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The enzymatic synthesis of membrane glucolipids in *Acholeplasma laidlawii*

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In membranes of the prokaryote *Acholeplasma laidlawii*, the physiological regulation of the two major membrane lipids, monoglucosyldiacylglycerol (MGlcDAG) and diglucosyldiacylglycerol (DGlcDAG), is governed by factors affecting the equilibria between lamellar and non-lamellar phases of the membrane lipids. The synthesis of the glucolipids is considered to be a two-step glucosylation: (i) DAG + UDP-Glc → MGlcDAG + UDP, and (ii) MGlcDAG + UDP-Glc → DGlcDAG + UDP. This was corroborated by in vivo pulse labelling experiments showing turnover of MGlcDAG but not DGlcDAG. The enzymatic synthesis of MGlcDAG was localized to fresh or freeze-dried membranes in vitro. Synthesis of DGlcDAG was minor in such membranes but of substantial magnitude in intact cells. Synthesis of MGlcDAG was stimulated by small amounts of SDS but completely inhibited upon solubilization of the membranes by a variety of detergents. The inhibitory effect of several UDP-Glc analogs on glucolipid synthesis demonstrated the importance of UDP-Glc as the sugar donor. Synthesis of both glucolipids was lost in freeze-dried plus lipid-extracted cells but restored when lipids were transferred back to the extracted cell membrane. By selectively adding specific lipids, a strong dependence on the acceptor lipid DAG, as well as the need for general matrix lipids for enzyme activity, was established. In addition, the anionic phosphaticylglycerol (PG), but not the other phospholipids, had a strong stimulatory effect. The presence of different phosphorylating agents stimulated the synthesis of DGlcDAG and partially inhibited that of MGlcDAG. This, together with the lipid dependency, may constitute mechanisms for the regulation of the enzyme activities in vivo.

Introduction

Biological membranes consist of lipid bilayers with more or less embedded proteins. In many cells or organelles the lipid composition is actively regulated to maintain certain structural and compositional features. A minimum fraction of the lipid hydrocarbon chains must be in the liquid crystalline state in order for cells to grow and divide properly [1]. However, the regulation of the polar headgroup composition is less well understood, although this constitutes the structural basis for bilayer assembly and maintenance [2–4].

In the cell wall-less prokaryote (mycoplasma) *Acholeplasma laidlawii*, the membrane polar lipids are regulated so that similar phase equilibria with respect to the transition between lamellar and non-lamellar phases are maintained for the lipids, irrespective of the growth or supplementation [5–7]. This regulation is accomplished primarily by a metabolic variation in the proportions between the two dominating glycolipids monoglucosyldiacylglycerol (MGlcDAG) and diglucosyldiacylglycerol (DGlcDAG). MGlcDAG can form lamellar as well as reversed cubic and hexagonal phases, while DGlcDAG forms lamellar phases only [6–9]. The regulation responds to: (i) the structure of the incorporated acyl chains [6,10]; (ii) the growth temperature [11]; (iii) the presence of different foreign molecules like alcohols, organic solvents and detergents [7], different sterols [10] and chlorophyll [12]; and (iv) the transmembrane electric potential, i.e., $\Delta\psi$ [13,14]. This is in agreement with the prevailing theories for the formation of different aggregate structures by amphiphilic molecules [15,16]. In addition, the balance between the charged (anionic) phospholipids and the non-charged glucolipids is regulated so that a constant

Abbreviations: DAG, diacylglycerol; MGlcDAG, monoglucosyldiacylglycerol; DGlcDAG, diglucosyldiacylglycerol; PG, phosphatidylglycerol; 18:1c, oleic acid; 14:0, myristic acid; TLC, thin-layer chromatography; LSC, liquid scintillation counting; NP-40, Nonidet P-40; SDS, sodium dodecyl sulfate; NaDOC, sodium deoxycholate, CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; MEGA 9, nonanoyl-*N*-methylglucamide; MEGA 10, decanoyl-*N*-methylglucamide.

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surface charge density and potential is maintained for the lipids [17].

What are the mechanisms for lipid polar headgroup regulation in *A. laidlawii* membranes? A few studies have been made regarding the biosynthesis of the glucolipids [18,19] and membrane lipid turnover [20,21]. The biosynthetic pathways are considered [22] to be similar to the ones in related gram-positive bacteria, like, e.g., *Streptococcus faecalis* [23]. A proposal for how a membrane protein might sense different packing properties (like curvature) of a lipid bilayer has been made recently [24]. It is obvious that these mechanisms need to be clarified. As a first step towards this, the present work describes some characteristics of the *A. laidlawii* enzymes responsible for the synthesis of MGlcDAG and DGlcDAG.

Materials and Methods

Growth conditions. A. laidlawii A (EF 22) was grown in a medium containing 2% (w/v) tryptose (Difco), 0.4% (w/v) bovine serum albumin (fraction V, Sigma), 98.8 kU/l penicillin-G (Pen-na, Sigma), 41 mM Tris, 39 mM glucose, 86 mM NaCl and 0.12 mM oleic acid (18:1*c*). The cells were grown for 16 h at 28°C (2% v/v inoculum), harvested by centrifugation at 18000 × *g* and 5°C for 20 min, washed once in 0.2 M Tris-maleate buffer (pH 8.0), and stored frozen at −80°C at a concentration of 5 mg membrane protein per ml buffer (protein determination, see below).

Preparation of cell membranes. Cells harvested from 1 liter of growth medium were lysed at room temperature by stirring for 45 min in 50 ml of 2 mM Tris-maleate buffer (pH 8.0). The membranes were separated from the cytoplasmic fraction (supernatant) by centrifugation at 48000 × *g* and 5°C for 60 min, washed once in 2 mM Tris-maleate, and stored at −80°C.

