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β(1,3)-Glucanosyl-Transferase Activity Is Essential for Cell Wall Integrity and Viability of Schizosaccharomyces pombe

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Abstract

Background: The formation of the cell wall in Schizosaccharomyces pombe requires the coordinated activity of enzymes involved in the biosynthesis and modification of β-glucans. The β(1,3)-glucan synthase complex synthesizes linear β(1,3)-glucans, which remain unorganized until they are cross-linked to other β(1,3)-glucans and other cell wall components. Transferases of the GH72 family play important roles in cell wall assembly and its rearrangement in Saccharomyces cerevisiae and Aspergillus fumigatus. Four genes encoding β(1,3)-glucanosyl-transferases -gas1p, gas2p, gas4p and gas5p- are present in S. pombe, although their function has not been analyzed.

Methodology/Principal Findings: Here, we report the characterization of the catalytic activity of gas1p, gas2p and gas5p together with studies directed to understand their function during vegetative growth. From the functional point of view, gas1p is essential for cell integrity and viability during vegetative growth, since gas1Δ mutants can only grow in osmotically supported media, while gas2p and gas5p play a minor role in cell wall construction. From the biochemical point of view, all of them display β(1,3)-glucanosyl-transferase activity, although they differ in their specificity for substrate length, cleavage point and product size. In light of all the above, together with the differences in expression profiles during the life cycle, the S. pombe GH72 proteins may accomplish complementary, non-overlapping functions in fission yeast.

Conclusions/Significance: We conclude that β(1,3)-glucanosyl-transferase activity is essential for viability in fission yeast, being required to maintain cell integrity during vegetative growth.

Introduction

The fission yeast Schizosaccharomyces pombe has a rod-like shape and grows asymmetrically at the poles. At the onset of mitosis, polarized growth abates and septum deposition occurs in the middle of the cell, followed by medial fission [1]. The cell wall is an extracellular structure that serves as an exoskeleton for fungi. Its main function is to preserve the osmotic integrity of the cells, but it also contributes to cellular morphology. When the cell wall is removed by lytic enzymes, or in certain cell wall biosynthesis mutants, the cells become ovoid or round, indicating that the cell wall is essential for maintaining cell shape [2,3]. All the morphogenetic changes, such as tip elongation, septation, mating or sporulation, require continuous cell wall synthesis and remodeling.

In S. pombe, the cell wall is composed of mannoproteins (9–14%), α-glucan (18–28%) and β-glucan (46–54%) [4,5,6]. β(1,3)-glucan is a major structural component of the fungal cell wall and it forms a fibrillary network, which is thought to be responsible for the mechanical strength of the cell wall. β(1,3)-glucans are present in the inner portion of the wall [7]. The biosynthesis of β(1,3)-glucan is carried out by a β(1,3)-glucan synthase complex, whose catalytic subunit in S. pombe is encoded by the bgs genes [3,8]. S. pombe contains four proteins of this family; cps1/bgs1p, bgs3p and bgs4p are essential for cell viability during vegetative growth [9,10,11,12], while bgs2p performs an essential role during spore wall formation [13,14]. It has been postulated that the nascent β(1,3)-glucan chains should be cross-linked to other components of the cell wall by the action of glycoside hydrolases (GH) and transglycosidases [15,16]. However, in vivo evidence of such mechanism has only been shown for the Saccharomyces cerevisiae Cbr1 and Cbr2 proteins, which are involved in the cross-linking of β(1,3)-glucan to chitin [17,18].
glucans through chain elongation. Originally described in *Aspergillus fumigatus*, this activity has been identified since then in many yeasts [19] and is encoded by the orthologous genes *GAL1, GAS1* and *PDR* in *A. fumigatus*, *Saccharomyces cerevisiae* and *Candida albicans*, respectively. *S. cerevisiae* contains five genes — *GAL1* to *GAL5* — encoding proteins of the GH72 family [20]. Gas1p is one of the most abundant glucosephosphatidylinositol (GPI)-anchored cell surface proteins [21,22]. Gas1p plays an active role in fungal cell wall synthesis during vegetative growth, since *GAL1* deletion results in cells with abnormal morphology and reduced β(1,3)-glucan content that is compensated by an increase in chitin and mannan [23,24,25]. Recently, it has been described that *GAL2* and *GAL4* are expressed exclusively during sporulation and that the double *gas2 gas4* diploid mutant shows a severe reduction in sporulation efficiency. An analysis of spore ultrastructure indicate that the loss of *Gas2p* and *Gas4p* proteins affects the proper attachment of the glucan to the chitosan layer, probably as a consequence of the lack of coherence of the glucan layer [26].

Four genes encoding putative β(1,3)-glucanosyl-transferases — *gas1*, *gas2*, *gas4* and *gas5* — are present in the *S. pombe* genome, suggesting that each protein might perform specific functions at different moments of the life cycle. Indeed, we have recently reported that gaslp is essential for ascospore wall maturation and spore viability in fission yeast [27]. Here we report the characterization of the other three members of the GH72 family in *S. pombe*, which exploit their functions during vegetative growth and whose expression is cell-cycle regulated. The three proteins show β(1,3)-glucanosyl-transferase activity with different substrate specificities. Interestingly, *gas1* is an essential gene in fission yeast, since gas1Δ mutants are unable to survive in the absence of an osmotic stabilizer and undergo cell lysis, mainly during septum degradation.

