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TEMPERATURE-INDUCED MODIFICATIONS OF GLYCOSPHINGOLIPIDS IN PLASMA MEMBRANES OF *NEUROSPORA CRASSA*

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Plasma membranes isolated from a cell-wall-less mutant of *Neurospora crassa* grown at 37 and 15°C display large differences in lipid compositions. A free sterol-to-phospholipid ratio of 0.8 was found in 37°C membranes, while 15°C plasma membranes exhibited a ratio of nearly 2.0. Membranes formed under both growth conditions were found to contain glycosphingolipids. Cultures grown at the low temperature, however, were found to contain 6-fold higher levels of glycosphingolipids and a corresponding 2-fold reduction of phospholipid levels. The high glycosphingolipid content at 15°C compensates for the reduced levels of phospholipids in such a way that sterol/polar lipid ratios are almost the same in plasma membranes under the two growth conditions. Temperature-dependent changes in plasma-membrane phospholipid and glycosphingolipid species were also observed. Phosphatidylethanolamine levels were sharply reduced at 15°C, in addition to a moderate increase in levels of unsaturated phospholipid fatty acids. Glycosphingolipids contained high levels of long-chain hydroxy fatty acids, which constituted 75% of the total fraction at 37°C, but only 50% at 15°C. Compositional changes were also observed in the long-chain base component of glycosphingolipids with respect to growth temperature. Fluorescence polarization studies indicate that the observed lipid modifications in 15°C plasma membranes act to modulate bulk fluidity of the plasma-membrane lipids with respect to growth temperature. These studies suggest that coordinate modulation of glycosphingolipid, phospholipid and sterol content may be involved in regulation of plasma-membrane fluid properties during temperature acclimation.

Introduction

Many microorganisms are known to modify their lipid components in response to changes in growth temperatures. Although it is generally assumed that such changes are part of a mechanism for modulating membrane fluidity [1,2], relatively few detailed studies exist concerning the actual changes which occur in lipids of different membrane fractions in eukaryotic organisms. This is especially true of plasma-membrane fractions, which in many respects are the most unique with respect to cellular lipid compositions.

We have recently shown that the Ascomycete fungus, *Neurospora crassa,* produces unusually large changes in both the phospholipids and sterol components of microsomal and mitochondrial membranes which appear to compensate for the effects of temperature on membrane fluidity [3]. We have now examined the lipid composition of the plasma membrane in *Neurospora* using a cell-wall-less mutant which was grown at either high or low temperatures. Our initial observations sug-

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gested that this membrane contains unusually high levels of sterols, with sterol/phospholipid ratios as high as 2.0 at low-temperature growth conditions. In this report, we show that this effect is due to large increases in non-phosphorus-containing glycosphingolipids at the lower growth temperature, which produce similar sterol/polar lipid ratios under both conditions. In addition to the temperature-induced changes in the glycosphingolipid levels, these lipids also exhibit striking changes in their long-chain base and fatty acid components with respect to growth temperature, which also act to compensate for the effects of temperature on the bulk fluid properties of the membrane.

Materials and Methods

The cell-wall-less mutant of *Neurospora crassa, slime* (FGSC No. 326) was kindly provided by Dr. E. Somberg. Cells were grown in Vogel's minimal medium [4] supplemented with 7.5% sorbitol and 10 mM arginine. Vegetative cultures were maintained by daily transfer of 5 ml glass wool filtered cells to 50 ml fresh medium. Stocks were stored on agar slants or in liquid culture at −70°C. Cells for plasma membrane isolation were grown in 500 ml medium in 2 liter flasks for 20 h at 37°C or for 12 h at 30°C followed by 16 h at 15°C in rotary shakers.

Isotope labelling

^{32}P-labelling of cells was performed by growing cells in 500 ml medium containing 5 mCi [^{32}P]orthophosphate (ICN, Irvine, CA). Glycolipids were tritium-labelled using glucose oxidase and galactose oxidase (Sigma, St. Louis, MO) and NaB^3H_4 (New England Nuclear, Boston, MA) according to the method of Gamberg [5]. Labelling was performed on washed cells and on plasma membrane vesicles.

