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The structure of cell wall α-glucan from fission yeast

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Morphology and structural integrity of fungal cells depend on cell wall polysaccharides. The chemical structure and biosynthesis of two types of these polysaccharides, chitin and (1→3)-β-glucan, have been studied extensively, whereas little is known about α-glucan. Here we describe the chemical structure of α-glucan isolated from wild-type and mutant cell walls of the fission yeast *Schizosaccharomyces pombe*. Wild-type α-glucan was found to consist of a single population of linear glucose polymers, ~260 residues in length. These glucose polymers were composed of two interconnected linear chains, each consisting of ~120 (1→3)-linked α-D-glucose residues and some (1→4)-linked α-D-glucose residues at the reducing end. By contrast, α-glucan of an α-glucan synthase mutant with an aberrant cell morphology and reduced α-glucan levels consisted of a single chain only. We propose that α-glucan biosynthesis involves an ordered series of events, whereby two α-glucan chains are coupled to create mature cell wall α-glucan. This mature form of cell wall α-glucan is essential for fission-yeast morphogenesis.

Key words: α-glucan/cell wall/fission yeast/morphogenesis/polysaccharides

Introduction

Cell morphogenesis involves distinct molecular processes, such as establishment and maintenance of morphology. An excellent model eukaryote for studying cell morphogenesis is the fission yeast *Schizosaccharomyces pombe*, because this unicellular fungus has a cylindrical rod-like morphology, many mutants are available or can be engineered, and its complete genome sequence is known (Wood *et al*., 2002). Fission yeast cells establish their characteristic cell morphology by growing out from their cell ends in a linear polarized fashion. Mutants have been isolated that are unable to establish proper cell polarity: They fail to localize zones of growth properly and therefore display aberrant cell morphologies, such as bent, branched, or rounded morphologies (Hayles and Nurse, 2001).

A second aspect of cell morphogenesis is maintenance of cell morphology. After zones of growth have been localized, fission yeast cells must assemble their extracellular cell wall correctly to maintain cell morphology. The cell wall of fungi (including fission yeast) consists of polysaccharides with associated glycoproteins, the polysaccharides being directly responsible for cell wall rigidity. Disruption of the assembly process of cell wall polysaccharides may lead to cell lysis. For instance, mutations in synthases for cell wall polysaccharides can cause cells to swell or lyse (Hochstenbach *et al*., 1998; Ishiguro *et al*., 1997). Because most fungi possess similar structural polysaccharides, enzymes involved in their assembly form ideal targets for the development of new classes of drugs against pathogenic yeasts and other fungi.

The major polysaccharides present in most fungal cell walls are chitin, (1→3)-β-glucan, and (1→3)-α-glucan. Chitin and (1→3)-β-glucan have been generally accepted as polysaccharides that are indispensable for maintaining rigidity and structural integrity of fungal cells, and their respective chemical structures and assembly processes have been characterized in detail (Klis *et al*., 2002). Furthermore, drugs have been identified that inhibit their synthase activities, causing osmotic fragility or lysis of fungal cells (Beauvais and Latgé, 2001; Georgopapadakou and Tkacz, 1995). By contrast, the role of (1→3)-α-glucan is still controversial. On the one hand, α-glucan appears to be a nonessential component of the cell wall in *Aspergillus nidulans* and *Schizophyllum commune* (Sietsma and Wessels, 1988; Zonneveld, 1972, 1973). On the other hand, Latgé and co-workers recently suggested that cell wall α-glucan may have a structural role in the pathogenic fungus *A. fumigatus*, based on the observation that it may replace chitin in a chitin synthase double mutant (Mellado *et al*., 2003). Moreover, for the pathogenic dimorphic fungi *Histoplasma capsulatum* (Kanetsuna *et al*., 1974; Klimpel and Goldman, 1988; Rappleye *et al*., 2004), *Blastomyces dermatitidis* (Hogan and Klein, 1994), and *Paracoccidioides brasiliensis* (San-Blas *et al*., 1977a), the virulent yeast forms contained substantial levels of α-glucan (35–46% of total cell wall carbohydrates), whereas the avirulent mycelial forms or

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avirulent yeast form mutants contained dramatically decreased levels of α-glucan. Interestingly, a mutant of *P. brasiliensis* with increased levels of α-glucan showed a higher degree of virulence than the wild-type strain (San-Blas *et al*., 1977b).

Despite its potential relevance for virulence, little is known about the biosynthesis of cell wall α-glucan. The cell wall of fission yeast contains galactomannan (9–14% of total cell wall), β-glucan (42–55%), and α-glucan (28%) (Bacon *et al*., 1968; Bush *et al*., 1974). The α-glucan fraction consists mainly of (1→3)-linked α-glucose, with also ~7% of (1→4)-glycosidic linkages. Whether these (1→4)-linkages are part of cell wall α-glucan or represent contamination by intracellular glycogen had remained uncertain.

Previously, we described a temperature-sensitive (ts) mutant, *ags1-1*^{ts}, with a point mutation in a gene, denoted *ags1*, responsible for α-glucan biosynthesis (Hochstenbach *et al*., 1998). This mutant displays a temperature-dependent cell morphology: At a permissive temperature of 19°C, cells have a rod-like morphology similar to that of wild-type cells, whereas at a semipermissive temperature of 34°C, they are rounded, indicating that their cell walls have weakened. This change in cell morphology correlates with a threefold reduction in cell wall α-glucan levels. At a restrictive temperature of 37°C, *ags1-1*^{ts} cells lyse, demonstrating that α-glucan is essential for maintaining the structural integrity of fission yeast cells.

Based on amino acid sequence similarities, we proposed a model for the function of the putative α-glucan synthase, Ags1p. In brief, we suggested that Ags1p consists of three domains: an intracellular domain for synthesis of α-glucan, a multipass transmembrane domain that might form a pore-like structure for transport of α-glucan across the plasma membrane, and an extracellular transglycosylase domain, which contains a point mutation (G696S) in the *ags1-1*^{ts} mutant, for linking or remodeling of α-glucan. This model provides a framework for addressing the molecular mechanism of α-glucan biosynthesis, in terms of chain initiation, elongation, and termination. For example, we wish to know whether a primer is used in chain initiation, how many glucose residues are added during chain elongation, and whether chain termination is subject to tight control.

Here we focus on the chemical structure of cell wall α-glucan of fission yeast to gain insight into the molecular mechanism of its biosynthesis. By using high-performance size-exclusion chromatography (HPSEC) in combination with chemical analyses and nuclear magnetic resonance (NMR) spectroscopy, we found that α-glucan of both wild-type cells and *ags1-1*^{ts} mutant cells with a rod-like morphology (grown at 19°C) consisted of two interconnected chains. By contrast, α-glucan isolated from *ags1-1*^{ts} mutant cells with a rounded morphology (grown at 34°C) consisted of a single chain only. These data suggest that the Ags1 protein is involved in both synthesis and coupling of α-glucan chains. We conclude that the proposed synthase and transglycosylase domains of Ags1p are both essential for α-glucan biosynthesis and propose that they may be suitable targets for the development of novel antifungal drugs.

Results

α-*Glucan has an essential role in maintaining cell morphology*

To gain insight into the role of cell wall α-glucan in cell morphogenesis, we took advantage of a mutant strain, *ags1-1*^{ts}, which is defective in the synthesis of α-glucan. When grown at the semipermissive temperature of 34°C, cells of this mutant strain, FH021, display a rounded morphology, osmotic sensitivity, and a threefold reduction in cell wall α-glucan levels (Hochstenbach *et al*., 1998). By using glass beads, we mechanically broke these rounded cells as well as rod-shaped cells of a matching wild-type strain, FH023, and visualized isolated cell walls by transmission electron microscopy. This lysis method preserved the typical rod-like morphology of wild-type cell walls and the rounded morphology of the mutant cell walls (Figures 1A and 1B), distorting them only by marks of glass-bead impact (Figures 1A and 1C, main micrographs, see arrows).

