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Novel roles for ATP-binding cassette G transporters in lipid redistribution in *Toxoplasma*

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Summary

Toxoplasma is a protozoan parasite proficiently adapted to thrive in a parasitophorous vacuole (PV) formed in the cytoplasm of a large variety of mammalian cells. As an actively dividing organism, the parasite must adjust the lipid composition of its membranes to preserve organelle vitality and expand the size of the PV membrane to accommodate growing progeny. We showed that *Toxoplasma* takes up host lipids and can expel major lipids in an ATP-dependent process. In order to provide detailed mechanistic insights into lipid trafficking phenomena relevant to *Toxoplasma* biology, we characterized six parasite ATP-binding cassette (ABC) G family transporters and investigated their potential contribution to lipid homeostatic processes. All these transporters are expressed in the parasite and five of them are upregulated upon exposure to sterols. Four ABCG are localized to secretory organelles and the plasma membrane, and promote cholesterol and phospholipid efflux, reflecting the importance in exportation of large amounts of lipids into the PV. Interestingly, one ABCG that is associated with vesicles in the PV and the plasma membrane acts as a cholesterol importer. This last finding expands our current view on the role of some ABCG transporters in eukaryotic sterol influx.

Introduction

Intracellular pathogenesis is a subset of the large world of host-microbe interactions that forces the pathogen to use unique strategies in order to persist and replicate within a host

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cell. *Toxoplasma gondii*, an obligate intracellular parasite of the phylum Apicomplexa, invades and successfully multiplies in a large variety of mammalian cells. The uniqueness of *Toxoplasma*-host cell interactions resides in the nature of the parasite-created compartment that allows bountiful access to host resources and ensures protection for the parasite. As the interface between *T. gondii* and the host cytoplasm, the delimiting membrane defining the parasitophorous vacuole (PV) plays strategic roles in securing *Toxoplasma* in a replication-permissive niche (Sinai, 2008). At the time of invasion, the PV is initially composed of lipids originating from the host plasma membrane (Suss-Toby *et al.*, 1996) and of vesicular contents released from the parasite secretory organelle rhoptry into the host cytoplasm (Håkansson *et al.*, 2001).

During its intracellular stay, *Toxoplasma* faces the challenge to expand the size of the vacuole by adding new lipids into the PV membrane to accommodate growing progeny. Almost nothing is known about the nature and sources of the lipids that shape the PV membrane post-invasion, or in general about the mechanisms of lipid transport to the PV, coming either from the parasite or the host cell. As dictated by its intracellular lifestyle, *T. gondii* has lost numerous genes needed for lipid biosyntheses, but in return, it has gained genes promoting lipid diversion from the host cell and lipid redistribution in its own cell. For example, cholesterol is scavenged intact by the parasite from the host endocytic pathway after low-density lipoprotein (LDL) internalization (Coppens *et al.*, 2000). Interference with LDL endocytosis or cholesterol egress from host lysosomes arrests parasite development. We demonstrated that *Toxoplasma* can sequester nutrient-filled lysosomes within invaginations of the PV membrane, which allows access to components, e.g. lipids supplied by the endocytic network (Coppens *et al.*, 2006). We showed that *T. gondii* is able to internalize cholesterol from the external medium, in a saturable and energy-dependent process (Sehgal *et al.*, 2005). Cholesterol incorporation into the parasite is abolished after treatment with various proteases, which suggests the presence of a transport system of cholesterol at the parasite plasma membrane, involving protein binding sites. The inability of *Toxoplasma* for endocytic uptake of exogenous molecules precludes a role for plasma membrane-derived vesicles in cholesterol internalization. Alternatively, lipid translocators distributed to the plasma membranes may contribute to the import of cholesterol in the parasite. Other major lipids such as phosphatidylcholine can be produced *de novo* by *Toxoplasma*, but their synthesis does not come close to satisfying the entire parasite's needs (Gupta *et al.*, 2005). In this case, these lipids must also be adeptly retrieved from the host environment. We previously reported a role for a parasite D-bifunctional protein containing two sterol-carrier protein-2 domains in promoting the circulation of phospholipids, cholesterol and fatty acids between parasite organelles and the plasma membrane (Lige *et al.*, 2009).

Survival of eukaryotic cells depends on the regulated and selective transport of lipids, and the waste of excess lipids from cells, which are processes often mediated by integral membrane proteins. *Toxoplasma* must also have developed homeostatic mechanisms to coordinate lipid acquisition and trafficking in and out of the parasite in order to adjust optimal levels of lipids within membranes of organelles and the PV. Among the lipid-translocating importers and exporters described in various systems, some members of the ATP-binding

cassette (ABC) transporter G subfamily (ABCG family) and of the ABC transporter A subfamily (ABCA family) are involved in the translocation of sterols and/or phospholipids across membranes. These lipid transporters include ABCG1, ABCG4, ABCG5, ABCG8 and ABCA1 (Velamakanni *et al.*, 2007; Tarr *et al.*, 2009). Active transport of their lipid substrates requires energy released by the binding and hydrolysis of ATP by two ABC molecules, known as the nucleotide-binding domain (NBD). Spanning ~100 amino acids, each NBD is composed of three non-continuous conserved domains termed Walker A, Signature motif and Walker B. The ABCG proteins contain only one single NBD, implying that they need to dimerize to form functional units. The NBD is followed by at least six putative transmembrane domains, referred as the TMD. ABCG1 and ABCG4 form homodimers and are mainly associated with the ER and Golgi membranes while ABCG5 and ABCG8 form heterodimers that are localized to the plasma membrane. Containing two NBD, ABCA1 is an ABC full-transporter comprising 12 putative transmembrane domains and it distributes to endosomes and cell surface (Neufeld *et al.*, 2004; Cavelier *et al.*, 2006; Hirata *et al.*, 2009). ABCG1 and ABCA1 play a pivotal role in mediating cellular efflux of phospholipids and cholesterol to extracellular lipid acceptors that are lipid-free apoA-I and apoA-I-containing lipoproteins respectively (Cavelier *et al.*, 2006). In mammalian cells, all these lipid traffic ATPases play fundamental roles in lipid homeostasis as mutations in their genes lead to aberrant lipid levels and distribution, resulting in several significant inherited human diseases (Stefková *et al.*, 2004).

Based on motif identifications, a detailed inventory of the ABC transporter superfamily in *Toxoplasma* in 2006 had retrieved five putative ABCG transporters from the *Toxoplasma* database (<http://www.ToxoDB.org>) and no ABCA homologue (Sauvage *et al.*, 2006). Among the human ABCG transporters, ABCG1 was the closest homologue for one parasite ABCG-like protein (42% identity). Four parasite ABCG transporters have the highest identity for human ABCG2 (ranging from 35% to 42%). Located at the plasma membrane, ABCG2 form homodimers that seem to be involved in the disposition of a large variety of hydrophobic compounds that do not include sterols or phospholipids (Hardwick *et al.*, 2007). Our presented study sought to bring novel information on the molecular mechanisms implicated in the influx and efflux of lipids across the membranes of *T. gondii*. We hypothesize that a protein-mediated inward movement of lipids would require essential transporters implicated in lipid supply for the parasite. In addition, a mechanism of protein-mediated export of lipids from the parasite may represent an adaptive system to deliver lipids to the PV necessary for its expansion. To this end, we examined the potential functionality of all the *Toxoplasma* ABCG in lipid transport activities. Among them, four ABCG are implicated in the release of both cholesterol and phosphatidylcholine from the parasite. Interestingly, our data also report an ABC transporter-like process that promotes sterol intake.

Toxoplasma gondii is a leading opportunistic parasite in immunosuppressive conditions. By entering into the confines of a mammalian cell, the parasite places itself in an intracellular milieu whose composition is different from what is experienced by mammalian cells. The presence of rudimentary homologues of mammalian lipid translocators in the parasite reveals that lipid homeostatic pathways are ancient. Nonetheless, our results suggest that *T.*

gondii may employ some lipid homeostatic proteins in unconventional ways. Peculiarities in parasite lipid regulatory mechanisms may expose new vulnerabilities.

