Isoprenoid-mediated changes in the glycerophospholipid molecular species of the sterol auxotrophic fungus *Lagenidium giganteum*

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The mosquito pathogenic fungus *Lagenidium giganteum* (Oomycetes: Lagenidiales) is a sterol auxotroph that can grow vegetatively in the absence of these compounds, but requires an exogenous source of sterols to enter its sexual and asexual reproductive cycles. Electrospray mass spectrometry (MS) and electrospray MS/MS were used to examine three major glycerophospholipid molecular species – glycerophosphocholine (GPC), glycerophosphoethanolamine (GPE) and glycerophosphoinositol (GPI) – from fungal mycelium and nuclei grown in defined medium with and without isoprenoids which induce (cholesterol and ergosterol) or do not induce (squalene, cholestane) reproduction. Testosterone supplementation of defined media inhibited growth of *L. giganteum*, so the effect of this steroid on phospholipid metabolism could not be assessed. Mycelium grown in defined media supplemented with these isoprenoids produced significantly different quantities of total phospholipid relative to unsupplemented media and to each other, ranging from a mean of 292 µg phosphate per g wet weight for cholesterol-supplemented media to 56 µg phosphate per g wet weight for mycelium grown in the presence of squalene. A very large percentage of the GPC (69–80 mol%) and GPI (74–79 mol%) molecular species from mycelia and nuclei contained ether linkages. GPE molecular species had 13–20 mol% ether-containing moieties. The elevated levels of ether lipids may be related to the sterol auxotrophic nature of the fungus. Isoprenoid supplementation of defined growth media resulted in many significant changes in molecular species for all three lipid classes. Significant differences ($P < 0.05$) in the percentage of total cell ether lipids in GPC and GPE were generated by isoprenoid supplements to culture media. Mycelium grown in the presence of the two sterols which induce asexual and sexual reproduction in *L. giganteum*, cholesterol and ergosterol, had a significantly greater percentage of ether-containing GPE moieties. The glycerolipid species from nuclei isolated from cultures grown with cholesterol and ergosterol were similar to the composition of nuclei isolated from fungus cultured in defined medium without any supplement or supplemented with squalene. The nuclear membrane from mycelia grown in cholestane-supplemented media, however, had a very different glycerophospholipid composition relative to either whole cells or nuclei from cells grown on other media. It appears that one of the reasons that cyclic isoprenoids such as cholestane do not induce fungal reproduction is that they drastically alter the nuclear membrane glycerophospholipid composition.

Keywords: *Lagenidium giganteum*, glycerophospholipids, sterol auxotroph, isoprenoids, nuclei

Abbreviations: GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPI, glycerophosphoinositol.

INTRODUCTION

Sterols are a basic component of eukaryotic cells and have structural and regulatory roles in cell metabolism. They can make up 30 mol% or more of the lipid fraction of some outer membranes (Yeagle, 1989, 1990), a concentration at which they undoubtedly play an important structural role. In some instances this bulk structural role can be met by sterols with a range of structures (Rodriguez *et al.*, 1985). In contrast, the regulatory functions of sterols require very specific ring and side-chain structures and stereochemistry (Pinto *et al.*, 1983; Bloch, 1981). In mammals the regulatory functions are satisfied by cholesterol (Bloch, 1983; Yeagle, 1989), while in yeast and many other fungi ergosterol is required (Rodriguez *et al.*, 1982; Rodriguez & Parks, 1983; Van den Bossche, 1990). These latter requirements can often be satisfied by sterol concentrations several orders of magnitude less than those required for their structural roles.

The roles of sterols in growth and morphogenesis have also been investigated in sterol auxotrophic members of the Oomycetes. These fungi can grow vegetatively in the absence of sterols, but require an exogenous source of these compounds to enter their sexual reproduction cycle (Elliot, 1983). Although they have long been regarded as a primitive group of fungi, Oomycetes have recently been more closely allied with heterokont algae in the Kingdom Chromista (Cavalier-Smith, 1989; Barr, 1992). This may explain in part why sterol structural requirements for the regulatory function in sterol auxotrophic Oomycetes are different from those of most other fungi. The developmental event with the strictest structural requirements is the production of oospores, the sexual stage of these organisms; however, structurally diverse sterols such as Δ -5 (cholesterol, sitosterol), Δ -5,7 (ergosterol), Δ -5,22 (stigmasterol) and Δ -5,24 (fucosterol, avenasterol) (Elliot, 1972, 1983) can, albeit with varying efficiency, induce oosporogenesis. The mechanism of sterol-induced reproduction in these fungi is not known, although sterols appear to play a pivotal role at the onset of meiosis (Elliot & Sansome, 1977).

Lagenidium giganteum (Oomycetes: Lagenidiales), a facultative parasite of mosquito larvae, is a member of this primitive group of fungi which cannot synthesize sterols (Warner & Domnas, 1981; Domnas & Warner, 1991). It can grow vegetatively in the absence of sterols, but requires an exogenous source of these compounds to enter its asexual (Domnas *et al.*, 1977) or sexual (Kerwin & Washino, 1983) reproductive cycles. It is interesting that the mosquito hosts of *L. giganteum* also lack the ability to synthesize sterols, which are required for larval growth and development. Mosquito larvae obtain their sterols from the diet on which they are reared. These dietary sterols, following uptake and metabolism by the larvae, are then used by the fungus to promote its sexual and asexual reproduction. Both reproductive stages culminate in the production of motile zoospores, the infective stage of *L. giganteum*, and allow it to persist for weeks or years following its introduction into a suitable natural habitat.

Supplementation of growth media with specific phospholipids enhances oosporogenesis by *L. giganteum* (Kerwin *et al.*, 1991) and other sterol auxotrophic Oomycetes (Ko, 1986; Kerwin & Duddles, 1989). For some species there is a synergistic effect between phospholipids and sterols on oosporogenesis. Interactions between sterols and phospholipids have been examined in many artificial and biological membranes (Chia *et al.*, 1993; Davidson *et al.*, 1993). Sterol modulation of the fluidity of phospholipid acyl chains is well documented, but since microgram per ml quantities of these compounds in culture media also supplemented with phospholipids can exert profound developmental changes in sterol auxotrophic Oomycetes, at least some of their effects are not due to changes in bulk membrane properties.

The action of steroid hormones in regulating sexual reproduction by *Aeblyia* spp., which are Oomycetes capable of synthesizing sterols and related isoprenoids, has been extensively investigated (Barksdale, 1969; Horgen *et al.*, 1983; Brunt & Silver, 1991; McMorris *et al.*, 1993). Although the possible role of sterols as steroid hormone precursors in sterol auxotrophic Oomycetes is the subject of controversy (Elliot, 1983), these compounds or their metabolites must directly or indirectly be involved in events in the nucleus associated with developmental regulation. The mechanism of sterol-induced reproduction in Oomycetes unable to synthesize sterols is not known, but this class of lipid also appears to play a pivotal role at the onset of meiosis (Elliot & Sansome, 1977). One possible mechanism of isoprenoid-induced reproduction is to regulate the composition of glycerophospholipid molecular species in the nuclear membrane. Coordinated regulation of the nuclear composition of different lipid classes has been shown to occur in mammalian nuclei following dietary supplementation with highly polyunsaturated fatty acids (Chapkin *et al.*, 1992). Sterols and other isoprenoids can alter membrane composition independently of developmentally regulated changes. As the initial step in understanding the role of isoprenoids in fungal morphogenesis, we have investigated the effects of isoprenoid supplementation of culture media on whole cell and nuclear glycerophospholipids from vegetative cultures of *L. giganteum*.

