Characterization of (1,3)- β -glucan synthase in *Candida albicans*: microsomal assay from the yeast or mycelial morphological forms and a permeabilized whole-cell assay

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A systematic evaluation of the *in vitro* (1,3)- β -glucan synthase assay parameters was performed using microsomes prepared from *Candida albicans* from either yeast or mycelial phase cells. Enzyme activities of both yeast and mycelial phase microsomes depended on the presence of guanosine-5'-O-(3-thiophosphate) and either bovine serum albumin or a detergent [W-1 (polyoxyethylene ether detergent) or Brij-35 (polyoxyethylene ether, 23 lauryl ether)]. Brij-35 was included in standard assays as it was compatible with the permeabilized whole-cell assay. Microsomes derived from both the yeast and mycelial phases generally yielded similar glucan synthase activities under a range of different assay conditions. Brij-35 significantly stabilized the enzyme, yielding a half-life of 5.6 d at 4 °C, compared with 0.9 d without detergent. The addition of detergent during mechanical breakage of yeast cells dramatically improved glucan synthase stability and activity. Enzyme catalysis was linear for at least 75 min with 100 μ g protein from microsomes of yeast cells grown to mid-exponential phase, with an apparent K_m for UDP-glucose of 1.1 mM. The pH and temperature optima were 7.75 and 30 °C, respectively. Glucan synthase activity was highest in cells derived from early mid-exponential phase and declined to a basal level by stationary phase. A permeabilization-based *in situ* assay for glucan synthase was developed. Cells were permeabilized with 2% (v/v) solution of toluene/methanol (1:1) and assayed for glucan synthase activity using standard reaction mixtures. Reactions were linear for 30 min and were inhibited by known inhibitors of glucan synthesis. This study represents the first characterization of glucan synthase using yeast and mycelial phase microsomes and adaptation of a permeabilized system using a single *C. albicans* strain with standardized assay conditions.

Keywords: *Candida albicans*, (1,3)- β -glucan synthase, permeabilized whole-cell assay

INTRODUCTION

Fungal infections from *Candida albicans* and other fungi pose a serious risk to immunocompromised individuals such as human immunodeficiency virus (HIV) patients and cancer patients rendered neutropenic by chemotherapy. Currently available fungicidal drugs are only marginally effective and new compounds are actively being sought (St Georgiev, 1988). The fungal cell wall serves as a protective barrier and is essential for viability

in a hypotonic environment. A major component of the *C. albicans* cell wall is (1,3)- β -glucan, and since the enzyme that biosynthesizes this polymer is not present in higher eukaryotes, it represents an ideal target for the development of antifungal agents.

(1,3)- β -Glucan synthase (EC 2.4.1.34) is a plasma-membrane-localized enzyme that transfers glucose from UDP-glucose to a growing polymer of (1,3)- β -glucan. GTP is an important activator of cell-free glucan synthase preparations and probably plays a critical role in the regulation of cell-wall biosynthesis and cell growth (Kang & Cabib, 1986; Shematek & Cabib, 1980; Szaniszló *et al.*, 1985). In addition to GTP, many other effectors must be

Abbreviations: UDP-glucose, uridine diphosphate glucose; BSA, bovine serum albumin; GTP γ S, guanosine-5'-O-(3-thiophosphate); Brij-35, polyoxyethylene ether, 23 lauryl ether; W-1, polyoxyethylene ether detergent.

added to microsomal glucan synthase assays to maximize *in vitro* catalytic activity. BSA activates the enzyme from *Saccharomyces cerevisiae* (Shematek *et al.*, 1980), *C. albicans* (Orlean, 1982) and from other fungal sources (Ruiz-Herrera, 1992). BSA could be substituted with β -lactoglobulin in *S. cerevisiae* (Frost *et al.*, 1992). Recently, the detergent W-1 was reported to activate the *C. albicans* enzyme (Tang & Parr, 1991). NaF and ATP have also been added to *in vitro* assays as activators (Shematek *et al.*, 1980; Orlean & Ward, 1983). Direct comparisons of activating factors for glucan synthases in *C. albicans* have not been conducted for the yeast and mycelial forms. Both forms are important in pathogenicity (Odds, 1988). Assay conditions as well as sensitivity to drugs targeted against glucan biosynthesis could vary depending on the morphological form of the organism.