Results

Toxoplasma can export lipids in an ATP-dependent process

We previously documented the transport of isotopic cholesterol from intracellular *Toxoplasma* to sterol acceptors in the medium (Sehgal *et al.*, 2005). This suggests that specific mechanisms must be operational to translocate free cholesterol across the parasite plasma membrane, the PV membrane and ultimately to the host plasma membrane. This efflux of cholesterol may result either from a net exchange of free cholesterol between the PV and the host cytoplasm, or from an active transfer mediated by the parasite. To assess the capability of *Toxo-plasma* for cholesterol export from its cell, mammalian cells were infected, then incubated with [³H]cholesterol for a period of 24 h in order to preload intracellular parasites with radioactive cholesterol. Parasites were subsequently isolated from the cells and resuspended in chase medium to monitor cholesterol deloading. After the chase period, the amount of radioactive cholesterol was measured both in the medium and in the parasites (Fig. 1A). Data showed that the fraction of cholesterol effluxed from *Toxoplasma* corresponded to 0.9% and 1.7% of total radioactive cholesterol associated with the parasites after 30 and 60 min of incubation respectively. To characterize further the process of cholesterol release by *T. gondii*, we added during the chase period both sodium azide and the glucose analogue 2-deoxyglucose (2DG) to block the mitochondrial and glycolytic production of ATP, respectively, to extracellular parasites preloaded with radioactive cholesterol. A significant inhibition of cholesterol release into the medium was observed. Cholesterol efflux was 61% and 85% inhibited by ATP poisons after 30 min and 60 min respectively. To assess the specificity of cholesterol export by the parasites, we monitored the movement of radioactive fatty acids from *Toxoplasma* in a dual labelling experiment with [¹⁴C]palmitic acid and [³H]cholesterol preloaded in the parasites. In contrast to the effect of energy poisons on sterol efflux, the release of radioactive palmitic acid was negligible and unchanged upon ATP depletion ($0.015 \pm 0.008\%$ and $0.013 \pm 0.007\%$ in the absence and the presence of sodium azide/2DG respectively).

In mammalian cells, ABCA1 and ABCG1 can mediate both sterol and phospholipid export (Gelissen *et al.*, 2006). We next wanted to determine whether sterol and phospholipid efflux are coupled in *Toxoplasma*. We monitored the export of radiolabelled choline-containing phospholipids after loading intravacuolar *T. gondii* with [³H]choline and deloading the parasites in the presence or the absence of sodium azide/2DG (Fig. 1B). Data revealed that during a 30 min of chase, *T. gondii* released ~3% of the total radioactive choline-containing phospholipids that were associated with the parasites. This process was abolished by 67% upon treatment with energy poisons. After 60 min of chase, 5.9% and 0.6% of total radioactive choline-containing phospholipids were found in the medium in the absence and the presence of sodium azide/2DG respectively. These results demonstrate that *Toxoplasma* is capable of selectively expelling cholesterol and phospholipids under conditions that require ATP.

Cloning and molecular characterization of six ABCG transporters in *Toxoplasma*

The next step was to determine whether a reaction coupled to ATP supply and hydrolysis as described for a classical ABC transport-like process is responsible for lipid efflux by *T. gondii*. To explore this possibility, we cloned all the members of the ABCG family of *Toxoplasma*. By RT-PCR from tachyzoite total RNA, we succeeded in amplifying the open reading frame (ORF) of six ABCG, including the five ABCG that have been previously identified by bioinformatics screening (Sauvage *et al.*, 2006). Fig. S1 compiles their genomic and molecular characteristics. From start to stop codons, the gene that corresponds to ABC-G1 (GenBank, accession #: DDQ099410) spans 7777 nucleotides on chromosome VI as confirmed by Southern blot analysis (Fig. S2), and the coding region is 2385 nucleotides long. Translation of the ORF of the cloned gene predicts a protein of 87.3 kDa. We therefore named this protein TgABCG₈₇. The gene that corresponds to ABC-G2 (accession #: DQ099407) encompasses 11 699 nucleotides on chromosome IX. The ORF for this gene contains 2301 nucleotides and the size of the predicted protein product is 83.8 kDa (named TgABCG₈₄). The gene corresponding to ABC-G3 (accession #: DQ099411) spans 13 817 nucleotides of chromosome IX. The ORF contains 2439 nucleotides and the predicted protein product from this gene is 89.1 kDa (named TgABCG₈₉). The ABCG gene corresponding to ABC-G4 (accession #: DQ099412) spans 4952 nucleotides on chromosome IX, and has an ORF of 2649 nucleotides, which predicts a protein of 95.6 kDa (named TgABCG₉₆). The gene corresponding to ABC-G5 (accession #: DQ099413) encompasses 9764 nucleotides on chromosome XII. The ORF contains 2945 nucleotides and the predicted translated sequence has a size of 106.5 kDa (named TgABCG₁₀₇). Finally, the gene corresponding to ABC-H3 (accession #: DQ099417) spans 5719 nucleotides on chromosome XI. The ORF for this gene contains 2106 nucleotides, and the predicted protein is 76.6 kDa (named TgABCG₇₇). These transporters have all the characteristic features of the G family of ABC transporters (Fig. 2), which includes the canonical NBD with Walker A, Signature motif and Walker B, preceding a large TMD consisting of multiple membrane-spanning α -helices (see Fig. S1 for the NBD-TMD domain structural arrangement).

Interestingly, the ORFs of TgABCG₉₆, TgABCG₁₀₇ and TgABCG₇₇ obtained by cloning and verified by sequencing were slightly different from the equivalent sequences predicted in the *Toxoplasma* database. The major difference between predicted ABC-H3 and cloned TgABCG₇₇ was the absence of the TMD for ABC-H3, resulting in its mistaken classification in the H family of ABC proteins. ABCH proteins only harbour a single NBD and their function has not been established yet (Dean *et al.*, 2001). All the cloned sequences of TgABCG revealed similitude in their NBD residues with the corresponding sequences deposited in GenBank. Additionally, the TgABCG sequences include, between the Walker A and Signature residues, the Q-loop glutamine that contributes to the conformational changes involved in signal transmission for ATP binding, and downstream of the Walker B, the H-loop histidine that stabilizes the ATP hydrolysis mechanism (Linton and Higgins, 2007).

All the TgABCG are expressed in *Toxoplasma* and located to distinct compartments

We next explored whether these TgABCG are expressed in *T. gondii* using antibodies raised against specific sequences of TgABCG₉₆, TgABCG₇₇, TgABCG₁₀₇, TgABCG₈₄ and

TgABCG₈₉ that we generated (Fig. S3). Lysates of wild-type parasites were probed by Western blotting using these antibodies. Immunoblots shown in Fig. 3A revealed a major band at high molecular weight as expected for each transporter, although some differences with the size of predicted translated sequences were noticeable, likely due to the presence of multiple hydro-phobic segments at the C-terminus of the transporters (Fig. 2 and Fig. S1). As noted for mammalian ABCG, we also detected the presence of bands into lower molecular mass species, suggestive of post-translational modifications. In parallel, we engineered a stable line of parasites expressing TgABCG₈₇ with a C-terminal HA tag. Gene expression in transgenic *T. gondii* was confirmed on immunoblots using anti-HA antibodies revealing a unique band around 85 kDa in the solubilized fraction of parasites (Fig. 3B).

In mammalian cells, functional ABCG form obligate dimers to promote lipid transport across membranes (Kusuhara and Sugiyama, 2007). To examine potential dimerization of TgABCG, these transporters were subjected to cross-linking using the thiol-cleavable and membrane-permeable DSP prior to immunodetection (Fig. 3C). Under this condition, immunoreactive bands between 220 and 260 kDa were detected. To assess the specificity of DSP treatment, parasite lysates were treated with DTT in parallel assays. We observed a separation of the cross-linked products upon DSP cleavage by DTT, which suggests that TgABCG may also form homo- or heterodimers.

We next analysed the subcellular distribution of TgABCG₉₆, TgABCG₇₇, TgABCG₁₀₇, TgABCG₈₄ and TgABCG₈₉ in wild-type *Toxoplasma* using anti-TgABCG antibodies and in TgABCG₈₇-HA expressing parasites using anti-HA antibodies by fluorescence microscopy (Fig. 4) and immunoEM (Fig. 5 and Table S1). We observed a specific staining of the ER, internal vesicles and cell periphery for TgABCG₈₉, TgABCG₈₄, TgABCG₈₇ and TgABCG₉₆ in intracellular parasites. By comparison, TgABCG₇₇ was more concentrated to the parasite Golgi and the plasma membrane. These data suggest a vesicular shuttling of all these transporters between the secretory pathway and the surface where they may be exerting their function. Interestingly, TgABCG₁₀₇ staining was visible in the PV and on the plasma membrane, which may suggest a role for TgABCG₁₀₇ in lipid movement inside the PV.

