Synthesis of yeast cell wall glucan and evidence for glucan metabolism in a *Saccharomyces cerevisiae* whole cell system

Michael L. Coen, Claude G. Lerner, John O. Capobianco
and Robert C. Goldman

Author for correspondence: Robert C. Goldman. Tel: +1 708 937 4477. Fax: +1 708 938 6603.
e-mail: goldmanr@randb.abbott.com

Anti-infective Research
Division, Abbott
Laboratories, Abbott Park, IL
60064-3500, USA

The synthesis and metabolism of yeast cell wall glucan were studied using a *Saccharomyces cerevisiae* construct in which radiolabelled galactose is metabolized to UDP-glucose and preferentially incorporated into glucan. Greater than 85% of the incorporated radiolabel was found within insoluble cell wall material. Our study also demonstrated that radiolabelled wall glucan is released from cells growing exponentially, and that the released radiolabel is reutilizable low molecular mass material. Size exclusion chromatography and enzymic analysis indicate that laminaribiose comprises approximately 50% of the released fraction. This is consistent with *in vitro* findings that laminaribiose is a by-product of a newly identified glucosyltransferase (R. P. Hartland, G. W. Emerson & P. A. Sullivan, 1991, Proc R Soc Lond B 246, 155–160) associated with fungal cell walls. Our results also suggest that pre-existing glucan undergoes less metabolic processes than newly synthesized material as evidenced by a decrease in released radiolabel over time. Pulse double labelling of glucan and total cellular protein indicate that glucan metabolism and protein synthesis (ps) are not tightly coupled although they do parallel each other during exponential growth. Inhibitors of glucan synthesis (gs) decrease the glucan to protein ratio. Measurement of ps allows normalization for non-specific decreases in the rate of cell wall synthesis due to general cessation of growth. Cilofungin and papulacandin B, two putative inhibitors of gs, inhibited galactose incorporation into glucan and thus showed a decrease in the glucan to protein ratio, although ps was affected. In contrast, cycloheximide, a known ps inhibitor, displayed an elevated ratio. This whole cell construct affords a simplified system for elucidating the synthesis and metabolic activity of the yeast cell wall and enables the discrimination between specific effectors of gs and ps.

Keywords: *Saccharomyces cerevisiae*, glucan metabolism, yeast cell wall, whole cell system

INTRODUCTION

Compared to bacteria, little is known about cell wall synthesis, assembly, and metabolism in yeast and other fungi. It is generally believed that the yeast cell wall undergoes many essential metabolic events during growth and maturation. *De novo* synthesis of cell wall components (Hector, 1993), cell wall restructuring and modification during morphogenesis (Wessels *et al.*, 1990) and in response to external stimuli (Ruiz-Herrera, 1992), and selective degradation (Hector, 1993) of wall materials are presumed to be important processes in cell growth. The

yeast cell wall consists of an inner electron-transparent layer composed of intertwined glucan and chitin fibrils surrounded by an outer dense layer predominately composed of mannoprotein (Ruiz-Herrera, 1992). Covalent cross-linkages between these components may exist, i.e. glucan and chitin (Gooday *et al.*, 1992; Gopal *et al.*, 1984; Shepherd *et al.*, 1984; Sietsma & Wessels, 1981, 1979; Surarit *et al.*, 1988) or glucan and/or chitin and manno-protein (Beaudouin *et al.*, 1993; Peberdy, 1990). More detailed reviews of yeast cell wall structure can be found elsewhere (Cabib *et al.*, 1991, 1982; Peberdy, 1990; Ruiz-Herrera, 1992; Wessels *et al.*, 1990). Many membrane- and

wall-associated enzymes have been identified and their catalytic activity with regard to the cell wall structural elements characterized. Among them are β -glucan and chitin synthetases (Peberdy, 1990), endo- and exo- β -glucanases (Chambers, 1993; Klebl & Tanner, 1989; Mrsa *et al.*, 1993), endochitinases (Gooday *et al.*, 1992; Hien & Fleet, 1983), and glucosyltransferases (P. A. Sullivan, personal communication). Characterization of these enzyme activities, the enzymic sequences, and the consequences of the multitude of metabolic events involved within the yeast cell wall architecture remain the topic of intense investigation.