METHODS

Source and maintenance of fungal cultures. The California isolate of *L. giganteum* (ATCC 52675) was maintained on the following completely defined medium, designated DM2 (g per litre distilled water): glucose 4.0, sodium glutamate 2.0, asparagine 1.0, Na₂EDTA 0.05, KH₂PO₄ 0.05, K₂HPO₄ 0.05, thiamin.HCl 0.1, KNO₃ 0.1, methionine 0.05, MgSO₄.7H₂O 0.1, CaCl₂.2H₂O 0.15, Fe(NO₃)₃.9H₂O 0.0081, MnCl₂.4H₂O 0.036, ZnSO₄.7H₂O 0.01, H₃BO₃ 0.063, CuSO₄.5H₂O 0.008, (NH₄)₆Mo₇O₂₄.4H₂O 0.01, CoCl₂.6H₂O 0.017, 250 µl tergitol NP-40/ethanol (1:1, v/v), adjusted to pH 7.0 with 1 M NaOH. The tergitol/ethanol, which was used to enhance solubilization of added isoprenoids, was included in the control media to ensure that the observed changes in glycerophospholipid species were due to addition of the isopentenoids rather than to the

detergent. DM2 is a variation of defined media previously used to grow Oomycetes (Gleason, 1968; Ko, 1986). The medium was supplemented with excess glucose, which inhibits both sexual and asexual reproduction (Domnas *et al.*, 1977; Kerwin & Washino, 1983), so that altered glycerophospholipid species were not induced by developmental changes. Isoprenoids were added to DM2 at a concentration of 50 mg l⁻¹. All compounds were added prior to autoclaving except for ergosterol, which was added after sterilization to minimize breakdown of this sterol, which is relatively labile due to the presence of conjugated double bonds at the Δ -5 and -7 positions. Cultures were grown in 1 l of medium in 2.8 l Fernbach flasks inoculated with 10 ml of stock culture, at 120 r.p.m. on a rotary shaker at 23 $\pm$ 3 °C. Cultures used for comparative lipid analyses were 6–8 d old, in the stationary phase of their development. Fungal cultures were maintained for at least three generations on a given medium before extraction of phospholipids for mass spectral analysis.

Whole cell phospholipid extraction and fractionation.

Stationary phase cultures (6–8 d old) were collected by filtration on 100 μ m nylon mesh and washed in 1% (v/v) aqueous Tergitol NP-40/ethanol (1:1, v/v) followed by distilled water (Lorenz *et al.*, 1986). Total cell lipid was extracted using slight modifications (Kerwin & Duddles, 1989) of the method of Folch *et al.* (1957). The extract was concentrated and the glycerophospholipids separated by thin layer chromatography (TLC) on silica gel G, using hexane/diethyl ether/acetic acid (90:10:1, by vol.) as the developing solvent. The glycerophospholipids were extracted from the origin using approximately 10 ml chloroform/methanol (2:1, v/v), filtered from the silica gel and stored in chloroform at -20 °C until used for quantification or electrospray MS.

Phospholipid quantification. Total phospholipid was measured using the procedure of Mrsny *et al.* (1986). Following separation by TLC, the phospholipid fractions were dried then resuspended in 50 μ l water and 0.5 ml perchloric acid. Samples were digested for a minimum of 6 h in open-top tubes at 130 °C. After digestion, 1 ml 2.5% (w/v) ammonium molybdate, 3 ml distilled water and 0.5 ml 10% (w/v) ascorbic acid were added to the tubes, which were incubated at 37 °C for 1 h. Using NaH₂PO₄ to generate a standard curve, phosphomolybdate concentration was monitored at 820 nm.

Purification of nuclei. Lysing solution (LS) was prepared using (per litre distilled water): 91.1 g sorbitol, 0.609 g MgCl₂, 0.442 g CaCl₂, 0.372 g EDTA, 170 mg PMSF, 150 mg DTT and 5.0 ml absolute ethanol in 100 ml distilled water. DTT and PMSF were added just prior to use. Washing solution (WS) consisted of (per litre distilled water): 1.576 g Tris/HCl and 1.015 g MgCl₂·6H₂O. Final washing solution (FWS) was prepared by adding 0.5 ml of a solution of 0.023 g NaCl (20 mM) and 10 mg PMSF dissolved in 2.5 ml ethanol to 20 ml of WS.

LS, WS and FWS were placed at -20 °C until ice began to form, and then placed at 5 °C. Two 10 cm diameter Buchner funnels with six layers of miracloth were chilled at 5 °C. Mycelium from stationary phase cultures was filtered and washed in distilled water. Approximately 10 g of cells was placed in a chilled metal blender cup. In order to initially disperse the mycelium, 60–80 ml of ice cold LS was added to the cup and blended for 15–30 s at the highest setting using a Virtis Tempest polytron. Liquid N₂ (1–2 ml) was added to the mixture and homogenization continued until the mixture thawed, usually 1–2 min. The process was repeated with liquid N₂ added as necessary for 6–8 min. When a minimum of 70% of the cells had been disrupted as determined microscopically, the lysate was filtered twice using the miracloth and the chilled Buchner

funnels. The filtrate was poured into centrifuge tubes and centrifuged for 10 min at about 8000 g. The supernatant was decanted and the pellet resuspended in about 8 ml ice-cold WS. The pellet was centrifuged and washed twice more. A Percoll step gradient was prepared using 5 ml of each of 50, 40, 20 and 10% (v/v) Percoll solutions diluted with LS. The pellet from the final wash was resuspended in approximately 1 ml ice cold FWS, gently layered on top of the Percoll, and centrifuged at 4 °C for 15 min at 6000 g. A small amount of each individual band from the gradient was carefully removed and placed on a microscope slide with a drop of acridine orange (prepared just prior to use by dissolving 10 mg acridine orange in 10 ml distilled water). Following examination of the individual bands under a Zeiss fluorescence microscope, the band containing purified nuclei was extracted with a Pasteur pipette and resuspended in a small volume of FWS in a 3 ml Reacti-vial (Pierce Chemical Co.). The vial was centrifuged for 5–10 min at about 8000 g, and the supernatant removed with a Pasteur pipette. The pellet was resuspended in CHCl₃/MeOH (2:1, v/v) and stored at -20 °C until mass spectral analysis. Some preparations were resuspended in 0.1 M Tricine buffer, pH 7.8, for marker enzyme assays.

In addition to microscopic confirmation of the purity of nuclear preparations, the following enzyme marker assays (Chapkin *et al.*, 1992) were used to confirm the absence of contaminating organelles: 5'-nucleotidase for plasma membranes (Michell & Hawthorne, 1965), modified according to Chapkin *et al.* (1992), using the method of Fiske & Subbarow (1925) to quantify phosphate release; glucose-6-phosphatase for microsomes; and cytochrome c oxidase for mitochondria (Smith, 1953). Pellets containing over 1000-fold more nuclei than was used for each molecular species analysis were used for the enzymic assays to ensure that any contaminating organelles would be detected.

A representative nuclear preparation was examined by transmission electron microscopy. Nuclei were fixed in 3% (v/v) glutaraldehyde in 1.0 M phosphate buffer, pH 7.3, for 4 h, followed by 2% (w/v) OsO₄ in 1.0 M phosphate buffer, pH 7.3, for 1 h. The organelles were embedded in Epon 812, stained for 1 min in uranyl acetate and then lead citrate, and viewed at 80 kV using a JEOL 100B microscope.

Phospholipid extraction from nuclei. Several hours before mass spectral analysis, samples were removed from the freezer, and the nuclear pellet extracted by vortexing the CHCl₃/MeOH mixture twice for 2 min, with 2 min periods of cooling on ice between vortexing. Longer extraction times removed unidentified (non-glycerolipid) components which interfered with the mass spectral analyses. The vials were centrifuged for 5 min at about 8000 g, the CHCl₃/MeOH was removed under a stream of nitrogen, and the lipids resuspended in 5 mM methanolic ammonium acetate for mass spectral analysis.

Electrospray mass spectrometry. The major glycerophospholipid molecular species were identified using a combination of positive and negative ion electrospray mass spectrometry (MS) and MS/MS as described by Kerwin *et al.* (1994). Chloroform was evaporated from stored samples and dissolved in 5 mM methanolic ammonium acetate at approximately 0.5–1 ng μ l⁻¹. This concentration was such that the peaks corresponding to the glycerophospholipids from most samples could be collected in 5–10 scans with minimal folding over of the [M + H]⁺ (or M⁺ or [M + 60]⁺ for choline-containing species) peaks. Lipid samples were analysed on a triple-quadrupole Sciex API III instrument (PE/SCIEX). Samples were infused into the electrospray source via a 50 μ m i.d. fused silica transfer line using a Harvard Apparatus pump at 1–3 μ l min⁻¹. Positive ion

MS and MS/MS was run with an orifice voltage of 70 or 80 V. Negative ion electrospray MS and MS/MS used an orifice voltage from -70 to -90 V. The interface temperature was maintained at 52°C . For tandem mass spectrometry (MS/MS), precursor ions were selected with the first of three quadrupoles (Q1) for collision-induced dissociation with argon in the second quadrupole (Q2). The third quadrupole (Q3) was scanned with a mass step of 0.20 Da and 1 ms per step. Parent ion transmission was maximized by reducing the resolution of Q1 to transmit a $2\text{--}3\text{ }m/z$ window about the selected parent ion, and Q3 resolution was adjusted to approximately 50% valley between peaks 3 Da apart. Spectra were collected and analysed using proprietary software from Sciex Corporation.

