Host cell lipids control cholesteryl ester synthesis and storage in intracellular Toxoplasma

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Summary

The intracellular protozoan Toxoplasma gondii lacks a de novo mechanism for cholesterol synthesis and therefore must scavenge this essential lipid from the host environment. In this study, we demonstrated that T. gondii diverts cholesterol from low-density lipoproteins for cholesteryl ester synthesis and storage in lipid bodies. We identified and characterized two isoforms of acyl-CoA:cholesterol acyltransferase (ACAT)-related enzymes, designated TgACAT1α and TgACAT1β in T. gondii. Both proteins are coexpressed in the parasite, localized to the endoplasmic reticulum and participate in cholesteryl ester synthesis. In contrast to mammalian ACAT, TgACAT1α and TgACAT1β preferentially incorporate palmitate into cholesteryl esters and present a broad sterol substrate affinity. Mammalian ACAT-deficient cells transfected with either TgACAT1α or TgACAT1β are restored in their capability of cholesterol esterification. TgACAT1α produces steryl esters and forms lipid bodies after transformation in a Saccharomyces cerevisiae mutant strain lacking neutral lipids. In addition to their role as ACAT substrates, host fatty acids and low-density lipoproteins directly serve as Toxoplasma ACAT activators by stimulating cholesteryl ester synthesis and lipid droplet biogenesis. Free fatty acids significantly increase TgACAT1α mRNA levels. Selected cholesterol esterification inhibitors impair parasite growth by rapid disruption of plasma membrane. Altogether, these studies indicate that host lipids govern neutral lipid synthesis in Toxoplasma and that interference with mechanisms of host lipid storage is detrimental to parasite survival in mammalian cells.

Introduction

Sterols are found in all eukaryotic organisms, and are membrane components that regulate the fluidity and the permeability of phospholipid bilayers. Sterol homeostasis is a complex event under subtle regulatory controls. Esterification of sterols by fatty acids, one of the critical homeostatic responses, is upregulated upon elevated cellular cholesterol or fatty acid levels (Chang et al., 1997). The esterification reaction is mediated by O-acyltransferases providing an important steryl ester storage depot, which overcomes any membrane perturbations that accrue from excessive sterol or free fatty acid levels (Warner et al., 1995; Tabas, 1997; Kellner-Weibel et al., 1998).

In mammalian cells, two acyl-CoA:cholesterol acyltransferases (ACAT; EC 2.3.2.26), ACAT1 and ACAT2, are involved in cholesteryl ester (CE) biosynthesis (summarized in Buhman et al., 2000; Chang et al., 2001). Both enzymes are mainly located to the endoplasmic reticulum (ER). The expression of ACAT1 is ubiquitous whereas that of ACAT2 is tissue-restricted (small intestine and liver). Nevertheless, the enzymatic properties of human ACAT1 and ACAT2 are quite similar (Chang et al., 2000).

Mammalian ACAT1 and ACAT2 descended from ancestral genes in yeast (Yang et al., 1996). Surprisingly, these mammalian enzymes do not have the same intron-exon structures despite they share nearly 40% identity at the amino acid level (Buhman et al., 2000). This implies that the two enzymes diverged quite early during evolution, possibly to perform different cellular functions. Based on ACAT-knockout mice observations (Meiner et al., 1996), CE synthesis mediated by ACAT1 may be preferentially coupled with ester storage in cytosolic lipid bodies while ACAT2 may be functionally linked to the lipoprotein-mediated secretion of CE (reviewed in Buhman et al., 2000). Conversely, in Saccharomyces cerevisiae, two ACAT-related enzymes are present within the same cell, and both yield steryl ester pools in yeast (Yang et al., 1996). In eukaryotic cells, steryl esters are stored in cytoplasmic lipid droplets, which are metabolically active inclusions (reviewed in Sandager et al., 2002).
mammalian cells, impairment in lipid body functions leads to several serious human diseases (summarized in Murphy, 2001).

Less clear are the functions of lipid droplets as well as the importance of neutral lipid storage in primitive eukaryotes. We have recently characterized the activity of triacylglycerol biosynthetic enzymes homologues to mammalian acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1) in two related apicomplexan parasites: Plasmodium falciparum, aetiologic agent of malaria (Vielemeyer et al., 2004) and Toxoplasma gondii (Quittnat et al., 2004). The latter is an obligate intracellular parasite capable of infecting nearly all types of nucleated cells and is responsible for fatal diseases in neonates and immunocompromised individuals (Lutf and Remington, 1992). An ACAT activity has been formerly detected in the free living ciliate Tetrahymena pyriformis (Billeheimer et al., 1989) and in T. gondii (Sonda et al., 2001; Charron and Sibley, 2002). Interestingly, two different sites of cholesterol accumulation have been identified in T. gondii: (i) the apical secretory organelle rhoptries implicated in the biogenesis of the parasitophorous vacuole (PV) membrane during parasite invasion and (ii) lipid inclusions similar to mammalian lipid bodies (Charron and Sibley, 2002; Coppens and Joiner, 2003).

We have previously shown that T. gondii is auxotrophic from low-density lipoproteins (LDL)-derived cholesterol and that interfering with host cholesterol acquisition by T. gondii impairs parasite growth (Coppens et al., 2000). As this parasite has no capability to synthesize sterols when needed, we therefore hypothesize that impairing cholesterol storage will also be detrimental to parasite viability. To test this hypothesis, we characterized the sterol esterification reaction in both functional and molecular terms, emphasizing on the unique features in steryl ester synthesis developed by T. gondii. Namely, the parasite expresses two unusual ACAT isoforms. Compared with mammalian ACAT, the parasite homologues differ in their substrate affinity and specificity, as well as in their mechanisms of regulation. In addition, some inhibitors of mammalian ACAT are particularly cytotoxic to Toxoplasma, reflecting differences in the steryl ester cycle between mammalian cells and this protozoan.

### Results

Toxoplasma is competent to synthesize cholesteryl esters using host cell-derived cholesterol

We assayed ACAT activity and quantified the synthesis of CE in Toxoplasma. Intracellular parasites were incubated with labelled substrates, including [3H]-cholesterol incorporated into LDL, [3H]-oleate associated with albumin, or [3H]-phosphatidylcholine (PC) labelled on the fatty acid moiety for different times. After isolation of parasite lipids by chromatography, we demonstrated that T. gondii was able to incorporate exogenous cholesterol and fatty acid into the CE fraction (Table 1), which confirms previous studies using radioactive oleate (Sonda et al., 2001). The production of CE was proportional to the amount of lipid taken up by the parasite over the time. Incubation in the presence of the lysosomotropic agent chloroquine that impairs the release of cholesterol within LDL (Brown et al., 1975), abolished cholesterol availability for the parasite (Coppens et al., 2000), and therefore CE synthesis.