Plasma membrane vesicles were isolated by the method of Scarborough [6]. Initial studies using this method indicated the presence of large amounts of phosphatidic acid and sharply reduced levels of phosphatidylcholine in plasma membrane vesicles (unpublished data). Similar effects have been previously reported in studies by other investigators using this method [7]. The addition of 10 mM EDTA to all buffers except that used in the homogenization step with DNAase eliminated the suspected phospholipase D activity, however, as indicated by reduced levels of phosphatidic acid and a corresponding increase in levels of phosphatidylcholine.

Lipid analysis

Phospholipids. Lipids were extracted from cells and plasma membranes as previously described [3]. Total lipids were fractionated by silicic acid column chromatography. Neutral lipids were eluted from columns in 75 ml chloroform. Polar lipids were then eluted in 100 ml chloroform/methanol, 1 : 1 (v/v), followed by 10 ml 1 : 9 (v/v) chloroform/methanol. Phospholipid phosphorus was determined by the colorimetric assay of Bartlett [8], as modified by Marinetti [9]. Phospholipid polar headgroup distribution was determined with ^{32}P-labelled lipid by two-dimensional thin-layer chromatography as previously described [10]. Phospholipid fatty acids were obtained by hydrolysis of polar lipids in 0.1 M NaOH in methanol for 30 min at 37°C, and analyzed as methyl esters by GLC on a 10 m SP-2330 glass capillary column (Supelco, Bellefonte, PA) in a Hewlett-Packard model 5710A gas chromatograph at 170°C.

Sterols. Free sterols were precipitated from neutral lipids with digitonin [11]. Digitonides were quantified by the colorimetric assay of Moore and Baumann [11] using recrystallized ergosterol as a standard.

Glycosphingolipids. Glycosphingolipids were obtained from polar lipids by the mild alkaline hydrolysis procedure described above. Tritium-labelled glycosphingolipids were separated by TLC on silica gel G in chloroform/methanol/water (65 : 25 : 4, v/v). Bands were identified by staining with naphthol [12], scraped and analyzed for ^{3}H by liquid scintillation counting. Glycosphingolipid components were obtained by hydrolysis in 2 M methanolic HCl for 2 h at 60°C with reflux. Fatty acid methyl esters were extracted three times in 5 ml petroleum ether. Long-chain bases were then extracted from deacidified aqueous phase in petroleum ether, as above. Long-chain bases were identified by GC-MS fragmentation patterns of trimethylsilyl derivatives [13] and by gas chromatographic comparison of fatty acid products produced by periodate-permanganate oxidation [14]

with authentic fatty acid standards as described above. Hydroxy fatty acids were identified by comparison with authentic standards using capillary gas chromatography on a 10 m SP-2230 and SE-30 capillary columns. The GSL fraction was quantified by gas chromatographic analysis of the fatty acid methyl ester products of the acid hydrolysis with methyl behenate (22 : 0) as an internal standard, assuming 1 mol of fatty acid to be produced by hydrolysis of 1 mol of glycosphingolipid.

Fluorescence measurements

Fluorescence polarization studies were conducted on plasma-membrane total lipids using the fluorescent probe 1,6-diphenylhexatriene (DPH). Liposomes were constructed with the probe at a lipid-to-probe ratio of 500 : 1. Fluorescence measurements were made with an Aminco-Bowman spectrofluorimeter fitted with a Glan prism in the excitation path and parallel and perpendicular film polarizers in the emission path. Liposomes were excited in a temperature-controlled cuvette at 340 nm. Fluorescence was detected at 420 nm. Data were collected by an Apple computer using a fast A/D converter (Interactive Microware, State College, PA). Triplicate determinations were made of the polarization value, P, at each temperature point between 5 and 50°C inclusive using the formula

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities parallel and perpendicular to the plane of the excitation beam.

Results

One of the more striking differences we observed between lipids in the *slime* plasma membrane and the total cellular lipid fraction appeared to involve an increase in free sterol levels. Plasma-membrane sterol phospholipid ratios represented a 4–10-fold increase over the whole cell free sterol/phospholipid ratio found in both the mutant and in phenotypically wild strains [3]. The plasma membrane free sterol/phospholipid ratio also showed a strong temperature dependence, ranging from 0.81 at 37°C to 1.98 at 15°C growth temperatures. In light of what were apparently unusually high levels of sterols at the lower growth temperature, we conducted further examinations of the plasma membrane lipids in an attempt to identify any non-phosphorus-containing polar lipids which may be present.