To visualize the α-glucan fraction of the cell wall, we specifically removed all cell wall components except α-glucan, using the following mild isolation method that does not degrade its chemical structure. This method is based on the observation that the *S. pombe* cell wall lacks significant amounts of chitin and consists mainly of water-insoluble α-glucans and β-glucans with associated glycoproteins. Digestion of the cell wall with zymolyase,

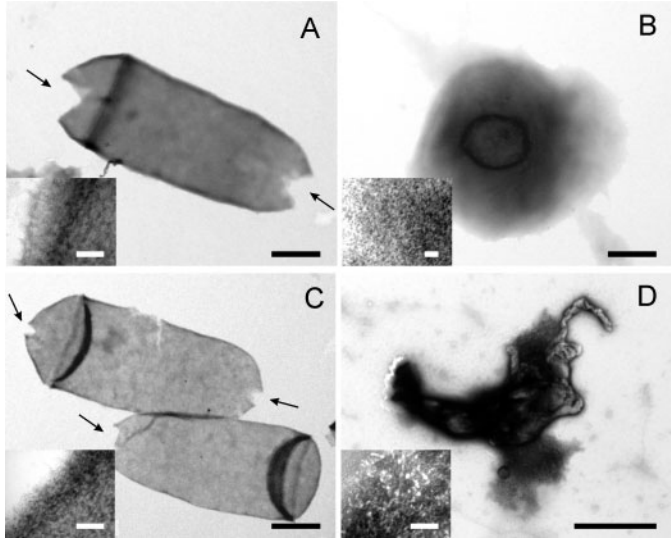


Fig. 1. α-Glucan of wild-type cell walls maintains a rod-like morphology, whereas α-glucan of rounded *ags1-1*^{ts} cells collapses after removal of β-glucan and glycoproteins. Electron micrographs of whole cell walls (**A**) and zymolyase-treated cell walls (**C**) of wild-type strain FH023, and whole cell walls (**B**) and zymolyase-treated cell walls (**D**) of *ags1-1*^{ts} mutant strain FH021 grown at 34°C. Note that after the zymolyase treatment, wild-type cell walls maintained their rod-like morphology, whereas mutant cell walls collapsed into loose fragments (displayed in main micrographs). Lysis by using glass beads introduced only localized destruction (indicated by arrows), preserving most cell wall polysaccharides. Insets are magnifications of the corresponding cell wall preparations. Bars represent 2 μm in the main micrographs and 100 nm in the insets.

a purified mixture of β-glucanases and proteases, solubilizes the β-glucans and glycoproteins but keeps α-glucans intact. After zymolyase digestion, the α-glucan fraction was isolated by centrifugation and the pellet fraction was extracted with a hot sodium dodecyl sulfate (SDS) solution to remove noncovalently linked material. This α-glucan preparation did not contain detectable β-glucans, as determined by NMR spectroscopy (see later discussion). When analyzed by transmission electron microscopy, the α-glucan fraction of wild-type cell walls retained a rod-like morphology (Figure 1C, main micrograph), whereas mutant cell walls disintegrated on zymolyase digestion (Figure 1D, main micrograph). In both cases, the α-glucans retained a microfibrillar structure (Figures 1C and 1D, insets), indicating that this isolation method preserves their native structures. Together, these data demonstrate that wild-type α-glucan is essential for maintenance of the rod-like morphology of wild-type fission yeast cells.

α-*Glucan consists of a single population of polymers*

We wondered whether α-glucan is linked covalently to other cell wall components or whether it forms a separate population of molecules. To pursue this, we analyzed cell walls of wild-type strain FH023 by HPSEC. Cell wall preparations were dried completely, dissolved in dimethyl sulfoxide (DMSO), and analyzed on a calibrated mixed-bed column with a fractionation range of 0.2–2000 kDa. The size-exclusion chromatogram of whole wild-type cell walls shows three strongly overlapping peaks in the range of ~10–1000 kDa (Figure 2A, profile I), suggesting that the *S. pombe* cell wall consists of three distinct populations of polymers. We then used HPSEC to analyze the α-glucan preparation isolated from wild-type cells (see previous discussion). Digestion of whole cell walls with zymolyase reduced the chromatogram to a single Gaussian-curved distribution (Figure 2A, profile II, see arrow), eluting at the same volume as the population of whole cell walls with the lowest molecular mass. These data indicate that α-glucan consists of a single population of glucose polymers. Based on retention times, we infer that the cell wall polymer population with the lowest molecular mass corresponds to α-glucan, indicating that it is not linked covalently to other polymers, such as β-glucans.

The discrete Gaussian-curved nature of the α-glucan peak allowed the calculation of the molecular mass distribution of the α-glucan population (Figure 2B), yielding a number-average molecular mass (Mn) of 42.6 ± 5.2 kDa (mean ± SD; $n = 3$). This molecular mass is equivalent to a number average degree of polymerization (DP_n) of 263 ± 32 (Table I). These data are in accordance with data obtained by Manners and Meyer (1977), who reported a DP_n of 207 for one of their α-glucan-containing cell wall fractions derived from *S. pombe* strain C-277.

To assess the variation in α-glucan length, we determined the polydispersity of the α-glucan population. Polydispersities (Mw/Mn) of naturally occurring polysaccharides vary between 1.1 for alkali-soluble β-glucans from *Saccharomyces cerevisiae* (Williams *et al*., 1994) to 35 for cornstarch (Chuang, 1990). For α-glucan of strain FH023, we found a polydispersity of 2.41 ± 0.25 (Table I), indicating a relatively narrow molecular mass distribution. Together, these results show that in the *S. pombe* cell wall, α-glucan polymers consist of ~260 glucose residues. The length of these polymers is regulated tightly, suggesting that only a single enzyme system may be responsible for α-glucan biosynthesis.

α-*Glucan consists of (1→3)-linked and (1→4)-linked glucose residues*

The uniform nature of α-glucan enabled us to investigate its chemical structure. By using monosaccharide analysis, including the determination of absolute configurations, we found that D-glucose was almost the only monosaccharide present. Besides D-glucose, only variable trace amounts of mannose were found, whereas no *N*-acetylglucosamine was detected (data not shown). Linkage analysis showed that α-glucan consisted for $88.9 \pm 1.0\%$ (mean ± SD; $n = 3$) of (1→3)-linked glucose residues, with $9.0 \pm 0.3\%$ of (1→4)-linked residues (Table II). These data are in good agreement with the data of Bush and colleagues (1974), who observed ~7% of (1→4)-linked glucose residues in α-glucan of *S. pombe* strain CBS351.

To independently verify the data from our chemical analyses and determine the anomeric configuration of the constituents, we used 1D ^{1}H-NMR and 2D ^{1}H-^{13}C (heteronuclear single-quantum coherence; HSQC) and ^{1}H-^{1}H (total correlation spectroscopy; TOCSY) NMR spectroscopy. The ^{1}H-NMR and ^{13}C-NMR data are summarized in Table III. Two anomeric signals are present in the ^{1}H-NMR spectrum (Figure 3, horizontal axis), a signal of high intensity at 5.092 ppm (denoted H-1_A) and one of low intensity at 5.139 ppm (denoted H-1_B). These signals indicate the presence of two distinct structural elements, A and B, respectively. For the major constituent A, signals at 4.834, 4.435, and 4.128 ppm could be assigned to hydroxyl protons because they do not produce cross-peaks in the HSQC spectrum (Figure 3). Using TOCSY, these signals were assigned to OH-4_A, OH-2_A, and OH-6_A, respectively (data not shown). The absence of an OH-3_A hydroxyl signal in the ^{1}H-NMR spectrum together with a down-field shift of C-3_A at 82.7 ppm in the ^{13}C-NMR spectrum demonstrate that the major constituent A is (1→3)-linked. Because the low-field anomeric proton signal at 5.092 ppm and the $^{3}J_{1,2}$ coupling constant of 3.9 Hz are typical for an α-anomeric configuration, we conclude that the major constituent is (1→3)-linked α-glucan.

The minor constituent B also has an α-anomeric configuration because of its low-field anomeric proton signal at 5.139 ppm and its $^{3}J_{1,2}$ coupling constant of 3.8 Hz (Figure 3, peak denoted H-1_B). In TOCSY and HSQC experiments, we identified C-4_B at a low-field chemical shift of 78.5 ppm, demonstrating that in constituent B, C-4 was involved in a glycosidic linkage. In addition, ^{13}C chemical shifts of all observed carbon atoms were found to be in good agreement with those obtained from amylose (data not shown). Together these data demonstrate that the minor constituent is (1→4)-linked α-glucose. Signals corresponding to β-glucan were not detected by NMR spectroscopy (i.e., below a detection limit of 0.5%).