The fatty acid substrate specificity for Toxoplasma was then examined. Intravacuolar parasites were incubated with radioactive oleate, palmitate, arachidonate, stearate or linoleate for 2 h. All the tested free fatty acids (FFA) were incorporated into parasite CE, suggestive of broad fatty acid specificity (not shown). However, in contrast to mammalian cells, T. gondii showed a nearly twofold higher incorporation of palmitate into CE as compared with other FFA. This palmitate specificity has been previously observed for triacylglycerol synthesis in T. gondii and was ascribed to a preferential uptake of palmitate over other FFA by the parasite (Quittnat et al., 2004). To

| Pulse conditions at 37°C | Lipid uptake (cpm per μg cell protein) | CE formed (cpm per μg cell protein) |
|--------------------------|--------------------------------------|----------------------------------|
| 1 h, [3H]-C-LDL          | 523 ± 22                             | 3140 ± 110 (n = 4)               |
| 1 h + chloroquine, [3H]-C-LDL | 56 ± 12                          | ND (n = 3)                       |
| 3 h, [3H]-C-LDL          | 850 ± 44                             | 4910 ± 165 (n = 4)               |
| 24 h, [3H]-C-LDL         | 1310 ± 62                            | 10 890 ± 180 (n = 5)             |
| 30 min, [3H]-oleate-BSA  | 390 ± 32                             | 2099 ± 123 (n = 3)               |
| 3 h, [14C]-PC-BSA        | 877 ± 54                             | 3388 ± 230 (n = 3)               |
| 3 h, [14C]-PC-LDL        | 243 ± 30                             | 989 ± 56 (n = 3)                 |

Intravacuolar parasites have been pulse-labelled with 1 mg ml⁻¹ tritiated cholesterol incorporated into LDL (C-LDL), tritiated oleate at 0.2 mM bound to albumin or 1 mM [14C]-PC for the indicated times. For chloroquine treatment, infected monolayers were incubated for 10 min with 200 μM chloroquine before the pulse and then maintained in the presence of 100 μM chloroquine during the 1 h pulse with radioactive LDL. Results of lipid uptake and CE production expressed in cpm per μg cell protein are means ± SD of three to five independent experiments. ND, not detected.
circumvent the consequences of FFA access or metabolism when added to infected cells, T. gondii was incubated axenically in the presence of the same radioactive FFA for 2 h. A higher incorporation of palmitate into parasite CE was also observed in extracellular parasites (not shown).

Identification of two isoforms of a T. gondii ACAT-related enzyme

Through homology searches of the T. gondii EST databases using coding sequences from O-acyltransferases, we identified an EST clone that shared homology with ACAT C-terminus sequences. The full cDNA corresponding to this EST was 1950 bp in length and contained an open reading frame (ORF) encoding a 650 aa protein (Fig. 1A). Northern blot analysis on total RNA revealed a mature transcript of ~2.6 kb (Fig. 1B). Based on phylogenetic analysis, the full length of the predicted protein was more closely related to various ACAT1 (~18% identical) than to ACAT2 (~14% identical), although the differences were minor (not shown). However, based on the ACAT functional motifs described below, the parasite enzyme was closely related to yeast homologues (Fig. 1C), which preferentially use ergosterol as substrate (Yang et al., 1997).

In the course of reverse transcription polymerase chain reaction (RT-PCR) cloning of T. gondii ACAT1-related enzyme, we observed that half of the clones had a smaller size than expected. Sequence analysis revealed that the smaller transcript of ~1.3 kb (Fig. 1B) was identical to the parasite ACAT1-related enzyme sequence, but lacked 738 nt encoding a predicted hydrophilic serine-rich region at the N-terminus. Of interest, the transcript also differed in the first 45 nucleotides of the truncated ORF. The full cDNA corresponding to this ACAT1-related enzyme was 1209 bp in length and contained an ORF encoding a 403 aa protein (Fig. 1B). We termed these isoforms TgACAT1α (650 aa) and TgACAT1β (403 aa).

Both isoforms shared common features of the superfamily of membrane-bound O-acyltransferases (Cases et al., 1998a,b; Hofmann, 2000) and had an invariant histidine positioned within a long hydrophobic region (His570 and His323 for TgACAT1α and TgACAT1β respectively; Fig. 1A). Two signatures shared between all ACATs and DGAT1 (Bouvier et al., 2000), have been identified in TgACAT1α and TgACAT1β: (i) a putative invariant serine (Ser376 and Ser136 for TgACAT1α and TgACAT1β respectively) and (ii) the remarkably conserved motif [AExxRFGDRxFYxDWWM] potentially ascribed to include the fatty acid binding site, which corresponds to [AEITNFANRNFYDDWNN] in the TgACAT1α and TgACAT1β sequences, starting at Ala604 and Ala357 respectively.

Finally, the TgACAT1 sequences had the putative cholesterol binding site [HYSF] including a serine surrounded by an aromatic and/or basic amino acids (Guo et al., 2001) present in all the identified ACATs so far (His375SY and His135SY in TgACAT1α and TgACAT2β respectively). Analysis by a transmembrane region prediction program (http://www.ch.embnet.org/) favours eight membrane helix spanning domains for both isoforms. Hence, the two parasite TgACAT1 isoforms have the major hallmarks of the ACAT family previously found in various species.

Coexpression and endoplasmic reticulum localization TgACAT1α and TgACAT1β

Parasite lysates probed on immunoblots with anti-TgACAT1 antibodies against the C-terminus of TgACAT1α revealed two bands, one at Mr 73 kDa and the other at 42.5 kDa (Fig. 2A, lane 1). On immunoblots using the same parasite lysates incubated with antibodies against the specific N-terminus of TgACAT1α, only the 73 kDa band was observed (Fig. 2A, lane 3), leading to the conclusion that the upper band probably corresponds to TgACAT1α and the lower one to TgACAT1β. This indicates that both parasite isoforms were fully expressed in T. gondii. Nevertheless, quantitative immunoblot analysis revealed that parasites expressed 3.5-fold lower levels of TgACAT1β protein compared with TgACAT1α. Metabolic labelling of TgACAT1α-HA and TgACAT1β-HA expressing cells with radioactive methionine followed by immunoprecipitation of the two enzymes with anti-HA antibodies showed that the half-lives of TgACAT1α and TgACAT1β enzymes were 8 h and 30 min respectively (data not shown).

To visualize the distribution of these enzymes in T. gondii, parasite lines expressing either TgACAT1α or TgACAT1β fused with a C-terminal nine-residue HA epitope tag were engineered. In either transiently or stably transfected Toxoplasma, TgACAT1α-HA and TgACAT1β-HA were predominantly localized to a network of membranes dispersed throughout the parasite cytoplasm and around the nucleus (Fig. 2B), which was identified as the ER by immunoelectron microscopy (Fig. 2C). Both the cortical and perinuclear ER were intensely labelled. No labelling was observed on other parasite structures such as at the surface of lipid bodies or in rhoptries. In addition, anti-TgACAT1α or -β antibodies colocalized with antibodies against TgDAGT1 to the parasite ER (Quittnat et al., 2004). To confirm that the serine-rich region at the N-terminus of TgACAT1α was fully translated, a c-myc tag was inserted in the middle of the N-terminal sequence (aa 110). In parasites expressing this construct, a clear co-labelling of the ER with antibodies against c-myc and HA was observed (not shown).
Sequence alignment for TgACTA1α and TgACTA1β, transcriptional analysis and phylogeny.