**Results**

Gas1p, Gas2p and Gas5p expression is cell-cycle regulated

*S. pombe* contains four genes encoding proteins belonging to the GH72 family, which contain the conserved transfase domain present in these proteins [19,28]. In addition to this conserved domain, GH72 proteins have a modular structure and may contain additional domains, such as a Cys-rich region that bears sequence similarity with proteins of the carbohydrate-binding module family 43 (CBM43) [20,29] and/or a Ser-rich region (Figure S1A). A feature of most of the proteins of this family is the presence of a hydrophobic region at the C-terminus that is part of a GPI attachment site. The gaslp and gas3p proteins contain a putative GPI-anchor site, whereas gas2p apparently lacks the hydrophobic C-terminal region that acts as a signal for this post-translational modification (Figure S1B). To rule out the existence of a frameshift or a sequencing error, the C-terminal region of gas2p was PCR-amplified from different strain backgrounds and sequenced. The results indicated that the sequence present in the *S. pombe* genome database is correct.

In agreement with earlier studies [30], Northern analysis indicated that only *gas1*, *gas2* and *gas5* were expressed during vegetative growth and that their expression was periodic along the cell cycle (Fig. 1). Interestingly, the maximum level of expression occurred at different moments. Thus, while *gas1* and *gas5* showed a peak during mitosis, just prior to septation, *gas2* transcript levels were higher during G2. These results suggest that these genes may exert their function at different moments of the vegetative cell cycle.

gas proteins localize to the cell periphery

In order to determine the localization of gaslp, gas2p and gas5p, the proteins were tagged with fluorescent epitopes. Since the N-terminal signal sequence and the putative C-terminal GPI anchor site could be important for correct localization of the proteins, the epitope was inserted in different regions of the proteins. For *gas1*, the YFP (yellow fluorescent protein) sequence was inserted between the putative signal sequence and the GH72 domain by recombinant PCR and cloned into vector pAL-KS. This plasmid partially suppressed the slow growth rate of *gas1Δ*. The growth rate of the *gas1Δ* null mutant transformed YFP-gaslp was 0.18 h⁻¹, compared with a value of 0.25 for the wild-type and 0.05 for the *gas1Δ*. A similar partial complementation has been recently described for *S. cerevisiae* Gas1p-GFP [31]. Since the YFP-gas1p construct was present in a plasmid, we confirmed that the protein was periodically synthesized during the cell by Western analysis, with a pattern similar to the transcription profile of the chromosomal copy (data not shown). Microscopic observation of the transformants revealed that YFP-gaslp localized to the cell periphery and, more precisely, to regions of active growth. The fluorescent signal was clearly observed at the poles of bipolar cells and at the septum of dividing cells (Fig. 2A). However, in monopolar cells the signal was more intense at the non-growing end, perhaps as a remnant of the protein present at the previous septum. For gas5p, a similar strategy was used, since its modular structure was very similar to that of gaslp (an N-terminal signal sequence and a putative C-terminal GPI-anchor site), although no clear localization could be observed. The fluorescence was mainly intracellular, in a vesicular pattern (data not shown), suggesting that the fusion protein had not been correctly processed.

Because *gas2* lacks the putative C-terminal GPI anchor signal, the GFP (green fluorescent protein)-coding sequence was fused in-frame to the last amino acid of gas2p at its chromosomal locus using the cassettes described by Bahler and coworkers [32]. Since gas2Δ mutants do not have any clear phenotype (see below), functionality of the gas2p-GFP fusion could not be assessed. Gas2p-GFP also localized to the cell periphery, with a localization pattern similar to that of gaslp; i.e., at the tips of bipolar cells and the
However, gas1p and gas2p localization to the septum region was not identical. gas1p first localized as a ring surrounding the septation region and then rapidly localized as a disc at the nascent septum, which expanded as its synthesis proceeded (Fig. 2B). In contrast, gas2p localized as a ring surrounding the septum and remained inside the cell wall that
surrounds the septum: the septum edging (Fig. 2B). Only at later stages, when septum had completely assembled, gas2p could be detected associated with the septum. These results therefore indicate that gas1p and gas2p localize to similar regions of the cell, the growing tips and the septum, even though gas2p lacks a GPI attachment site, and that gas1p and gas2p might play different functions during septum synthesis.

gas1p is required for correct morphogenesis

To elucidate whether gas1p, gas2p or gas5p play a role in cell wall remodeling during the cell cycle, mutants lacking each gene were generated with a PCR-based system [33]. Only mutants lacking gas2p or gas5p were obtained, suggesting that the third gene might be essential for growth in fission yeast. Accordingly, to confirm this possibility a heterozygous diploid strain -gas1Δ::KanMX4/gas1Δ- was constructed. Correct deletion of one of the alleles was confirmed by Southern blot, and the heterozygous strain was sporulated and tetrads were dissected on rich medium plates (YES). Tetrad analysis revealed two viable and two inviable spores, all the viable spores being kan’ (data not shown).

Microscopic observation of the unviable spores showed that most of them germinated to form two or three rounded cells before growth stopped. When tetrads were dissected on plates containing 1.2 M sorbitol, the spores bearing the gas1Δ::KanMX4 deletion allele were able to germinate and form colonies that were apparently indistinguishable from those of the wild-type strain. gas1Δ deletion mutants were only able to grow in the presence of osmotic support (Fig. 3A), thus confirming that gas1p is an essential gene during vegetative growth in fission yeast. In liquid medium, gas1Δ mutant cells grew slower than the wild-type strain in the presence of sorbitol (Fig. 3B) and were unable to grow when transferred to rich or minimal media without an osmotic stabilizer. In contrast, gas2Δ and gas5Δ mutants, and even the double gas2Δ gas5Δ mutant, were viable at all temperatures and showed no apparent growth defect in rich medium or minimal medium (data not shown). Taken together, these findings suggest that gas1p, but not gas2p or gas5p, is essential for vegetative growth in absence of osmotic protection.