The substrate for (1,3)- β -glucan synthase, UDP-glucose, does not diffuse through intact membranes; thus the plasma membrane must be permeabilized to develop a whole-cell-based assay. Organic solvents have been used to permeabilize fungal cells (Ram *et al.*, 1983; Sentandreu *et al.*, 1975). In this paper we have modified the organic solvent permeabilization procedure to obtain active glucan synthase preparations which incorporate added UDP-glucose into (1,3)- β -glucan product. Assay conditions for glucan synthase utilizing cell-free extracts from both the yeast and mycelial forms as well as a whole-cell *in situ* assay system using a single *C. albicans* strain (CCH 442) are described. Structure-activity relationships of glucan synthesis inhibitors derived from these assay systems can then be compared to antifungal activity levels *in vitro* and efficacy *in vivo* in mouse pathogenicity models.

METHODS

***C. albicans* cultures.** The ATCC strains were obtained from the American Type Culture Collection, Rockville, MD, USA. All other strains were obtained as clinical isolates from Cook County Hospital (CCH) and are standard cultures at Abbott Laboratories.

Culture conditions. Yeast cells (*C. albicans* CCH 442) were inoculated into 375 ml YEPD medium [1% (w/v) yeast extract (Difco), 2% (w/v) peptone (Difco), and 2% (w/v) glucose] and shaken at 30 °C for 24 h. Cells were then transferred to 15 l YEPD medium and grown to the mid-exponential phase (OD_{600} 2.5). The mycelial form was obtained according to the method described by Lee *et al.* (1975) with some modifications. Cells were grown in Lee's medium at 25 °C for 48 h on a rotary shaker at 200 r.p.m. A 16 ml portion of the preculture was used to inoculate 166 ml prewarmed (37 °C) medium. The cells were incubated as above at 37 °C for 6–8 h. Microscopic examination of the cultures revealed that over 98% of the cells contained at least a germ tube by 4 h and mycelium by 6.5 h. Incubations longer than 8 h gave increasingly higher proportions of yeast present in the culture. Both yeast and mycelia were harvested by centrifugation at 3000 *g* for 10 min.

Isolation of microsomal membranes. Cells were disrupted with a Bead-Beater, 340 ml vessel size (Biospec Products). Cell paste (70–90 g wet wt) and 160 g glass beads (0.5 mm) were suspended in 250 mM sucrose, 3 mM EDTA, 3 mM EGTA, 4 mM DTT, 20 mM β -mercaptoethanol, 25 μ M GTP and 70 mM Tris/HCl, pH 8.0 (homogenization buffer). The Bead-Beater was operated

five times with 30 s bursts and 5 min cooling intervals. Crude extracts were recovered and beads were washed with an equal volume of 1 mM EDTA, 1 mM EGTA, 4 mM DTT, 25 μ M GTP and 50 mM Tris/HCl, pH 8.0 (washing buffer). Fractions were combined and centrifuged at 1000 *g* for 10 min to remove cell debris. The suspension was then centrifuged at 23000 *g* for 10 min. To recover microsomal membranes the resultant supernatant was recentrifuged at 80000 *g* for 1 h at 4 °C. Microsomal membranes were then resuspended in 1.0 mM EDTA, 1.0 mM DTT, 33% (v/v) glycerol and 50 mM Tris/HCl, pH 7.5 (storage buffer) for storage at –80 °C. Protein was assayed by Coomassie blue dye-binding with BSA as standard (Bradford, 1976).

(1,3)- β -Glucan synthase assay. Unless otherwise stated, the standard assays were performed in a final volume of 100 μ l 2.0 mM UDP-[¹⁴C]glucose (0.625 mCi mmol⁻¹), 1 mM EDTA, 8% (v/v) glycerol, 20 μ M GTP γ S, 0.5% Brij-35, 80 mM Tris/HCl, pH 7.75, and enzyme (100 μ g protein). Assay mixtures were incubated for 30 min at 30 °C and stopped by addition of 100 μ l (w/v) 10% trichloroacetic acid. Samples were transferred to a 96-well MilliBlot-D (Millipore) containing a Type G-10 glass-fibre filter (Inotech). Each well was washed three times with 300 μ l distilled deionized water. The filters were removed, air-dried and counted on a direct beta counter (Matrix-9600, Packard Instruments). Units of activity (U) are defined as nmol glucose incorporated into trichloroacetic acid-insoluble glucan per min.

