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Polar glycerolipids of *Chlamydomonas moewusii*

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Abstract

The fatty acid and polar lipid compositions of the unicellular green alga *Chlamydomonas moewusii* were characterized. Since this organism is an important plant model for phospholipid-based signal transduction, interest was focused on the lipids phosphatidic acid, phosphatidylinositolphosphate and phosphatidylinositolbisphosphate. A phosphatidylinositol:phosphatidylinositolphosphate:phosphatidylinositolbisphosphate ratio of 100:1.7:1.3 was found. The polyphosphoinositides accounted for 0.8 mol% of the total phospholipids and their fatty acid compositions were similar to that of phosphatidylinositol except for the enrichment of linolenic acid in phosphatidylinositol phosphate. Phosphatidic acid accounted for 0.67 mol% of the phospholipids. Major structural glycerolipids were monogalactosyldiacylglycerol (35 mol%), digalactosyldiacylglycerol (15 mol%), sulfoquinovosyldiacylglycerol (10 mol%), diacylglyceryltrimethylhomoserine (16 mol%), phosphatidylglycerol (9 mol%), phosphatidylethanolamine (8 mol%) and phosphatidylinositol (6 mol%). Relative changes in the total fatty acid compositions found during growth on nutrient-limited medium reflected mainly alterations in the compositions of the chloroplast lipids phosphatidylglycerol and monogalactosyldiacylglycerol. [^{32}P]Pi-incorporation studies revealed that it took 6 days before the amount of label in the major phospholipids was proportional to their abundance.

Keywords: *Chlamydomonas moewusii*; Volvocales; Fatty acid composition; Glycerolipids; Phosphoinositides; Phosphatidic acid

1. Introduction

In recent years, lipids have emerged as specific regulators of physiological processes in plants. The unicellular green algae *Chlamydomonas* and *Dunaliella* have proved excellent model systems for the study of lipid-based signal transduction. The uniformity of these cell systems allows such metabolic pathways to be studied in great detail and the findings made can often be extrapolated to higher plants due to the marked similarities between these organisms at the cellular level (Thompson, 1996; Munnik, Irvine & Musgrave, 1998a).

While the advantages of green algae in this novel field of exploration are becoming clear, only a few species have been examined in detail for their lipid and fatty acid compositions. Moreover, most studies have ignored phosphatidylinositolphosphate (PtdInsP), phosphatidylinositolbisphosphate ($PtdInsP_2$) and phosphatidic acid (PtdOH), because of their low abundance and the difficulty of purifying sufficient amounts. These phospholipids have attracted much interest in the study of signal transduction in plants, because different kinds of stress stimulate phospholipase C (PLC, EC 3.1.4.11) and phospholipase D (PLD, EC 3.1.4.4) activity resulting in the breakdown of $PtdInsP_2$ by PLC, and the formation of PtdOH by both phospholipases (Munnik et al., 1998a). Using [^{32}P]Pi-labeling techniques, these activities have been characterized in *C. moewusii* (Munnik, Arisz, De Vrije & Musgrave, 1995; Munnik et al., 1998b).

Here, we present the polar glycerolipid and fatty

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Table 1
Changes in fatty acid composition of *C. moewusii* after 1–5 weeks of growth[a]

Week no.	Fatty acid (mol%)											
	14:0	16:0	16:1(Δ3*t*)	16:1ω9	16:2	16:3ω3	16:4	18:1ω7	18:1ω9	18:2	18:3ω3	18:4
1	6.7	13.7	1.5	1.7	1.5	7.1	16.4	1.3	7.2	6.8	31.8	3.0
3	4.6	16.4	0.9	2.4	2.7	3.4	18.1	2.1	12.0	7.0	25.7	3.9
5	4.1	22.2	0.7	1.2	2.7	2.9	12.4	3.2	15.6	9.1	21.1	3.8

[a] Fatty acids found in trace amounts, including 14:3, 18:0 and 18:3ω6, are omitted.

acid composition of this alga. Since our interest was focused on the polyphosphoinositides (PPIs), procedures for their purification, solid-phase extraction and 2D-TLC separation were developed. Because of an apparent discrepancy in the measured phospholipid masses with previous in vivo labeling experiments, a [^{32}P]Pi-incorporation study is included, showing labeling trends towards molar ratios.