Although cell wall α-glucan forms a single polymer population, as observed in HPSEC, we wished to exclude

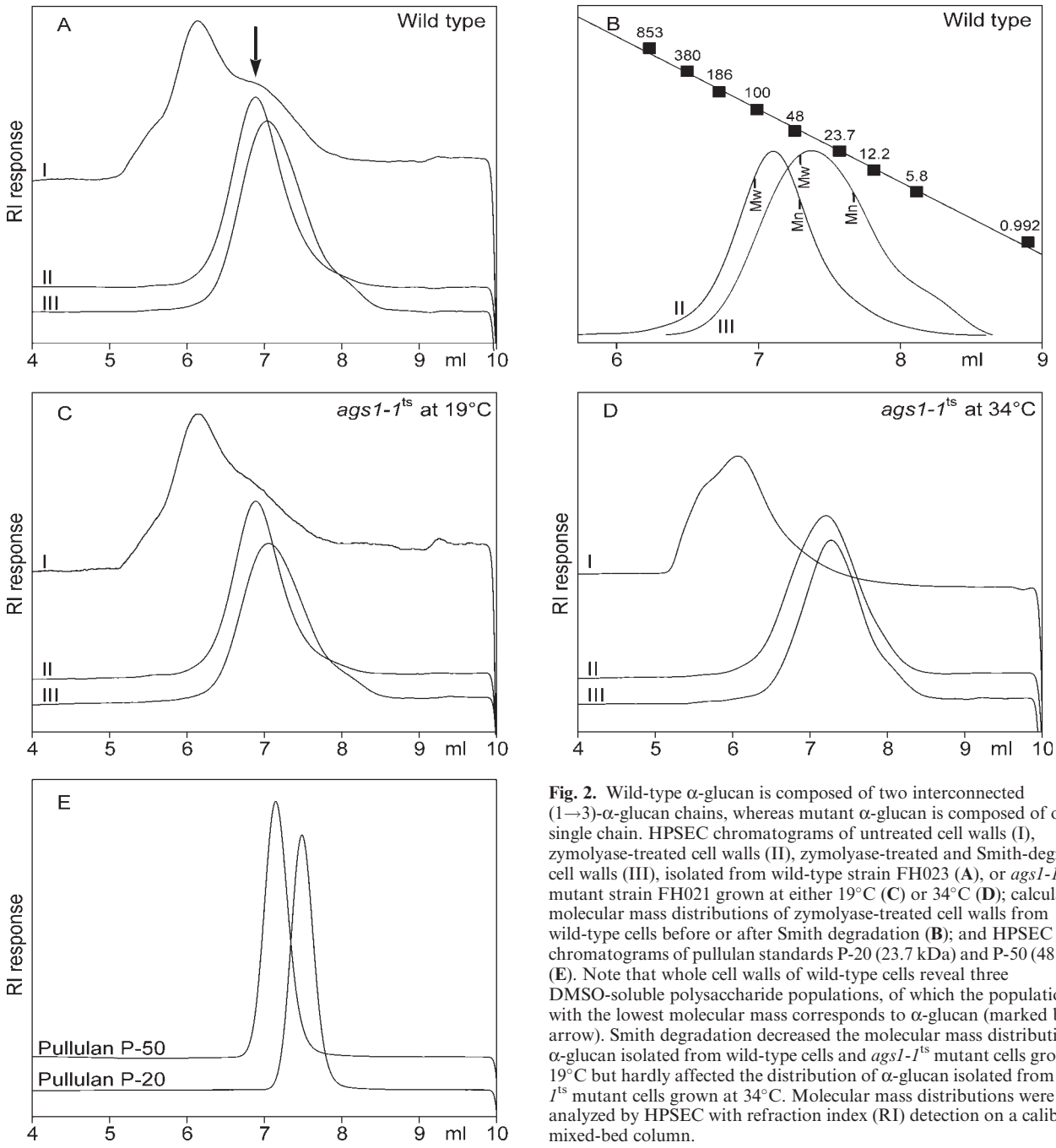


Fig. 2. Wild-type α-glucan is composed of two interconnected (1→3)-α-glucan chains, whereas mutant α-glucan is composed of only a single chain. HPSEC chromatograms of untreated cell walls (I), zymolyase-treated cell walls (II), zymolyase-treated and Smith-degraded cell walls (III), isolated from wild-type strain FH023 (**A**), or *ags1-1*^{ts} mutant strain FH021 grown at either 19°C (**C**) or 34°C (**D**); calculated molecular mass distributions of zymolyase-treated cell walls from wild-type cells before or after Smith degradation (**B**); and HPSEC chromatograms of pullulan standards P-20 (23.7 kDa) and P-50 (48 kDa) (**E**). Note that whole cell walls of wild-type cells reveal three DMSO-soluble polysaccharide populations, of which the population with the lowest molecular mass corresponds to α-glucan (marked by arrow). Smith degradation decreased the molecular mass distribution of α-glucan isolated from wild-type cells and *ags1-1*^{ts} mutant cells grown at 19°C but hardly affected the distribution of α-glucan isolated from *ags1-1*^{ts} mutant cells grown at 34°C. Molecular mass distributions were analyzed by HPSEC with refraction index (RI) detection on a calibrated mixed-bed column.

contamination by an independent but overlapping population of glycogen or amylose polymers. By staining cell lysates with iodine, we showed that glycogen and amylose contents were negligible in exponentially growing haploid cells, whereas they were easily detectable in sporulating diploids (data not shown). Furthermore, digestion with α-amylase or glucoamylase did not solubilize any detectable material (data not shown). These results indicate that the (1→4)-linked glucose residues form an integral part of cell wall α-glucan molecules. In conclusion, our results show that α-glucan of fission yeast is a polysaccharide consisting mainly of (1→3)-linked α-glucose residues, with also ~9% of (1→4)-linked α-glucose residues.

α-*Glucan is a linear polysaccharide consisting of two (1→3)-α-glucan chains*

The (1→4)-linked residues must be embedded within linear polymers of (1→3)-linked residues, because no branching points could be detected in our linkage analysis (Table II). To determine whether the (1→4)-linked glucose residues were distributed randomly along the α-glucan chain or whether they were located at specific positions, we removed them selectively by periodate oxidation followed by acid hydrolysis (Smith degradation) and analyzed the remaining (1→3)-linked α-glucan by using HPSEC. If the (1→4)-linked glucose residues were distributed randomly, Smith

Table I. Molecular mass averages and polydispersities of wild-type and mutant cell wall α-glucan

	Mn (kDa)	Mw (kDa)	Mw/Mn	DP_n
Wild type	42.6 ± 5.2	101.8 ± 8.9	2.41 ± 0.25	263 ± 32
Wild type, Smith-degraded	19.1 ± 2.4	55.3 ± 2.3	2.95 ± 0.46	118 ± 15
ags1-1^{ts}	22.1 ± 1.5	66.5 ± 4.4	3.01 ± 0.26	137 ± 9
ags1-1^{ts}, Smith-degraded	19.9 ± 2.6	54.1 ± 9.6	2.74 ± 0.40	123 ± 16

Values are the mean ± SD of three independently isolated α-glucan preparations.

Table II. Linkage analysis of wild-type and mutant cell wall α-glucan

	Molar amounts (%)	
Residue	Wild type	*ags1-1*^{ts}
Glc*p*-(1→	1.1[a]	2.6[a]
→3)-Glc*p*-(1→	89.9	91.6
→4)-Glc*p*-(1→	9.0	5.8
→3,6)-Glc*p*-(1→[b]	trace	trace
→3,4)-Glc*p*-(1→[b]	trace	trace
→3,2)-Glc*p*-(1→[b]	trace	trace

[a]Percentages of nonreducing ends appeared to be too high due to overlap with a contaminating compound.
[b]Using GC-EI-MS, variable traces of triple substituted glucose residues were detected, indicating undermethylation rather than branching points.

Table III. Chemical shifts for wild-type cell wall α-glucan in DMSO-d_6 at 80°C

Constituent **A** ((1 → 3)-α-Glc*p*)				Constituent **B** ((1 → 4)-α-Glc*p*)			
Proton	ppm	Carbon	ppm	Proton	ppm	Carbon	ppm
$H\text{-}1_A$	5.092	$C\text{-}1_A$	99.2	$H\text{-}1_B$	5.133	$C\text{-}1_B$	99.8
$H\text{-}2_A$	3.453	$C\text{-}2_A$	70.5	$H\text{-}2_B$	3.352	$C\text{-}2_B$	71.7
$H\text{-}3_A$	3.665	$C\text{-}3_A$	82.7	$H\text{-}3_B$	3.714	$C\text{-}3_B$	72.9
$H\text{-}4_A$	3.453	$C\text{-}4_A$	69.3	$H\text{-}4_B$	3.390	$C\text{-}4_B$	78.5
$H\text{-}5_A$	3.863	$C\text{-}5_A$	71.8	$H\text{-}5_B$	3.652	$C\text{-}5_B$	71.2
$H\text{-}6a_A$	3.669	$C\text{-}6_A$	60.0	$H\text{-}6a_B$	3.701	$C\text{-}6_B$	ND[a]
$H\text{-}6b_A$	3.541			$H\text{-}6b_B$	3.612		
$OH\text{-}2_A$	4.435			$OH\text{-}2_B$	ND[b]		
$OH\text{-}4_A$	4.834			$OH\text{-}3_B$	ND[b]		
$OH\text{-}6_A$	4.128			$OH\text{-}6_B$	4.231		

[a]The chemical shift corresponding to $C\text{-}6_B$ could not be determined due to overlap by $C\text{-}6_A$.
[b]Chemical shifts of $OH\text{-}2_B$ and $OH\text{-}3_B$ could not be determined. By analyzing a (1 → 4)-α-glucan preparation (amylose), these signals were found to occur around 5.1 ppm and therefore overlap with the H-1 signal of (1 → 3)-linked α-glucose.