TgABCG mRNA are differentially expressed upon sterols added in the medium

ABCG1 is transcriptionally regulated by cholesterol and endogenous agonists of liver X receptors including 22(R)-hydroxycholesterol (Koldamova *et al.*, 2003; Kusuhara and Sugiyama, 2007). We examined whether TgABCG transcripts are responsive to exogenous sterols, which would suggest a participation of TgABCG in sterol metabolism in *Toxoplasma*. Intravacuolar parasites were incubated in medium containing 10 μ M 22(R)-hydroxycholesterol or 2 μ g ml⁻¹ LDL to monitor the upregulation of ABCG transcripts, or in medium devoid of sterols to examine potential downregulation of these transcripts. Control experiments were performed on parasites incubated in the presence of 10% fetal bovine serum (FBS; nominal concentration of LDL: 0.2 μ g ml⁻¹). After 48 h of treatment, RNA was extracted from parasites and subjected to quantitative RT-PCR using transcript-specific primers to examine mRNA expression for each TgABCG. Data were normalized by parasite actin transcripts. Figure 6 shows that exposure to 22(R)-hydroxycholesterol

increased the transcript levels of *TgABCG₇₇* and *TgABCG₁₀₇* by ~eightfold, and those of *TgABCG₈₄* by ~fourfold. A ~2.4 and twofold induction of transcription were observed on *TgABCG₈₉* and *TgABCG₈₇*, respectively, while *TgABCG₉₆* mRNA levels remained unchanged. Only *TgABCG₇₇* was transcriptionally upregulated under conditions of excess LDL (~threefold). Removal of lipoproteins from the medium had no significant influence on any *TgABCG* transcript levels. Results from these experiments provide evidence that expression of five *TgABCG* is influenced by the sterol composition in the medium and these transporters may act as sterol regulators in the parasite.

Validation of the model of COS cells expressing *TgABCG* as a surrogate system for functional studies of *TgABCG*

We sought next to determine the function of each *TgABCG*. In a first attempt, we exploited the stable line of parasites expressing *TgABCG₈₇*-HA to investigate the role of this transporter in lipid movement in a gain-of-function phenotype-driven approach. Compared with wild-type parasites, no significant difference in efflux of cholesterol or phospholipid was monitored in the transfectants (Table S2). Experiments were then conducted to induce the overexpression of *TgABCG* in *Toxoplasma* by transient transfection; however, only ~0.5–1% of overexpressors was obtained, which precludes the exploitation of *Toxoplasma* for functional studies.

We therefore envisaged to use mammalian COS cells expressing *TgABCG* as a surrogate system to investigate the function of each transporter. We first examined the expression level and localization of *TgABCG* in these cells after transient transfection of plasmids containing individual *TgABCG* tagged with either HA or V5. Immunofluorescence assays using anti-tag antibodies revealed that ~20–25% of the cells were positively labelled for the parasite transporters. *TgABCG₉₆*, *TgABCG₈₉*, *TgABCG₈₄* and *TgABCG₈₇* were uniformly distributed to the ER and to some portions of the cell surface of COS cells (Fig. S4). In COS cells transfected with two plasmids each carrying a different transporter in fusion with either HA or V5 tag, the detection of the transporters on the cell surface was more evident (Fig. S5). In COS cells expressing *TgABCG₁₀₇*, a punctate fluorescence pattern was systematically noticeable in the cytoplasm, in addition to a well-defined staining that demarcates the cell surface (Fig. S6). Double staining of COS cells cotransfected with *TgABCG₁₀₇* and *TgABCG₉₆*, *TgABCG₈₉*, *TgABCG₈₄* or *TgABCG₈₇* clearly revealed the peripheral staining of *TgABCG₁₀₇*. The nature of some *TgABCG₁₀₇*-containing vesicles was identified as endosomes by using antibodies against the endosomal marker EEA1. Altogether, these observations indicate that COS cells express *TgABCG₉₆*, *TgABCG₈₉*, *TgABCG₈₄*, *TgABCG₈₇* and *TgABCG₁₀₇* in compartments equivalent to those containing these transporters in *Toxoplasma*, which is a prerequisite for studying the role of *TgABCG* in a mammalian heterologous system.

The morphology of the plasma membrane is altered in mammalian cells expressing *TgABCG₇₇*

Expression of *TgABCG₇₇* in COS cells for 16 h revealed the presence of the transporter in the secretory pathway and at the plasma membrane (Fig. 7A). Surprisingly, *TgABCG₇₇* was detected in thin protrusions emanating from the cell body. Approximately 70% of HA-

positive cells displayed this striking morphology of the cell surface, which may reflect changes in lipid composition of the plasma membrane. When TgABCG₇₇-expressing cells were incubated in medium containing human serum/ apoA-I to deload lipids for the cells, their morphology appeared normal. Overexpression of TgABCG₇₇ in COS cells for a period extending to 36 h resulted in irreversible cell damage, characterized by cell atrophy and ultimately death. Similar cellular degeneration was observed in mammalian cells expressing both TgABCG₈₄ and TgABCG₇₇ whereas cells expressing TgABCG₈₄ alone exhibited normal phenotype, which confirms that TgABCG₇₇ produced by the cells is likely altering the cell.

To enlighten the molecular basis of TgABCG₇₇ cytotoxicity, we engineered TgABCG₇₇-HA constructs harbouring punctual mutations in the NBD sequence either mimicking amino acid changes associated with diseases or substituting residues within highly conserved motifs (Gregory *et al.*, 1991; Aparicio *et al.*, 1996; Szentpétery *et al.*, 2004). After transfection of TgABCG₇₇ mutants, cell size and morphology were then inspected by immunofluorescence and compared with cells expressing wild-type TgABCG₇₇ (Fig. 8B). In a first construct, the conserved glycine in the fourth position of the signature motif was substituted for aspartic acid (G335D). Expression of ABCG₇₇^{G335D} in COS cells resulted in the localization of the protein to the ER without any noticeable cell surface deformation compared with cells expressing TgABCG₇₇. Second, we substituted conserved lysine located within the Walker A motif either by glutamine (K233Q) or arginine (K233R). Transfection of ABCG₇₇^{K233Q} partially rectified the phenotype of TgABCG₇₇-expressed COS cells as the cells expressing the mutant were larger than those expressing the wild-type protein, and an ER staining was clearly discernible for the transporter. By contrast, cells transfected with TgABCG₇₇^{K233R} appeared similar to those expressing TgABCG₇₇ with altered morphology and nuclear staining. Introduction of double mutations in the Signature and the Walker A motifs either G335D + K233Q or G335D + K233R resulted in the ER localization of the mutant transporters and the transfected cells were morphologically unaffected. These data suggest that Gly335 and Lys233 in the NBD of TgABCG₇₇ are essential residues for the transporter function. The mutation of K233R is silent, which may indicate that TgABCG₇₇ can properly function when Lys233 is replaced by a same charge residue. In conclusion, these observations indicate that TgABCG₇₇ expression for a long period of time progressively results in COS cell toxicity. To minimize the cytotoxic effects resulting from TgABCG₇₇ expression, we will then conduct our functional studies for TgABCG₇₇ on a short period post-transfection. In parallel, we will also examine the role of TgABCG₇₇ mutants expressed by COS cells.

TgABCG₇₇, TgABCG₈₄, TgABCG₈₉ and TgABCG₈₇ are selectively involved in cholesterol and phosphatidylcholine export

We investigated the respective role of the different TgABCG in lipid distribution in COS cells. These cells were transfected with plasmids harbouring individual TgABCG and allowed to express the parasite transporters for 24 h, with the exception of TgABCG₇₇ for which the expression was limited to 16 h. In order to monitor lipid efflux in the medium, transfected cells were exposed to [³H]cholesterol or [³H]choline for 4 h in the presence of human serum/apoA-I (efflux condition) or not (control condition) before analysis of the

radioactive lipid content in the medium and in cells (Fig. 8A and B). Cells overexpressing TgABCG₇₇, TgABCG₈₄, TgABCG₈₉ and TgABCG₈₇ exhibited a significant stimulation by 270–440% in cholesterol release, and by 250–520% in phosphatidylcholine export in efflux conditions compared with control conditions. In order to validate our morphological observations on COS cells expressing TgABCG₇₇ or derived-mutants, which identify potential residues required for TgABCG₇₇ activity (Fig. 7), we monitored the release of radioactive cholesterol and phosphatidylcholine from cells expressing the TgABCG₇₇ mutants. Table 1 shows that mutations ABCG₇₇^{G335D}, ABCG₇₇^{G335D+k223R} and ABCG₇₇^{G335D+k233Q} strongly abolished lipid export activities (by 88–96%) while mutation ABCG₇₇^{K233Q} had a less moderate inhibitory effect (by 18–50%). Mutant parasites for ABCG₇₇^{K233R} exhibited normal lipid egress activities. These data reveal that *Toxoplasma* possesses four ABCG transporters that function as cholesterol and phosphatidylcholine effluxers.

