Subcellular localization, abundance and stability of chitin synthetases 1 and 2 from *Saccharomyces cerevisiae*

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The existence of more than one chitin synthetase in fungal cells poses the question of whether these enzymes have similar or different localization. The subcellular distribution of chitin synthetases 1 and 2 (Chs1 and Chs2) was determined in cell-free extracts of *Saccharomyces cerevisiae* fractionated by sucrose density gradient sedimentation. Chs1 was examined in two strains: ATCC 26109, a wild-type strain, and D3C (*MAT α ura3-52*). Chs2 was investigated in a strain (D3B) freed of Chs1 by gene disruption (*MAT α his4 ura3-52 chs1::URA3*). A prolonged, strong centrifugation (20 h at 265 000 g) was necessary to cleanly resolve two major populations of chitin synthetase particles: chitosomes (a population of microvesicles of low buoyant density, $d = 1.15 \text{ g ml}^{-1}$) and plasma membrane (a population of vesicles of high buoyant density, $d = 1.21 \text{ g ml}^{-1}$). Chs1 and Chs2 were both present in chitosomes and plasma membrane, but the relative distribution of each chitin synthetase in these two membranous populations varied. Chs2 was much less abundant than Chs1 and required Co^{2+} rather than Mg^{2+} as a cofactor. A salient finding was the high sensitivity of chitosomal Chs2 to high centrifugal forces. The subcellular distribution of 1,3- β -glucan synthetase was the same in the three strains studied, i.e. unaffected by the presence or absence of Chs1. Culture conditions affected the profiles of chitin and glucan synthetases: the relative abundance of Chs1 in chitosomes or plasma membrane was quite different in cells grown on two different media but the buoyant density was not affected; in contrast, there was shift in the buoyant density of the two peaks of 1,3- β -glucan synthetase. We concluded that the subcellular localization of Chs1 and Chs2 remains the same despite genetic and other differences in the properties of these enzymes. We confirmed that 1,3- β -glucan synthetase and chitin synthetase exhibit a partially different subcellular distribution – an indication that these two enzymes are mobilized through different secretory pathways.

Keywords: Chitin synthetases Chs1 and Chs2, chitosomes, 1,3- β -glucan synthetase, plasma membrane, *Saccharomyces cerevisiae*

INTRODUCTION

Studies on chitin synthetase (EC 2.4.1.16) in different fungi have identified two major subcellular locations: chitosomes (Bracker *et al.*, 1976; Bartnicki-Garcia *et al.*,

1978; Hanseler *et al.*, 1983; Flores Martinez & Schwencke, 1988; Gozalbo *et al.*, 1992) and plasma membrane (Duran *et al.*, 1975; Jan 1974; Dalley & Sonneborn, 1982). Initially, in *Saccharomyces cerevisiae* Cabib and co-workers showed a plasma membrane localization for the enzyme later identified as chitin synthetase 1 (Chs1) (Cabib & Farkas, 1971; Duran *et al.*, 1975; Cabib *et al.*, 1983). With improved methods for separating membranous organelles by density gradient sedimentation, we demonstrated that Chs1 was present in both chitosomes and plasma mem-

Abbreviations: Chs1, chitin synthetase 1; Chs2, chitin synthetase 2; UDP-GlcNAc, uridine diphosphate *N*-acetyl-D-glucosamine; YPG, yeast extract/peptone/glucose medium. YNBG, yeast nitrogen base/glucose medium.

brane of growing cells of *S. cerevisiae* (Leal-Morales *et al.*, 1988). A similar dual location was observed in protoplasts of *S. cerevisiae* (Flores Martinez & Schwencke, 1988).

Molecular genetic studies on chitin synthetase from *S. cerevisiae* (Bulawa *et al.*, 1986; Sburlati & Cabib, 1986; Bulawa & Osmond, 1990; see review by Bulawa, 1993) revealed several genes coding for three chitin synthetases (Chs1, Chs2, and Chs3). Gene disruption experiments suggested that each enzyme governs a different function (Bulawa *et al.*, 1986; Silverman *et al.*, 1988; Bulawa & Osmond, 1990; Shaw *et al.*, 1991; Bowen *et al.*, 1992). It has been proposed that Chs1 has a secondary role as a repair enzyme, while Chs2 is mainly responsible for septum formation (Cabib *et al.*, 1989) and Chs3 is thought to be the main enzyme involved in chitin synthesis in yeast (lateral walls and spore wall chitosan) (Shaw *et al.*, 1991; Bulawa, 1993).