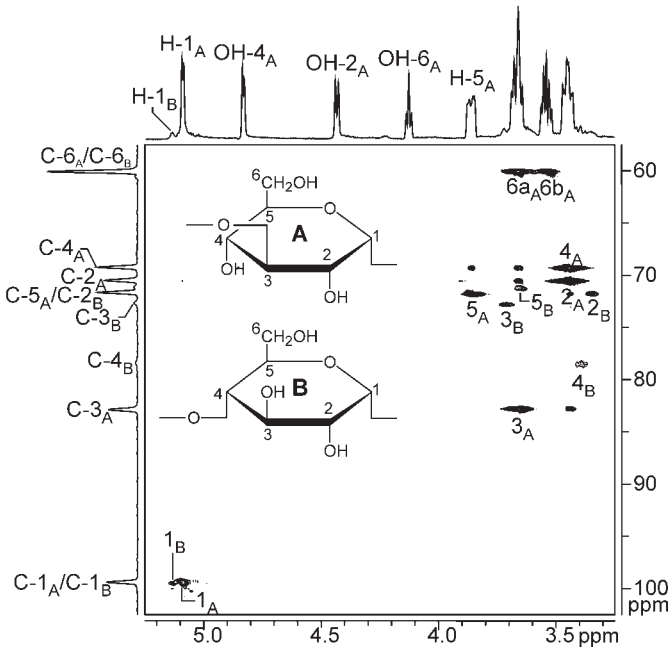


Fig. 3. α-Glucan consists of (1→3)-linked α-glucose residues with some (1→4)-linked residues. ^1H (horizontal axis), ^{13}C (vertical axis), and HSQC NMR spectra of α-glucan from wild-type strain FH023. Samples were dissolved in DMSO-d_6, and the spectra were recorded at 500.08 MHz at 80°C. The major constituent (denoted **A**) is (1→3)-linked α-glucose, whereas the minor constituent (denoted **B**) is (1→4)-linked α-glucose. Note that the down-field chemical shifts of $C\text{-}3_A$ and $C\text{-}4_B$ typify (1→3)-linked and (1→4)-linked glucose residues, respectively. Also note that the relative positions of the anomeric signals for both constituents (denoted $H\text{-}1_A$ and $H\text{-}1_B$) are typical for an α-anomeric configuration. Due to the high signal-to-noise ratio required to study the minor constituent, long-range magnetization transfer signals of the major constituent were observed in addition to the single bond magnetization transfer signals. In fact, these two-bond couplings facilitated the assignment of the spectrum.

degradation would result in a population of reaction products with a polydispersity increased significantly over that of native α-glucan, which was 2.41 ± 0.25. However, the Smith-degraded material eluted as a Gaussian-curved peak with a polydispersity of only 2.95 ± 0.46 (Table I), indicating that the (1→4)-linked residues must be located at specific positions.

To determine whether α-glucan contains internal (1→4)-linked residues, we measured the molecular mass of the Smith-degraded reaction products. Smith-degraded α-glucan had an Mn of 19.1 ± 2.4 kDa (mean ± SD; $n = 3$) (Figure 2A, profile III, and Figure 2B), which is equivalent to a DP_n of 118 ± 15 (Table I). Remarkably, this molecular mass is approximately half that of native α-glucan, which was 42.6 ± 5.2 kDa, and equivalent to 263 ± 32 glucose residues (see previous discussion). Data indistinguishable from these on α-glucan of wild-type strain FH023 were obtained for α-glucans of another *S. pombe* wild-type strain, 972, and a nonsporulating diploid strain, FH058 (data not shown). Importantly, similar data were obtained for α-glucan of the *ags1-1*^{ts} mutant grown at the permissive temperature of 19°C (Figure 2, compare profiles II and III of C with those of A). These data show that Smith degradation cleaves wild-type

α-glucan, dividing it into two halves. They therefore suggest that α-glucan contains (1→4)-linked glucose residues located in the center where they interconnect two chains of ~120 (1→3)-linked glucose residues.

(1→4)-Linked α-glucose residues at the reducing end of α-glucan

Our results so far do not exclude the presence of additional (1→4)-linked glucose residues at the non-reducing or the reducing end. Treatment of the α-glucan preparation with glucoamylase, an *exo*-glucanase that hydrolyzes α-glycosidic (1→4)-linkages starting from the nonreducing end, did not result in release of glucose, providing no evidence for the presence of (1→4)-linked glucose residues at the nonreducing end of cell wall α-glucan. To determine the type of linkage at the reducing end, we subjected α-glucan to controlled β-elimination, a chemical cleavage reaction that occurs under alkaline conditions and starts from the reducing end, progressing slowly toward the nonreducing end. β-Elimination can give detailed information on the type of linkages at the reducing end because degradation products released into the medium are characteristic for the type of linkage. Specifically, isosaccharinic acids are formed from 4-substituted glucose at the reducing end, whereas metasaccharinic acids are formed from 3-substituted glucose at the reducing end (Kennedy and White, 1971; Whistler and BeMiller, 1958) (Figure 4, reaction scheme).

As a reference for (1→4)-linked glucan, we incubated amylose in a saturated solution of calcium hydroxide for 5 days and analyzed the hydrolysis products by gas chromatography electron impact mass spectrometry (GC-EI-MS) after trimethylsilylation. In this analysis, both isosaccharinic acid isomers could be resolved as individual peaks of equal size (Figure 4A, peaks I_a and I_b). As a reference for (1→3)-linked glucan, we used Smith-degraded cell wall α-glucan (Figure 4B, peak $II_{a,b}$) or laminaran, a (1→3)-linked β-glucan (data not shown). The released metasaccharinic acid isomers, however, could not be resolved individually, producing a single peak at a retention time identical to that of one of the isosaccharinic acid isomers. Thus, when we analyzed nigeran, an α-glucan with alternating (1→4)-linkages and (1→3)-linkages, we observed an equal mixture of isosaccharinic and metasaccharinic acids, eluting as two unequal-sized peaks (Figure 4C, peaks $I_a + II_{a,b}$ and I_b).

We then carried out β-elimination of cell wall α-glucan and analyzed the hydrolysis products at different time points. Immediately after the start of the reaction, no isosaccharinic or metasaccharinic acids were detected (Figure 4D). However, after an incubation period of 1 day, we observed the release of both isosaccharinic acids and metasaccharinic acids in approximately equal amounts (data not shown). This nearly equal ratio hardly changed in the course of 5 days (Figure 4E). Linkage analysis showed that after a 5-day incubation ~25% of the (1→4)-linked glucose residues had been hydrolyzed, whereas HPSEC analysis showed that the molecular mass distribution of the treated α-glucan had hardly changed (data not shown). Together, these data demonstrate that (1→4)-linked α-glucose residues are present at the reducing end.