2. Results and discussion

Plant glycerolipids are synthesized in the chloroplast and the endoplasmic reticulum, where phosphatidic acid moieties are formed as precursors of all diglycerides (Frentzen, 1986; Ohlrogge & Browse, 1995). As a result of the activities of specific acyltransferases and desaturases in these compartments, each lipid class has its own characteristic fatty acid content. In glycerolipids of the Chlorophytae, which are considered very close to higher plants with respect to lipid composition and metabolism, polyunsaturated fatty acids (PUFAs) of 16 and 18 carbon atoms predominate (Thompson, 1996).

In order to characterize the fatty acid content of *C. moewusii* gametes, cells were grown on nutrient-limited medium, which enhances the production of gametes which have been used in most studies on cell signaling. Since this environmental restriction could affect lipid composition, cells were sampled after 1 to 5 weeks of culture (see Table 1). The PUFAs 16:3, 16:4 and 18:3, characteristic of the plastidic galactolipids, and 16:1 (Δ3-*trans*), specific for plastidic phosphatidylglycerol (PtdGro) (Thompson, 1996), decreased from week 1 to week 5. Changes in monogalactosyldiacylglycerol (MGDG) and plastid PtdGro have been found as a corollary to changes in temperature, nutrient availability and irradiance level (Kuiper, 1985; Sukenik, Yamagutchi & Livne, 1993), and may be regarded as a general response to unfavourable growth conditions. The unusual fatty acid 16:1 (Δ3-*trans*), which is believed to play a structural role in chloroplast lipid–protein complexes (Trémolières, Roche, Dubertret, Guyon & Garnier, 1991), accounted for 24 mol% of the total fatty acids in PtdGro after 1 week and 9 mol% after 5 weeks (not shown). This may be related to the morphological changes in thylakoids described for other *Chlamydomonas* cells (Martin & Goodenough, 1975), and is consistent with the reduction in chloroplast lipids described elsewhere (Piorreck & Pohl, 1984). The concomitant rise in 16:0 and 18:1 may reflect synthesis of neutral storage lipid

Table 2
Major lipids of *C. moewusii* and their fatty acid compositions[a]

Lipid	% of total[b]	Fatty acid (mol%)[a]											
		14:3	16:0	16:1[c]	16:2	16:3	16:4	18:0	18:1ω7	18:1ω9	18:2	18:3ω3	18:4
MGDG	35	< 1	2	1	2	4	36	< 1	–	2	9	43	–
DGDG	15	–	28	2	8	11	2	–	2	19	10	18	–
SQDG	10	–	81	–	–	–	–	1	2	3	5	9	1
DGTS	16	–	71	–	< 1	2	3	< 1	2	4	3	8	7
PtdGro	9	12	35	25	–	< 1	–	1	2	5	10	11	1
PtdEtn	8	–	5	–	–	< 1	–	< 1	–	92	1	2	–
PtdIns	6	–	50	–	–	1	–	–	20	14	9	7	–

[a] Two- to three-week-old cultures. Values represent the averages of at least four independent analyses ; s.d. ≤ 4 for glycolipids and s.d. ≤ 2 for other lipids. Fatty acids only found in trace amounts including 16:1ω7, 16:3ω3 and 18:3ω6, are omitted.
[b] Values represent mole percentages of total polar glycerolipids as determined by reference to internal GC standard and/or P-determination.
[c] 16:1ω9 or, in the case of PtdGro, 16(Δ3-*trans*).

Table 3
Minor phospholipids of *C. moewusii*[a]

Lipid	Mole percentages of phospholipids	Fatty acids (mol%)[b]				
		16:0	18:1ω7	18:1ω9	18:2	18:3ω3
PtdInsP	0.45	44	15	14	13	13
$PtdInsP_2$	0.34	53	19	15	9	5
PtdOH	0.67	20	2	73	2	3

[a] Two- to three-weeks-old cultures.
[b] Values represent the averages of at least four independent analyses (s.d. $\leq$ 2).

(Piorreck & Pohl, 1984), since these fatty acids predominate in neutral lipids of *C. moewusii* (data not shown).

