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Inhibition of yeast (1,3)-β-glucan synthase by phospholipase A_2 and its reaction products

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Abstract

Fungal (1,3)-β-glucan synthases are sensitive to a wide range of lipophilic inhibitors and it has been proposed that enzyme activity is highly sensitive to perturbations of the membrane environment. Yeast membranes were exposed to phospholipases and various lipophilic compounds, and the resultant effects on glucan synthase activity were ascertained. Glucan synthase from *Saccharomyces cerevisiae* was rapidly inactivated by phospholipase A_2 (PLA_2), and to a lesser extent by phospholipase C. Inactivation was time and dose-dependent and was protected against by EDTA and fatty-acid binding proteins (bovine and human serum albumins). Albumins also partially protected against inhibition by papulacandin B. PLA_2 reaction products were structurally characterized and it was shown that fatty acids and lysophospholipids were the inhibitory moieties, with no novel inhibitory compounds apparent. Glucan synthase was inhibited by a range of fatty acids, monoglycerides and lysophospholipids. Inhibition by fatty acids was non-competitive, and progressive binding of [^{14}C]oleic acid correlated with activity loss. Fluorescence anisotropy studies using diphenylhexatriene (DPH) confirm that fatty acids increase membrane fluidity. These results are consistent with proposals suggesting that glucan synthase inhibition is due in part to non-specific detergent-like disruption of the membrane environment, in addition to direct interactions of lipophilic inhibitors with specific target sites on the enzyme complex.

Key words: (1,3)-β-Glucan synthase; Phospholipase A_2; Fluorescence anisotropy; Antifungal compound; Fatty acids; Membrane perturbation

1. Introduction

The biosynthesis of cell wall polymers in yeast, fungi and higher plants is important for cell growth and development. In the yeast *Saccharomyces cerevisiae*, a predominantly (1,3)-β-linked glucan is a major cell wall component. This glucan is synthesized by the plasma membrane-bound glycosyl transferase (1,3)-β-glucan synthase (GS) (EC 2.4.1.34), which catalyzes the transfer of glucose units from UDP-Glc (uridine diphosphate glucose) onto a growing polysaccharide chain [1–3]. Fungal GSs are thought to exist as multimeric complexes containing both integrally-bound subunits as well as a peripherally-bound GTP binding subunit which is essential for activity [4,5]. In contrast to the higher plant GSs, which are readily solubilized by either digitonin or CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) and have been subsequently partially purified [6–11], it has not been possible to obtain detailed structural information for fungal GSs due to rapid activity losses during solubilization from the membrane [12–14]. Therefore it is important to understand factors which influence the stability of fungal GSs.

Yeast GS is of great interest because it represents a molecular target for antifungal antibiotics to treat systematic fungal infections associated with AIDS, cancer, and other diseases that result in immunocompromised states. A number of antifungal drugs have been targeted against GSs from various fungi including the lipopeptide echinocandin-like class of inhibitors [15–18], aculeacin A [19,20], and the papulacandins [21–24] which are lipid-like saccharides. Each of these com-

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pounds contains fatty acid (FA) side chains, which has been shown to be essential for the action of cilofungin [25] and papulacandin B [23]. Although various lipophilic compounds are known to rapidly inactivate fungal glucan and chitin synthases [26], the mechanism by which these compounds interfere with GS has received little attention, and it has been suggested that interactions with the membrane may in part be responsible for observed inhibitory effects [24,27,28].

In this study, membranes from *Saccharomyces cerevisiae* were disrupted by phospholipase digestion and exposure to lipophilic compounds, and the resultant effects on GS activity were ascertained. Our results are consistent with the idea that drug-induced inhibition of (1,3)-β-GS is due in part to non-specific perturbations of the membrane environment.

2. Materials and methods

2.1. Materials

Saccharomyces cerevisiae GS-1–36, a glycogen synthase defective mutant [29], was used in all experiments. Uridine diphosphate glucose (UDP-[^{14}C]Glc) (256 mCi mmol^{-1}), [^{14}C]oleic acid (50 mCi mmol^{-1}), [^{3}H]mannitol (30 Ci mmol^{-1}) and liquid scintillation supplies were obtained from ICN Radiochemicals (Irvine, CA). Papulacandin B was a gift from Schering-Plough (Bloomfield, NJ). Guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) was obtained from Boehringer Mannheim (Indianapolis, IN). Phospholipase A_2 (PLA_2) (porcine pancreas), phospholipase C (*Bacillus cereus*), phospholipase D (cabbage, type I), serum albumins (fatty acid-free) and all other chemicals were purchased from Sigma (St. Louis, MO). HPLC grade solvents were from Fisher Scientific (Pittsburgh, PA). Growth media was obtained from Difco (Detroit, MI). 1,6-Diphenylhexa-1,3,5-triene (DPH) was obtained from Molecular Probes (Eugene, OR). Nitrocellulose transfer membranes (0.22 μm) were obtained from Micron Separations (Westboro, MA) and Nitex nylon cloth (10 μm mesh) was from Tetko (Elmsford, NY).

