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Sphingolipid C4 hydroxylation influences properties of yeast detergent-insoluble glycolipid-enriched membranes

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Abstract Sphingoid base C4 hydroxylation is required for syringomycin E action on the yeast plasma membrane. Detergent-insoluble glycolipid-enriched membranes (DIGs) from a yeast strain lacking C4 hydroxylated sphingoid bases (*sur2Δ*) are composed of linear membrane fragments instead of vesicular structures observed for wild-type DIGs, though they have similar lipid compositions and amounts of DIG marker proteins. Light-scattering bands collected from *sur2Δ* after centrifugation of Triton X-100-treated cell lysates in continuous density gradients have lower buoyant densities than that of the wild-type. The results show that C4 hydroxylation influences the physical and structural properties of DIGs and suggest that syringomycin E interacts with lipid rafts.

Keywords: Syringomycin E; Lipid raft; Sphingolipid; *SUR2*; *Saccharomyces cerevisiae*

1. Introduction

Sphingolipids are important ceramide-containing membrane lipids with structural and regulatory functions [1]. They are composed of a long chain base with an amide-linked fatty acid at the C2 position and a polar constituent at the C1 position. *Saccharomyces cerevisiae* produces primarily the long chain base known as phytosphingosine (PHS), which is hydroxylated at the C4 position.

The gene responsible for sphingoid base C4 hydroxylation in *S. cerevisiae* has been identified and studied [2–4]. It was recognized as *SUR2* [2], but also as *SYR2* [5], since it is necessary for the antifungal action of syringomycin E, a small lipodepsinonapeptide secreted by the bacterium *Pseudomonas syringae*.

Sphingolipids hydroxylated at the C4 position were shown to promote pore formation by syringomycin E in planar lipid bilayers [6] and these hydroxylated lipids appear to be an integral part of the syringomycin E channel pore structure (lipidic pores) [7]. Other structural features of yeast sphingolipids also promote syringomycin E action [8–10]. These, as well as C4 hydroxylation, contribute to the hydrogen bonding abilities of the sphingolipids. Since sphingolipid hydrogen bonding is involved in the formation of liquid-ordered domains in biomembranes (lipid rafts) [11], it was speculated that these membrane domains are sites for syringomycin E binding and channel formation [6].

Lipid rafts are regions in eukaryotic membranes that are enriched in sterols and sphingolipids. They are distinct from the remaining liquid-disordered phases of membranes that are more fluid and rich in unsaturated glycerolipids. One of the very important features of lipid rafts is that they selectively include certain membrane proteins such as GPI-anchored proteins [12], and double acylated and palmitoylated proteins [13]. These microdomains are implicated in many cellular processes such as cell adhesion and migration [14], cellular signaling [15], protein sorting, and as docking sites for pathogens and toxins [16]. Biochemically, lipid rafts are characterized by their insolubility in cold non-ionic detergents such as Triton X-100. This property allows the extraction of detergent-insoluble glycolipid-enriched membranes (DIGs) that are considered to be the structural equivalent of functional lipid rafts [17]. DIGs have been isolated from many mammalian membranes and also from yeast [18,19]. Studies with lipid biosynthetic mutants of yeast showed that both sphingolipids and sterols are important for raft association [18,20,21].

Since C4 sphingoid base hydroxylation in yeast is necessary for syringomycin E action and possible sites for this fungicide's action are lipid rafts, we investigated whether this step in yeast sphingolipid biosynthesis affects the physical and biochemical properties of DIGs. Using a yeast mutant strain deficient in *SUR2*, we observed that the C4 hydroxyl group on the sphingoid base influences the physical and structural properties of DIGs isolated from yeast.

2. Materials and methods

2.1. Yeast strains and growth conditions

S. cerevisiae strains used in this study were W303C (MAT**a** *ade2 his3 leu2 trp1 ura3*) (wild-type) and W303A (MAT**a** *ade2 his3 leu2 trp1 ura3 sur2*::URA3) (*sur2Δ*). The strains were grown in 250-ml capacity Erlenmeyer flasks (50 ml culture) at 28–30 °C with shaking at 200 rpm. Growth media used were YPD [22] and low-phosphate medium [23].