Glucan is the most abundant structural polymer of the cell wall of all yeasts and fungi. Yeast glucan is composed of glucose oligomers having (1,3)- β and (1,6)- β configurations (Peberdy, 1990, Ruiz-Herrera, 1992). Synthesis of fungal glucan can be studied *in vitro* using isolated microsomes; however, technical constraints have made it difficult to study wall glucan metabolism *in vivo*. To date, analysis of glucan synthesis has been hampered by cumbersome methodologies which involve cell breakage, harsh extraction conditions, or both (Ruiz-Herrera, 1992). Three different types of glucan have been identified from yeast cell walls (Fleet & Manners, 1976; Manners *et al.*, 1973a, b; Peberdy, 1990; Shepherd *et al.*, 1984) but it is not proven whether these three species are distinguishable within the intact cell or their separation is simply an artifact of the harsh extraction procedures employed. Following the initial synthesis of glucan there are likely post-synthetic processing/restructuring events which occur at various points within the cell cycle. Such events likely occur during bud emergence, hyphal branching, wall expansion, cell separation, spore formation and germination (Kurranda & Robbins, 1991; Peberdy, 1990; Ruiz-Herrera, 1992; San Segundo *et al.*, 1993; Wessels *et al.*, 1990) but are poorly understood.

In the present study a simplified whole cell model for assessing glucan biosynthesis and metabolism was developed utilizing a *Saccharomyces cerevisiae* construct which allows selective double labelling of cell wall glucan and cellular protein. The resultant genetic manipulations afford a method for specific labelling of glucan with radiolabelled galactose rather than glucose. Inhibition of synthesis of either of the major carbohydrate polymers (glucan or chitin) is detrimental to fungal cell survival; however, the affects of blocking morphological rearrangements or assembly of these polymers into the wall structure are presently unknown. Cilofungin (a derivative of echinocandin B) and papulacandin B are members of two chemical groups which inhibit glucan synthesis by an unknown mechanism of action (Cabib *et al.*, 1991). Additionally, many azole and polyene compounds used as antifungal agents have effects on numerous plasma membrane enzymes including those involved in glucan synthesis (Surarit & Shepherd, 1987). No definitive studies have been conducted on the effects of such agents on the synthetic rates of glucan versus total cellular protein. Advantages of this system are: (i) enzymes involved in glucan synthesis (gs) and protein synthesis (ps) remain in their natural state; (ii) rates of gs and ps are

simultaneously monitored by direct TCA precipitation; (iii) cell supernatants can be easily analysed for by-products (metabolites) of gs; and (iv) the effect of putative inhibitors of gs and ps and the metabolism of cell wall glucan can be studied in detail using pulse double labelling techniques. We present *in vivo* evidence for the metabolism and subsequent release of cell wall glucan oligosaccharides and using inhibitors investigated the coupling between gs and ps.

METHODS

Yeast strains and plasmids. The parent *Saccharomyces cerevisiae* strain, Sc488 (*a ura3-52 leu2-3, 112 ade1 gal80 Δ hxc2::URA3*) and plasmid pBM34BX-HD (source of *GAL5*) were kindly provided by Dr James E. Hopper (Department of Biological Chemistry, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA, USA). Plasmid Ep146 (source of *LEU2*, Ap^R) was kindly provided by Dr Alan Hinnebusch (NIH, Bethesda, MD, USA). Plasmid pB34BX-LD (source of *gal5*) used for insertional inactivation of the major phosphoglucosyltransferase (*PGM2* = *GAL5*) isoenzyme was constructed by ligation of the *LEU2* gene into the pBM34BX-HD vector and selected for ampicillin resistance in *Escherichia coli* DH5a. Briefly, the plasmid vector, pBM34BX-HD, was linearized and the *LEU2* gene excised from plasmid Ep146 by restriction enzyme digestion with *Hind*III. The fragments were purified by agarose gel electrophoresis and ligated with T4 ligase. *E. coli* was transformed by standard methods and colonies selected for ampicillin resistance (Maniatis *et al.*, 1982). Plasmids were isolated and confirmed by *Hind*III digestion and agarose gel electrophoresis. The plasmid pBM34BX-LD was restriction digested with *Xba*I and *Bam*HI to yield a 4.5 kb fragment containing the *LEU2* gene flanked by *GAL5* sequences. The parent strain Sc488 was transformed with the resulting digest mixture by a modified lithium acetate method (Ito *et al.*, 1983). The transformed construct Sc488-11A (*a ade1 leu2-3, 112 ura3-52 gal80 Δ gal5::LEU2 hxc2::URA3*) was selected by leucine-independent growth. The phenotypes of all yeast strains were verified by growth on specific media. The *hxc2* inactivation and leucine auxotrophy in the parent strain Sc488 were confirmed by growth on galactose as the sole carbon source, uracil-independent growth, and leucine-independent growth, respectively. *PGM2* (*gal5*) inactivation in construct Sc488-11A was confirmed by the inability to grow on galactose as the sole carbon source and leucine-independent growth.

Culture conditions. All strains were grown and maintained on YPD medium (Difco) containing 40 μ g adenine ml⁻¹ (YPAD) at 30 °C. Sc488-11A labelling experiments were carried out in yeast nitrogen base minimal media (1 $\times$ YNB with amino acids + (NH₄)₂SO₄; Difco) containing 2% (w/v) glucose and 40 μ g adenine ml⁻¹ in a 30 °C shaking water bath unless otherwise noted. OD was measured at a wavelength of 420 nm. The mean generation time of Sc488-11A growing in YNB containing 2% glucose as described above was determined to be approximately 2.1 h during mid-exponential growth.