Percentage composition of molecular species was generated using the peak intensities of ions from positive ion electrospray MS, scanning from either m/z 450 or 600 to m/z 1000 . In positive ion electrospray MS and MS/MS, ions generated by parent species occurred as $[M+H]^+$ adducts, except for choline-containing species. In the positive ion mode, diacyl-phosphatidylcholine species occurred as M^+ due to the quaternary ammonium present in the headgroup. Ether-containing glycerophosphocholine occurred primarily as $[M+60]^+$ adducts due to association with acetate ion. Confirmation of $[M+60]^+$ was readily obtained using positive ion MS/MS. In the negative ion mode, all choline-containing species formed acetate adducts. When multiple molecular species were represented by a single peak in positive ion electrospray, negative ion MS/MS spectra were used to determine the approximate contribution of individual species to each peak by summing the respective contributions of constituent fatty acids. For example, the peak at m/z 764 in negative ion electrospray MS contained both $18:1/20:4$ and $18:2/20:3$ phosphatidylethanolamine (PE). The contribution by each species to the peak intensity was obtained by adding the signal intensities at m/z 281 and 303 for $18:1/20:4$ PE and at m/z 279 and 305 for $18:2/20:3$ PE. For the few peaks that contained both diacyl and acyl-ether species, the peak intensity corresponding to the fatty acyl constituent in the *sn*-2 position of the ether lipids was doubled to obtain an approximate contribution since there was no signal generated by the ether-linked moiety (Kerwin *et al.*, 1994).

Electrospray MS and MS/MS is a powerful and versatile technique for structural studies of glycerophospholipids, and it is probable that many or all of the unique species reported below for, e.g. the cholestane-supplemented nuclei, are produced by cells cultured in the other media. Since we collected hundreds of electrospray MS/MS spectra for this study, we had to limit the amount of time spent scanning each peak. The great majority of MS/MS spectra were collected in $15\text{--}30$ s, and data for less than 10% of the peaks required $45\text{--}120$ s to obtain a reliable signal. We arbitrarily chose not to collect data for periods longer than 2 min. Molecular species reported as 0% or not reported either were not present or made up an insignificant percentage (usually less than 0.1 mol %) of a given class of glycerophospholipid.

Sterol extraction and gas chromatography (GC). Nuclei isolated from mycelial cultures grown with ergosterol, cholesterol or cholestane supplements were analysed for the presence of sterols. Nuclear pellets were suspended in 2 ml 0.5% pyrogallol, 2 ml 60% (w/v) KOH, and 3 ml methanol. The mixture was refluxed with boiling stones for 2 h at $70\text{--}80^{\circ}\text{C}$, and the saponified lipids extracted three times with 10 ml hexane. The pooled hexane extracts were dried and resuspended in a minimal volume of methanol for GC (Lorenz *et al.*, 1986). GC of underivatized sterols was done with a Hewlett Packard 5890 series II gas chromatograph using a 15 m DB-5 (5% phenyl, J&W Scientific) column, 0.32 mm i.d. The injector

temperature was 250°C , the FID temperature 310°C . An initial temperature of 200°C was held for 2 min, then raised to 240°C at $40^{\circ}\text{C min}^{-1}$, and held for 20 min. Helium gas carrier flow was maintained at 2 ml min^{-1} by operating the electronic pressure-controlled injector in the constant flow mode. Sterols were identified using relative retention times, with cholestane as the internal standard for ergosterol- and cholesterol-supplemented cultures, and cholesterol as the internal standard for cholestane-supplemented cultures. Sterol identification was confirmed using GC-MS.

The relative mol % composition of glycerophospholipid molecular species is reported as the mean ± 1 sd. Statistical differences were evaluated using Student's *t*-test. All statistical evaluations were done using the SYSTAT package.

RESULTS

Total cell glycerophospholipids

Isoprenoids alter the growth and morphogenesis of *L. giganteum* and related sterol auxotrophic Oomycetes. The compounds used in these investigations were chosen because of their effect on fungal development. Cholesterol and ergosterol can induce sexual and asexual reproduction, while cholestane and squalene, the acyclic precursor of sterols and related compounds, do not promote reproduction. The defined medium used in these investigations does not permit reproduction even with appropriate sterol supplementation, because of its high concentration of glucose. This medium was used so that observed changes in glycerophospholipid molecular species were not due to developmental changes.

One effect of isoprenoid supplementation was regulation of the amount of phospholipid produced by the fungus. In μg phosphate per g wet weight, mycelia cultured on the different media produced: defined medium (DM2), 164 ± 19 ; DM2 plus cholesterol, 292 ± 50 ; DM2 plus ergosterol, 120 ± 23 ; DM2 plus cholestane, 73 ± 5 ; and DM2 plus squalene, 56 ± 12 , all of which are significantly different from one another ($P < 0.05$). Although cholesterol-supplemented mycelium produced almost twice as much phospholipid as that grown on defined medium, the other sterol which induces sporulation, ergosterol, supported growth of mycelium with less phospholipid than that with no sterol supplement. Attempts to assess the effect of testosterone supplementation were unsuccessful because this steroid inhibited fungal growth.

Recently we demonstrated that electrospray MS and MS/MS can be used to identify glycerophospholipid molecular species with minimal sample preparation (Figs 1–3). With proper adjustment of operating parameters, over 90% of signal intensity in a typical positive ion spectrum was due to $[M+H]^+$ (or $[M+60]^+$ for choline-containing species) ions. For diacyl species, this technique does not identify which fatty acids are found in the *sn*-1 or *sn*-2 positions. We follow the accepted practice of reporting the less unsaturated fatty acid as occurring in the *sn*-1 position. Another limitation involves isobaric ether lipids. As an example, it is not possible without subclass separation to differentiate between a mono-

Fig. 1. Positive electrospray mass spectrum from m/z 735 to m/z 850 of glycerophospholipids from *L. giganteum* nuclei isolated from mycelium grown with a cholestane supplement. Representative peaks are marked, and six are labelled with the corresponding component molecular species that were identified using positive and negative ion electrospray MS/MS. PE, glycerophosphoethanolamine; PC, glycerophosphocholine; PI, glycerophosphoinositol; a, alkyl-acyl species; e, alkenyl-acyl species; all others are diacyl compounds. Peaks correspond to $[M+H]^+$ ions except for ether-containing glycerophosphocholine species, which are found either as $[M+60]^+$ or $[M+44]^+$ due to acetate adduct formation with or without loss of $-CH_3$ from the phosphocholine headgroup.

Fig. 2. (a) Positive ion electrospray MS/MS of m/z 718, produced by 16:0/18:1 phosphatidylethanolamine and a small amount of 14:0/18:0 alkenyl-acyl glycerophosphocholine. Loss of phosphoethanolamine (-141) from m/z 718 produces the peak at m/z 577. (b) Positive ion electrospray MS/MS of m/z 828, the acetate adduct of m/z 768, corresponding to M^+ of 16:0/20:4 alkyl-acyl glycerophosphocholine. The phosphocholine headgroup produces a well-defined peak at m/z 184.

unsaturated 18-carbon alkyl-linked moiety and a saturated 18-carbon alkenyl-linked species. Species reported in this paper are considered the most likely combinations to be synthesized based on fatty acid profiles of the *L. giganteum* phospholipid fraction. For instance, the fungus produces very small amounts of myristoleic acid (14:1 ω 5), so it is unlikely that the glycerophosphocholine species reported as 14:0/20:4 alkenyl-acyl, which makes up a significant percentage of this lipid class (see Table 2), is actually a 14:1/20:4 alkyl-acyl species. Interpretation of spectra for

this fungus is somewhat simplified since it does not synthesize 18:4, 20:5 or any 22-carbon fatty acids.

Scanning over a mass range of m/z 450 or 600 to m/z 1000, relative peak intensities for molecular species of GPC, GPI, GPE and monomethylphosphatidylethanolamine for *L. giganteum* mycelium and nuclei were as reproducible as those found for analyses of commercially available glycerophospholipids (Kerwin *et al.*, 1994; data not shown). This served as a check for the reliability of using electrospray MS and MS/MS to characterize *L. giganteum* glycerophospholipid composition. Three classes of glycerophospholipid were chosen for detailed analysis: GPC and GPE, which together comprise a minimum of 70% of total cell glycerophospholipid, and GPI, which has a pivotal role in regulation of cellular processes in many eukaryotic cells. The most surprising result was the almost unprecedented high levels of ether lipids found in GPC and GPI, with less, but still appreciable quantities, in GPE (Table 1). This could be an artefact of electrospray MS, but there was no evidence of preferential ionization of ether lipids in commercially available glycerophospholipids, all of which had been previously analysed using other methods (Kerwin *et al.*, 1994). This included a preparation of bovine brain GPE which contained approximately 60% ether lipids.

