

Identification of two cell cycle regulated genes affecting the β 1,3-glucan content of cell walls in *Saccharomyces cerevisiae*

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Abstract The Calcofluor white-hypersensitive mutants *cwh52* and *cwh53* are severely reduced in β 1,3-glucan. *CWH52* was equivalent to *GAS1*. *CWH53* represented a new gene, located on the right arm of chromosome XII, and predicted to encode a 215 kDa protein with multiple transmembrane domains. The transcription of *CWH53* was cell cycle-dependent and, similar to *GAS1*, *CWH52*, increased in late G₁, indicating that the formation of β -glucan is cell cycle-regulated. Further, in some mutant alleles of both *gas1/cwh52* and *cwh53* lethal concentrations of Calcofluor induced growth arrest at a specific phase of the cell cycle.

Key words: *GAS1*; *GGPI*; Calcofluor white; Cell wall; Cell cycle arrest; β -Glucan

1. Introduction

The cell wall of *Saccharomyces cerevisiae* is composed of equal amounts of mannoproteins and β -glucan, and a small amount of chitin [1,2]. Mannoproteins limit the permeability of the cell wall thereby retaining periplasmic enzymes and protecting the cell against degradation by enzymes from other organisms [3,4]. β -Glucans play an important role in maintaining the osmotic stability of the yeast cell. Eighty percent of cell wall β -glucan consist of β 1,3-linked glucose residues, and the remaining twenty percent are accounted for by β 1,6-linked glucose residues [1]. The synthesis of β -glucan is carried out by an enzyme complex in the plasma membrane and requires UDP-glucose as substrate and GTP as activator [5]. The active site of the enzyme is at the cytosolic side of the plasma membrane [6], and the nascent glucan chain is therefore believed to be transported to the outside of the plasma membrane, possibly by a channel-like protein.

The presence of a GTP-binding protein in the β -glucan synthase complex suggests that the synthesis of β -glucan is strongly regulated. Indeed, it has been shown that the rate of β -glucan synthesis varies during the cell cycle [7]. In addition, it has been shown autoradiographically that in budding cells glucan (and mannan) are mainly deposited in the wall of the growing bud and not in the wall of the mother cell [8,9], suggesting that when a new bud is formed, the β -glucan synthase activity in the mother cell is rapidly turned off. Recently, we developed a

general method to isolate cell wall mutants, which is based on the disordering effect of Calcofluor white (CFW) on cell wall structure [10]. In this paper, we describe the identification of two genes (*CWH52* and *CWH53*) that are cell-cycle regulated and are involved in the formation of β (1–3)-glucan. In addition, we present evidence that their function is specifically needed during the first stages of bud growth.

2. Materials and methods

2.1. Yeast strains, bacterial strains, and growth media

Saccharomyces cerevisiae AR27 (*MATa ura3-52*) and AR49 (*MATa lys2*) were used as reference strains [10]. Growth conditions and growth media for yeast were as described [10]. Yeast was transformed by the lithium acetate method [11]. Hypersensitivity to CFW was determined as described previously [10]. For screening of hypersensitivity to CFW on SD-plates the medium was buffered to pH 6.0 with 50 mM MES. K2944 (*MATa bar1::URA3 cdc15-2*) was used for synchronization experiments. *Hansenula mrakii* killer strain IFO 0897 was kindly provided by Dr. Beth DiDomenico. *Saccharomyces cerevisiae* killer strain A4772-15D (*MATa ade2-1 his4-15 p⁺[KIL-k]*) was obtained from Cold Spring Harbor Laboratory. *Escherichia coli* DH5 α was used for propagation of all plasmids.

2.2. Plasmids, DNA purification, and recombinant DNA techniques

The YCp50-based yeast genomic library [12] was used to clone the *CWH52* and *CWH53* genes. YCplac33 [13] was used for subcloning experiments. Plasmid DNA was prepared from *E. coli* as described [14]. Yeast DNA was isolated by the method of Hoffman and Winston [15]. Restriction endonucleases, Klenow and T4 DNA polymerases, alkaline phosphatase, and T4 ligase were from Pharmacia, and were used according to the instructions of the manufacturer. DNA fragments were isolated from agarose gels using a GeneClean II kit (Bio 101, La Jolla, CA).