Pulse labelling of lipids. Cells were grown in a lipid-depleted medium [25] supplemented with 0.12 mM 18:1*c* and 30 μM myristic acid (14:0). After 16 h of growth at 30°C, [^{3}H]14:0 (36 Ci/mmol in a small volume of growth medium) was added to a final concentration of 54 mCi/l and the cells were incubated as before. Samples were withdrawn at different points of time (10, 30, 60, 120 and 360 min) and the turbidity at 540 nm was measured before harvest. Washed cells were lyophilized, lipids extracted with chloroform/methanol (2:1, v/v) and separated by thin-layer chromatography (TLC) (0.2 mm silica gel 60; Merck) developed with chloroform/methanol/water (65:25:4, v/v). Individual lipids were identified as described [26], excised and analyzed by liquid scintillation counting (LSC). In control experiments cells were grown (as above) in medium supplemented with 0.12 mM [^{14}C]18:1*c* (2 mCi/l) and 30 μM [^{3}H]14:0 (5.4 mCi/l) and lipids were analyzed as above.

Solubilization of cell membranes. The following detergents were used: Nonidet P-40 (NP-40, Sigma); Tween 20 (Bio-Rad); Triton X-100 (Sigma); *n*-octyl β-D-glucopyranoside (octyl glucoside, Serva); sodium dodecyl sulfate (SDS, Bio-Rad); sodium deoxycholate (NaDOC, Fluka AG); 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS, Calbiochem); nonanoyl-*N*-methylglucamide (MEGA 9); and decanoyl-*N*-methylglucamide (MEGA 10) (the two latter synthesized according to the method of Hildreth [27]). Membranes (prepared as above from cells grown in presence of 0.2 mCi ^{3}H-labelled amino acid mixture (Amersham) per liter growth medium), at a final concentration of 1.3 mg protein/ml, were agitated (shaking) at room temperature for 60 min in 10 mM Tris-maleate buffer (pH 8.0) with detergents added to the final concentrations indicated in Fig. 2. The incubation mixtures were then centrifuged at 40000 × *g* and 10°C for 60 min. The supernatants (solubilized fraction) were collected and assayed for enzyme activity. Protein concentrations were determined by colourimetric assay [28] and LSC. Removal of detergents (MEGA 10 or CHAPS) from completely solubilized membranes could be achieved by dialysis. The material was placed in a dialysis bag (cut-off 12–14 kDa) and dialyzed against 10 mM Tris-maleate (pH 8.0) at 10°C for 24 or 48 h.

Substrate lipids. Synthetic 1,2-dioleoyldiacylglycerol was purchased from Larodan (Sweden). The A-EF22 strain grows well with 95% oleoyl chains [26], and since the acyl chains on MGlcDAG from *A. laidlawii* occupy the *sn*-1 and *sn*-2 positions according to a circular dichroism analysis [29], we only tested the *sn* 1,2-dioleoylglycerol as the primary sugar acceptor. MGlcDAG (containing more than 95% oleoyl chains) was prepared from *A. laidlawii* A cells grown in a lipid-depleted medium [25] containing 0.15 mM 18:1*c*. Labelled lipids were prepared by adding [^{3}H]18:1*c* (0.3 mCi/l) to the growth medium. After cell harvest, washing and freeze-drying, the lipids were extracted with chloroform/methanol (2:1, v/v). Nonlipid contaminants were removed by chromatography on Sephadex G-25 Fine [30]. A fraction of the lipids were dried under a stream of N_2 and emulsified (3.2 mg/ml) in 0.1 M Tris-maleate buffer (pH 7.2) by a mild sonic treatment. To 5 ml of this emulsion were added 1 mg $ZnCl_2$, 5 μmol mercaptoethanol, 1.0 kU phospholipase C (Boehringer Mannheim) and 0.1 ml diethyl ether. After 16 h at 37°C the mixture was freeze-dried and the dry residue was extracted with chloroform/methanol (2:1, v/v). Neutral lipids (including native and liberated DAG) and MGlcDAG were separated from the other lipids by TLC on 1-mm plates of silica gel H-60 (Merck), developed with chloroform/methanol/water (65:25:4, v/v). Lipids (including references) on wet plates were visualized by light and eluted from the gel by chloroform/methanol (2:1,

TABLE I

Conditions for in vitro synthesis of glucosyldiacylglycerols in A. laidlawii strain A

Constituents	Concentrations
Tris-maleate (pH 8.0)	0.12 M
$MgCl_2$	20 mM
UDP-[^{14}C]glucose	1–5 mM (1–4 mCi/mmol)
Substrate lipids (DAG or MGlcDAG)	0–1 mM
Membranes/Cells membrane protein)	0.20–1.0 mg/ml

v/v). DAG was separated from other neutral lipids on 1mm plates of silica gel H-60 developed with chloroform/acetone (96:4, v/v) and eluted from the gel as described above.

In vitro synthesis of glucosyldiacylglycerols. Two different methods were used. Method 1: A lipid emulsion of 10 mM lipid substrate and 2 mM SDS in water was prepared by sonication. To 10 μl of this emulsion were added membranes or cells (25–100 μg membrane protein), 12 μmol Tris-maleate (pH 8.0), 2.0 μmol $MgCl_2$ and 0.1 μmol UDP-[^{14}C]glucose (0.4 μCi) to a final volume of 0.1 ml. After incubation for 60 min at 37°C, the reactions were stopped by freezing (−20°C). Method 2: Aliquots of cells or membranes were removed (25 μl, 25–50 μg membrane protein) and lyophilized. Exogenous substrate lipids dissolved in 25 μl benzene were added to the lyophilized membranes or cells. The benzene was removed by evaporation under a stream of N_2, leaving the substrate lipids in direct contact with the dry enzymes [31]. UDP-[^{14}C]glucose (0.1 μmol, 0.2 μCi) and $MgCl_2$ (2 μmol) in 0.1 ml 0.12 M Tris-maleate (pH 8.0) were added and the mixtures were incubated for 30–60 min at 28 or 37°C. The reactions were stopped by freezing (−20°C). The assay conditions are presented in Table I.