2.2. DIG isolation

Yeast strains were grown at 28–30 °C and cells equivalent to 30 OD_{600} units were collected. Cells were spheroplasted using Zymolyase 100T (Seikagaku Corp.), disrupted by sonication, and the cell lysate was used to isolate DIGs according to the protocol described by Bagnat et al. [18].

2.3. Flotation of DIGs in continuous Optiprep (Nycomed, Oslo) gradient

Cell lysates were prepared as described above and overlaid with a continuous Optiprep gradient (0–40% v/v, 11 ml total volume) and centrifuged in a Beckman SW41 rotor at 37 000 rpm for 6 h. Fraction containing the light-scattering band from the first gradient was col-

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lected, then subjected to a second round of Triton X-100 treatment and centrifugation. 46 fractions (250 μl each) were collected from the bottom using a peristaltic pump (P-3, Pharmacia). The distribution of light-scattering bands within each fraction was analyzed by measuring the OD_{595} profile using a UV–Vis spectrophotometer (UV1201, Shimadzu). Gas1p distribution was analyzed using rabbit anti-Gas1p antibody.

2.4. Lipid analysis

DIGs and total membranes were prepared for lipid analysis as previously described [18]. For phospholipid and sphingolipid analyses, cells were grown in 10 ml of low phosphate YPD in the presence of 1 mCi of [^{32}P]orthophosphate (ICN Biomedicals, Inc.) as described by Bagnat et al. [18]. Radiolabeled phospholipids were extracted and analyzed according to published methods [24,25]. Radiolabeled sphingolipids were extracted by addition of 1.4 ml of ethanol–ether–pyridine (1:0.33:0.067; v/v) and incubated at 57 °C for 30 min [26]. The samples were dried under N_2 and deacylated as follows: Dried lipid extracts were dissolved in 1 ml of solvent A (chloroform–methanol–water (16:16:5; v/v). An equal volume of 0.2 N NaOH in methanol was added to each sample and the mixture was incubated at 30 °C for 45 min. To each sample, 1.1 ml of 0.5% (w/v) EDTA was added and the mixtures were neutralized by addition of 0.2 ml of 1 N acetic acid. Non-deacylated lipids were extracted with 0.5 ml of chloroform, dried under N_2, and suspended in solvent A and resolved by thin layer chromatography on silica gel plates (layer thickness, 0.25 mm, Whatman) using chloroform–methanol–4.2 N NH_4OH (9:7:2; v/v) as chromatographic solvent.

Sterols were labeled by growing cells in YPD with 10 μCi of [^{14}C]acetate (ICN Biomedicals) for 2 h, extracted as described [18], and analyzed by thin layer chromatography on silica gel 60 plates (layer thickness, 0.25 mm, Merck) using hexane-ethyl ether (1:1; v/v) as chromatographic solvent. Radiolabeled lipids were detected by autoradiography.

2.5. Immunoblotting and protein analysis

Proteins were precipitated from Optiprep fractions by adding two volumes of 15% trichloroacetic acid. Western blot analysis was performed using rabbit anti-Gas1p antibody (obtained from H.Riezman, Geneva) diluted 1:10 000 and rabbit anti-Pma1p antibody (obtained from A. Chang, New York) diluted 1:500.

2.6. Electron microscopy of DIGs

Fractions containing DIGs isolated from Optiprep gradients as described above [18] were centrifuged at 100 000 rpm in a Beckman 100.3Ti rotor for 20 min. Samples were prepared and analyzed as previously described [19] at the Advanced Microscopy and Imaging Laboratory of Auburn University (Auburn, Alabama).

