Host cells: mobilizable lipid resources for the intracellular parasite *Toxoplasma gondii*

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Accepted 17 May 2002
Journal of Cell Science 115, 3049-3059 (2002) © The Company of Biologists Ltd

Summary

Successful replication of the intracellular parasite *Toxoplasma gondii* within its parasitophorous vacuole necessitates a substantial increase in membrane mass. The possible diversion and metabolism of host cell lipids and lipid precursors by *Toxoplasma* was therefore investigated using radioisotopic and fluorophore-conjugated compounds. Confocal microscopic analyses demonstrated that *Toxoplasma* is selective with regards to both the acquisition and compartmentalization of host cell lipids. Lipids were compartmentalized into parasite endomembranes and, in some cases, were apparently integrated into the surrounding vacuolar membrane. Additionally, some labels became concentrated in discrete lipid bodies that were biochemically and morphologically distinct from the parasite apical secretory organelles. Thin layer chromatography established that parasites readily scavenged long-chain fatty acids as well as cholesterol, and in certain cases modified the host-derived lipids. When provided with radiolabeled phospholipid precursors, including polar head groups, phosphatidic acid and small fatty acids, intracellular parasites preferentially accrued phosphatidylcholine (PtdCho) over other phospholipids. Moreover, *Toxoplasma* was found to be competent to synthesize PtdCho from radiolabeled precursors obtained from its environment. Together, these studies underscore the ability of *Toxoplasma gondii* to divert and use lipid resources from its host, a process that may contribute to the biogenesis of parasite membranes.

Key words: Cholesterol, Endocytosis, Exocytosis, Fatty acid, Organelle association, Parasitophorous vacuole, Phospholipid, Phosphatidylcholine, Vesicular transport

Introduction

Eukaryotic cells invaded by *Toxoplasma gondii* acquire a unique and dynamic membranous organelle, the parasitophorous vacuole, the design of which accounts for the success of *T. gondii* as an intracellular pathogen. As the causative agent of human toxoplasmosis, this crescent-shaped protozoan is encompassed along with a variety of intracellular parasites within the phylum Apicomplexa. Unified by a complex battery of apical organelles, apicomplexans sequentially discharge these specialized organelles upon host cell invasion (Dubremetz et al., 1993). The initial attachment to host cells is mediated by the secretion of micronemes, which is succeeded immediately by the discharge of rhoptries (Carruthers and Sibley, 1997). Rhoptry proteins are injected into the membranous indentation directly subjacent to the invading parasite, and several investigators have speculated that rhoptries also contribute *Toxoplasma* lipids to the forming parasitophorous vacuole membrane (PVM) (Bannister et al., 1986; Foussard et al., 1991; Håkansson et al., 2001; Joiner, 1991; Nichols et al., 1983). Concurrent with these processes, the parasite squeezes through a region of intimate membrane apposition between the host and parasite termed the moving junction (Aikawa et al., 1977). The parasite is progressively translocated through the moving junction by its actinomyosin motor and it enters a compartment bounded by the PVM (Dobrowolski et al., 1997; Dobrowolski and Sibley, 1996). Once established intracellularly, parasites extensively modify the PV by discharging their dense granules, the contents of which are probably instrumental in the acquisition of host cell molecules (Carruthers and Sibley, 1997; Dubremetz et al., 1993). Throughout the extremely rapid course of invasion, host cell integral membrane proteins are largely excluded from the PVM (Mordue et al., 1999b). This exclusion results in the formation of a membrane unique in its biochemical and biophysical properties and the creation of a nonfusogenic vacuole, securing parasites in a niche sequestered from host cell endo/exocytic transport pathways (Mordue et al., 1999a; Sibley et al., 1985). Ironically, the nonfusogenic PVM quickly becomes physically associated with sites of host cell lipid biosynthesis, the endoplasmic reticulum (ER) and mitochondrial membranes, a phenomenon that may be critical for the survival of the parasite (Endo et al., 1981; Jones et al., 1972; Sinai and Joiner, 2001; Sinai et al., 1997). These coordinated events design an organelle poised for selective transactions with host cell organelles, which may facilitate the intracellular survival and growth of *T. gondii*.

The inability of *T. gondii* to grow in vitro in the absence of a host cell suggests that, in addition to protection from extracellular immune confrontations, its intracellular lifestyle provides the parasite with some essential factor(s) unobtainable in the extracellular milieu. The isolation from host cell endo/exocytic pathways negates the possibility that the parasites require proteins or lipids whose trafficking is restricted to those pathways and suggests that the needed host components are selectively mobilized via non-vesicular transport routes. Candidate components include nucleobases,

amino acids, cytosolic proteins, and lipids. *T. gondii* is in fact auxotrophic for both purines and tryptophan (Pfefferkorn, 1984; Schwartzman and Pfefferkorn, 1982), and is afforded access to small molecules (<1400 Da) via pores in the PVM (Schwab et al., 1994). In addition, low density lipoprotein (LDL)-complexed cholesterol is integrated into intracellular parasite membranes by an undefined mechanism (Coppens et al., 2000), indicating that *T. gondii* is competent to scavenge this lipid from its host. Because rapid parasite replication must coincide with significant biogenesis of parasite membranes and the concomitant enlargement of the PVM, it is plausible that parasites are adept at diverting and/or metabolizing host cell membrane lipids or lipid precursors. This hypothesis was tested in the current study, which made use of fluorescent and radioactive lipid probes in conjunction with fluorescence microscopy and thin layer chromatography.

Materials and Methods

Reagents

The fluorescent lipid probes 5-butyl-4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-nonanoic acid (C4-BODIPY-C9), cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate (cholesteryl BODIPY C12), 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD-cholesterol), 2-(4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY-phosphatidylcholine), 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphate, diammonium salt (BODIPY-phosphatidic acid), and 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid (BODIPY-C12) were obtained from Molecular Probes (Eugene, OR). Also from Molecular Probes were the far red-emitting nucleic acid dye TOPRO3 and the neutral lipid dye Nile red. All unlabeled lipids (cholesterol, L-α-phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, and sphingomyelin) were purchased from Avanti Polar Lipids (Alabaster, AL). The radiolabeled lipids and lipid precursors ([^{14}C-methyl] choline chloride, 55 mCi/mmol; [1-^{14}C]butyric acid, sodium salt, 55 mCi/mmol; [1,2-^{14}C]acetic acid, sodium salt, 55 mCi/mmol; [^{14}C(U)]L-serine, 120 mCi/mmol; [1,2-^{14}C]ethanolamine hydrochloride, 110 mCi/mmol; and [oleic-1-^{14}C] L-α-phosphatidic acid, dioleoyl, 55 mCi/mmol) were supplied by American Radiolabeled Chemicals (St Louis, MO). Fluorophore-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Labs (West Grove, PA) or Molecular Probes (Eugene, OR). Delipidized bovine serum was purchased from Biocell Laboratories (Rancho Domingo, CA). Rabbit antiserum directed against the parasite rhoptry protein ROP2 was kindly provided by Con Beckers (University of Alabama, Birmingham, AL) (Beckers et al., 1994). Mouse monoclonal ascites directed against the microneme protein MIC4 and an antiserum directed against the dense granule protein GRA2 were generated in this laboratory (Brecht et al., 2001) (L.D.S., unpublished). Vectashield mounting media was obtained from Vector Laboratories (Burlingame, CA). Merck silica gel 60 thin layer chromatography sheets were supplied by EM Science (Gibbstown, NJ). Thermanox coverslips were purchased from Electron Microscopy Sciences (Fort Washington, PA). En^{3}Hance spray was purchased from Perkin-Elmer Life Sciences (Boston, MA). Organic solvents for thin layer chromatography were from Sigma (St Louis, MO) and were of the highest grade available. All cell culture reagents were supplied by Gibco Life Sciences (Bethesda, MD).