The lipids from the reaction mixtures were extracted according to Bligh and Dyer [32] and separated by TLC (0.2 mm silica gel 60; Merck) developed with chloroform/methanol/water (65:25:4, v/v). Individual lipids were identified as described [26] and labeled glucolipid spots were excised and quantified by liquid scintillation counting (LSC). Synthesis of MGlcDAG and DGlcDAG (in cells or cell fractions) was measured as nmol glucose incorporated per mg membrane protein per-hour. The protein content of membranes was determined by the method of Hartree [28].

Results

In vivo synthesis of glucosyldiacylglycerols by A. laidlawii A

Synthesis of the major lipids MGlcDAG and DGlcDAG in *A. laidlawii* is considered to involve the transfer of a glucose moiety from the glucose donor, uridine 5′-diphosphoglucose (UDP-Glc), to the acceptor molecules diacylglycerol (DAG) or MGlcDAG [18].

UDP-Glc + 1,2-diacylglycerol → MGlcDAG + UDP

UDP-Glc + MGlcDAG → DGlcDAG + UDP

However, pulse labelling analysis of the lipid biosynthesis [20,21] has shown little or no turnover of the major lipids (including MGlcDAG) in *A. laidlawii*, which is inconsistent with the reactions above. Since *A. laidlawii* is sensitive to centrifugation which is necessary in a pulse-chase experiment, we designed a pulse labelling experiment where [^{3}H]14:0 was added to growing cells in the late exponential phase. After 16 h of growth with 120 μM 18:1c plus 30 μM 14:0, the 14:0 in the medium was already consumed and therefore the addition of small molar amounts (1.5 μM) of [^{3}H]14:0 was metabolized within a short time by the cells. The majority of the radioactivity in the cells were found in the lipids, since *A. laidlawii* can not degrade fatty acids [22], and reached a maximum value within 10 to 30 min (data not shown). By measuring the changes in the relative amount of radioactivity in the lipids, at different points of time after the addition of the [^{3}H]-14:0, the synthesis and turnover of the different lipids could be followed. Shortly (10 min) after the addition of [^{3}H]14:0 70% of the radioactivity incorporated was found in MGlcDAG, while only 5% was found in DGlcDAG (Fig. 1) which was the major constituent of the membrane lipids (legend to Fig. 1). The relative amount of [^{3}H]14:0 in MGlcDAG was decreased to 48% during the first two hours of the pulse, concomitant with an increase of [^{3}H]14:0 in DGlcDAG to 21%. The glycerophosphoryl derivatives of MGlcDAG and DGlcDAG showed the same slow uptake of radioactivity as DGlcDAG, while uptake of radioactivity into PG was rapid with a maximum value after 10 min. The distribution of radioactivity in the lipids during the later 4 h of the pulse was more or less constant. It was confirmed by the increase of cell turbidity and total lipid content that cells were growing during the time for the pulse (legend to Fig. 1). These results strongly indicate that there is a turnover of the *A. laidlawii* lipids, but it varies for different lipids. These results are compatible with the suggestion that DGlcDAG arises by glucosylation of MGlcDAG.

Conditions for in vitro synthesis of glucosyldiacylglycerols in membranes

Studies on the synthesis of glucosyldiacylglycerols in *A. laidlawii* B has been described earlier [18]. It was shown that synthesis of both MGlcDAG and DGlcDAG was associated with the membrane. Here, in vitro rates of synthesis in frozen and thawed membranes (Method 1 in Materials and Methods), prepared from *A. laidlawii* A grown in a fatty acid-depleted medium supple-

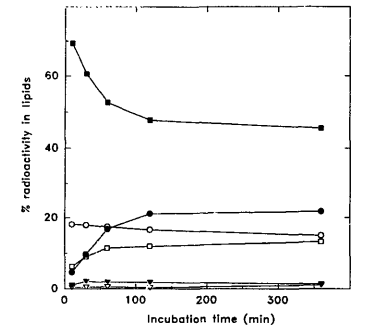

Fig. 1. Pulse labelling of *A. laidlawii* lipids during growth. The relative amount of radioactivity in glycerophosphoryl-MGlcDAG and -DGlcDAG (□), PG (○), DGlcDAG (●), MGlcDAG (■), monoacyl-MGlcDAG (▼) and neutral lipid fraction (▽), was analyzed at different points of time after addition of [^{3}H]14:0 (1.5 μM) to cells grown for 16 h in a medium supplemented with 120 μM 18:1*c* plus 30 μM 14:0 (see Materials and Methods). When the pulse started the total content of membrane lipids were 14.2 nmol per ml growth medium with the following composition; 18.5% glycerophosphoryl-MGlcDAG and -DGlcDAG; 25.1% PG; 35.8% DGlcDAG; 19.6% MGlcDAG; 0.4% monoacyl-MGlcDAG and 0.6% neutral lipid-fraction. During the pulse period (360 min) there was an increase of the culture turbidity at 540 nm by 30% and total lipid amount by 5%, while the relative amount of MGlcDAG decreased to 13.6% and DGlcDAG increased to 42.7% (see above).

mented with 18:1*c*, showed similar values as described by Smith [18]. Typical values obtained for the biosynthesis of MGlcDAG were 15 nmol glucose incorporated per mg membrane protein per h, but only around 2 nmol per mg per h for the biosynthesis of DGlcDAG. This was also valid for a B strain of *A. laidlawii* obtained from Dr. R.N. McElhaney, Edmonton, Canada (data not shown). About twice as high synthesis rate of MGlcDAG was obtained by using freshly prepared (not frozen) membranes as the enzyme source, while the biosynthetic activity of DGlcDAG in such membranes remained at a low level. A 2-fold increase in biosynthetic activity obtained for the biosynthesis of MGlcDAG by adding the anionic detergent SDS to the assay mixture could not be achieved with non-ionic detergents such as NP-40. Addition of exogenous 1,2-dioleoylglycerol to the assay mixture markedly enhanced the rate of synthesis of MGlcDAG, while addition of MGlcDAG had no stimulatory effect on the synthesis of DGlcDAG, although MGlcDAG is claimed to be the lipid substrate (cf above). Assay conditions are presented in Table I.