To determine whether two chitin synthetases coded by different genes have the same subcellular distribution, we examined the distribution of Chs2 in fractionated cell-free extracts to compare it with our previous findings on Chs1. Since Chs1 is the dominant chitin synthetase in wild-type cells, it was necessary to use a yeast strain (D3B) lacking Chs1, through disruption of its *CHS1* gene (*chs1::URA3*), to determine the localization and properties of Chs2. Possible interference by Chs3 was deemed unlikely since it is present only in small amounts and does not respond to activating protease (Shaw *et al.*, 1991). We found that Chs2 was also distributed in two major subcellular fractions with different buoyant densities and sedimentation velocities.

METHODS

Strains. For studies on Chs2 we used the D3B strain of *Saccharomyces cerevisiae* freed of Chs1 by gene disruption (*MATa his4 ura3-52 chs1::URA3*) (Sburlati & Cabib, 1986). For studies on Chs1, two strains of *S. cerevisiae* were used: ATCC 26109, a wild-type strain we previously examined (Leal-Morales *et al.*, 1988), and D3C (*MATa ura3-52*), a strain arising from the same tetrad in the above gene disruption (Bulawa *et al.*, 1986; Sburlati & Cabib, 1986). The D3B and D3C strains were kindly provided by E. Cabib (NIH, Bethesda, MD 20892, USA).

Culture conditions. Strain ATCC 26109 was cultivated in two liquid media: (i) a filter-sterilized medium containing 0.7% (w/v) yeast nitrogen base (Difco) and 2% (w/v) glucose (YNBG), and (ii) a YPG medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose. Media were autoclaved at 121 °C for 15 min. Strains D3C and D3B were grown only on YPG medium because they were auxotrophic. Cultures were grown at 30 °C in a shaker bath at 150 r.p.m. Preinoculum (25 ml) was started in the corresponding medium from a loopful of cells taken from a slant culture less than 15 d old. Approximately 12 h later, cells were transferred into fresh medium; usually 1200 ml of either medium was used (initial density 250 000 cells ml⁻¹). Cultures were grown for about 12 h and harvested in exponential phase at a cell density of 4–7 × 10⁷ cells ml⁻¹.

Preparation of cell-free extracts. Cells were collected by centrifugation at 1500 g for 5 min, washed in 100 ml 1 mM EDTA, resuspended in 20 ml 1 mM EDTA, and filtered

through a 5 µm pore size Millipore nitrocellulose membrane. Suction was applied until all the liquid was drained. The cells were weighed (approx. 3.3–5.7 g) and placed into a 75 ml Duran bottle; 2 ml of glass beads (0.45–0.50 mm diameter) and 1 ml 17% (w/v) sucrose in 1 mM EDTA were added per g of wet cells. Cells were then disrupted in a Braun MSK homogenizer for 12–15 s while the flask was cooled with CO₂. After breakage, the crude extract was removed and the glass beads were washed with 1 mM EDTA [1 ml (g wet cells)⁻¹]. The crude extract and washings were combined and centrifuged at 1500 g for 10 min. The supernatant was used for sucrose density gradient centrifugation.

Density gradient centrifugation. A 5–7 ml sample of the 1500 g supernatant was layered on top of a 33 ml linear sucrose gradient (10–65%, w/v) in 50 mM Tris/HCl, pH 7.5. Beckman Quick-Seal polyallomer ultracentrifuge tubes (1 in. × 3.5 in.) were used. The tubes were centrifuged at 265 000 g in a Beckman 70Ti rotor for 3 h or 20 h. In some experiments the centrifugation was done under milder conditions at 75 100 g in a Beckman SW-27 rotor. Where indicated, selected samples used for electron microscopy were further purified by isopycnic flotation (Lending *et al.*, 1990). Pairs of adjacent gradient fractions were combined, and diluted with 2 vols 65% (w/v) sucrose, to adjust the sucrose concentration to about 51–54% (w/v). The samples were then applied to a 35 ml linear sucrose gradient (10–65%, w/v) with a Pasteur pipette (inserted into the gradient at a depth with the same density as that of the sample), and centrifuged in a SW-27 rotor at 75 100 g for 20 h. All gradients were fractionated from the top with an ISCO fractionator; samples of 1 ml were collected. All centrifugations were done at 4 °C. Centrifugal forces are given as r_{av} .

Enzyme assays. Chitin synthetase activity was measured as the incorporation of radioactivity from UDP-[¹⁴C]GlcNAc into 5% (w/v) TCA-insoluble material. The assay mixture contained 1 mM UDP-GlcNAc (specific activity 31.4 mCi mol⁻¹), 32 mM GlcNAc and 4 mM MgCl₂. Chs2 was assayed similarly, but 3 mM Co(NO₃)₂ was added instead of magnesium. Except where indicated, all assays for Chs1 or Chs2 were done in the presence of 80 µg trypsin ml⁻¹ to activate zymogenic chitin synthetase. One unit of enzyme is the amount that catalyses the incorporation of 1 nmol GlcNAc min⁻¹.

