Glycoprotein molecules in the walls of Schizosaccharomyces pombe wild-type cells and a morphologically altered mutant resistant to papulacandin B

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Schizosaccharomyces pombe cell walls contain two major glycoprotein species, I and II, with molecular masses of $2 \times 10^6$ and $5 \times 10^5$ Da respectively, as determined by gel filtration chromatography and PAGE. The ratio of sugar to protein is higher in species I than in species II. Much of the sugar in both glycoproteins (about 85% in wild-type cells) is O-linked to the peptide moiety. The morphological sphl mutant is resistant to papulacandin B, and its cell wall contains less glycoprotein II (but not less glycoprotein I) than the parental wild-type strain, although glycoprotein II is still synthesized and released into the growth medium. Papulacandin B largely reverses the morphological alteration of the mutant, and returns the ratio between species I and II to about that found in the parental strain, although the absolute amount of species II is still lower in the mutant. The results point to the importance of the relative amounts of the different wall polymers in determining cell morphology.

Introduction

The fission yeast Schizosaccharomyces pombe differs from budding yeasts such as Saccharomyces cerevisiae in aspects of its cell cycle and gene structure, as well as in cell morphology (Russell & Nurse, 1986). In Sacch. cerevisiae and Candida albicans, changes in cell morphology correlate with qualitative and quantitative differences in the mannoproteins of the cell wall released by the glucanase complex Zymolyase (Elorza et al., 1985; Herrero et al., 1985), and the major mannanprotein components thus liberated are larger than 150 kDa (Valentin et al., 1987; Elorza et al., 1988). This mannanprotein material is more heterogeneous in size in Sacch. cerevisiae than in C. albicans, where it may be resolved in defined bands by gel electrophoresis. This heterogeneity is due to the variable length of the saccharide component of the molecules (Valentin et al., 1987; Elorza et al., 1988).

No detailed studies have previously been made on the nature of the Zymolyase-released glycoprotein material from isolated walls of S. pombe, although the existence of a high-molecular-mass glycoprotein component has been reported (Herrero et al., 1987). More is known about the chemistry of the sugar moiety (mannan) of S. pombe cell-wall glycoprotein. It consists of a (1→6)-linked backbone of mannose residues, with (1→2)-linked mannose chains having α-linked terminal galactose units (Bush et al., 1974; Horisberger & Rosset, 1977). The existence of galactose in the cell wall glycoprotein is characteristic of S. pombe among yeast species, as is the absence of chitin, and the presence of an alkali-soluble α-glucan, together with the more extensively present β-glucan (Manners et al., 1974; Manners & Meyer, 1977).

J. C. Ribas and co-workers (personal communication) have isolated a collection of S. pombe mutants resistant to papulacandin B, an inhibitor of glucan synthesis (Baguley et al., 1979; Durán et al., 1984). One of these mutants, sphl (spherical shape), exhibits gross morphological alterations, and contains less galactomannan than the parent strain. Genetic studies indicate that the papulacandin B resistance and the altered morphology are closely-linked defects, although it has still to be shown that they are due to a single mutation (J. C. Ribas and co-workers, personal communication). The present study examines the glycoprotein composition of cell walls from the sphl mutant and its parent strain, hoping to improve our knowledge of S. pombe cell wall structure, and its relation to cell morphology.
Methods

Strains, growth conditions and radioactive labelling. Wild-type strain S. pombe 972h- (ATCC 24843) was used, and a papulacandin B-resistant sphl mutant obtained from it (J. C. Ribas and co-workers, personal communication).

To label the sugar moiety of glycoproteins, cells were grown in YPD medium (containing 1% yeast extract plus 2% peptone and 2% glucose, w/v) at 30 °C to exponential phase (about 250 μg dry weight ml⁻¹). At this point, they were sedimented, and resuspended at the same concentration in fresh medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 1.8% (w/v) galactose, 0.2% glucose and 0.15 μCi ml⁻¹ (11.1 kBq ml⁻¹) of [U-¹⁴C]mannose (0.3 Ci mmol⁻¹) (Amersham), and incubation was continued for 3 h at 30 °C before cell processing.