Detergent solubilization of membranes

A critical step in purifying and identifying membrane-bound enzymes is to solubilize the membrane without inactivating the enzymes. Thus the release of undenatured proteins from membranes calls for a detergent that is able to solubilize lipid bilayers without altering the structure of the desired proteins.

The effect of different detergents on the solubilization of membrane proteins from *A. laidlawii* A are shown in Fig. 2A. Different types of detergent such as nonionic (Tween 20, Triton X-100, MEGA 9, MEGA 10 and octyl glucoside), anionic (SDS and NaDOC), and zwitterionic (CHAPS) were used. The most efficient solubilization was achieved using anionic detergents. A concentration of 6 mM of SDS resulted in totally solubilized membranes. MEGA 9 and octyl glucoside were least efficient, less than 25% of the mem-

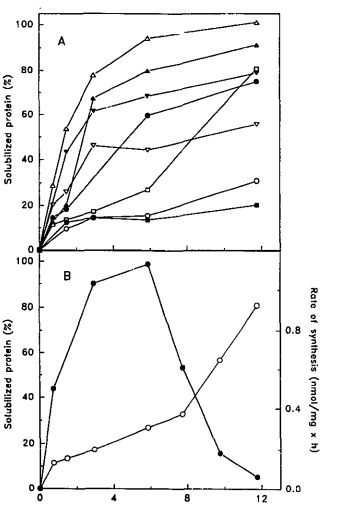

Fig. 2. Solubilization of *A. laidlawii* membranes. See Materials and Methods for experimental conditions. (A) Effect of detergent concentration on the solubilization of membrane proteins. MEGA 9 (■), octyl glucoside (○), Tween 20 (▽), CHAPS (●), Triton X-100 (▼), MEGA 10 (□), NaDOC (▲) and SDS (△). (B) Rate of synthesis of MGlcDAG (●) in solubilized membranes. The membrane proteins were solubilized with MEGA 10 (○).

brane proteins were solubilized at a detergent concentration of 12 mM. Synthesis of glucosyldiacylglycerols were not detectable in totally solubilized membranes, which also was shown by Smith using SDS or NP-40 as solubilizing agents [18]. Even at detergent concentrations causing only 50% solubilization, the synthesis of glucosyldiacylglycerols in the solubilized fraction was almost totally inactivated. MEGA 10 was able to partially solubilize the membranes and still maintain the synthesis of glucosyldiacylglycerols (Fig. 2B) An optimal synthesis rate of MGlcDAG synthesis in partially solubilized membranes was achieved at a MEGA 10 concentration of 6 mM with 30% of the total membrane proteins in the solubilized fraction.

Removal of detergents (MEGA 10 or CHAPS) by dialysis from completely solubilized membranes partially reactivated the enzymes responsible for synthesis of MGlcDAG. The synthesis rate of MGlcDAG was quantified to 2–4 nmol per mg protein per h (DGlcDAG synthesis was not detectable) after dialysis (48 h at 10°C) of membranes solubilized with 15 mM MEGA 10.

A method for enzymatic detection of lipid biosynthetic enzymes after protein electroblotting has been described by Poole et al. [33]. Samples of membranes from *A. laidlawii* A were subjected to SDS-polyacrylamide slab gel electrophoresis and electroblotted onto nylon filters [34]. The filters were incubated in a renaturation buffer containing *A. laidlawii* membrane lipids and cut into 1 cm strips. No recovery of synthesis of MGlcDAG or DGlcDAG were found on such strips when assayed for synthesis of the glucosyldiacylglycerols.

Synthesis of glucosyldiacylglycerols in delipidated membranes

The synthesis of MGlcDAG in *A. laidlawii* membranes was stimulated by the presence of SDS at low concentration (Method 1). Most probably this reflects a solubilizing effect of the detergent on the added (emulsified) substrate lipid. Such effect was not found for the synthesis of DGlcDAG when MGlcDAG was added together with SDS.

In order to better promote the interaction between the glucolipid synthesizing enzymes and exogenously added substrate lipids, a modified technique described by Pieringer [31] was used (Method 2 in Materials and Methods). Lyophilized membranes of *A. laidlawii* were partially delipidated by a repeated (3×) washing in acetone. When biosynthesis in such delipidated membranes was analyzed, almost no synthesis of MGlcDAG or DGlcDAG was detected (see Table II). Addition of DAG dissolved in benzene to dry delipidated membranes, followed by removal of the benzene, resulted in a high rate of synthesis of MGlcDAG. No such effect was achieved for the synthesis of glucosyldiacylglycerols when MGlcDAG was added as substrate (Table II).

TABLE II

Rates of synthesis of MGlcDAG and DGlcDAG in partially delipidated membranes from A. laidlawii A

Rates of synthesis of MGlcDAG and DGlcDAG in partially delipidated membranes (35 μg membrane protein), prepared by stirring lyophilized membranes in acetone 3×0.5 ml for 10 min at 4°C, were assayed at 37°C for 60 min as described by Method 2 in Materials and Methods. Data presented are mean values from two experiments.