Liposomes/lipids

Liposomes comprising NBD-cholesterol or BODIPY-phosphatidylcholine were prepared by combining either fluorescent cholesterol with unconjugated phosphatidylcholine (PtdCho) and phosphatidylglycerol or fluorescent PtdCho with unconjugated cholesterol and phosphatidylglycerol to yield a final ratio of 1 cholesterol:0.9 PtdCho:0.1 phosphatidylglycerol. Lipid solutions were combined in round-bottom flasks and immediately dried under a stream of argon with constant agitation with glass beads in a 40°C water bath. After drying, the flask containing the lipid cake, phosphate-buffered saline (PBS), and the glass beads was rapidly rotated in a water bath sonicator set at 40°C. Liposomes were collected and stored under argon at room temperature in glass vials. Immediately prior to each use the liposomes were sonicated in a water bath sonicator for 10 minutes at 37°C. Dried lipids and non-liposome lipid solutions were stored dessicated at –20°C in single-use aliquots until needed. The optimal dilution of each liposome and organic lipid solution was determined empirically for each experimental procedure.

Cell culture

Tachyzoites of the RH strain of *T. gondii* were maintained by serial passage in human foreskin fibroblasts (HFFs) as previously described (Morisaki et al., 1995). HFFs and intracellular parasites were routinely cultured in D10 media (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10 mM Hepes, 2 mM L-glutamine, and 2 μg/ml gentamycin). Mutant 89.1 cells and parental CHO-K1 cells were cultured in H10 (Hams F12 media containing 10% fetal bovine serum, 10 mM Hepes, 2 mM L-glutamine, and 2 μg/ml gentamycin). In preparation for experiments, host cells were grown to confluence in 12-well dishes or 100 mm diameter dishes or to near confluence on glass coverslips.

Infection and labeling

HFFs

Host cells were washed with D10 in which lipid-containing serum was substituted with delipidized bovine serum (DLS-D10). Rinsed cells were either prelabeled with lipids before parasite infection or preinfected with parasites before lipid labeling. In the first case, lipids diluted in DLS-D10 were added to each well and the labeling performed for either 1 hour (for fluorescence experiments) or 3 hours (for radioactive experiments) in a tissue culture incubator. Following replacement of the lipid-containing solution with DLS-D10, parasites suspended in DLS-D10 were added to each well at a multiplicity of infection (MOI) of 20. Infection was performed for 3 hours (for radioactive experiments) or as otherwise noted (for fluorescence experiments) in a tissue culture incubator. In the second case, the order of labeling/infection was simply reversed. [^{14}C]Ethanolamine, [^{14}C]serine, [^{14}C]choline, and [^{14}C]phosphatidic acid were all used at a final concentration of 2 μCi/ml, whereas [^{14}C]acetic acid and [^{14}C]butyric acid were both used at 20 μCi/ml.

89.1s/CHOs

Monolayers were either mock-infected or infected at an MOI of 20 with *T. gondii*. Approximately 6 hours before imminent infected cell lysis, host cells were gently washed with H10 in which lipid-containing serum was substituted with delipidized bovine serum (DLS-H10). DLS-H10 containing 2 μCi/ml [^{14}C]choline was added to each well and labeling performed for 30 minutes in a tissue culture incubator. The labeling solution was then removed and the cells chased with label-free DLS-H10.

Extracellular parasites

Approximately 1×10^8 parasites were collected from a lysing-out culture and purified by sequential passage through 20, 22 and 25 G needles (to disrupt monolayer debris) and finally through a 3 μm pore polycarbonate filter (Nuclepore, Whatman, Clifton, NJ). Purified parasites were pelleted by centrifugation at 1200 ***g*** for 10 minutes,

resuspended in a small volume of DLS-D10, and incubated with lipid label for 3 hours at either 2°C or 37°C. Both phosphatidic acid and choline were used at a final concentration of 2 μCi/ml, while acetic acid was used at 20 μCi/ml. Following labeling, the label was removed, the parasites were washed in PBS and pelleted by centrifugation, and the pellet prepared for thin layer chromatographic analysis as described below.

Fluorescence microscopic analysis of lipid recruitment

The subcellular distribution of fluorescent lipid analogs was examined in uninfected hosts exposed to lipids following mock infection, hosts exposed to lipids prior to parasite infection, and hosts exposed to lipids subsequent to parasite infection. Infection and lipid labeling was carried out as described above. The coverslips were then rinsed in ice-cold PBS^+ (PBS containing 1 mM each of $CaCl_2$ and $MgCl_2$). The coverslips were mounted upside down on microscope slides in ice-cold PBS^+, sealed onto the slide with nail polish, placed on ice, and immediately imaged live using a Zeiss LSM510 laser scanning confocal microscope. Fluorescence and brightfield images were obtained using a 63× oil plan-apochromat objective lens (NA 1.4, Zeiss) and He-Ne and Kr-Ar lasers. Each image depicts a 0.4 μm-thick focal slice. Images were processed using Zeiss ImageBrowser software and imported into Adobe PhotoShop for final arrangement.

Immunofluorescence microscopy of the *T. gondii* lipid compartment

Host cells were inoculated with parasites at an MOI of 20 and replaced into a tissue culture incubator. After 6 hours the uninvasive parasites were removed and the media was replaced with DLS-D10 containing C4-BODIPY-C9. This solution was removed ~4 hours before host cell lysis and substituted with lipid-free DLS-D10. The lysed-out parasites were collected, forced through 20 G and 25 G needles (to disrupt monolayer debris), and purified by passage through a 3 μm pore filter. Parasites were resuspended in DLS-D10 and either deposited upon poly-L-lysine-coated glass coverslips or added to coverslips seeded with host cells. After a 1 hour incubation at 20°C, the extracellular parasites on coverslips were rinsed in PBS and fixed with 4% (w/v) paraformaldehyde dissolved in PBS^+. After either 10 minutes (for MIC4 and ROP2) or 30 minutes (for GRA2) the media and parasites in the host-containing samples was removed and replaced with fresh DLS-D10 for either 1 hour or 6 hours. At the appropriate time, these samples were rinsed with PBS and fixed. The autofluorescence in all samples was quenched by a 10 minute incubation in 50 mM NH_4Cl dissolved in PBS^+. The samples were permeabilized with 0.05% (w/v) saponin dissolved in PBS containing 0.2% (v/v) fish skin gelatin (PBS/FSG/sap) for 10 minutes. The permeabilized samples were rinsed and incubated in a humidified chamber for 1 hour at 37°C in PBS/FSG/sap in which one of the following antibodies was diluted to a final IgG concentration of 1 μg/ml: pAb anti-ROP2, pAb WU1228 (anti-GRA2), or mAb 5B1 (anti-MIC4) ascites. The coverslips were rinsed extensively in PBS/FSG/sap and stained with Cy5-conjugated anti-mouse or anti-rabbit antibodies for 30 minutes at 37°C. Alternatively, coverslips not incubated with primary antibodies were labeled with either the nucleic acid stain TOPRO3 or the lipophilic dye Nile red for 30 minutes at 37°C. Finally, all coverslips were rinsed, mounted in Vectashield, and confocal images collected as described above.