The morphology of mutant cells was also analyzed during growth at different temperatures by microscopic observation using aniline blue to stain the β(1,3)-glucan of the cell wall [34]. gas1Δ cells grown on plates with sorbitol were shorter and rounder than the wild-type, and some lysed cells were also observed (Fig. 4A). In some cases, cell wall staining was not uniform, an abnormal deposition of cell wall material that stained more intensely with aniline blue being observed (Fig. 4A, arrows). When transferred to media without an osmotic stabilizer for two hours, a large fraction of the gas1Δ mutant cells (around 60%) underwent lysis. Of the surviving cells, half of them had lost their polarity and were rounded, with large accumulations of abnormal cell wall material that were even visible with Nomarsky optics (Fig. 4B and C). Interestingly, although in some cases we observed lysis at the cell tips, many lysed cells displayed a similar terminal phenotype, appearing as pairs of lysed cells, as if they had undergone lysis during the cell separation process (Fig. 4A and C). To confirm this hypothesis, a time-lapse experiment was performed to monitor the growth over time of a gas1Δ mutant. When gas1Δ cells were inoculated in rich media without sorbitol, cells were able to grow normally, although some of them displayed polarity defects, and they were also able to assemble the separation septum properly (Figure S2). However, when cell separation started and the newly generated ends started to appear round, lysis occurred suddenly (minutes 19, 27, 80, 98). These results suggest that gas1p is essential for the maintenance of cellular integrity and viability during vegetative growth and also for cell morphogenesis. In the absence of gas1p, cell lysis takes place in actively growing regions (poles and septum), and cells that do not lyse display morphogenetic defects, manifested as widened poles or widened medial regions (Figure S2, asterisks).

gas1p, gas2p and gas5p display β(1,3)-glucanosyltransferase activity

The similarity between S. pombe gas proteins and S. cerevisiae Gas1p or A. fumigatus Gel1p, and the sequence conservation around the two aspartic acids that form the catalytic pair -FF(A/S)GNE*V (the acid-base donor) and F/F/LE*Y/F/GCN (the nucleophilic residue) [35,36]- suggest that gas1p, gas2p and gas5p could display the same glucanosyl-transferase activity as other GH72 proteins [20,27,28,36]. To test this notion, recombinant versions of gas1p and gas5p (lacking the GPI moiety and containing 6 His at the C-terminus: r-gas1p and r-gas5p) and gas2p (containing two sets of 6 His, one before the GH72 domain and another at the C-terminus: r-gas2p) were analyzed. The purified proteins were incubated with reduced laminari-oligosaccharides of 13 or 20 glucose residues (G13r and G20r) for different times, and the products of the reaction were analyzed by high-performance anion exchange chromatography coupled to pulse electrophoresis.
electrochemical detection (HPAEC-PED) (Fig. 5A and Figure S3B). The resulting products were smaller and larger than the starting substrate, indicating that all enzymes were catalyzing a transfer reaction. The results revealed that the products of r-gas1p were very similar to those generated by ScGas1p, ScGas4p, AfGel1p, AfGel2p or Spgas4p [19,20,27,28,37].

The chromatogram shows that the enzyme preferentially released an oligosaccharide of 6-8 glucose units from the reducing end of G13r and transferred it to the acceptor laminari-oligosaccharide (Figure S3A). Thus, the major initial products were G6–8r and rG18–20r, in agreement with the two-step reaction scheme previously described by Hartland et al. [38]. Analysis of r-gas2p and r-gas5p indicated a slight difference with r-gas1p activity. Mainly, G13r was a poor substrate for r-gas2p (Figure S3B), which only weakly transferred glucan fragments smaller than 10 glucose residues (Fig. 5A), while gas5p catalyzed the transfer of a glucan with a degree of polymerization $\Delta \sim 4$ (Fig. 5A and Figure S3A). These results indicated that although the catalytic reaction was the same, the three enzymes had slight differences in substrate recognition. In all cases, the pattern observed after prolonged incubation times indicated that the initial transfer products could be subsequently re-used either as donors or acceptors, resulting in a broad range of transfer products of increasing size (degree of polymerization $> 30$) until they became water-insoluble. The minimum oligosaccharide that could be used in the reaction was an oligosaccharide of 9 glucose units (data not shown), and the optimum pH was slightly acid: pH 5.0. In light of the foregoing, we conclude that gas1p, gas2p and gas5p are true $\beta$(1,3)-glucanosyl-transferases in vitro, but that the three enzymes differ in their affinities for different substrates, the cleavage point, and also in their transfer products.