2.2. Growth conditions

Cultures of *Saccharomyces cerevisiae* GS-1–36 were maintained on YEPD (1% yeast extract, 2% peptone and 2% dextrose) media plates containing 2% agar. Cells were grown at 30°C in 200 ml of YEPD media for 12 h at 200 rpm. Cells (200 ml) were then transferred to 2 liter of YEPD in a 4 liter flask and grown to mid-log phase (4–6 h) at 30°C and 200 rpm. Cells were harvested by centrifugation at $3000 \times g$ for 10 min.

2.3. Membrane isolation

All steps were conducted as described [14] on ice unless otherwise indicated. Cells were disrupted with a Bead-Beater (Biospec Products Bartlesville, OK). Wet paste (50 g) was suspended in 250 ml of 250 mM sucrose, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride and 70 mM Tris-HCl (pH 7.5) (buffer A) in a 340 ml vessel. Prechilled glass beads (160 g) were added and the vessel was fully filled with buffer A. The Bead-Beater was operated for five 20 s bursts, with 5 min cooling intervals. The crude extract was recovered and the beads were washed with an equal volume of buffer A (minus sucrose). The fractions were combined and centrifuged at $1500 \times g$ for 10 min to remove cell debris.

The microsomal membrane fraction was prepared by differential centrifugation [14]. Mitochondria were removed by centrifugation of the extract at $23\,000 \times g$ for 10 min. Microsomes were recovered by centrifugation of the supernatant at $100\,000 \times g$ for 1 h. The microsomes were resuspended to concentrations of 12–48 mg ml^{-1} in 1 mM EDTA, 1 mM DTT, 33% (v/v) glycerol and 50 mM Tris-HCl (pH 7.5), and were stored at −80°C. Protein was determined by the Coomassie brilliant blue dye-binding method [30] with bovine serum albumin (BSA) as standard. Membrane phospholipid was quantified by determining inorganic phosphate [31].

2.4. Enzyme assay

GS assays were conducted in mixtures of 100 μl containing 0.1 mM GTPγS, 1 mM EDTA, 1.4% (v/v) glycerol, β-lactoglobulin (β-LG), BSA or human serum albumin (HSA) each at 8 mg ml^{-1}, as specified, 75 mM Tris-HCl (pH 8.0), microsomal membranes (60–240 μg protein) and 1 mM UDP-[^{14}C]Glc (0.12 mCi mmol^{-1}) [14]. Mixtures were incubated at 30°C for 30 min and reactions were terminated by spotting mixtures onto Whatman GF/A, 2.4 cm filters under a heat lamp. Ethanol-insoluble [^{14}C]glucan was measured after successive washes with 66% (v/v) ethanol containing 85 μM EDTA, 66% ethanol, and 70% ethanol [32].

2.5. Effects of phospholipase digestion on GS activity

PLA_2 (1 unit (mg protein)$^{-1}$), phospholipase C (20 units mg^{-1}) and phospholipase D (20 units mg^{-1}) were preincubated with 180 μg of membranes in 50 μl of 4 mM $CaCl_2$, 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (Hepes), pH 8.0, (buffer B) at 30°C for 15 min [33]. To stop the reactions, EGTA (2 μl of a 200 mM stock) was added to a level of 8 mM. PLA_2-treated membranes were then combined with

effectors and substrate (above) and assayed for GS activity.

Time-dependence experiments (Fig. 1) were initiated in scaled-up reaction mixtures of 300 μl containing 1.08 mg of protein. For each concentration of phospholipase (0–2.5 units mg^{-1}), 50 μl aliquots were withdrawn at 15 and 30 min, and brought to 8 mM EGTA. Effectors and substrate were then added and residual GS activity determined. Mixtures without added phospholipase served as controls.

2.6. Protective effects of serum albumins and β-LG against PLA_2-induced inhibition

Three types of protection experiments were conducted. In the first type (Fig. 2A), active microsomes (75 μg) in a volume of 50 μl were preincubated with PLA_2 (0 or 1 unit mg^{-1}) in the absence or presence of β-LG or BSA (both at 8 mg ml^{-1}), in buffer B at 25°C. At 13, 22 and 35 min, hydrolysis was terminated by addition of 2 μl of 200 mM EGTA. Following hydrolysis, activity assays were then initiated by addition of effectors (all assay mixtures were brought to 8 mg ml^{-1} BSA) and substrate, and run for 30 min at 30°C. In the second type (Fig. 2B), preheated (at 50°C for 30 min) membranes devoid of endogenous GS activity were incubated with PLA_2. The hydrolysis mixtures (500 μl) contained 3 mg of membranes, 3 units of PLA_2 in buffer B, and were reacted at 30°C for 30 min. Following termination by adding EGTA to 8 mM, aliquots (0–30 μl) were added to GS assay mixtures (containing either β-LG, BSA or HSA at 8 mg ml^{-1}). UDP-Glc was added and reactions were run for 30 min. In the third protection experiment (Fig. 2C), a PLA_2 hydrolysate (prepared as described above), or a corresponding control with PLA_2 omitted, was centrifuged at 7000 × *g* for 30 min at 15°C. To determine if inhibitory moieties were released into the supernatant, aliquots of (0, 10, 40, 80 and 100 μl) were added to 200 μl assay mixtures containing either β-LG, BSA or HSA (8 mg ml^{-1}) and were assayed for GS activity.