A. Alignments were performed using the CLUSTALW program. The deduced protein sequences of TgACTA1α and TgACTA1β were aligned with the following sequences: HsACTA1, the Homo sapiens ACAT1 (Accession No. L21934; Chang et al., 1993), MmACTA1, the Mus musculus ACAT1 (Accession No. AF059202; Delker et al., 1998), MmACAT2, the Mus musculus ACAT2 (Accession No. I49454; Uelmen et al., 1998), SsACAT1, the S. cerevisiae ACAT1-related enzyme (Accession No. P29628; Yang et al., 1996) and SsACAT2, the S. cerevisiae ACAT2-related enzyme (Accession No. U51790; Yang et al., 1996). Identical amino acids are shaded in black; similar and conserved amino acids are shaded in light and dark grey respectively. Signs are placed under the important amino acids (see their description in the text). The potential sites of N-glycosylation are located on Asn162, Asn407 and Asn520 for TgACTA1α and between amino acids 17–38, 58–82, 97–129, 199–219, 229–249, 308–312, 325–352, 361–379 for TgACTA1β.

B. Analysis of the TgACTA1α and TgACTA1β transcripts and translated products. Northern blotting of total RNA probed with radiolabelled TgACTA1α cDNA. Twenty micrograms of total RNA were resolved on a denaturing gel, blotted onto a nylon membrane and hybridized with a [32P]-dCTP-TgACTA1α probe (lane 1; full length: 1–1950 bp; lane 2: 1–336 bp). The size of RNA markers are indicated at the right margin. The arrows denote the TgACTA1α 2.6 kb and TgACTA1β 1.3 kb transcripts.

C. Phylogeny of TgACTA1. Neighbour-joining phylogenetic tree constructed from the conserved domains in the protein sequences of TgACTA1 (starting at Lys373 for TgACTA1α) and the indicated homologues.
Fig. 2. Coexpression and localization of TgACAT1α and TgACAT1β in T. gondii.
A. Immunoblots of parasite lysates. After lysis of the parasites in a solution of deoxycholate/PC, resolution of the proteins on gel and transfer to a nitrocellulose membrane, the Western blot analysis of the gel was probed with anti-TgACAT1α
474-650 antibodies (lane 1) and preimmune (lane 2), or anti-TgACAT1α
14-117 antibodies (lane 3) and preimmune (lane 4). The molecular weight markers are indicated at the left margin.
B. Fluorescence microscopy of stably expressed Toxoplasma with TgACAT1α-HA and TgACAT1β-HA. Intravacuolar parasites were stained with anti-HA antibodies, followed by FITC-conjugated goat anti-mouse IgG, revealing an ER staining. The parasite nuclei were stained with DAPI.
C. Immunogold labelling of TgACAT1α-HA and TgACAT1β-HA. Cryosections of stably expressed Toxoplasma with TgACAT1α-HA and TgACAT1β-HA were incubated with anti-HA antibodies, followed by incubation with protein A adsorbed to 10 nm gold particles. The gold particles were concentrated in the parasite ER area. Control cryosections on wild-type parasites with equivalent anti-HA antibody dilution were completely free of immunogold label (not shown). G, Golgi; n, nucleus. Scale bars are 0.150 μm.
Both TgACAT1α and TgACAT1β can restore cholesteryl ester production in mammalian ACAT-deficient cells and present broad sterol substrate specificity

The presence of two ACAT isoforms is likely to have functional implications for CE production in T. gondii. To investigate the relative function of TgACAT1α and TgACAT1β, mammalian ACAT-deficient cell lines were transiently transfected with expression plasmids encoding each isoform of TgACAT1-HA. Both constructs localized to the ER in mammalian cells and exhibited extensive colocalization with calnexin, an endogenous ER marker (TgACAT1α, Fig. 3A; TgACAT1β, not shown). We first monitored oleate uptake and CE formation in MEF–/–, an ACAT-deficient cell line (Meiner et al., 1996), after transfection with pTgACAT1α or pTgACAT1β (Fig. 3B). The uptake of oleate by the mutant cells expressing one isoform of TgACAT1 was similar over a period of 2 h. As a result of TgACAT1α or TgACAT1β expression in both cell lines, a significant ACAT activity was reconstituted, leading to the production of CE. The addition of exogenous cholesterol to the cells greatly increased esterification by approximately twofold in TgACAT1α- and TgACAT1β-expressing cell lines. This indicates that the two Toxoplasma isoforms are functional ACAT enzymes, although a threefold lower incorporation of oleate into CE was observed in TgACAT1β-expressing cells as compared with TgACAT1α-expressing cells. This observation might be ascribed to a lower percentage of MEF–/– expressing the β compared with the α isoform. A lower CE activity for TgACAT1β compared with TgACAT1α was also detected in mammalian AC29 cells, another cell line lacking ACAT activity (Cadigan et al., 1988; data not shown). Additionally, parasites transiently transfected with pTgACAT1α or pTgACAT1β showed an increase in CE production by 230 ± 73% and 146 ± 45%, respectively, compared with the wild-type strain upon incubation with tritiated oleate (not shown).

Having demonstrated the activity of the Toxoplasma ACAT1 cDNA in ACAT-mammalian cells, we extended these observations to assess the affinity of the parasite ACAT enzymes for various sterols in comparison to mammalian ACAT (Fig. 3C). An in vitro system was utilized to measure ACAT activity in the presence of radioactive oleate and different potential fatty acyl acceptors for the esterification reaction. Both Toxoplasma and mammalian enzymes esterified the cholesterol precursor 7-dehydrocholesterol, but not lanosterol. The β-sitosterol, a specific substrate for the plasma lecithin:cholesterol acyltransferase (LCAT), was esterifiable by the parasite ACAT while mammalian enzymes had a limited cellular synthesis of sitosterol esters. TgACAT1 also showed an esterification activity for 25-hydroxycholesterol, a hydroxylated derivative of cholesterol known to both inhibit cholesterol synthesis (Adams et al., 2005) and activate ACAT by post-translational mechanisms (Cheng et al., 1995; Chang et al., 1997), in the same extent as mammalian ACAT.

In contrast to mammalian enzyme, the parasite ACAT were competent to readily esterify ergosterol. Nevertheless, we determined that Toxoplasma was unable to synthesize ergosterol in physiological conditions (Fig. 3D).
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A

TgACAT1α calnexin merge

B

\[
\begin{align*}
\text{ WT } & \quad \text{ Mut. } \\
\text{ WT } + TgACAT1\alpha & \quad \text{ Mut. } + TgACAT1\beta
\end{align*}
\]

C

Sterol \[^{3}H\]-oleate formed (% of control)

D

Yeast strain

E

ACAT activity (pmoles per mg protein per min)

\[
\begin{align*}
\text{ WT } & \quad \text{ Mut. } \\
\text{ Mut. } + TgACAT1\alpha & \quad \text{ Mut. } + TgACAT1\beta
\end{align*}
\]
under growth condition in the absence of lipoproteins for 36 h.

