

## NOTE

# Synthesis of Chloroplast Galactolipids in Apicomplexan Parasites

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**Monogalactosyldiacylglycerol and digalactosyldiacylglycerol are major chloroplast lipids of algae and land plants and are synthesized within the plastid envelope. Here we report that in *Toxoplasma gondii* and *Plasmodium falciparum* lysates, radiolabeled UDP-galactose is incorporated into monogalactosylcerebrosides, monogalactosyldiacylglycerol, and digalactosyldiacylglycerol due to distinct enzymological activities. Furthermore, DGDG is immunologically detected in apicomplexans.**

Apicomplexan parasites contain a vestigial plastid, the apicoplast, limited by multiple membranes (4, 9, 11, 19). Apicoplast proteins encoded in the nucleus exhibit bipartite N-terminal targeting sequences comprised of a signal peptide and a chloroplast-like transit peptide (1, 6, 17, 18). Therefore, the envelope membranes that are shared between green and non-green plastids in land plants might be among the multiple membranes limiting the apicoplast (10). A striking feature of the plastid envelope is the synthesis of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which constitute more than 70% of chloroplast lipids of algae and land plants as well as cyanobacteria (3). In this report, we analyzed galactolipid synthesis in *Toxoplasma gondii* (RH strain grown 72 h in confluent Vero cells, initially infected with  $3 \times 10^7$  tachyzoites in Dulbecco modified Eagle medium with 2% fetal calf serum) and *Plasmodium falciparum* (FCBR strain, maintained as previously described [15]).

Free *T. gondii* tachyzoites were released from host cell debris by passing twice through a 25-gauge needle and through a 20-ml glass wool column. *T. gondii* tachyzoites and *P. falciparum* cells (50% schizonts in unsynchronized culture) were hypotonically lysed (15). Fresh lysates ( $2 \times 10^8$  *T. gondii* tachyzoites or  $5 \times 10^8$  *P. falciparum* cells) were washed three times in 500  $\mu$ l of reaction buffer (10 mM MOPS [morpholinepropanesulfonic acid], pH 7.8, 1 mM dithiothreitol, 2% [wt/vol] glycerol, 50 mM KCl) and incubated with 4  $\mu$ Ci of UDP-[<sup>3</sup>H]galactose (75  $\mu$ M in 100  $\mu$ l of reaction buffer). Incubation was stopped by glycolipid extraction (12), and thin-

layer chromatography (TLC) on 60- $\mu$ m-thick silica gel plates resolved with chloroform-methanol-water (65:25:4, vol/vol/vol). Labeled lipids were detected with a TLC analyzer (LB2842 automatic TLC scanner). Complete identification of lipids included glycolipid orcinol staining, comigration with standards, hydrolysis of the polar head groups by green coffee bean  $\alpha$ -galactosidase and bovine testes  $\beta$ -galactosidase, and deacylation by mild alkaline hydrolysis (5). We identified three types of radiolabeled galactolipids: type 1 comigrated with MGDG from spinach chloroplasts, type 2 comigrated with monogalactosylcerebrosides (MGCB) from bovine brain, and type 3 comigrated with DGDG from spinach chloroplast (Fig. 1).

The types 1 and 2 lipids were equally sensitive to  $\beta$ -galactosidase treatments, indicating that galactose was incorporated with a  $\beta$ -linkage like those in MGDG and MGCB (Fig. 1D). The type 1 lipid was completely deacylated by mild alkaline hydrolysis, demonstrating that its hydrophobic moiety was a diglyceride. Additionally, the type 1 lipid comigrated with spinach chloroplast MGDG in two-dimensional TLC, as described previously (12). These points indicate that type 1 is similar to plant chloroplast MGDG. The type 2 lipid showed three peaks in *T. gondii* (Fig. 1A and B); relative quantities were stable from one experiment to another. These were not affected by alkaline treatment, showing that their hydrophobic moiety was not a diglyceride. In *T. gondii*, the type 2 lipids peaks comigrated with the three complex ceramide structures of the bovine brain standard: the upper band contained nonhydroxylated fatty acids, whereas the middle and lower bands contained  $\alpha$ -hydroxylated fatty acids, the lowest band being enriched in shorter chains (13). We report therefore MGCB production in *P. falciparum* and *T. gondii* and show that the ceramide composition between these two species differs since the lowest MGCB band was absent in *P. falciparum* (Fig. 1C). Synthesis of MGDG was two times more sensitive to EDTA