2.7. Lipid extraction

Heat-treated membranes (1.2 mg in 0.2 ml), prepared as described above, were digested with PLA_2 (1 unit mg^{-1} for 30 min) and were then extracted with an equal volume of chloroform/methanol (2:1, v/v) [34]. The aqueous phase was extracted again, and the organic layers were pooled and dried under N_2. A parallel sample not subjected to PLA_2 digestion served as control.

2.8. HPLC

Polar lipids (phospholipids) were resolved by HPLC in a Model 660 liquid chromatograph (Waters, Milford, MA) using a 25 cm Waters Partisil 5 microparticulate silica gel column [35]. The column was run at a flow rate of 1 ml min^{-1} for 40 min at room temperature, with a linear gradient of 80% solvent A/20% solvent B, to 100% solvent B. Solvent A consisted of hexane/isopropanol (4:5, v/v) and solvent B hexane/isopropanol/water (6:8:1.5, v/v). Less polar lipid species (e.g., triglycerides (TGs), diglycerides (DGs), monoglycerides (MGs) and FAs were separated using a 12.5 cm version of the above column [35]. The column was run at a flow rate of 1 ml min^{-1} for 40 min at room temperature, with a linear gradient of 90% hexane/10% solvent C to 100% solvent C. Solvent C consisted of 0.5% acetic acid in diethyl ether [35]. Peaks were detected using a mass detector (ACS model 750/1, Peris Industries, State College, PA) with nebulizer temperatures settings of 45°C (polar lipids) and 40°C (non-polar lipids) at an air pressure of 20 PSIG. Peak areas were integrated using a Varian CDS 401 chromatography data system. Lipid peaks were tentatively identified by comparison of retention times with authentic lipid standards. To screen for inhibition, HPLC fractions were recovered, concentrated, and then added back to GS assay mixtures. Chemical identities of inhibitory fractions were then determined by mass spectrometry [36].

2.9. Inhibitor screening and kinetics

IC_{50} values were obtained by adding selected compounds in either water or dimethylsulfoxide (DMSO) (to 5% v/v) to standard assay mixtures containing 240 μg of membranes and 8 mg ml^{-1} β-LG. The mixtures were held for 20 min on ice and reactions were initiated by addition of substrate and allowed to proceed for 30 min at 30°C. DMSO at levels of 5% was non-inhibitory. IC_{50} values were obtained from plots of residual activity vs. the log of inhibitor concentration.

2.10. Fluorescence anisotropy

Steady-state fluorescence anisotropy was measured at ambient temperature (22 ± 2°C) using microsomes derived from cells grown for 6 h as described [37] with minor modifications. Membranes, with a phospholipid content of 1.23 mg phospholipid per mg protein [31], were adjusted to an A_{360} of 0.1 with 40 mM Tris-HCl (pH 7.4) and 100 mM NaCl (TBS buffer) [38]. DPH (stock conc. 0.3 mM in DMSO) was added to 0.2 μM (the phospholipid to DPH molar ratio maintained at 300:1). DPH at concentration of 0.2 μM was not inhibitory. The DPH/membrane mixtures were equilibrated for 30 min. Stock solutions of the tested compounds dissolved in DMSO were then added (final volume 3 ml), and DMSO at its final concentration of 0.2% had no effect on anisotropy values. Fluorescence

measurements were taken in a SPEX F1T11 spectrofluorimeter (SPEX Industries, Metuchen, NJ) equipped with Glan-Thompson polarization optics with excitation and emission wavelengths of 360 and 430 nm (both with 3.8 nm bandwidth), respectively.

Fluorescence anisotropy (r) was calculated by the following equation:

$$r = (I_{VV} - G \cdot I_{VH})/(I_{VV} + 2G \cdot I_{VH})$$

where $G = I_{HV}/I_{HH}$, I_{VV} and I_{VH} are the fluorescence intensities determined at vertical and horizontal orientations of the emission polarizer, when the excitation is set in the vertical position. I_{HV} and I_{HH} are fluorescence intensities determined at the vertical and horizontal positions of the emission polarizer when the excitation polarizer is set horizontally. G is a correction factor for the differential responses of the monochromators and detectors to vertically and horizontally polarized light. Fluorescence intensities were corrected for background and light scattering from the unlabeled sample [37].

2.11. Binding study

FA binding analysis was conducted using a microcentrifugation-filtration method [39]. In direct binding experiments, binding mixtures of 360 μl contained 1.08 mg microsomes, [^{14}C]oleic acid (0, 6.25, 12.5, 20, 40, 80 μM), 8 mg ml^{-1} β-LG, and other cofactors normally present in GS reaction mixtures. Each binding mixture also contained [^{3}H]mannitol (3.75 μCi ml^{-1}) which was dried into each tube from an ethanol stock solution prior to addition of the above components. In displacement experiments each tube contained a 10- or 50-fold excess of [^{12}C]oleic acid, with respect to the indicated [^{14}C]oleic acid levels. After preincubation of binding mixtures on ice for 45 min, four aliquots (80 μl each) were withdrawn and pipetted into the microfilter device. This device, mounted within a 0.4 ml centrifuge tube (3.7 × 45 mm), consisted of a 1 ml syringe barrel (cut at 0.5 ml mark) serving as an upper reservoir, a nitrocellulose filter (0.22 μm) seated on a 0.5 cm^2 nylon cloth and a 0.4 ml microcentrifugation tube serving as the lower reservoir. Each was centrifuged at 9000 × g for 30 s and the nitrocellulose discs were then suspended in 500 μl of a 1% Triton solution, shaken for 30 min at 250 rpm, and counted for ^{3}H and ^{14}C on a Beckman LS-3801 scintillation counter. [^{3}H]Mannitol retained on the filter was used to calculate the volume of liquid trapped on the filter. Net [^{14}C]oleic acid binding was calculated as follows:

Trapped volume fraction = Filter ^{3}H/Total ^{3}H

Background ^{14}C = Trapped volume fraction × Total ^{14}C

Net ^{14}C on filter = Filter ^{14}C − Background ^{14}C

3. Results

3.1. Phospholipase-induced inactivation of (1,3)-β-GS

Microsomal membranes from yeast were incubated with phospholipases A_2, C and D. PLA_2, which is Ca^{2+}-dependent [40] and cleaves FAs from the second position of phospholipids, gave almost total inhibition of GS at 1 unit of enzyme mg^{-1} of protein (Table 1). EDTA protected against PLA_2-induced inactivation, since more than 85% of GS activity remained in its presence. Protection by EDTA indicates that inhibition is due to the catalytic activity of PLA_2, rather than due to non-specific-interaction of PLA_2 with the GS complex, as was the case with prothrombinase [41]. Under similar conditions, phospholipase C which cleaves the ester bond at the third position of glycerol, inhibited GS but not as strongly as PLA_2, since 20% activity still remained when phospholipase C levels were 20-fold higher than PLA_2. Phospholipase D exhibited no inhibition, even at 20 U mg^{-1}. Since PLA_2 is most inhibitory, it was used for all subsequent experiments.

Inactivation of GS by PLA_2 was both concentration- and time-dependent (Fig. 1). Incubation of PLA_2 with 1 unit mg^{-1} of membrane protein caused almost 100% activity loss after incubation for 15 min and is consistent with Table 1. The marginal stability of yeast GS is apparent here, since controls, incubated without PLA_2, lost about 15–20% of their initial activity within the 30 min incubation period.

3.2. Protection against inactivation by PLA_2 and papulacandin B by serum albumins

To determine if inhibition was the result of FA release from the membrane, three types of protection experiments were conducted. In the first type, protectants (BSA and β-LG), membranes and PLA_2 were combined in a single mixture. To show that FAs play a role in the inhibition, BSA, a FA-binding protein [42] should sequester FAs therefore protecting against inactivation. On the other hand, β-LG, a retinol-binding

Table 1
Effect of various phospholipases on GS activity

Phospholipase	Units mg^{-1}	Residual activity (%)
None		100.0
A2	1	0.02
	10 + EDTA	85.3
C	20	19.6
D	20	96.9

Incubation conditions are described under Materials and methods. For the EDTA protection experiment, 4 mM EDTA was incubated with 10 units mg^{-1} PLA_2. Incubations were conducted at 30°C for 15 min.

protein [43], should have minimal protective effects. When directly compared (Fig. 2A), this was indeed the case. Relative to the PLA_2 treated sample without any protectant, β-LG afforded little or no protection against inactivation while BSA, in contrast, provided approximately 50% protection.

The protective effects of albumin were confirmed in additional experiments where inhibitors were generated from membranes which had been exposed to a mild heat-treatment in order to eliminate any endogenous GS activity. When the hydrolysates were added back to assay mixtures containing active GS, dose-dependent inactivation was observed, with maximal inhibition in the presence of β-LG (Fig. 2B). Relative to β-LG, significant protection was provided (50–65%) by either BSA or HSA [44], relative to β-LG. When the supernatant from the hydrolysate was recovered by high-speed centrifugation, BSA and HSA both gave complete protection against PLA_2-induced inhibition (Fig. 2C).

Protection experiments were also conducted with papulacandin B, an inhibitor of yeast GS which contains a lipophilic acyl side chain [21]. Papulacandin B was screened for GS inhibition in the absence (β-LG control) and presence of BSA. Partial protection, ranging from 10 to 20%, was observed at concentrations less than 0.3 mM (Fig. 3A). BSA significantly increased the IC_{50} value of papulacandin B (16 to 44 μM). Serum albumins also provided protection of in vitro reaction mixtures against inactivation by FAs and lysophospholipids (LPL), both of which are PLA_2 reaction products. With BSA present, the IC_{50} value of lysophosphatidylcholine (LPC) increased from 120 to 460 μM (Fig. 3B).

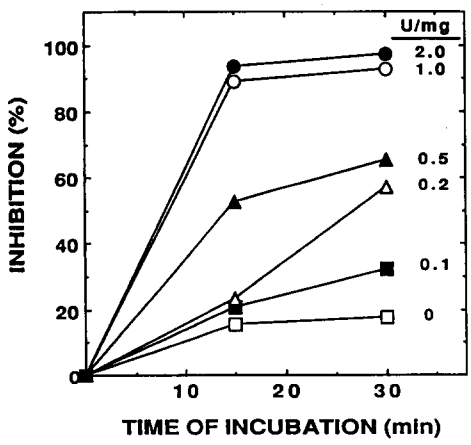

Fig. 1. Time- and concentration-dependence of PLA_2 inhibition. Membranes were preincubated with various amounts of PLA_2 and GS activity was determined as indicated under Materials and methods.