Electron microscopy

Host cells were inoculated with parasites at an MOI of 20 and replaced into a tissue culture incubator. After 6 hours, the media and uninvasive parasites were replaced with DLS-D10 containing NBD-cholesterol liposomes. This solution was removed ~4 hours before host cell lysis and substituted with DLS-D10. The lysed-out parasites were collected, forced through 20 G and 25 G needles, and purified by passage through a 3 μm pore filter. Parasites were resuspended in DLS-D10 and deposited onto thermanox coverslips seeded with host cells. After 1 hour at 37°C, all coverslips were rinsed in PBS and fixed for 1 hour at 4°C with chilled 4% (w/v) paraformaldehyde, 2% (w/v) glutaraldehyde (both from Polysciences, Warrington, PA) dissolved in PBS^+. Coverslips were rinsed with 125 mM Pipes buffer (4×5 minutes). The samples were then incubated for 10 minutes in diaminobenzadine (DAB) at a concentration of 1.5 mg/ml in 0.1 M Tris pH 7.6. The coverslips were placed cell-side up in DAB solution on a slide and covered with a second, larger coverslip resting on nail polish posts. This assembly was immediately subjected to photoactivation: the light from the FITC filter cube of a Zeiss Axioskop epifluorescence microscope was focused on the parasites/HFFs with the 10× dry objective until fluorescence was undetectable. Samples were washed in 100 mM Pipes, pH 7 and fixed in a freshly prepared mixture of 1% (w/v) glutaraldehyde and 1% (w/v) osmium tetroxide (Polysciences, Warrington, PA) in 100 mM Pipes buffer at 4°C for 30 minutes. The samples were then rinsed extensively in dH_2O prior to en bloc staining with 1% (w/v) aqueous uranyl acetate (Electron Microscopy Sciences, Ft. Washington, PA) for 3 hours at 4°C. Following several rinses in dH_2O, samples were dehydrated in a graded series of ethanol and embedded in Eponate-12 (Ted Pella, Redding, CA). Sections of 70-80 nm were cut, stained with uranyl acetate, and viewed on a JEOL 1200 EX transmission electron microscope.

Thin layer chromatographic analyses of lipid acquisition

Thin layer chromatography was performed on lipid-labeled extracellular parasites, uninfected hosts, uninfected host elements potentially contaminating purified parasite preparations, parasites exposed to lipid subsequent to the establishment of a parasitophorous vacuole, and parasites allowed to invade hosts previously exposed to lipid.

Immediately subsequent to labeling and infection/mock infection, fresh DLS-D10 was added to each culture well and the cultures returned to the incubator until infected cell lysis. The uninfected, scraped monolayers or infected culture debris was forced sequentially through 20 G and 25 G needles. One set of uninfected hosts was reserved as the host sample until the following centrifugation step. In order to provide a mock-infected (–ctl) sample representing host cell lipids contaminating the purified parasite (TL and LT) samples, another set of uninfected hosts was passed through a 3 μm pore polycarbonate filter and reserved until the following centrifugation step. Finally, infected culture debris was passed through a 3 μm pore polycarbonate filter, resulting in a purified parasite preparation. All samples were pelleted by centrifugation at 1200 ***g*** for 10 minutes. The pellets were resuspended in PBS, transferred to microfuge tubes, and pelleted again by centrifugation at 11,000 ***g*** in a swinging bucket rotor (10 minutes, 4°C). The resulting pellet was resuspended in either 15 μl (for radioactive experiments) or 100 μl (for fluorescence experiments) of 150 mM NaCl. Samples were then assayed for protein concentration using the BCA protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. Sample spotting onto silica gel 60 TLC plates was normalized for protein concentration (with the exception of the host cell contaminant samples, which were spotted with a volume equal to that of the lysed-out parasite samples). BODIPY-fatty-acid-labeled samples were resolved by developing TLC plates in hexane/ether/acetic acid (70:30:1), whereas NBD-cholesterol-labeled samples were resolved by developing first with hexane/ether/acetic acid (70:30:1), then with toluene/acetone (70:10), and finally with chloroform/acetic acid (96:4), allowing the plates to dry between each development. The dried fluorescent plates were imaged using a gel documentation camera (Alpha Innotech, San Leandro, CA) and a UV transilluminator following the completion of each development step. Plates spotted with radioactive samples were developed in chloroform/methanol/acetic acid/water (25:15:4:2),

dried, sprayed with the fluorographic agent En^{3}Hance (Perkin Elmer, Boston, MA), and exposed to X-ray film at –80°C. Densitometric analyses of digitalized autoradiograms were performed using AlphaImager 2000 software (Alpha Innotech, San Leandro, CA).

Results

Intracellular *Toxoplasma* diverts lipids from its host

The potential for diversion of host cell lipids by *Toxoplasma* was assessed by confocal microscopic detection of several fluorescent lipid probes incubated with host cells either prior to or subsequent to infection with *T. gondii*. Strikingly, despite the segregation of the PV from the host cell endo/exocytic pathways, a variety of probes was accessible to the parasites, influenced by the order of infection versus addition of lipids. Upon *Toxoplasma* infection, NBD-cholesterol was transported from perinuclear membranes of pre-labeled host cell (Fig. 1, cholesterol hosts panels) to intracellular parasites, in which large puncta were intensely labeled (Fig. 1, cholesterol toxo+lipid and lipid+toxo panels). A parasite-delimiting membrane and reticular parasite membranes were also demarcated following exposure of already intracellular parasites to NBD-cholesterol (Fig. 1, cholesterol toxo+lipid panel), revealing sites of deposition not evident when the label was added before infection (Fig. 1, cholesterol lipid+toxo panel). While BODIPY-PtdCho moved from the plasma membrane and/or dispersed puncta of host cells (Fig. 1, PC hosts panel) to similar locations within their established intracellular parasites (Fig. 1, PC toxo+lipid panel), the lipid was excluded from parasites that had invaded prelabeled hosts (Fig. 1, PC lipid+toxo panel). BODIPY-phosphatidic acid was mobilized to a circumscribing membrane and a Golgi-like pattern within established intracellular parasites (Fig. 1, PA toxo+lipid panel) from host organelles reminiscent of the ER and mitochondria (Fig. 1, PA hosts panel). The fatty acid C4-BODIPY-C9 was routed to large puncta in parasites (where it was concentrated) as well as in a membrane delineating the parasite (Fig. 1, C4C9 toxo+lipid panel), presumably via the large, discrete puncta and/or Golgi-like organelle of the host cell (red/yellow staining in Fig. 1, C4C9 hosts panel). Interestingly, diversion of both BODIPY-phosphatidic acid and C4-BODIPY-C9 from prelabeled host cells invaded by *T.*