3. Results

3.1. Sedimentation properties of Triton X-100-treated cell lysates in Optiprep continuous density gradients

Because of the potential hydrogen bond contribution of the sphingolipid C4 hydroxyl group, it was suspected that the lack of this group might influence lipid raft properties. The sedimentation properties of Triton X-100-treated cell lysates from the wild-type and *sur2Δ* strains after two rounds of extraction with Triton X-100 and flotation in a continuous Optiprep gradient (0–40%) were examined. Discontinuous gradients are commonly used for isolation of lipid rafts [18], but to increase our ability to observe subtle differences between strains, we chose to first employ continuous gradients. For both strains, a prominent light scattering band was observed in the upper to middle region of the gradient. However, material of the *sur2Δ* band sedimented to a position equivalent to a lower buoyant density than that of the wild-type band (Fig. 1A). Similar results were obtained with continuous gradients with centrifugation times of up to 30 h (data not shown). To determine if the observed bands could represent DIGs, we examined if

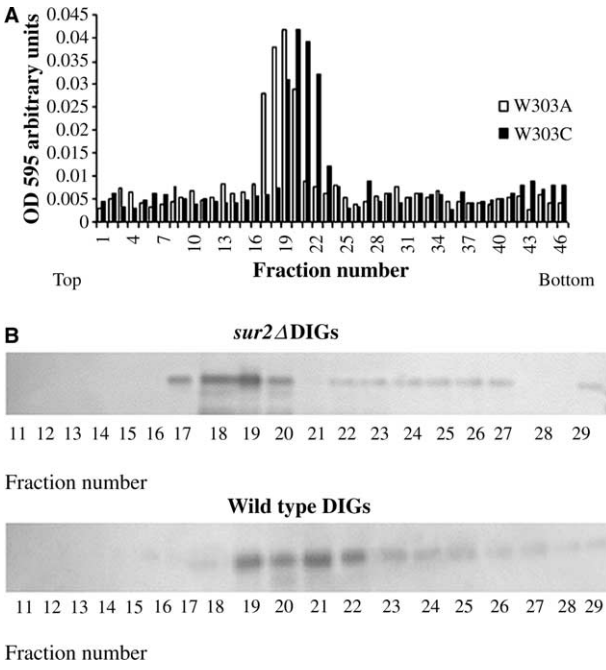

Fig. 1. OD_{595} (A) and Gas1p (B) Optiprep density gradient profiles of Triton X-100-treated cell lysate from wild-type and *sur2Δ* strains. Triton X-100-treated cell lysate was subjected to continuous (0–40%, v/v) Optiprep density gradients as described in Section 2. (A) Gradient fractions were collected from the bottom of the gradient and the OD_{595} of each fraction was determined (white bars, *sur2Δ*; black bars, wild-type). (B) Fractions 11–29 were analyzed for the presence of Gas1p. The proteins were TCA-precipitated, separated by $NaDodSO_4$ polyacrylamide gel electrophoresis, and Gas1p was immunodetected by Western blot analysis.

Gas1p [18] (a GPI-anchored protein associated with yeast DIGs) was present in these bands. Presence of Gas1p was determined by Western blot analysis after $NaDodSO_4$ polyacrylamide gel electrophoresis (Fig. 1B). Gas1p from *sur2Δ* was detected in lower density fractions compared to the Gas1p from the wild-type strain. Based on these data, we speculate that the major light scattering band in the Optiprep density gradients represents yeast DIGs and that lack of sphingoid base C4 hydroxylation has the effect of lowering the buoyant density of this light-scattering band. Previously published analyses of DIGs have utilized discontinuous Optiprep density gradients [18]. We initially used a continuous Optiprep gradient to ensure detection of subtle differences in the distribution of Gas1p. Further comparison of properties of DIGs from *sur2Δ* and wild-type yeast strains was done using a standard protocol for DIG isolation [18] so that results would be directly comparable with the work of others.

3.2. Effect of sur2Δ on the lipid composition of DIGs

The lipid compositions of DIGs (isolated using standard Bagnat's protocol [18]) from the wild-type and *sur2Δ* strains were studied. The phospholipids, sphingolipids, and sterols were analyzed. DIG fractions from the wild-type and *sur2Δ* strains were highly enriched in sphingolipids and sterols (Fig. 2) compared to the level of phospholipids. In addition, the relative levels of the individual sphingolipids and phospholipids and ergosterol were similar for the DIGs of both strains (Fig. 2). As expected, the DIGs of the *sur2Δ* strain possessed the non-C4 hydroxylated sphingolipid species in