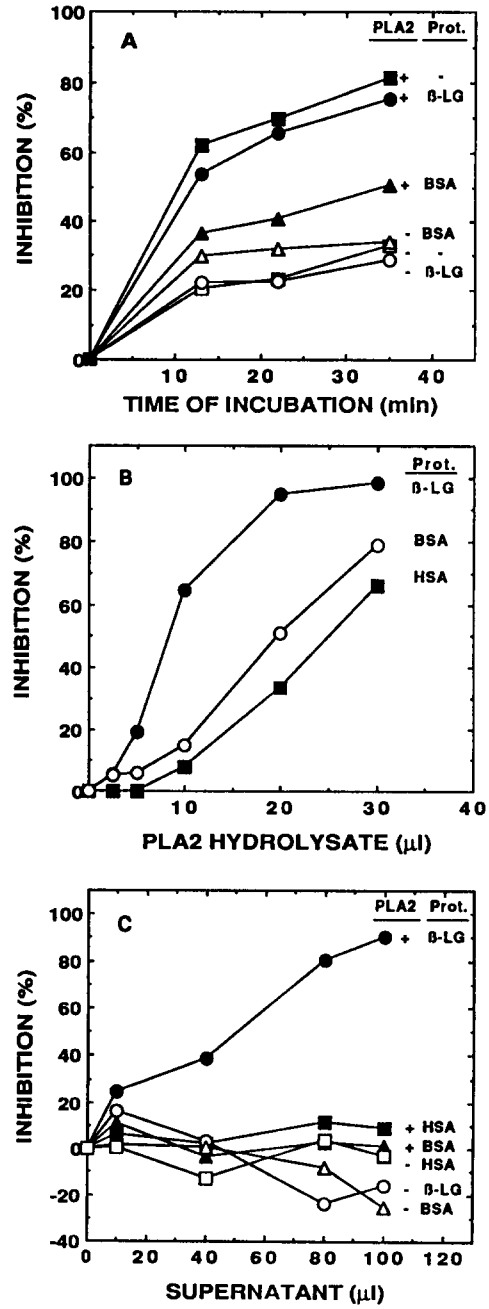

Fig. 2. Protective effects of serum albumins and β-LG against PLA_2-induced inhibition. (A) Protective effects during time course of PLA_2 digestion. Membranes were incubated with various combinations of PLA_2, and the protectants BSA and β-LG and assayed as indicated under Materials and methods. (B) Membranes in which GS was heat-inactivated were hydrolyzed with PLA_2 as indicated under Materials and methods and the hydrolysates were added to GS reaction mixtures containing either β-LG, BSA or HSA. (C) The supernatant from the PLA_2 hydrolysate was recovered by centrifugation as indicated under Materials and methods and aliquots were added to assay mixtures containing either β-LG, BSA or HSA.

3.3. Structural characterization of PLA_2-released products

HPLC of lipid extracts from PLA_2-treated membranes demonstrated extensive hydrolysis of each of

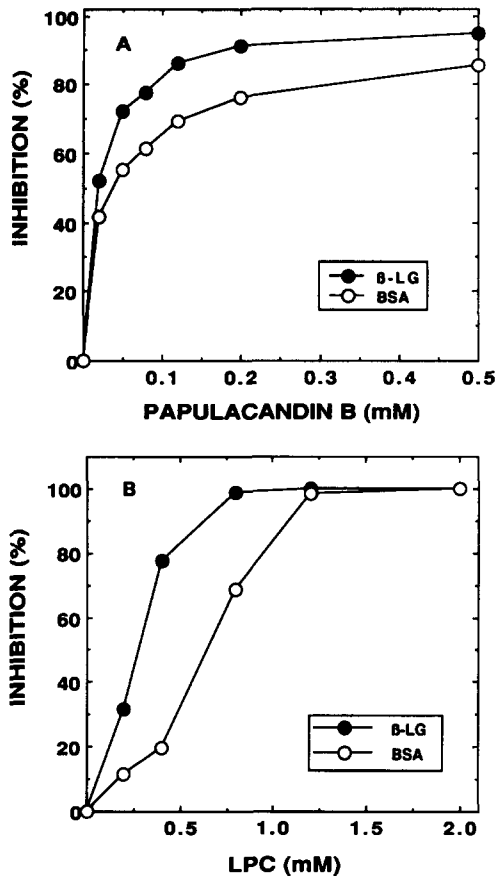

Fig. 3. Protection against GS inactivation by BSA. Dose dependence of papulacandin B (Panel A) and LPC (Panel B) in GS assays containing either β-LG or BSA, as indicated.