Growth phase study and strain comparison. A 5 ml overnight culture of each strain was used to inoculate 200 ml batches of YEPD which were shaken at 30 °C. At defined intervals (Fig. 1) cells were harvested by centrifugation. Cells were disrupted with glass beads using a Mini-bead Beater (Biospec Products). Paste (0.6 g wet wt) was added to 1.85 g chilled glass beads (0.5 mm) and then suspended in homogenization buffer. The Bead-Beater was operated six times with 20 s bursts and 2 min cooling intervals. Crude extracts were recovered, the beads were washed with an equal volume of washing buffer and combined fractions were centrifuged at 1000 *g* for 10 min to remove cell debris. Supernatants were then centrifuged at 40000 *g* for 45 min. Resultant pellets (particulate samples) were resuspended in storage buffer.

Permeabilized whole-cell glucan synthase assay. The *in situ* assay for (1,3)- β -D-glucan synthesis was based on previous work of Ram *et al.* (1983). Cells were grown to an OD_{600} of 2.0 in YEPD (300 ml). The cells were centrifuged at 8000 *g* for 5 min, resuspended in 4 ml storage buffer and recentrifuged at 3000 *g* for 5 min at room temperature. The pellets were resuspended in 4 ml storage buffer and permeabilized with 2% (v/v) toluene/methanol (1:1) at 22 °C for 5 min. A nonpermeabilized control was also conducted. The samples were washed twice with storage buffer at 4 °C and resuspended to 6 ml with storage buffer (4 °C). Glucan synthase assays were performed as described above except that 58 μ l permeabilized cells (5×10^7 c.f.u., 3–4 mg dry wt) were used in the assay. Non-permeabilized cells were used as baseline controls. Assay mixtures were incubated for 30 min at 30 °C and stopped by addition of 100 μ l 10% (w/v) trichloroacetic acid. Precipitates were placed on GF/F glass-fibre filters (Whatman) and washed successively with 3 $\times$ 5 ml cold 5% (w/v) trichloroacetic acid and cold 95% (v/v) ethanol (1 $\times$ 5 ml). The radioactivity was determined with a liquid scintillation spectrometer. All data were expressed as nmol glucose incorporated per mg dry cells. To determine dry weight, 58 μ l cells were diluted in 1 ml of water and the cells were then trapped on GF/F filters and dried in a microwave oven at high setting with six 40 s bursts (Koliander & Hampel, 1989).

Table 1. Activity of microsomal glucan synthase fractions derived from yeast and mycelia (CCH 442)

Assay conditions*								Relative activity (%)†	
GTP	Brij-35	W-1 (%)		BSA	ATP	NaF (mM)		Yeast	Mycelium
		0.025	0.50			10	100		
—	—	—	—	—	—	—	—	0	0
+	—	—	—	—	—	—	—	1.0	5.6
+	+	—	—	—	—	—	—	100.0	100.0
+	—	+	—	—	—	—	—	4.5	54.2
+	—	—	+	—	—	—	—	42.2	52.4
+	—	—	—	+	—	—	—	69.2	47.9
+	—	—	—	+	—	+	—	76.5	48.3
+	—	—	—	+	—	—	+	61.6	60.0
—	—	—	—	+	+	—	—	38.0	10.8

* Effector concentrations added were 20 μ M GTP γ S, 0.5% Brij-35, 0.8% BSA and 5 mM ATP. Products were washed on 2.5 cm Whatman GF/A glass-fibre filters.

† Maximal specific activities were 2.89 and 2.86 nmol min⁻¹ (mg protein)⁻¹ for the yeast and mycelial samples, respectively.