Only TgACAT1α expressed in a yeast mutant strain lacking neutral lipid synthesis can form lipid bodies and produce cholesteryl esters

The capability of T. gondii ACAT to esterify ergosterol has been directly addressed in a yeast strain devoid of DGAT and ACAT activities (Sandager et al., 2002). This strain was transformed with the parasite ACAT enzymes under the control of a yeast GAL1 promoter and expressed under induced and non-induced conditions. Wild-type yeast accumulated lipid droplets in stationary phase as visualized by Nile Red staining and fluorescence microscopy (Fig. 3E). In contrast, the JCY500 strain with null alleles for LRO1, DGA1, ARE1 and ARE2 genes failed to accumulate lipid droplets. When the JCY500 strain transformed with the parasite enzymes, only TgACAT1α produced the wild-type fluorescence pattern under induced conditions. This suggests that yeast endogenous sterols (primarily ergosterol) could be used as substrates for TgACAT1α. Strikingly, the transformed mutant yeast expressing TgACAT1α acquired a filamentous phenotype, reminiscent of a pseudohyphal growth of S. cerevisiae. Coincidently, only yeast homogenates from JCY500 transformed with TgACAT1α produced CE under galactose induction after incubation with cholesterol and radioactive palmitate as substrates (Fig. 3E). No CE was observed in JCY500 yeast extracts after transformation with TgACAT1α in the absence of galactose (data not shown). As monitored by immunofluorescence assay, mutant yeast was unable to express TgACAT1β, therefore resulting in an absence of production of CE and formation of lipid bodies.

Mutations in the putative substrate binding sites of TgACAT1 decrease cholesteryl ester synthesis

We assessed the importance of two motifs present in O-acyltransferase sequences, FYxGWNN and (H/Y)S(F/Y), which are conserved among various ACAT, including TgACAT1 (Fig. 1A). The corresponding residues in TgACAT1α (Fig. 4A) were mutated, and the constructs were expressed in T. gondii and in mammalian ACAT-deficient cells. The ER localization of TgACAT1α variants was verified by immunofluorescence (Fig. 4A). A comparable expression level in the wild-type and mutant TgACAT1α was quantified (legend of Fig. 4). The TgACAT1α-S376L and TgACAT1α-WW518,519AS mutants produced no CE in ACAT-deficient cells, and the TgACAT1α-S376L mutant synthesized one-tenth of the CE compared with the wild-type TgACAT1α (Fig. 4B). The enzymatic activity of TgACAT1α-WW518A was reduced by twofold compared with wild type while the activity of TgACAT1α-Y377F was retained. These results indicate that the two conserved [FYxGWNN] and [HYS] sequences are necessary for CE synthesis in T. gondii, although the contribution of different residues varies, as found for mammalian and yeast ACAT (Guo et al., 2001).

LDL-derived cholesterol can serve as a substrate and physiological activator of Toxoplasma ACAT

Nile Red strongly fluoresces in the presence of triacylglycerol or steryl esters stored in cytoplasmic lipid bodies. We previously demonstrated that in normal culture conditions (i.e. 10% of complete serum), intravacuolar T. gondii contained 4 ± 2 Nile Red positive-neutral lipid bodies (Quittnat et al., 2004). Ultrastructural observations illustrated the presence of homogenous 0.25 μm electron-dense structures without membranous profiles in T. gondii, which were morphologically similar to mammalian lipid bodies.

The addition of LDL to mammalian cells leads to an increase in ACAT activity as a consequence of the expansion of cellular cholesterol pools (Gavigan and Knight, 1983; Xu and Tabas, 1991). We investigated the capability of cholesterol storage by intravacuolar Toxoplasma under conditions of excess LDL. Figure 5A shows that as observed for fibroblasts, there was a remarkable increase in parasite lipid body number, which is proportional to the LDL concentration added in the culture medium (until 10 ± 3 Nile Red positive structures at 200 μg ml⁻¹ LDL after 24 h). This lipid body biogenesis in response to LDL paralleled an activity of CE formation (Fig. 5B), thus indicating that large amounts of LDL-derived cholesterol could be efficiently diverted by the parasites from the host cytoplasm and immediately converted to CE before storage in lipid inclusions. Neither lipid bodies nor CE were found in parasites under lipoprotein starvation. Alternatively, incubation of parasites in the absence of lipoproteins from 6 h to 48 h induced a gradual disappearance of lipid bodies (Fig. 5C) and a decrease in ACAT activity (Fig. 5D).

The TgACAT1α mRNA expression and cholesterol esterification are co-ordinately regulated: effect of excess fatty acids

Incubation of Toxoplasma-infected fibroblasts with a large excess of oleate bound to albumin resulted in an increase in the number of lipid bodies in the cytoplasm of both host cells (Fig. 6A, panel a) and parasites (5–8 per parasite; Fig. 6A, panel b). Interestingly, the Toxoplasma vacuole was also intensively labelled with Nile Red upon incubation with excess oleate, thus revealing lipid deposits in the...
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Fig. 4. Expression of mutant TgACAT1α and esterification analysis in mammalian ACAT-deficient cells.

A. Conserved motifs and mutations in TgACAT1α. Punctual mutations were created in the conserved motifs common to the ACAT gene family, the FYxDWNW heptapeptide, and the tripeptide consisting of an invariant serine surrounded by aromatic and/or basic amino acids. The expression and ER localization of the missense variants were confirmed in both *T. gondii* and two ACAT-deficient cell lines, MEF−/− and AC29, by immunofluorescence assay as described above.

B. Cholesterol esterification activity. The MEF−/− were transiently transfected with either the wild-type or mutant pTgACAT1α and vector controls for 24 h, before incubation with 0.2 mM tritiated oleate for 2 h. The uptake of oleate was similar between the wild-type TgACAT1α and variants (less than 10% variation). A comparable percentage transfection was observed between wild-type TgACAT1α (5.1 ± 2.3%), TgACAT1α-Y377F (4.9 ± 1.9%), TgACAT1α-S376L (3.9 ± 1.3%), TgACAT1α-W518A (5 ± 2.4%) and TgACAT1α-WW518,519AS (4.4 ± 2.2%). Results of oleate incorporation into CE expressed in cpm per μg protein are means ± SD of three independent experiments. Differences between values of CE production in MEF−/− between wild-type TgACAT1α versus some mutants are statistically significant (*P < 0.005; **P < 0.0005).
Fig. 5. Effect of excess or absence of LDL on CE synthesis and lipid body biogenesis.
A. Nile Red staining of HFF (a–c) or T. gondii (d–f) after incubation 24 h in LPDS or in the presence of 20 or 200 μg ml\(^{-1}\) LDL. The parasites illustrated in d–f are extracellular as they have been isolated from the fibroblasts shown in a–c before staining with the lipid dye to spot solely parasite lipid bodies and avoid host lipid body overlapping in the same field.
B. Cholesterol esterification activity. After 24 h incubation in LPDS or with the indicated LDL concentrations, intravacuolar parasites were exposed to 0.2 mM tritiated oleate for 2 h to monitor CE synthesis. Results of lipid incorporation into CE expressed in cpm per μg cell protein, are means of two separate experiments, with less than 15% variation.
C. Nile Red staining of intracellular T. gondii after incubation during 6–48 h in LPDS.
D. Cholesterol esterification activity. Intravacuolar parasites were starved in LPDS at the indicated times before incubation with 0.2 mM tritiated oleate for 2 h to assess CE synthesis. Results of lipid incorporation into CE expressed in cpm per μg cell protein are means of two separate experiments, with less than 10% variation.

