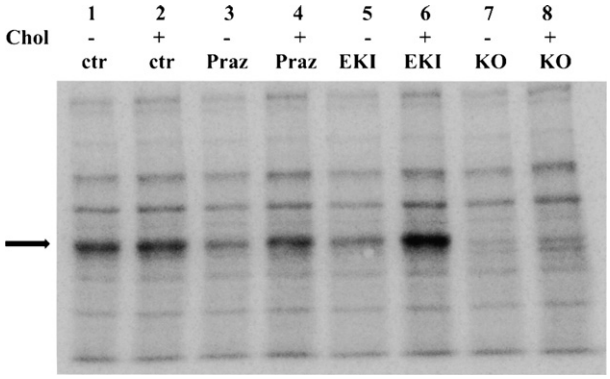


Fig. 7. Effect of cholesterol loading on the formation of the catalytic intermediate (nucleotide trapping) of human ABCG2 in isolated Sf9 membrane preparations. 8-azido-nucleotide trapping by the human, wild-type ABCG2 protein, expressed in Sf9 cell membranes, was measured as described in Materials and methods, in the presence of 2.5 μM Co-8-azido-ATP (containing alpha-^{32}P-8-azido-ATP) at 37 °C for 2 min. Following UV-irradiation, gel-electrophoresis and electroblotting, the labeled bands were visualized by Phospho-Imager. The arrow indicates the position of the ABCG2 protein identified by immunoblotting. Isolated Sf9 cell membranes, containing the same amount of wild-type ABCG2 protein were loaded with cholesterol by preincubation with 4 mM C-CD, as described in the Materials and methods. Cholesterol-loaded membrane vesicles contained 50 μg cholesterol/mg membrane protein, while the control membranes contained 8 μg cholesterol/mg membrane protein. The concentration of EKI or Ko143 was 1 μM, the concentration of prazosin was 20 μM in the assay. The figure shows a representative experiment.

Fig. 6. Effect of cholesterol loading on the ATPase activity of ABCG2 in isolated Sf9 membrane preparations. Panel A Effect of cholesterol loading on the vanadate-sensitive ATPase activity in isolated Sf9 membrane preparations. ATPase activity in the vesicles was measured for 20 min at 37 °C in membranes containing the human wild-type ABCG2 (WT), the R482G–ABCG2 (R482G), or the R482T–ABCG2 (R482T) transporter. The basal ATPase activity and the effects of two potential substrates of the ABCG2 transporter were examined. Prazosin was applied in 20 μM concentration, EKI was used in 1 μM concentration. The effect of Ko143, a specific ABCG2 inhibitor was measured at 1 μM concentration. The mean values±SD are presented. Control membranes contained 8 μg cholesterol/mg membrane protein, while cholesterol-loaded membranes contained 56 μg cholesterol/mg membrane protein in the case of the wt ABCG2, 62 μg cholesterol/mg membrane protein in the case of the ABCG2–R482G, and 65 μg cholesterol/mg membrane protein in the case of the ABCG2–R482 T variant. Empty columns: basal ATPase activity, black columns: 1 μM EKI, light gray columns: 20 μM prazosin, striped columns: 1 μM Ko143. Panel B Concentration dependence of the stimulatory effect of different drugs on the ABCG2 ATPase activity in control (Panel I) and in cholesterol loaded (Panel II) Sf9 cell membrane preparations. ATPase activity was measured for 20 min at 37 °C in membranes containing the human wild-type ABCG2, either without cholesterol-loading (left panel) or loaded with cholesterol (right panel). The ABCG2-specific ATPase activity was determined as the Ko143-sensitive fraction of the activity. Cholesterol loading by C-CD was achieved as described in the Materials and methods. Each drug was tested in at least two independent membrane preparations, the mean±SD values are presented in a representative experiment with triplicate measurements. The control membranes contained 8 μg cholesterol/mg membrane protein, while cholesterol-loaded membrane vesicles contained 42 μg cholesterol/mg membrane protein. □—Quercetin, ●—EKI-785, △—Iressa, ▼—Prazosin, ◇—Topotecan, ◆ Flavopiridol, ×—SN-38. Panel C Effect of membrane cholesterol content on the substrate stimulation of the ABCG2 ATPase activity in Sf9 membranes. ATPase activity was measured for 20 min at 37 °C in membranes containing the human wild-type ABCG2 (WT), or the R482G–ABCG2 (R482G) transporter. The specific ATPase activity for ABCG2 was calculated as the Ko143-sensitive fraction. Cholesterol loading by C-CD was achieved as described in Materials and methods. The mean values obtained in three independent experiments±SD are presented. ●—Per cent stimulation of the Ko143-sensitive ATPase activity by 20 μM Prazosin in membranes containing the human wild-type ABCG2 (WT) transporter, ■—Per cent stimulation of the Ko143-sensitive ATPase activity by 1 μM EKI in membranes containing the human wild-type ABCG2 (WT) transporter, ○—Per cent stimulation of the Ko143-sensitive ATPase activity by 20 μM Prazosin in membranes containing the human R482G ABCG2 (G) transporter, □—Per cent stimulation of the Ko143-sensitive ATPase activity by 1 μM EKI in membranes containing the human R482G ABCG2 (G) transporter.

decrease produced by these drugs in the unloaded control membranes. Similar results were obtained at two different time-points (2 min and 5 min) in these nucleotide trapping studies. In all cases the addition of Ko143 eliminated ABCG2-dependent nucleotide trapping.