Chemicals. D-[1-¹⁴C]galactose (50–60 mCi mmol⁻¹, catalogue no. CFA.435; 1 Ci = 3.7 $\times$ 10¹⁰ Bq) and L-[4,5-³H]leucine (120–190 Ci mmol⁻¹, catalogue no. TRK.636) used for double radiolabelling experiments, and D-[6-³H]galactose (20–40 Ci mmol⁻¹, catalogue no. TRK.651) used for glucan release experiments were obtained from Amersham, Arlington Heights, IL, USA. [³H]Laminaribiose (250 mCi mmol⁻¹) was prepared by enzymic hydrolysis and isolated by column chromatography by John Capobianco and Dorothy Zakula at

Abbott Laboratories, Abbott Park, IL, USA. Briefly, β -(1,3)-[^3H]glucan was prepared enzymically from UDP-D-[6- ^3H]glucose (13 Ci mmol $^{-1}$, catalogue no. TRK.385) and *Candida albicans* microsomes as described by Frost *et al.* (1992). The radiolabelled glucan was digested with zymolyase 100T and the hydrolytic products were separated by Sephadex G-50 (Pharmacia, Uppsala, Sweden) column chromatography. The radioactive oligosaccharides migrating in the included volume were recovered by lyophilization, and laminaribiose was then purified by HPLC on a 4.6 mm $\times$ 25 cm Dynamax-60A NH $_2$ column (Rainin Instruments) run in acetonitrile/water (1:1). Zymolyase 100T and laminarioligosaccharides were obtained from Seikagaku America Inc. Cilofungin was obtained from Eli Lilly. Papulacandin B was obtained from Ciba-Geigy. Cycloheximide and all other reagents were obtained from Sigma and were of the highest possible grade. The minimal inhibitory concentrations (MIC) for cilofungin, papulacandin B, and cycloheximide in strain Sc488-11A are 50 $\mu\text{g ml}^{-1}$, 2.5 $\mu\text{g ml}^{-1}$, and 39 ng ml $^{-1}$, respectively, as determined by microtitre broth dilution. In duplicate, compounds were twofold serially diluted to a final volume of 50 μl medium per well. A culture was grown, as described, to an OD $_{420}$ = 0.4, diluted 1:50 in medium, and 50 μl added to each well. Growth inhibition was recorded after overnight incubation at 32 °C. MIC values are defined as the lowest drug concentrations necessary for complete growth inhibition with a control well reaching maximal density.

Radiolabel distribution analysis. Sc488-11A was continuously labelled with 2 μCi [^{14}C]galactose ml $^{-1}$ and 1 mg galactose carrier ml $^{-1}$ to an approximate OD $_{420}$ = 3.0. Cells were broken by vortexing with an equal volume of 500 μm diameter glass beads previously acid washed and rinsed in 0.01 M ammonium acetate buffer, pH 5.6. The buffer-insoluble cell walls were resuspended in 10 mM Tris/HCl, pH 7.5, containing 2% (w/v) SDS and heated to 100 °C for 5 min to solubilize mannoprotein (Valentin *et al.*, 1984). Samples of buffer-insoluble, SDS-washed walls were treated with α -amylase, proteinase K or zymolyase, respectively, and analysed for released radioactive counts. The β -(1,3) and β -(1,6) linkage ratio was estimated by zymolyase digestion and size separation by Sephadex G-25 (Pharmacia) column chromatography. Zymolyase-100T contains predominantly β -(1,3)-glucan laminaripecta-hydrolase in addition to significant activities of β -(1,3)-exoglucanase, protease and mannanase. All of the soluble supernatants resulting from the above digestions were TCA-precipitated, after which any TCA-soluble material was ethanol-precipitated. Radioactive counts were quantified in a Wallac 1410 liquid scintillation counter (Pharmacia).

Continuous double radiolabelling of glucan and protein. An Sc488-11A culture (20 ml) was grown to early exponential phase (approx. OD $_{420}$ = 1.0), at which time labelling mix [10 μg leucine ml $^{-1}$, 1 mg galactose ml $^{-1}$, 5 μCi [^3H]leucine ml $^{-1}$ (final specific activity = 65.6–103.9 mCi mmol $^{-1}$), and 2 μCi [^{14}C]galactose ml $^{-1}$ (final specific activity = 0.358–0.430 mCi mmol $^{-1}$)] was added. The OD $_{420}$ was monitored and every hour 1.0 ml samples of the labelling culture were precipitated with 1.0 ml cold 10% (w/v) TCA containing leucine and galactose (each at 1 mg ml $^{-1}$), divided for duplicate measurements, and incubated on ice for 40 min. Samples were filtered onto glass fibre filters (Whatman GF/F) and washed three times with 5% TCA containing leucine and galactose (each at 1 mg ml $^{-1}$), once with 95% (v/v) ethanol, and counted for TCA-precipitable incorporated radioactive counts as described above.