To minimize these culture-age-dependent changes, two- to three-week-old cultures were routinely used for the analysis of lipids and fatty acids (see Table 2). Quantities were deduced from GC mass analysis of the component fatty acids and, for phospholipids, by phosphorus determination. The plastid glycolipids MGDG, digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) accounted for 60 mol% of total polar glycerolipids. The structural phospholipids PtdGro, phosphatidylethanolamine (PtdEtn) and phosphatidylinositol (PtdIns) accounted for 23 mol% and were found in a molar ratio of 39:35:27, in agreement with phosphorus determinations. While phosphatidylcholine is absent from *Chlamydomonas* (Harwood & Jones, 1989), diacylglyceryltrimethylhomoserine (DGTS), another zwitterionic lipid common to green algae (Eichenberger, 1993), accounted for 16 mol% of the total polar glycerolipid.

Galactolipids, as compared to phospholipids, were characterized by an abundance of C16- and C18-polyunsaturates (Table 2), largely accounting for the high PUFA content of the total extracts (Table 1). PtdEtn contained mainly oleic acid (18:1ω9), consistent with its extraplastidic glycerolipid synthesis (Frentzen, 1986). Only one isomer of linolenic acid, 18:3ω3, was consistently found in the polar glycerolipid classes, although the 18:3ω6 isomer was found in trace amounts in total extracts and sometimes in DGTS. This is in contrast to *C. reinhardtii* (Giroud, Gerber & Eichenberger, 1988), where 18:3ω6 is a major component and the exclusive isomer in PtdEtn and DGTS, while 18:3ω3 is the exclusive isomer in MGDG and DGDG. Apart from these interspecies differences, their glycerolipid and fatty acid compositions are similar. In both species, PtdGro was characterized by the presence of 16:1 (Δ3-*trans*). The fatty acid 14:3, which is almost limited to PtdGro in *C. moewusii*, has also been found in PtdGro of *C. reinhardtii* (Janero & Barnett, 1981). In *C. moewusii*, the relative fatty acid compositions of vegetative cells and gametes of both mating types were found to be the same (data not shown).

Due to their roles in intracellular signal transduction, the minor phospholipids PtdInsP, $PtdInsP_2$ and PtdOH are interesting new subjects for lipid and fatty acid analysis. In *C. moewusii*, $PtdInsP_2$-hydrolyzing PLC activity has been characterized under various stimulatory conditions (Munnik et al., 1998a, 1998b). Although PtdIns is the precursor to the PPIs, its fatty acid composition is not necessarily the same. For example, in some mammalian cells, it is enriched in arachidonic acid (Augert, Blackmore & Exton, 1989).

The small amount of PtdInsP, $PtdInsP_2$ and PtdOH compared with the large background of structural lipids makes their analysis a difficult task. Combinations of solid-phase extraction and TLC were utilized to resolve sufficient quantities for analysis. We, therefore, developed a modified elution protocol for silica columns that resulted in the recovery of 70% of the total PPIs and 92% of the PtdOH. The results of these analyses were confirmed using neomycin-coated glass beads (Schacht, 1978) for purification. Due to the high selectivity of neomycin, this method proved more efficient in the extraction of PtdInsP and $PtdInsP_2$ (recovery 96%).

The quantities of these lipids are expressed in Table 3 as mole percentages of the total phospholipids, which were quantitated from the same extract. Per 10^9 cells, *C. moewusii* contained 1.4, 1.1 and 2.1 nmoles of PtdInsP, $PtdInsP_2$ and PtdOH, respectively. The PtdIns:PtdInsP:$PtdInsP_2$ molar ratio was calculated to be 100:1.7:1.3. No significant differences were found in the fatty acid compositions of PtdIns, PtdInsP and $PtdInsP_2$ except for an enrichment of 18:3 in PtdInsP. In contrast to reports on mammalian PPIs (Augert et al., 1989), but concurring with those on carnation (Munnik, Musgrave & De Vrije, 1994a) and carrot (Van Breemen, Wheeler & Boss, 1990), these lipids had no distinctive fatty acids such as long-chain PUFAs. Also, analysis of *D. salina* showed the presence of mainly 16:0 and 18:1 (Ha & Thompson, 1991). Previous analyses with contrary results, are likely to have employed insufficient purification protocols