In the case of the ABCG2–R482G mutant variant, prazosin and EKI stimulation of nucleotide trapping was already present in the control membranes [17], and in this case we did not find any significant difference by cholesterol enrichment of the Sf9 cell membranes in the present study (data not shown).

These experiments indicate a significant effect of membrane cholesterol on the rate of nucleotide trapping by the human ABCG2 protein. In correlation with the observed acceleration of direct vesicular substrate transport, and the appearance of drug-stimulated ATPase activity, cholesterol loading was found to promote the drug stimulation of the formation of the catalytic intermediate, indicating a cholesterol stimulation of the turnover of the wild-type transporter.

4. Discussion

Our present experiments document a major effect of membrane cholesterol on the activity of the human ABCG2 protein. We found that in intact mammalian cells a rapid and mild cholesterol depletion, without causing a significant cellular damage, strongly reduced ABCG2-dependent transport activity. This effect was fully reversible, and selective for the wild-type ABCG2, while the function of the highly active mutant variant ABCG2–R482G, found in drug-selected tumor cells, was not influenced by a similar cholesterol depletion. It is important to note that cell viability or membrane localization of ABCG2 was unchanged in the course of these cholesterol modulating experiments. Moreover, under similar conditions we found no measurable effect of cholesterol depletion or cholesterol loading on the activity of another ABC multidrug transporter, the MDR1 (ABCB1) protein.

In intact cells several non-specific effects may occur, including the removal of various hydrophobic membrane components by cyclodextrin. Moreover, long-term cholesterol depletion significantly reduces survival, thus drug-resistance experiments could not be performed under these conditions. Therefore, the molecular details of cholesterol effects could be much better examined in isolated membrane preparations. In order to perform such studies we used Sf9 cell membrane vesicles, obtained from insect cells expressing high levels of the human ABCG2 or its variants. Sf9 cell membranes contain relatively low levels of endogenous cholesterol, thus variable levels of increased membrane cholesterol, approaching or even exceeding those in mammalian cell membranes, could be achieved.

Direct vesicular transport studies performed in such preparations indicated that the maximum transport rate of ABCG2 can be increased up to 20 fold by increasing membrane cholesterol levels. By using various sterol-cyclodextrin complexes we found that this effect on ABCG2 was selective for alterations in membrane cholesterol. Although the structurally similar ergosterol or sitosterol also have the capacity to increase membrane order in artificial membranes, and they have a stabilizing role in drosophila (ergosterol) or plant (sitosterol) membranes [see refs. [34,42,50], none of them were able to mimic the effect of cholesterol in our experiments. Similarly to that seen in intact cells, this effect of membrane cholesterol was absent in the case of the R482G mutant variant of the transporter.

Vanadate- and inhibitor-sensitive membrane ATPase activity in ABC transporter expressing cell membrane preparations has been shown to correlate with the transport activity of these proteins [5]. However, according to our earlier experiments, in the case of the wild-type ABCG2 transporter, in spite of a pronounced basal ATPase activity, the substrate drug stimulation in the Sf9 cell membranes was relatively small [14]. In contrast, in isolated mammalian cell (e.g. MCF7) membranes, ABCG2-ATPase activity could be significantly stimulated by various drug substrates. It has been suggested for MDR1 and for ABCG2 as well, that the basal ATPase activity, measured in the absence of added substrates, may be due to activation by endogenous substrates, or may reflect a partially uncoupled ATPase activity of the transporter [1,4]. Interestingly, the ATPase activity of the R482G or R482T mutant variants of ABCG2 could be significantly enhanced by the respective substrate drugs both in the Sf9 and the mammalian cell membrane preparations [10,14].

Since membrane proteins, when expressed in Sf9 cells, are underglycosylated, we first speculated that the loss of glycosylation may result in these alterations of the ABCG2-ATPase activity. However, it has been demonstrated that glycosylation of ABCG2 has no effect either on its activity, processing, or membrane localization [46–48]. The present experiments strongly suggest that alterations in ABCG2-ATPase are mostly due to the different membrane cholesterol levels, that is cholesterol enrichment of the Sf9 cell membranes enables a substrate-stimulation of the wild-type ABCG2-ATPase activity. An important result of these experiments is that by using cholesterol preloaded Sf9 cell membranes this technology allows the screening of a wide variety of ABCG2-specific substrate molecules by measuring ABCG2-ATPase activity. This is clearly shown by the large effects of submicromolar concentrations of e.g. Gefitinib (Iressa), or quercetin in this system (see Fig. 6B-II).