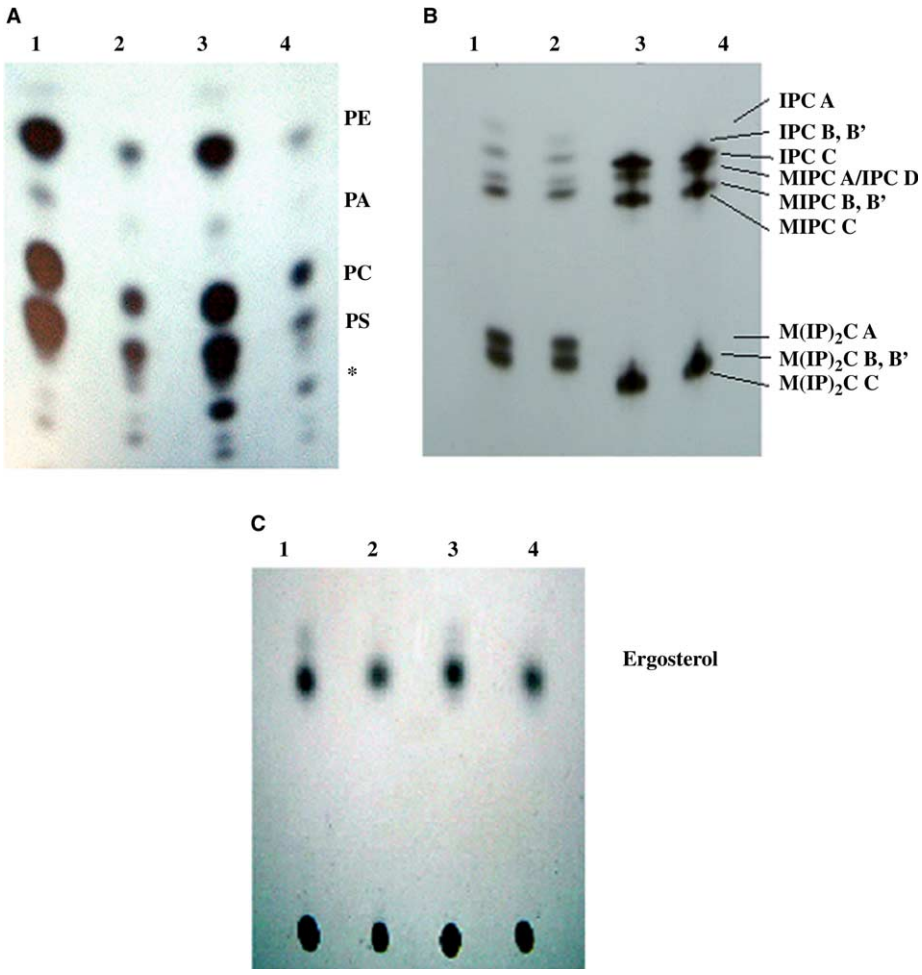

Fig. 2. Lipid analyses of total membranes and DIGs fractions of wild-type and *sur2Δ* strains. Lipids were extracted as described in Section 2, resolved by thin-layer chromatography, and visualized by autoradiography. Phospholipids (A), sphingolipids (B), and sterols (C) were analyzed. 1, Lipids extracted from *sur2Δ* total membranes; 2, lipids extracted from *sur2Δ* DIGs; 3, lipids extracted from wild-type total membranes; and 4, lipids extracted from wild-type DIGs. PE, phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidtylcholine; PS, phosphatidylserine; *, sphingolipids; IPC A, B, B′, C, D – inositolphosphoceramide species, MIPC A, B, B′, C-mannosylinositolphosphoceramide species, $M(IP)_2C$ A, B, B′, C: mannosyldiinositolphosphoceramide species. Species A–D differ in the degree of hydroxylation of the sphingolipid long chain base and/or fatty acid [36].

place of the normal C4-hydroxylated version [3] that are less polar and have higher mobility in the TLC system used.

3.3. DIGs morphology

Since the DIGs from the wild-type and *sur2Δ* strains were observed to have different buoyant densities, we speculated that they also differ structurally. DIGs isolated by standard protocol [18] were collected, fixed, and embedded and stained thin-sections were examined by transmission electron microscopy. The electron micrographs of the wild-type DIGs showed membrane structures similar in appearance to those previously reported by Kubler et al. [19]. These were closed vesicular structures with variable diameters ranging between 100 and 500 nm (Fig. 3A). In contrast, the electron micrographs displaying *sur2Δ* DIGs showed linear membrane fragments ranging in length between 100 and 600 nm (Fig. 3B).