**Cell wall composition of gasΔ mutants**

To test whether GH72 proteins had any role in cell wall construction in vivo in fission yeast, the cell wall composition of the different mutants was analyzed. Cell walls were purified and fractionated in alkali-soluble (AS) and alkali-insoluble (AI) fractions. The major cell wall fraction was the AS. Total carbohydrate analysis showed no quantitative differences in the AS fraction of all strains (Figure S3B). The AS fraction was partially digested by treatment with endo-$\beta$(1,3)-glucanase (Laminarinase A, LamA). Gel filtration of the AS fractions carboxymethylated before or after LamA treatment indicated that $\beta$(1,3)-glucans corresponded to the fractions with the highest molecular weight. The LamA resistant fraction eluted later (Fig. 5B). This fraction most probably contained the $\alpha$ (1,3)-glucans, which are the other major cell wall component resistant to endo-$\beta$1,3 glucanase. The same digestion and elution profiles were seen for...
Figure 5. Cell wall composition of gasΔ mutants and analysis of enzymatic activity. (A) Fission yeast gas proteins display β(1,3)-glucanosyl-transferase activity. Recombinant gas1p, gas5p and gas2p expressed P. pastoris were incubated with G20r in 50 mM acetate buffer (pH 5.0) for the indicated times. The reaction products were analyzed by HPAEC-PED on a CarboPAC-PA200 column. The main reaction products are indicated. (B) For the preparation of cell walls, strains h20 (WT), YMMR16 (gas2Δ), YMMR32 (gas5Δ) and YMMR40 (gas2Δ gas5Δ) were grown in YES medium. YMMR106 (gas1Δ) was grown in YES medium supplemented with 1.2 M sorbitol (+S) and compared with a wild-type strain (h116) grown under the same conditions. Gel filtration chromatography of carboxymethylated cell wall fractions on an HR500S column with or without digestion with Laminarinase A (LamA). Sugars were detected by the phenol sulphuric method. (C) Percentage of β(1,3)-glucan branching in cell wall fractions of gasΔ mutants. The amount of branching was estimated after laminarinase A digestion and HPAEC analysis. doi:10.1371/journal.pone.0014046.g005
the AS fraction from the wild-type strain and the gas2Δ, gas5Δ and
gas2Δ gas5Δ mutants (Fig. 5B). However, the AS fraction from the
gas1Δ mutant grown in medium containing 1.2 M sorbitol had a
lower amount of the high molecular weight β(1,3)-glucans, as
compared to the wild-type strain grown under the same
conditions. It has recently been described that LamA digestion
and HPAEC analysis allows to estimate the level of β(1,6)-
branched of the cell wall β(1,3)-glucans [39]. As shown in Fig. 5C,
a low percentage of branching was observed in gas5Δ and gas2Δ
gas5Δ mutants and the decrease was even more pronounced in
gas1Δ cell walls. No differences were observed for the gas2Δ
mutant. The differences in cell wall composition in the gas1Δ
mutant are also in good agreement with the high degree of staining
with aniline blue (Fig. 4), a dye that gives stronger fluorescence
with linear β(1,3)-glucan chains than with β(1,3)/β(1,6)-
glucans.

For the alkali-insoluble fraction, only a significant amount could be
recovered and analyzed in the gas1Δ mutant and in the double
gas2Δ gas5Δ mutant in comparison with the other strains (Figure
S3B). The AI fraction was however very limited, since it did not
exceed 8% of the AS fraction. This AI fraction did not contain
GlcNac (data not shown). Gel filtration analysis of the carboxymethylated AI polysaccharides revealed that the LamA
digested material of the gas1Δ and the double gas2Δ gas5Δ mutants
contained the largest glucans (Fig. 5C). Thus, the overall structural
organization of the cell wall of the gas2Δ gas5Δ and gas1Δ mutants
were significantly modified from that of the wild type. The
reduction in the size of the β(1,3)-glucan chains of the major cell
wall fraction seen in the gas1Δ mutant is in agreement with the
biochemical function of the gas1 protein.

β(1,3)-glucanosyl-transferase activity is essential during
vegetative growth

We have previously shown that another member of this family,
gaslp, is essential for spore wall assembly and that it catalyzes a
transfer reaction with similar specificity to that found for gaslp
[27]. We therefore wondered whether gaslp could complement
the cell lysis phenotype and/or the morphogenetic defects of a
gas1Δ mutant. In order to allow the expression of gasf4 during
mitotic growth, the coding region was cloned under the control of the
nmt1Δ promoter, which is induced in the absence of thiamine.
Since gas1Δ is periodically expressed during the cell cycle, gasf4
was also placed under the control of the gas1Δ promoter (Pgas1)
allowing its cell cycle-regulated expression. Strains gas1Δ and the wild-type
carrying the vector were used as controls to analyze growth in
liquid media, with or without osmotic support. gas1Δ cells carrying
the vector were able to grow in the presence of sorbitol, but not
when the osmotic stabilizer was absent (Fig. 6A). However,
overexpression of gasf4 under the control of the nmt1Δ promoter
or the gas1Δ promoter was able to complement the growth defect of
gas1Δ cells without osmotic support, growth rates similar to those
of wild-type cells being observed.

To test whether the morphogenetic defects of gas1Δ cells were
also complemented by gasf4, the morphology of the transformants
was analyzed and the different defects were quantified. Similar to
what happened in rich media without osmotic support, gas1Δ cells
harboring the vector were rounder and shorter than the wild-type
(Fig. 6B), and around half of the cells underwent lysis in these
culture conditions (Fig. 7). In contrast, cells ectopically expressing
gasf4 (under the control of Pmmt1 or Pgas1) were similar to the wild-
type strain (Fig. 6B) and the number of dead cells was severely
reduced with respect to gas1Δ cells (Fig. 7). Thus, gaslp is able to
fully complement the defects of gas1Δ cells. Interestingly, the Pgas1-
gasf4 construct was more efficient at reverting gas1Δ viability
than Pmmt1-gasf4, suggesting that proper temporal expression is
important for better complementation.

In the gas1Δ mutant strain, gas2Δ and gas5Δ are present
although they are not able to compensate the loss of gaslp. To test
whether higher levels of gas2Δ and gas5Δ than those present in
vegetative cells would be able to revert the gas1Δ phenotype, gasf4
and gasf5 were also cloned under the control of the nmt1Δ
promoter and introduced in gas1Δ haploid cells. Interestingly,
overexpression of these two genes also complemented the lysis and
morphogenetic defects of gas1Δ cells (Figure S4 and Fig. 7).
Together, these results indicate that a certain level of β(1,3)-
glucanosyl-transferase activity is required for proper morphogen-
esis and viability in fission yeast, regardless of the length of the
transferred oligosaccharide.

Discussion

Proteins of the GH72 family display glucanosyl-transferase activity. They are thought to be involved in β(1,3)-glucan remodeling and this activity is of great importance in fungal biology [16]. In S. pombe, 4 genes encoding GH72 proteins have been identified. We have previously characterized the gas4p function in S. pombe as a glucanosyl-transferase necessary for spore wall assembly and spore viability [27]. The other three genes are expressed during vegetative growth and their functions were investigated in this study.