We determined 1,3-β-glucan synthetase activity by measuring the incorporation of radioactivity from UDP-[¹⁴C]Glc into 5% TCA-insoluble material, but the incubation was done in the presence of 5 mM ATP as described earlier (Leal-Morales *et al.*, 1988; Leal-Morales & Ruiz-Herrera, 1985). Enzyme activity is expressed in units. One unit is the amount of enzyme that catalyses the incorporation of 1 nmol glucose min⁻¹. Radioactivity was counted in an LS 3801 liquid scintillation spectrometer (Beckman).

Data processing. To obtain average enzyme profiles from replicated sucrose density gradients, the original data points in units of chitin synthetase or 1,3-β-glucan synthetase activity for each individual fraction were first converted to percentage of the total activity in the entire gradient and plotted against specific gravity. The resulting curves were digitized to generate ordinate values for 75 or 150 common specific gravity points with the aid of a Hewlett-Packard plotter model 7475 driven by the Graph PAD program, version 2.0 (Institute for Scientific Information). Mean values were calculated and plotted for each specific gravity.

To estimate the relative distribution of chitin synthetase on the sucrose gradients, the usual midway density point (1.185 g ml⁻¹) in the valley between the peaks of chitosomes and the peak of plasma membrane was regarded as the dividing line. Enzyme

activity below this value was considered as chitosomal; activity above this point pertained to plasma membrane.

Staining for electron microscopy. Samples were negatively stained with 2.5% (w/v) uranyl acetate as already described (Leal-Morales *et al.*, 1988).

Specific gravity. This was calculated from the sucrose concentration values measured with an Abbe refractometer (Carl Zeiss).

Chemicals. Ultra-pure grade sucrose was from Schwarz/Mann Biotech. GlcNAc, UDP-GlcNAc and UDP-Glc were obtained from Sigma. UDP-[Glc-¹⁴C]GlcNAc (4–5 Ci mol⁻¹), UDP-[Glc-¹⁴C]Glc (240 Ci mol⁻¹) and Zymolyase-20T were purchased from ICN (Irvine, California, USA).

RESULTS

Chs1 in wild-type strain ATCC 26109

Growing cells. The chitin synthetase of this wild-type strain can be consistently separated into two major subcellular fractions, chitosomes and plasma membrane, by prolonged centrifugation (20 h) on sucrose density gradients (265 000 *g* in a 70Ti rotor; Fig. 1). Because of unavoidable minor variations in the profiles of chitin synthetase from one gradient to the next, we averaged the profiles from several separately centrifuged gradients to get a more reliable estimate of buoyant density and the relative distribution of the chitin synthetase populations (Fig. 1). Accordingly, after 20 h at 265 000 *g*, the chitosome fraction equilibrated at 1.146–1.150 *g* ml⁻¹, and the chitin synthetase associated with plasma membrane equilibrated at 1.213–1.214 *g* ml⁻¹ (Table 1; Fig. 1). The culture medium affected the relative abundance of Chs1 in chitosomes and plasma membrane populations (see below).

Effect of culture medium. Because it was necessary to grow the auxotrophic mutant used for studies of Chs2 in a richer culture medium than that used before to study Chs1 in the wild-type strain ATCC 26109 (Leal-Morales *et*

al., 1988), we compared the effect of the medium on the distribution of chitin synthetase in homogenates of this strain. The discrepant culture media did not affect the total amount of enzyme activity recovered in the gradients or the buoyant density of the two chitin synthetase fractions, but they did alter the relative proportions of chitosomal and plasma-membrane-bound chitin synthetase (Table 1; Fig. 1). In cells grown in rich medium (YPG), the chitosomal Chs1 was more abundant (range 59–80%; mean 70%) than plasma membrane Chs1 (range 20–41%; mean 30%). In cells grown in a poorer medium (YNBG) both populations were of similar abundance (Table 1).

Fig. 1. Profiles of Chs1 from *S. cerevisiae* wild-type strain ATCC 26109 and mutant strain D3C. The former was grown in two different culture media, YNBG and YPG. Cell-free extracts (5–7 ml; 1500 *g* supernatant) were applied to linear sucrose gradients and centrifuged for 20 h at 265 000 *g*. Each profile is the average of between three and seven individual runs. C, chitosome peaks; P, plasma membrane peaks. Chs1 activity is expressed as a percentage of the total activity recovered in the gradient. To avoid overlapping, the upper two profiles were displaced vertically by 3 and 7%, respectively.