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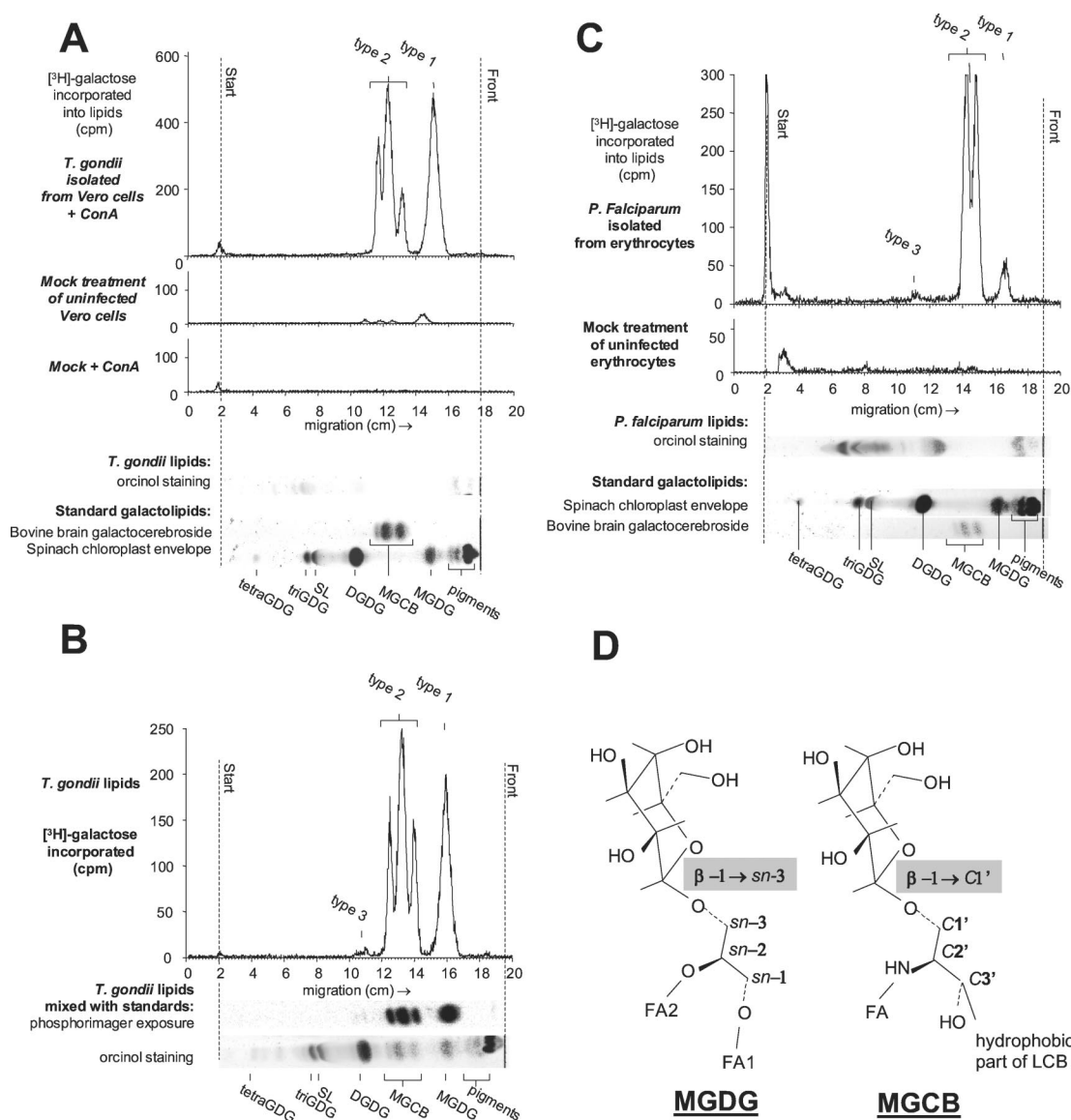