2.3. Analysis of *CWH53*

The *CWH53* DNA sequence was determined by a combination of nested deletions and oligo primer walking. Subclones of *CWH53* were inserted into YCplac33, and nested deletions were constructed using the Erase-a-base system (Promega). Oligonucleotide primers for sequencing were obtained from Eurogentec (Seraing, Belgium). The nucleotide sequence of both DNA strands was determined by the dideoxy-chain termination method [16] using T7 DNA polymerase (Pharmacia) and [α -³⁵S]dATP (Amersham) as a substrate. The *CWH53* sequence was deposited in the EMBL data base with the Accession Number X80817. To physically map *CWH53*, a chromosomal blot of *S. cerevisiae* was used (Clontech, Palo Alto, CA). Specific DNA probes were randomly labeled using [α -³²P]dATP (Amersham) as a substrate [17]. The *CWH53* probe was also hybridized to the λ phage library of mapped yeast genomic DNA inserts [18], obtained from ATCC.

2.4. Synchronization procedure and Northern analysis

Synchronous division of *cdc15-2* cells was induced as described [19]. Samples for Northern analysis were withdrawn every 12 min. Procedures for mRNA analysis were as described previously [20]. DNA restriction fragments for probing were excised from low melting point agarose and labeled by random-prime labeling using a Prime-It kit (Stratagene). For *CWH53* a 2.2-kb *EcoRI* fragment, and for *CWH52*

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Abbreviations: CFW, Calcofluor white; SCB, *SWI4*,6-dependent cell cycle box; MCB, *MluI* cell cycle box; MES, 2-[*N*-morpholino]-ethanesulfonic acid; GPI-anchor, glycosylphosphatidylinositol anchor; ConA, concanavalin A; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

a 1.3-kb *SalI*–*XhoI* fragment were used, both containing parts of the open reading frames. For *H2A*/protein 1 and *CLN2* transcript analysis, restriction fragments were used as described previously [19].

2.5. Cell wall analysis

Isolation of cell walls from logarithmically growing yeast cells, and determination of their sugar composition by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) were performed as described previously [10]. Resistance to HM-1 killer toxin was determined by a seeded plate assay, using 2% Bacto Agar and YPD medium containing 0.003% methylene blue and 0.1 M sodium citrate buffer, pH 4.3. Five ml of cell suspension of a stationary phase culture of *Hansenula mrakii* killer strain IFO 0897 were spotted on a seeded plate and incubated at 19°C for 2–3 days. Sensitive strains showed a halo of no-growth surrounded by a blue zone of dead cells. Resistance to K1 killer toxin was assayed as described [21].

β 1,3-Glucan synthase activities were determined as outlined [22], and modified as described [23]. Cell extracts were prepared as described [23]. Protein concentrations were estimated with the BCA protein assay reagent from Pierce.

2.6. Fluorescence microscopy

Cells were grown overnight in YPD in the presence of lethal concentrations of CFW (50 μ g/ml), washed twice with PBS, and incubated in ConA-FITC (0.25 mg/ml PBS). After 2 h, cells were again washed twice with PBS and examined using an Olympus (BH2-RFC) fluorescence microscope equipped with a 100 W Mercury Arc lamp (SPlan 100PL 1.25 oil-immersion lens) in combination with a cooled CCD camera (Astromed TE3/W; Cambridge, UK). A Hewlett Packard/Apollo 425 series workstation with Scilimage software [24] was used for image processing. Photographs were taken directly from the computer screen using Kodak EPN 100 ASA slide films.

3. Results

3.1. *cwh52* and *cwh53* have reduced levels of β -glucan

cwh52 and *cwh53* were isolated as Calcofluor white hypersensitive cell wall mutants [10]. Three different alleles of *cwh52*, and four alleles of *cwh53* were isolated. Since *cwh52* and *cwh53* did not complement each other completely in a heterozygote diploid, complementation groups were assigned by crossing the different mutants to each other followed by tetrad analysis. Further characterization showed that all mutant alleles were reduced in cell wall glucose (Table 1). This was further investigated by determining the sensitivity of the mutant cells to killer toxins. All mutants remained sensitive to K1 killer toxin which specifically binds to β 1,6-glucan [25,26] indicating that the levels of β 1,6-glucan were unaffected (Table 1). *Hansenula mrakii* killer toxin HM-1 binds to both β -glucan and β 1,6-glucan [27,28]. Table 1 shows that *cwh53-1* and *cwh52-2* had become more resistant to HM-1 toxin, whereas *cwh52-3* remained sensitive; *cwh53-1*, *cwh53-2*, and *cwh53-3* were also resistant, whereas *cwh53-4*, the weakest allele, was only partially resis-

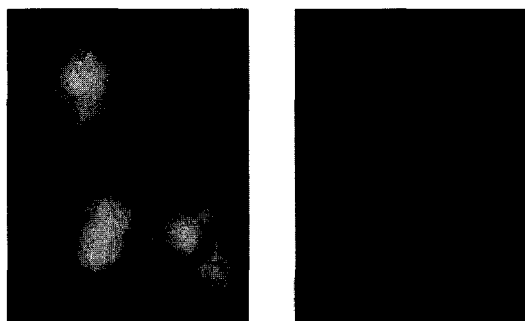


Fig. 1. Terminal phenotype of *cwh52* (left) and *cwh53* (right) grown in the presence of lethal concentrations CFW. Cells were stained with ConA-FITC and observed using a fluorescence microscope.