Product characterization. The glucan synthase reaction mixtures which were labelled with UDP-[¹⁴C]glucose (250 μ l reaction volume) were heat-terminated and mixed with 5 vols distilled deionized water. Samples were centrifuged at 15000 *g* for 15 min at 4 °C and pellets were washed with distilled deionized water. Pellets were resuspended in either (1) α -amylase (1 mg ml⁻¹) (Sigma) in 50 mM Tris/HCl, pH 7.5, (2) Zymolyase 100T (80 μ g ml⁻¹) (ICN Biochemicals) in 50 mM MES/NaOH, pH 6.5 and 1 mM DTT, (3) pronase (50 μ g ml⁻¹) (Calbiochem) in 50 mM Tris/HCl, pH 7.5, or (4) laminarinase, mollusk (1 mg ml⁻¹) (Sigma) in 50 mM sodium acetate, pH 5.5, to a final volume of 200 μ l. Mixtures were incubated for 20 h and the samples were washed and counted on glass-fibre filters as described for the standard enzyme assays.

Following reaction with UDP-[¹⁴C]glucose, permeabilized cells (200–300 mg wet wt) were washed and resuspended in 0.3 ml 20 mM sodium phosphate, pH 7.0, 6 mM sodium chloride buffer (PBS). The cells were disrupted by vortex mixing in a glass tube with 0.5 mm glass beads (4 °C). Lysed fractions were centrifuged and the resulting pellets were dissolved in 2% SDS in 10 mM Tris/HCl, pH 7.0, and heated at 100 °C for 5 min to solubilize mannoproteins. The SDS-insoluble material was incubated with (1) α -amylase (1 mg ml⁻¹) in PBS, (2) proteinase K (1 mg ml⁻¹) in PBS, or (3) Zymolyase-100T (100 units) in sodium acetate, pH 5.5 in a final volume of 500 μ l at 30 °C for 17–24 h. Samples were centrifuged and ¹⁴C radioactivity remaining in each fraction was determined.

Reproducibility of results. All *in vitro* experiments were repeated at least twice with different batches of microsomal membranes. Mean values with standard deviations are given in each table and figure as representative values from experiments.

RESULTS

Activity of different strains

Several *C. albicans* strains were evaluated for glucan synthase activities. Strain ATCC 10231 had the lowest activity [7.9 ± 0.65 U (mg protein)⁻¹, *n* = 4] while strain

ATCC 38247 had the highest activity [35.6 ± 0.91 U (mg protein)⁻¹, *n* = 4]. Except for strain ATCC 38247, the other strains were either clinical isolates or ones typically used in mouse pathogenicity models. Strain CCH 442 was chosen for all subsequent experiments because it was a clinical isolate that exhibited high glucan synthase activities [19.0 ± 0.64 U (mg protein)⁻¹, *n* = 4]. Moreover, CCH 442 easily transforms from the yeast to the mycelial morphology and is amenable for use in permeabilized assays.

Effects of assay conditions

Selected assay mixtures previously developed for (1,3)- β -glucan synthase from various fungi were evaluated using both the yeast and mycelial forms of *C. albicans* (Table 1). As expected, glucan synthase activity was stimulated by either GTP or ATP in both morphological forms (Orlean, 1982). However, activation by GTP required an additional factor, either BSA or a detergent. BSA (0.8%) is added typically to assays with *S. cerevisiae* (Shematek *et al.*, 1980) and *C. albicans* (Orlean, 1982) glucan synthase, while the detergents Brij-35 (0.5%) and W-1 (0.025%) have stimulated activities from *S. cerevisiae* (Frost *et al.*, 1992) and *C. albicans* (Tang & Parr, 1991), respectively. Brij-35 was superior to W-1 in activating CCH 442 glucan synthase [2.89 U (mg protein)⁻¹ versus 1.22 U (mg protein)⁻¹]. Although NaF has been cited as a glucan synthase activator in fungi (Orlean & Ward, 1993; Larriba *et al.*, 1981; Ruiz-Herrera, 1992), NaF gave minimal stimulation compared to the BSA/GTP treatment. Overall, microsomes isolated from either the mycelial or yeast morphological form yielded similar activities. However, one exception was the response to treatment with GTP/0.025% W-1 and with the ATP/BSA. Mycelial glucan synthase was activated at a lower concentration of

Table 2. Effect of NaF on glucan synthase activities in microsomal membranes derived from the yeast and mycelial forms of CCH 442