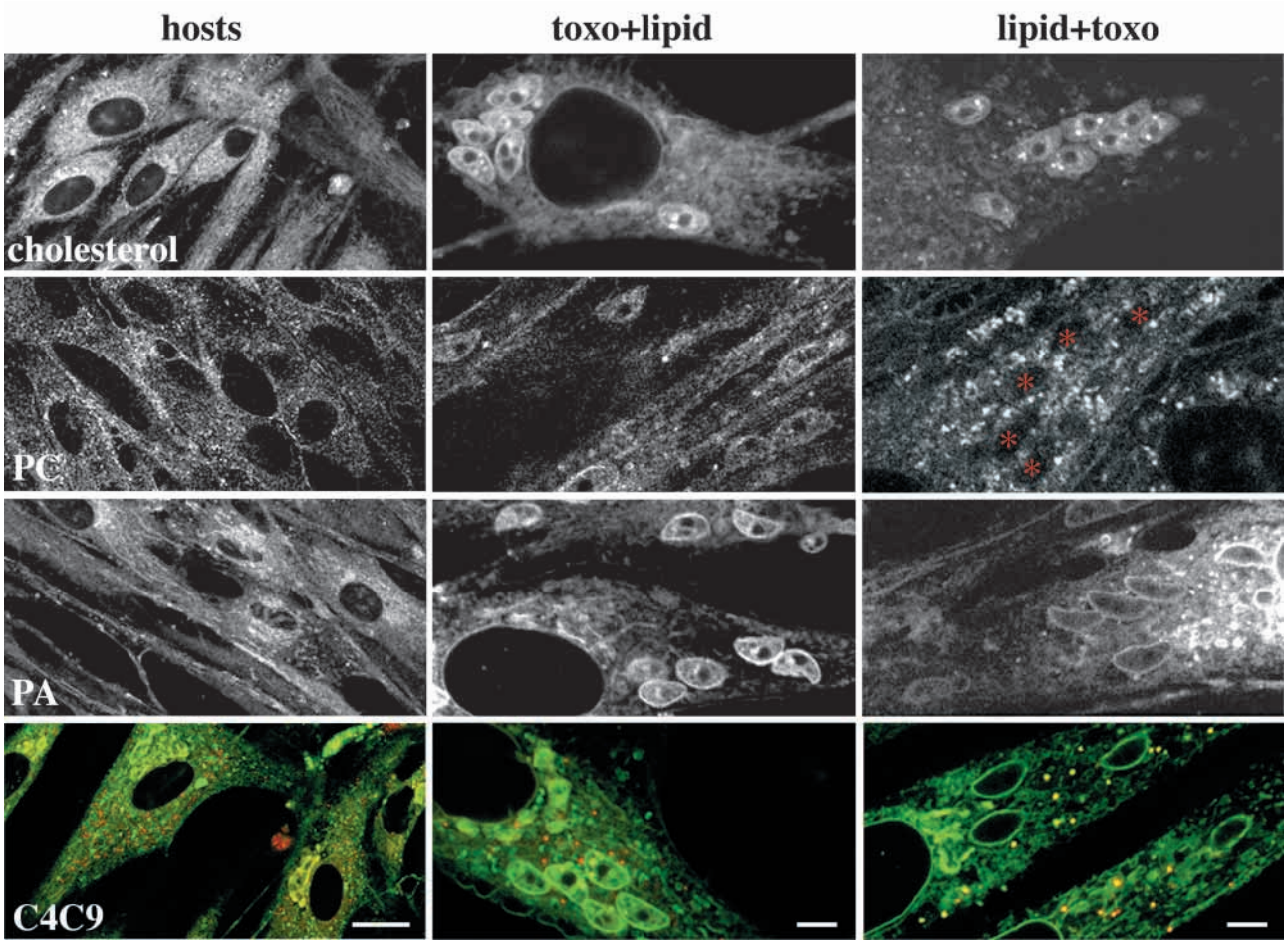


Fig. 1. Selective diversion of host cell lipids. Coverslip-grown host cells were incubated with fluorescent probes alone (hosts), infected with *T. gondii* prior to the introduction of the probe (toxo+lipid), or labeled with the probe before infection with *T. gondii* (lipid+toxo). Images of live cells were immediately acquired by confocal microscopy. Single, 0.4 μm thick optical sections are shown. Cholesterol, NBD-cholesterol; PC, BODIPY-phosphatidylcholine; PA, BODIPY-phosphatidic acid; C4C9, C4-BODIPY-C9. Red emission in the C4C9 panels corresponds to excimer shift of highly concentrated BODIPY. Intracellular parasites in the PC lipid+toxo panel are indicated with asterisks. Bars, 20 μm (hosts); 5 μm (toxo+lipid, lipid+toxo).

gondii resulted in the enrichment and exclusive labeling of a membrane surrounding the parasite that likely represents the PVM (Fig. 1, PA and C4C9 lipid+toxo panels). Collectively, the results described above demonstrated that *T. gondii* mobilizes lipids from various host cell compartments and is selective regarding its compartmentalization of acquired lipids.

Host cell neutral lipids are metabolized by intracellular *T. gondii*

The concentration of cholesterol and fatty acids by *T. gondii* (Fig. 1; and data not shown) raised the question of whether stored lipids were substrates for modification. To investigate the metabolism of acquired neutral lipids, potential metabolites of NBD-cholesterol and the BODIPY-conjugated fatty acids C4C9 and C12 (differing principally in the position of the fluorophore) were resolved by thin layer chromatography (TLC) (Fig. 2). Although integrated into host cell membranes, neither NBD-cholesterol nor BODIPY-C12 served as metabolic substrates (Fig. 2, cholesterol and C12 host lanes).

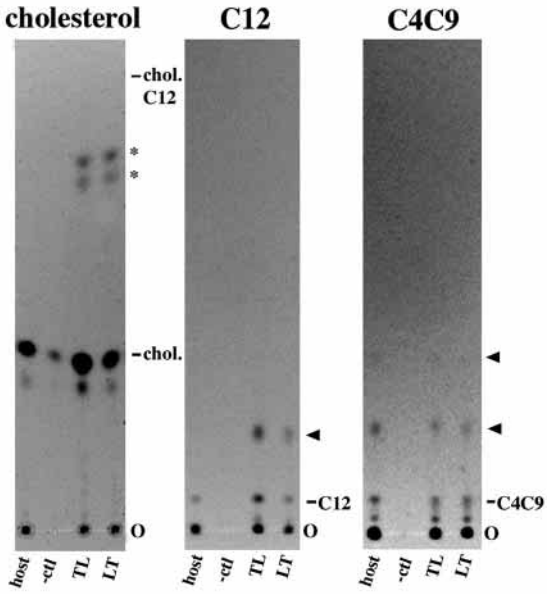


Fig. 2. Neutral lipid handling by intracellular parasites. The indicated fluorophore-conjugated probes were introduced to hosts alone (host and -ctl) or hosts infected with *T. gondii* before (TL) or after (LT) labeling. Lipids of hosts (host), isolated parasites (TL, LT), or contaminating membranes (-ctl) were resolved by thin layer chromatography. BODIPY-fatty-acid-labeled samples were resolved by developing TLC plates in hexane/ether/acetic acid (70:30:1), whereas NBD-cholesterol-labeled samples were resolved by developing first with hexane/ether/acetic acid (70:30:1), then with toluene/acetone (70:10), and finally with chloroform/acetic acid (96:4), allowing the plates to dry between each development. Lipids were detected by illumination of the plates with UV light. O, origin. The migration of the fluorescent probes NBD-cholesterol (chol.), BODIPY-C12 (C12), and C4-BODIPY-C9 (C4C9), as well as the cholesteryl ester standard cholesteryl BODIPY-C12 (chol. C12) are noted. Presumed cholesteryl esters are indicated with asterisks and metabolites of the BODIPY-fatty acids are indicated with arrowheads. The prominent species at the origin of the C12 and C4C9 panels is suspected to be a BODIPY-phospholipid on the basis of its migratory behavior in very polar solvents.