Vacuolar space (Fig. 6A, arrow in panel b). The presence of lipid inclusions in the Toxoplasma-containing vacuole was confirmed by electron microscopy using malachite green incorporated into the fixative solution to retain lipid elements, and therefore enhance staining of lipid-containing structures. Large and abundant malachite green structures corresponding to depots of neutral lipids were clearly visible between parasites (Fig. 6A, arrows in panel c).

The regulatory mechanisms of cholesterol esterification were probed in T. gondii. These regulatory studies were performed solely on TgACAT1α because of the higher expression levels of this isoform in both Toxoplasma and heterologous systems. Synthesis of CE was monitored in...
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the presence of excess oleate, palmitate or bromo-palmitate, which is chemically inert to form esters, by incubating intravacuolar parasites with radioactive cholesterol (Fig. 6B). Excess oleate or palmitate in the extracellular medium resulted in a three- and fivefold increase in CE synthesis, respectively, compared with basal conditions or when bromo-palmitate was used. This increase in ACAT activity correlated with a 2.2- and 1.7-fold stimulation of

Fig. 6. Effect of excess FFA on parasite CE synthesis and lipid body biogenesis.
A. Morphological detection of neutral lipid bodies in HFF (a) and intravacuolar Toxoplasma (b and c). After 24 h incubation in the presence of 0.5 mM oleate (a and b), cells were stained with Nile Red revealing cytosolic lipid bodies (LB) and lipid deposits in the vacuolar space (arrows). In (c), transmission electron microscopy in the presence of malachite green of intracellular T. gondii, showing the ultrastructural details of cytosolic lipid bodies (LB) and lipid aggregates inside the PV. PVM, PV membrane; hLB, host lipid bodies; rh, the rhoptry secretory organelle; P, parasite. Scale bar is 0.3 μm.
B. Cholesterol esterification activity. Intravacuolar parasites have been incubated with 1 mg ml⁻¹ [³H]-cholesterol incorporated into LDL with the indicated unlabelled fatty acids (0.6 mM) for 5 h to assess radioactive cholesterol incorporation into CE. Results expressed in cpm per μg cell protein are means ± SD of three independent experiments. Differences between values of CE synthesis in control conditions versus incubation with oleate or palmitate are statistically significant (*P < 0.01; **P < 0.005).
C. Expression of TgACAT1α mRNA in intravacuolar parasites incubated for 24 h with the indicated fatty acids or 25-hydroxycholesterol (25-OH-chol) as measured by Northern blot. After total RNA extraction from parasites, 20 μg was subjected to electrophoresis and blot hybridization with [²⁰P]-labelled probes. A typical Northern blot representative of three independent preparations of parasites is shown.
mRNA parasite ACAT expression by oleate and palmitate, respectively, as demonstrated by Northern blot analysis of total RNA probed with radiolabelled 5′ end of TgACAT1 cDNA (Fig. 6C). By comparison, 25-hydroxycholesterol that can stimulate ACAT activity in T. gondii (Fig. 3C) has no effect on TgACAT1α RNA expression.

**Inhibitors of cholesterol esterification induce the rupture of parasite plasma membrane**

Derivatives of trisubstituted urea (e.g. DuP128), derivatives of fatty acid amides (e.g. CI976) and sulphonylurea (e.g. glibenclamide) are known to inhibit cholesterol esterification in mammalian cells as well as to decrease the number of lipid bodies. This, in turn, results in the disruption of cellular lipid metabolism and eventually cell toxicity (Higley et al., 1994; Harte et al., 1995; Ohgami et al., 2000). However, the mechanism of action as well as the cellular target/s of these compounds are still undetermined. Regardless, we have tested selected cholesterol esterification inhibitors on CE synthesis in T. gondii. Data revealed differences in drug sensitivity and selectivity between Toxoplasma and mammalian cells (Fig. 7A, panel a). Treatment of intravacuolar parasites with DuP128 at 1 μM showed a marked reduction in CE synthetic rate proportional to the incubation time when compared with untreated parasites; no change in TgACAT1 mRNA levels was displayed (not shown). By comparison, 1 μM DuP128 had a negligible effect on inhibition of CE synthesis in HFF (not shown). CI976 and glibenclamide had a moderate inhibitory effect on CE synthesis in Toxoplasma while they are effective inhibitors of the ACAT reaction in mammalian cells (Higley et al., 1994; Ohgami et al., 2000).

As monitored by uracil incorporation assays, 24 h exposure of intravacuolar parasites to DuP128 resulted in impairment of parasite growth (IC50 of 5 μM) whereas CI976 and glibenclamide showed no cytotoxicity up to 5 and 100 μM respectively (Fig. 7A, panel b). Although differential effects were observed for DuP128 and CI976 on CE formation in Toxoplasma, the values of uracil incorporated into parasites were more severe in the presence of excess LDL. This suggests that the parasite ACAT reaction was probably targeted by these drugs. As illustrated by electron microscopy, 24 h incubation with DuP128 at 5 μM induced severe cytopathic effects on parasites, such as qualitative changes in lipid bodies and loss of plasma membrane integrity associated with release of parasite content into the vacuolar space (Fig. 7B). The morphological changes probably resulted from the accumulation of excess cholesterol in the plasma membrane as free cholesterol was significantly increased by 1.6- and 2.4-fold in parasites treated with DuP128 at 1 and 5 μM respectively. A dramatic dilatation of parasite mitochondrial matrices with swelling of cysteae was also visible, as previously reported for mammalian cells (Vernetti et al., 1993). With the exception of some lamellar bodies, host cells showed no ultrastructural changes after 24 h incubation in the presence of 5 μM DuP128.

**Discussion**

We provide here the first characterization of ACAT-related proteins present in a protozoan, T. gondii. Two isoforms, TgACAT1α and TgACAT1β, an N-terminus truncated form of TgACAT1α are identified. Both isoforms are co-expressed in the ER of the parasite. Their predicted sequences contain the hallmarks of the ACAT family members including the putative fatty acid and sterol binding sites. Both TgACAT1α and TgACAT1β drive parasite CE synthesis, utilizing host lipids as substrates.

The esterification reaction requires the 3β-hydroxy group but unlike in higher eukaryotes or in yeast, which synthesize a predominant sterol ester (Yang et al., 1997), the ACAT reaction in Toxoplasma is not sensitive towards changes in the structure of the sterol side-chain. Indeed, in vitro assays demonstrate that Toxoplasma ACAT is able to efficiently esterify both ergosterol and cholesterol. In vivo, at least one parasite isoform, TgACAT1α is competent to esterify yeast endogenous sterols as lipid bodies are formed in mutant yeast strain lacking neutral lipids after TgACAT1α expression. This observation correlates with the highest degree of identity of Toxoplasma ACAT with yeast homologues, but remains intriguing as ergosterol is not synthesized by T. gondii and cannot be provided by mammalian cells where the parasites reside.