Added lipid	nmol of glucose incorporated per h per mg membrane protein	
	synthesis of MGlcDAG	synthesis of DGlcDAG
None	0.21	0.04
DAG	80.4	0.20
MGlcDAG	0.20	0.03

Judged by the 3–4 fold stimulation of MGlcDAG synthesis upon addition of DAG as compared with using a surfactant adjuvant such as SDS (Method 1), this technique (Method 2) probably better promoted the interaction between the enzyme and the exogenously added lipid substrates. It also made the removal of endogenous lipids possible with a retained enzymatic activity. The rate of synthesis of DGlcDAG was low both in partially lipid-depleted membranes to which substrate lipids (MGlcDAG dissolved in benzene) were added directly to dry membranes, and in the previously described method (Method 1 in Materials and Methods) where substrate lipids were added emulsified in water to intact or solubilized membranes. These results raise the question if there are some components missing in the membrane for the synthesis of DGlcDAG.

Synthesis of glucosyldiacylglyceroles in different cellular fractions

Synthesis of glucosyldiacylglycerols was analyzed in different cellular fractions in order to elucidate if synthesis of DGlcDAG is dependent on other non-membrane components. In whole cells (harvested immediately before the analysis), the rates of synthesis of both MGlcDAG and DGlcDAG were high (Table III). The synthesis rate of MGlcDAG increased upon osmotic lysis of the cells, but the rate of synthesis of DGlcDAG dropped dramatically to around 2% of the synthesis rate in intact cells. The synthesis rate of DGlcDAG remained at this low level in the membrane fraction (pellet from centrifugation of lysed cells at 50000×*g* for 1 h). Synthesis of MGlcDAG and DGlcDAG were extremely low in the cytoplasm fraction or in heat inactivated membranes, indicating that the synthesis of both MGlcDAG and DGlcDAG are catalyzed by membrane-bound proteins. Furthermore, the synthesis of

TABLE III

Rates of synthesis of MGlcDAG and DGlcDAG in different cellular fractions of A. laidlawii A

Rates of synthesis of MGlcDAG and DGlcDAG in intact cells or cell fractions (32 μg membrane protein) were assayed at 37°C for 60 min with exogenous DAG added as described by Method 1 in Materials and Methods. Data presented are mean values from two experiments.

Cell fraction	nmol of glucose incorporated per h per mg membrane protein	
	synthesis of MGlcDAG	synthesis of DGlcDAG
Cell	19.8	90.2
Lysed cell	33.7	2.0
Membrane	19.4	1.5
Cytoplasma [a]	0.4	0.4
+ membrane	28.7	0.7
Heat inactivated membrane	1.0	0.2

[a] Rate of synthesis measured as nmol of glucose incorporated per h per mg protein.

DGlcDAG is probably dependent on compounds that are rapidly degraded or irreversibly released from the membranes upon lysis of cells. The synthesis of MGlcDAG was increased in lysed cells or the combined cytoplasm and membrane fractions (Table III). The rate of synthesis of DGlcDAG in different cellular fractions was not enhanced by addition of exogenous MGlcDAG, cf. above (data not shown).

Synthesis of glucosyldiacylglycerols in lyophilized cells

In order to better characterize the synthesis of glucosyldiacylglycerols in *A. laidlawii*, analyses were performed using lyophilized cells having an optimal rate of synthesis for both MGlcDAG and DGlcDAG. No exogenous lipids were added. Linearity between formed products and amount of cells (measured as mg membrane protein) added were obtained with a membrane protein content less than 0.1 mg per assay (Fig. 3). A time course (Fig. 4) indicates that MGlcDAG synthesis continued for at least 1 h and that formation of MGlcDAG was proportional with time during 40 min, except for the first minutes. Synthesis of DGlcDAG did not show the same linearity with respect to time and the rate of synthesis started to decrease after 20 min and after 40–60 min there was no further synthesis of DGlcDAG (data not shown).

Optimal synthesis rates of both MGlcDAG and DGlcDAG were achieved at a concentration of UDP-glucose around 5 mM (data not shown) and at a temperature of 28°C (activity measured at 25, 28, 37 and 40°C; data not shown), hence this was chosen for standard conditions (cf. below). Under these conditions about 1% of the added UDP-glucose were incorporated into MGlcDAG and about 0.5% into DGlcDAG.

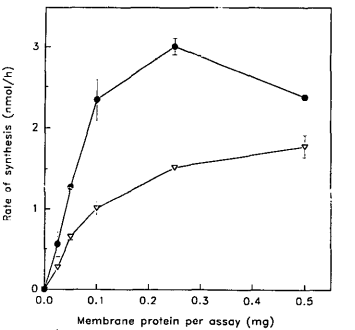

Fig. 3. Effect of cell concentration on the synthesis of glucosyldiacylglycerols. Rates of synthesis of MGlcDAG (●) and DGlcDAG (▽) in lyophilized cells were assayed at 37°C for 60 min as described by Method 2 in Materials and Methods. Amounts of cells were determined as mg membrane protein. Data presented are mean values from two experiments.