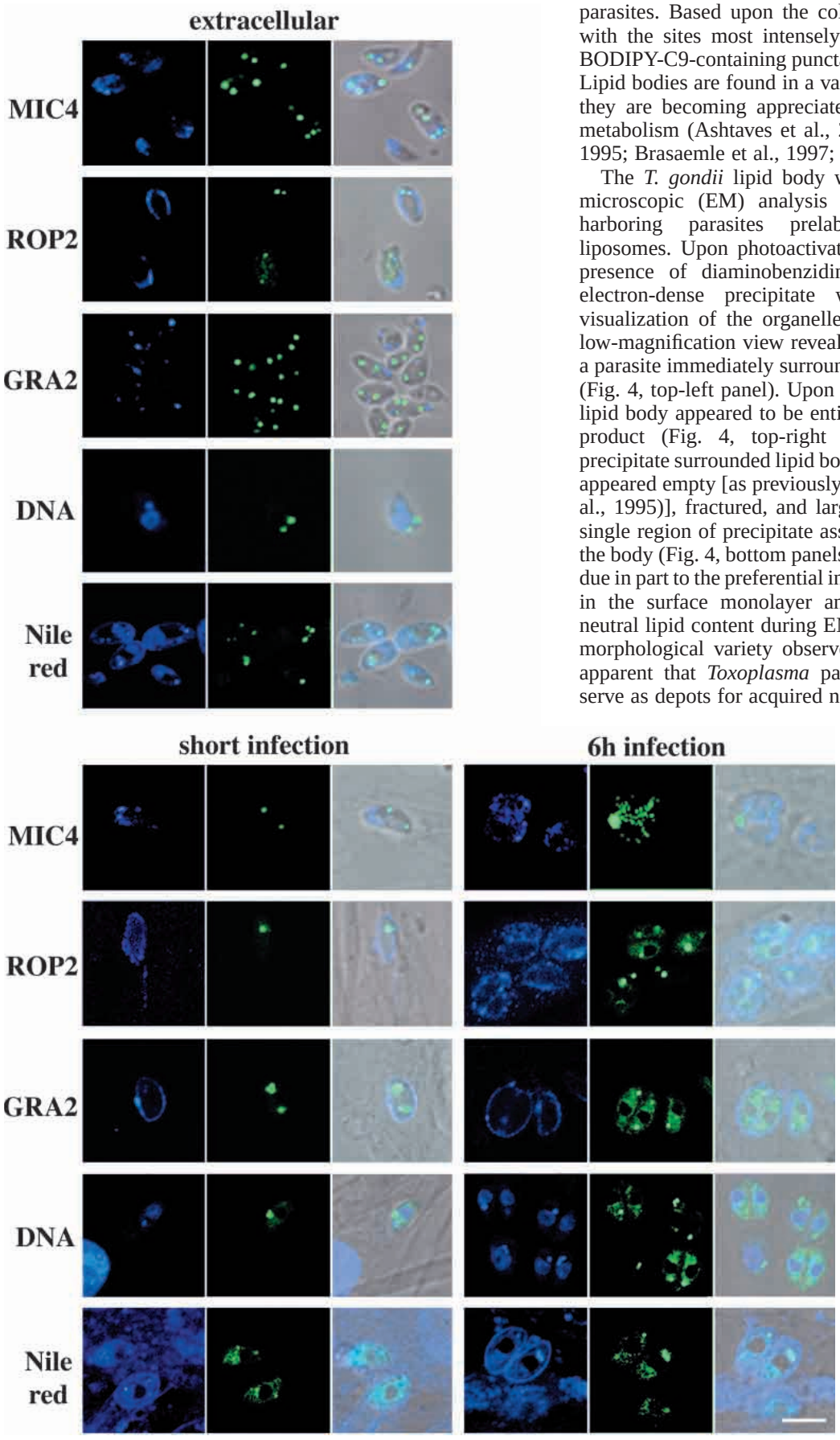
Remarkably, *T. gondii* not only acquired these probes from its host, but also metabolized them (Fig. 2, TL and LT lanes). Host-derived cholesterol was modified by the parasites to generate two faster migrating species that probably represent one or more cholesteryl esters (Fig. 2, cholesterol TL and LT lanes, asterisks), consistent with the recent discovery of acyl-CoA:cholesterol acyltransferase (ACAT) activity in *T. gondii* (Sonda et al., 2001). While parasites contained C4-BODIPY-C9 lipids that migrated similarly to those of host cells (Fig. 2, C4C9 lanes, arrowheads), BODIPY-C12 was perceptibly altered only by the parasites to generate two faster migrating products (Fig. 2, C12 lanes, arrowheads). These experiments therefore establish that *Toxoplasma* can metabolize some neutral lipids acquired from the host.

T. gondii sequester host-cell-derived lipids in a non-secretory compartment

The prospect that lipid constituents of the large puncta were mobilized by *T. gondii* was investigated by confocal microscopy. The profile of the parasite secretory organelles was compared with that of C4-BODIPY-C9 in parasites freshly egressed from host cells, those very briefly established in an intracellular niche, and those that had modified their parasitophorous vacuole and replenished their secretory organelles (Fig. 3). Whereas the lipid puncta were dispersed throughout the parasites, the microneme protein MIC4 in freshly egressed, newly invaded, and established intracellular parasites was polarized to the parasite apex (Fig. 3, MIC4 panels). When the relationship between the rhoptry protein ROP2 and the lipid puncta was assessed, under no circumstance did ROP2 colocalize with C4-BODIPY-C9; instead ROP2 contoured the apical region of extracellular parasites or delineated the PVM of intracellular parasites (Fig. 3, ROP2 panels). Likewise, C4-BODIPY-C9 staining was in all cases distinct from that of the dense granule protein GRA2, which demarcated dense granules in extracellular parasites and PVs surrounding intracellular parasites (Fig. 3, GRA2 panels). Notably, within parasites that had replicated intracellularly, the fluorescent lipids appeared in organelles reminiscent of the ER and Golgi (Fig. 3, 6h infection panels). These observations established that the puncta represent a unique non-secretory organelle, that the concentrated lipids were not redistributed en masse to replenish the secretory organelles, and that lipids were gradually mobilized from the puncta to other organelles.

Given the discrete staining pattern of C4-BODIPY-C9, it was important to determine whether these puncta represented the apicoplast, a site of fatty acid metabolism in apicomplexans (Waller et al., 1998; Waller et al., 2000). When apicoplast DNA was visualized using the nucleic acid probe TOPRO3, none of the C4-BODIPY-C9 puncta coincided with the singular apicoplast (Fig. 3, DNA panels).

Nile Red has been widely used to detect neutral lipid stores, and is considered the hallmark of lipid bodies in higher eukaryotes (Greenspan et al., 1985). When the staining patterns of Nile Red and C4-BODIPY-C9 were examined, partial overlap under all circumstances was conspicuous (Fig. 3, Nile Red panels). Nile Red prominently decorated lipid bodies and to a lesser extent the ER membranes of both host cells and parasites, the PVM and plasma membrane of briefly intracellular parasites, and the plasma membrane of established



parasites. Based upon the colocalization of C4-BODIPY-C9 with the sites most intensely stained by Nile Red, the C4-BODIPY-C9-containing puncta were judged to be lipid bodies. Lipid bodies are found in a variety of other eukaryotes, where they are becoming appreciated for their role in cellular fat metabolism (Ashtaves et al., 2001; Blanchette-Mackie et al., 1995; Brasaemle et al., 1997; Servetnick et al., 1995).

The *T. gondii* lipid body was next examined by electron microscopic (EM) analysis of freshly invaded host cells harboring parasites prelabeled with NBD-cholesterol liposomes. Upon photoactivation of the NBD moiety in the presence of diaminobenzidine (Pagano et al., 1989), an electron-dense precipitate was formed, facilitating the visualization of the organelle storing NBD-cholesterol. The low-magnification view revealed a single, intact lipid body in a parasite immediately surrounded by liposomes and the PVM (Fig. 4, top-left panel). Upon closer inspection, this spherical lipid body appeared to be entirely surrounded by the reaction product (Fig. 4, top-right panel). More often, reaction precipitate surrounded lipid bodies that were irregular in shape, appeared empty [as previously reported (Blanchette-Mackie et al., 1995)], fractured, and largely extracted, and contained a single region of precipitate associated with the outer limits of the body (Fig. 4, bottom panels). These variations are probably due in part to the preferential incorporation of NBD-cholesterol in the surface monolayer and the difficulty in preserving neutral lipid content during EM processing. Regardless of the morphological variety observed, from this examination it is apparent that *Toxoplasma* parasites house lipid bodies that serve as depots for acquired neutral lipids.

Fig. 3. Storage of neutral lipids. Parasites grown in the presence of the fluorescent fatty acid C4C9 were added either to poly-L-lysine-coated coverslips (extracellular) or coverslip-grown host cells. After 1 hour (extracellular and short infection) or 6 hours (6h infection), coverslips were fixed and processed for immunocytochemistry using antibodies directed against MIC4, ROP2 or GRA2. Primary antibodies were detected by subsequent incubation with Cy5-conjugated anti-mouse or anti-rabbit antibodies. Additional coverslips were incubated with the lipophilic dye Nile Red or the nucleic acid stain TOPRO3. Images were acquired by confocal microscopy. Single, 0.4 μm thick optical sections are shown. The immunochemical staining of parasite organelles appears blue, whereas C4C9 is depicted in green. The two channels merged onto the brightfield image are shown to facilitate the comparison of labeling patterns. Bar, 5 μm.