Table 1. Relative distribution of Chs1 and Chs2 in sucrose density gradients

Cell-free extracts (1500 *g* supernatants) from cells grown in either YPG or YNBG medium were centrifuged on sucrose gradients (10–65%) for 20 h in either a 70Ti rotor (265 000 *g*) or a SW-27 rotor (75 100 *g*). Total enzyme present in the chitosomal (1.11–1.185 *g* ml⁻¹) and plasma membrane (1.187–1.26 *g* ml⁻¹) regions was averaged from several experiments and the relative abundance of chitin synthetase was calculated.

Enzyme	Strain	Culture medium	Rotor	No. of runs	Chitosomes		Plasma membrane	
					Relative abundance (%)	Peak density (g ml ⁻¹)	Relative abundance (%)	Peak density (g ml ⁻¹)
Chs1	ATCC 26109	YPG	70 Ti	6	69.5	1.146	30.5	1.214
		YPG	SW-27	2	67.9	1.150	32.1	1.190
		YNBG	70 Ti	4	48.4	1.150	51.6	1.213
	D3C	YPG	70 Ti	3	53.1	1.150	46.9	1.210
Chs2	D3B	YPG	70 Ti	5	25.0	NP	75.0	1.210
		YPG	SW-27	5	55.9	1.165	44.1	1.205

* NP, No peak.

Table 2. Recovery of Chs1 or Chs2 and 1,3- β -glucan synthetase activity in a prolonged centrifugation

Total enzyme units were measured in cell-free extracts from cells grown in YPG and centrifuged on sucrose density gradients at 265 000 *g*. The percentage of enzyme activity recovered after 20 h centrifugation is compared to that after 3 h centrifugation.

Enzyme	Strain	Chitosomes		Total recovery (%)	Plasma membrane		Total recovery (%)
		Total units after centrifugation for:	Recovery (%)		Total units after centrifugation for:	Recovery (%)	
		3 h	20 h		3 h	20 h	
Chs1	ATCC 26109	57.8	48.5	83.9	29.6	17.4	58.8
	D3C	67.2	52.3	77.8	49.9	30.8	61.7
Chs2	D3B	3.4	1.4	41.2	4.0	3.3	82.5
1,3- β -Glucan synthetase	ATCC 26109				88.0	56.6	64.3
	D3C				27.4	37.3	136.2
	D3B				28.8	27.5	95.5

Fig. 2. Profiles of Chs2 from strain D3B after (a) strong and (b) mild centrifugation. A 5–7 ml sample of cell-free extract (1500 *g* supernatant) was applied on top of a sucrose gradient and centrifuged for 3 h (fine trace) or 20 h (heavy trace) in: (a) a 70Ti rotor at 265 000 *g*; (b) a SW-27 rotor at 75 100 *g*. Chs2 activity was assayed in the presence of 3 mM Co^{2+} and 80 μg trypsin ml^{-1} . In (a) each profile is the average of five individual runs. In (b) each profile corresponds to a single run. C, chitosome peak; P, plasma membrane peak. The positions of fractions 22 and 32 are indicated in (b).

Chs1 in mutant strain D3C

The Chs1 of strain D3C was compared with the Chs2 activity of its sister strain (D3B) used below. As with the Chs1 of ATCC 26109, two distinct peaks of chitin synthetase were clearly resolved after a prolonged 20 h

centrifugation (Fig. 1). Both chitin synthetase peaks from D3C had nearly identical buoyant densities to those from ATCC 26109 (Table 1). In this mutant strain, chitosomes and plasma membranes contained about equal amounts of the chitin synthetase recovered in the gradient. After a 20 h centrifugation, the overall recovery was 71 % of the chitin synthetase activity present at 3 h. Chitosomal and plasma membrane forms of Chs1 of D3C were about as stable as those from ATCC 26109 strain (Table 2).

Chs2 in mutant strain D3B

A homogenate of strain D3B (1500 *g* supernatant) was subjected to the same sucrose density gradient centrifugations used to separate Chs1 from wild-type cells (265 000 *g*, for 3 or 20 h). In contrast to Chs1, prolonged centrifugation at 265 000 *g* (20 h) failed to reveal two populations of Chs2 (Fig. 2a); there was only one major peak of chitin synthetase in the plasma membrane region (1.21 g ml^{-1}) and little evidence of chitin synthetase activity in the chitosome region (1.11–1.185 g ml^{-1}). In a shorter centrifugation (3 h), a small amount of Chs2 appeared in the chitosomal region (Fig. 2a; shoulder on the left of the main peak) but this was nearly wiped out upon continued centrifugation (Fig. 2a; Table 2).