Influence of UDP-glucose analogs on the synthesis of the glucosyldiacylglycerols

The effect of UDP-glucose analogues on the synthesis of glucolipids may give some insight into the molecular mechanisms of the glucolipid-synthesizing en-

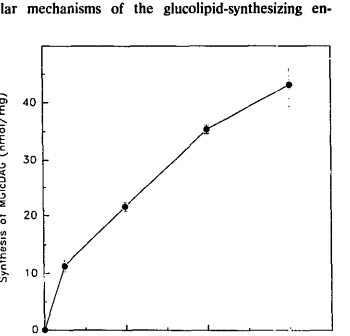

Fig. 4. Time course of MGlcDAG synthesis. Lyophilized cells (50 μg membrane protein) were analyzed at 28°C (Method 2 in Materials and Methods). Data presented are mean values from three experiments.

zymes. Hence, the formation of glucosyldiacylglycerols were analyzed in the presence of several potential inhibitors (5 mM) and the results are listed in Table IV. UDP and UDP-galactose had a slight inhibitory effect (20–30%) on the synthesis of both MGlcDAG and DGlcDAG. Although Smith has earlier shown [18] that UDP-galactose could not function as a hexose donor for MGDG, it seems to compete with binding of UDP-glucose to the enzyme. UTP stimulated DGlcDAG and partially inhibited MGlcDAG synthesis, respectively (Table IV). Other different sugar nucleotides, glucose, or glucose 1-phosphate had no clear effect on the formation of the glucosyldiacylglycerols, except that GDP-glucose slightly inhibited DGlcDAG synthesis. Both glucolipid synthesizing enzymes in *A. laidlawii* seem to especially recognize the uridine-phosphate moiety of their substrate. This has also been shown for other glycosyltransferases [35,36].

Periodate oxidized UDP (oUDP) and UDP-glucose (oUDP-glucose) are useful when studying enzymes utilizing UDP-glucose as substrate. It has been observed with other systems that periodate oxidized nucleotides becomes covalently bound to their respective enzymes [37]. Here, oUDP inhibited the synthesis of glucosyldiacylglycerols more strongly than UDP. Synthesis of MGlcDAG was inhibited by 69% when 5 mM oUDP was included in the reaction mixture, compared to a 25% inhibition upon addition of 5 mM UDP (Table IV). Preincubation of lyophilized cells with 5 mM oUDP for 30 min at 28°C increased the extent of inhibition of MGlcDAG synthesis to 84% (data not shown). A similar but less pronounced effect was obtained for the synthesis of DGlcDAG. These results indicate that oUDP becomes covalently bound to the enzymes responsible for synthesis of MGlcDAG and DGlcDAG.

TABLE IV

Effects of UDP-glucose analogues on the synthesis of MGlcDAG and DGlcDAG in lyophilized cells

Rates of synthesis of MGlcDAG and DGlcDAG were assayed at 28°C for 40 min as described by Method 2 in Materials and Methods, in a mixture of lyophilized cells (50 μg membrane protein), 12 μmol Tris-maleate (pH 8.0), 2 μmol $MgCl_2$, 0.5 μmol UDP-[^{14}C]Glc (0.2 μCi) and 0.5 μmol of analogue in a total volume of 0.1 ml. Data presented are mean values from four experiments.

Compound added [b]	Relative rates of synthesis [a] (%)	
	MGlcDAG	DGlcDAG
None	100	100
UDP-Gal	64	67
UDP-GlcUA	98	86
UDP-Glc-NAc	82	74
Glc	108	104
Glc-1-phosphate	102	112
ADP-Glc	102	110
TDP-Glc	102	114
GDP-Glc	93	69
CDP-Glc	114	113
UMP	83	85
UDP	75	77
UTP	41	159
oUDP	31	62
oUTP	41	79

[a] The values have been normalized against the rate of synthesis in lyophilized cells where no analogues of UDP-glucose were added. 100% rate of synthesis of MGlcDAG and DGlcDAG correspond to 64 and 94 nmol/mg per h, respectively.

[b] Abbreviations: Gal, galactose; GlcUA, glucoronic acid; Glc-NAc, *N*-acetylglucosamine; Glc, glucose; oUDP, periodate-oxidized UDP; oUTP, periodate-oxidized UTP.

TABLE V

Effects of phosphorylating agents on the synthesis of MGlcDAG and DGlcDAG in lyophilized cells

Rates of synthesis of MGlcDAG and DGlcDAG were assayed at 28°C for 30 min in a mixture of lyophilized cells (50 μg membrane protein), 12 μmol Tris-maleate (pH 8.0), 2 μmol $MgCl_2$, 0.5 μmol UDP-[^{14}C]Glc (0.2 μCi) and 0.5 μmol of a phosphorylating agent (final concn. 5 mM) in a total volume of 0.1 ml as described by Method 2 in Materials and Methods. Data presented are mean values from three experiments.

Compound added [b]	Relative rates of synthesis [a] (%)	
	MGlcDAG	DGlcDAG
None	100	100
ATP	35	160
PP_i	55	144
P_i	73	118
PEP	59	108

[a] Values have been normalized against the rate of synthesis in lyophilized cells with no phosphorylating agent added. 100% rate of synthesis for MGlcDAG and DGlcDAG corresponds to 83 and 105 nmol/mg per h, respectively.

[b] Abbreviations: PP_i, pyrophosphate; P_i, ortophosphate; PEP, phosphoenolpyruvate.

Influence of phosphorylating agents on the synthesis of the glucosyldiacylglycerols

One condition likely to change dramatically upon cell lysis (cf. Table III) is the phosphorylation potential. The ability of different phosphorylating compounds to modulate the synthesis of glucosyldiacylglycerols in lyophilized cells were therefore examined. ATP (Table V), GTP and CTP (data not shown) seemed to have similar effects as UTP (Table IV) on the synthesis of glucosyldiacylglycerols, stimulating the synthesis of DGlcDAG by 50–60% and inhibiting MGlcDAG synthesis by 60–70%. Phosphoenolpyruvate (PEP), pyrophosphate (PP_i), and orthophosphate (P_i) had similar effects on glucolipid synthesis but not to the same extents. cAMP had no effect (data not shown).