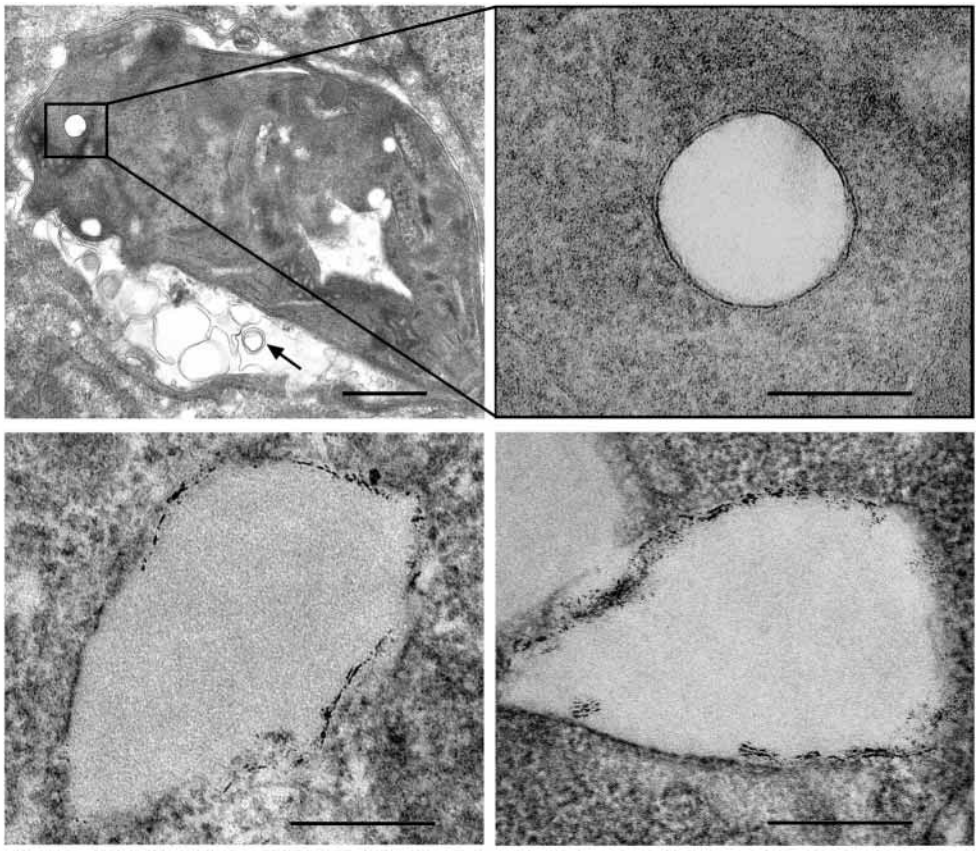


Fig. 4. The *T. gondii* lipid body. Parasites were grown in host cells exposed to the fluorescent probe NBD-cholesterol throughout the course of infection. Following host cell lysis, the purified, labeled parasites were allowed to infect fresh coverslip-grown host cells. After a 1 hour infection, coverslips were fixed, photoactivated in the presence of diaminobenzidine, and processed for electron microscopic analysis. An intracellular parasite is shown in low magnification in the top-left panel, and its prominent lipid body demarcated with electron-dense reaction product is shown in high magnification in the top-right panel. Two additional profiles of typical lipid bodies are depicted in the lower panels. The arrow in the top-left panel denotes an intravacuolar liposome. Bars, 500 nm (low magnification); 100 nm (high magnification).

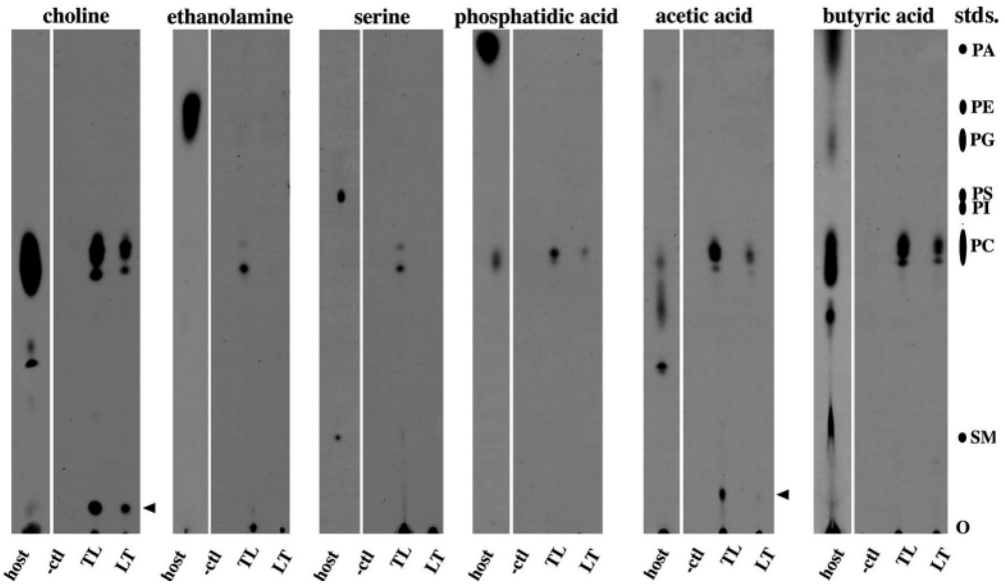


Fig. 5. Use of phospholipid precursors by *T. gondii*. The indicated ^{14}C-labeled lipid precursors were introduced to hosts alone (host and -ctl) or hosts infected with *T. gondii* before (TL) or after (LT) labeling. Lipids of hosts (host), isolated parasites (TL, LT), or contaminating membranes (-ctl) were resolved by thin layer chromatography using the solvent system chloroform/methanol/acetic acid/water (25:15:4:2), and detected by autoradiography after visualizing standards. The migration of standards (stds.) is indicated: PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin. O, origin. Arrowheads denote the migration of radiolabeled precursors. Both within and between individual precursor label samples, spotting was normalized for protein concentration (with the exception of the host cell contaminant samples, which were spotted with a volume equal to that of the parasite samples). Because the host lanes were exposed to film for periods briefer than the -ctl, TL and LT lanes, the host lanes are separated from the others. All other lanes were exposed to film for equal time periods.

Intracellular *T. gondii* preferentially accrue choline-containing phospholipid species

The diversion of host cell phospholipids, considered together with the metabolism of host-derived neutral lipids by intracellular *T. gondii*, raised the possibility that parasites use host phospholipids/phospholipid precursors as a source of membrane materials. This prospect was investigated using three classes of radioactive phospholipid precursors: the head groups (ethanolamine, serine and choline), phosphatidic acid, and two short-chain fatty acids (acetic and butyric acids).