Mild alkaline hydrolysis of the polar lipid fraction resulted in a virtually complete hydrolysis of the phospholipids, yielding free fatty acids and a phosphate-free polar lipid fraction. Prolonged acid hydrolysis of the latter fraction produced fatty acids, free carbohydrate and nitrogen-containing long-chain bases, all of which indicate the presence of glycosphingolipids. Molar ratios of the glycosphingolipids to the phospholipids and sterol components were found to be extremely temperature-dependent, with the glycosphingolipid fraction comprising 6.2 mol% of 37°C plasma membrane lipid, while at 15°C they constituted 36.9 mol% (Table I). These large changes in glycosphingolipid levels and the corresponding decrease in phospholipids at the low temperature had the effect of producing a sterol/polar lipid ratio which was relatively constant at both temperatures, with values of 0.72 and 0.70, respectively, at 37 and 15°C. Table I summarizes the relative proportions of plasma membrane polar and neutral lipid species found at both growth temperatures. In addition to the large changes in the relative proportions of phospholipid and glycosphingolipid species, the distribution of components within each of these classes was also found to vary with respect to growth temperature. This involved changes in both the relative proportions of lipid species within each polar lipid class and in the distribution of fatty acyl and long-chain base distributions of those classes.

TABLE I

RELATIVE PROPORTIONS OF MAJOR PLASMA-MEMBRANE LIPIDS OF THE *SLIME* MUTANT

Values are mol% of total lipid determined as described in Materials and Methods.

	37°C	15°C
Phospholipids	51.3	20.8
Free sterols	41.7	40.6
Glycosphingolipids	6.2	36.9

Effects of growth temperature on plasma-membrane phospholipid composition

Slime plasma membranes were found to have phospholipid compositions markedly different from their total cellular lipid fractions, as summarized in Table II. Total phospholipid fatty acid distributions were found to be virtually identical between the mutant and those we previously reported for phenotypically wild-type strains [3]. The whole-cell phospholipid distributions shown in the table are also similar to those we have previously reported for the phenotypically wild strains [3,10], with the exception of elevated levels of PE and lowered PC in the *slime* mutant. The latter effect may reflect a relative increase in a specific membrane fraction, such as mitochondria, which show similar trends in PC and PE levels. An increase in the relative levels of mitochondria in the *slime* mutant is, in fact, suggested by elevated levels of cardiolipin.

The major changes observed in plasma membrane phospholipid species at high and low growth temperature involve phosphatidylethanolamine. At 37°C, phosphatidylethanolamine is the most abundant of the plasma-membrane phospholipids (Table II). These levels are sharply reduced at 15°C, however, with a corresponding increase in the levels of the acidic phospholipids phosphatidylserine and phosphatidylinositol. Phosphatidylcholine levels remain virtually unchanged between the two temperature extremes. Another phospholipid species which has been undetected in intracellular membranes and whole-cell extracts, but is present in plasma membranes, is sphingomyelin. Although only a minor component, sphingomyelin levels are observed to vary with respect to growth temperature, being higher at 15°C.

As previously observed in intracellular membranes, phospholipid fatty acid composition differs markedly between the two growth temperatures (Table III). Growth at low temperature results in an increased content of unsaturated fatty acids in phospholipids, characterized most notably by a 5-fold increase in the levels of linolenic acid (18 : 3). Plasma-membrane fatty acids tend to be more highly saturated at both temperatures compared to whole cell lipids in terms of the number of double bonds found per 100 molecules. *Slime* total cellular phospholipids contain 138 and 208 double bonds/100 molecules at the high and low temperatures, which corresponds to 10% and a 24% higher levels of double bonds, respectively. The increased saturation in the plasma membrane

TABLE II

PHOSPHOLIPID COMPOSITION OF PLASMA MEMBRANES AND TOTAL CELLULAR LIPIDS OF THE *SLIME* MUTANT

Values are given in mol% total lipids as described in Materials and Methods. Each value represents the average of two independent experiments.