Two possible explanations for the preferential loss of chitosomal Chs2 were considered: (i) the low-density activity (presumptive chitosomal enzyme) detected after 3 h was already at its equilibrium density but became unstable, or (ii) the low-density activity was not chitosomal but a denser form of chitin synthetase that had not yet reached equilibrium by 3 h. To test these alternatives, a parallel sample of cell-free extract was centrifuged on a 10–65 % sucrose gradient under milder conditions, a velocity sedimentation in a SW-27 rotor at 75 100 *g*. After 3 h, two distinct chitin synthetase populations, a slow-

Table 3. Properties of Chs1 and Chs2 in two strains of *S. cerevisiae*: activation by trypsin, Mg^{2+} and Co^{2+}

Crude extracts of strains made from the same amount of cells (65×10^6) of the ATCC 26109 and D3B strains were fractionated on a sucrose density gradient centrifuged for 20 h at 75 100 *g*. Chitin synthetase activity in the presence or absence of trypsin and Mg^{2+} or Co^{2+} was measured in the fractions. The values shown are the sum of all individual fractions in the gradient.

Enzyme	Assay condition	Chitin synthetase activity (units)			
		Chitosomal	Plasmalemma	Total	Relative (%)
Chs1 (from wild-type strain ATCC 26109)	Trypsin (80 μ g ml ⁻¹) + Mg^{2+} (4 mM)	44.1	19.5	63.6	100
	Trypsin (80 μ g ml ⁻¹) + Co^{2+} (3 mM)	9.7	3.4	13.1	21
	Mg^{2+} (4 mM)	1.1	1.1	2.2	3
Chs2 (from mutant D3B)	Trypsin (80 μ g ml ⁻¹) + Mg^{2+} (4 mM)	1.7	2.1	3.8	6
	Trypsin (80 μ g ml ⁻¹) + Co^{2+} (3 mM)	5.0	4.4	9.4	15
	Co^{2+} (3 mM)	2.2	2.1	4.3	7

Table 4. Effect of centrifugal force on Chs2 activity from chitosomal and plasma membrane fractions of strain D3B

The chitosomal and plasma membrane populations separated from a cell-free extract of strain D3B on the SW-27 rotor centrifuged for 20 h at 75 100 *g*, were recentrifuged at two different centrifugal forces, 75 100 *g* in the SW-27 rotor and 265 000 *g* in the 70Ti rotor for 20 h.

Fraction	Centrifugal force (g)	Initial activity applied (units)	Activity recovered (%)
Chitosome	75 100	0.56	70.6
	265 000	0.56	38.9
Plasma membrane	75 100	0.77	68.9
	265 000	0.77	63.3

Fig. 3. Comparative activity of Chs1 and Chs2 from strain ATCC 26109 and mutant D3B, respectively. Samples of 1500 *g* supernatant, prepared from the same number of cells of each strain, were layered on sucrose density gradients and subjected to a mild centrifugation in the SW-27 rotor at 75 100 *g* for 20 h. ●, Chs1; ○, Chs2. The positions of fractions 22 and 25 are indicated (see Fig. 4).

moving (chitosome) peak and a fast-moving (plasma membrane) peak, were detected (Fig. 2b). After 20 h, the chitosome peak became compacted into a sharp peak at $d = 1.149$ g ml⁻¹, while the plasma membrane equilibrated at 1.207 g ml⁻¹. In general, the profiles of Chs2 were not as smooth as those of Chs1, but this is probably a result of experimental variation in view of the low levels of Chs2 being measured. The buoyant density for the peaks of chitosomal Chs2 oscillated in the range 1.149–1.175 g ml⁻¹ and 1.195–1.210 g ml⁻¹ for the plasma membrane. The chitosomal and plasma membrane populations comprised 56 % and 44 %, respectively, of the Chs2 activity recovered in the gradient (Fig. 2b; Table 1).

The detection of two populations of Chs2 after prolonged centrifugation of cell-free extract in the swinging bucket (SW-27) rotor but not in the fixed angle (70Ti) rotor (Fig. 2a and 2b, respectively) led us to suspect that the stronger centrifugal force generated by the latter might have caused the selective destruction of the chitosomal population of Chs2. To compare the effect of different centrifugal forces (hydrostatic pressure), on chitin synthetase particles, we collected and pooled the suspected chitosome fractions (21–27) and plasma membrane fractions (28–34) from two identical gradients that had been centrifuged for 20 h at 75 100 *g* (similar to the 20 h run in Fig. 2b). Duplicate samples of 5.7 ml from the two pools were loaded into four (33 ml, 10–65 %) sucrose gradients. The samples were deposited with a Pasteur pipette inserted into the gradient at a depth with the same density as that of the sample. Each duplicate sample was centrifuged for 20 h under two different conditions: (i) SW-27 rotor at 75 100 *g* and (ii) 70Ti rotor at 265 000 *g*. In both rotors, the recentrifuged samples equilibrated at the same densities as in the first centrifugation, approximately 1.17 g ml⁻¹ for chitosomes and 1.21 g ml⁻¹ for plasma membrane. In the SW-27 rotor there was no preferential destruction. Both the chitosomes and plasma membrane lost about 30 % of activity during centrifugation (Table 4). However, in the 70Ti rotor, 61 % of the activity of the

Table 5. Morphometry of vesicle populations from wild-type and mutant strain D3B of *S. cerevisiae*

A minimum of 100 vesicles were measured from each of the samples shown in Fig. 4(e–h). Samples were from two places in the chitosome region: $d = 1.14$ and $d = 1.16$.