GTP	Assay conditions*		Specific activity [nmol min ⁻¹ (mg protein ⁻¹)]†			
			No Brij in assay		Brij present in assay	
	NaF (mM)					
	10	100	Yeast	Mycelium	Yeast	Mycelium
—	—	—	0	0	0.33 ± 0.02	0.25 ± 0.01
—	+	—	0	0	0.36 ± 0.02	0.30 ± 0.02
—	—	+	0	0	0.43 ± 0.01	0.50 ± 0.04
+	—	—	0.03 ± 0.01‡	0.16 ± 0.01	2.93 ± 0.23	2.62 ± 0.08
+	+	—	0.05 ± 0.01	0.17 ± 0.01	2.80 ± 0.20	2.72 ± 0.32
+	—	+	0.12 ± 0.02	0.25 ± 0.01	3.40 ± 0.16	3.45 ± 0.13
—	AMF‡		0	0	0	0

* The final effector concentrations used in the assay were 20 µM GTPγS and 0.5 % Brij-35.

† Means ± SD, *n* = 4.

‡ AMF consists of 50 µM AlCl₃, 5 mM MgCl₂ and 20 mM NaF.

Fig. 1. Effects of Brij-35 and W-1 on CCH 442 microsomal glucan synthase assays. Yeast microsomal membranes were assayed with Brij-35 (○) or W-1 (□). Microsomes were isolated from cells broken in the presence of 0.5 % Brij-35 and assayed with either Brij-35 (●) or W-1 (■). Error bars represent SD, *n* = 4.

W-1 and with the ATP/BSA treatment activity was only 23 % of the GTP/BSA control. The combination of Brij-35 and GTP was then used routinely for assays since it provided maximum activities and is compatible to the permeabilized whole-cell assay (see below).

Since NaF gave only a minor stimulation in our assays, we investigated the possibility that Brij-35 was interfering or substituting the action of NaF. Perhaps either NaF or Brij-35 could inhibit deleterious enzymes or stimulate directly the glucan synthase complex. Even without Brij-35 or GTP, NaF still provided minimal activation (Table 2). Therefore, we examined the possibility of a new role for fluoride in the activation of glucan synthase in fungi.

Fluoride is a potent activator of the heterotrimeric G-proteins (Gilman, 1987; Sternweis & Gilman, 1982). Since the type of GTP-binding protein that interacts with glucan synthesis is unknown, we hypothesized that a G-protein could be involved. Fluoride interacts with aluminium to form an AlF_4^- complex which mimics the γ -phosphate of GTP when GDP is bound to the G_α subunit, thus activating the protein (Bigay *et al.*, 1987). However, in the absence and presence of 50 µM AlCl₃ the response was the same (data not shown). Furthermore, addition of AMF (50 µM AlCl₃, 5 mM MgCl₂ and 20 mM NaF), a standard activation mixture of many G-proteins (Kahn, 1991), totally inactivated the enzyme (Table 2). Inhibition from AMF was due to the presence of Mg²⁺ since 5 mM Mg²⁺ alone inhibited glucan synthase activity (data not shown).

Brij-35 greatly stimulated activity with at least a twofold increase in activity relative to W-1 with maximal activities obtained at levels of 0.5 % (Fig. 1). Since Brij-35 strongly activated glucan synthase in the *in vitro* assay, its addition during bead breakage was examined. Indeed, the specific activity of microsomal membranes with Brij-35 during breakage rose to 5.0 U mg⁻¹ (with 0.5 % Brij present in the assay). Only 0.25 % Brij-35 was required in the assay with detergent-isolated microsomes rather than 0.5 %. In addition, W-1 inhibited enzyme activity of Brij-isolated microsomes when W-1 levels were greater than 0.06 % in the assay. Inhibition by W-1 is probably due to additive effects of the two detergents since assay mixtures contain carry-over from the cell homogenates.