In mammals, in addition to the formation of CE catalysed by intracellular ACAT, CE can be also formed in the plasma by a separate enzyme, LCAT. The sterol substrate specificity of LCAT is similar to that of ACAT except that the substrate to be esterified is β-sitosterol (Nordby and Norum, 1975). Toxoplasma ACAT can produce sitosterol esters although in a lesser extent than cholesterol or ergosterol esters. The identification of a homologue to LCAT in the Toxoplasma genome database (http://ToxoDB.org) raises the possibility that this enzyme may also participate in sitosterol esterification and/or secretion. Finally, parasite ACAT show activity with cholesterol oxidation derivatives. Oxysterols found in abundance in macrophages (Jialal et al., 1991) are potentially toxic to cells (Kandutsch et al., 1978). The conversion of free oxidized cholesterol to oxysterol esters could represent a detoxification mechanism for Toxoplasma infecting macrophages.

Human ACAT has broad acyl-CoA substrate specificity with a preference for oleate, while the yeast homologues have a marked preference for unsaturated C18 forms (Yang et al., 1997). In contrast, the Toxoplasma ACAT-like enzymes prefer palmitate over other fatty acids. This might
**Fig. 7.** Effect of cholesterol esterification inhibitors on parasite CE synthesis, viability and morphology.

A. Cholesterol esterification inhibition (a) and parasite viability (b). Intravacuolar parasites have been incubated with cholesterol esterification inhibitors at the indicated conditions before incubation with 0.2 mM tritiated oleate for 2 h to assess CE synthesis. Results expressed in percentage of controls without drug are means ± SD of three independent experiments. Uracil incorporation was assayed on Toxoplasma-infected HFF treated with various cholesterol esterification inhibitors at the indicated drug concentrations to monitor parasite viability. Results expressed in percentage of controls performed in the presence of the solvent alone are the means of three different assays, with less than 15% variation. *P < 0.01; **P < 0.005; ***P < 0.001.

B. Transmission electron microscopy illustrating the cytopathic effects of DuP28 on parasites. Intravacuolar *T. gondii* have been incubated for 24 h in the presence of 5 μM DuP128 before processed for electron microscopy. A large lipid body (LB) is visible. The arrows pinpoint deformations of the parasite plasma membrane leading to a discharge of parasite content into the PV. The arrowheads show the lamellar bodies in the host cytoplasm. P, parasite; mt, mitochondrion; hcell, host cell. Scale bars are 0.5 μm.
be the result of a preferential uptake of exogenous palmi-
itate over other fatty acids and/or a selective biosynthesis
of palmitate by the parasite. Interestingly, another
Toxoplasma enzyme belonging to the superfamily of
membrane-bound O-acyltransferases (Hofmann, 2000),
TgDGAT1 also incorporates predominantly palmitate into
triacylglycerol (Quittnat et al., 2004). One explanation for
this fatty acid specificity may be different membrane envi-
ronments, making the accessibility of CoA to the active
sites dependent on the length and desaturation of the acyl
chains. Nevertheless, mutagenesis in the conserved res-
idences of the putative lipid binding sites abolishes the ACAT
activity, either by impairing the accessibility of sterols and
acyl-CoA to these sites (Guo et al., 2001), or by inducing
a gross structural alteration of the enzyme.

In Toxoplasma, both LDL-derived cholesterol and FFA
can serve as ACAT substrates and activators, and cellular
cholesterol esterification and lipid droplet biogenesis are
directly co-ordinated. FFA can increase ACAT1 mRNA
levels in Toxoplasma as is the case for two of the four
ACAT transcripts in mammalian cells with some acyl-CoA
preferences (Seo et al., 2001). The massive accumulation
of lipid bodies in parasite cytoplasm in response to excess
LDL in the medium seems to indicate an absence of
selective mechanisms regulating the trafficking of host
cholesterol to the parasite, at both the PV membrane and
the parasite plasma membrane levels. However, managed
proteolysis of isolated Toxoplasma PV result in an abol-
ishment of cholesterol delivery to the parasite, indicative
of the presence of cholesterol transporters (e.g. ABCG
transporters) on the PV membrane (A. Seghal, T.T. Sted-
man, I. Coppers and K.A. Joiner, in preparation). Simi-
larly, the accumulation of malachite green positive
material in the PV lumen in response to excess FFA raises
the intriguing issue on the regulation of host fatty acid
supply in Toxoplasma.

TgACAT1α is more efficient at esterifying sterols than
TgACAT1β. One explanation for the difference in cellular
ACAT activity between the two isoforms could be the more
rapid turnover of TgACAT1β versus TgACAT1α. This dif-
ference in protein stability probably results in few
TgACAT1β molecules as demonstrated by immunoblotting
and immunofluorescence microscopy, and lower activity in
TgACAT1β compared with TgACAT1α-expressing cells.
The TgACAT1α sequence predicts a unique hydrophilic
serine-rich region at the N-terminus (~50% serine
between amino acids 24 and 107), absent from
TgACAT1β. This polyserine tract in the TgACAT1α may
contribute to overall protein stability. Additionally,
TgACAT1β shows no activity in transformed yeast, as a
result of an absence of gene expression, protein degra-
dation or enzymatic incompetence. Further studies will
be needed to determine the exact basis for the difference
in TgACAT1α and TgACAT1β stability.

In mammalian cells, the cycle of cholesterol esterifica-
tion, hydrolysis and re-esterification is necessary for cho-
lesterol homeostasis, and ultimately for proper cell
function. Perturbing the balance between CE and free
cholesterol by inhibiting ACAT with a pharmacological
agent forces the accumulation of intracellular excess free
cholesterol. Once a critical mass of free cholesterol is
reached, free cholesterol crystallization occurs in the
membrane bilayer (Kellner-Weibel et al., 1999). Free
cholesterol crystal formation is inhibited proportionally to
the concentration of cholesterol acceptors in the medium
(mainly apolipoproteins), presumably by removing
excess intracellular free cholesterol. Alternatively, nucle-
ation of cholesterol crystallization is increased by agents
stimulating the cellular rate of CE hydrolysis. In T. gondii,
inhibition of sterol esterification blocks parasite growth.
The apparent rupture of the parasite plasma membrane
is probably linked to the free cholesterol build-up coincid-
ing with a dramatic increase in parasite toxicity, at sub-
toxic concentrations for mammalian cells. Upon a
massive incorporation of cholesterol into parasite mem-
branes a rapid destabilization of the plasma membrane
is provoked, followed by the release of parasite cyto-
plasm inside the vacuolar space, including large
organelles.