Phospholipid species	Plasma membrane	Total lipid
37°C cultures		
Phosphatidic acid	7.8	1.8
Phosphatidylserine + phosphatidylinositol	5.9	10.8
Phosphatidylcholine	25.2	41.5
Sphingomyelin	3.7	–
Phosphatidylethanolamine	53.7	39.8
Cardiolipin	–	3.7
15°C cultures		
Phosphatidic acid	10.8	5.3
Phosphatidylserine + phosphatidylinositol	21.1	15.4
Phosphatidylcholine	28.0	38.9
Sphingomyelin	8.5	–
Phosphatidylethanolamine	32.3	33.7
Cardiolipin	–	6.7

TABLE III

PHOSPHOLIPID FATTY ACID COMPOSITIONS OF *SLIME* PLASMA MEMBRANES

Values are weight percent of total fatty acids in the phospholipid fractions determined as described in Materials and Methods. Species comprising less than 1% of the total are not shown.

Fatty acid	37°C	15°C
14:0	1.0	1.2
16:0	19.9	20.0
16:1	1.4	0.8
18:0	2.1	3.2
18:1	29.7	6.8
18:2	43.1	52.9
18:3	2.8	15.1
Double bonds/100 molecules	126	159

lipids appears to be due primarily to lowered levels of linoleic acid (18 : 2) at the high temperature and α-linolenic acid at the lower temperature, when compared to the whole-cell phospholipid fraction.

Effects of growth temperature on plasma-membrane glycosphingolipid composition

Glycosphingolipids in *Neurospora* appear to be largely localized in the plasma membrane, which represents a 10- to 15-fold enrichment of this fraction relative to phospholipids over whole cells. We have detected at least three major classes of glycosphingolipid by thin-layer chromatographic methods. The most abundant class is that of the monoglycosylceramides or cerebrosides. These lipids make up nearly 75% of the glycosphingolipids at 37°C and approx. 50% in 15°C membranes as measured by [^{3}H]borohydride labelling of the total isolated lipid fraction. The other classes, as yet unidentified, most likely contain more polar complex oligosaccharide moieties, with at least one species containing phosphorus.

Analysis of glycosphingolipid fatty acid composition revealed that this fraction is distinct from the other polar lipids in *Neurospora* (Table IV). While phospholipids are found to contain straight-chain saturated and unsaturated fatty acids from 14–18 carbons in length, the majority of glycosphingolipid fatty acids are hydroxylated species with chain lengths up to 24 carbons. Large compositional differences were also observed in these lipids at the two growth temperatures. In 37°C membranes, the majority of the glycosphingolipid fatty acids are the HO-20 : 0 and HO-24 : 0 species. At that temperature, the hydroxylated species constitute nearly 75% of the total glycosphingolipid fraction. At 15°C, the levels of the long-chain hydroxy-fatty acids are reduced, with a concomitant increase in the levels of the shorter-chain saturated species 16 : 0 and 18 : 0. These changes reduce the net proportion of the hydroxy-fatty acids to 50%.

Compositional changes in the long-chain base composition of the glycosphingolipids were also observed in response to altered growth temperatures. The most prominent long-chain base species found is sphinganine, the relative levels of which remain constant at 37 and 15°C (Table V). Other long-chain base species show considerable variation with growth temperature, with sharply reduced levels of sphingosine and a more than 2-fold increase in the levels of a 20-carbon sphinganine found at the lower growth temperature. Glycosphingolipids at both temperatures were also found to contain hydroxysphinganine or phytosphingosine as well as a 19-carbon sphinganine.

Effects of lipid composition on membrane fluid properties

The differences in lipid composition found between the two growth temperatures which involve large changes in the levels of the glycosphingolipid

TABLE VI

FATTY ACID COMPOSITION OF PLASMA-MEMBRANE GLYCOSPHINGOLIPIDS

Values are expressed as weight percent of total fatty acids. Species comprising less than 1% of the total are not shown.