For protein labelling, cells were grown at 30 °C to exponential phase in Yeast Nitrogen Base (YNB) medium without amino acids (Difco) plus 2% (w/v) glucose. [¹⁴C]Protein hydrolysate (56 mCi per matom carbon) (Amersham) was then added at a concentration of 0.3 μCi ml⁻¹ (22.2 kBq ml⁻¹), and incubation was continued for 3 h.

To fractionate cell wall polysaccharides, cells grown at 30 °C in YPD medium to exponential phase were sedimented and resuspended in fresh YPD medium containing a lower concentration of non-radioactive glucose (0.2%) and 0.3 μCi ml⁻¹ (22.2 kBq ml⁻¹) (0.25 Ci mmol⁻¹) of [¹³C]glucose (Amersham). Incubation was continued for a further 4 h at 30 °C.

In some experiments, papulacandin B (Ciba Geigy) (50 μg ml⁻¹, from a stock solution at 100 mg ml⁻¹ in methanol) was added to the cultures one hour before addition of the corresponding isotope; an identical volume of methanol alone was added to the control cultures.

Isolation of cell walls and culture supernatants. Cells were broken mechanically and walls were purified by repeated washings, as described by Elorza et al. (1988). Supernatant fluids were separated from the cells by centrifugation (120 000 g, 10 min) and further filtration through nitrocellulose filters (1.2 μm pore size). The filtrates were concentrated by lyophilization, and then dialysed against double distilled water at 4 °C with repeated changes; the dialysed material was precipitated in 75% (v/v) ethanol (overnight at 4 °C) and the precipitate was resuspended in a small volume of 10 mM-sodium phosphate buffer, pH 7.0.

Fractionation of wall polysaccharides. Purified walls and culture supernatants labelled with [¹⁴C]glucose were fractionated as described by Murgui et al. (1985). Alkali-insoluble material corresponds to β-glucan, while the alkali-soluble material is further divided into a Fehling-solution-precipitable fraction (galactomannan) and a non-precipitable fraction that includes α-glucan plus some β-glucan (Manners & Meyer, 1977).

Solubilization of wall glycoproteins. Purified walls (the equivalent obtained from 10 mg dry weight of cells) were incubated with Zymolase 20T (Miles Laboratories) (100 μg) or Novozym 234 (Sigma) (200 μg) in 1 ml of 10 mM-citrate/phosphate buffer, pH 5.8, at 30 °C for 2 h. This period was sufficient for maximal liberation of sugar from the walls (data not shown). The following protease inhibitors (all from Sigma) were included in the digestion mixtures: 1 mM-phenylmethyl-sulphonyl fluoride for Zymolyase; and 2 μg ml⁻¹ each of leupeptin, antipain, chymostatin and pepstatin A for Novozym. The material liberated by one of the enzymes was separated from the insoluble fraction by centrifugation (2500 g, 10 min), and then was treated with the other enzyme for 2 h or was immediately precipitated with 3 vols of ethanol. The precipitated molecules were resuspended in the adequate buffer solution for further analysis.

Endoglycosidase treatments. Digestion of glycoproteins with Endo H (Boehringer) was carried out as described by Pastor et al. (1984).

Treatment with PNGase F (Boehringer) was done on samples that had previously been boiled for 5 min in the presence of 0.2% SDS, and were then diluted to reach the final conditions for digestion: 20 mM-phosphate buffer, pH 7.2, with 50 mM-EDTA, 0.1% SDS, 1% (w/v) 2-mercaptoethanol and 0.75% Triton X-100; digests were continued for 18 h at 37 °C.

Gel chromatography of glycoproteins. Glycoproteins solubilized from the walls and resuspended in 10 mM-sodium phosphate buffer (pH 7.0) were passed through a Sephacryl S-400 column (1.6 × 90 cm) equilibrated with 10 mM-sodium phosphate buffer (pH 7.0) plus 50 mM-NaCl. Elution was carried out at a flow rate of 20 ml h⁻¹ and fractions of 1.75 ml were collected. The column had been previously calibrated by running several dextrans (Sigma) and globular proteins (Pharmacia) of known size. In some experiments, fractions were pooled, concentrated by ethanol precipitation and analysed by SDS-PAGE.