TgABCG₁₀₇ is implicated in cholesterol influx in cells

Because transfection of cells with TgABCG₁₀₇ and TgABCG₉₆ did not show any significant difference with mock-transfected cells in terms of lipid export from cells, experiments were carried to determine whether TgABCG₁₀₇ and TgABCG₉₆ could be implicated in inward lipid movement into cells. Transfected cells with individual TgABCG were incubated for 16 h in lipoprotein-deficient serum (LPDS), then incubated for 3 h in the presence of [³H]cholesterol or [¹⁴C]phosphatidylcholine before monitoring the amounts of radioactive lipids associated with cells (Fig. 8C). Only TgABCG₁₀₇-overexpressed cells showed a significant accumulation of cholesterol that was four- to fivefold higher than mock-transfected cells or cells expressing any other TgABCG. All TgABCG-transfected cells showed a slight increase in [¹⁴C]phosphatidylcholine uptake that was similar to mock-transfected cells (not shown). Addition of sodium azide or dicyclohexylcarbodiimide to cells overexpressing TgABCG₁₀₇ during the cholesterol uptake experiment resulted in an eightfold inhibition. In contrast to the effect of sodium azide on cholesterol influx, the uptake of [¹⁴C]phosphatidylcholine in a dual labelling assays with [³H]cholesterol were unchanged. The values of [³H]cholesterol uptake were $11\,120 \pm 1158$ and 1446 ± 247 cpm mg⁻¹ cell protein, in the presence or the absence of sodium azide, respectively, while the values of [¹⁴C]phosphatidylcholine uptake were 2120 ± 140 and 1855 ± 301 cpm mg⁻¹ cell protein with or without sodium azide respectively. These data suggest that TgABCG₁₀₇ is uniquely involved in cholesterol intake.

Discussion

Like macrophages, *T. gondii* scavenges large amounts of cholesterol by uncontrolled uptake mechanisms (Coppens *et al.*, 2000). To circumvent the cytotoxicity of cholesterol, conversion of cholesterol into cholesteryl esters and packaging of these lipids into lipid bodies is a mechanism by which parasites control cholesterol accumulation (Nishikawa *et al.*, 2005). In addition, efflux of cholesterol by *Toxoplasma* may represent an efficient sink for cholesterol disposal to correct imbalance in lipid supply and distribution within the parasite. We examined this possibility by characterizing six *Toxoplasma* ABCG membrane transport proteins and investigating their potential relevance for lipid export processes. Our

results reveal a direct involvement of at least four transporters, TgABCG₇₇, TgABCG₈₄, TgABCG₈₉ and TgABCG₈₇, in cholesterol and phospholipid efflux. The sequences of these TgABCG contain a single hydrophilic NBD at the N-terminus and a lipophilic TMD encompassing several transmembrane α -helices at the C-terminal end, which are characteristic features shared by the G family of ABC transporters (Kusuhara and Sugiyama, 2007). Mammalian cells expressing TgABCG₇₇, TgABCG₈₄, TgABCG₈₉ or TgABCG₈₇ show an enhanced outward transport of cholesterol and choline-containing phospholipids, as observed for cells overexpressing mammalian ABCG1, ABCG4 or ABCG5/ABCG8. Synthesized as half-transporters, these TgABCG may also form dimeric complexes like other ABCG members. Because expression of TgABCG₇₇, TgABCG₈₄, TgABCG₈₉ or TgABCG₈₇ alone in cells promotes lipid export, it seems likely that these transporters may function as homodimers, as reported for ABCG1 and ABCG4. In addition to their surface localization, the TgABCG were detected inside the parasite, predominantly in the perinuclear compartment. Although this may be reflective of their normal transit through the bio-synthetic pathway, it is plausible that they can also be involved in intracellular lipid transport processes, as known for ABCG1, ABCG5/ABCG8. Similar to ABCG1 that is regulated by sterol loading in cells, expression of TgABCG₇₇, TgABCG₈₄, TgABCG₈₉ and TgABCG₈₇ is highly responsive to cholesterol status in the parasite. Collectively, these observations pinpoint that TgABCG₇₇, TgABCG₈₄, TgABCG₈₉ and TgABCG₈₇ may be analogous to mammalian ABCG1, acting in synergy to promote cholesterol and phospholipid export from *Toxoplasma*. Therefore, we named these transporters TgABC.G1a (TgABCG₇₇), TgABC.G1b (TgABCG₈₄), TgABC.G1c (TgABCG₈₉) and TgABC.G1d (TgABCG₈₇).

TgABC.G1a-overexpressing cells exhibit long thin projections emanating from their surface, which contain strong immunofluorescent signals for TgABC.G1a. This phenotype may reflect modifications in the lipid composition at the plasma membrane. Similar alterations in cell morphology have been reported for mammalian blood cells after massive incorporation of lipids into the outer leaflet of their plasma membrane (Sheetz and Singer, 1974; Ferrell *et al.*, 1985), and for fibroblasts from Tangier disease patients, which have a marked defect in lipid efflux due to low concentrations of lipid acceptors, e.g. apolipoproteins (Remaley *et al.*, 1997). When lipid acceptors are added in the medium of TgABC.G1a-overexpressing cells, no protrusions from cells are noticeable. This suggests that these long membrane evaginations may also arise from increased amounts of lipids at the plasma membrane. In this case, TgABC.G1a would be implicated in the transfer of intracellular cholesterol and phospholipids from the secretory pathway to the plasma membrane at sites accessible to lipid acceptors. Confirmed, lipid transport-incompetent mutants of TgABC.G1a do not induce any cell alterations. The upregulation of TgABC.G1a expression upon sterol loading in intracellular *Toxoplasma* could thus be a physiological response to remove excess cellular cholesterol. Identification of potential parasite lipid acceptors in the PV might help to clarify the cellular mechanisms by which lipids are effluxed from *T. gondii*. Interestingly, the parasite secretes a lecithin : cholesterol acyltransferase (LCAT) homologue into the PV (V. Pszeny, B. Lige and I. Coppens, in preparation). Similar to mammalian LCAT (Rousset *et al.*, 2009), this enzyme may then contribute to the process of lipid efflux in maintaining cholesterol homeostasis and regulating cholesterol transport in the parasite environment.

The active exportation of lipids mediated by *Toxoplasma* may be regarded as an evolutionary conserved mechanism by which eukaryotic cells control the fluidity of their plasma membrane by adjusting the lipid levels and composition within this membrane. In addition to that function, it is reasonable to assume that the parasite needs to export large amounts of lipids into the PV in order to expand the size of the PV membrane during replication (parasite doubling time of ~7 h). So far, neither the mechanisms by which *Toxoplasma* adds new lipids to the PV membrane nor the lipid composition of this membrane are known. It has been described that intravacuolar *T. gondii* can secrete large sheets of tubular membranes, which form a tubulo-vesicular network (TVN) that extends into the PV (Sibley *et al.*, 1995). One attractive possibility would be that this TVN fuse with the PV membrane, which would result in a bulky increase of the PV surface. An alternative scenario would be the incorporation of selective lipids that are effluxed by the parasites into the PV membrane. In this case, lipids originating from the host cell must first be transported to the parasite before being redistributed among organelles and the PV. This process implies sensing mechanisms to precisely control membrane lipid content and to deliver specific lipids to proper compartments. It would also necessitate lipid transport machineries operational in the PV lumen. To this point, the TVN may play a role in accepting the lipids deloaded from the plasma membrane and transporting them to the vacuolar membrane. The large repertoire of TgABC.G1 proteins expressed by the parasite may reflect the importance of lipid efflux activities for the PV maintenance and/or the PV membrane expansion during the intracellular tenure of *Toxoplasma*.

TgABCG₉₆ mRNA levels are unresponsive to exogenous sterols and this protein does not show any activity in moving cholesterol and choline-containing phospholipids across the plasma membrane. TgABCG₉₆ shares the structural features and functional motifs harboured by all TgABC.G1. It is plausible that TgABCG₉₆ is then involved in the intake and/or disposition of lipophilic molecules other than sterols and phospholipids, as reported for ABCG2 that pumps out of the cells steroids and hydro-phobic xenobiotics (Kusuhara and Sugiyama, 2007). Interestingly, the protozoan parasite *Leishmania* expresses two different ABCG-like transporters (ABCG4 and LiABCG6) that confer resistance to several leishmamicidal agents. This phenotype has been ascribed to enhance outward transport of anti-*Leishmania* drugs due to the efflux activity of ABCG4 and LiABCG6 (Castanys-Muñoz *et al.*, 2007; 2008). From a pharmacological point-of-view, identification of the nature of the substrates transported by TgABCG₉₆ might reveal novel activities for this protein. Additionally, it could be interesting to examine whether TgABCG₇₇, TgABCG₈₄, TgABCG₈₉ and TgABCG₈₇ can transport various lipophilic molecules different from cholesterol and phosphatidylcholine. At least, our data reveal that *Toxoplasma* does not expel fatty acids through a classical ABC transport-like process.