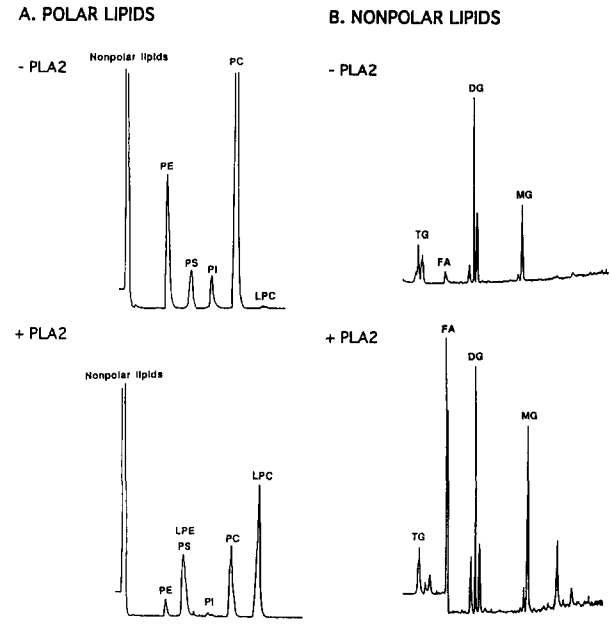

Fig. 4. Fractionation of polar and non-polar membrane lipid components by HPLC following PLA_2 digestion. (A) Polar lipids. Positions of the major phospholipids and lysophospholipids are indicated. (B) Nonpolar lipids. Positions of TGs, FAs, DGs and MGs are indicated. Top panels: untreated controls; bottom: PLA_2-treated samples.

the major membrane phospholipids (Fig. 4), coupled with the appearance of FAs, MGs and LPLs. Mass spectra of reaction products yielded commonly occurring membrane lipid components such as palmitic acid, palmitioleic acid, stearic acid, oleic acid, LPC, lysophosphatidylethanolamine (LPE) and MG. No reaction products with novel or unique structures [45,46] were identified. The phospholipid composition of the microsomal fraction from strain GS-1–36 (60.3% phosphatidylcholine (PC), 24.8% phosphatidylethanolamine (PE), 8.7% phosphatidylserine (PS) and 6.1% phosphatidylinositol (PI)) is consistent with values obtained for wild-type strains [47]. The HPLC peaks were dried, resuspended and added back to standard GS assay

Table 2
Inhibitor screening table

Compound			IC_{50} (μM)	Compound		IC_{50} (μM)
Fatty acids		16:0	20	*1-Monoglycerides-*	14:0	190
		16:1	90		16:0	> 200
		18:0	100		16:1	200
		18:1	80		18:0	> 200
		18:2	30		18:1	160
		20:4	140		18:2	120
		22:6	60			
Lysophospholipids	LPC-	14:0	160	*2-Monoglyceride-*	16:0	200
	LPC-	16:0	80			
	LPC-	18:0	420	*Miscellaneous*		
	LPC-	18:1	90		Papulacandin B	40
	LPC-	PAF	100		Platelet activating	325
	LPE-	16:0	400		factor (PAF)	
	LPS-	18:0	1000			
	LPI-	16:0,18:0	600		DG	< 4000

IC_{50} values are defined as concentrations inhibiting 50% of GS activity in assay mixtures containing 240 μg of protein, 8 mg ml^{-1} β-LG and 1 mM UDP-[^{14}C]Glc assayed for 30 min at 30°C.

mixtures. Most were found to be inhibitory, therefore screening of FAs, LPLs and MGs of defined structure was conducted in order to determine relative IC_{50} values.

3.4. Inhibitor screening

To determine if any specific structural requirement was necessary for GS inhibition, a wide range of compounds were screened for GS inhibition. In the presence of β-LG, IC_{50} values were generally in the micromolar range (Table 2). FAs were the most inhibitory lipid species, with little variation between the species tested. IC_{50} values were generally comparable with earlier screenings [26]. Of the LPLs tested, LPC (16:0 and 18:1) and LPE were more inhibitory than lysophosphatidylinositol (LPI) or lysophosphatidylserine (LPS). A range of MGs were also inhibitory, however as with the other classes, no discernible pattern of specificity was evident.

3.5. Effects of lipophilic compounds on membrane fluidity

The inhibition of yeast GS by a wide range of lipophilic compounds as well as detergents [14] suggests that the enzyme is highly sensitive to alterations in the membrane environment. To address this question, the effects of lipophilic compounds on membrane fluidity were determined by fluorescence anisotropy measurements using the probe DPH. DPH is a highly hydrophobic probe that inserts into the membrane bilayer and has been used to assess the lipid mobility in many kinds of membranes, including yeast [37,47,48]. The fluorescence anisotropy or r value is taken as an index of membrane fluidity, with decreasing r values indicating increases in membrane fluidity. Oleic acid, 1-MG (18:1) and LPC (18:1), each of which inhibits GS (Table 2) caused dose-dependent decreases in anisotropy (r) values, which is reflective of a decrease in membrane order (Fig. 5). However, the magnitude of these decreases do not strictly correlate with the IC_{50} values reported in Table 2. Interestingly, the DG (1,2 18:1) which does not inhibit GS, had no effect on r values. It was not possible to interpret similar experiments with papulacandcin B, cilofungin, BSA or β-LG, since these compounds appeared to displace DPH from the membranes.