Pulse double radiolabelling of glucan and protein. Sc488-11A master cultures (10 ml) were grown as above. The appropriate drug concentrations (see Figs 1a, 2 and 3) of cilofungin, papulacandin B and cycloheximide, respectively, were added at

OD $_{420}$ = 1.0. Samples (1.5 ml) of the cultures were pulse-labelled for 40 min (approximately 0.3 of a cell generation) beginning at 10, 40 and 70 min post drug addition. Prior to each labelling, 1.5 ml of each master culture was removed to a 15 ml flask in a shaking water bath. At the designated labelling start time, labelling mix (see above) was added to individual 15 ml flasks. Following a 40 min labelling period, 1.0 ml of each 1.5 ml labelled culture was precipitated and duplicates counted as described above. The ratio of [^{14}C]:[^3H] was calculated for analysis of inhibitor specificity.

Cell wall glucan release. Sc488-11A cultures (2 ml) were grown to a mid-exponential OD $_{420}$ = 2.5 (before glycogen accumulation) containing labelling mix [250 μCi [^3H]galactose ml $^{-1}$ and 1.0 mg galactose carrier ml $^{-1}$ (final specific activity = 23.7–47.4 mCi mmol $^{-1}$)]. The culture was washed three times with medium by centrifugation or with warmed medium by filtration. The cell pellet was resuspended 1:10 (OD $_{420}$ = 0.25) in fresh low glucose YNB medium (1 $\times$ YNB with amino acids + (NH $_4$) $_2$ SO $_4$ containing 0.4% glucose and 40 μg adenine ml $^{-1}$) without label. Immediately, 1 ml of the culture was pelleted and 0.5 ml of the supernatant counted in a scintillation counter (time 0). The OD $_{420}$ was monitored and 0.5 ml of the supernatant counted as before each hour after washing. At roughly OD $_{420}$ = 1.0 the cells were pelleted, the supernatant removed, heated to 80 °C for 5 min and adjusted to neutral pH (supernatant 1). The remaining pellet was resuspended in the original volume of fresh low glucose medium and growth continued until OD $_{420}$ = approx. 3.0. The supernatant was harvested as before (supernatant 2). The final pellet was resuspended in twice the original volume of growth medium (2% glucose), grown overnight for additional release of labelled material, and processed as before (supernatant 3). Supernatants (2 ml) were analysed by Bio-Gel P2 (Bio-Rad) column chromatography for liberated radiolabelled material. The remaining supernatants were lyophilized and saved for reutilization and digestion experiments (see below). Additionally, a labelled culture was monitored for growth and radiolabel release to obtain a kinetic profile for radiolabel release. A portion of this culture was also washed as above and resuspended in an equal volume of fresh medium to further demonstrate a second release of radiolabelled material.

Analysis of released material. The radioactive material released in supernatant 2 was digested with zymolyase 100T (approx. 100 units) overnight at 30 °C with rotation. Sodium azide (0.02%) was included to inhibit growth of any contaminating organisms. The digested supernatant 2 and each of the three undigested supernatants were analysed by Bio-Gel P2 size exclusion column chromatography. Two columns (0.7 $\times$ 30 cm and 1.5 $\times$ 50 cm) packed with Bio-Rad P2 size exclusion chromatographic resin hydrated in water were connected in series, equilibrated with 0.01 M ammonium acetate, pH 5.6. Individual samples (2 ml) of supernatant 1, 2 (before and after digestion) and 3 were loaded and fractions approximately 0.25 ml in size were collected. Fractions were counted as before and the sample profiles compared to profile standards of [^{14}C]glucose and [^3H]laminaribiose. [^{14}C]Glucose ($\sim$ 40000 d.p.m.) was added to supernatants 2 and 3 as an internal control.

Reutilization of released material. A portion of supernatant 2 was lyophilized and resuspended in water. Approximately 7000 d.p.m. in 1 ml water were added to an unlabelled 2 ml culture (2% glucose) of Sc488-11A and grown to OD $_{420}$ = approx. 3.0. A sample (0.5 ml) of the culture was TCA-precipitated and counted as described above.