Our previous studies demonstrated that cholesterol uptake by *Toxoplasma* requires high affinity-binding sites (Sehgal *et al.*, 2005). Cells expressing TgABCG₁₀₇ selectively accumulate larger amounts of cholesterol in an ATP-dependent process compared with untransfected cells. This entails that TgABCG₁₀₇ may be responsible for cholesterol internalization across the parasite plasma membrane. The concept that some ABC transporters can mediate the opposite (i.e. inward) transport of lipids has been previously

assessed for yeast in which two ABCG1-like transporters (AUS1 and PDR11) are implicated in sterol uptake under conditions when sterol biosynthesis is compromised (e.g. under anaerobiosis; Wilcox *et al.*, 2002). By analogy to anaerobic yeast, *Toxoplasma* cannot produce any sterol molecules and the parasite survival in mammalian cells depends on its ability to divert and internalize host sterols to its own cell. The localization of TgABCG₁₀₇ in the PV and at the plasma membrane is coherent with a function of cholesterol trafficking in the vacuole and the delivery of this lipid to the parasite interior mediated by TgABCG₁₀₇. These data strengthen the assertion that ABCG transporters could have been exploited to perform diverse roles in function of specific needs of a cell. Evidently, current functional data on ABCG transporters in lipid auxotroph-organisms expand our view on the versatile role of ABC transporters in eukaryotic sterol influx.

Experimental procedures Chemicals and antibodies

All chemicals were obtained from Sigma (St Louis, MO) or Fisher (Waltham, MA) unless indicated otherwise. Solvents and standards for chromatography were of the highest analytical grade. Silica gel 60 thin layer chromatography plates were from EM Science (Gibbstown, NJ). Radiolabelled reagents included [1,2-³H]cholesterol (sp. act: 48 mCi mmol⁻¹), [1-¹⁴C]cholesterol (sp. act: 24 mCi mmol⁻¹), phosphatidyl [N-methyl-¹⁴C]choline (sp. act: 55 mCi mmol⁻¹) and [methyl-¹⁴C]choline (sp. act: 52 mCi mmol⁻¹) from Amersham Corp. (Arlington Heights, IL), [1-¹⁴C]palmitic acid (sp. act: 55 mCi mmol⁻¹) and [5,6-³H]uracil (sp. act: 40 Ci mmol⁻¹) from PerkinElmer (Waltham, MA). Human apoA-I was commercially obtained (BioDesign, Saco, ME). Commercial primary antibodies were: rabbit anti-V5 antibodies were a generous gift from Immunology Consultants Laboratory (Newberg, OR), mouse anti-HA antibodies were purchased from Covance (Emeryville CA), and anti-EEA1 antibodies were from Abcam (Cambridge, MA). The primary antibody against ROP2.3.4 is a generous gift of J-F Dubremetz. Goat anti-rabbit and goat anti-mouse antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 used as secondary antibody for immunofluorescence assays were from Molecular Probes (Eugene, OR).

Mammalian cell lines, culture conditions and parasite propagation

Mammalian cell lines used included: primary human foreskin fibroblasts (HFF) and COS-7 cells. All these cells were grown as monolayers and cultivated in α -minimum essential medium (α -MEM) supplemented with 10% FBS, 2 μ M glutamine and penicillin/streptomycin (100 units ml⁻¹ per 100 μ g ml⁻¹) as described (Coppens *et al.*, 2000). The tachyzoite RH strain of *T. gondii* was propagated *in vitro* by serial passage in HFF, and parasite viability was assessed by uracil incorporation assays (Roos *et al.*, 1994).

Cloning of full-length cDNA encoding TgABCG

Several sequences corresponding to specific domains of the ABCG superfamily were identified in the genome of *T. gondii* and in ESTs (*Toxoplasma* database: <http://www.ToxoDB.org>). The ORF of six TgABCG-like transporters was directly amplified from *T. gondii* RNA. Total RNA was extracted from *Toxo-plasma* with the Qiagen RNeasy MiniKit (Valencia, CA), treated with DNase (RNase-free) to digest any contaminating

genomic DNA and then amplified using primer pairs spanning the predicted coding sequences of *ABCG* with the Invitrogen SuperScript III First Strand Synthesis System (Carlsbad, CA). Amplified fragments were ligated via TA cloning into TOPO vector pCDNA2.1 (Invitrogen Life Technologies). Vector specific M13 forward and M13 reverse oligonucleotides were used for priming the sequencing reactions. The *TgABCG* sequences were verified by the Johns Hopkins Sequencing Facility by comparison with contigs from the *Toxoplasma* database. The coding sequences were: TGME_49 chrIX minus strand nucleotides 2900608–2905559 for *TgABCG₉₆*, TGME_49 chrXI minus strand nucleotides 4811505–4817223 for *TgABCG₇₇*, TGME_49 chrXII minus strand nucleotides 3379250–3389013 for *TgABCG₁₀₇*, TGME_49 chrIX plus strand nucleotides 3309984–3321682 for *TgABCG₈₄*, TGME_49 chrIX minus strand nucleotides 3828389–3842205 for *TgABCG₈₉* and TGME_49 chrVI minus strand nucleotides 3115836–3123612 for *TgABCG₈₇*. The identification of spliced RNA products was determined with the Invitrogen Super-Script One-Step RT-PCR with Platinum Taq. The resulting sequences were compiled with care to identify any unspliced products. The genomic and cDNA sequences coding for *TgABCG₉₆*, *TgABCG₇₇*, *TgABCG₁₀₇*, *TgABCG₈₄* and *TgABCG₈₉* have been deposited in GenBank under the accession numbers of GU296659, GU296662, GU797442, GU296660 and GU296661 respectively.

RACE identification of 5' and 3' regions for *TgABCG₉₆*, *TgABCG₇₇* and *TgABCG₈₄*

RNA was isolated as mentioned above and the First Choice RLN-RACE kit from Ambion (Austin, TX) was used to extend and identify the 5' and 3' untranslated regions of *TgABCG₉₆*, *TgABCG₇₇* and *TgABCG₈₄* obtained from cDNA, in addition to the poly A tail and upstream UTRs. Briefly, nested inner and outer gene specific primer pairs for both 5' and 3' regions (denoted in Table S3 as: 5' or 3' and I (inner) or O (outer) oligonucleotides) were used to generate cDNA in tandem with kit supplied 5' RACE and 3' RACE adapter primers. Results were compared with the genomic sequences surrounding the cDNA sequences of the predicted genes.

Recombinant peptide expression in *Escherichia coli*

The pQE-30 plasmids (Qiagen, Hilden, Germany) containing 6xHis tags have been engineered to produce recombinant peptides derived from each *ABCG* gene in fusion with the *His* sequences at the 5' end of the inserts. Table S3 summarizes the list of primers used in this study. pQE-*ABCG₉₆* contains the nucleotide sequence coding for amino acids 2–53 of *TgABCG₉₆* that have been inserted into the KpnI and HindIII restriction sites of the vector for amplification using the oligo-nucleotides *fPQE30A* and *rPQE30A*. pQE-*ABCG₇₇* harbours the sequence encoding amino acids 2–55 of *TgABCG₇₇* inserted into the BamHI and HindIII sites using the oligo-nucleotides *fPQE30B* and *rPQE30B*. pQE-*ABCG₁₀₇* contains the sequence corresponding to amino acids 109–162 of *TgABCG₁₀₇*, cloned into the BamHI to HindIII site with the oligonucleotides *fPQE30C* and *rPQE30C*. pQE-*ABCG₈₄* contains the sequence for amino acids 2–76 of *TgABCG₈₄* inserted into the BamHI and HindIII sites using the oligo-nucleotide *fPQE30D* and *rPQE30D*. Finally, pQE-*ABCG₈₉* contains the sequence coding for amino acids 2–61 of *TgABCG₈₉* that was introduced into the BamHI and HindIII sites using the oligonucleotide *fPQE30E* and *rPQE30E*. The *E. coli* M15[pREP4] strain was used to produce the recombinant peptides that were then purified

under native condition on Ni²⁺-NTA resin according to the Qiagen protocol. The His-tagged peptides were used to generate rabbit polyclonal antisera (Covance, Denver, PA). Before use, the antibodies were affinity-purified using the AminoLink Immobilization Kit (Thermo Scientific, Waltham, MA) following manufacturer's instructions.