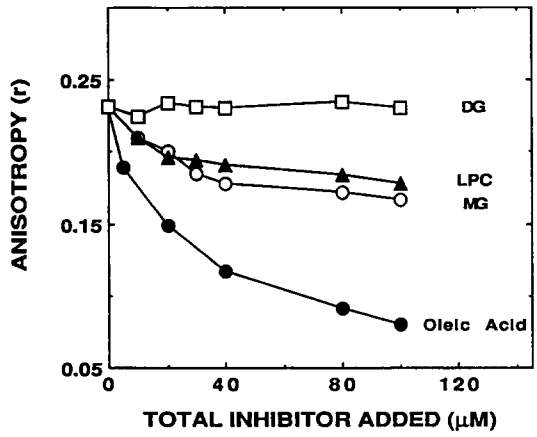

Fig. 5. FA and lipophilic compounds-induced effects on membrane fluidity. Yeast membranes were equilibrated with 0.2 μM DPH for 30 min (phospholipid to DPH ratio: 300 to 1) and incubated with FAs or lipophilic compounds at the levels indicated. Compound (□) 1,2-18:1 DG, (▲) LPC-18:1, (○) 1-MG-18:1, and (●) oleic acid.

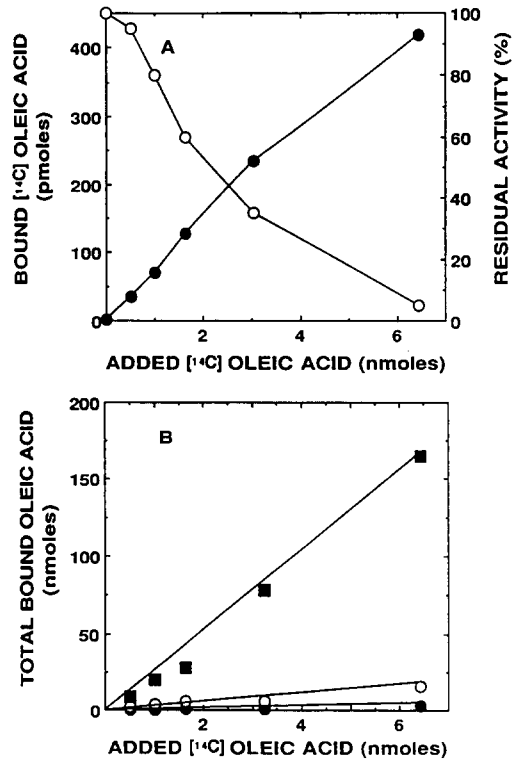

Fig. 6. Correlation of [^{14}C]oleic acid binding with the loss of GS activity and kinetics of [^{14}C]oleic acid binding. (A) Oleic acid binding vs. GS activity. Symbols: ○, residual activity; ●, bound [^{14}C]oleic acid. (B) Effect of increasing levels of unlabeled oleic acid on the binding of [^{14}C]oleic acid. Symbols: ●, binding of [^{14}C]oleic acid at levels indicated on the abscissa; ○, binding of [^{14}C]oleic acid with a 10-fold excess of unlabeled oleic acid relative to the indicated levels of added [^{14}C]oleic acid; ■, 50-fold excess as above.

3.6. Fatty acid binding to yeast membranes

To further assess possible inter-relationships between FA binding to membranes and GS activity, binding experiments were conducted with [^{14}C]oleic acid. The binding of [^{14}C]oleic acid over the range of 6.3–80 μM strongly correlated with the loss of GS activity (Fig. 6A). As expected from studies of the binding of oleic acid to cytotoxic T lymphocytes [49], displacement experiments confirm that binding of [^{14}C]oleic acid

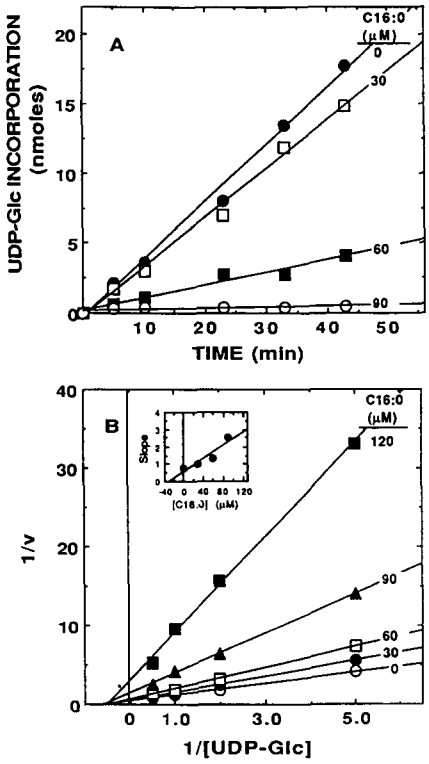

Fig. 7. Kinetics of GS inhibition by palmitic acid. (A) Time-dependence of ethanol insoluble product formation in the presence of the indicated levels of added palmitic acid. (B) Lineweaver-Burk plot showing GS inhibition by palmitic acid as a function of UDP-Glc concentration. The inset is a secondary plot of slope versus inhibitor concentration. The K_m for UDP-Glc was 1.3 mM, and the K_i for palmitic acid was 35 μM.

occurs by non-specific insertion into the membrane, rather than by interaction with specific membrane proteins such as GS. In fact, preincubation of membranes with an excess of unlabeled oleic acid (10–50-fold) resulted in greatly enhanced binding of oleic acid (^{12}C and ^{14}C) to the membrane (Fig. 6B). Furthermore, binding was not saturable. Since FAs rapidly partition between the aqueous phase and the membrane [50], FA binding is not saturable until the critical micelle concentration (CMC) is reached.