Characterization of yeast glucan synthase

Glucan synthase properties vary widely among different fungi (Ruiz-Herrera, 1992) and glucan synthase of *C. albicans* has not been characterized completely. Enzyme

Table 3. Summary of properties of glucan synthase in microsomal membranes derived from the yeast form of CCH 442 with or without Brij-35 during cell breakage

Property	Brij absent	Brij present
Optimum pH	7.75	7.75
Optimum temperature ($^{\circ}\text{C}$)	30	30
Linearity with time (min)	90	105
Linearity with protein (μg)	110	175
Half-life		
With Brij during preincubation		
4 $^{\circ}\text{C}$ (d)	5.6	12.1
30 $^{\circ}\text{C}$ (h)	15.9	18.4
Without Brij during preincubation		
4 $^{\circ}\text{C}$ (d)	0.92	5.5
30 $^{\circ}\text{C}$ (h)	0.5	11.8
$K_{\text{m app}}$ (mM)	1.1	0.9
$V_{\text{max app}}$ [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$]	4.1	12.5
Product sensitivity:		
α -Amylase (% degraded)	< 1	< 1
Protease (% degraded)	< 1	< 2
1,3- β -Glucanase		
Zymolyase (% degraded)	> 87	> 80
Laminarinase (% degraded)	> 99	> 99

Fig. 2. Effect of growth phase on glucan synthase activities of CCH 442. Yeast cells were harvested at each time point indicated and microsomal membranes were isolated and assayed as described in Methods. Error bars represent SD, $n = 4$. $\circ$, Specific activity; $\bullet$, OD_{600} .

characteristics of *C. albicans* in this study are summarized in Table 3. Assays conducted using Brij-35 gave temperature and $K_{\text{m app}}$ values that were comparable with those reported previously for *C. albicans* (Orlean, 1982). The half-life of glucan synthase was extended over 6- and 31-fold at 4 $^{\circ}$ and 30 $^{\circ}\text{C}$, respectively with Brij-35 in the buffer used for preincubation of microsomal membranes. A similar response was also reported when the enzyme

Fig. 3. Permeabilized whole-cell assays of CCH 442 glucan synthase. The main figure shows the incorporation of substrate into product over time using different permeabilized treatments [$\square$, no treatment; $\blacktriangle$, 4% toluene/ethanol (1:1); $\triangle$, 4% toluene/methanol (1:1)]. The inset shows product formation with the 4% toluene/methanol (1:1) treatment with increasing amount of cells. Error bars represent SD, $n = 3$.

was extracted with W-1 (Tang & Parr, 1991). However, the addition of Brij-35 during the breakage of cells further extended the half-life. The enzyme was stable for more than 5 d without additional detergent and 12 d when 0.5% Brij-35 was added during stability studies (4 $^{\circ}\text{C}$). Most of the other glucan synthase characteristics of Brij-35 breakage cells were similar to those without detergent during breakage.

Growth phase

Glucan synthase activities were highest during the early exponential phase of growth of strain CCH 442 and decreased by 10-fold as growth progressed towards the stationary phase (Fig. 2). Thus, cells are typically during mid-exponential phase so that adequate cell mass and moderate specific activities are obtained. Moreover, we always obtained the highest glucan synthase activities when cells were grown in small shake flasks rather than large-scale fermenters (15 l or greater). This could be due to differences in the fermentation conditions since the geometry of the vessels is different. In addition, cell breakage is conducted with different bead-beaters which may alter enzyme activity.

Development of a permeabilized whole-cell assay

Incorporation of UDP-glucose into product was linear for 30 min in each treatment (Fig. 3). Toluene/methanol (T/M) permeabilized cells had the highest incorporation of radioactivity compared to toluene/ethanol treatment. Product formation was roughly linear for 4 mg (dry wt)

Fig. 4. Effect of different activators on the permeabilized glucan synthase assay (CCH 442). The amount of glucose incorporated into product was measured using the 4% toluene/methanol (1:1) permeabilized treatment (see Methods). Error bars represent sd, $n=3$. Treatments 1–4 involved permeabilized cells whereas treatment 5 involved nonpermeabilized cells. 1, +Brij +GTP; 2, –Brij +GTP; 3, +Brij –GTP; 4, –Brij –GTP; 5, –Brij +GTP.

of cells (Fig. 3, insert). The permeabilized assay required the presence of Brij-35 and GTP with maximal activation when both activators were present (Fig. 4). Combinations of BSA and GTP did not activate the enzyme (data not shown). A product analysis showed that over 80% of the added UDP-[¹⁴C]glucose was incorporated into the SDS-insoluble pellet. This fraction was digested by Zymolyase (> 87%) and was resistant to either α -amylase or proteinase K, thus indicating that the majority of product is (1,3)- β -glucan.