Plasmid constructions for expression in *Toxoplasma*

To monitor the expression of *TgABCG*₉₆, *TgABCG*₇₇, *TgABCG*₁₀₇, *TgABCG*₈₄ and *TgABCG*₈₉ in the parasite by direct fluorescence, plasmids containing the sequence of *YFP* at the C-terminus (pACP-YFP-sagCatsag) or *GFP* at the N-terminus (pACP-GFP-sagCatsag) from the Roos laboratory (University of Pennsylvania) were used to express the fusion proteins under the *T. gondii* tubulin promoter. The *TgABCG* genes were individually amplified from parasite RNA by reverse transcription-PCR (Table S3). The *fYFPA* and *rYFPA* oligonucleotides were used to insert *TgABCG*₉₆ into the BglII and AvrII restriction sites of vector pACP-YFP-sagCatsag, creating the plasmid pYFP-ABCG₉₆. *TgABCG*₁₀₇ was similarly cloned into pACP-YFP-sagCatsag using the oligonucleotides *fYFPC* and *rYFPC* to create the plasmid pYFP-ABCG₁₀₇. pACP-GFP-sagCatsag was used to clone *TgABCG*₇₇, *TgABCG*₈₄ and *TgABCG*₈₉ into the BglII and AflIII sites with the oligonucleotide *fGFPB* and *rGFPB* (for pGFPABCG₇₇), *fGFPD* and *rGFPD* (for pGFP-ABCG₈₄), and *fGFPE* and *rGFPE* (for pGFP-ABCG₈₉). For expression analysis in *T. gondii*, *Toxoplasma* were transfected by electroporation using the Genetronics ECM 630 Electro Cell Manipulator (San Diego, CA) according to published protocols (Roos *et al.*, 1994) and fluorescence associated with parasites was detected between 16–36 h post-transfection.

Generation of a stable line of *T. gondii* expressing *TgABCG*₈₇

The plasmid pHX-NTPHA was used to generate a stable line of *Toxoplasma* expressing *TgABCG*₈₇ with a carboxy-terminal HA tag under the nucleoside triphosphatase III (NTPase) promoter. The *TgABCG*₈₇ gene was cloned into the NcoI and NheI restriction sites using the primers *fPHXF* and *rPHXF*, creating the plasmid pHX-ABCG₈₇. Parasites expressing *TgABCG*₈₇-HA were selected using mycophenolic acid and xanthine, and cloned by limiting dilution to ensure stable integration of the construct as described (Roos *et al.*, 1994).

Plasmid construction for expression in mammalian cells

The pCDNA3.1(+) plasmid from Invitrogen was engineered for localization and functional studies of *TgABCG* in mammalian cells. Two different 3' sequence tags, *HA* and *V5*, were cloned individually into the XbaI and ApaI sites, creating plasmids pCDNA3.1HA and pCDNA3.1V5 in order to visualize two different *TgABCG* after cotransfection of their genes in a single cell. The *V5* tag was inserted into pCDNA3.1(+) using annealed oligonucleotides *fV5* and *rV5*, whereas the *HA* tag was inserted into the plasmid using annealed oligo-nucleotides *fHA* and *rHA*. For *TgABCG*₉₆ cloning into the two vectors, the gene was amplified with primer pairs, *fPCDNA3.1A* and *rPCDNA3.1A*, and inserted into the HindIII and XbaI sites of both pCDNA3.1V5 and pCDNA3.1HA, creating the plasmids pCDNA3.1-ABCG₉₆V5 and pCDNA3.1-ABCG₉₆HA. For *TgABCG*₇₇ cloning, the gene was

amplified using the *f*PCDNA3.1B and *r*PCDNA3.1B, and inserted into the HindIII and XhoI sites, creating plasmids pCDNA3.1-ABCG₇₇V5 and pCDNA3.1-ABCG₇₇HA. For *TgABCG*₁₀₇ cloning, the gene was amplified with oligonucleotides *f*PCDNA3.1C and *r*PCDNA3.1C, then cloned into the HindIII and XbaI sites, creating plasmids pCDNA3.1-ABCG₁₀₇V5 and pCDNA3.1-ABCG₁₀₇HA. pCDNA3.1-ABCG₈₄HA and pCDNA3.1-ABCG₈₄V5 were created by amplification of the gene with oligonucleotides *f*PCDNA3.1D and *r*PCDNA3.1D, and cloned into the HindIII and XbaI sites. For *TgABCG*₈₉ cloning, pCDNA3.1-ABCG₈₉HA and pCDNA3.1-ABCG₈₉V5 were synthesized by PCR amplification of DNA using oligo-nucleotides *f*PCDNA3.1-E and *r*PCDNA3.1E, and these fragments were cloned into the NheI and NotI sites. Finally *TgABCG*₈₇ DNA was amplified with oligonucleotides *f*PCDNA3.1F and *r*PCDNA3.1F and inserted into the HindIII and XbaI sites creating pCDNA3.1-ABCG₈₇HA and pCDNA3.1-ABCG₈₇V5. All of the plasmids contained the mammalian Kozak sequence GCC located directly upstream of each ATG initiation methionine, and were fused in frame with the tag peptides, HA or V5. For expression analysis in mammalian cells, the HA and V5 tagged constructs of the *TgABCG* genes in pCDNA3.1(+) were transfected in COS-7 cells using lipofectamine (Invitrogen) following manufacturer's instructions.

Mutations of the Walker A/Signature sequence of *TgABCG*₇₇

To introduce mutations in the Walker A and/or Signature sequences, *TgABCG*₇₇ was used as a template. The invariant lysine 233 in Walker A was replaced by arginine by fusion PCR. A 5' PCR fragment was generated using *f*PCDNA3.1B and *r*WAB(K To R) while a 3' PCR fragment was generated using *f*WAB(K To R) and *r*PCDNA3.1B. These two PCR products were added together and reamplified with *f*PCDNA3.1B and *r*PCDNA3.1B. The complete PCR product was cloned into pCDNA3.1HA and pCDNA3.1V5 at the HindIII and XhoI restriction sites to create plasmids pmutWARHA and pmutWARV5. The second mutation in Walker A was the replacement of K²³³ by glutamine. The 5' PCR fragment was generated using *f*PCDNA3.1B and *r*WAB(K To Q). The 3' PCR fragment was generated by the amplification of *TgABCG*₇₇ with *f*WAB(K To Q) and *r*PCDNA3.1B. These two PCR products were joined and amplified with the oligonucleotides *f*PCDNA3.1B and *r*PCDNA3.1B to generate plasmids pmutWAQHA and pmutWAQV5. We created a mutation of glycine 335 within the Signature sequence of *TgABCG*₇₇ by generating a 5' product using *f*PCDNA3.1B and *r*SIGB(G To D). The 3' portion was generated with the *f*SIGB(G To D) and *r*PCDNA3.1B oligonucleotides. These two products were ligated and amplified with *f*PCDNA3.1B and *r*PCDNA3.1B to create pmutSHA and pmutSV5. Utilizing the upstream HindIII site and a unique AfeI site between the Walker A and Signature sequences, we created plasmids containing *TgABCG*₇₇ in which a double mutation has been introduced: one in Walker A (K To R or Q) and another one in the Signature sequence (G to D). We named these plasmids pmutWAR + SHA for mutant ABCG₇₇^{G335D+K233R} and pmutWAQ + SHA for mutant ABCG₇₇^{G335D+K233Q}.

Immunoblot analysis of parasites

For immunodetection, $1-2 \times 10^7$ parasites were lysed in RIPA solution containing 100 $\mu\text{g ml}^{-1}$ PMSF, 1 μM sodium ortho-phosphate and 50 $\mu\text{g ml}^{-1}$ aprotinin. Parasite extracts were suspended in SDS gel-loading buffer (50 μM Tris-HCl at pH 6.8) containing 2% SDS and

50 μ M 2-mercaptoethanol plus 0.1% bromophenol blue and 10% glycerol. The samples were subjected to SDS-PAGE, and the proteins were electro-phoretically transferred to a membrane (Immobilon Transfer Membranes, Millipore, Bedford, MA). The membranes were immersed in blocking buffer (PBS containing 3% skim milk) for 60 min, and then exposed to anti-TgABCG antibodies (1:1000 dilution) for 60 min before incubation with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Amersham Pharmacia Biotech; dilution, 1:15 000) and detection by chemiluminescence using ECL-Plus (GE Health, UK).