The biochemical activity of GH72 proteins was first demonstrated for AfGel1p, and this was reported to catalyze in vitro a two-
step β(1,3)-glucanosyl transferase reaction: first, an internal glycosidic linkage of a donor β(1,3)-glucan chain is cleaved and the new reducing end is transferred to the non-reducing end of an acceptor β(1,3)-glucan chain through a β(1,3)-linkage, resulting in its elongation [19,38]. Our results indicate that S. pombe gaslp, gas2Δ and gas5Δ are also endowed with the same β(1,3)-glucanosyl transferase activity. Differences between these proteins were observed in the pattern of the products released. Recombinant
gasl p displayed the same β(1,3)-glucanosyl transferase activity as
those described for ScGas1p, ScGas4p, AfGel1p, AfGel2p and
ScGas4p [19,20,27,38,37], catalyzing the transfer of an oligosac-
charide of 6-8 glucose units, while the activity of gas5Δ was similar to
that described for ScGas2p [20], transferring oligosaccharides of
more than 4 monomers between glucan molecules. Finally, gas2Δ
preferentially used longer oligosaccharides as substrates (G20r
vs. G13r) and the oligosaccharides transferred from the donor
substrate to the acceptor molecule were also longer.

All yeasts and fungal species sequenced so far have members of this family in their genomes, and they usually form a redundant
family of enzymes [20]. Molecular and biochemical analysis of
gaslp, gas2Δ and gas5Δ during fission yeast vegetative growth has
revealed similarities and differences with S. cerevisiae, possibly
reflecting differences in the biology or variations in cell wall
composition between these two yeasts. In spite of their
homologous sequences and the similar enzymatic activities of the
encoded proteins, disruption of these three genes resulted in very
different phenotypes in S. pombe. gas2Δ and gas5Δ mutants had a
phenotype similar to that of wild-type cells, whereas gas1Δ mutant
and in the double
mutants have no apparent phenotype, while gel2Δ mutants have
severe growth defects and abnormal mycelial morphology and
GEL4 is essential [37], or for C. albicans, where PHR1 and PHR2 genes are differentially expressed depending on the extracellular pH [40,41,42].

A major result of this study is that the β(1,3)-glucanosyl transferase gas1p of S. pombe is an essential protein for vegetative growth in fission yeast. The structural organization of the β(1,3)-glucan of the cell wall is clearly altered in gas1Δ cells. Disruption of gas1 resulted in an abnormal β-glucan deposition and a reduction of the size of β(1,3)-glucan chains in the cell wall of S. pombe, in agreement with the biochemical function of these proteins. In S. cerevisiae, deletion of GAS1 also affects cell wall composition [21]. gas1Δ mutants have a decrease in β(1,3)-glucan levels that is compensated by an increase in mannan and chitin contents [24,25,43,44]. An interesting observation is that a decrease in β-glucan branching has also been observed in other gas mutants. A similar result was found for the S. cerevisiae gas1Δ mutant (Aimanianda et al., personal communication). Since no branching activity has been detected in any of the GH72 members in vitro, these observations suggested that the decrease in branching is a compensatory reaction to the Gas1 deletion as it has been seen also for chitin in other yeasts [25]. It also suggests that the synthase, the branching enzymes and the elongases could interact in the remodeling of the cell wall glucans. No such complex has yet been identified in fungi. This is the next challenge in the cell wall field and the data presented in this study show that S. pombe is an interesting model for understanding β-glucan synthesis and remodeling.

**Materials and Methods**

**Strains and growth conditions**

The S. pombe strains used in this study are listed in Table 1. Yeast cells were grown on YES media or minimal media (EMM) with appropriate supplements [45]. When required, sorbitol was added at final concentration 1.2 M. Synchronization of strains
genes complements the defects of the gas1 mutant. Percentage of isolated cells showing polarity or lytic defects with and without osmotic support. Wild-type fresh media with and without 1.2 M sorbitol and incubated at 32°C growing overnight in minimal media with sorbitol were inoculated in doi:10.1371/journal.pone.0014046.g007

Healthy isolated cells, and cells with polarity defects were calculated. 4 hours before the percentage of isolated lysed cells, pairs of lysed cells, and samples were taken every 20 minutes.

Table 1. Yeast strains used in this study.

| Strain   | Genotype                        | Source      |
|----------|--------------------------------|-------------|
| h20      | h+ leu1-32                      | Lab stock   |
| h116     | h+ leu1-32 ura4-Δ18 ade6-M216   | Lab stock   |
| h54      | h+ leu1-32 ura4-Δ18 ade6-M210   | Lab stock   |
| YMMR16   | h+ leu1-32 gas2Δ::kanMX4        | This study  |
| YMMR17   | h+ leu1-32 gas4Δ::kanMX4        | This study  |
| YMMR32   | h+ leu1-32 ura4-Δ18 ade6-M216 gas5Δ::kanMX4 | This study |
| YMMR37   | h+ leu1-32 ura4-Δ18 ade6-M216 gas5Δ::kanMX4 + pMMR18 | This study |
| YMMR40   | h+ leu1-32 gas2Δ::kanMX4 gas5Δ::kanMX4 (YMMR16 x YMMR32) | This study |
| YMMR41   | h+ leu1-32 ade6-M216 gas2Δ::kanMX4 gas5Δ::kanMX4 (YMMR16 x YMMR32) | This study |
| YMMR104  | h+/h- leu1-32 ura4-Δ18 ura4-Δ18 ade6-M210/ade6-M216 gas1Δ::kanMX4/gas1* | This study |
| YMMR105  | h- leu1-32 gas2-GFP              | This study  |
| YMMR106  | h+ leu1-32 ura4-Δ18 ade6-M216 gas1Δ::kanMX4 | This study |
| YMMR133  | h+ leu1-32 ura4-Δ18 ade6-M216 gas1Δ::kanMX4 + pC530 | This study |
| YMMR134  | h+ leu1-32 ura4-Δ18 ade6-M216gas1Δ::kanMX4 + pMMR39 | This study |
| YMMR135  | h+ leu1-32 ura4-Δ18 ade6-M216 + pC530 | This study |
| YMMR138  | h- leu1-32 ura4-Δ18 ade6-M216 gas1Δ::kanMX4 + pMMR41 | This study |
| YMMR139  | h+ leu1-32 ura4-Δ18 ade6-M216 gas1Δ::kanMX4 + pMMR42 | This study |
| YMMR140  | h+ leu1-32 ura4-Δ18 ade6-M216 gas1Δ::kanMX4 + pMMR43 | This study |