There were significant differences in relative molecular species composition in all three classes of glycerophospholipid from mycelium grown on the different media (data not shown). Many of these were minor components, but others such as the 14:0/20:4 ether GPC, 18:3/20:4 alkenyl-acyl GPC and 18:1/20:4 and 18:2/20:4 diacyl GPC made up a significant percentage of their lipid class. Of the three lipid classes, the GPE molecular species varied the least among mycelia cultured on the different growth media, although there was one

Fig. 3. Negative ion electrospray MS/MS of m/z 802, the acetate adduct of m/z 742. Peaks at m/z 255, 279 and 281 corresponding to RCOO⁻ of 16:0, 18:2 and 18:1, respectively, confirm that the peak at m/z 742 corresponds to three ether-linked glycerophosphocholine moieties: 18:3/16:0 alkyl-acyl, 16:1/18:2 alkyl-acyl and 16:1/18:1 alkenyl-acyl.

Table 1. Percentage ether glycerophospholipids in three lipid classes from *L. giganteum* grown on defined media

Values are (mol%) mean $\pm$ SD from three separate cultures; figures in a given row with matching superscript letters are significantly different from one another ($P < 0.05$). Values for nuclear preparations followed by an asterisk are significantly different in percentage composition from those found in total cell phospholipids. Data were generated by dividing peak intensities corresponding to alkyl-acyl plus alkenyl-acyl moieties by total peak intensity for a given lipid class. DM2, defined medium; DM2CHO, defined medium + cholesterol; DM2ERG, defined medium + ergosterol; DM2CHN, defined medium + cholestane; DM2SQN, defined medium + squalene.

Lipid class	Percentage composition after growth on:				
	DM2	DM2CHO	DM2ERG	DM2CHN	DM2SQN
Total cell glycerophospholipid					
Glycerophosphocholine	74.0 $\pm$ 2.65 ^a	74.3 $\pm$ 2.1 ^b	80.0 $\pm$ 2.0 ^{c,d}	69 $\pm$ 1.7 ^{a,b,c}	73.0 $\pm$ 1.7 ^d
Glycerophosphoethanolamine	12.6 $\pm$ 2.6 ^{a,b}	19.8 $\pm$ 3.6 ^{a,c}	16.8 $\pm$ 1.9 ^{b,d}	13.0 $\pm$ 1.2 ^{c,d,e}	15.4 $\pm$ 1.1 ^e
Glycerophosphoinositol	74.3 $\pm$ 4.7	74.3 $\pm$ 3.2	75.0 $\pm$ 2.0	78.7 $\pm$ 1.5	77.7 $\pm$ 0.6
Nuclear membrane glycerophospholipid					
Glycerophosphocholine	71.3 $\pm$ 3.1 ^a	58.3 $\pm$ 2.1 ^{*a,b,c,d}	76 $\pm$ 5.2 ^b	78.0 $\pm$ 1.0 ^{*c}	81.3 $\pm$ 2.1 ^{*a,d}
Glycerophosphoethanolamine	10.3 $\pm$ 2.1 ^a	12.7 $\pm$ 3.8	15.7 $\pm$ 4.0	7.3 $\pm$ 2.1 ^{*a}	12.3 $\pm$ 2.1
Glycerophosphoinositol	81.7 $\pm$ 0.6 ^{a,b}	84.7 $\pm$ 5.1	79.3 $\pm$ 2.5 ^c	86.7 $\pm$ 1.5 ^{*a,c,d}	77.3 $\pm$ 0.6 ^{b,d}

significant difference as discussed below. Some of the molecular species were unusual in having polyunsaturated ether-linked fatty acids, especially 18:3 ω 6, which was found in alkyl-acyl and alkenyl-acyl species of GPC (Table 2), GPE (Table 3) and GPI (Table 4) in both whole cell and nuclear fractions.

We were most interested in differences in molecular species produced by *L. giganteum* grown on media supplemented with cholesterol and ergosterol, which induce sporulation, relative to the other three media. The one consistent difference observed when comparing mycelium grown on ergosterol and cholesterol with that grown on other media was an elevated percentage of total ether lipids in the GPE fraction compared to mycelium from cultures grown on defined medium alone or on defined medium plus cholestane (Table 1).

Nuclear glycerophospholipids

Initial attempts to isolate *L. giganteum* nuclei followed the standard protocol of incubation with lytic enzymes in order to obtain protoplasts, which could subsequently be gently disrupted as the first step in obtaining these organelles. Unexpectedly, the cell walls of this fungus proved to be resistant to degradation by a wide variety of enzymic treatments with or without various preincubations or subsequent osmotic shock. Enzymes which proved to be ineffective either singly or in various combinations included three proteases, glusulase, laminarinase, three cellulases, Novozym 234, β -glucuronidase, pectinase, driselase, chitinase, lyticase, macerozyme, zymolase and lyticase. After trying a number of alternatives, it was found that a combination of chilling in liquid nitrogen and mechanical abrasion would break

Table 2. Glycerophosphocholine molecular species from nuclei of *L. giganteum* grown on defined media

Values are (mol %) mean $\pm$ SD from three separate cultures; figures in a given row with matching superscript letters are significantly different from one another ($P < 0.05$). Values followed by an asterisk are significantly different in percentage composition from those found in total cell phospholipids. Alk, alkyl-acyl molecular species; enyl, alkenyl-acyl species; all others are diacyl species. DM2, defined medium; DM2CHO, defined medium + cholesterol; DM2ERG, defined medium + ergosterol; DM2CHN, DM2 + cholestane; DM2SQN, DM2 + squalene.