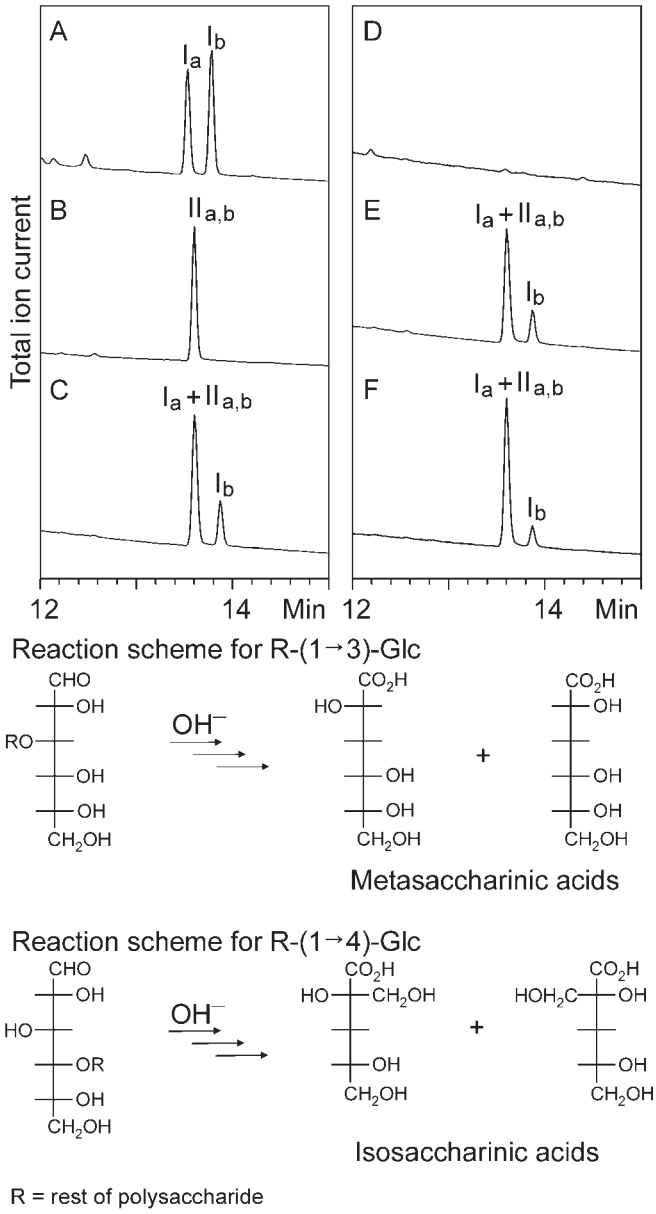


Fig. 4. α-Glucan of wild-type and mutant cell walls have (1→4)-linked glucose residues at their reducing ends. GC chromatograms of β-elimination products of the following glucans: amylose ((1→4)-α-glucan) (**A**); Smith-degraded *S. pombe* cell wall α-glucan ((1→3)-α-glucan) (**B**); nigeran (α-glucan with alternating (1→3) and (1→4) linkages) (**C**); wild-type α-glucan at $t = 0$ (**D**) or after 5 days (**E**); α-glucan of *ags1-1*^{ts} mutant cells grown at 34°C after 5 days (**F**). Note that β-elimination of terminal 4-substituted glucose released two isosaccharinic acid isomers (denoted I_a and I_b), whereas β-elimination of terminal 3-substituted glucose released two metasaccharinic acid isomers (denoted $II_{a,b}$). Note that with both wild-type and mutant α-glucan, a mixture of metasaccharinic and isosaccharinic acids were released at a ratio of 1.2:1 and 2.5:1, respectively. Samples were incubated in a saturated calcium hydroxide solution at room temperature, and reaction products in supernatants were analyzed as their trimethylsilyl-derivatives by GC-EI-MS. The reaction scheme indicates that the metasaccharinic acids, 3-deoxy-D-*arabino*-hexonic acid and 3-deoxy-D-*ribo*-hexonic acid, are formed from 3-substituted glucose in stoichiometric amounts, whereas the isosaccharinic acids, 3-deoxy-2-*C*-(hydroxymethyl)-D-*threo*-pentonic acid and 3-deoxy-2-*C*-(hydroxymethyl)-D-*erythro*-pentonic acid, are formed from 4-substituted glucose in stoichiometric amounts.

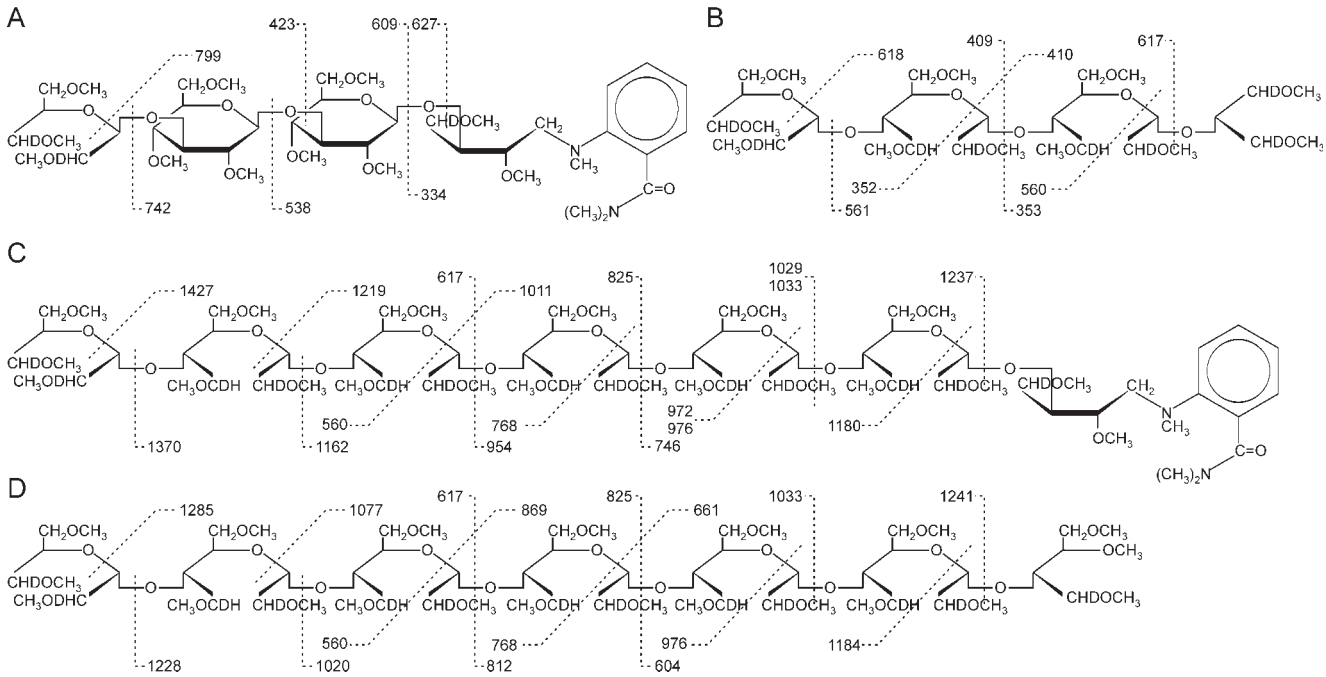


Fig. 5. The 4-substituted α-glucose residues are present in the center and at the reducing end of cell wall α-glucan. Structures of 2-AB-labeled, oxidized, and then reduced and per-*O*-methylated laminaritetraose (**A**), maltotetraose (**B**), and typical (1→3)-α-glucanase-resistant oligosaccharides originating from the center (**C**) or from the reducing end of cell wall α-glucan (**D**). Structures were derived from positive-ion mode electrospray collision-induced dissociation mass spectra (MS-MS). Note that 2-AB label at 3-substituted reducing ends is retained after oxidation, whereas 2-AB label at 4-substituted reducing ends will be cleaved off. Note also that oxidation and subsequent reduction of (1→4)-linked interresidues increases the nominal mass of each residue by 4 Da compared to (1→3)-linked interresidues.

(1→4)-Linked α-glucose residues in cell wall α-glucan are homo-oligomers

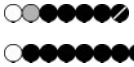
To determine whether the 4-substituted glucose residues of α-glucan are part of a stretch of alternating (1→3)-linked and (1→4)-linked residues, or whether they form (1→4)-linked homo-oligomers, we digested α-glucan from *S. pombe* wild-type cells with purified (1→3)-α-glucanase, isolated (1→3)-α-glucanase-resistant oligosaccharides using high-performance anion-exchange chromatography (HPAEC), analyzed the fractions by ^{1}H-NMR spectroscopy, and selected fractions that contained 4-substituted glucose residues for further analysis. To unambiguously discriminate between oligosaccharides with 3-substituted glucose residues at their reducing ends and those with 4-substituted glucose residues, we labeled their reducing ends with 2-aminobenzamide (2-AB) and oxidized the reaction products with sodium periodate. The samples were then per-*O*-methylated after reduction with sodium borodeuteride and analyzed by MS.

As shown for a 3-substituted glucose-oligosaccharide control, laminaritetraose, the 2-AB label was retained at the reducing end and internal glucose residues were not affected by periodate treatment (Figure 5A), whereas for a 4-substituted glucose-oligosaccharide control, maltotetraose, the 2-AB label was lost on oxidation and internal glucose residues were oxidized resulting in a molecular mass increase of 4 Da per residue (Figure 5B). When analyzing the (1→3)-α-glucanase-resistant oligosaccharides, we identified two classes of oligosaccharides: those with one, two, or three (1→3)-linked α-glucose residues at the reducing end followed by one (or more) (1→4)-linked α-glucose residues (Figure 5C and Table IV), and those with five to eight consecutive (1→4)-linked α-glucose residues at the reducing end (Figure 5D and Table IV). We propose that the former class is derived from the center of cell wall α-glucan, whereas the latter class is derived from the reducing end. No alternating (1→3)-linked and (1→4)-linked residues were identified. The release of metasaccharinic acids found during β-elimination (Figure 4E) is explained by trace amounts of contaminating (1→3)-β-glucan that had not been removed completely by zymolyase digestion and were not observed by NMR spectroscopy. Even very small amounts would largely influence our β-elimination results, especially because 3-substituted glucose residues are degraded ~10 times faster than 4-substituted glucose residues (Kennedy and White, 1971). Taken together, these data indicate that both the center and the reducing end of cell wall α-glucan contain a homo-oligomer of (1→4)-linked glucose residues. We interpret these data to mean that in *S. pombe*, cell wall α-glucan consists of two interconnected linear chains, each composed of ~120 (1→3)-linked α-glucose residues and some (1→4)-linked α-glucose residues at the reducing end.

ags1-1^{ts} mutant is unable to synthesize mature cell wall α-glucan

We characterized the chemical structure of α-glucan present in low amounts in the cell walls of rounded *ags1-1*^{ts} cells

Table IV. (1 → 3)-α-Glucanase-resistant oligosaccharides from wild-type cell wall α-glucan