Wall glycoproteins were purified by affinity chromatography in a Con A-Sepharose 4B (Pharmacia) minicolumn made in a Pasteur pipette. The column was washed with 20 ml of Con A buffer (40 mM-Tris/HCl pH 7.4 containing 1 mM-CaCl₂, 1 mM-MgCl₂, 1 mM-MnCl₂ and 0.5 mM-NaCl), the samples were then applied, and the column was washed again with 10 ml of the buffer. Retained glycoproteins were eluted with 4 ml of Con A buffer containing 0.3 M-methyl a-D-mannoside, and were then concentrated in a smaller volume by ethanol precipitation.

β-Elimination and paper chromatography. β-Elimination of the carbohydrate moiety of [¹⁴C]mannose-labelled glycoproteins was done in 0.1 M-NaOH at 22 °C for 24 h. The released sugars were separated by descending paper chromatography in ethyl acetate/butanol/acetic acid/water (3:4:2.5:4, by vol.). Chromatograms were cut into 1 cm pieces, and the radioactivity was measured. Counts remaining at the origin were considered to be due to carbohydrate N-glycosidically linked to protein, and therefore resistant to β-elimination; radioactivity in peaks migrating through the paper was due to O-linked saccharides.

SDS-PAGE and autoradiography of gels. Glycoproteins labelled with [¹⁴C]mannose or [¹³C]protein hydrolysate were separated by SDS-PAGE. Standards of high and low molecular mass (Sigma) were run in parallel to estimate the apparent molecular mass of the bands. After Coomassie blue staining, the gels were fluorographed with Amplify (Amersham), dried, and exposed for adequate periods of time to X-ray films. The radioactivity in individual bands or zones of the gels was determined after cutting the band areas and digesting them with NCS tissue solubilizer (Amersham).

Analytical determinations. Total sugars were determined by the phenol/sulphuric acid method, using a mixture of glucose, mannose and galactose (85%, 10% and 5%, respectively, w/v) as standard. The aminoacidic moiety in glycoprotein molecules was estimated by measuring the absorbance at 280 nm of the suspensions.

Results

Synthesis of wall polysaccharides in the parental and mutant strains

Mutant sphl was isolated from its parent strain S. pombe 972h- as a papulacandin B-resistant strain with reduced amounts of cell wall galactomannan (J. C. Ribas and co-workers, personal communication). When growing exponentially in YPD medium, sphl cultures contained about 80% of cells with spherical morphology. However, after 6 h in the presence of papulacandin B (50 μg ml⁻¹)
only 45% maintained the abnormal phenotype. The antibiotic therefore partially reverses the spherical morphology of the mutant.

The effect of papulacandin B on wall polysaccharide synthesis in the parental and mutant strains was examined (Table 1). As in previous studies (Varona et al., 1983), the alkali-insoluble acid-insoluble fraction (β-glucan) was the most diminished in wild-type cell walls, although the incorporation of other polysaccharides was affected to a lesser extent. In contrast, papulacandin B stimulated galactomannan incorporation into sphl cell walls more than fourfold, raising the proportion of this polymer to approximately that of wild-type cell walls in the absence of the antibiotic.

Polysaccharides liberated into the growth medium were also analysed (Table 2). Whether papulacandin B was present or absent, both parent and mutant strains lost significant amounts of polysaccharide to the medium. The proportion of mannan in the growth medium was almost twofold higher for the mutant than for the parent strain. In the presence of papulacandin B, the mutant lost less sugar in absolute terms, although the relative proportion of mannan in the medium was increased.
Fig. 1. Elution profile from a Sephacryl S-400 column (a, c, e) and electrophoretic pattern (b, d, f) of [14C]mannose-labelled wall glycoproteins from S. pombe 972h− (●) and the mutant sphl (○). Glycoproteins were solubilized by treatment of the walls with Zymolyase (a, b), Novozym (c, d) or Zymolyase followed by Novozym treatment of the released material (e, f). In all experiments, 150000 c.p.m. were loaded in the column. Fractions eluted from the column were pooled and subjected to SDS-PAGE according to the following $V_e$ (elution volume) values: 79–102 ml, lanes 1 of the gels, 103–116 ml, lanes 2; 117–130 ml, lanes 3; 131–144 ml, lanes 4; 145–158 ml, lanes 5; and 159–172 ml, lanes 6. SDS-PAGE was done with 4% and 7% (w/v) acrylamide in the stacking and separating regions of the gels, respectively (the acrylamide:bisacrylamide ratio was 30:0.8). The following standards were employed to calibrate the Sephacryl column: blue dextran (BD), dextran (D) with mean sizes of 523 and 35-6 kDa, thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (150 kDa). The migration position of monomers of thyroglobulin (330 kDa) and ferritin (220 kDa) in the gels is also indicated.
Solubilization of wall glycoproteins by Zymolyase and Novozym