Laminaribiose transport. Sc488-11A was grown at 32 °C in YPAD to OD $_{420}$ = 1.0. Cells were harvested by centrifugation (10000 g, 10 min), then washed twice with transport buffer

containing 0.1% glucose (Capobianco *et al.*, 1993). Cells were resuspended in transport buffer at one-tenth the original volume. Cell suspensions were mixed with an equal volume of [^3H]laminaribiose (0.01 μCi at two specific activities, 0.6 or 60 mCi mmol^{-1} , giving external concentrations of 85 μM and 8.5 mM total laminaribiose, respectively) at 32 °C for 0, 5, 10 and 20 min. Cells were separated from the transport buffer by centrifugation through silicone oil as previously described (Capobianco *et al.*, 1993). Cells were measured for uptake of radioactive counts.

Growth on laminarioligosaccharides. A 5 ml starter culture of Sc488-11A was grown in YNB growth medium containing 2% glucose and 40 $\mu\text{g adenine ml}^{-1}$ to an $\text{OD}_{420} = 1.0$ in a shaking 30 °C water bath. Individual test cultures (1 ml) were inoculated to a starting $\text{OD}_{420} = 0.05$ in YNB medium lacking glucose or supplemented with glucose (G1), laminaribiose (G2), laminarihexaose (G6) or laminarin (each at 10 mg ml^{-1}), respectively. Duplicate cultures were grown in the presence of two glucanase inhibitors, deoxynojirimycin ($10 \times \text{IC}_{50} = 1.13 \text{ mM}$) and castanospermine ($10 \times \text{IC}_{50} = 16 \mu\text{M}$). Growth of each culture was monitored over several days. Maximal growth yields and growth rate were reported qualitatively as compared to G1.

RESULTS AND DISCUSSION

Strain construct and analysis of [^{14}C]galactose labelling

In the present study a genetic construct of *S. cerevisiae* was utilized to evaluate yeast cell wall synthesis and metabolism. Based on the construct phenotype, galactose is

Fig. 1. Galactose metabolism in *S. cerevisiae* Sc488-11A.

constitutively utilized in the presence of glucose and is preferentially incorporated into cell wall glucan. A deletion in the *GAL80* regulatory gene allows constitutive expression of the genes necessary for galactose utilization (Nehlin *et al.*, 1991; Yun *et al.*, 1991). Galactose utilization is also not subject to glucose repression due to insertional inactivation of the major hexokinase PII isozyme (*hxx2::URA3*) (Rose *et al.*, 1991; Gancedo, 1992). Briefly, galactose is transported via a specific permease, phosphorylated, converted to UDP-galactose, and epimerized to UDP-glucose. The resulting UDP-glucose is then incorporated into glucan without further dissemination through the glycolytic pathway. Further dissemination is prevented by the inactivation of the *GAL5* gene (*gal5::LEU2*) encoding the major phosphoglucumutase (PGM2) isozyme (Bevan & Douglas, 1969; Oh & Hopper, 1990). UDP-glucose arising from glucose continues to be incorporated into glucan as a result of the activity of a minor PGM isozyme which converts glucose-6-phosphate to glucose-1-phosphate (Fig. 1).

The incorporation of [^{14}C]galactose into glucan and other macromolecules was assessed to verify the specificity of labelling in this particular construct. Following a continuous labelling period, the buffer-insoluble cell wall pellet contained 86% of the total radioactive counts. Buffer-soluble radioactive counts (14% of the total cell-associated counts) likely arise from unincorporated UDP-glucose which is not chased into product. Additionally, some of the counts may be due to small glucan fragments. A small amount of radiolabelled material (9.5% of the total cell-associated counts) was released when the insoluble cell wall pellet was boiled with SDS. This may be a result of small glucan oligomers covalently attached to the mannan (Peberdy, 1990). The majority of radioactive counts (60% of the total cell-associated counts) in the insoluble cell walls were released by zymolyase digestion. Zymolyase is a crude mixture of (1,3)- β -glucan laminaripenta-hydrolase, (1,3)- β -exoglucanase, proteinase and mannanase. Prolonged incubation of (1,3)- β glucan (as laminarin) with zymolyase 100T results in nearly complete hydrolysis to glucose (data not shown). Sephadex G-25 separation of the zymolyase-solubilized radiolabel resulted in two major peaks. One peak contained predominantly glucose plus small (1,3)- β oligomers (70% of the zymolyase-solubilized label) as confirmed by thin layer chromatography. A second peak of larger undigestible oligomers contained (1,6)- β linkages (30% of the zymolyase-solubilized label). Qualitatively, this distribution is consistent with previous findings (Surarit & Shepherd, 1987). Minimal amounts of label (1.1% of the total cell-associated counts) were found in glycogen, digested with α -amylase, which may be from a low constitutive level of glycogen biosynthesis (*GSY1* = glycogen synthase 1) (Farkas *et al.*, 1991). During late exponential growth, however, activation of the major glycogen synthase (*GSY2*) results in a rapid accumulation of radiolabel (data not shown). A small amount (1.4% of the total cell-associated counts) of radiolabel was released by proteinase K digestion. Owing to the slow growth rate of this construct and the cellular

Fig. 2. Effects of cilofungin ($\mu\text{g ml}^{-1}$: $\square$, 0; $\triangle$, 50; $\circ$, 100; $\blacktriangle$, 200) on *S. cerevisiae* Sc488-11A growth (a), rate of ps (b), rate of gs (c) and ratio of gs to ps (d). Arrows indicate 40 min labelling period.