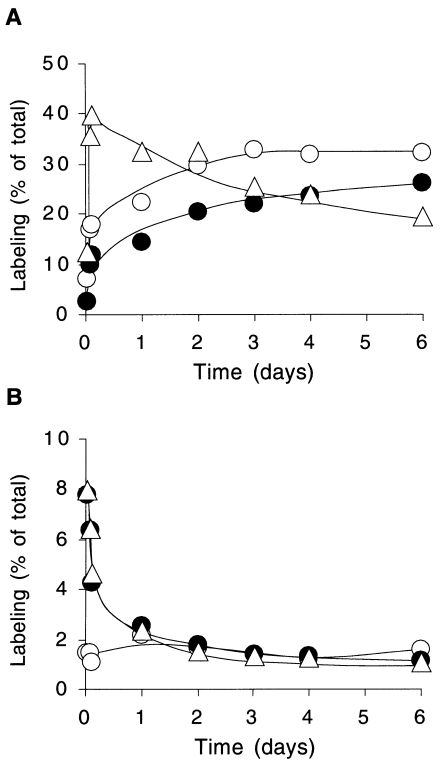

Fig. 1. [^{32}P]Pi-labeling of *C. moewusii* phospholipids with time. Labeling is expressed as a percentage of the total labeled lipid. (A) Structural lipids; PtdGro (○), PtdEtn (●) and PtdIns (△). (B) Minor lipids; PtdOH (○), PtdInsP (●) and PtdInsP$_2$ (△).

(Brederoo, De Wildt, Popp-Snijders, Irvine, Musgrave & Van den Ende, 1991). The discrepancy with mammals may be related to specific physiological functions of these lipids in plants (Munnik et al., 1998a).

While PtdOH can function as a second messenger in signaling cascades and can be generated via both PLD (Munnik et al., 1995) and PLC activities (Munnik et al., 1998b), its fatty acid composition in non-stimulated cells is likely to reflect its role as intermediate in de novo glycerolipid synthesis. The predominance of 16:0 and 18:1 (Table 3) supports this idea since these fatty acids are esterified to glycerolphosphate in newly formed PtdOH. This lipid is the precursor to various complex glycerolipids which serve as substrates for further fatty acid desaturation, for which evidence has been found in *C. reinhardtii* (Giroud & Eichenberger, 1989).

Metabolic radiolabeling strategies have been utilized to investigate pathways of glycerolipid biosynthesis (Giroud & Eichenberger, 1989; Harwood & Jones, 1989) and signal transduction in plants (Einspahr, Peeler & Thompson, 1988; Munnik et al., 1998b). The molar ratios determined here by fatty acid and phosphorus analysis are not consistent with the ratios seen in [^{32}P]Pi-labeling studies of *C. moewusii*, which show heavily labeled phosphoinositides and relatively small amounts of PtdEtn (Munnik et al., 1998b; Brederoo et al., 1991). However, due to divergent rates of label incorporation and lipid turnover, amounts of radioactivity need not reflect the molecular abundance of different lipid classes. For example, short-term labeling of phospholipids with [^{32}P]Pi has been shown to reflect turnover rates rather than abundance (Einspahr et al., 1988; Munnik et al., 1994a; Munnik et al., 1994b; Munnik et al., 1998b). However, by extending the time of labeling and adding unlabeled carrier-phosphate, the influence of different metabolic rates is overruled by differences in pool sizes. To investigate whether such a labeling protocol can generate a phospholipid labeling pattern equivalent to the mass ratios, a cell suspension was labeled with [^{32}P]Pi in a 1 mM K–Pi buffer and sampled at subsequent time points (Fig. 1(A)). After 6 days the [^{32}P]PtdGro:[^{32}P]PtdEtn:[^{32}P]PtdIns ratio was 42:34: 25, a close approximation to their molar ratios, as determined by fatty acid and phosphorus analysis, indicating that the lipids had become equally labeled.

[^{32}P]Pi-labeling of the PPIs is usually very rapid due to their high turnover rates (Munnik et al., 1994a; Munnik et al., 1994b; Munnik et al., 1998b). This is illustrated in Fig. 1(B) as the high proportion of label they contain during the first hours of labeling. However, over a six-day period, labeling of PtdOH, PtdInsP and PtdInsP$_2$ changed to represent 1.76, 0.92 and 0.77%, respectively, of the total [^{32}P]phospholipids, approaching their molar ratios in the cell. The apparent overestimation relative to the major phospholipids may be due to the particular conditions of the labeling experiment.

While radiolabeling is the method of choice for analyzing rapid changes in phospholipid metabolism in response to environmental stimuli, knowledge of the lipid mass levels and fatty acid compositions will be useful for further characterization of the signal transduction pathways. Such information may reveal precursor-product relations and thus elucidate the metabolic origin and fate of lipid signals.