Vesicle diameter (nm)	Relative abundance (%) of vesicles from strain:			
	ATCC 26109		D3B	
	$d = 1.14$ (Fig. 4e)	$d = 1.16$ (Fig. 4g)	$d = 1.14$ (Fig. 4f)	$d = 1.16$ (Fig. 4h)
40–70	37.7	27.2	32.5	29.8
70–100	64.0	47.4	58.8	53.5
100–330	36.0	52.6	41.2	46.5
Median diameter (nm)	80.7	91.7	76.9	79.3

chitosomal sample applied was lost after centrifugation at 265 000 *g* for 20 h; activity in the plasma membrane region was less affected by the centrifugal force, and only 37% of the activity was lost.

To estimate the relative abundance of Chs1 and Chs2, we broke equal amounts of cells of the ATCC 26109 strain and the D3B mutant. The cell-free extracts were centrifuged at 71 000 *g* for 20 h on sucrose density gradients. We found that the amount of Chs2 recovered from the gradient was only 11–15% of the amount of Chs1 recovered from the wild-type strain (Table 3, Fig. 3).

Electron microscopy

To ascertain if the large quantitative differences in chitin synthetase activity between Chs1 and Chs2 strains in the chitosome fractions reflected changes in abundance or morphology of particles, parallel samples from both gradients shown in Fig. 3 were compared. In both gradients, samples from two places in the chitosome region, at $d = 1.14$ (Fig. 4a, b) and at $d = 1.16$ (Fig. 4c, d) had a similar appearance: vesicles of widely different sizes were scattered among numerous smaller particles resembling ribosomes. To obtain a clearer view of vesicle size and distribution, selected samples were recentrifuged by isopycnic flotation. Most of the ribosomes and

background contaminants disappeared, leaving a heterogeneous collection of vesicles (Fig. 4e–h, Table 5). There were no appreciable differences in vesicle abundance between the Chs1 strain (Fig. 4e, g) and the Chs2 strain (Fig. 4f, h). The median vesicle diameter was between 77–92 nm; the proportion of microvesicles of typical chitosome size (40–70 nm) was similar (27–37%) for the Chs1 strain and the Chs2 strain (Table 5). No attempt was made to separate the microvesicles from the larger vesicles as was done previously (Leal-Morales *et al.*, 1988).

Activators of chitin synthetase

Chs2 required both trypsin and cobalt for maximum activity, and both the chitosomal and plasma membrane populations of Chs2 behaved similarly in zymogenicity and metal requirement (Table 3). In contrast, Chs1 was better activated by magnesium than by cobalt. The activation by trypsin was much more pronounced for Chs1 than for Chs2: whereas Chs2 was activated 2–3-fold, Chs1 was increased 17–40-fold. From the values in Table 3, it follows that nearly all of Chs1 (97%) was zymogenic (only 2.2 out of 63.6 units were expressed in the absence of trypsin). For Chs2, 55% of the enzyme was zymogenic at the time of assay; the rest (4.3 out of 9.4 units) required no activation by trypsin.

1,3- β -Glucan synthetase

ATCC 26109. Two fractions of 1,3- β -glucan synthetase were detected in gradients from cells grown in YNMG medium (Fig. 5a). A major peak with a buoyant density of 1.220 g ml^{-1} and 62.7% of the total activity was in the plasma membrane region. A somewhat smaller peak was found at a buoyant density of 1.180 g ml^{-1} (Table 6). The major peak of glucan synthetase coincided with the plasma membrane peak of chitin synthetase, but the minor peak did not (Fig. 5a, Tables 1, 6). In fact, there was practically no 1,3- β -glucan synthetase activity in the chitosome peak region (1.14–1.16 g ml^{-1}). The profile of 1,3- β -glucan synthetase was markedly affected by the culture medium. Thus, in cells grown on YPG, the profile of 1,3- β -glucan synthetase was much more compact and the peaks were closer together (Fig. 5b, Table 6).