Molecular species	Percentage composition after growth on:				
	DM2	DM2CHO	DM2ERG	DM2CHN†	DM2SQN
14:0/18:1 alk	0.38 $\pm$ 0.02 ^a	0.62 $\pm$ 0.31*	0.95 $\pm$ 0.09 ^a	0* ^{a,b}	0.86 $\pm$ 0.25 ^b
14:0/18:0 alk	2.80 $\pm$ 0.12 ^a	3.95 $\pm$ 0.65*	1.98 $\pm$ 0.40* ^b	5.00 $\pm$ 0.20* ^{a,b}	2.95 $\pm$ 1.07
16:0/16:0	0.22 $\pm$ 0.07	0.26 $\pm$ 0.11	0.21 $\pm$ 0.08	0.32 $\pm$ 0.08 ^a	0.25 $\pm$ 0.08 ^a
14:0/20:4 enyl	14.62 $\pm$ 2.02*	16.98 $\pm$ 5.49	17.88 $\pm$ 2.50 ^a	14.52 $\pm$ 2.89	23.23 $\pm$ 1.91 ^a
14:0/20:4 alk	4.48 $\pm$ 0.90	9.87 $\pm$ 1.68 ^{a,b}	6.76 $\pm$ 2.48	5.94 $\pm$ 1.55 ^a	4.80 $\pm$ 1.37 ^b
16:0/18:1 enyl	0.25 $\pm$ 0.04*	0.22 $\pm$ 0.12 ^c	0.25 $\pm$ 0.16 ^b	0.72 $\pm$ 0.18 ^{a,b}	0.57 $\pm$ 0.24 ^c
16:0/18:2 alk	0.34 $\pm$ 0.06 ^a	0.29 $\pm$ 0.15 ^b	0.33 $\pm$ 0.21	0*	0.75 $\pm$ 0.31 ^b
18:1/16:0 enyl	0.08 $\pm$ 0.01 ^a	0.07 $\pm$ 0.04 ^b	0.08 $\pm$ 0.05	0* ^{a,c}	0.26 $\pm$ 0.18 ^{b,c}
16:0/18:1 alk	0.21 $\pm$ 0.09 ^a	0.28 $\pm$ 0.30	0.16 $\pm$ 0.09 ^a	0.67 $\pm$ 0.24 ^a	0.31 $\pm$ 0.05
18:0/16:1 alk	0.18 $\pm$ 0.08 ^a	0.25 $\pm$ 0.26	0.13 $\pm$ 0.08 ^a	0* ^b	0.27 $\pm$ 0.05 ^b
18:1/16:0 alk	0.09 $\pm$ 0.04 ^a	0.12 $\pm$ 0.13	0.11 $\pm$ 0.06	0* ^{a,b}	0.13 $\pm$ 0.02 ^b
18:0/16:0 alk	0.08 $\pm$ 0.04 ^a	0.06 $\pm$ 0.02 ^b	0.08 $\pm$ 0.05 ^c	9.87 $\pm$ 3.29* ^{a,b,c,d}	0.15 $\pm$ 0.05 ^d
16:1/20:3 enyl	0.17 $\pm$ 0.02*	0.18 $\pm$ 0.06 ^b	0.13 $\pm$ 0.08	0.22 $\pm$ 0.03*	0.27 $\pm$ 0.06* ^{a,b}
16:1/20:4 alk	0.76 $\pm$ 0.12 ^a	0.80 $\pm$ 0.25 ^b	0.60 $\pm$ 0.34	0.99 $\pm$ 0.13*	1.25 $\pm$ 0.26* ^{a,b}
18:3/18:2 alk	0.23 $\pm$ 0.04 ^a	0.24 $\pm$ 0.08	0.19 $\pm$ 0.11	0.30 $\pm$ 0.04*	0.38 $\pm$ 0.08* ^a
18:3/18:1 enyl	0.22 $\pm$ 0.03*	0.23 $\pm$ 0.07 ^b	0.18 $\pm$ 0.10	0.29 $\pm$ 0.03*	0.36 $\pm$ 0.07* ^{a,b}
18:0/18:3 enyl	0.89 $\pm$ 0.16*	0.49 $\pm$ 0.21	0.43 $\pm$ 0.21	0.25 $\pm$ 0.02*	1.65 $\pm$ 0.66
18:1/18:2 enyl	2.07 $\pm$ 0.37*	1.15 $\pm$ 0.48	1.00 $\pm$ 0.50	0* ^{a,b}	3.85 $\pm$ 1.55 ^b
18:0/18:2 enyl	9.34 $\pm$ 0.53* ^{a,c}	0.38 $\pm$ 0.06* ^{a,b}	8.46 $\pm$ 1.54 ^b	0* ^{b,c}	7.04 $\pm$ 0.74* ^{a,c}
18:1/18:1 enyl	6.60 $\pm$ 0.37*	0.27 $\pm$ 0.04* ^{a,b}	6.06 $\pm$ 1.14 ^b	3.74 $\pm$ 1.30 ^b	4.97 $\pm$ 0.53 ^a
20:3/16:0 alk	4.12 $\pm$ 0.23*	0.17 $\pm$ 0.03* ^{a,b,c}	3.73 $\pm$ 0.68 ^{b,c}	1.02 $\pm$ 0.36* ^{a,c}	3.11 $\pm$ 0.33 ^a
18:0/18:2 alk	2.63 $\pm$ 0.26* ^{a,b}	0.27 $\pm$ 0.16* ^{a,d}	3.24 $\pm$ 1.23 ^c	0* ^{b,c,e}	2.36 $\pm$ 0.77 ^{d,e}
18:1/18:1 alk	2.07 $\pm$ 0.21* ^{a,b}	0.21 $\pm$ 0.13* ^{a,d}	2.54 $\pm$ 0.97 ^c	0* ^{b,c,e}	1.85 $\pm$ 0.60 ^{d,e}
18:3/20:4 enyl	7.41 $\pm$ 1.07* ^{a,c}	3.59 $\pm$ 0.23* ^{a,b}	9.49 $\pm$ 1.66 ^{b,d}	2.24 $\pm$ 0.55* ^{a,d,e}	6.10 $\pm$ 1.37* ^e
18:3/20:4 alk	2.10 $\pm$ 0.81	1.78 $\pm$ 0.58 ^a	2.97 $\pm$ 1.28	0.33 $\pm$ 0.17* ^{a,b}	2.06 $\pm$ 0.66 ^b
18:2/20:4 alk	1.22 $\pm$ 0.25 ^a	3.03 $\pm$ 0.81* ^b	1.71 $\pm$ 0.63 ^c	8.21 $\pm$ 0.39* ^{a,b,c,d}	2.08 $\pm$ 0.13* ^d
20:3/18:2 alk	0.36 $\pm$ 0.22 ^a	0.56 $\pm$ 0.13* ^b	0.22 $\pm$ 0.06 ^{b,c}	1.19 $\pm$ 0.06* ^{a,b}	1.12 $\pm$ 0.31* ^c
18:1/20:4 alk	0.58 $\pm$ 0.11* ^{a,c}	1.46 $\pm$ 0.35* ^{a,b}	0.57 $\pm$ 0.15 ^b	4.72 $\pm$ 0.68* ^{a,b,c}	0.80 $\pm$ 0.10* ^c
18:1/20:3 enyl	0.25 $\pm$ 0.05* ^{a,c}	0.64 $\pm$ 0.15* ^{a,b}	0.25 $\pm$ 0.07 ^b	0.36 $\pm$ 0.02	0.35 $\pm$ 0.04* ^c
18:0/20:4 alk	1.09 $\pm$ 0.29* ^{a,b}	2.34 $\pm$ 0.73 ^a	1.09 $\pm$ 0.50	1.96 $\pm$ 0.28	0.96 $\pm$ 0.73 ^b
18:0/20:3 enyl	0.12 $\pm$ 0.03	0.26 $\pm$ 0.08	0.12 $\pm$ 0.05	0.12 $\pm$ 0.02	0.16 $\pm$ 0.03*
18:3/16:0 alk	0.17 $\pm$ 0.03 ^a	0.12 $\pm$ 0.04 ^b	0.12 $\pm$ 0.08 ^c	1.26 $\pm$ 0.10* ^{a,b,c,d}	0.16 $\pm$ 0.03* ^d
16:1/18:2 alk	0.35 $\pm$ 0.07 ^a	0.26 $\pm$ 0.08* ^{a,b}	0.25 $\pm$ 0.17	0* ^{a,b}	0.35 $\pm$ 0.08* ^b
16:1/18:1 alk	0.52 $\pm$ 0.10 ^a	0.39 $\pm$ 0.12* ^{a,b}	0.36 $\pm$ 0.25	0* ^{a,b}	0.51 $\pm$ 0.11* ^b
18:3/20:4	5.29 $\pm$ 0.53* ^{a,b}	6.32 $\pm$ 4.65	2.84 $\pm$ 0.78 ^a	3.27 $\pm$ 0.73 ^b	4.17 $\pm$ 0.46
18:2/20:4	9.62 $\pm$ 2.50*	11.78 $\pm$ 1.30* ^{a,b,c}	6.28 $\pm$ 0.74 ^a	7.79 $\pm$ 1.25* ^b	6.08 $\pm$ 0.78* ^c
18:1/20:4	6.71 $\pm$ 1.42 ^a	11.17 $\pm$ 2.25 ^b	6.48 $\pm$ 2.45	0.99 $\pm$ 0.25* ^{a,b}	3.67 $\pm$ 0.83* ^{a,b}
18:2/20:3	2.36 $\pm$ 0.49 ^a	3.92 $\pm$ 0.79 ^b	2.28 $\pm$ 0.86 ^c	0* ^{a,b,c}	1.29 $\pm$ 0.30* ^{a,b}
18:0/20:4	4.29 $\pm$ 1.50 ^a	8.10 $\pm$ 0.92 ^b	6.10 $\pm$ 0.93* ^c	9.55 $\pm$ 1.11* ^{a,c,d}	3.31 $\pm$ 0.37* ^d
20:3/20:4 alk	0.97 $\pm$ 0.22 ^a	1.63 $\pm$ 0.81	0.81 $\pm$ 0.39	0* ^{a,b}	1.13 $\pm$ 0.19* ^b
20:0/20:4 enyl	1.85 $\pm$ 0.52 ^a	2.53 $\pm$ 0.06* ^b	1.39 $\pm$ 0.66	0.78 $\pm$ 0.11* ^{a,b}	1.75 $\pm$ 0.43*
20:0/20:4 alk	1.68 $\pm$ 0.45	2.42 $\pm$ 0.81	1.08 $\pm$ 0.64	0.57 $\pm$ 0.07* ^a	1.57 $\pm$ 0.09* ^a
20:0/20:3 enyl	0.22 $\pm$ 0.06	0.32 $\pm$ 0.11	0.14 $\pm$ 0.09	0.17 $\pm$ 0.02	0.21 $\pm$ 0.01*

† Other molecular species identified in nuclei from cholestane-supplemented media were: 16:1/18:0 alkenyl acyl, 0.60 $\pm$ 0.15%; 18:2/18:3 alkenyl-acyl, 1.60 $\pm$ 0.19%; 18:3/18:2 alkenyl-acyl, 2.28 $\pm$ 0.27%; 16:1/20:4 alkenyl-acyl, 0.57 $\pm$ 0.07%; 18:3/18:1 alkyl-acyl, 0.41 $\pm$ 0.03%; 18:3/16:0 alkyl-acyl, 6.57 $\pm$ 2.29%; 18:2/20:4 alkenyl-acyl, 0.58 $\pm$ 0.29%.