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Plasmid constructions and DNA manipulations

The oligonucleotides used in this study are listed in Table 2. Construction of plasmid pMMR15 carrying the gas1" coding sequence under its own promoter was achieved by PCR amplification of the coding sequence using oligonucleotides 1353 and 1392 that introduced BamHI and Xhol sites at the ends. The sequence was then cloned between the BamHI and XhoI sites of the pAL-KS vector. Plasmid pMMR39 (P3xmutergas2") was constructed by PCR amplification of the coding sequence of gas2" using primers 1427 and 1428 that introduced BamHI and XhoI at the ends, and cloning the resulting fragment between the BamHI and XhoI of the pJCR1-3XL vector [46]. The gas2" coding sequence was placed under the control of gas1" promoter (Pgas1) by PCR amplification of the gas1" promoter sequence with oligonucleotides 1431 and 1432 that introduced SphI and XhoI sites at the ends, and cloning the resulting fragment between the same sites of plasmid pMMR39 to generate plasmid pMMR43. Construction of plasmids pMMR41 (P3xmutergas2") and pMMR42 (P3xmutergas5") was achieved by PCR amplification of the coding sequence of gas2" and gas5" using specific primers 1433–1434 (that introduced XhoI and BamHI at the ends), and 1435–1436 (that created XhoI and NotI sites), respectively. These fragments were cloned into the pJCR1-3XL vector.

Construction of null mutants and strains

The coding sequence of gas1" corresponding to GH72 domain was replaced by the KanMX cassette using the recombinant PCR approach described by Wach [33]. For this purpose, DNA fragments corresponding to the 5' and 3' regions were PCR-amplified using oligonucleotides 1353-1354 and 1355-1356, respectively. The resulting fragments were fused by recombinant PCR to the KanMX cassette from plasmid pFA6a-KanMX4 [47]. The entire coding sequences of gas2" and gas5" were deleted to create the null mutants by replacing them by the KanMX cassette.

Figure 7. Over-expression of gas2" genes complements the defects of the gas1 mutant. Percentage of isolated cells showing polarity or lytic defects with and without osmotic support. Wild-type and gas1Δ cells overexpressing gas2", gas4" or gas5" that had been growing overnight in minimal media with sorbitol were inoculated in fresh media with and without 1.2 M sorbitol and incubated at 32°C for 4 hours before the percentage of isolated lysed cells, pairs of lysed cells, healthy isolated cells, and cells with polarity defects were calculated. doi:10.1371/journal.pone.0014046.g007
using the same approach. For gas2', oligonucleotides 1025, 1026, 1027 and 1028 were used; for gas5', the cassette was constructed using oligonucleotides 1047, 1048, 1049 and 1050. The N-terminally YFP-tagged gas1' fusion was constructed by PCR amplification of 570 and 210 bp DNA fragments with oligonucleotide pairs 1076–1140 and 1141–1142, respectively. The resulting fragments were fused by recombinant PCR to the YFP coding sequence obtained from plasmid pBS7 (Yeast Resource Center). The amplified fragment contained the YFP coding region fused in-frame to the 23rd codon of the gas1' gene and was then cloned at the HindIII sites of plasmid pMMR13, generating pMMR18. For C-terminal tagging of gas2p with GFP, the pFA6a-GFP(S65T)-KanMX6 plasmid [32] was used as a template to amplify the GFP-KanMX6 cassette flanked by gas2' homology regions with oligonucleotides 1371 and 1372.

### RNA isolation and Northern blot analyses

Total RNA was prepared from \(1.3 \times 10^9\) cells using the method described previously [27]. For Northern blot analyses, 12.5 µg of RNA was used. The DNA probes used were 400–500 bp internal