Chemical cross-linking assays

To study the formation of TgABCG dimers, freshly lysed-out parasites ($2-3 \times 10^7$ wild-type or transgenic parasites expressing TgABCG₈₇-HA) were subjected to cross-linking using the homobifunctional, cleavable cross-linking reagent dithiobis(succinimidylpropionate) (DSP; Pierce-ThermoFisher, Rockford, IL) at 1 μ M for 30 min at room temperature, according to manufacturer's instructions before analysis by SDS-PAGE and immunodetection by Western blotting with anti-ABCG antibodies (at 1/100–1,2000) or anti-HA antibodies (at 1/2500). In some experiments, samples were incubated with DTT after the DSP treatment to reductively break the cross-link of TgABCG.

Quantitative reverse transcription-PCR

The potential regulation of *TgABCG* in *Toxoplasma* in response to sterol loading and deloading was assessed by quantitative reverse transcription-PCR. HFF were heavily parasitized (MOI of 6) and washed after 2 h to remove non-invaded parasites and to replace the medium by α -MEM containing: (i) 10% FBS, (ii) 10% LPDS (prepared as described in Coppens *et al.*, 2000), (iii) 10% FBS plus 10 μ M 22(R)-hydroxycholesterol, or (iv) 2 μ g ml⁻¹ LDL (purified according to Poumay and Ronveaux-Dupal, 1984). After 48 h, the parasites were isolated from cells and purified as described (Sehgal *et al.*, 2005). Total RNA was isolated from *Toxoplasma* as described above. DNase-treated RNA was reverse-transcribed using oligodT primers, and 180 ng was used for cDNA synthesis. One hundred ng of cDNA was used for real-time PCR experiments. Oligonucleotides designed for annealing the unique regions of each respective *TgABCG* included (Table S3): *fRTABCG*₉₆ and *rRTABCG*₉₆, *fRTABCG*₇₇ and *rRTABCG*₇₇, *fRTABCG*₁₀₇ and *rRTABCG*₁₀₇, *fRTABCG*₈₄ and *rRTABCG*₈₄, *fRTABCG*₈₉ and *rRTABCG*₈₉, and *fRTABCG*₈₇ and *rRTABCG*₈₇. Amplification of the housekeeping gene *actin* by *fRTactin* and *rRTactin* served to normalize input RNA amounts. Quantitative PCR was performed in triplicates using SyBr Green-based Applied Biosystems' protocols on the ABI PRISM 7000 (Applied Biosystems, Foster City, CA). The sizes of the PCR products were: 258 bp (TgABCG₉₆), 180 bp (TgABCG₇₇), 594 bp (TgABCG₁₀₇), 249 bp (TgABCG₈₄), 215 bp (TgABCG₈₉), 248 bp (TgABCG₈₇) and 198 bp (Tgactin). To determine the fold induction of the real-time PCR, the following two formulas were used: $2^{(\text{average actin value of the treated sample} - \text{average actin value of the untreated sample})}$ was determined. The second value was $2^{(\text{average actin value of the untreated sample (10% FCS)} - \text{average actin value of the gene untreated})}$. The fold induction is the treated value divided by the untreated value. Values of relative fold induction above 1 indicate upregulation, whereas values lower than 1 indicates downregulation.

Cellular lipid efflux assays

From *Toxoplasma*: HFF were infected with *T. gondii* at the MOI of 5–10 and cultured in α -MEM plus 10% FBS. Six hours post-infection (p.i.), infected cells were labelled with 2 mCi ml⁻¹ of [³H]cholesterol for cholesterol efflux, 2 μ Ci ml⁻¹ [³H]choline for phospholipid efflux or 25 μ Ci ml⁻¹ [1-¹⁴C]palmitic acid for fatty acid efflux. Next day, infected cells were washed three times with PBS, and parasites were isolated from cells and purified as described (Sehgal *et al.*, 2005). Parasites were resuspended at the density of 10⁷ ml⁻¹ in α -MEM plus 10% FBS in the presence or the absence of 15 μ M sodium azide + 15 μ M 2DG for 30 or 60 min at 37°C. After centrifugation, the media were collected and counted for radioactivity by liquid scintillation counting (fatty acid and cholesterol efflux assays) or extracted with hexane/ isopropanol (3:2) solution and then counted (phospholipid efflux assays). Cells were dissolved with 0.1 N NaOH and 0.2% SDS (fatty acid and cholesterol efflux assays) or extracted with hexane/isopropanol solution (phospholipid efflux assays), and radioactivity remaining in cells was determined. For each assay, the reduction in intracellular ATP content in extracellular parasites treated with ATP poisons was properly verified by firefly bioluminescence using the luciferase assay kit according to the manufacturer's protocols (Promega, Madison, WI). Briefly, 100 ml of parasite suspensions from each sample were mixed with 100 ml of luciferinluciferase solution and the mixture was incubated at room temperature. After 10 min, the produced bioluminescence signal was measured in a Tecan spectrophotometer (Tecan, Männedorf, Switzerland). When ATP is the limiting component in a luciferase reaction, the intensity of light emitted is proportional to the concentration of ATP in parasite extracts.

From transgenic COS cells: mock transfected COS cells or COS cells transfected with plasmids harbouring the sequences of individual *TgABCG* were used to assess the function of *TgABCG*. Twelve to 24 h post-transfection, cells were incubated with 0.5 μ Ci ml⁻¹ of [³H]cholesterol or 0.5 μ Ci ml⁻¹ [³H]choline for 4 h in an efflux medium (α -MEM containing 10% human serum and 10 μ g ml⁻¹ human apoA-I) or in a medium containing α -MEM plus 1% bovine serum albumin (BSA) before washing and analysis of radioactive lipid content in the media and in cells as described above.

Cellular lipid accumulation assays

Mock transfected COS cells or *TgABCG*-transfected COS cells were transferred in α -MEM plus 10% LPDS 16 h after transfection. The next day, cells were incubated in the same medium containing [³H]cholesterol-cyclodextrin complexes, [¹⁴C]phosphatidylcholine conjugated to BSA (Coppens *et al.*, 1995; Sehgal *et al.*, 2005) for 3 h. The net accumulation of radioactive lipids in cells was measured by scintillation counting. The energetics of lipid transport were investigated by inclusion of 2 μ M dicyclohexylcarbodiimide (DCCD) or 1 μ M sodium azide for a 20 min pretreatment period followed by co-incubation with the [³H]cholesterol or [¹⁴C]phosphatidylcholine for 1 h.

Light and electron microscopy studies

Light and epifluorescence microscopy were performed on infected cells seeded on sterile coverslips in 24-well culture dishes using a Nikon Eclipse E800 microscope equipped with a Spot RT CCD Camera and processed using Image-Pro-Plus software (Media Cybernetics,

Silver Spring, MD) before assembly using Adobe Photoshop (Adobe Systems, Mountain View, CA). Immunofluorescence assays on parasites or mammalian cells were performed as previously described (Lige *et al.*, 2009) by using as primary antibodies: affinity-purified anti-TgABCG (1:100–250) antisera, anti-EEAI (1:10), anti-V5 (1:40) or anti-HA (1:1000) antibodies, revealed by secondary antibodies conjugated to either Alexa 488 or Alexa 594 (1:2000). For immunoelectron microscopy (immunoEM) on transgenic parasites, *Toxoplasma*-infected cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.25 M HEPES (pH 7.4) for 1 h at room temperature, then in 8% paraformaldehyde in the same buffer overnight at 4°C. They were infiltrated, frozen and sectioned as previously described (Coppens *et al.*, 2006). The sections were immunolabelled with anti-HA antibodies (1:100 in PBS/1% fish skin gelatin), then with mouse anti-rabbit IgG antibodies, followed directly by 10 nm protein A-gold particles (Department of Cell Biology, Medical School, Utrecht University, the Netherlands) before examination with a Philips CM120 Electron Microscope under 80 kV.

Protein determination

Protein content was determined by the Bradford (Smith *et al.*, 1985) assay, using serum bovine albumin (BSA) as a standard.

Statistical analysis

Student's *t*-test was used for analysis of statistical significance between means.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Quantitative analysis of lipid export from *Toxoplasma*. After host cell infection for 6 h, intracellular parasites were preloaded with either [³H]cholesterol (A) or [³H]choline (B) for 24 h, then isolated from mammalian cells, washed and resuspended in medium plus serum for 30 or 60 min with or without sodium azide/2-deoxyglucose (NaN₃/2DG) to assess the efflux of radioactive cholesterol or choline-containing phospholipids. Arbitrary values of luminescence/emission (lum/E) that are proportional to the intracellular ATP content are indicated. Percentages of ATP decrease in parasites exposed to ATP poisons compared with untreated parasites are shown in parentheses. After centrifugation, supernatant and parasites were separately collected, and the radioactivity was measured by liquid scintillation counting. The mean values in cpm in the media and parasite fractions are presented in Supporting Information. Efflux was determined as a fraction of the cpm in media over the total (cpm in media ± cpm in parasites). Data represent means ± SD of two independent experiments, each done in triplicate. Statistical values for comparison between conditions with and without sodium azide are **P* < 0.05 and ***P* < 0.01.