Mannoproteins have been solubilized from cell walls of Sacch. cerevisiae, C. albicans and other yeasts (Pastor et al., 1984; Elorza et al., 1985; Frevert & Ballou, 1985; Herrero et al., 1987) by digestion of the walls with Zymolyase, a complex with β-glucanase as the major enzyme activity (Kitamura et al., 1974). Since the walls of S. pombe also contain α-glucan, we did not expect Zymolyase treatment to solubilize them completely. Wall digestions were therefore done in parallel with Zymolyase and Novozym, the latter being a complex containing α- and β-glucanases, besides other enzyme activities (Peberdy, 1985). When acting on purified walls from [14C]mannose-labelled cells of the parent strain, Zymolyase liberated 78% of cell-wall radioactivity, and Novozym liberated 93%.

The [14C]mannose-labelled wall material liberated by Zymolyase or Novozym was analysed by Sephacryl S-400 chromatography (Fig. 1a, c). Successive pools were formed with the eluted fractions and each was analysed by SDS-PAGE (Fig. 1b, d). The labelled material eluted in two peaks with average molecular masses of approximately $2 \times 10^6$ (peak I) and $5 \times 10^5$ Da (peak II); these sizes correlated well with the migration of the respective molecules in the denaturing gels, an indication of the absence of interactions between different molecules in peaks I and II. No differences in the elution or gel migration positions were observed between the parent and mutant strains; however, the mutant had about twice as much label in peak I as did the parent strain (also see below). Both peaks were better defined in the Novozym-solubilized than in the Zymolyase-solubilized material; when the material liberated by Zymolyase was redigested with Novozym, the resolution of the peaks resembled that obtained by a single Novozym digestion (Fig. 1e, f).

In another experiment, walls from [14C]protein-hydrolysate-labelled cells were digested with Zymolyase or Novozym. The material solubilized from the parent strain eluted in the column in the same volumes as the [14C]mannose-labelled molecules, and the gel migration positions were also similar (Fig. 2). The same results were obtained with the mutant strain (data not shown). However, although their positions do not alter, the relative intensities of peaks I and II depend on which moiety is labelled (compare Figs. 1 and 2), peak I being relatively more intense with [14C]protein hydrolysate labelling.

The glycoprotein nature of species I and II (corresponding respectively to peaks I and II) was confirmed by being retained in a Con A-Sepharose column and being quantitatively eluted with methyl a-D-mannoside (data not shown).

Sugar/protein ratio in wall glycoproteins

To compare the ratio of sugar to protein in species I and II, walls from unlabelled parental cells were treated with Novozym, and the liberated material was passed through the Sephacryl S-400 column. Eluted peaks of species I
Fig. 3. (a, c) Chromatography on a Sephacryl S-400 column of glycoproteins released into the growth medium by cultures of *S. pombe* 972h− (●) and the mutant sphl (○), labelled with [14C]mannose (a, 85,000 c.p.m. loaded) or [14C]protein hydrolysate (c, 320,000 c.p.m. loaded). (b, d) Electrophoretic patterns of the fractions eluted from the column, corresponding to the samples labelled with [14C]mannose (b) or [14C]protein hydrolysate (d). The fractions were pooled as follows: lanes 1, Vc from 70–107 ml; lanes 2, 108–120 ml; lanes 3, 121–133 ml; lanes 4, 134–146 ml; lanes 5, 147–159 ml; lanes 6, 160–173 ml. Standards and electrophoresis conditions are as for Fig. 1.

and II were pooled. The glycoproteins were adsorbed on a Con A-Sepharose 4B column and then liberated with methyl α-D-mannoside. The ratio of mg sugar to units of absorbance at 280 nm in the samples purified in this way was 2.25 and 4.21 for species I and II respectively. Thus, species II is about 80% more glycosylated than species I; this explains why species II was less labelled by [14C]protein hydrolysate.