HPAEC fraction	*m/z* after oxidation	Structure[a]	Proposed origin
3.5	924		Center
4.5	924		Center
4.8	1128		Center
5.3	1128		Center
5.5	990		Reducing end
	1198		Reducing end
	1548[b]		Center
5.6	1198		Reducing end
5.8	1406		Reducing end
6.3	1198		Reducing end
6.4	1198		Reducing end
	1742[b]		Center
6.7	1402		Reducing end
7.5	1819[b]		Reducing end

Mass-to-charge ratios (*m/z*) correspond to $[M + Na]^+$ ions. Note that this analysis was limited to oligosaccharides of $m/z < 2000$.
[a]White spheres indicate nonreducing ends; black spheres, 4-substituted residues; gray spheres, 3-substituted residues; diagonal lines, reducing ends.
[b]Underoxidation was observed resulting in aberrant molecular masses.

grown at 34°C. Based on dry weights, we determined that α-glucan levels at 34°C had decreased by a factor of 2.8, from 14.8% of whole cell walls at 19°C to 5.3%. These data confirm our previous results on cell wall carbohydrate content using phenol-sulfuric acid measurements (Hochstenbach *et al.*, 1998). Linkage analysis and ^{1}H-NMR spectroscopy showed that α-glucan of *ags1-1*^{ts} cell walls consisted mainly (91.6%) of (1→3)-linked α-glucose residues, with 5.8% of (1→4)-linked α-glucose residues (Table II), indicating that α-glucan of the *ags1-1*^{ts} mutant has a basic composition similar to that of wild-type α-glucan. However, when we analyzed its molecular mass distribution, we made an unexpected observation. Although the HPSEC profile of the mutant α-glucan showed a single peak, which was also Gaussian-shaped, its Mn was calculated to be only 22.1 ± 1.5 kDa, which is equivalent to a DP_n of 137 ± 9 (Figure 2D, profile II; Table I). This molecular mass is similar to that of Smith-degraded wild-type α-glucan, which was 19.1 ± 2.4 kDa and equivalent to 118 ± 15 glucose residues (see earlier discussion). The polydispersity of the mutant α-glucan was 3.01 ± 0.26, indicating a uniform population of polymers. Smith degradation slightly affected its characteristics, resulting in an Mn of 19.9 ± 2.6 kDa (equivalent to a DP_n of 123 ± 16 glucose residues) with a polydispersity of 2.74 ± 0.40 (Figure 2D, profile III; Table I). Together, these results indicate that mutant α-glucan consists of a single (1→3)-α-glucan chain of ~120 residues and some (1→4)-linked glucose residues at one of its ends.

To determine whether these (1→4)-linked glucose residues are present at the nonreducing or reducing end, mutant α-glucan was subjected to glucoamylase digestion or β-elimination. Glucoamylase treatment did not result in the release of any glucose residues, providing no evidence for the presence of (1→4)-linked glucose residues at the nonreducing end. Next, we performed a β-elimination on mutant α-glucan in the same way as was done with wild-type α-glucan (see previous description). After 5 days of incubation, both isosaccharinic and metasaccharinic acids were released, as is evident from GC-EI-MS analysis of the trimethylsilylated products (Figure 4F). Linkage analysis of the mutant α-glucan remaining after 5 days of incubation showed a 30% decrease in (1→4)-linked residues, whereas its HPSEC profile showed a slight shift in Mn from 22 to 18 kDa. These results demonstrate the presence of (1→4)-linked glucose residues at the reducing end of mutant α-glucan.

In summary, our data show that in the cell wall of rounded *ags1-1*^{ts} cells, α-glucan was composed of only a single chain of ~120 (1→3)-linked α-glucose residues and some (1→4)-linked α-glucose residues at the reducing end. We conclude that *ags1-1*^{ts} mutant cells grown at a semipermissive temperature are unable to couple α-glucan chains.

Discussion

Here we show that cell wall α-glucan of vegetatively grown fission-yeast cells consists of a single population of polysaccharides composed of two interconnected linear chains, each consisting of ~120 (1→3)-linked α-glucose residues and some (1→4)-linked α-glucose residues at the reducing end (Figure 6A). Remarkably, cell wall α-glucan of a mutant with a point mutation in the extracellular domain of the α-glucan synthase, Ags1p, is composed of only a single chain when grown at the semipermissive temperature (Figure 6A), suggesting that α-glucan chains were not coupled in this mutant.

To explain how the Ags1 protein may be involved in producing mature cell wall α-glucan, we propose the following speculative model (Figure 6B). First, the intracellular domain of Ags1p contributes to the synthesis of a first chain. This chain is then transported across the plasma membrane, perhaps via the multipass transmembrane domain of Ags1p. Once transported, the extracellular domain of Ags1p retains this first chain. Then the same Ags1 protein initiates a second round of synthesis and also transports the second chain across the plasma membrane. Finally, the extracellular domain of Ags1p acts as a transglycosylase and couples both chains, thereby forming a mature cell wall α-glucan molecule. This mature α-glucan molecule is then released into the cell wall, allowing synthesis of a following α-glucan molecule.

We speculate that the (1→4)-linked α-D-glucose residues at the reducing end of each α-glucan chain might constitute a primer for chain initiation (Figure 6B, step 1). Biosynthesis of several polysaccharides requires an oligosaccharide as primer for initiation, either conjugated to another molecule or as a free moiety: Cellulose biosynthesis requires sitosterol-β-glucoside (Peng *et al.*, 2002), streptococcal

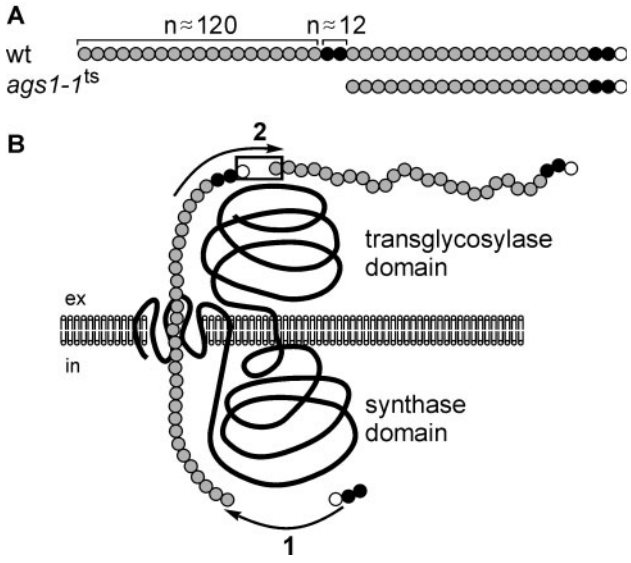


Fig. 6. Speculative model for synthesis and coupling of α-glucan chains by the Ags1 protein. Schematic representation of the chemical structure of cell wall α-glucan from wild-type and *ags1-1*^{ts} cells (**A**). The model (**B**) proposes that the intracellular domain of Ags1p is involved in the synthesis of α-glucan chains (1), whereas the extracellular domain of Ags1p is involved in the coupling of α-glucan chains (2). Note that this coupling activity is absent from *ags1-1*^{ts} mutant cells grown at 34°C, causing deposition of only single α-glucan chains. Black circles indicate a postulated primer of (1→4)-linked α-glucose residues; gray circles, (1→3)-linked chains; and, white circles, reducing ends.

(1→3)-α-glucan biosynthesis requires (1→6)-linked α-glucose (Germaine *et al*., 1974), and glycogen and starch biosynthesis require (1→4)-linked α-glucose oligosaccharides (Smith, 1999). Importantly, the *Escherichia coli* glycogen synthase, GlgA, with which the intracellular domain of the Ags1 protein shares sequence similarity (Hochstenbach *et al*., 1998), requires a (1→4)-linked α-glucose primer (Fox *et al*., 1976). Based on the relative contribution of these residues to a mature α-glucan polymer, we predict that ~12 (1→4)-linked α-glucose residues are present in each postulated primer. These residues may form a (1→4)-linked α-glucose homo-oligomer primer, as is used in glycogen biosynthesis.

In our model, we speculate that the postulated primer is elongated at its nonreducing end by addition of (1→3)-linked α-glucose residues (Figure 6B, step 1), consistent with the chemical structure of α-glucan. Whether the intracellular domain of Ags1p is involved in synthesis of the (1→4)-linked α-glucose primer or the (1→3)-linked α-glucose chain remains to be determined. To distinguish between these possibilities, biochemical assays need to be developed. First attempts to synthesize α-glucan *in vitro*, by using isolated cell membranes as a source of Ags1 synthase and UDP-glucose as a substrate, produced large quantities of (1→3)-β-glucan but no detectable α-glucan as analyzed by ^{1}H-NMR spectroscopy (unpublished data).