3.7. Kinetics of fatty acid inhibition

Inhibition kinetics with palmitic acid further substantiates the non-specific nature of GS inhibition. At all levels of added palmitic acid, reaction rates remained linear (Fig. 7A); however, kinetic constants derived from Lineweaver-Burk double reciprocal plots (Fig. 7B) show that increasing amounts of palmitic acid resulted in a progressive decrease in V_{max}, with little change in K_m (1.3 mM), demonstrating non-competitive inhibition (apparent K_i value, 35 μM, Fig. 7B, inset). Each of the other compounds (oleic acid, LPC (18:0), and 1-MG (18:2) also exhibited noncompetitive inhibition (data not shown).

4. Discussion

Over the past two decades, a broad range of GS inhibitors have been identified, which include potential antifungal drugs [15,19,21], detergents [14] and FAs [26]. However, the mechanism by which GS is inhibited has not been extensively investigated, and it has been speculated that GS inhibition by a variety of compounds goes beyond direct interaction between the target enzyme and the inhibitor, pointing rather towards a complex type of interaction between the inhibitor and the membrane [24,27,28]. This study provides further evidence suggesting that yeast GS is highly sensitive to perturbations of the membrane environment. The importance of membrane fluidity and related biophysical properties with regard to drug-membrane interactions is becoming increasingly recognized [51,52].

We initially focused on phospholipase-induced inactivation of yeast GS. The observation that PLA_2-induced inactivation could be protected against by FA-binding proteins such as BSA provided preliminary indication that FAs and possibly LPLs were directly responsible for GS inactivation. Mechanisms of inactivation were then further probed. First, reaction products were analyzed to determine whether any unusual or novel [45,46] PLA_2-induced reaction products contributed to GS inactivation; reaction products were all commonly occurring lipid components. A variety of FAs, MGs and LPLs were then screened (Table 2) to determine if structural specificity could be determined and the results clearly indicated that inhibition was largely non-specific. Since the intercalation of FAs and other lipophilic compounds into the membrane environment is well documented [50,53], our results are consistent with the notion that GS activity declines in response to minor perturbations of the phospholipid bilayer, such as the insertion of FAs into the membrane (Fig. 6A). Thus, the lipophilic side chains of GS inhibitors such as cilofungin [25] or papulacandin [23], which are essential for inhibition, may function in part by affecting membrane fluidity. A study of GS inhibition by papulacandin B [24] concluded that 'in addition to some inhibitory action on GS, papulacandin B has a hitherto unknown effect on some other essential function of the fungal cell.' Additional evidence supporting the importance of membrane integrity as a regulator of GS derives from a study showing that aculeacin A resistant mutants of *Candida albicans* possess altered membrane lipid compositions [54].

The effects of FAs on membrane fluidity were then investigated (Fig. 5) using DPH, an intrinsic fluorescent probe which seats deeply within the hydrophobic core of the membrane bilayer. Incubation of membranes with either FAs, MGs or LPCs resulted in a concentration-dependent decrease in fluidity (Fig. 5),

which is consistent with morphologically observed deterioration of vesicular structure and permeability induced by FAs [53]. Several points are worth noting. First, at the highest concentration of oleic acid utilized here (100 μM), most becomes associated with the membrane [49]. Only a small amount (approx. 5 nM), remains free, which is well below the CMC of 6 μM [55]. Thus, it is unlikely that oleic acid micelles draw DPH out of the membrane. Interestingly, DG, which is non-inhibitory (Table 2), was unable to alter apparent membrane fluidity. Although a mixed microsomal fraction was used for this experiment, we believe that purified plasma membranes would behave similarly. The poor stability of GS precluded the use of more highly-purified membranes in this study.

Another potential issue with lipophilic drugs arising from this study centers on the high affinity of serum albumins for FA-containing compounds. For example, most fungal GS assay mixtures contain a proteinaceous activator. BSA has been most widely utilized [4,24], however, β-LG is an equally effective alternative [14]. Whereas BSA is a strong FA-binding protein [38,56,57], β-LG is not, although β-LG does have a strong binding affinity for retinols [43]. We therefore hypothesized that IC_{50} values of compounds such as papulacandin B assayed with β-LG would be significantly decreased relative to assays conducted with BSA. Fig. 3 confirms that BSA significantly protects against inhibition by papulacandin B and LPC, in addition to FAs. Thus, serum albumins can significantly mask the presence of, and lower the efficacy of acyl-chain-containing antifungal drugs targeted against membrane-bound enzymes.

In light of the lipophilic nature of antifungals targeted against GS, this study supports suggestions that the membrane environment plays a central role in the modulation of fungal GS activity. To test this hypothesis, it will be necessary to purify and reconstitute this enzyme. Because GS from *S. cerevisiae* has not been purified despite years of extensive solubilization efforts, methodologies to study this enzyme in situ will become increasingly important. Purification of the GS complex is further complicated by the presence of a peripheral GTP-binding subunit [4,5]. Since it is critically important to eliminate side effects from potential antifungal drugs, quantification of the effects of selected compounds on parameters such as membrane fluidity, could represent an important step in pre-clinical drug screening efforts.

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