FIG. 1. Synthesis of galactolipids in *T. gondii* and *P. falciparum*. After incubation with tritiated UDP-galactose, lipids were extracted and separated by TLC (chromatograms [A, B, and C]), followed by phosphorimager detection after a 2-week exposure (B). Glycolipids were compared with standard MGCB from bovine brain and MGDG and DGDG from spinach chloroplast envelope membranes. (A) A mock preparation of parasites, using uninfected Vero cells, incorporated galactose (mock treatment of uninfected Vero cells). When we preincubated the membrane suspension with concanavalin A (50  $\mu$ g for 30 min at 37°C), a lectin with affinity for the rich mannose decoration of mammalian CGaIT (UDP-galactose:ceramide galactosyltransferase), aggregates appeared in the suspension and the pattern of incorporation of tritiated UDP-galactose due to the broken Vero cells disappeared (mock + ConA). *T. gondii* incorporation of galactose was insensitive to concanavalin A treatment (*T. gondii* isolated from Vero cells + ConA). (C) Erythrocytes uninfected with *P. falciparum* did not yield significant amounts of labeled glycolipids (mock treatment of uninfected erythrocytes). Standards were either loaded in separate lanes (A and C) or mixed with the parasite sample to demonstrate comigration of the radiolabeled parasite lipids with the galactolipid standards (B). Shown are syntheses of the following: MGDG and MGCB in *T. gondii* lysates after a 1-h incubation in the absence of  $Mg^{2+}$  (A), digalactolipids in *T. gondii* lysates after a 1-h incubation in the presence of 5 mM  $MgCl_2$  (B), and MGDG, MGCB, and DGDG in *P. falciparum* after a 1-h incubation in the absence of  $Mg^{2+}$  (C). Counts were integrated for 60 min (A), 30 min (B), and 120 min (C). SL, sulfoquinovosyldiacylglycerol; triGDG, trigalactosyldiacylglycerol; tetraGDG, tetragalactosyldiacylglycerol. (D) Structures of polar portions of MGDG and MGCB. MGDG is made of a glycerol backbone (*sn* nomenclature of the three carbons) esterified by two fatty acids in the *sn*-1 and *sn*-2 positions (FA1 and FA2) and *O*-galactosylated in the *sn*-3 position. In MGCB, only the hydrophilic part of the long chain base (LCB) of the ceramide portion is indicated (C1' to C3' nomenclature of the first three carbons). The LCB is *N*-esterified to a fatty acid in C2', making up the ceramide moiety, and *O*-galactosylated in the C1' position.

inhibition than the MGCB synthetic activity. In addition, only MGDG synthesis was enhanced by  $Mg^{2+}$  whereas only MGCB synthesis was inhibited by  $Ca^{2+}$ . An MGDG/MGCB synthesis ratio of  $0.48 \pm 0.01$  was measured in control conditions, de-

creasing to  $0.21 \pm 0.06$  in the presence of 1 mM EDTA and increasing to 0.8 ( $0.71 \pm 0.08$  on average) in the presence of 5 mM  $Mg^{2+}$ . These data strongly suggest that an MGDG synthase and a ceramide galactosyltransferase occur in *T. gondii*.