Effects of inhibitors

Papulacandin B, cilofungin and echinocandin B each inhibited glucan biosynthesis (Table 4). The IC_{50} values of the glucan synthesis assay *in situ* were slightly higher than the glucan synthase assay *in vitro*. This response has been frequently observed using a range of compounds comparing the assays *in vitro* and *in situ* (data not shown).

DISCUSSION

Several different assay conditions have been published for an *in vitro* (1,3)- β -glucan synthase reaction in fungi. Only one report of glucan synthase activities has appeared using similar assay conditions in the mycelial and yeast phases of *C. albicans* but a permeabilized system has not been reported (Orlean, 1982). In order to evaluate antifungal agents we needed a uniform assay system that could be used not only with isolated membranes, but also with a whole-cell system. A systematic evaluation of the assay parameters was therefore conducted with *C. albicans* strain CCH 442 and a permeabilized whole-cell assay was developed. Further characterization of the enzyme led to the development of a stable enzyme preparation that achieved optimal activities.

GTP is a common activator of glucan synthase but assay reactions also required either BSA or a detergent (Table 1). The combination of Brij-35 and GTP was most effective for several reasons. BSA can bind drugs and obscure potential leads during screening efforts of antifungal agents. Furthermore, BSA could not be used in the permeabilized system and finally addition of the extra protein to assays can clog the glass-fibre filters and increase the background counts. Mechanism of action is unknown for either BSA or Brij-35, but they may act in a similar manner. BSA may function by sequestering free fatty acids or other endogenous inhibitors lysed during mechanical breakage (Ko *et al.*, 1994). BSA could also slow down proteolysis and detergents could inhibit proteases. Several detergents have been found to activate fungal glucan synthase (Beauvais *et al.*, 1993; Frost *et al.*, 1992; Quigley *et al.*, 1988; San-Blas & San-Blas, 1982; Tang & Parr, 1991). Detergents could alter enzyme conformation or membrane fluidity, or act by permeabilizing the membrane to enable better accessibility of substrate and effectors.

Addition of detergent during cell breakage further increased the specific activity and stability of the enzyme while not affecting other enzyme characteristics (Table 3). The addition of detergent during breakage is quite important since specific activities of *C. albicans* glucan synthase preparations prepared in the absence of detergent

Table 4. IC_{50} values for inhibitors of glucan synthesis in yeast, mycelial, and permeabilized cell assays (CCH 442)

Assays were conducted as described in Methods. MIC values of *C. albicans* CCH 442 were 3.0, 3.0 and 1.5 $\mu\text{g ml}^{-1}$ using the microtitre dilution method for papulacandin B, cilofungin and echinocandin B, respectively.

Assay condition	IC_{50} ($\mu\text{g ml}^{-1}$)		
	Papulacandin B	Cilofungin	Echinocandin B
Yeast microsomes	4	30	7
Mycelial microsomes	4	52	9
Permeabilized yeast cells	15	75	18

can be low (Taft *et al.*, 1988; Schmatz *et al.*, 1990). Although W-1 has been reported to solubilize glucan synthase from *C. albicans* membranes, we could not solubilize the enzyme from the microsomal membranes using either W-1 or Brij-35 (data not shown). However, Brij-35 improved the stability and activity of the enzyme in a similar manner to that reported with W-1 treated membranes (Tang & Parr, 1991).

NaF is an important activator for many fungi, with the F⁻ ion acting as the active component (Ruiz-Herrera, 1992). Yet NaF treatment provided only minimal activation in our assays and Brij-35 did not substitute its action (Table 2). Several mechanisms have been proposed for the action of NaF. Among these F⁻ could protect an endogenous activator distinct from GTP, inactivate phosphatases, stimulate directly the enzyme or inhibit endogenous inhibitors (Orlean & Ward, 1983; Ruiz-Herrera, 1992). We examined the possibility that F⁻ was acting by a totally different mechanism, that is, through activation of a G-protein involved in glucan biosynthesis. Although a GTP-binding protein is involved in glucan synthesis, the type of GTP-binding protein is unknown (Kang & Cabib, 1986). It has not been determined whether the GTP-binding protein of glucan synthase is a G-protein (heterotrimeric protein), a small GTP-binding protein (Ras-like) or another type. Additions of Al³⁺ with NaF did not affect glucan synthase activity. Aluminium forms a complex with F⁻ that mimics the γ -phosphate of GTP when GDP is bound to the G _{α} subunit and activates the protein (Bigay *et al.*, 1987). These data suggest that a classical G-protein is not present in the CCH 442 glucan synthase complex. The mechanisms proposed previously best fit the activation role of NaF in fungal glucan synthases (Ruiz-Herrera, 1992).