Mutant D3C. After 20 h centrifugation at 265 000 *g*, most of the 1,3 β -glucan synthetase present in this strain appeared as a single broad peak with a density of

Fig. 4. Comparative morphology of parallel cell fractions containing Chs1 and Chs2 from *S. cerevisiae* strains ATCC 26109 (a, c, e, g) and mutant D3B (b, d, f, h), respectively. Parallel fractions from the gradients in Fig. 3 were examined before (a, b, c, d) and after (e, f, g, h) an additional centrifugation (isopycnic flotation) at 75 100 *g* for 20 h. Fractions were selected from two different densities in the gradients: $d = 1.14$ (a, b, e, f) and $d = 1.16$ (c, d, g, h). The initial samples (a, b, c, d) were taken from the gradients in Fig. 3 as follows: (a) ATCC 26109 sample at $d = 1.14$ (combined fractions 21+22); (b) D3B sample at $d = 1.14$ (combined fractions 22+23); (c) ATCC 26109 sample at $d = 1.16$ (combined fractions 25+26); (d) D3B sample at $d = 1.16$ (combined fractions 25+26). For isopycnic flotation, samples a, b, c, and d were each independently mixed with two volumes of 65% sucrose, introduced near the bottom of linear 10–65% sucrose density gradients, and recentrifuged at 75 100 *g* for 20 h. Samples from the four gradients were withdrawn at densities corresponding to those in the original samples and examined microscopically: $d = 1.14$ (e, f) and $d = 1.16$ (g, h). Bar, 100 nm.

Fig. 5. Distribution of 1,3- β -glucan synthetase activity from the wild-type strain ATCC 26109 grown in two different culture media: (a) YNBG medium, (b) YPG medium. The cell-free extracts were processed as described for Fig. 1, and centrifuged for 20 h at 265 000 *g*. Average 1,3- β -glucan synthetase profiles (—) are compared with the average chitin synthetase profiles (---) from Fig. 1.

1.208 g ml^{-1} . This peak co-sedimented with the plasma membrane peak of chitin synthetase (Tables 1, 6). A shoulder of 1,3- β -glucan synthetase was at ca 1.180 g ml^{-1} (Fig. 6). The enzyme profiles of this mutant resembled those of the wild-type strain ATCC 26109 (Fig. 6).

Mutant D3B. As with strain D3C, most of 1,3- β -glucan synthetase present in the cell-free extract (1500 *g* supernatant) of sister strain D3B appeared in a single broad peak with a density of 1.205 g ml^{-1} after 20 h centrifugation at 265 000 *g* (Table 6). This 1,3- β -glucan synthetase co-sedimented with the plasma membrane peak of chitin synthetase (Tables 1, 6), including a shoulder at approximately 1.180 g ml^{-1} as with the other strains (Fig. 6).

Fig. 6. Profiles of 1,3- β -glucan synthetase from homogenates of the ATCC 26109 and the two sister strains, D3C and D3B, of *S. cerevisiae* grown on YPG medium. The profiles were obtained after a 20 h centrifugation as described for Fig. 1. To avoid overlapping, the profiles of D3C and ATCC 26109 were displaced vertically by 5 and 10 %, respectively.

DISCUSSION

Subcellular localization of Chs1 and Chs2

We found that Chs2 occurs in the same two vesicle populations that were previously identified for Chs1 in *S. cerevisiae* (Leal-Morales *et al.*, 1988): (i) chitosomes, microvesicles (diameter typically 40–70 nm) with a low buoyant density (1.15 g ml^{-1}), lacking 1,3- β -glucan synthetase activity; and (ii) plasma membrane vesicles of larger diameter (> 100 nm), higher buoyant density (1.21 g ml^{-1}), and rich in 1,3- β -glucan synthetase.

In our previous work on Chs1 (Leal-Morales *et al.*, 1988), we emphasized that an appropriate centrifugation protocol was the key to successful fractionation of different vesicle populations from cell-free extracts on sucrose density gradients. Thus, to isolate a relatively pure sample of chitosomes from *S. cerevisiae* two steps were required: a prolonged, strong, isopycnic centrifugation (20 h at 265 000 *g*) to separate chitosomes from the plasma-membrane-bound enzyme, and a velocity sedimentation to segregate the chitosome microvesicles from

Table 6. Relative distribution of 1,3- β -glucan synthetase in sucrose density gradients

Cell-free extracts (1500 *g* supernatants) from cells grown in either YPG or YNBG medium were centrifuged on sucrose gradients (10–65 %) at 265 000 *g* in the 70Ti rotor for 20 h. Total enzyme values were calculated for two regions of the gradient. Values from several experiments were averaged and the relative abundance of 1,3- β -glucan synthetase was calculated.