This particularly high sensitivity of T. gondii towards
cholesterol esterification inhibitors may be explained by
the absence of extracellular cholesterol acceptors (e.g.
serum, HDL, apolipoprotein E, apolipoprotein A-I/phos-
pholipid particles) in the PV. Such acceptors are known
in mammalian cells to desorb cholesterol from the plasma
membrane and to prevent a nucleation event from occur-
ing. Esterification of cholesterol may lead to the secretion
of CE into the vacuolar space of Toxoplasma, as observed
with mammalian apolipoprotein B secretion from hepato-
cytes (Spady et al., 2000) and lipoprotein-like particles
from astrocytes (Mutka et al., 2004). The large lipid
deposits visible in the lumen of the vacuole of intravacu-
ocular parasites upon excess oleate may be a direct conse-
quence of parasite ACAT overexpression, ensuing neutral
lipid secretion. In addition, T. gondii lacks caveolin and
caveolae (our personal data), known to transfer chole-
sterol from the ER to the plasma membrane in mammalian
cells, and so promoting the efflux of free cholesterol
(reviewed in Batetta et al., 2003).

In yeast, ergosterol esters in lipid bodies are dispens-
able for growth but may be utilized for membrane forma-
tion under conditions of lipid depletion (Sandager et al.,
2002). In mammals, there are a broader array of distribu-
tions and functions for esterified sterols. CE can be depos-
ted in cytoplasmic lipid droplets, as major reservoirs
of free cholesterol for membrane biogenesis and/or for ste-
roid hormone biosynthesis (reviewed in Murphy, 2001).
Alternatively, CE can also become part of the neutral lipid

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core of circulating lipoproteins in cells specialized in lipoprotein assembly and secretion. When taken in combination, our data suggest key functions for esterification and storage of cholesterol molecules in \textit{T. gondii} viability in host cells. The identification of ACAT cDNA in \textit{T. gondii} has significant implications for understanding the regulation/importance of the CE biosynthetic pathway and the intracellular cholesterol metabolism in this parasite. \textit{TgACAT1} mRNA is encoded by a single copy gene, which the disruption by knockdown strategy will definitely inform on the central role of the ACAT reaction for \textit{Toxoplasma} infection. The esterification of cholesterol by acyl-CoAs provides an excellent target for the pharmacological reduction of CE. Toxic build-up of free cholesterol in \textit{T. gondii} impairs parasite development. Mutant expressing \textit{TgACAT1} can be helpful for rapid screening of new ACAT inhibitors.

### Experimental procedures

#### Chemicals and antibodies

All chemicals were obtained from Sigma Chem. (St Louis, MO) or Boehringer Mannheim Biochemicals (Indianapolis, IN), unless indicated otherwise. Solvents and standards for chromatography were of the highest analytical grade. Silica gel 60 TLC plates were from EM Science (Gibbstown, NJ). CI 976 and DuP 128 were kindly provided by Dr Annabelle Rodriguez (Johns Hopkins University). Radiloabelled reagents included: [5,6-\textsuperscript{3}H]-uracil (sp. act: 45 Ci mmol\(^{-1}\)), [1,2-\textsuperscript{3}H]-cholesterol (sp. act: 48 Ci mmol\(^{-1}\)), [3H,10-\textsuperscript{3}H]-oleic acid (sp. act: 10 Ci mmol\(^{-1}\)), [1-\textsuperscript{14}C]-palmitic acid (sp. act: 55 mCi mmol\(^{-1}\)), [1-\textsuperscript{14}C]-stearic acid (sp. act: 58 mCi mmol\(^{-1}\)), [1-\textsuperscript{3}H]-linoleic acid (sp. act: 55 mCi mmol\(^{-1}\)).

#### Cell lines and culture conditions

The mammalian cell lines used in this study are the primary HFF (ATCC CRL-1635), CHO cells (ATCC CCL-163), and the MEF derived from either mutant lacking ACAT activity from mutagenized CHO cells (Cadi, 1988) generously given by Dr Ta-Yuan Chang (University of California). HFF and CHO cells were grown as monolayers (Coppens et al., 1988). The AC29 mutant was obtained from the American Type Culture Collection (ATCC CRL-1635). Cells were grown in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS) and gentamicin at 10 m\(\mu\)g ml\(^{-1}\).

#### Parasite propagation and purification

The RH strain tachyzoite of \textit{T. gondii} was used throughout this study and was propagated \textit{in vitro} by serial passages in monolayers of HFF (Roos et al., 1994). Intracellular parasites were purified by density gradient using Nycodenz and isopycnic centrifugation as detailed previously (Coppens et al., 2000). Parasite concentration, replication and viability were routinely determined for all studies using standard methods (Coppens et al., 1996).

#### Preparation of LDL, LPDS and LDL-associated lipids

Production of human LDL (density 1.019–1.063 g ml\(^{-1}\)) and LPDS, and incorporation of radioactive cholesterol into LDL were performed as described (Coppens et al., 2000). Specific radioactivity values of LDL-associated [\textsuperscript{1}H]-cholesterol were 3000–6500 cpm per \(\mu\)g protein.

#### Cloning of full-length cDNA encoding \textit{TgACAT1}x and \textit{TgACAT1}β

Expressed sequence tags (EST) with sequence similarity to mammalian ACAT were identified from \textit{blast} data searches. The 3’ and 5’ ends of \textit{TgACAT1}x and \textit{TgACAT1}β cDNA were obtained by using rapid amplification of cDNA ends (RACE) and \textit{Toxoplasma} cDNA. A full length of cDNA was synthesized from RNA isolated with TRIzol reagent (Gibco-BRL, Grand Island, NY) using Superscript reverse transcriptase (Gibco-BRL). PCR amplification of \textit{TgACAT1}x and \textit{TgACAT1}β cDNA was performed with Puu DNA polymerase (Stratagene, La Jolla, CA), with cycling conditions of 94.5°C for 55 s (1 cycle); 66°C for 55 s, 72°C for 1 min 45 s (33 cycles). The cDNA sequence of \textit{T. gondii} ACAT1x and ACAT1β have been deposited in GenBank under the Accession No. AY562994 and AY562995 respectively.

#### DNA constructs and mutagenesis

\textit{TgACAT1}x and \textit{TgACAT1}β encoding sequences with a C-terminal HA-tag epitope (YPYDVPDYA) for immunodetection were subcloned into the NcoI–NheI sites of an NTpase 3 expression cassette plasmid, as previously described (Robibaro et al., 2002). An internal myc-tag epitope (EOQKISEELD) was inserted at the AflII restriction site (110–112 amino acids) of the \textit{TgACAT1}x-HA encoding sequence for double immunodetection. Four different mutants of the initial isolated \textit{TgACAT1}x wild type were engineered by PCR-based site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Stratagene). The corresponding point mutations introduced in the conserved sequences encoding potential binding sites for cholesterol (\textit{TgACAT1}x-Y277F and \textit{TgACAT1}x-S278L) or fatty acid (\textit{TgACAT1}x-W242A and \textit{TgACAT1}x-W251A, AS) have been reported to impair ACAT activity (Guo et al., 2001).