Earlier we examined the vanadate-dependent formation of a trapped nucleotide by ABCG2, reflecting the catalytic intermediate in ABC transporters in isolated Sf9 cell membrane preparations [17]. In these experiments we found that in the case of the wild-type ABCG2 protein, the transported substrates did not increase, rather slightly decreased the formation of this intermediate. In contrast, transported substrates significantly increased nucleotide trapping by the ABCG2-R482G variant [17]. In our present experiments we found that in cholesterol enriched Sf9 cell membranes transported substrates produced an increased rate of catalytic intermediate formation also in the wild-type ABCG2. This finding indicates that cholesterol enhances the drug substrate-dependent turnover of ABCG2.

In this study we observed that the effect of membrane cholesterol was specific for the wild-type ABCG2 protein, containing an Arg (R) at the proposed intracellular membrane region of the third transmembrane loop of the protein. Replacement of this

Arg by Gly or Thr significantly alters the substrate specificity of ABCG2, and seems to removes its cholesterol modulation. This finding excludes a non-specific stabilization or modulation of this protein by cholesterol, and suggests a special role of this protein region both in the substrate recognition and cholesterol modulation of ABCG2. A recent study [48,49] concluded that mutations at position 482 in ABCG2, while alter the transport and ATPase profile of this protein, do not affect drug binding. Thus cholesterol may interact with a region involved in the catalytic/transport region of ABCG2. An exploration of the exact molecular or conformational specificity of this cholesterol effect needs further experiments.

Based on Sf9 membrane ATPase measurements, earlier we proposed that the R482G and R482T variants of ABCG2 may have "gain-of-function" properties [14]. Our present experiments indicate that these mutant ABCG2 variants may show a higher ATPase and transport activity only in the cholesterol-poor membranes, and at increasing membrane cholesterol levels in the Sf9 cell membranes the wild-type protein may achieve an ATPase and transport capacity approaching that in the mutant variants. We speculate that in spite of the reduced xenobiotic recognition profile of the wild-type ABCG2, its cholesterol-modulation may explain the evolutionary conservation of Arg at position 482.

In these experiments we observed gradual effects of the Sf9 cell membrane cholesterol on the transport and ATPase activity of ABCG2. The range where membrane cholesterol had the most significant effect, in mammalian cells corresponds to the values observed between the low-cholesterol intracellular (e.g. ER) membranes, and the high-cholesterol plasma membranes [50]. Certain plasma membrane microdomains (e.g. "membrane rafts") were shown to have especially high cholesterol levels [34,50]. Thus in mammalian cells a complex, reversible cholesterol modulation of the ABCG2-dependent xenobiotic extrusion or cancer drug resistance may be expected. The routing of the ABCG2 protein to specific plasma membrane regions, or the internalization of the protein to intracellular membrane compartments, may alter ABCG2 transport activity by more than an order of magnitude.

Several members of the ABCG family are involved in active cholesterol transport [51–53], and this may be true for ABCG2 as well. However, direct vesicular cholesterol transport measurements could not be performed in our system. Still, the present study does not suggest that cholesterol itself is a transported substrate for ABCG2. We did not observe any cholesterol competition in the transport assays, and found no major stimulation of the ABCG2-ATPase activity or nucleotide trapping by cholesterol in the absence of added substrates. However, a possible co-transport of cholesterol with specific ABCG2 substrates cannot be excluded at present.

Cholesterol modulation of multidrug transporters, especially that of MDR1 (ABCB1) has been reported in the literature [29,30]. In our present study, under similar conditions as examined for ABCG2, we found no major effect of the mild cholesterol depletion of mammalian cell membranes on the MDR1 transport activity (see Fig. 1A). In experiments to be reported in detail elsewhere, we have examined the effects of membrane cholesterol both on the human MDR1 and the MRP1 proteins, expressed in Sf9 cell membrane preparations. However, under conditions used in the present study, the effect of membrane cholesterol on these transporters was almost negligible, as compared to the wild-type human ABCG2. In earlier studies with isolated MDR1 protein it has been shown [54] that cholesterol increased the basal ATPase activity but did not significantly modulate the drug-stimulated MDR1-ATPase in proteoliposomes. In a similar system, recent experiments documented that cholesterol directly and variably modulated the drug-affinity of the isolated MDR1 protein, while did not affect its maximum turnover rate in the case of most substrates, having high stimulatory activity. Paclitaxel, a substrate with a low stimulatory effect, was found to be an exception [55].

As a summary, here we found that membrane cholesterol depletion inhibits, while cholesterol enrichment greatly augments the active transport and ATPase activity, as well as the catalytic intermediate formation of the ABCG2 protein. Detailed studies on these cholesterol effects, regarding various toxin and drug substrates, as well as the polymorphic variants of ABCG2, are underway in our laboratory. These combined studies may allow the exploration of a functional, complex regulation of this medically important ABC transporter protein.

5. Note added in proof

While this manuscript was under reviewing, a communication (Ref. [56]) described a stimulating effect of cholesterol loading on the ABCG2 ATPase and transport activity in isolated membrane preparations.

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