3.4. Effect of sur2Δ on DIGs marker proteins

Two major plasma membrane proteins, GPI-anchored Gas1p and the ATPase Pma1p, are associated with DIGs in yeast [18]. To determine whether hydroxylation at the sphingoid base C4 position is important for these associations, DIGs were isolated from both wild-type and *sur2Δ* strains. The presence of Gas1p protein within the light-scattering bands that are thought to represent DIGs examined after centrifugation in continuous Optiprep gradients was described above.

In this experiment, we focused on the distribution of both Gas1p and Pma1p after isolation of DIGs following a standard protocol described by Bagnat et al. After two rounds of extraction with Triton X-100 and flotation in three-layered (cell lysate adjusted to 40% and overlaid with 1.2 ml of 30% w/v, and 0.2 ml of TNE buffer) Optiprep medium, the majority of both marker proteins were found in the top fraction of the gradients that corresponded to DIGs (Fig. 4). No significant difference was observed in the density gradient profiles of the two marker proteins between the DIGs of wild-type and *sur2Δ* strains. This is consistent with data from continuous density gradients shown in Fig. 1, where Gas1p is present within the light-scattering fraction of both wild-type and *sur2Δ*.

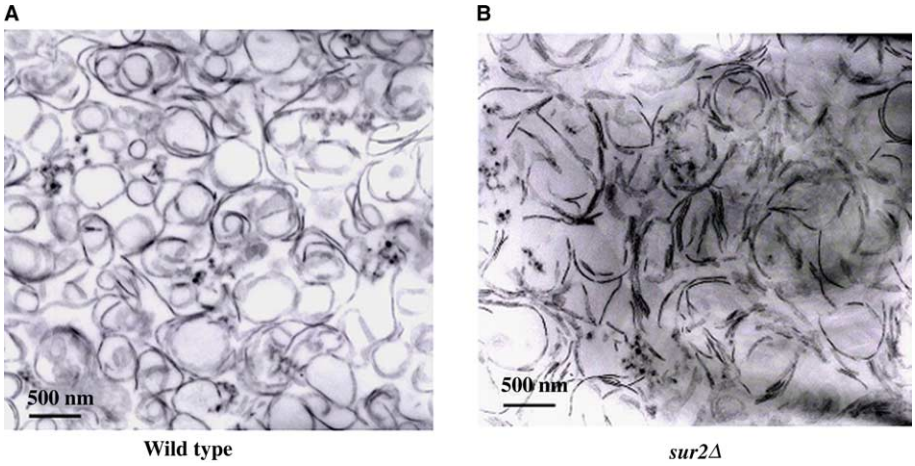

Fig. 3. Transmission electron micrographs of embedded thin sections of DIGs isolated from wild-type (A) and *sur2Δ* (B) strains.

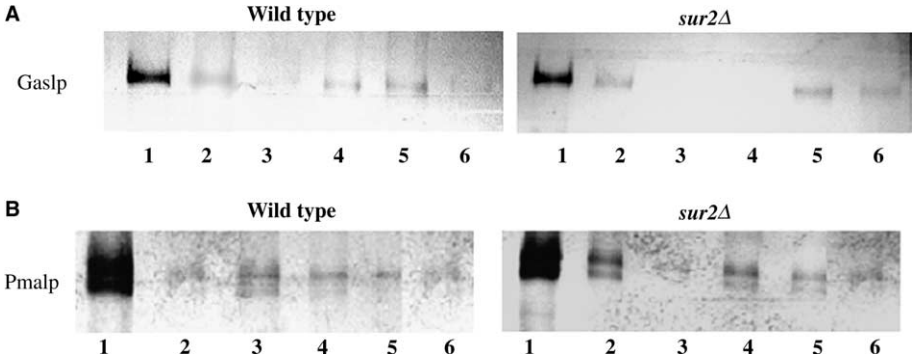

Fig. 4. Western blot analysis of Gas1p (A) and Pma1p (B) distribution in wild-type and *sur2Δ* strains. Fractions of equal volume were collected from the top (1) to the bottom (6) of a second density gradient during DIGs isolation.