TABLE 1. Effects of bivalent cations and UDP-glucose on MGDG and MGDG syntheses in *T. gondii*

Galactolipid synthesized	$[^3\text{H}]$ galactose incorporation (% of control)					
	Control	+ EDTA, 1 mM	+ $\text{MgCl}_2$ , 5 mM	+ $\text{CaCl}_2$ , 5 mM	+ $\text{ZnCl}_2$ , 1 mM	+ UDP-Glc, 10 mM
MGCB	100	57	107	76	66	ND <sup>a</sup>
MGDG	100	16	152	108	73	ND

<sup>a</sup> ND, not detected.

As for plant chloroplast MGDG synthase (8) and mammal endoplasmic reticulum CGaT (16), UDP-glucose was a competitive inhibitor (Table 1), but while large amounts of glucosylcerebrosides were synthesized by *T. gondii* lysates, mono-glucosyldiacylglycerol (MGlcDG) was barely detected. Therefore, the MGDG synthase from *T. gondii* resembles the land plant MGDG synthase, which is inhibited by UDP-glucose but does not utilize this sugar donor for MGlcDG synthesis (8). In addition, *T. gondii* MGDG synthesis exhibits enzymological features similar to those of the activity measured in plant chloroplast envelope membranes (8), i.e., an inhibition by EDTA, an enhancement by  $\text{Mg}^{2+}$ , and an inhibition by  $\text{Zn}^{2+}$ .

The type 3 lipids were not affected by  $\beta$ -galactosidase treatment and decreased after an  $\alpha$ -galactosidase incubation and mild alkaline hydrolysis, like plant DGDG. In *T. gondii*, the weakly labeled peak 3 (Fig. 1B) was broad and was never completely hydrolyzed by mild alkaline hydrolysis, indicating

that the hydrophobic moiety partially contained diacylglycerol. We produced specific polyclonal antibodies by immunizing rabbits with 2.5 mg of pure spinach chloroplast DGDG, and immunoblottings were performed as previously described (5). The antibody reacted with DGDG from spinach chloroplast and from cyanobacteria (Fig. 2B) and did not react with MGDG, trigalactosyldiacylglycerol (triGDG) (Fig. 2A), or lipids from *Escherichia coli* (Fig. 2D), which are rich in phospho- and glycolipids but devoid of DGDG (2). Attempts to label subcellular structures of *T. gondii* with the polyclonal anti-DGDG antibody failed. When lipid extracts from *T. gondii* ( $2 \times 10^9$  tachyzoites) or *P. falciparum* ( $10^9$  cells) were analyzed by TLC, no orcinol-stained glycolipid could be detected at the precise level of DGDG (Fig. 1A and C). However, after a blind scraping of the TLC plate at the  $R_f$  level of DGDG and blotting on nitrocellulose, the anti-DGDG antibody reacted with the corresponding spots from *T. gondii* and *P. falciparum* (Fig. 2C and D). In addition, when the scraped lipids from *T. gondii* or