3. Experimental

3.1. Cell cultures

Chlamydomonas moewusii strain UTEX 10 (mating type minus) from the Culture Collection of Algae, University of Texas (Austin) and strain 17.17.2 (mating type plus) were autotrophically grown as described before (Schuring, Smeenk, Homan, Musgrave & Van den Ende, 1987). Swimming gamete suspensions were obtained by flooding each plate culture with 20 ml HMCK (10 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM KCl; pH 7.4). After 16 h, cells were harvested.

Vegetative cells were grown in the liquid medium described by Kates and Jones (1964).

3.2. Lipid isolation

Lipids were extracted by a modification of the Bligh and Dyer method (Munnik, De Vrije, Irvine & Musgrave, 1996) and separated by TLC (silica gel 60) using the following solvent systems (volume ratios given): A: $CHCl_3$–MeOH–NH_4OH–H_2O (90:70:4:16); B: $CHCl_3$–Me_2CO–MeOH–HAc–H_2O (80:30:28:26:15); C: $CHCl_3$–MeOH–H_2O, (65:25:4); D: $CHCl_3$–MeOH–NH_4OH (65:35:5); E: EtOAc-*iso*-octane–HCO_2H–H_2O (13:2:3:10). PtdGro, PtdEtn and PtdIns were separated by 2D-TLC using solvent A in the first dimension and solvent B in the second dimension. Other structural lipids were separated by 2D-TLC using solvent C in the first and D in the second dimension (Allen & Good, 1971). Lipids were localized by autoradiography, iodine or dichlorofluoresceine staining and identified on the basis of their migration, specific staining and ^{32}P-labeling properties.

For the purification of minor phospholipids, the extract was fractionated by column-adsorption chromatography on a 2 g-silica column. Briefly, 10 mg lipid was applied to the column in *n*-hexane. ^{32}P-lipid markers were included to monitor phospholipids and to calculate the recovery. Elution solvents were (1) hexane–Et_2O (99:1, 18 ml), (2) hexane–Et_2O (4:1, 15 ml), (3) $CHCl_3$ (10 ml), (4) Me_2CO–$CHCl_3$ (2:1, 25 ml), (5) Me_2CO–MeOH (29:1, 10 ml), (6) Me_2CO–MeOH (19:1, 30 ml), (7) Me_2CO–MeOH (2:1, 25 ml), (8) $CHCl_3$–MeOH–H_2O–HCl (50:100:40:1, 10 ml). Lipids were extracted from the last fraction by a normal two-phase lipid extraction, treating the eluate as a homogeneous phase. After drying the eluates, the PPIs were resolved by TLC using solvent C, followed by solvent A in the same direction. PtdOH from fraction 7 was separated using solvent E.

Alternatively, PtdInsP and PtdInsP$_2$ were purified by solid-phase extraction with neomycin-coated glass beads (Schacht, 1978), kindly provided by R.F. Irvine (Cambridge, UK). The extract was dissolved in $CHCl_3$–MeOH-170 mM HCO_2NH_4 (5:10:2) and mixed with beads for 15 min at 4°C. After several washes with the solvent, the beads were clear of all lipids, except the PPIs that were quantitatively recovered by washing with $CHCl_3$–MeOH-2.4 N HCl (2:4.5:2). These lipids were extracted from the aqueous phase into $CHCl_3$ and resolved by TLC using solvent A.

3.3. Fatty acid derivatization and analysis

Lipid spots were scraped off the plates directly into the transmethylation reagent. Known concentrations of heptadecanoic (17:0) and heneicosanoic (21:0) acid methyl esters served as internal standards. The concentrated FAME extract was analyzed in a GC equipped with a 50 m WCOT fused silica column and FID with carrier gas N_2 at 30 ml min^{-1} and a split ratio of 1:75. Injection volume was 1 μl and operating conditions were 180°C isothermal or temperature programmed 180–220°C at 0.5°C min^{-1} with injector and detector temperatures at 250 and 270°C, respectively. Phosphorus determination was by the method of Rouser, Fleischer and Yamamoto (1970).

3.4. Metabolic radiolabeling

40 μCi [^{32}P]Pi in a 1 mM K–Pi buffer pH 7.4 was added to 4 ml of cell suspension in HMCK. Samples were taken at the time points indicated, lipids extracted and separated by TLC using solvent A as described earlier (Munnik et al., 1994a). Radioactivity was quantitated by phosphoimaging.

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