Table 3. Glycerophosphoethanolamine molecular species from nuclei of *L. giganteum* grown on defined media

Values are (mol %) mean $\pm$ SD from three separate cultures; figures in a given row with matching superscript letters are significantly different from one another ($P < 0.05$). Values followed by an asterisk are significantly different in percentage composition from those found in total cell phospholipids. Alk, alkyl-acyl molecular species; enyl, alkenyl-acyl species; all others are diacyl species. DM2, defined medium; DM2CHO, defined medium + cholesterol; DM2ERG, defined medium + ergosterol; DM2CHN, DM2 + cholestane; DM2SQN, DM2 + squalene.

Molecular species	Percentage composition after growth on:				
	DM2	DM2CHO	DM2ERG	DM2CHN†	DM2SQN
14:0/18:2	1.49 $\pm$ 0.61 ^{a,b,c}	2.02 $\pm$ 0.79 ^a	1.68 $\pm$ 0.62	3.17 $\pm$ 0.20 ^{*b}	1.32 $\pm$ 0.37 ^c
14:0/18:1	1.24 $\pm$ 0.11 [*]	3.61 $\pm$ 1.07	1.75 $\pm$ 0.77	5.62 $\pm$ 1.84	2.39 $\pm$ 0.48
16:1/16:1	0.80 $\pm$ 0.33 ^a	1.09 $\pm$ 0.43	0.91 $\pm$ 0.33	0.79 $\pm$ 0.05 ^a	0.71 $\pm$ 0.20
16:0/16:1	2.42 $\pm$ 0.21 ^{a,b}	7.01 $\pm$ 2.08	3.40 $\pm$ 1.49	10.71 $\pm$ 3.79 ^a	4.63 $\pm$ 0.91 ^a
16:0/16:0	5.53 $\pm$ 2.13	6.46 $\pm$ 3.21 ^a	5.42 $\pm$ 1.04	7.22 $\pm$ 2.09	9.91 $\pm$ 3.71 ^a
18:3/16:0 enyl	4.20 $\pm$ 0.92	5.26 $\pm$ 2.00 ^{*a}	9.12 $\pm$ 3.09 ^a	2.38 $\pm$ 0.61 [*]	5.13 $\pm$ 1.43
16:1/18:2 enyl	2.36 $\pm$ 0.52 ^a	2.96 $\pm$ 1.12 ^{*b}	5.13 $\pm$ 1.74 ^b	1.14 $\pm$ 0.30 ^{*a}	2.89 $\pm$ 0.80
14:0/20:4	0.67 $\pm$ 0.10	1.27 $\pm$ 0.69 ^a	0.84 $\pm$ 0.22	2.03 $\pm$ 0.58 ^a	0.63 $\pm$ 0.24
16:1/18:3	0.36 $\pm$ 0.06 ^a	0.68 $\pm$ 0.37 ^b	0.45 $\pm$ 0.12	0 ^{*a,b}	0.34 $\pm$ 0.13
16:0/18:2	1.38 $\pm$ 0.28	1.30 $\pm$ 0.33	1.25 $\pm$ 0.06	2.39 $\pm$ 0.50	1.56 $\pm$ 0.23
16:0/18:1	3.72 $\pm$ 0.14 ^a	7.56 $\pm$ 1.19 ^{*a}	13.51 $\pm$ 4.71	5.11 $\pm$ 2.05	5.29 $\pm$ 0.44
16:0/18:0	0.75 $\pm$ 0.04 ^{a,b}	1.95 $\pm$ 0.17 ^{*a,c}	2.35 $\pm$ 1.09	10.46 $\pm$ 3.33 ^{*b,c,d}	1.49 $\pm$ 0.67 ^d
16:0/20:4	5.13 $\pm$ 0.79 ^{*a}	7.73 $\pm$ 2.87 ^b	6.72 $\pm$ 3.17 ^c	33.82 $\pm$ 1.86 ^{*a,b,c,d}	5.80 $\pm$ 0.65 ^{*d}
16:0/20:3	1.96 $\pm$ 0.54 ^a	1.75 $\pm$ 0.32 ^b	1.89 $\pm$ 0.42 ^c	0.23 $\pm$ 0.05 ^{*a,b,c}	2.47 $\pm$ 0.44 ^b
18:0/18:2	1.65 $\pm$ 0.35	1.87 $\pm$ 0.30 ^a	2.11 $\pm$ 0.52 ^b	0 ^{*a,b,c}	2.20 $\pm$ 0.34 ^c
18:3/20:4 enyl	0.45 $\pm$ 0.21	0.49 $\pm$ 0.14 ^a	0.43 $\pm$ 0.10 ^b	0 ^{*a,b,c}	0.42 $\pm$ 0.11 ^c
18:2/20:4 enyl	0.38 $\pm$ 0.17	0.30 $\pm$ 0.27	0.43 $\pm$ 0.17	1.51 $\pm$ 0.99	0.44 $\pm$ 0.13
18:2/20:4 alk	2.87 $\pm$ 0.24 ^a	3.37 $\pm$ 0.50 ^b	3.81 $\pm$ 1.57	1.42 $\pm$ 0.43 ^{*a,b,c}	3.44 $\pm$ 0.15 ^c
18:3/20:4	2.99 $\pm$ 0.30	4.78 $\pm$ 1.02	6.91 $\pm$ 3.30	2.17 $\pm$ 1.51	2.20 $\pm$ 0.50
18:2/20:4	10.48 $\pm$ 0.80 ^{*a}	7.11 $\pm$ 0.89 ^a	7.76 $\pm$ 1.38 ^b	0.92 $\pm$ 0.44 ^{*a,b,c}	7.59 $\pm$ 1.00 ^c
18:1/20:4	10.14 $\pm$ 0.56 ^a	12.29 $\pm$ 2.53 ^b	9.34 $\pm$ 0.82 ^c	1.21 $\pm$ 0.77 ^{*a,b,c}	10.51 $\pm$ 0.92 ^c
18:2/20:3	3.38 $\pm$ 0.19 ^a	4.09 $\pm$ 0.84 ^b	3.11 $\pm$ 0.27 ^c	0 ^{*a,b,c}	3.50 $\pm$ 0.31 ^c
18:0/20:4	4.30 $\pm$ 0.50 ^a	3.97 $\pm$ 1.33 ^b	2.68 $\pm$ 0.56 ^a	0 ^{*a,b}	4.79 $\pm$ 0.82
18:1/20:3	4.92 $\pm$ 0.56 ^a	4.53 $\pm$ 1.52	3.07 $\pm$ 0.64 ^a	1.15 $\pm$ 0.88 ^b	5.48 $\pm$ 0.94 ^b
20:3/20:3	26.40 $\pm$ 1.15 ^{*a,b}	6.55 $\pm$ 2.82 ^a	5.14 $\pm$ 1.83 ^b	0 ^{*b}	14.86 $\pm$ 6.17

† Other molecular species identified in nuclei from cholestane-supplemented media were: 16:1/20:3 diacyl, 5.60 $\pm$ 0.26 %; 18:2/20:3 alkenyl-acyl, 1.10 $\pm$ 0.33 %.

the mycelial cell walls without significant disruption of nuclei. As monitored by fluorescence microscopy and marker enzyme assays, the final preparations of purified nuclei were not contaminated by other organelles or membrane fragments including mitochondria, microsomes and plasma membranes. The marker enzyme assays used a minimum of 1000-fold more nuclei than were used for each electrospray MS analysis, and even using these much greater quantities of nuclei, there was no enzyme activity associated with other organelles in the nuclei preparations. Purity of a representative nuclear preparation was also confirmed by transmission electron microscopy.

Using GC-MS, it was confirmed that cholesterol, ergosterol and cholestane are incorporated by nuclei; presumably a proportion of these is located in the nuclear membrane. Although we did not quantify sterol uptake, when a roughly equal volume of nuclei was extracted from the three sterol-supplemented cultures, it

was evident from the GC peak intensity that a significantly greater amount of cholestane was present in nuclei relative to nuclei from cells grown with cholesterol or ergosterol supplements. We did not rigorously monitor metabolism of the exogenous sterols, but there was no evidence of appreciable sterol metabolism from the GC chromatograms or GC-MS spectra.