UDP-glucose pool, with which the radiolabel must equilibrate, a labelling period of approximately 0.3 generations (40 min) was indicated for pulsed-label analysis.

Lack of glycogen accumulation below OD₄₂₀ = 3

Continuous labelling was performed to demonstrate the growth range in which this construct can be utilized for double labelling experiments without radiolabel dissemination into the glycogen biosynthetic pathway. Continuous [³H]leucine incorporation into TCA-precipitable cellular protein paralleled the exponential growth curve reaching near-stationary phase growth (OD₄₂₀ = 8) (data not shown). [¹⁴C]Galactose incorporation into TCA-precipitable glucan displayed linear incorporation until OD₄₂₀ = approx. 3 when incorporation markedly increased due to radiolabel accumulation into glycogen. Plotting the ratio of [¹⁴C] to [³H] initially yielded a zero slope indicating equal rates of glucan and protein synthesis. At OD₄₂₀ = approx. 3 the labelling culture demon-

strated a rapid elevation of [¹⁴C]galactose incorporation into glycogen causing the ratio to progressively increase (data not shown). It was concluded that specific double labelling can be performed confidently until mid-exponential phase growth (OD₄₂₀ = 3) with the majority of radioactive material accumulating in the cell wall glucan.

Effect of inhibitors of glucan and protein synthesis

Pulse double labelling of cell wall glucan and cellular protein was performed in the presence of specific inhibitors. The antifungal agent, cilofungin, had no apparent effect on growth (Fig. 2a) or [³H]leucine incorporation into total protein (Fig. 2b) until 200 $\mu\text{g ml}^{-1}$ (4 × MIC). However, [¹⁴C]galactose incorporation into glucan decreased in a dose-dependent manner (Fig. 2c). While glucan and protein were both affected, the rate at which these two parameters were affected was unequal resulting in a decreased ratio of glucan to protein (Fig. 2d). The decreased ratio indicates a faster and more drastic

Fig. 3. Effects of papulacandin B ($\mu\text{g ml}^{-1}$: $\square$, 0; $\triangle$, 1.0; $\circ$, 2.0; $\blacktriangle$, 4.0) on the ratio of glucan to protein. Arrows indicate 40 min labelling period.

Fig. 4. Effects of cycloheximide ($\mu\text{g ml}^{-1}$: $\square$, 0; $\triangle$, 0.004; $\circ$, 0.02; $\blacktriangle$, 0.10) on the ratio of glucan to protein. Cell growth, ps and gs display a dose-dependent decrease. Arrows indicate 40 min labelling period.

decrease in the rate of glucan synthesis compared to protein synthesis. This suggests that the effects on protein synthesis are secondary to that of glucan.

The antifungal agent, papulacandin B, demonstrated a slightly different profile than cilofungin. Cell growth displayed a dose-dependent decrease different from that observed with cilofungin (data not shown). Decreases in glucan and protein synthesis appeared to be affected at similar rates at low doses, and only at the highest concentration ($4 \mu\text{g ml}^{-1} = 1.6 \times \text{MIC}$) was a clearly decreased glucan to protein ratio observed (Fig. 3). This may indicate that papulacandin B has other toxic effects on

the yeast cell which affect protein synthesis and that there is less of a selectivity for glucan synthesis than cilofungin.

The effects of cycloheximide were assessed to demonstrate the specificity and selectivity of this construct and help validate this construct as having the ability to discriminate between specific effectors of gs and ps. Cycloheximide, a known inhibitor of ps, caused an increase in the ratio of glucan to protein (Fig. 4). This is an opposite effect of that observed with cilofungin and papulacandin B. Growth was decreased in a dose-dependent manner, as were gs and ps (data not shown). However, with complete inhibition of ps, OD and gs increased slightly for a brief period. This further supports findings of continued gs with inhibition of ps (Elorza & Sentandreu, 1969). This suggests that the decrease in gs is secondary to the inhibition of ps and that thickening of the glucan layer may result in an apparent continued growth when measured by OD.