| Name | SEQUENCE |
|------|----------|
| 998  | CTTCCATCGTGAGCGTGTCTTT |
| 999  | ACCATGATCTCAGGAGCTTT |
| 1000 | ACTCTCTTCATCCTCTCTTCA |
| 1001 | AGACCGCGCTGCTCTT |
| 1004 | TGCTAATCATACTGAGAGAGTGGT |
| 1005 | AAGCAGAAGAGTTAAAAGAAGC |
| 1025 | TAAGCAGCGCGCTATCGTATTACGTTGG |
| 1026 | GGGATCCGTCGACCTGCAGCGTACGTAAATTTGGTAAAGGAGCATG |
| 1027 | AACGAGCTCGAATTATCGATGATTGTTGGTGAGCTTAAGGCATAATTTCC |
| 1028 | AAAACTATTTGTCGATGAACTATAGGTTG |
| 1032 | TAAACTCGAGCGAATATATCAATAATGAACTTGAGAAG |
| 1047 | AAAGACTGCGAATGTTTCTT |
| 1048 | GGGATCCGTCGACCTGCAGCGTACGAGGAAGTTCATAGCAGATATAGGTTC |
| 1049 | AACGAGCTCGAATTATCGATGATTGTTGGTGAGCTTAAGGCATAATTTCC |
| 1050 | TAAACTCGAGCGAATATATCAATAATGAACTTGAGAAG |
| 1076 | GCTTCTTCACCTGATGAAATGAAAGGAGGACGAGTTCC |
| 1140 | TAAATCTAGAACCGCAGGTGATGAACTATACAAAGCTACAGTATGGAAGAG |
| 1141 | GGGATTACACATGCGATGAACTATACAAAGCTACAGTATGGAAGAG |
| 1142 | AGTAGGATCAGAGCTGAAAA |
| 1259 | GAAGCTGAAATTCTCAGTTTCTCTT |
| 1260 | GCTGCGGCCGCCGCTGCTCTT |
| 1263 | GAAGCTGAAATTCTCAGTTTCTCTT |
| 1264 | GTTCTTATGAAAGTTAAAGAAGGACGAGTTCC |
| 1333 | ATAGGATCAGAGCTGAAAA |
| 1354 | GGGATCCGTCGACCTGCAGCGTACGAGGAAGTTCATAGCAGATATAGGTTC |
| 1355 | AACGAGCTGAAATTCTCAGTTTCTCTT |
| 1356 | ATAGGATCAGAGCTGAAAA |
| 1371 | TACACTCAGGATCAGTGCGATGCGCTCAGT |
| 1372 | TTACACATGAACTGATGAGCAGGGAGTTTACCTTTCTCAGGAGAGG |
| 1386 | TTGTCTTTCGACCTGACAGGACGAGT |
| 1395 | GAAGCTGAAATTCTCAGTTTCTCTT |
| 1427 | AGTCTGCGGAGAGAGGAGAGGAGAG |
| 1428 | AGCTGCGGCCGCCGCTGCTCTT |
| 1431 | CTGATGGGTTTCTGATGAGCAGGAG |
| 1432 | TCCCTGAGGTTAAACAGAAAGAGGAGTTAAAAAG |
| 1433 | AGCTTGGAGAACAGAAAAAGGAGAGGAG |
| 1434 | AGCTGCGGCCGCCGCTGCTCTT |
| 1435 | AGCTTGGAGAACAGAAAAAGGAGAGGAG |
| 1436 | AGCTGCGGCCGCCGCTGCTCTT |
fragments amplified by PCR with oligonucleotides 1000–1001 for gas1*, 998–999 for gas2* and 1004–1005 for gas3*, or a 1.7 kb BowII-HindIII fragment obtained from plasmid pSK-ura4* for gas4*. Probes were labeled using the random priming method.

Microscopy techniques

For light microscopy, the β(1,3)-glucan of the cell wall was stained with aniline blue [34]. Samples were viewed using a Leica DMRXA microscope equipped for bright-field and Nomarski optics and epifluorescence and photographed with a Leica DFC325 FX camera or an ORCA-ER camera. To determine the percentage of cell lysis, cells were stained with 0.6% methylene blue. Dead cells accumulate the dye and appear dark on a phase contrast microscope.

Cell wall analysis

Fractionation of cell wall polysaccharides was achieved as follows. A portion of 200 mg of dry cell walls was treated twice with 10 ml of 25 mM Tris-HCl, 5 mM EDTA, pH 7.4, 2% SDS and 40 mM β-mercaptoethanol at 100°C for 10 minutes. Insoluble materials were recovered by centrifugation (10 minutes, 3000 g) and washed extensively with water. Alkali-soluble materials were extracted twice with 1M NaOH at 65°C for 1 hour. Alkali-insoluble materials were removed by centrifugation (10 minutes, 3000 g) and washed extensively with water. Alkali-soluble fractions were neutralized by the addition of acetic acid and then freeze-dried. Precipitates were recovered by centrifugation (10 minutes, 3000 g), dialyzed against water, and freeze-dried. Protein amounts were estimated using the BCA reagent (Pierce). Total hexose contents were estimated using the phenolsulfuric acid method [46]. Determination of α (1,3)-glucan and β(1,3)-glucan content was achieved by digestion of the cell wall material with specific glucanases (enzymatic unit: 1 μmol Glc equiv/min). In the case of β(1,3)-glucan, 20 μl of cell wall extracts (5 mg/ml) was incubated in 100 μl of 100 mM NaOAc, pH 6.2, containing 0.4 units of laminarinase A (from Anaeromyces elegans, expressed in E. coli, and a kind gift from Dr. Vladimir V. Zverlov, Institute of Molecular Genetics, Moscow, Russian Federation) at 37°C for 6 hours [49]. Glucon contents were estimated as the amount of reducing sugars released in each treatment by the PAHBAH method [30].

Estimation of β(1,3)-glucan branching

The branching point of β(1,3)-glucan is resistant to laminarinase A digestion, resulting in a specific trisaccharide that can be measured by HPAEC. Cell wall fractions were subjected to laminarinase A digestion as described above and the products were analyzed with HPAEC on a CarboPAC-PA200 column (4.6 × 250 mm, Dionex), as described [39]. Branching was correlated to the presence of a specific peak absent when a linear β(1,3)-glucan was digested.

Gel filtration chromatography

The molecular size of cell wall polysaccharides was estimated by gel filtration chromatography. Cell wall fractions were carboxymethylated to solubilise the polymers. 10 to 20 mg of cell walls was suspended in 1 ml of 1.6 N NaOH. After the addition of 1 ml monochloroacetic acid (MCA, 0.3 mg/ml, pH 10–11 by addition of NaOH), samples were incubated at 75°C for 3 hours. Then, another 2 ml of MCA were added and the samples were incubated at 75°C overnight. After neutralization by the addition of acetic acid, samples were dialyzed against water and freeze-dried. Carboxymethylated fractions were dissolved in 50 mM NaOH, and 250 mM AcONa at a final concentration of 10 mg/ml, and 300 μl were loaded onto a HR500 gel filtration column (GE Healthcare, 50 × 1.6 cm) and eluted at a flow rate of 0.5 ml/min. Sugars were detected with the phenolsulfuric acid method [40].