TABLE VI

Effects of lipids on the synthesis of MGlcDAG and DGlcDAG in partially delipidated cells

Partially delipidated cells were prepared by stirring lyophilized cells of *A. laidlawii* A in acetone (6 × 1 ml per mg membrane protein) at −20°C for 30 min, followed by sedimentation at 20000 × *g*. Pelleted cells were dried in vacuum at 20°C. To these cells (35 μg membrane protein) were added lipids dissolved in 25 μl benzene: (A) 0.2 mg *A. laidlawii* A membrane lipids (12% DAG, 16.9% MGlcDAG, 34.6% DGlcDAG, 16.0% PG, 0.7% DPG and 19.7% GPDGlcDAG, w/w), (B–G) as in A except that the indicated lipids were not included in the lipid mixture. (I–M) 25 μg DAG, 35 μg MGlcDAG and/or 150 μg PG. The rates of synthesis of MGlcDAG and DGlcDAG were assayed at 28°C for 30 min as described by Method 2 in Materials and Methods. Data presented are mean values from two experiments.

Added lipids [b]		Relative rates of synthesis [a] (%)	
		MGlcDAG	DGlcDAG
A.	*A. laidlawii* A membrane lipids	100	100
B.	minus DAG	8	38
C.	minus MGlcDAG	93	76
D.	minus DGlcDAG	137	146
E.	minus PG	13	27
F.	minus DPG	88	91
G.	minus GPDGlcDAG	75	143
H.	None	4	15
I.	PG	9	78
J.	DAG	95	60
K.	MGlcDAG	0	27
L.	PG and DAG	151	376
M.	PG and MGlcDAG	21	228

[a] Values have been normalized against the rate of synthesis in delipidated cells with all *A. laidlawii* A membrane lipids added (sample A). 100% rate of synthesis for MGlcDAG and DGlcDAG corresponds to 182 and 41 nmol/mg per h, respectively.

[b] DAG, diacylglycerol; MGlcDAG, monoglucosyldiacylglycerol; DGlcDAG, diglucosyldiacylglycerol; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; GPDGlcDAG, glycerophosphoryldiglucosyldiacylglycerol.

Influence of membrane lipid environment on the synthesis of the glucosyldiacylglycerols

To examine the influence of each different polar membrane lipid of *A. laidlawii* on the biosynthesis of MGlcDAG and DGlcDAG, we added lipid mixtures with different lipid compositions back to lyophilized and partially delipidated cells and measured the synthesis of the glucosyldiacylglycerols.

The syntheses of glucolipids were restored in partially delipidated cells when *A. laidlawii* membrane lipids were transferred back to extracted cell membranes (Table VI). The rates of synthesis of MGlcDAG and DGlcDAG when all *A. laidlawii* membrane lipids (in vivo lipid composition plus DAG substrate) were added to delipidated cells was used as a control (Table VI, sample A). If DAG was not included in the *A. laidlawii* membrane lipid mixture, the rate of synthesis of MGlcDAG was reduced by more than 90% to the background values obtained with no lipids (Table VI, samples A, B and H). This strongly indicates that most of the endogenous DAG substrate for synthesis of MGlcDAG must have been extracted from the delipidated cells and that exogenously added DAG could function as a glucose acceptor.

Phosphatidylglycerol (PG) seemed to have a strong effect on the synthesis of both MGlcDAG and DGlcDAG. The rate of synthesis of MGlcDAG and DGlcDAG was reduced by 70–90% in cells where PG had been withdrawn from the added lipid mixture (Table VI, sample E). The stimulating effect of PG could also be seen when PG was added together with DAG or MGlcDAG to the delipidated cells (Table VI, samples L and M). Addition of PG only (to delipidated cells) increases the rate of synthesis of DGlcDAG from 15 to 78% showing that in delipidated cells still there is a significant amount of MGlcDAG substrate left. A higher rate of synthesis of DGlcDAG when DAG is added instead of MGlcDAG could be an indication that the preferred substrate for synthesis of DGlcDAG is newly synthesized MGlcDAG instead of exogenously added MGlcDAG. Absence of GPDGlcDAG in the added *A. laidlawii* lipid mixture had an differential effect on the synthesis of MGlcDAG and DGlcDAG (Table VI, sample G).

This study demonstrates the importance of a specific lipid composition for the optimal activities of the enzymes involved in the syntheses of glucosyldiacylglycerols in *A. laidlawii* strain A, where PG seemed to have a key role in maintaining active enzymes.

Discussion

Synthesis of glucosyldiacylglycerols in vivo

The turnover of MGlcDAG shown in Fig. 1 supports the synthesis of MGlcDAG and DGlcDAG in *A. laidlawii* by a sequential glucosylation of DAG and MGlcDAG as suggested before [18]. A similar turn-over can be observed among, e.g., glycolipids in cyanobacteria [38]. We have recently shown that metabolic conversions can occur in vivo between most *A. laidlawii* lipids as traced at the acyl chain level [39]. Analogous glycolipid syntheses occur in a number of different cells but the type of sugar nucleotide varies (reviewed in Ref. 40). Here, UDP-Glc seems to be the donator, cf. Table IV.

Localization and activity of the enzymes

The differential effect of several experimental conditions on the synthesis of MGlcDAG and DGlcDAG, respectively, suggests that these sequential reactions are performed by two different enzymes. Fractionation of the cell contents (Table III) and the lipid dependence (Table VI) indicate that they both reside in the membrane. The two-step synthesis of analogous glyco-

lipids in the Gram-positive bacteria *Micrococcus lysodeikticus* [41] and *S. faecalis* [31] are performed by two membrane-bound, and one membrane-bound and one soluble enzyme, respectively. In the Gram-negative *Pseudomonas diminuta*, the enzyme synthesizing an anionic monoglycolipid is found distributed between the membrane and the cytoplasm [42]. The enzyme responsible for the synthesis of DGlcDAG in *A. laidlawii* is either inactivated, released and inactivated, or critically dependent upon a cytoplasmic factor which in itself is inactivated upon cell lysis and the separation of membrane and cytoplasm (Table III). Smith [18] obtained a higher rate of synthesis of DGlcDAG in washed membranes with a similar assay method (Method 1). The strong synthesis of DGlcDAG in whole cells (Tables III–VI) supports the presence of additional factors as suggested above. Several lipid-synthesizing enzymes consist of two non-identical protein subunits [3,43].