Analysis of glycoproteins liberated into the growth medium

The parent and mutant strains accumulated [14C]-mannose (Fig. 3a) or [14C]protein-hydrolysate-labelled (Fig. 3c) material in the growth medium. The high-molecular-weight species I and II were detected in both cultures; however, only very small amounts of species I appeared in the supernatant of cultures from the parent
Cell wall glycoproteins from *S. pombe*

Table 3. Amounts of glycoprotein species I and II in the cell walls of *S. pombe* strains 972h− and sph1 grown in the absence or presence of papulacandin B

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<tr>
<td></td>
<td>Species I</td>
<td>Species II</td>
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<tr>
<td>972h−</td>
<td>1707</td>
<td>6663</td>
</tr>
<tr>
<td>972h− (plus papulacandin B)</td>
<td>0</td>
<td>1580</td>
</tr>
<tr>
<td>sph1</td>
<td>2490</td>
<td>1980</td>
</tr>
<tr>
<td>sph1 (plus papulacandin B)</td>
<td>1174</td>
<td>1746</td>
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A large peak, which eluted in the chromatograms of [14C]mannose-labelled samples at a *V_e* of 150 ml and later, probably represents small saccharides not detected using acrylamide gels (Fig. 3b).

A material labelled in the peptide moiety, of lower molecular size than species I and II, was also observed (Fig. 3c). It migrated in the acrylamide gels as defined bands corresponding to molecular masses of 30–60 kDa (Fig. 3d). Since these species were not labelled with [14C]mannose (Fig. 3b), they probably do not contain polysaccharide chains.

**Effect of papulacandin B on the glycoprotein composition of the cell wall**

The amount of glycoprotein species I and II in cell walls of the parent and mutant strains was quantified from the material separated by SDS-PAGE (Table 3). The level of radioactivity incorporated into species I from either [14C]mannose or [14C]protein hydrolysate (after normalizing with respect to cell biomass) was not greatly altered in the mutant. In contrast, radioactivity in species II was much reduced (25–33%) relative to the wild-type. Together with the results from the chromatography experiments, this indicates that the main difference in glycoprotein composition of the wall between these strains is the reduced amount of species II in the mutant.

Papulacandin B drastically reduced the incorporation of both glycoprotein species into the cell wall of the parent (Table 3). However, only species I incorporation was severely reduced in the mutant. Consequently, the ratio between species I and II in the mutant grown in the presence of papulacandin B approached that usually found in the wild-type.

**β-Elimination of the wall glycoproteins**

Yeast glycoproteins contain two kinds of linkages between the saccharide and peptide moieties (Kukuruzinska et al., 1987): some saccharides are N-linked to Asn residues, while others are O-linked to Ser or Thr residues. The latter are released from the peptide by mild alkali treatment (β-elimination) and this allows the estimation of the relative amount of sugar in O-linked chains.

[14C]Mannose-labelled glycoprotein species I and II (liberated from the walls by Novozym digestion) were isolated from the eluted fractions of the Sephacryl S-400 column. They were treated in mild alkali, and the released material was separated by paper chromatography and quantified as described in Methods. More than 80% of the radiolabelled sugar in the parent strain, and 50% in the mutant, corresponded to O-linked saccharides (Table 4); in absolute terms, species II contained significantly lower amounts of radioactivity in both N-linked and O-linked sugars.

Peaks in the chromatograms differed from mannooligosaccharides from *Sacch. cerevisiae* cell walls which were run in parallel. There were also differences in the migration distances between the parent and mutant strains (data not shown). These O-linked saccharides were not further characterized.