Expression of recombinant gas proteins in Pichia pastoris

P. pastoris KM71H (Invitrogen) and the expression vectors pPICZαA and pPICZαB were used to express recombinant proteins. Truncated forms of gas1* and gas3* were generated by PCR-amplification of the gene with specific oligonucleotide pairs. gas1* was amplified with primers 1259 and 1260, which created EcoRI and NotI sites at the ends. For gas3*, the primers were 1263 and 1264, which incorporated PstI and XhoI sites at the ends. The gas1* PCR product was digested by EcoRI and NotI and cloned into the vector pPICZαA, generating plasmid pMMR22. The gas3* PCR product was digested with PstI and XhoI and cloned into the pPICZαB vector to obtain plasmid pMMR24. A gas2* recombinant form lacking the last 27 nucleotides was generated by PCR amplification of the gene with oligonucleotides 1395 and 1386. The forward primer introduced a 6xHis coding sequence and incorporated an EcoRI site at the 5’ end. The reverse primer created a XhoI site at the 3’ end. The PCR product was digested by EcoRI and XhoI and cloned into pPICZαA, generating plasmid pMMR35. The transformation and production of the recombinant proteins was performed according to manufacturer’s instructions (Invitrogen).

Enzymatic analysis of recombinant proteins

Culture filtrates were concentrated using Amicon Ultra Centrifugation Devices (Amicon) and dialyzed against Bind-Buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM imidazole). Then, they were incubated with Ni-NTA His-Bind Resin (Novagen), loaded into a column, and the flow-through was discarded. The column was washed twice with Wash Buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 20 mM imidazole) and the recombinant proteins were eluted with Elution buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 250 mM imidazole) in four fractions, which were analyzed by SDS-PAGE. Purified recombinant proteins were incubated at concentrations of 0.18, 0.95 and 0.53 mg/ml of protein for gas1p, gas2p and gas3p, respectively, with 4 mM of reduced laminari-oligosaccharides (G1u or G2mu) in 20 μl of 50 mM NaOAc, pH 5.0, at 37°C. Sequential aliquots of 2.5 μl supplemented with 40 μl of 50 mM NaOH were analyzed by HPAEC with a CarboPAC-PA200 column (Dionex, 3.2 × 250 mm), as previously described [39].

Supporting Information

Figure S1 Characteristics of S. pombe GH72 proteins. (A) Summary of the main characteristics of S. pombe GH72 proteins. (B) gas1p, gas4p and gas5p contain a hydrophobic signal at the C-termini for GPI-attachment. Hydrophobicity profile of gas1p, gas2p, gas4p and gas5p. The hydrophobic regions at the C-termini are highlighted in grey. To the right, the cleavage points for GPI attachment proposed by GPI-SOM are shown by an arrowhead. Found at: doi:10.1371/journal.pone.0014046.s002 (1.08 MB TIF)

Figure S2 Time-lapse microscopic analysis of the gas1Δ mutant. gas1Δ cells grown in liquid media with osmotic support were inoculated on YES solid medium and observed under a microscope equipped for Nomarski optics. Images were captured every minute. Growth, polarity defects and cell lysis along the time are shown. Black arrowheads point to cell lysis at each time point. Asterisks highlight cells with defects in polarity. Scale bars, 10 μm. Found at: doi:10.1371/journal.pone.0014046.s002 (1.08 MB TIF)
Figure S3 (A) β[1,3]-glucanosyl-transferase activity of recombinant gas1p, gas2p and gas5p using G13r as substrate. Reactions were incubated in 50 mM acetate buffer (pH 5.0) for the indicated times. The reaction products were analyzed by HPAEC-PED on a CarboPAC-PA200 column. (B) S. pombe cell walls were extracted with 1 M NaOH to separate alkali-soluble materials (AS) from the alkali-insoluble (AI) by centrifugation. The amount of each fraction was estimated by colorimetric assays of total proteins and hexoses. The percentage corresponds to the fraction of hexoses in each fraction relative to the total amount of hexoses detected in the dried cell walls. (C) Gel filtration chromatography of carboxymethylated cell wall fractions on an HR500s column. An aliquot of the different fractions was digested by Laminarinase-A before the carboxymethylation and was compared with the untreated control. Sugars were detected with the phenol-sulphuric assay.

Found at: doi:10.1371/journal.pone.0014046.s003 (0.16 MB TIF)

Figure S4 Over-expression of gas+ genes complements the defects of the S. pombe gas1 mutant. (A) Growth rate of gas+ strain carrying the vector (YMMR135) and gas1A cells harbouring vector (YMMR133), Pnmt1-gas2+ (YMMR139) or Pnmt1-gas5+ (YMMR139), in media with (right) and without (left) sorbitol. (B) Microscopic appearance of gas1A cells lacking ectopically expressing gas2+ or gas5+. Wild-type cells and gas1A strains harboring the different constructs were incubated in minimal media with or without osmotic support. Samples were stained directly with aniline blue before images were captured. Differential interference contrast (DIC) or fluorescence (AB) photographs are shown. Scale bars, 10 μm.

Found at: doi:10.1371/journal.pone.0014046.s004 (1.73 MB TIF)

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Author Contributions

Conceived and designed the experiments: MdMR FD r JPL CRVdA. Performed the experiments: MdMR YAP CC TF. Analyzed the data: MdMR CC FrD CVdA. Contributed reagents/materials/analysis tools: YAP TF. Wrote the paper: MdMR TF FrD JPL CVdA.

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Fission Yeast GH72 Proteins

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