The most unexpected finding in examining whole cell glycerophospholipid composition was the very high levels of ether-linked glycerophospholipids, especially in choline- and inositol-containing moieties. Comparable levels of alkyl-acyl and alkenyl-acyl moieties were found in nuclear membranes (Tables 1–4). There were statistically significant differences in many glycerophospholipid molecular species between whole cells and nuclei isolated from cells grown in the same medium.

The glycerophospholipid composition of nuclei isolated from cholestane-supplemented media for the three classes

Table 4. Glycerophosphoinositol molecular species from nuclei of *L. giganteum* grown on defined media

Values are (mol %) mean $\pm$ SD from three separate cultures; figures in a given row with matching superscript letters are significantly different from one another ($P < 0.05$). Values followed by an asterisk are significantly different in percentage composition from those found in total cell phospholipids (J. L. Kerwin and others, unpublished). Alk, alkyl-acyl molecular species; enyl, alkenyl-acyl species; all others are diacyl species. DM2, defined medium; DM2CHO, defined medium + cholesterol; DM2ERG, defined medium + ergosterol; DM2CHN, DM2 + cholestane; DM2SQN, DM2 + squalene.

Molecular species	Percentage composition after growth on:				
	DM2	DM2CHO	DM2ERG	DM2CHN†	DM2SQN
14:0/18:1 alk	4.19 $\pm$ 0.37 ^a	2.61 $\pm$ 0.15 ^a	3.71 $\pm$ 1.04	1.61 $\pm$ 0.19 ^{a,b}	5.00 $\pm$ 1.03 ^b
18:0/14:0 alk	0.66 $\pm$ 0.45	0.28 $\pm$ 0.06 ^a	0.58 $\pm$ 0.16 ^b	0 ^{a,b,c}	0.78 $\pm$ 0.30 ^c
16:0/16:0	1.58 $\pm$ 0.09 ^a	1.80 $\pm$ 1.07	2.88 $\pm$ 1.84	0 ^{a,b}	2.63 $\pm$ 0.55 ^b
18:1/16:1 alk	0.22 $\pm$ 0.01 ^a	1.07 $\pm$ 0.27 ^{a,b,c}	0.25 $\pm$ 0.15 ^b	2.37 $\pm$ 0.26 ^{a,b,c}	0.23 $\pm$ 0.03 ^c
18:2/16:0 alk	0.11 $\pm$ 0.01 ^{a,c}	0.51 $\pm$ 0.13 ^{a,b,d}	0.13 $\pm$ 0.07 ^b	0.18 $\pm$ 0.02 ^c	0.12 $\pm$ 0.02 ^d
16:0/18:2 alk	0.44 $\pm$ 0.03 ^{a,c}	2.14 $\pm$ 0.54 ^{a,b,d}	0.51 $\pm$ 0.30 ^b	0.62 $\pm$ 0.14 ^c	0.46 $\pm$ 0.06 ^d
16:1/18:1 alk	0.39 $\pm$ 0.02 ^a	1.92 $\pm$ 0.48 ^{a,b,c}	0.44 $\pm$ 0.26 ^b	0 ^{a,c}	0.41 $\pm$ 0.06 ^c
18:0/16:1 alk	0.78 $\pm$ 0.07 ^a	2.22 $\pm$ 1.07	0.64 $\pm$ 0.23 ^b	0 ^{a,b,c}	0.83 $\pm$ 0.13 ^c
18:1/16:0 alk	0.53 $\pm$ 0.05 ^a	1.25 $\pm$ 0.60	0.44 $\pm$ 0.16 ^b	2.39 $\pm$ 0.18 ^{a,b,c}	0.57 $\pm$ 0.09 ^c
18:0/16:0 alk	0.47 $\pm$ 0.07 ^a	1.88 $\pm$ 0.82	0.44 $\pm$ 0.18	0.13 $\pm$ 0.01 ^a	0.87 $\pm$ 0.30
16:0/18:2	0.54 $\pm$ 0.06 ^a	2.57 $\pm$ 0.35 ^{a,b}	0.83 $\pm$ 0.04 ^a	0 ^{a,b}	1.04 $\pm$ 0.14 ^b
16:0/18:1	0.26 $\pm$ 0.05 ^a	1.50 $\pm$ 0.79	0.33 $\pm$ 0.06 ^b	0 ^{a,b,c}	0.41 $\pm$ 0.11 ^c
16:1/20:4 alk	6.80 $\pm$ 0.31 ^a	4.22 $\pm$ 1.88	5.06 $\pm$ 0.63 ^b	3.37 $\pm$ 0.77 ^{a,b}	5.37 $\pm$ 0.57
16:0/20:4 alk	6.86 $\pm$ 0.18 ^{a,c}	8.60 $\pm$ 2.48	7.24 $\pm$ 0.24 ^{b,d}	4.36 $\pm$ 0.23 ^{a,b}	5.14 $\pm$ 0.26 ^{c,d}
18:0/18:3 alk	1.18 $\pm$ 0.19 ^a	1.29 $\pm$ 0.71	1.18 $\pm$ 0.06 ^b	0 ^{a,b,c}	1.12 $\pm$ 0.23 ^c
18:0/18:2 enyl	2.63 $\pm$ 0.43	2.87 $\pm$ 1.59	2.69 $\pm$ 0.21 ^a	1.26 $\pm$ 0.43 ^a	2.50 $\pm$ 0.51
18:0/18:2 alk	1.76 $\pm$ 0.08 ^a	3.85 $\pm$ 1.20 ^c	1.59 $\pm$ 0.34 ^c	0 ^{a,b,c}	1.16 $\pm$ 0.10 ^a
18:0/18:1 enyl	1.93 $\pm$ 0.09 ^a	4.17 $\pm$ 1.30 ^b	1.74 $\pm$ 0.38 ^b	0 ^{a,b}	1.27 $\pm$ 0.10 ^a
18:0/18:1 alk	1.68 $\pm$ 0.18 ^a	5.16 $\pm$ 2.57	2.29 $\pm$ 1.15	0.29 $\pm$ 0.36 ^a	1.89 $\pm$ 0.42 [*]
16:0/20:4	4.32 $\pm$ 0.28 ^a	2.97 $\pm$ 1.76	3.91 $\pm$ 0.25 ^a	2.79 $\pm$ 0.15 ^a	5.39 $\pm$ 1.17 [*]
18:3/20:4 enyl	3.07 $\pm$ 0.65	1.77 $\pm$ 0.51 ^a	2.94 $\pm$ 0.48 [*]	2.81 $\pm$ 0.30 ^{a,b}	4.45 $\pm$ 0.55 ^{a,b}
18:3/20:4 alk	5.86 $\pm$ 0.63	3.16 $\pm$ 1.60 ^a	4.45 $\pm$ 0.78 ^b	7.32 $\pm$ 1.95 ^a	8.13 $\pm$ 1.85 ^b
18:2/20:4 alk	7.94 $\pm$ 1.24 ^a	5.97 $\pm$ 1.49 ^a	6.22 $\pm$ 1.92	5.27 $\pm$ 1.24	8.11 $\pm$ 0.79 [*]
18:1/20:4 alk	7.91 $\pm$ 0.65 ^a	6.67 $\pm$ 2.06	7.35 $\pm$ 1.04	4.91 $\pm$ 1.03 ^a	6.20 $\pm$ 0.88
18:0/20:4 alk	5.64 $\pm$ 0.28	5.17 $\pm$ 1.09 ^a	6.65 $\pm$ 1.42 ^a	4.96 $\pm$ 0.82	4.92 $\pm$ 0.34
18:2/20:4	2.61 $\pm$ 0.28 ^a	1.89 $\pm$ 0.82 [*]	2.51 $\pm$ 0.93	1.81 $\pm$ 0.30 ^{a,b}	3.81 $\pm$ 0.26 ^b
18:1/20:4	3.11 $\pm$ 0.34 ^a	1.66 $\pm$ 0.60 ^{a,b}	4.05 $\pm$ 2.28	2.06 $\pm$ 0.63 ^c	3.44 $\pm$ 0.13 ^{b,c}
18:0/20:4	3.96 $\pm$ 0.01 ^a	1.70 $\pm$ 0.27 ^{a,b}	3.69 $\pm$ 0.77	3.04 $\pm$ 0.39 ^b	3.26 $\pm$ 0.21 ^a
20:3/20:4 alk	7.27 $\pm$ 0.37 ^a	4.52 $\pm$ 1.57	5.37 $\pm$ 1.54 ^b	2.97 $\pm$ 0.32 ^a	7.73 $\pm$ 1.41 ^{a,b}
20:0/20:4 enyl	7.85 $\pm$ 0.50 ^{a,b}	7.13 $\pm$ 0.85	9.09 $\pm$ 1.96 ^c	5.23 $\pm$ 0.61 ^a	4.27 $\pm$ 0.90 ^{b,c}
20:0/20:4 alk	5.67 $\pm$ 0.96	6.05 $\pm$ 1.66	8.47 $\pm$ 2.10	4.97 $\pm$ 0.33 ^a	5.92 $\pm$ 0.52 ^a
20:0/20:4	1.77 $\pm$ 0.25	1.45 $\pm$ 0.46 ^a	2.32 $\pm$ 0.75	2.43 $\pm$ 0.57 [*]	2.56 $\pm$ 0.79 ^a