Detergent sensitivity and lipid dependence

Small amounts of the anionic detergent SDS, but not the non-ionic NP-40, stimulated the synthesis of MGlcDAG (Method 1). In several other systems a dependence on either an anionic or a non-ionic detergent has been recorded [40]. This has been attributed to the ability of the detergents to solubilize the lipid substrates and may well be the case with Method 1 used here.

The sensitivity of the synthesis of glucosyldiacylglycerols to complete solubilization by a variety of detergents (Fig. 2) has prevented an affinity purification of the enzymes (data not shown), since this necessitates a high extent of solubilization. The synthesis rate of glucosyldiacylglycerols in partially solubilized membranes was only 5–10% of the rate obtained in intact membranes. This effect could be explained by one or more of the following reasons: (i) proteins are selectively solubilized [44] and at a low detergent concentration only a minor fraction of the enzymes responsible for synthesis of glucosyldiacylglycerols are solubilized; (ii) essential protein–protein and/or protein–lipid interactions are disrupted; and (iii) nonspecific inactivation of the enzymes by detergent interaction occurs.

The stimulation of MGlcDAG and DGlcDAG synthesis in cells after acetone extraction and the readdition of various lipids (Method 2) suggests that critical properties of the enzymes (cf above) are restored or not disturbed by freeze-drying, and by acetone and benzene treatment. Although acetone does not remove all endogenous lipids, the strong dependence on exogenous lipids for activity is evident from Tables II and VI and the essential character of DAG is also obvious. Exogenously added MGlcDAG to fresh or frozen *A. laidlawii* membranes had no significant effect on the synthesis of DGlcDAG which is inconsistent with results presented by Smith [18] but could be explained by differences (e.g., packing properties of membrane lipids) in the strains used (unpublished observation). However, it is shown in this paper that exogenously added MGlcDAG, especially together with PG, to partly delipidated cells have a stimulatory effect on the synthesis of DGlcDAG (Table VI). The strongly stimulatory effect of the anionic lipid PG on the synthesis of both MGlcDAG and especially DGlcDAG is only partially achieved by the other (anionic) phospholipids (Table VI). There are many reports about the activation of different membrane-bound enzymes by specific polar lipids but PG is less common among these. However, the requirement of PG for optimal activity is also described for the analogous monogalactosyldiacylglycerol synthesizing enzyme in spinach chloroplast [45]. For the membrane-bound $(Na^+ + Mg^{2+})$-ATPase of *A. laidlawii*, small amounts of PG stimulate and large amounts irreversibly inactivate the enzyme in vitro [46]. We have previously shown that the fraction of anionic lipids (including PG) in *A. laidlawii* membranes is regulated as a function of several stimuli, so that a constant surface charge density and potential is maintained [17]. It is therefore possible that the stimulatory effect of PG on the glucolipid synthesis in vitro (Table VI) is due to the restoration of the proper surface potential. The stimulatory effect of (anionic) SDS, but not NP-40, with Method 1 (see above) supports this proposal.

Modification of enzyme activity

The physiological regulation of lipid composition in *A. laidlawii* is affected by a variety of conditions and agents that change the packing properties and phase equilibria of the membrane lipids (see, for example Ref. 6), cf. Introduction. At least two different mechanisms for how the responsible enzymes sense these changes can be proposed: (i) a direct effect by a structural or dynamic property of the lipid bilayer on the structure, and hence activity, of a glucolipid synthesizing enzyme; or (ii) an indirect effect from a change in certain membrane or metabolic properties of the cell (e.g. membrane permeability, redox or phosphorylation potential). Table VI shows that the mechanism in point (i) is probably involved (see also the discussion above). We have previously shown a correlation in vivo between the transmembrane potential, energy charge and the regulation of the metabolic balance between the two glucolipids in *A. laidlawii* [13,14], cf. point (ii) above. This is further supported by the data in Tables IV and V which show that the presence of triphosphate nucleotides reduces the rate of synthesis of MGlcDAG and stimulates that of DGlcDAG. Other phosphorylating agents have a similar but less pronounced effects (Table V). The assay concentrations of these compounds (i.e. 5 mM) are similar to the in vivo concentrations of ATP recorded in *A. laidlawii* [14,47]. Storage

of washed *A. laidlawii* cells in buffer for several hours, a condition known to deplete the energy stores, increases the synthesis rate for MGlcDAG 3-fold (data not shown), which supports the data in Table V. All four nucleotides tested (i.e. ATP, CTP, GTP and UTP) had similar effects on the glucolipid synthesis. This might be similar to the acetate kinase of *A. laidlawii*, which can use these four nucleotides as phosphate donors [48]. Pyrophosphate-dependent kinases are also more common in mycoplasmas than in other bacteria [49]. Two phosphate-labelled membrane proteins have been identified in *A. laidlawii* [50]. Several labelled peptides can also be separated and detected from *A. laidlawii* after incubation of cells with [^{32}P]ATP followed by enzymatic hydrolysis of the membrane proteins [51].

The results obtained here support the proposal that the activity of the enzymes in *A. laidlawii* responsible for the synthesis of the major membrane lipids MGlcDAG and DGlcDAG can be effected by the properties of the surrounding lipid matrix and perhaps also by the phosphorylation potential.

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