After chain termination, two newly synthesized chains must be coupled to form mature α-glucan. We propose that the extracellular domain of the Ags1 protein is involved directly in the coupling of the two chains (Figure 6B, step 2). This domain shares sequence similarity with bacterial amylases that have been shown to function as transglycosylases (MacGregor *et al*., 2001). These amylases can hydrolyze (1→4)-linked α-glucan oligosaccharides and transfer the newly generated reducing ends to the nonreducing ends of other α-glucan molecules.

Evidence corroborating our hypothesis that the extracellular domain of Ags1p may act as a transglycosylase and couple α-glucan chains came from the analysis of the α-glucan structure of the *ags1-1*^{ts} mutant. We showed that the *ags1-1*^{ts} mutant has a temperature-dependent α-glucan structure correlating with its cell morphology. When grown at the permissive temperature of 19°C, *ags1-1*^{ts} cells display a rod-like morphology comparable to that of wild-type cells and possess a wild-type α-glucan structure. By contrast, when grown at the semipermissive temperature of 34°C, *ags1-1*^{ts} cells are rounded and possess an α-glucan structure consisting of only a single chain. We infer from these data that at the semipermissive temperature the point mutation in the extracellular domain of the Ags1 protein inhibits transglycosylase activity such that α-glucan chains cannot be coupled (Figure 6).

In addition to the defect in α-glucan coupling, the *ags1-1*^{ts} mutant grown at the semipermissive temperature has a defect in α-glucan biosynthesis, given the threefold reduction in α-glucan levels. We presume that this latter defect is caused by a decrease in Ags1 protein levels at the plasma membrane. Katayama and colleagues (1999) observed that when their ts mutant with a mutation in the Ags1 protein (denoted Mok1p in their study) was grown at its semipermissive temperature of 35.5°C, mutant Ags1p was mislocalized to intracellular locations, depleting the plasma membrane of Ags1 protein. Which of these two events, the change in the structure of cell wall α-glucan or the reduction in its levels, is directly responsible for the disorganization of cell wall structure, the weakening of the cell wall, and the rounded cell morphology, remains unresolved. Nonetheless, our data clearly indicate that the Ags1 protein is involved in both synthesis and coupling of cell wall α-glucan.

Although compounds against fungal pathogens have been developed that inhibit synthesis of chitin and (1→3)-β-glucan, no inhibitors of cell wall α-glucan synthesis have been identified as yet. Of the major fungal pathogens, *Candida albicans* lacks cell wall α-glucan and contains no homologs of the Ags1 protein. By contrast, other fungal pathogens, such as *Aspergillus fumigatus*, *Cryptococcus neoformans*, *H. capsulatum*, *P. brasiliensis*, *B. dermatitidis*, and *Coccidioides immitis*, contain cell wall α-glucan. For *A. fumigatus*, two homologs (with GenBank accession numbers AAL18964 and AAL28129) of the *S. pombe* Ags1 protein have already been identified. They possess a multidomain structure that is similar to that of the *S. pombe* Ags1 protein, indicating that also their mechanisms of action may be similar. In particular, the α-glucan synthase of *Cryptococcus* has been indicated as a potentially interesting target, because this pathogenic yeast is resistant to chitin and (1→3)-β-glucan synthase inhibitors (Georgopapadakou and Tkacz, 1995; Georgopapadakou, 2001) and requires cell wall α-glucan to anchor its capsule (Reese and Doering, 2003), which is critical for virulence. Our present data suggest that inhibition of either the synthase activity or the

transglycosylase activity of the Ags1 protein may weaken fungal cell walls. Consequently, we propose that both domains may be suitable targets for the development of novel antifungal drugs.

Materials and methods

S. pombe *strains and cell culture*

Strains FH021 (h^- *ags1-1*^{ts}) and FH023 (h^-) were described before (Hochstenbach *et al.*, 1998); strain FH058 (h^-/h^- ade6-M210/ade6-M216) was a spontaneous, nonsporulating derivative of diploid strain FH045 (h^+/h^- ade6-M210/ade6-M216). Strains FH023, FH058, and 972 (h^-) were grown in YEA medium (5 g/L yeast extract, 30 g/L glucose, 250 mg/L adenine sulfate) at 28°C, whereas strain FH021 was grown in YEA medium containing 1.2 M sorbitol at 19°C or 34°C for 2 days. The addition of sorbitol ensured that *ags1-1*^{ts} cells grown at 34°C remained physically intact, whereas the 2-day culture period ensured that virtually all cells displayed a rounded morphology (Hochstenbach *et al.*, 1998).

Isolation of whole cell walls and cell wall α*-glucan*

For whole cell wall preparations, cells were grown in 1.5 L of the indicated medium to a final OD_{595} of 4 (yielding ~16 g wet weight), cooled in an ice bath, collected by centrifugation, and washed twice in breaking buffer (5 mM sodium azide, 20 mM Tris–HCl, pH 7.6). Cells were resuspended in breaking buffer and subjected to mechanical breakage in a Bead-Beater (BioSpec Products, Bartlesville, OK) by using glass beads (0.45 mm diameter). Twelve rounds of 1-min homogenization were alternated with 1-min cooling periods, lysing more than 95% of the cells. The cell lysate was collected and centrifuged. This and all subsequent centrifugation steps were carried out at 7500 × *g* at 4°C for 20 min. After two washing steps in MilliQ-H_2O (Millipore, Bedford, MA), the pellet was resuspended in 200 ml of SDS extraction buffer (40 mM 2-mercaptoethanol, 2% [w/v] SDS, 100 mM Na-EDTA, 50 mM Tris–HCl, pH 7.6), and incubated in a boiling water bath for 20 min to remove cytoplasmic contaminants. The suspension was centrifuged, washed twice in MilliQ-H_2O, and stored in 5 mM sodium azide at 4°C.

For α-glucan isolations, whole cell wall preparations were resuspended in 200 ml digestion buffer (5 mM sodium azide, 40 mM 2-mercaptoethanol, 50 mM citrate-phosphate, pH 5.3) containing 15 mg of zymolyase-100T (Seikagaku, Tokyo), and incubated in a rotary shaker at 37°C for at least 12 h. After centrifugation, the pellet was resuspended in breaking buffer, glass beads were added, and the cell wall material was treated in a Mikro-Dismembrator (B. Braun Biotech International) at 3000 rpm for 3 min to break cells that had remained intact during the first homogenization. Then zymolyase digestion was repeated, followed by a final extraction with SDS. After two washing steps in MilliQ-H_2O, purified α-glucan was stored in 5 mM sodium azide at 4°C.

Transmission electron microscopy

Cell walls were adsorbed on Pioloform-carbon-coated copper grids and negatively stained with 1% (w/v) uranyl acetate. The ultrastructure was analyzed with a Tecnai 12 electron microscope (FEI Electron Optics, Eindhoven, The Netherlands) at 120 kV acceleration voltage.

Monosaccharide and linkage analysis

For monosaccharide analysis, samples were subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85°C), followed by trimethylsilylation (5:1:1 [v/v/v] of pyridine/chlorotrimethylsilane/hexamethyldisilazane, 30 min, room temperature) and were analyzed by GC and GC-EI-MS (Kamerling and Vliegenthart, 1989). Absolute configurations were determined by (-)-2-butanolysis (Gerwig *et al.*, 1978).

For linkage analysis, per-*O*-methylation was performed using the method of Hakomori (1964). Then, per-*O*-methylated polysaccharides were hydrolyzed in aqueous 90% (v/v) formic acid (1 h, 100°C), followed by evaporation and incubation in 2 M trifluoroacetic acid (1 h, 120°C). Samples were reduced with excess $NaBD_4$ in 0.5 M NH_4OH for 90 min at room temperature, followed by acetylation with acetic anhydride (3 h, 120°C).

GC analyses were performed on a WCOT CP-SIL 5CB fused-silica capillary column (25 m × 0.32 mm) (Chrompack, Bergen op Zoom, The Netherlands) using a CP 9002 gas chromatograph (Chrompack) and a temperature program of 140–240°C at 4°C/min. GC-EI-MS of partially methylated alditol acetates was carried out on an MD800/8060 system (Fisons Instruments, Manchester, U.K.) equipped with a WCOT CP-SIL 5CB fused-silica capillary column (25 m × 0.25 mm) (Chrompack), also using a temperature program of 140–240°C at 4°C/min.