† Other molecular species identified in nuclei from cholestane-supplemented media were: 14:0/16:0 alkyl-acyl, 1.04 $\pm$ 0.14 %; 16:1/16:1 alkenyl-acyl, 1.57 $\pm$ 0.25 %; 14:0/18:2 alkenyl-acyl, 0.22 $\pm$ 0.04 %; 14:1/18:2 alkyl-acyl, 0.22 $\pm$ 0.04 %; 18:0/14:0 alkenyl-acyl, 0.73 $\pm$ 0.08 %; 16:0/16:0 alkenyl-acyl, 2.93 $\pm$ 0.34 %; 14:0/18:0 alkenyl-acyl, 1.10 $\pm$ 0.13 %; 16:0/16:0 alkyl-acyl, 1.31 $\pm$ 0.63 %; 14:0/18:0 alkyl-acyl, 1.39 $\pm$ 0.66 %; 16:0/16:1 diacyl, 2.12 $\pm$ 0.15 %; 16:1/18:0 alkenyl-acyl, 1.76 $\pm$ 0.19 %; 20:0/14:1 alkyl-acyl, 0.99 $\pm$ 0.07 %; 20:0/14:0 alkenyl-acyl, 0.77 $\pm$ 0.06 %; 16:0/18:1 alkyl-acyl, 0.56 $\pm$ 0.04 %; 16:1/18:0 alkyl-acyl, 1.20 $\pm$ 0.09 %; 16:0/18:0 alkyl-acyl, 0.24 $\pm$ 0.02 %; 16:1/20:4 alkenyl-acyl, 2.37 $\pm$ 0.32 %; 18:1/18:1 alkenyl-acyl, 2.39 $\pm$ 0.82 %; 18:3/18:0 alkyl-acyl, 1.40 $\pm$ 0.48 %; 18:2/18:0 alkyl-acyl, 3.66 $\pm$ 0.27 %; 18:1/18:0 alkyl-acyl, 0.29 $\pm$ 0.36 %; 20:0/16:0 alkenyl-acyl, 1.41 $\pm$ 0.25 %.

of lipid we examined was very different from that of nuclei isolated from mycelium grown in the other media (Tables 2–4). Nuclei from cholestane-supplemented cultures had seven GPC species not found, or present in very small amounts, in nuclei from cells grown in the other media (Table 2). Other significant differences found in cholestane-containing nuclei in this lipid class included elevated levels of 18:0/16:0 alkyl-acyl, 18:2/20:4 alkyl-

acyl and 18:1/20:4 alkyl-acyl species; and a significant decrease in 18:1/20:4 diacyl glycerophosphocholine. The most significant difference from nuclei isolated from the cholestane-supplemented cultures was the production of 22 GPI molecular species not present, or present in trace quantities, in nuclei from mycelia grown in the other media (Table 3). For the GPE species, cholestane supplementation resulted in significant increases in

16:0/18:0 and 16:0/20:4 diacyl moieties, and large decreases in 18:1/20:4 and 18:2/20:4 diacyl molecular species (Table 4).

As was found for total cell fractions, ether-linked lipids made up a very high percentage of GPC and GPI molecular species in nuclear membranes (Table 1). Cholesterol supplementation of media produced the greatest change in ether-linked species between total cell and nuclear glycerophosphocholine species from *L. giganteum* grown on the same medium, with an average of 74% ether-linked species in the former and 58% in the latter.

DISCUSSION

One effect of isoprenoid supplementation of *L. giganteum* culture media is to alter the relative composition of mycelial and nuclear glycerophospholipid molecular species. This is not unexpected since sterols including cholesterol are readily incorporated into eukaryotic membranes, where they profoundly influence membrane integrity, fluidity and related physical properties (Bloch, 1983; Pasenkiewicz-Gierula *et al.*, 1990, 1991; Bittman *et al.*, 1992; Spector & Yorek, 1985; Yeagle, 1989, 1990).

The most unexpected result of this investigation was the very high levels of GPC and GPI ether lipids. Oomycetes have long been considered a group of primitive fungi, but it is becoming clear that they evolved independently of true fungi. They are now considered to be related to heterokont algae in the Kingdom Chromista (Cavalier-Smith, 1989; Barr, 1992). In either case, there is limited information on the glycerophospholipid molecular species from these taxonomic groups that can be used for comparison. Ascomycete yeasts including *Saccharomyces cerevisiae* and *Lipomyces starkeyi* produce no or trace amounts of ether lipids (McElroy & Stewart, 1967; Jollow *et al.*, 1968). Higher quantities of ether-linked lipids (12% GPC and 32% GPE) were found in *Pullularia pullulans* (Dembitsky, 1989), but the few other yeasts that have been examined have insignificant quantities of this lipid class (Weete, 1980). *Pythium prolatum*, a sterol auxotroph related to *L. giganteum*, was found to produce 12–16% ether lipids in GPC and GPE fractions (Dembitsky, 1989).

Ether lipids are apparently synthesized by at least some members of all major kingdoms (Dembitsky, 1989). Archaeobacteria are characterized by production of ether-linked phytanyl isoprenoids rather than acylglycerols (de Rosa & Gambacorta, 1988). Levels of ether lipids comparable to those found in *L. giganteum* have been documented in the anaerobic spirochaete *Treponema hyodysenteriae* (Meyer & Meyer, 1971); in the protozoan *Paramecium tetraurelia*, where these lipids make up 80% of the GPC fraction and 90–95% of ceramideaminoethylphosphonate (Kaneshiro, 1980); and in some major groups of the animal kingdom including Porifera (sponges), Mollusca and Echinodermata (Dembitsky, 1989). Ether-linked glycerophospholipids have been found in varying amounts in insects (Lambremont, 1972; Lambremont *et al.*, 1976; Kulkarni *et al.*, 1971), but we

have found no reference to these compounds in mosquitoes, the host for *L. giganteum*.

L. giganteum and two genera of plant parasites in the Pythiaceae are the only documented sterol auxotrophs among Oomycetes. Elevated ether lipid content could be related to sterol auxotrophy. Ether-linked lipids generally have lower phase transition temperatures than the corresponding diacyl analogues (Lee & Fitzgerald, 1980). Although there may be minimal differences in membrane properties induced following sterol binding to diacyl relative to ether-linked phospholipids (Bittman *et al.*, 1984), it has been suggested that hydrogen bonding of cholesterol is enhanced in ether-linked glycerophospholipids relative to diacyl compounds due to the absence of a carbonyl at the *sn*-1 position of the former (Brockhoff, 1974). The importance of sterols to the morphogenesis of *L. giganteum* and other sterol auxotrophs may have resulted in selection for a membrane glycerophospholipid composition with enhanced affinity for this class of compound.

Coordinated changes in isoprenoid supplementation or metabolism with fatty acid and phospholipid composition have been documented in fungi (Aaronson *et al.*, 1982; Casey *et al.*, 1992) and other organisms (Schmitz *et al.*, 1990; Cinci *et al.*, 1993; Maziere *et al.*, 1990). It appears that isoprenoid and glycerophospholipid metabolism are also coordinated in *L. giganteum*. We have recently found that effects of cholesterol and other isoprenoids on *L. giganteum* morphogenesis are due in part to regulation of phospholipase A₂ activity (MacKichan *et al.*, 1994; unpublished observations), a pivotal class of enzyme involved in synthesis and remodelling of membranes. We are now investigating the interaction of sterols and glycerophospholipid metabolism in reproductively competent cultures.

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