Strain	Culture medium	No. of runs	Minor peak		Major peak	
			Relative abundance (%)	Peak density (g ml^{-1})	Relative abundance (%)	Peak density (g ml^{-1})
ATCC 26109	YPG	3	32.2	1.195	67.8	1.218
	YNBG	3	37.3	1.180	62.7	1.220
D3C	YPG	1	37.7	1.180	62.3	1.208
D3B	YPG	2	42.0	1.180	58.0	1.205

larger vesicles of equal density. Such an elaborate protocol for separation of vesicles containing Chs2 was not feasible because of the small amounts of Chs2 present in the cell plus the lack of stability of chitosomal Chs2 during high-speed centrifugation.

Differences between Chs1 and Chs2

We confirmed that Chs2 is present in yeast cells in much lower proportions than Chs1 (11–15%). Our procedure probably enabled greater recovery of Chs2 activity than the procedure used by Sburlati & Cabib (1986), who reported a Chs2 content of 5%. We also confirmed that Chs2 is preferentially stimulated by Co^{2+} rather than by Mg^{2+} (Sburlati & Cabib, 1986). We also found, as did the latter authors, that Chs2 is a zymogen which can be activated by trypsin, although Chs2 zymogen is activated much less than Chs1. We interpret this reduced zymogenicity of Chs2 as an indication that the smaller pool of chitin synthetase is more prone to undergo spontaneous activation by endogenous proteases in the cell-free extract.

Orlean (1987) found that Chs2 was not activated by trypsin. However, Shaw *et al.* (1991) believed that the enzyme studied by Orlean was actually Chs3, which according to Bulawa & Osmond (1990) does not respond to protease activation.

Our values for Chs2 may include some Chs3 activity since the latter is also present in the D3B mutant. However, given that the total amount of Chs3 detectable in cell-free extracts is much smaller than that of Chs2, and that our assays were done under conditions that favoured Chs2 (Co^{2+} rather than Mg^{2+} as a cofactor) and destroyed Chs3 (trypsin treatment), we conclude that only a small percentage of the total activity detected in the D3B mutant may be ascribed to Chs3. A novel difference between Chs1 and Chs2 was the relative instability of chitosomal Chs2 during high-speed centrifugation.

Destruction of Chs2 by hydrostatic pressure

The destruction of Chs2 during long centrifugation at 265 000 *g* was unexpected. Previous studies with the Chs1 of *S. cerevisiae* had shown no such destruction of chitosomal chitin synthetase during prolonged high speed centrifugation. Similarly, the chitin synthetase in chitosomes from *Mucor rouxii* (Flores-Martinez *et al.*, 1990; Kamada *et al.*, 1991) exposed to even higher forces (373 000 *g*, r_{av}) survived with no apparent destruction. Since the Chs1 and Chs2 of *S. cerevisiae* are equally stable at 1 *g* (non-centrifuged samples), the selective loss of activity of Chs2 during high-speed centrifugation must have been caused by the enormous hydrostatic pressure exerted by the high centrifugal force. At the place in the gradient where chitosomes equilibrate, the pressure was at 460 and 1000 atm at 75 100 and 265 000 *g*, respectively.

There are a number of reports on the deleterious effect of hydrostatic pressure on enzymes. Champeil *et al.* (1981) found that high pressure during high-speed centrifugation irreversibly inactivated the ATPase of sarcoplasmic reticulum vesicles. High pressure rendered fish lactate

dehydrogenase more susceptible to proteolytic degradation (Hennessey & Siebenaller, 1987) and caused the reversible inactivation of bacterial tryptophan synthetase (Seifert *et al.*, 1984) and mitochondrial ATPase synthase (Dreyfus *et al.*, 1988). Since plasma-membrane-bound Chs2 was much more stable than the chitosomal Chs2, the instability may not be caused solely by the structural changes in the Chs2 molecule but by other factors present in the chitosomes of the D3B strain that may render Chs2 more susceptible to degradation. We ruled out the possibility that the selective destruction of Chs2 was caused by a soluble protease that moved into the chitosome band during protracted centrifugation as occurred when chitosomes of *M. rouxii* were isolated by high-speed centrifugation (Kamada *et al.*, 1991).

Changes induced by the culture medium

Although the relative abundance of the two vesicle populations of Chs1 was affected by culture conditions, the buoyant density, a gross indicator of composition, remained the same. In contrast, the same changes in culture medium affected the buoyant density of the 1,3- β -glucan synthetase peaks. This discrepant behaviour suggests that these two polysaccharide synthetases may be contained in different vesicle subpopulations.

Conclusion

This study showed that Chs1 and Chs2 are located in the same subcellular sites despite structural (genetic) differences that affect other properties of these enzymes. We confirmed that chitin synthetase and 1,3- β -glucan synthetase, two enzymes that function at the cell surface, exhibit different subcellular distributions in fractionated homogenates. This is a strong indication that these two wall-synthesizing enzymes are mobilized to the cell surface through different secretory pathways.

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