tant. These observations indicate that in both *cwh52* and *cwh53* the β -glucan portion of the cell wall is affected. In order to determine whether *CWH52* and *CWH53* were directly involved in the synthesis of β -glucan, we determined in vitro activities of β -glucan synthase. No differences were found in the in vitro activity of β -glucan synthase for wild type cells and *cwh53-1* and *cwh53-1* (data not shown) suggesting that *CWH52* and *CWH53* play an indirect role in the formation of β -glucan.

3.2. Morphology of *cwh52* and *cwh53* mutants

Since β -glucan is important for the mechanical strength and the shape of the cell, the cell morphology was examined. *cwh52* and *cwh53* cells were slightly more spherical and larger than wild type cells, but no striking morphological defects were observed. Also, staining with CFW was normal, except that the overall staining was more intense. When the terminal phenotypes of *cwh52* and *cwh53* mutants were analysed in the presence of CFW, we observed that *cwh53-1* and *cwh52-2*, and also *cwh53-1* and *cwh53-2*, showed a cell cycle-specific growth arrest. Whereas wild type cells stopped growing in all phases of the cell cycle, about 75% of the mutant cells stopped growing after having formed a small bud (small-bud phase) (Fig. 1). This indicates that the functions of *CWH53* and *CWH52* are required at that particular phase of the cell cycle.

3.3. *CWH52* is identical to GAS1/GPP1

The *CWH52* gene was cloned by complementation of the CFW hypersensitive phenotype of *cwh52-2*. Out of 90,000 transformants, seven transformants grew on plates containing 1.5 mg CFW/ml. All seven plasmids were isolated, and were able to restore the CFW hypersensitivity of all three *cwh52*

Table 1
Phenotypic characteristics of *cwh52* and *cwh53* mutant alleles

| Strain | Carbohydrate composition of the cell wall (%) | | | Glc/Man (Mean \pm S.E.M.)* | K1 killer resistance | HM-1 killer resistance |
|----------------|---|------|------|---------------------------------|-------------------------|---------------------------|
| | GN | Glc | Man | | | |
| AR27 | 1.1 | 49.8 | 49.1 | 1.014 \pm 0.077 (n = 8) | S | S |
| <i>cwh52-1</i> | 3.9 | 30.7 | 65.4 | 0.469 \pm 0.031 (n = 2) | S | R |
| <i>cwh52-2</i> | 2.7 | 32.7 | 64.6 | 0.506 \pm 0.033 (n = 2) | S | \pm R |
| <i>cwh52-3</i> | 1.8 | 45.0 | 53.2 | 0.846 \pm 0.049 (n = 2) | S | S |
| <i>cwh53-1</i> | 3.3 | 19.7 | 77.0 | 0.256 \pm 0.001 (n = 2) | S | R |
| <i>cwh53-2</i> | 2.9 | 20.4 | 76.7 | 0.266 \pm 0.028 (n = 2) | S | R |
| <i>cwh53-3</i> | 1.9 | 31.8 | 66.3 | 0.480 \pm 0.024 (n = 2) | S | R |
| <i>cwh53-4</i> | 1.6 | 36.4 | 62.0 | 0.587 \pm 0.008 (n = 2) | S | \pm R |

* n = number of observations. S, sensitive; R, resistant

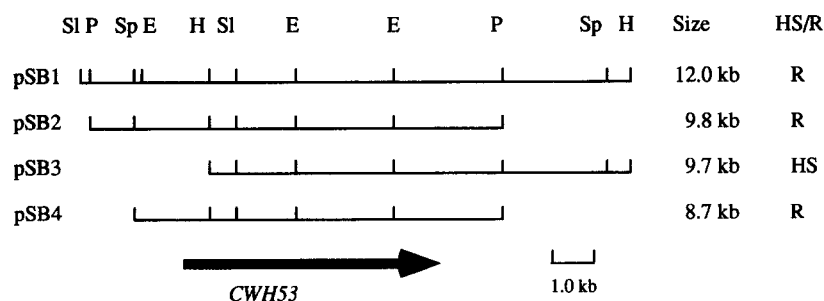


Fig. 2. Partial restriction map and subcloning of *CWH53*. The location of *CWH53* is indicated by the black line, and the direction is shown by the arrow. Subclones of the original genomic fragment were tested for their ability to complement the *cwh53* mutants. R, resistant to CFW; HS, hypersensitive to CFW. The results are shown together with the size of the genomic insert on the right. Restriction sites are mapped as follows: SI, *SalI*; P, *PstI*; Sp, *SphI*; E, *EcoRI*; H, *HindIII*.

mutants after retransformation. Restriction analysis showed that four different plasmids had been isolated with a 8.0-kb overlapping region.