A bias of phospholipid/phospholipid precursor diversion by *T. gondii* was immediately evident upon examination of TLC lanes in which host and parasite lipids were resolved (Fig. 5). Host cells fed [^{14}C]choline accumulated lipids that co-migrated with the PtdCho standard (Fig. 5, choline host lane). Strikingly, parasites isolated from these hosts were comprised of substantial quantities of choline-containing phospholipids (Fig. 5, choline TL and LT lanes). Based on co-migration with the host cell PtdCho as well as the PtdCho standard, these species were tentatively considered to be one or more species of PtdCho. The phosphatidylethanolamine synthesized by hosts fed [^{14}C]ethanolamine was not included in parasite membranes, whereas two spots that co-migrated with PtdCho, presumably formed by the methylation of phosphatidylethanolamine, were noted (Fig. 5, ethanolamine lanes). Parasites grown in [^{14}C]serine-fed hosts also contained similarly migrating species, which were likely generated by the enzymatic modification of serine-labeled host cell phospholipids (Fig. 5, serine lanes). The biased acquisition/metabolism of host cell phospholipids was further indicated upon labeling of cultures with phosphatidic acid, acetic acid and butyric acid. Whereas the efficient uptake of [^{14}C]phosphatidic acid was not coupled with a marked conversion into other phospholipids by hosts, phosphatidic acid was undetectable in parasites, which instead contained a spot co-migrating with PtdCho as the predominant phospholipid species (Fig. 5, phosphatidic acid lanes). Finally, while both [^{14}C]acetic and [^{14}C]butyric acids were anabolized into multiple host phospholipids, they were integrated solely into spots co-migrating with PtdCho in the parasite membranes (Fig. 5, acetic acid and butyric acid lanes). *T. gondii* thus appears to be particular in its scavenging of lipids and/or precursors from its host.

Parasite PtdCho is derived from both parasites and hosts

The relatively low level of diversion of BODIPY-PtdCho from host cells (Fig. 1), in concert with the preponderance of radiolabeled PtdCho lipids in parasite membranes (Fig. 5) intimated that *T. gondii* may be competent to metabolize precursors into PtdCho. This was tested by TLC analysis of host-free parasites incubated at either 2°C or 37°C with radiolabeled phosphatidic acid, choline or acetic acid (Fig. 6). As expected from its energy-independent insertion into plasma membranes, [^{14}C]phosphatidic acid was associated with parasite membranes at both 2°C and 37°C (Fig. 6, phosphatidic acid panel, EC lanes). However, extracellular parasites failed to convert [^{14}C]phosphatidic acid into more complex phospholipids, while a species co-migrating with PtdCho was again observed within parasites labeled intracellularly (Fig. 6, phosphatidic acid panel, compare the *37C* lanes). Intracellular parasites fed [^{14}C]choline converted it into several lipids that co-migrated with PtdCho standards and which are hence tentatively identified as forms of PtdCho (Fig. 6, choline panel, IC lane). Remarkably, host-free parasites not only internalized [^{14}C]choline at physiological temperature (Fig. 6, choline panel, EC lanes), but converted it into one of these lipid species (Fig. 6, choline panel, compare the 37C lanes). Moreover, the diffusion of [^{14}C]acetic acid into extracellular parasites (Fig. 6, acetic acid panel, EC lanes) was succeeded by the incorporation of two of the three spots co-migrating with PtdCho (Fig. 6, acetic acid panel, compare the 37C lanes). These results suggest that *T. gondii* is able to synthesize PtdCho from choline and a fatty acid unit.

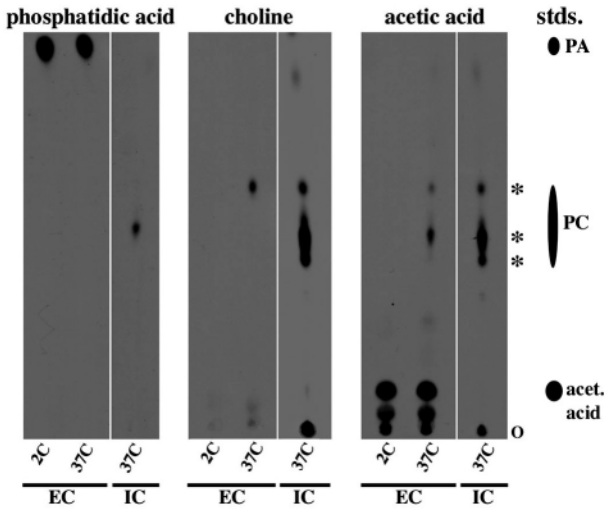


Fig. 6. De novo synthesis of phospholipids by *T. gondii*. The indicated ^{14}C-labeled phosphatidylcholine precursors were introduced to either untreated, purified extracellular parasites (EC) or intracellular (IC) parasites. Labeling was performed at either 2°C (2C) or 37°C (37C) before parasites were harvested for analysis by thin layer chromatography using the solvent system chloroform/methanol/acetic acid/water (25:15:4:2), and lipids detected by autoradiography after visualizing standards. The plates were exposed to X-ray film for identical time periods. The migration of standards (stds.) is indicated: PA, phosphatidic acid; PC, phosphatidylcholine; acet. acid, acetic acid. Asterisks indicate three clearly resolved phosphatidylcholine spots. O, origin.

Although extracellular *Toxoplasma* appears able to manufacture PtdCho, the extent to which parasites scavenge versus synthesize PtdCho when they are intracellular remained in question. To address this question, mutant fibroblasts (89.1) or parental Chinese hamster ovary (CHO) cells infected with *T. gondii* were pulsed with [^{14}C]choline and the kinetics of PtdCho synthesis monitored. 89.1 cells have a moderate deficiency in the de novo synthesis of PtdCho, owing to a defect in the first enzymatic reaction in this pathway, the phosphorylation of choline (Nishijima et al., 1984). This deficiency was evident in the TLC profiles of uninfected host cells: 89.1 cells were retarded in their synthesis of PtdCho (Fig. 7, compare H lanes in the CHO and 89.1 panels). The accumulation of a PtdCho-co-migrating species in parasites grown in CHO and 89.1 cells also differed (Fig. 7, compare T lanes in the CHO and 89.1 panels). Surprisingly, parasites

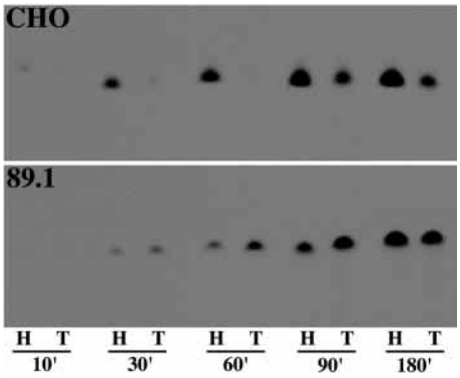


Fig. 7. Precursor requirements for *T. gondii* synthesis of phosphatidylcholine. Either CHO cells alone (CHO panel, H lanes), 89.1 cells alone (89.1 panel, H lanes), or hosts harboring *T. gondii* (CHO and 89.1 panels, T lanes) were pulse-labeled with [^{14}C]choline. Following the removal of the label, the cultures were chased for the indicated time periods with label-free media. The cells were then harvested and the parasites in the T samples were isolated. Samples were normalized for protein concentration, resolved by thin layer chromatography using the solvent system chloroform/methanol/acetic acid/water (25:15:4:2), and lipids detected by autoradiography after visualizing standards.

within 89.1 cells actually accumulated more (two- to threefold, quantitatively) of the radiolabeled PtdCho than those within CHO cells (Fig. 7, compare H and T lanes in the CHO panel). These results indicate that when environmental PtdCho resources are limited, resident parasites adeptly scavenge host cell choline and probably synthesize PtdCho.

Discussion

This report establishes that *Toxoplasma gondii* acquires lipid resources from its host and probably uses them for the construction of complex lipids. Scavenged lipid probes were compartmentalized into the lipid body as well as within endomembranes. Acquisition of NBD-cholesterol and BODIPY-C12 was accompanied by their modification. In host cells provided with labeled precursors, choline-containing species were the major phospholipids accrued by intracellular parasites. These phospholipids were tentatively judged to represent PtdCho based on co-migration with host cell PtdCho as well as PtdCho standards. Extracellular parasites were capable of incorporating acetate and choline into PtdCho and parasites inhabiting host cells compromised in their capability to synthesize PtdCho were able to generate PtdCho independently. Together, these studies indicate that the parasite both acquires lipid precursors from its environment and is capable of biosynthesis to provide lipids critical for membrane biogenesis.