**Sensitivity of the wall glycoproteins to endoglycosidases**

Glycoprotein species I and II radiolabelled in the protein moiety were treated separately with Endo H, an endoglycosidase that catalyses the hydrolysis of the chitobiose core of high-mannose N-linked saccharides (Maley et al., 1989). Both species were resistant to this
glycoproteins, such as the external invertase from \textit{Saccharomyces cerevisiae}, were completely deglycosylated. Glycoproteins I and II were also resistant to PNGase F, another endoglycosidase with a broader specificity than Endo H.

**Discussion**

The glycoprotein compositions of cell walls of different yeast species share several features. A high-molecular-mass mannoprotein material and a 33–34 kDa mannoprotein species are present in the walls of a number of ascomycetous yeasts (Herrero \textit{et al.}, 1987). Antibodies against the peptide moiety of the 33 kDa species of \textit{Saccharomyces cerevisiae} are cross-reactive against the equivalent species in \textit{Candida albicans} and \textit{Hansenula wingei} (Herrero \textit{et al.}, 1987). Recently, the gene coding for the 33 kDa wall mannoprotein of \textit{Saccharomyces cerevisiae} has been cloned, and the purified protein has been shown to possess $\beta$-1,3-exoglucanase activity (Klebi \& Tanner, 1989).

Glycoproteins from \textit{S. pombe} cell walls show several differences from other yeasts. First, in the fission yeast, the high-molecular-mass material has been resolved into two molecular species (herein named I and II), which are larger than the corresponding material in \textit{Saccharomyces cerevisiae} or \textit{Candida albicans}. In \textit{Saccharomyces cerevisiae}, this is a size-heterogeneous component between 120 and 500 kDa, the heterogeneity being due to the saccharide moiety (Valentin \textit{et al.}, 1987). In yeast cells of \textit{Candida albicans}, the chromatographic polydispersity was less evident, and this large material was resolved into four defined bands between 150 and 500 kDa by SDS-PAGE (Elorza \textit{et al.}, 1985, 1988). Species I and II from \textit{S. pombe} have molecular masses of 2000 kDa and 500 kDa respectively, and their glycoprotein nature has been confirmed because they are recognized by Con A.

A second difference is the large amount of O-linked ($\beta$-eliminable) sugar in \textit{S. pombe} wall glycoproteins (about 85% of the total) relative to \textit{Saccharomyces cerevisiae} or \textit{Candida albicans}, where only 10–15% of the sugar is susceptible to $\beta$-elimination (Valentin \textit{et al.}, 1987; Elorza \textit{et al.}, 1988).

The third main difference in \textit{S. pombe} wall glycoproteins is the absence of a prominent band in the region of 33–34 kDa in SDS-polyacrylamide gels, although a minor band of 30-5 kDa has been observed, which does not cross-react with antibodies against the 33 kDa species from \textit{Saccharomyces cerevisiae} (Herrero \textit{et al.}, 1987).

The morphological mutant \textit{sphl} contains less glycoprotein II in its cell wall than the parent strain, and this explains the lower galactomannan levels detected in the mutant cell walls. Synthesis of species II is not reduced in \textit{sphl} cells, since it is liberated into the growth medium as a major component; in fact, the \textit{sphl} mutant releases into the medium increased amounts of mannan relative to the parental strain. Thus, it is only the incorporation of species II into the cell wall that is altered by the mutation.

Papulacandin B inhibits the synthesis \textit{in vivo} of $\beta$-glucan in \textit{S. pombe} (Varona \textit{et al.}, 1983). We have shown here that incorporation of glycoproteins into the wall is also inhibited in the wild-type. The effect of the antibiotic on \textit{sphl} cells is completely different. The mutant was isolated as resistant to papulacandin B, and in the presence of the latter, a partial reversion of the spherical morphology is observed (unpublished data). Incorporation of sugars into cell walls in the presence of papulacandin B is much less reduced in the mutant than in the wild type, and more importantly, the relative proportions of the polysaccharides studied approximate to those usually found in the wild-type strain. This occurs because the incorporation of species I is partially
inhibited by papulacandin B, while that of species II is almost unaffected. These results suggest that the relative proportions of wall glycoproteins may be important in determining cell wall structure and morphology.

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