Fig. 2. Characteristic features of the G family of ABC transporters in TgABCG sequences and comparison of the conserved motifs by sequence alignment.

A. Schematic representation of the primary sequences of the six TgABCG obtained after cloning showing the position of the predicted Walker A domain (WA), the Gln loop (Gln), the Signature (Sig), the Walker B domain (WB), the His loop (His) and the transmembrane domains (in black).

B. Sequence alignment of the predicted Walker A domain (in blue), the Gln loop (in yellow), the Signature (in turquoise), the Walker B domain (in pink) and the His loop (in green) among the six TgABCG. Identical amino acids are pinpointed by an asterisk; similar and conserved amino acids are designated by two or one dot respectively.

Fig. 3.

Expression of TgABCG in wild-type and transgenic parasites.

A. Immunoblots of total lysates of wild-type *T. gondii* (lanes a and c) or HFF (lanes b).

Western blot analyses of the gel were probed with antibodies against recombinant ABCG peptides (lanes b and c) or preimmune serum (lanes a). No band was observed after probing lysates of HFF in which the parasites have been cultivated.

B. Immunoblots of total lysates of wild-type *T. gondii* (wt) parasites or parasites stably expressing the construct TgABCG₈₇-HA (HA-tg). After solubilization, cell preparations

were centrifuged, and the supernatant (S) and pellet fractions (P) were subjected to Western blot analyses using anti-HA antibodies. Arrows show the full-length protein in reductive conditions.

C. Dimerization of *Toxoplasma* ABCG. Parasites were exposed with DSP alone before lysis, or with DSP followed by DTT treatment. Proteins were then separated by 7.5% polyacrylamide gel electrophoresis and immunodetected. Arrowheads show the position of the dimers (D) in immunoblots relative to that of monomers (M).

Fig. 4.

Localization of TgABCG in intracellular parasites by fluorescence microscopy.

Immunofluorescence assays on intravacuolar *T. gondii* using anti-TgABCG antibodies on wild-type parasites except for TgABCG₈₇-HA-expressed parasites revealed by anti-HA antibodies. To assess the distribution of TgABCG₁₀₇ in the vacuolar space, double staining assays were performed using antibodies against TgABCG₁₀₇ and ROP2.3.4, discharged into the PV from the secretory organelle rhoptries. Preimmune sera did not react with the parasites (not shown). DAPI, in blue.

Fig. 5. Distribution of TgABCG in intracellular parasites by immunoEM. **A.** Immunogold staining with anti-TgABCG antibodies on wild-type parasites except for TgABCG₈₇-HA-expressed parasites revealed by anti-HA antibodies. The cryosection shown in **a** has been labelled for localizing TgABCG₉₆, **b** for TgABCG₈₇, **c** and **c'** for TgABCG₈₉ (arrows show labelled vesicles), **d** and **d'** for TgABCG₁₀₇ (arrow pinpoints the plasma membrane, PPM) and **f** for TgABCG₈₄. For each immunogold staining, representative pictures of parasites have been selected. n, nucleus; PVM, PV membrane. Bars are 150 nm except for **c'** and **d'** (50 nm). **B.** Stereological analysis of gold labelling demonstrating the specificity in localization of the TgABCG for various compartments in intracellular *T. gondii*. Density (gold particles per μM^2) of labelled structures was determined from 15 to 25 cellular cryosections. Percentage of individual intracellular compartment density was determined from the sum of gold density normalized for the variation in expression of each TgABCG. NE, nuclear envelop; PM, plasma membrane; nd, not detected.

Fig. 6.

Influence of exogenous sterols on TgABCG expression in *Toxoplasma*. Quantitative RT-PCR on TgABCG levels. Intracellular *T. gondii* were incubated for 48 h in medium containing 10% lipoprotein-deficient medium (LPDS), 10% LPDS plus 10 μ M 22(*R*)-hydroxycholesterol (+22-HC), 10% normal serum plus 2 μ g ml⁻¹ of LDL (+LDL) or 10% normal serum (control). Total parasite RNA was isolated and quantified by RT-PCR using specific primers against individual TgABCG and actin. The induction fold was measured as described in *Experimental procedures*. Data are means $\pm$ SD for six independent experiments, each done in triplicate.

Fig. 7.

Localization and cytotoxicity of TgABCG₇₇ expressed in COS cells.

A–B. Fluorescence microscopy. In (A), COS cells were transfected with a plasmid coding for *TgABCG₇₇-HA* for 16 h to determine the localization of the transporter with anti-HA antibodies. In another set of experiments, COS were cotransfected with two plasmids, one encoding *TgABCG₈₄-V5* and the other one *TgABCG₇₇-HA* for 36 h before immunofluorescence assays to detect V5 and HA signals, in red and green respectively. Arrow shows plasma membrane protrusions. In (B), COS cells were transfected with a plasmid harbouring the wild-type sequence of TgABCG₇₇ (control) or plasmids with punctual mutations either in the ABCG Signature (Sig) or the Walker A domain (Walk A), or both, as indicated. Constructs were HA tagged and 24 h post-transfection, cells were immunostained with anti-HA antibodies.

Fig. 8.

Biological activities of TgABCG expressed by COS cells. A–B. Quantitative assays for lipid efflux from cells. In (A), COS cells were transfected for 16–24 h with each individual *Toxoplasma* ABCG or the vector alone before incubation with [³H]cholesterol (A) or [³H]choline (B) in an efflux medium containing human serum and human apoA-I (EF; white histograms) or in a medium containing BSA only (ctl; black histograms) to monitor the efflux of radioactive cholesterol or phosphatidylcholine. After 24 h, media and cells were separately collected, and the radioactivity was measured by liquid scintillation counting. In (A), the mean values in cpm in the media and cells are presented in Supporting Information. Data represent means $\pm$ SD of three independent experiments, each done in triplicate. Statistical values for comparison between conditions in control and efflux medium are $*P < 0.05$ and $**P < 0.01$.

C. Quantitative assays for cholesterol intake by cells. TgABCG-transfected COS cells or cells transfected with the vector alone were transferred in medium without lipoproteins 24 h following transfection. Sixteen hours later, cells were incubated with [³H]cholesterol for 3 h, washed and the radioactivity associated to the cells was measured by scintillation counting. In inset, the same protocol has been applied on TgABCG₁₀₇-transfected COS cells except that dicyclohexylcarbodiimide (DCCD) or sodium azide was added to the medium before

and during the exposure to radioactive cholesterol for 1 h, compared with untreated cells (ctl). Data are means $\pm$ SD of three experiments done in duplicate each. The statistical value for comparison between mock- and TgABCG₁₀₇-transfected cells is $**P < 0.01$.

Table 1Lipid egress activities mediated by ABCG₇₇ and derived-mutants.

Transfection conditions	³ H]cholesterol (cpm x 100)			¹⁴ C]phosphatidylcholine (cpm x 100)		
	Medium	Cell	(Efflux %)	Medium	Cell	(Efflux %)
+Vector	31 ± 5	5741 ± 335	(0.5)	28 ± 5	3944 ± 299	(0.7)
+ABCG ₇₇	299 ± 31	4153 ± 202	(6.7)	119 ± 14	5148 ± 615	(2.3)
+Mutant ABCG ₇₇ ^{G335D}	25 ± 6	4895 ± 370	(0.5)*	13 ± 3	3982 ± 417	(0.3)*
+Mutant ABCG ₇₇ ^{K233Q}	154 ± 18	4251 ± 412	(3.5)*	76 ± 5	3846 ± 320	(1.9)
+Mutant ABCG ₇₇ ^{K233R}	345 ± 27	5562 ± 509	(5.8)	121 ± 13	5002 ± 236	(2.3)
+Mutant ABCG ₇₇ ^{G335D+K233R}	21 ± 5	5015 ± 214	(0.4)*	8 ± 0.7	3745 ± 193	(0.2)*
+Mutant ABCG ₇₇ ^{G335D+K233Q}	34 ± 5	4129 ± 319	(0.8)*	17 ± 3	4125 ± 348	(0.4)*

Quantitative assays for lipid efflux from cells were performed in an efflux medium as described in the legend of Fig. 8. Data are means ± SD of three experiments done in triplicate each. Statistical values comparing TgABCG₁₀₇[−] and mutant TgABCG₁₀₇[−]-transfected cells are

* P < 0.05 and

** P < 0.01.