Analysis of the ratio of rates of ps to gs by dual pulse labelling allowed discrimination of inhibitor specificity. To the best of our knowledge this is the first definitive study utilizing pulsed rate analyses. Previous labelling experiments by Tkacz (1984) were performed using a *pgm2 (gal5) glc1 GAL81-12 GAL82-R1* strain which was obtained by traditional genetic crosses; however, all glucan labelling experiments were continuous, and incorporation was not normalized to any other parameter (e.g. dry weight, protein, OD, etc.). Thus, the experimental data on the effects of aculeacin A and cycloheximide were difficult to interpret. This strain was not available for comparative studies. Our strain construction differed in that constitutive expression of the genes involved in utilization of galactose was obtained by gene inactivation and deletion. As with any double labelling procedure, certain limits exist for the discrimination between inhibitor modes of action, in that any massive perturbation of macromolecular synthesis will have profound effects on all cellular processes given enough time (i.e. cell growth will cease). Acknowledging this fact, our system can differentiate between inhibitors based on their specificity of action by analysis of the ratio of the rates of incorporation of galactose and leucine. Inhibitors of gs cause this ratio to decrease, while a ps inhibitor caused an increase. This may indicate that gs and ps are not tightly coupled. It should be stressed that the glucan to protein ratio should not be the sole criterion for establishing specificity. Complete data analysis could elucidate potential mechanistic differences between compounds which may impair, for example, transport of radiolabelled material (unpublished observations).

Kinetics and analysis of radiolabelled glucan release

Prelabelled yeast cells, washed and diluted into fresh medium, shed cell wall glucan fragments into the surrounding medium demonstrating that the cell wall is subject to metabolic flux. Cells displayed a 2–3 h lag period after washing before exponential growth was resumed. Interestingly, during this lag period of apparently little growth, radiolabelled glucan fragments (13.2% of the total cell-associated counts) were released into the

Fig. 5. (a) Release of radiolabelled cell wall glucan from prelabelled cells washed free of unincorporated label. A post-wash lag period of 2 h corresponded to release of radiolabel (13.2% of the total cell-associated counts) into the surrounding medium. An additional 17.1% of the total cell-associated counts (56.5% of the released radiolabel) were released after the cells had recovered and resumed exponential growth. □, OD₄₂₀; ▲, radioactivity, (b) Glucan release per cellular mass displays a peak of released radiolabel concurrent with the resumption of exponential growth.

medium. A portion of this release might be induced by the washing procedure. However, an additional 17.1% of the total cell-associated counts (56.5% of the released radiolabel) were released after the cells had recovered and resumed exponential growth (Fig. 5a). Washing by filtration with warmed medium yielded similar results. The amount of released radiolabel per mass of cells peaked at the time in which the cells resumed exponential growth (Fig. 5b). By approximately 10 h post wash, the amount of released material reached an apparent maximum level. Upon overnight incubation, during which time the cells reached stationary phase (OD₄₂₀ > 10), the amount of radiolabel remained relatively unchanged from the 10 h measurement.

Fig. 6. Bio-Gel P2 size exclusion separation of supernatant 2 from cells prelabelled and washed free of unincorporated label.

The supernatant of an actively growing culture, previously washed free of any unincorporated radiolabel, was harvested at three time points and analysed for any released radiolabel by Bio-Gel P2 chromatography. Supernatant 1 (collected after two cell doublings) contained one peak comigrating with that of a radiolabelled disaccharide (laminaribiose) standard and a second peak with that of a glucose standard (data not shown). The second peak, owing to the loss of this peak in subsequent supernatants 2 and 3, was thought to contain a small amount of residual galactose or glucose possibly released from the cell during washing. A small peak (less than 1.5% of the total cell-associated counts) of high molecular mass material was observed only in supernatant 1 which was likely to be larger fragments of glucan. Supernatant 2 (collected after an additional threefold increase in cell density) contained only one major peak (G2) comigrating with that of a disaccharide standard and eluting ahead of a glucose standard (G1 std) (Fig. 6). Zymolyase digestion of this peak resulted in a shift to the right corresponding with a glucose standard, demonstrating that it contained β -(1,3) linkages (data not shown). Quantitatively, the amount of released material was sequentially less over the course of cell growth: 14.1–24.4% of the total cell-associated counts (cells + medium) appeared in supernatant 1 (culture OD₄₂₀ = 1.0) of which 8.5–12.1% of the total cell-associated counts comigrated with a laminaribiose standard; supernatant 2 (culture OD₄₂₀ = 3.0) contained 3.7–6.4% of the total cell-associated counts with 2.9–4.1% of the total cell-associated counts as laminaribiose; supernatant 3 (culture OD₄₂₀ > 8) contained 2.1–2.9% of the total cell-associated counts with 1.3–1.7% of the total cell-associated counts as laminaribiose. The total amount of released radiolabel agrees with the kinetic release analysis demonstrated above.