After screening large numbers of *C. albicans* strains the polyene-resistant strain, ATCC 38247, exhibited the highest specific activity for glucan synthase. This strain accumulates methylsterols rather than ergosterol and its membrane is more rigid than that of the wild-type strain (Lees *et al.*, 1989). Membrane fluidity could be an important mechanism of enzyme regulation. Accordingly, chitin synthase activities were higher in polyene-resistant strains and with normal strains when treated with azoles (Pesti *et al.*, 1981; Georgopapadakou, 1992). The alterations in membrane fluidity were suggested to play a central role. The broad range of activity levels amongst different strains could also explain variable responses to activators reported in the literature (Ruiz-Herrera, 1992). Strain CCH 442 was chosen for our experiments because it is a clinical isolate containing high glucan synthase activities, it easily transforms from the yeast to the mycelial morphology and it is amendable for use in permeabilized assays.

C. albicans is a dimorphic fungus and specific morphological forms may play unique roles in pathogenesis (Odds, 1988). Glucan synthase activities from microsomal membranes derived from either mycelial or yeast morphological forms were similar under a wide range of assay conditions except that lower levels of W-1 detergent

activated the mycelial form of the enzyme. Furthermore, the mycelial glucan synthase was only marginally responsive to activation by BSA and ATP. These results could suggest that multiple forms of the enzyme are present. Chitin synthase has at least three forms and each one exhibits a differential response to various effectors and drugs (McCullough, 1992; Cabib *et al.*, 1989). One could also envisage specialized forms of glucan synthase which would be required by either the yeast and mycelial forms for both spatial and temporal localizations. Repair functions of the cell wall and specialized interactions with certain polymers such as chitin during septation could also require specialized forms of glucan synthase exhibiting unique regulatory properties.

Cell-wall biosynthetic enzymes are most frequently isolated from yeast cells grown to mid-exponential phase. Chitin synthase from *C. albicans* exhibited its highest activity during active budding and then declined during the stationary phase (Barrett-Bee *et al.*, 1982). Orlean (1982) compared the activities of glucan synthase during the first 4 h of growth after inoculation with both yeast and mycelia and found that activities rose during early exponential growth. We extended the yeast growth curve and found that activities were highest during the early exponential phase. This was followed by a plateau and subsequent decrease during the stationary phase. Glucan synthase levels therefore followed the expected pattern for cell-wall biosynthesis since cell-wall generation is highest during budding. Low levels of activity may only be required for basal repair or turnover of glucan.

The plasma membrane is a major barrier for many drugs. Certain compounds may be specific and potent inhibitors of glucan synthase assays yet be devoid of antifungal activity. We developed a permeabilized whole-cell assay for glucan synthase to address the permeability issue. In our assay, the toluene/methanol effectively permeabilized the membrane and the incorporation of UDP-glucose to glucan was linear for 30 min. This assay also required GTP and Brij-35. BSA did not activate the enzyme since its large size probably prohibited permeation through the membrane.

Both classes of glucan synthesis inhibitors, the glucolipids (papulacandin B) and lipopeptides (echinocandin B and cilofungin), inhibited the assays *in vitro* and *in situ*. The IC₅₀ values were close to the MIC values for papulacandin B and echinocandin B but higher for cilofungin. It should be noted that these compounds are inhibitors of glucan synthesis and may not interact directly with the glucan synthase catalytic subunit. Their precise mode of action has yet to be determined (Ruiz-Herrera, 1992).

Conclusions

In summary, β -(1,3)-glucan synthesis in fungi is a major pharmaceutical target. The assays described in this paper allow for comparison of compounds inhibiting glucan synthesis. Comparisons can then be extended to antifungal activity *in vitro* and efficacy in mouse pathogenicity models *in vivo*. In addition, the detailed characterization of glucan

synthase should facilitate further purification of the enzyme and determination of its enzymological properties.

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