Fatty acid	37°C	15°C
16 : 0	6.1	16.7
18 : 0	4.5	13.2
18 : 1	3.3	3.7
18 : 2	1.6	0.5
18 : 3	1.2	5.0
20 : 0	1.7	1.0
24 : 0	1.8	6.4
HO-16 : 0	0.4	0.7
HO-18 : 0	1.6	4.7
HO-20 : 0	29.3	12.9
HO-24 : 0	43.1	33.4

TABLE V

LONG-CHAIN BASE COMPOSITION OF PLASMA-MEMBRANE GLYCOSPHINGOLIPIDS

Values are expressed as weight percent of total long-chain bases. Numbers in parentheses following the species name refer to the number of carbon atoms in the long-chain base.

Species	37°C	15°C
Phytosphingosine (C18)	2.8	8.3
Sphingosine (C18)	34.5	19.6
Dihydrosphingosine	43.0	41.6
Sphinganine (C19)	11.2	10.7
Sphinganine (C20)	8.5	19.7

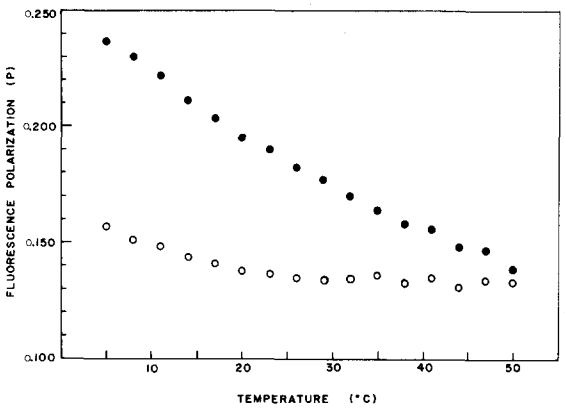

Fig. 1. Fluorescence polarization of 1,6-diphenylhexatriene in vesicles formed from total lipid extracts of purified plasma membranes of 37 (●) and 15°C (○) -grown cultures. Experiments were performed as described in Materials and Methods.

fraction led us to question whether these modifications could, in fact, compensate for temperature-induced changes in membrane fluidity. In order to gain some insight into this problem, we measured the fluorescence polarization of 1,6-diphenylhexatriene in vesicles of total lipids isolated from either 37 or 15°C-acclimated plasma membranes. Polarization measurements over a 5–50°C temperature range showed significantly lower values for the 15°C acclimated lipid fraction (Fig. 1). Comparison of polarization values from the two lipid preparations measured at 15°C are most revealing, showing the large differences in lipid mobilities of the two fractions at that temperature. Although this and other probes have been shown to exhibit complex rotational motions in lipid bilayers [15], which precludes precise measurement of membrane lipid mobility, the polarization values found at low temperatures between both lipid fractions show a marked difference in membrane lipid mobilities, consistent with a scheme in which the modifications in lipid composition act to modulate membrane fluidity.

Discussion

On the basis of the above data, it would appear that *Neurospora* plasma membranes are similar to those of other eukaryotic cells in containing high levels of sterols and sphingolipids compared to internal membrane fractions. The existence of sphingolipids in total lipid extracts of *Neurospora* has been previously reported [13,18]. The relative proportions of these lipids to total membrane lipid species or their subcellular locations, however, do not appear to have been previously determined. In light of our present studies, it would appear that although glycosphingolipids in *Neurospora* represent a minor component of total cellular lipids, they comprise a major fraction of plasma-membrane lipid species.

Our earlier studies of *Neurospora* mitochondrial and microsomal membrane fractions showed that acclimation to temperature changes primarily involved alterations in phospholipid fatty acid saturation and changes in membrane sterol levels. In light of those data, we were surprised to find that, while plasma-membrane sterol levels remained constant relative to total lipids, temperature-induced modifications of plasma-membrane lipids involved radical changes in phospholipid and glycosphingolipid levels in addition to changes in their fatty acid and long-chain base constituents.

Virtually all studies to date on the temperature-induced modulation of membrane fluidity in eukaryotic cells have been reports on the changes observed in either sterol or phospholipid components. To our knowledge, this is the first report of temperature-induced modification of glycosphingolipids in eukaryotic cells. This may be due, in fact, to the lack of studies of this nature on the eukaryotic plasma membrane. Given the enormity of change observed in phospholipid and sphingolipid levels between the two growth conditions, it is clear that glycosphingolipids must play an important role in the modulation of plasma-membrane fluid properties.