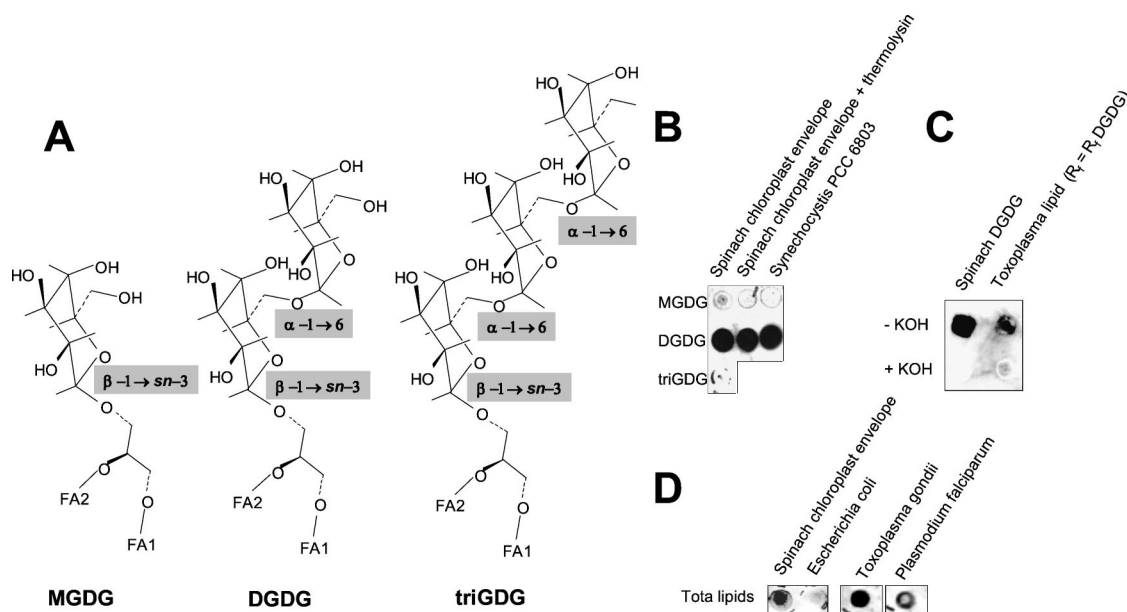


FIG. 2. Immunodetection of DGDG in lipid extracts from *T. gondii* and *P. falciparum*. (A) Comparison of MGDG, DGDG, and triGDG polar heads. (B) The specificity of the anti-DGDG antibody for the [ $\alpha$ -D-galactopyranosyl-(1 $\rightarrow$ 6)-O- $\beta$ -D-galactopyranosyl] polar head was investigated by dot blot immunostaining (1/100 dilution of anti-DGDG antibody) of 10  $\mu\text{g}$  of lipids purified from spinach chloroplast envelope membranes (first two lanes) or *Synechocystis* PCC 6803, a cyanobacterium. The anti-DGDG antibody was detected on DGDG spots whereas it did not react with MGDG or triGDG. (C) *T. gondii* lipids extracted from  $2 \times 10^9$  free tachyzoites were separated by TLC as shown in Fig. 1. Silica at the level of the DGDG standard was scraped, and lipids were extracted (those with an  $R_f$  equal to the  $R_f$  of DGDG) and analyzed by dot blot immunostaining. The anti-DGDG antibody reacted with this spot, whereas it did not when the extracted lipids were subjected to mild alkaline hydrolysis and reextracted (+KOH). A similar immunostaining pattern was obtained with a spinach chloroplast DGDG control subjected to identical treatments. The loss of immunostaining after mild KOH hydrolysis showed that the immunostained lipids were deacylated. (D) Dot blot immunostaining with the anti-DGDG antibody of total lipid mixtures from spinach chloroplast envelope membranes (10  $\mu\text{g}$  of lipids), *E. coli* membranes (10  $\mu\text{g}$  of lipids), *T. gondii* (total lipid extracts from  $2 \times 10^9$  tachyzoites), and *P. falciparum* (total lipid extracts from  $10^9$  cells).

from spinach chloroplast DGDG were hydrolyzed by mild alkaline treatment and the remaining lipids were reextracted, the anti-DGDG antibody failed to react with any spot (Fig. 2C), confirming that the hydrophobic moiety of the spotted lipid was a diacylglycerol. Together, these data show that at least a portion of the type 3 lipids correspond to a chloroplastic-like DGDG.

In conclusion, the *in vitro* synthesis of galactolipids in apicomplexans is reported for the first time. We searched *T. gondii* and *P. falciparum* databases but found no sequences having a statistically reliable similarity with the primary sequences of ER CGaIT, chloroplast envelope MGDG synthase, or DGDG synthase multigenic families. Future prospects therefore include the identification, characterization, and unambiguous subcellular localization of the apicomplexan enzymes responsible for MGCB, MGDG, and DGDG syntheses and the investigation on possible homologies with the corresponding plant enzymes.

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