The *Toxoplasma* lipid body

The present study substantiates the existence of lipid bodies within *T. gondii* and suggests that this organelle has a role in *Toxoplasma* lipid homeostasis. Lipid bodies described in plants, animals and yeast are composed of a monolayer of proteins and polar lipids circumscribing a core of neutral lipids (reviewed by Murphy and Vance, 1999). The polar lipids that shuttled from host cell organelles to the *Toxoplasma* lipid body (i.e. NBD-cholesterol and the BODIPY-fatty acids and derivative polar metabolites) are thus predicted to be stored within the surface monolayer until modified or transported elsewhere. An active role for the lipid body in the metabolism and trafficking of lipids has been implicated by the enrichment of several lipid enzymes and lipid-binding proteins within previously characterized lipid bodies in other systems (Ashtaves et al., 2001; Blanchette-Mackie et al., 1995; Brasaemle et al., 1997). The parasite lipid body has not yet been confirmed as the site of the lipid metabolism noted herein. However, trafficking between *Toxoplasma* lipid bodies and ER, as noted in other eukaryotes (Murphy and Vance, 1999), is suggested by the gradual disappearance of C4-BODIPY-C9 from the lipid body and its appearance in the ER and Golgi upon parasite growth and replication. The *Toxoplasma* lipid body thus appears to concentrate diverted host cell lipids, providing a source for the biogenesis of parasite membranes and possibly serving as a site of lipid metabolism.

The source of *Toxoplasma* lipids

Toxoplasma parasites acquired lipid resources from the host regardless of the order of labeling and infection. The order did, however, impact the compartmentalization of most acquired lipids. Although the reason behind this observation has yet to be elucidated, in either case the parasites readily modified host cell lipids. The esterification of acquired NBD-cholesterol and the modification of acquired BODIPY-fatty acids into related lipids demonstrates that parasites metabolize host-derived neutral lipids. Regarding the phospholipids, intracellular parasites metabolize scavenged choline and appear to convert scavenged phosphatidylethanolamine and phosphatidylserine into PtdCho, preferentially accruing choline-containing phospholipids. The manipulation of diverted host cell lipids almost certainly represents an important mechanism by which *Toxoplasma gondii* growth is accommodated.

Toxoplasma does not merely scavenge lipids, but is also capable of their biosynthesis. *T. gondii* expresses at least three components of the type II fatty acid synthesis (FAB II) pathway (Waller et al., 1998), and a recent study indicated that interference with a step in this pathway retards growth of intracellular *T. gondii* (McLeod et al., 2001). The current demonstration that *T. gondii* incorporates labeled acetic acid into fatty acyl chains indicates a capability for fatty acid biosynthesis, which is presumably mediated by the FAB II pathway. Furthermore, when considered together with the use of choline, it is likely that the parasite is fully able to biosynthesize PtdCho, the predominant phospholipid in *Toxoplasma* (Foussard et al., 1991). The synthesis of less abundant phospholipids is also probable although not directly demonstrated here. *A Toxoplasma* phosphatidylinositol synthase has been described (Seron et al., 2000), and the *T. gondii* dbEST database of NCBI contains entries with homology to key enzymes in the synthesis of phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol (http://www.ncbi.nlm.nih.gov; GenBank accession numbers BM132998, AW702647, BG657255) Moreover, the related apicomplexan *Plasmodium falciparum* contains a full complement of enzymes that synthesize

phospholipids during its residence in the biosynthetically inactive erythrocyte (Vial and Ancelin, 1998). Studies using information gleaned from the ongoing *Toxoplasma* genome project, in conjunction with preparative-scale lipid biochemistry, are expected to be instrumental in elucidating the precise pathways involved in *Toxoplasma* lipid metabolism.

Potential mechanisms of lipid scavenging

The mechanism by which host-cell-derived lipids are transferred across the PVM to the parasite remains uncertain. A physical interconnection may be provided by the intravacuolar network, a system of proteins and membranes constructed by the parasite shortly after entry (Mercier et al., 1998; Sibley et al., 1995). A role for this extensive membranous interface as a conduit for the transfer of materials from the host to the parasite is currently under investigation.

There are five potential mechanisms by which the *Toxoplasma*-containing PVM acquires host cell lipids. The first of these is acquisition during invasion, via the inclusion of host plasma membrane lipids into the forming PVM. Parasites indeed integrate the host plasma membrane lipids $DiIC_{16}$ and GM1 into the PVM during invasion (Mordue et al., 1999a). However, this possibility is not favored as the sole means of lipid scavenging, since in the present study parasites readily acquired lipids provided after the establishment of intracellular residence. Second, it is conceivable that *T. gondii* acquires lipids through the interception of a vesicular transport pathway(s). This possibility can be tentatively discarded, as *T. gondii* is segregated from the vesicular transport routes responsible for the trafficking of lipids as well as proteins, and vesicular transport carries neither host plasma membrane lipids nor host lysosomal NBD-cholesterol to PVs (Coppens et al., 2000). The third possibility is that lipids passively diffuse to the parasite. There are several indications that diffusion may play only a minor role in *Toxoplasma* lipid acquisition. Diffusion of cholesterol and PtdCho through aqueous environments (i.e. the host cell cytosol) is highly unfavorable. Moreover, ablation of the fatty acid translocase gene in *Saccharomyces cerevisiae* markedly reduces the uptake of BODIPY-C12, arguing against diffusion as the central means of mobilization of this probe (Faergeman et al., 1997). Finally, when energy dependent transport processes were precluded by fixation, diffusion of subsequently added BODIPY-phosphatidic and -fatty acids to the *Toxoplasma*-containing vacuole was undetectable (data not shown). The fourth potential mechanism of lipid acquisition involves carrier-protein-mediated lipid transfer, which has been previously invoked to account for the delivery of lysosomal cholesterol to *Toxoplasma* (Coppens et al., 2000). Although carrier protein-mediated transport is probably instrumental in parasite lipid acquisition, the differential compartmentalization of individual lipids (a function of the sequence of lipid labeling/infection) implied that PVs are not indiscriminant acceptors of carrier-coupled lipids.

The close opposition of host cell organelles with the PVM suggests an additional mechanism for PVM lipid acquisition: direct interorganelle transfer (Sinai and Joiner, 2001). The biochemical exchange between the mitochondrial-associated membrane (MAM) and mitochondria provides an analogy with which to consider direct transfer of host lipids to the *Toxoplasma* PVM. Lipids manufactured in the ER must transit from the ER to mitochondria, organelles that, like the PVM and host cell compartments, are not coupled by vesicular transport (Sprong et al., 2001). This quandary has been remedied by the specialization of the ER in a region known as the MAM. Lipid transfer from the MAM takes place via translocators that deliver phospholipids to the mitochondrial membranes for further modifications (Shiao et al., 1995). PVMs also physically associate with the host cell mitochondria and ER, doing so immediately after invasion, owing to the anchoring of a secreted parasite protein into both the PVM and mitochondrial membrane (Sinai and Joiner, 2001; Sinai et al., 1997). Lipid mobilization to the parasite may thus be facilitated by the physical association of the PVM and host cell organelles. If so, the specific recruitment of host organelles by the PVM may be an important adaptation for lipid acquisition that facilitates intracellular *Toxoplasma* growth.

We thank Con Beckers (University of Alabama, Birmingham) for the generous gift of the ROP2 antiserum and Masahiro Nishijima (National Institute of Infectious Diseases, Japan) for kindly providing the choline kinase mutant cell line. We also acknowledge Jaime Dant and Wandy Beatty for their electron microscopy support. This work was supported by a Scholars Award in Molecular Parasitology (to L.D.S.) from the Burroughs Wellcome Fund, National Institutes of Health grant R01 (AI-34036). A.J.C. was partially supported by an Institutional National Research Service Award T32(AI-0717221) and an Individual National Research Service Award F32(AI-5034901).

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