#### Northern blot analyses

Total RNA of \textit{T. gondii} was extracted, subjected to electrophoresis and transferred to a membrane as reported (Quittmat et al., 2004). Blots of total RNA were hybridized with [\textsuperscript{32}P]-labelled full-
length cDNA probes of TgACAT1α or TgACAT1β or a [32P]-labelled PCR fragment from the 5' coding region of TgACAT1α (1–336 bp) and revealed by autoradiography as described (Quittnat et al., 2004). For determination of TgACAT1α mRNA levels, intravacuolar parasites were grown in MEF ACAT-1−/− in the presence of either FFA (0.3 mM) bound to BSA (1% BSA; FFA:BSA ratio of 2:1) or 1 μg ml−1 25-hydroxycholesterol before extraction of parasite total RNA. The DNA probes included the [32P]-labelled 336 bp fragment of TgACAT1α (nucleotides 1–336) and the [32P]-labelled Toxoplasma α-tubulin cDNA as an internal control.

Production of antibodies against TgACAT1α-derived peptides

Anti-TgACAT1α42–351 and TgACAT1α42–445 antibodies generated using a fragment of 339 bp (42–351 bp from the 5' coding region of the TgACAT1α gene) and 528 bp (142–1950 bp from the 5' coding region of the TgACAT1α gene), respectively, were amplified by PCR. The PCR products, which were engineered to contain an EcoRI site at the N-terminus and a NotI site at the C-terminus, was cut with EcoRI and NotI, and then inserted into the EcoRI and NotI sites of pgEX-T1 (Pharmacia Biotech). The resulting products were used to express the TgACAT1α-derived peptides as fusion proteins of the glutathione S-transferase in Escherichia coli. Antiseras against the TgACAT1α fragments were produced by immunization of female BALB/c mice (Cocalico Biologicals, Reamstown, PA) with the recombinant protein purified by glutathione-Sepharose (Sigma). Protocols of parasite lysis and immunodetection of the TgACAT1α and TgACAT1β were performed as described (Quittnat et al., 2004) using anti-HA, anti-myc or anti-TgACAT1α antibodies.

Expression analysis in T. gondii and selection of stable lines

For transient expression and generation of parasites stably expressing TgACAT1α or TgACAT1β, expression plasmids containing the parasite sequences were engineered as described (Quittnat et al., 2004).

Transient TgACAT1α-HA and TgACAT1β-HA expression in mammalian cells

ACAT-deficient cells were transfected with the NTPase 3 expression cassette plasmid encoded TgACAT1α-HA or TgACAT1β-HA. Transfections and immunodetection of the parasite gene products were performed as described (Quittnat et al., 2004). The transfection percentage was measured by counting the number of fluorescent cells expressing TgACAT1α-HA or TgACAT1β-HA, revealing by immunofluorescence assays using anti-HA antibodies (see below).

Measurement of cholesteryl ester synthesis

Mammalian cells or parasites were incubated for 2 h in medium containing 0.2 mM radioactive FA at bound to albumin (1% BSA, oleate:BSA molar ratios of 2:1), 1 mg ml−1 [3H]-cholesterol incorporated into LDL, or 0.2 mM [14C]-PC to monitor the formation of radiolabelled CE. Cell isolation, lipid extraction and separation by TLC using hexane/diethyl ether/acetic acid (80:20:1) were realized as described (Coppens et al., 1995).

In vitro sterol esterification assay

Assay of the activity of reconstituted ACAT was performed as described (Cadigan et al., 1988). Briefly, a deoxycholate/PC solution (final concentration: 20/4 mg ml−1) was added to the Toxoplasma or CHO cell homogenates obtained by hypotonic shock and scraping. The mixture was incubated for 20 min at 4°C. The solubilized cell extract was then diluted 16-fold (v/v) with cholesterol-PC liposomes prepared by the cholesteryamine method (Cadigan et al., 1988) and incubated for 10 min at 4°C. The acyl acceptors were added to the mixture at the concentration of 20 μg at a constant acceptor:PC molar ratio (~0.2), followed by incubation for 10 min at 4°C. The reaction commenced by adding of [3H]-oleoyl CoA-BSA conjugate (final concentration: 50 mM radioactive oleoyl CoA-BSA containing 2.5 mg ml−1 fatty acid-free BSA in 0.02 M Tris-HCl pH 7.8), and incubated for 10 min at 37°C (Ohgami et al., 2000). The reaction was terminated by the addition of 3 ml of a solution of CHCl3/CH3OH (2:1), and the radioactive steryl oleate was determined by TLC.

Determination of sterol composition

Intravacuolar T. gondii were cultivated in medium containing either 10% FBS or 10% LPDS for 36 h at 37°C. After parasite isolation, lipid extraction and separation of unaponifiable material by chromatography as described above, the plates were sprayed with 50% H2SO4 and dried at 100°C. This colours cholesterol as a red spot, well-distinct from the brown spot of ergosterol. Parasite sterol composition was determined by reference to internal standards.

Construction of yeast vectors expressing Toxoplasma ACAT1 homologues

The yeast strain SCY910 (MATα trp1-1 ade2-1 ura3-1 his3-11,15 leu2-3112 are1Δ::HIS3 are2Δ::LEU2) was the generous gift of Dr Steven Sturley (Columbia University, NY). To express Toxoplasma proteins in this yeast strain defective in steryl esters and TAG formation, the putative TgACAT1α and TgACAT1β sequence in the pCl-TgACAT vector was released by HindIII and XbaI restriction digestion, purified and ligated into YEpGAL-URA3 vector digested with HindIII and XbaI. The resulting YEpGAL-TgACAT1α and TgACAT1β plasmids were transformed into the JCY500 strain.

Measurement of ACAT activity in yeast extracts

A wild-type yeast strain, the isogenic yeast quadruple knockout strains harbouring or not the YEpGAL-TgDGAT plasmid described above, were examined for ACAT activity. Each strain was diluted to an A600 of 0.1 and grown overnight in 40 ml of SC-uracil with 2% raffinose at 30°C. The absorbance of the different strains ranged from 1.2 to 2.7 following the initial growth period. The cultures were then diluted to an A600 of 0.15 in 100 ml of SC-uracil with 2% raffinose and grown for 4 h. At this stage, the
cultures were adjusted to 2% galactose, grown for another 8 h and harvested at an absorbance ranging from 1.2 to 1.5. The cells were sedimented by centrifugation and the resultant pellets were washed twice with distilled water and subsequently frozen at –70°C. Homogenates were prepared from frozen cells using aliquots corresponding to an absorbance of 0.45 in a buffer containing 50 mM Hepes, 1 mM 2-mercaptoethanol, 0.5 mM EDTA and 0.25 M sucrose (pH 7.2), in the presence of a protease inhibitor cocktail including 0.5 mM phenylmethylsulphonylfluoride. The cells were disrupted with 0.3 ml of glass beads in a Bead Beater for six cycles of 60 s, with 60 s cooling in between each cycle. Aliquots containing 50 μg of protein were utilized for the enzyme assay as described (Yang et al., 1997). Briefly, ACAT activity was determined by the rate of incorporation of [14C]-palmitoyl-CoA into cholesterol esters in the presence of unlabelled cholesterol. The reaction was terminated after 2.5 min by adding CHCl₃/CH₃OH (2:1), vigorously mixed and centrifuged to separate aqueous and organic phases after the addition of water. The recovered chloroform layer containing radioactive cholesteryl palmitate was proceeded for TLC and quantified as described (Yang et al., 1997).

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