At this point, it is somewhat difficult to reconcile our present findings with known thermotropic properties of glycosphingolipids. There are few data available on the thermotropic properties of sphingolipids, and most reports have involved physical-chemical observations of mixtures of sphingolipids containing several long-chain base and fatty acyl species. Model systems composed of natural cerebrosides from brain have been reported to exhibit high phase-transition temperatures ranging from 55 to 72°C [16]. Raman vibra-

tional spectra in that study indicated a high degree of order in these systems, suggesting that the high transition temperatures in liposomes composed solely of cerebrosides are due to the long fatty-acid chains of these lipids, which interdigitate with the other half of the bilayer in the gel state. Other investigators have reported that monoglucocerebrosides exhibit complex behavior in model liposome systems with dipalmitoylphosphatidylcholine, which suggests that specific interactions may occur between the two lipid species [17]. At this point, however, there appear to be no studies reported on the thermotropic properties of model systems composed of glycosphingolipids and with either naturally occurring phospholipids or sterols such as ergosterol, which is the major sterol component of *Neurospora* membranes. Given the asymmetric nature of the sphingolipids, it is conceivable that interaction of these molecules with complex mixtures of phospholipids or with sterols could create packing defects which would be favorable for the formation of *gauche* rotomers which could, in fact, have a fluidizing influence on the membrane lipid bilayer. It appears that considerable insight into the effects we have observed here could be obtained by studying such systems in a systematic way.

Although glycosphingolipids have long been recognized as important components of mammalian-cell plasma membranes, much of the study of glycosphingolipid function has focused on the polar moiety of the molecule. It appears from this study that the hydrophobic portion of the glycosphingolipid molecule may also serve an important function in cellular processes through modulation of membrane fluid properties. For this reason, it is important to understand the mechanisms by which glycosphingolipid metabolism is integrated with that of phospholipids and sterols in the biogenesis of the plasma membrane, as well as in the regulation of membrane fluidity in response to a perturbing force. In light of the results of the present study, we believe that *Neurospora* provides an excellent experimental system in which to study these phenomena.

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References

1 Thompson, G.A. (1979) in Low Temperature Stress in Crop Plants. The Role of the Membrane (Lyons, J.M., Graham, D. and Raison, J.K., eds.), pp. 347–363, Academic Press, New York
2 Quinn, P.J. (1981) Prog. Biophys. Mol. Biol. 38, 1–104
3 Aaronson, L.R., Johnston, A.M. and Martin, C.E. (1982) Biochim. Biophys. Acta 713, 456–462
4 Vogel, H.J. (1964) Am. Nat. 98, 435–446
5 Gamberg, C.G. (1978) Methods Enzymol. 50, 204–206
6 Scarborough, G.A. (1975) J. Biol. Chem. 250, 1106–1111
7 Friedman, K.J. and Glick, D. (1980) J. Membrane Biol. 54, 183–190
8 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466
9 Marinetti, G.V. (1962) J. Lipid. Res. 3, 1
10 Martin, C.E., Siegel, D. and Aaronson, L.R. (1981) Biochim. Biophys. Acta 665, 399–407
11 Moore, P.R. and Baumann, C.A. (1952) J. Biol. Chem. 195, 615–621
12 Siakatos, A.N. and Rauser, G. (1965) J. Am. Oil Chem. Soc. 42, 913
13 Maggese, M.C., Gros, E. and Torre, N.H. (1981) Neurospora Newsl. 28, 14–16
14 Von Rudloff, E. (1956) Can. J. Biochem. 34, 1413
15 Martin, C.E. and Foyt, D.C. (1978) Biochemistry 17, 3587–3591
16 Bunow, M.R. (1979) Biochim. Biophys. Acta 574, 542–546
17 Correa-Freire, M.C., Freire, E., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1979) Biochemistry 18, 442–445
18 Lester, R.L., Smith, Sharron, W., Wells, G.B., Rees, D.C. and Angus, W.W. (1974) J. Biol. Chem. 249, 3388–3394