NMR spectroscopy

All NMR spectra were recorded on a DRX500 spectrometer (Bruker Biospin, Karlsruhe, Germany). Polymeric samples were dissolved in 600 µl 99.6% DMSO-d_6 and were analyzed at 80°C. In 1D ^{1}H-NMR experiments, residual water signals were suppressed by applying a WEFT pulse sequence. 2D ^{1}H-^{1}H TOCSY was carried out in the phase-sensitive mode using the States-TPPI method and using MLEV-17 mixing sequences of 10–50 ms. Spectral width was 3501 Hz in both dimensions; 512 experiments of 1024 data points were acquired with 32 scans per increment. In the sensitivity-enhanced two-dimensional ^{1}H-^{13}C HSQC experiment, Echo/Antiecho gradient selection with decoupling was used. Spectral widths were 1600 Hz and 10,000 Hz for the proton and the carbon dimensions, respectively, and 950 free-induction decays of 1024 data points were acquired using 128 scans per decay. Chemical shifts were expressed in ppm relative to internal DMSO (^{1}H, 2.505 ppm; ^{13}C, 39.6 ppm). ^{1}H-NMR spectra of water-soluble oligosaccharides were recorded in 99.9% D_2O at 27°C. The residual HOD signal was suppressed by applying a WEFT pulse sequence. Chemical shifts were referred to internal acetate (^{1}H: 1.908 ppm). Data were processed using in-house-developed software.

Smith degradation

For Smith degradation (Smith and Montgomery, 1956), polysaccharides were suspended in 15 mM sodium periodate at a concentration of ~2 mg/ml. The mixture was placed in

the dark at 4°C under continuous mixing. After 48 h, the reaction was stopped by adding ethylene glycol to a final concentration of 350 mM. Oxidized polysaccharides were reduced with an excess of $NaBH_4$ for 24 h. Then, excess borohydride was removed by the addition of acetic acid. The product was washed three times with water and hydrolyzed in 100 mM hydrochloric acid at room temperature for 8 h.

HPSEC

The HPSEC system consisted of a Delta 600 pump (Waters, Milford, CT) with a DRI 2410 refractive index detector (Waters). For the mobile phase, sodium nitrate was added to DMSO to a final concentration of 3 mM to reduce aggregation of polymers and to eliminate ionic strength effects (Chuang, 1990). The mobile phase was delivered at a flow rate of 1.0 ml/min. A single PLgel 5 μm MIXED-C column (300 × 7.5 mm) (Polymer Laboratories, Amherst, MA) was connected in series with a PLgel 10 μm guard column (50 × 7.5 mm). Both columns were thermostated at 80°C. The system was calibrated using pullulan narrow standards of 853, 380, 186, 100, 48, 23.7, 12.2, and 5.8 kDa with polydispersities ranging from 1.06 to 1.14 (Standard P-82, Shodex, Showa Denko, Tokyo, Japan), plus maltohexaose and glucose. These linear polysaccharide standards are appropriate to determine molecular mass distributions of linear polysaccharides including cell wall α-glucan (Churms, 1996). Samples were lyophilized and then further dried overnight *in vacuo* over phosphorus pentoxide; then they were dissolved in the mobile phase to a concentration of 2 mg/ml and filtered through 0.45 μm PTFE filters. Injection volumes of 100 μl were used. Using Millenium^{32} software (Waters), each HPSEC profile was divided into a number of virtual time slices, n_i, that each corresponded to a certain molecular mass, M_i, obtained by calibrating the column, and from these values, the Mn and Mw were calculated according to:

$$Mn = \Sigma_i n_i M_i / \Sigma_i n_i \quad \text{and} \quad Mw = \Sigma_i n_i M_i^2 / \Sigma_i n_i M_i$$

The polydispersity is given by the ratio of Mw over Mn.

Glucoamylase digestion

Glucoamylase (AMG L300) was a kind gift from AVEBE (Veendam, Netherlands). Samples were digested for 24 h with 0.5 mass % of glucoamylase at 57.5°C and pH 4.3. Products were analyzed by thin-layer chromatography on Silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) using 1-butanol-ethanol-water (3:2:2, v/v/v) as eluent. Carbohydrates were stained using orcinol-sulfuric acid.

β-*Elimination*

Alkaline degradation was carried out at room temperature in an oxygen-free saturated calcium hydroxide solution. After the time intervals indicated, samples were taken and centrifuged. Supernatants were neutralized by sparging with carbon dioxide, evaporated to dryness under reduced pressure, and further dried *in vacuo* over phosphorus pentoxide. The dried reaction products were then trimethylsilylated and analyzed using GC-EI-MS as described for monosaccharide analysis. Residues were washed with water and prepared for analysis by linkage analysis or HPSEC. Reference compounds, amylose (Sigma, St. Louis, MO), laminaran (Koch-Light, NBS Biologicals, Huntingdon, UK), and nigeran (Koch-Light) were washed and lyophilized. (1→3)-α-Glucan was prepared by performing Smith degradation on *S. pombe* cell wall α-glucan, followed by partial hydrolysis in 2 M trifluoroacetic acid at 50°C for 30 min to reintroduce reducing ends.

Preparation and separation of α-glucan oligosaccharides

One hundred milligrams of α-glucan obtained from cell walls of *S. pombe* wild-type strain FH023 were suspended in digestion buffer (50 mM NaOAc, pH 5.6, 5 mM NaN_3). (1→3)-α-Glucanase MutAp purified using adsorption chromatography from an enzyme preparation obtained from *Trichoderma harzianum* (Dekker *et al*., 2004) was added and the reaction mixture was incubated at 37°C for 1 h. Glucanase digestion was repeated several times using fresh buffer and enzyme. Collected supernatants were desalted by solid-phase extraction using Carbograph SPE columns (Alltech Associates, Deerfield, IL) using the procedure described by Packer *et al*. (1998), which also separates monosaccharides from oligosaccharides. Products were applied on a thermostated Bio-Gel P4 size-exclusion column (1.6 × 90 cm, 55°C) (BioRad, Hercules, CA) and eluted with water at a flow rate of 4.8 ml/h. Carbohydrates in the eluate were quantitatively determined by the phenol-sulfuric acid assay (Dubois *et al*., 1956). Fractions of 2.0 ml were collected, and appropriate fractions were pooled and further purified by HPAEC.

HPAEC

HPAEC was performed on a Dionex DX 500 system equipped with a GP 40 gradient pump and an ED 40 electrochemical detector (Dionex, Sunnyvale, CA). A 4 × 250 mm Carbopac PA-1 column was used for analytical HPAEC. Appropriate linear gradients were applied for each component using 100 mM NaOH and 500 mM NaOAc in 100 mM NaOH as eluents. A flow rate of 1.0 ml/min was used. Purification of Bio-Gel P4 fractions was performed on a semipreparative 9 × 250 mm Carbopac PA-1 column using the same eluents at a flow rate of 4.0 ml/min. Fractions were desalted using Carbograph SPE columns, followed by evaporation of the solvent *in vacuo*.

2-AB labeling, periodate oxidation, and methylation

Oligosaccharides were labeled at their reducing ends with 2-AB according to Bigge *et al*. (1995). To the dried oligosaccharides, 5 μl of a solution of 2-AB (23.6 mg) and sodium cyanoborohydride (31.75 mg) in DMSO/acetic acid 7:3 (500 μl) was added. The reaction was carried out at 65°C for 2 h, after which the samples were cleaned up on Whatman QM-A chromatography paper. Reactants were removed by rinsing with acetonitrile (1 ml) and 4% water in acetonitrile (6 × 1 ml). Labeled products were eluted with water (4 × 0.5 ml).

Periodate oxidation of labeled oligosaccharides was basically performed as described by Angel *et al*. (1991). Briefly, 2-AB-labeled oligosaccharides were dissolved in 50 mM sodium acetate (pH 5.5) containing 0.8 M sodium periodate. Samples were stirred in the dark at 4°C for 24 h.

The pH was adjusted to 7.0 by addition of NaOH. $NaBD_4$ was added, and incubation was continued for 24 h at 4°C. Excess of $NaBD_4$ was removed by adjusting the pH to 4.5 with acetic acid. Boric acid was coevaporated with methanol under reduced pressure. The reduced products were acetylated in acetic anhydride/pyridine for 30 min at 70°C. After concentration to dryness, acetylated products were extracted by chloroform from water. The dried products were methylated according to Ciucanu and Kerek (1984).

MS

Experiments were performed on a LC-Q ion-trap mass spectrometer (Thermo-Finnigan, San Jose, CA) equipped with a nanoES sample probe (Protana, Odense, Denmark). 2-AB-labeled, oxidized, and then reduced and per-*O*-methylated samples were dissolved in methanol/water (7:3) to a concentration of ~10–30 pmol per µl. For each experiment, 2 µl were loaded into the gold-coated glass capillary. The capillary temperature was set to 180°C. Spectra were taken in the positive ion mode with a spray voltage of 1.5 kV and a varying capillary voltage of 31.5 to 46.0 V.

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Abbreviations

2-AB, 2-aminobenzamide; Ags1, α-glucan synthase I; DMSO, dimethyl sulfoxide; DP, degree of polymerization; GC-EI-MS, gas chromatography electron-impact mass spectrometry; HPAEC, high-performance anion-exchange chromatography; HPSEC, high-performance size-exclusion chromatography; HSQC, heteronuclear single-quantum coherence; Mn, number-average molecular mass; Mw, weight-average molecular mass; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; TOCSY, total correlation spectroscopy.

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