This result indicates that C4 hydroxylation of sphingoid bases is not required for the assembly of Pma1p and Gas1p into yeast lipid rafts.

4. Discussion

In this work, we provide data showing that sphingoid base C4 hydroxylation is important for the physical and structural properties of yeast DIGs. Triton X-100-treated cell lysates from a strain (*sur2Δ*) that does not synthesize PHS have lower densities than those from the wild-type after centrifugation in continuous Optiprep gradients. Based on the presence of Gas1p within these fractions, we can speculate that they represent DIGs and that C4 hydroxylation has an influence on their buoyant densities. Morphological differences were also observed. DIGs from *sur2Δ* formed linear membrane fragments or sheets and lacked significant vesicular or curved membranes characteristic of wild-type yeast DIGs [19]. The altered properties of the *sur2* DIGs suggest that the properties of lipid rafts of this strain differ from those of the wild-type. The reasons for the observed differences in buoyant densities and morphology are unknown, but it may be speculated that differences in protein content (see below) or in lipid/lipid interactions could contribute.

It was previously shown that disruption of early steps of sphingolipid biosynthesis led to impaired association of marker proteins Gas1p and Pma1p with lipid rafts [18]. Similar observations were made for Pma1p in yeast mutants defective in ergosterol synthesis and production of sphingolipids with very long chain fatty acids (*elo3Δerg6*^{ts}) [20]. In our studies, the distribution and amounts of Gas1p and Pma1p marker proteins were not altered in *sur2Δ* and thus not dependent on C4 hydroxylation of sphingoid bases. Consistent with this result, Balguerie et al. [35] found that in intact cells Pma1p tagged with green fluorescent protein is correctly localized at the cell surface in a *sur2Δ* mutant, again suggesting that lipid raft delivery of Pma1p is not altered. Nevertheless, preliminary results reveal that a few protein compositional differences occur between the DIGs of *sur2Δ* and the isogenic wild-type strain as revealed by two-dimensional differential gel electrophoresis (J. Idkowiak-Baldys, unpublished). Further examination of the extent and nature of these differences may reveal if they can account for the differences in buoyant densities and morphology of DIGs that were observed. Detailed analyses and identification of the differentially distributed proteins are currently under investigation.

Despite the importance of C4 hydroxylation for raft structure and the multiple proposed functions of lipid rafts, the *sur2Δ* deletion strain is still viable and does not appear to have

growth defects when grown under laboratory conditions. However, it does confer resistance to the antifungal agent syringomycin E. Other features of membrane sphingolipids and sterols promote syringomycin E activity and the same lipids are highly enriched in lipid rafts [27]. Thus, lipid raft microdomains are plausible targets for syringomycin E. Lipid rafts could promote the local concentration of syringomycin E to facilitate ion channel formation, and perturbation of lipid raft structures would then require higher concentrations of syringomycin E (wild-type strains are at least fivefold more sensitive to syringomycin E than *sur2Δ*, unpublished). The hydrogen bond contribution of C4-hydroxylation [28] provides an explanation for the observed differences in DIG properties. It is well known that PHS-based synthetic ceramides promote the condensation of lipid monolayers [28,29] and the strong headgroup hydrogen bonds of C4 hydroxylated sphingolipids [30] promote their aggregation. In light of this, it would be of interest to observe properties of DIGs in other sphingolipid strains altered in syringomycin E sensitivity. Especially interesting would be examination of *fah1Δ* and *fah1Δsur2Δ* double mutants. *FAH1* is necessary for α-hydroxylation of the fatty acid of yeast sphingolipids and thus, like *SUR2*, a significant contributor to sphingolipid hydrogen bonding capabilities. While common in yeast, C-4 hydroxylated sphingolipids are present in minor amounts in mammals. However, their amounts are increased in mammalian carcinomas [31–33] suggesting possible contributions of hydrogen bond-dependent sphingolipid clustering in cancer [34].

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