Radiolabel shed by actively growing cells can be reutilized

by yeast cells growing on glucose and is incorporated into TCA-precipitable material. However, the amount of incorporated material is only 3–5 % of the amount added to the growing culture. Laminaribiose does not appear to be transported as a disaccharide (data not shown). Based on the limit of detection, less than 3 pmol per reaction (culture OD₄₂₀ = 0.5 or 1.3 µl cell volume) was transported (< 2.3 µM internal laminaribiose at 85 µM and 8.5 mM external laminaribiose). However, Sc488-11A does have the ability to grow on laminaribiose as the sole carbon source, apparently after its hydrolysis to glucose (see below). Growth on a number of (1,3)- β -laminari-oligosaccharides as the sole carbon source was demonstrated, although the time to reach the maximal density was dependent on the size of the oligosaccharide. For example, the glucose-grown culture attained maximal density in less than 24 h while laminaribiose, laminari-hexaose, and laminarin required approximately 2, 3–4, and 4–5 d, respectively. Growth on (1,3)- β -laminari-oligosaccharides appeared to require functioning extracellular glucanases as demonstrated by the effects of deoxynojirimycin and castanospermine. These amino sugars act as competitive inhibitors with a variety of α - and β -glucosidases, including, for example, fungal exo- β (1,6)-glucanase (Winchester & Fleet, 1992). These inhibitors prevented growth in cultures grown with oligosaccharide substrates but did not have any effect on the glucose-grown culture. Incorporation of laminaribiose (following hydrolysis) accounts for very little of the TCA-precipitable material presumably owing to competition for transport with the cold glucose in the media. A basal level of extracellular laminaribiose may be maintained which may serve some, as yet unidentified, regulatory or protective purpose (i.e. glucan regulation, osmoregulation, immunoregulation) (Ruiz-Herrera, 1992).

Analysis of culture supernatants revealed that laminaribiose is a major wall component released from growing cells. Although a portion of the released radiolabel may be the result of changes in the wall architecture induced by washing procedures, this release continues well after exponential growth is resumed. Interestingly, laminaribiose is produced as one of the *in vitro* products of a newly identified 34 kDa wall-associated glucosyltransferase from *C. albicans* (Hartland *et al.*, 1991) and more recently from *S. cerevisiae* (P. A. Sullivan, personal communication). This enzyme transfers a linear (1,3)- β glucose oligomer to the non-reducing end of an acceptor (1,3)- β oligomer with the concomitant release of G2 from the reducing terminus of the donor molecule. Initially believed to represent a branch point, recent NMR studies revealed an intrachain (1,6)- β linkage ('kink') connecting the two (1,3)- β oligomers (Yu *et al.*, 1993). The release, during growth, of labelled laminaribiose from prelabelled yeast glucan is consistent with the *in vitro* functioning of such a glucosyltransferase, lending support to the hypothesis that the yeast wall is metabolically active. Other exo-(1,3)- β -glucanases and endo- β -glucanases have been described which release glucose, gentobiose and other oligosaccharides from glucan (Ruiz-Herrera, 1992). The role of such activities in wall biochemistry is unknown at

present. Mutants defective in specific exoglucanase activities are viable demonstrating that the products of certain exoglucanase genes are not essential for growth under normal conditions (Pitson *et al.*, 1993). However, effects on growth under non-laboratory conditions and on virulence of pathogenic strains have not been studied. One such viable mutant lacks the *BGL2* gene product, initially believed to be an exoglucanase but now considered an endoglucanase (Mrsa *et al.*, 1993). The *SFG1* gene encodes a sporulation-specific (1,3)- β -glucanase in *S. cerevisiae* (San Segundo *et al.*, 1993). From our studies of laminaribiose release and progressive decrease in the amount of shed material over time, we may speculate that newly synthesized wall material is more prone to enzymic modification than older pre-existing glucan, which would presumably have already undergone such events. It is reported that newly-formed cell wall from *Aspergillus* hyphae is more susceptible to autolysis thus increasing its plasticity during growth (Gooday *et al.*, 1992; Peberdy, 1990). Yet, with maturation, the sub-apical region has increased rigidity corresponding, presumably, to enzymic modification (Wessels *et al.*, 1990). Cell wall restructuring and modification involve a variety of activities which likely have roles in processes including: cell wall growth and extension, morphological events (i.e. in response to environmental conditions or nutrient status), autolysis, reproductive processes, and yeast-to-mycelial transition (for reviews see Peberdy, 1990; Ruiz-Herrera, 1992; Wessels *et al.*, 1990).

The ever increasing list of opportunistic fungal infections in recent years due to the increase and variety of immunocompromised patients has brought a need for better understanding of yeast cell wall biology. The unique structure of the cell wall and its importance for the maintenance of cellular integrity is clearly established in the fungal system. Fungal cell wall assembly is likely a continuous metabolically organized process made up of several intertwined and interconnected components; the importance and balance of these events need further elucidation.

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