Several lines of evidence indicated that the *CWH52* gene is identical to *GAS1/GPP1*, which encodes a 125-kDa glycoprotein anchored to the plasma membrane by a GPI anchor [29,30]. First, the restriction map of *CWH52* was the same as *GAS1*, except for the *XbaI* site, which is missing in our subclone. Second, partial DNA sequences derived from *CWH52* subclones were identical to the *GAS1/GPP1* sequence. Third, *GAS1/GPP1* is located on Chromosome XIII [31]. *CWH52* was also mapped onto chromosome XIII by probing a chromosome blot with a 1.1-kb *EcoRI* fragment (containing part of the open reading frame), and further onto λ clone 4,047 of the λ phage library of mapped yeast genomic DNA inserts which is located on the right arm of chromosome XIII. Fourth, *cwh53-1* and *cwh52-2* showed an incomplete separation of mother and daughter cell, as also described for *gas1/gpp1* mutants [32]. Thus, it appears that *CWH52* is identical to *GAS1* for the remainder of this paper. In the promoter region of *GAS1*, three perfect matches of *SWI4*-6-dependent cell cycle boxes (SCB) (consensus sequence: 5'-(C/A)(A/G/T)CGAAA-3'; [33]) were found at positions -749, -358, -314, respectively. In addition, two other possible cell cycle boxes were found at positions -771 and -116 which had one mismatch to the consensus sequence. It has indeed been shown that the transcription level of *GAS1* varies during the cell cycle with a maximum at the onset of budding [34]. It is therefore likely that these SCB elements play an important role in the transcription regulation of *GAS1*.

3.4. Isolation of the *CWH53* gene

The *CWH53* gene was also isolated by complementation of the hypersensitivity of *cwh53-1* to CFW. Approximately 45,000 *Ura*⁺ transformants were screened for CFW resistance on plates containing 1.5 mg CFW/ml. Four colonies grew like wild type. Restriction analysis showed that all four transformants contained the same plasmid which was able to complement the *cwh53-1* defect after retransformation. The three other *cwh53* alleles were also complemented by this plasmid. The complementing plasmid contained a 12-kb insert, and a restriction map of this fragment was generated (Fig. 2). Subcloning showed that the smallest complementing fragment was the 8.7-kb *SphI*–*PstI* subclone. *CWH53* was mapped onto chromosome XII by probing a chromosome blot with a 2.4-kb *SalI* fragment, and onto

λ clones 2019, 6165, and 3925 of the λ phage library of mapped yeast genomic DNA inserts, which are also located on chromosome XII.

3.5. Sequence of *CWH53*

The subcloning experiments indicated an *HindIII* site in the functional region of *CWH53* (Fig. 2), and the DNA sequence in this region was obtained. This revealed a continuous open reading frame of 5,628 nucleotides, spanning the *HindIII* site. The *CWH53* sequence predicts a gene encoding a protein of 1,876 amino acids with a molecular mass of 214.8 kDa (Fig. 3). Structurally, *CWH53* seems to be a transmembrane protein. Both the N-terminal and the C-terminal half contain multiple putative transmembrane domains separated from each other by a large, more hydrophilic domain (Fig. 4). Ten potential sites for N-glycosylation were found. In the promoter region of *CWH53*, five SCBs were found at positions -1112, -743, -598, -500, and -395, respectively. Two *MluI* cell cycle boxes (MCB) (consensus sequence: 5'-ACGCGT-3'; [36]) were found at positions -636 and -582, suggesting that transcription of *CWH53* is cell cycle coordinated and might be induced at the G₁/S boundary.

3.6. *CWH53* and *GAS1* transcripts are cell cycle regulated

Since the 5' upstream regions of both *CWH52* and *CWH53* contained cell cycle regulatory elements, the transcript levels of *CWH53* and *GAS1* were compared and monitored during the cell cycle (Fig. 5). By temperature shift to 37° followed by incubation at 25°C, *MATa bar1::URA3 cdc15-2* cells were synchronized. Samples for Northern analysis were taken every 12 min. *H2A* is transcribed throughout the S-phase and therefore its transcript served as an indicator of the beginning and the end of the S-phase. Protein1 transcript levels do not vary during the cell cycle and were used as a loading control. *CLN2* transcription is strongly cell cycle regulated, peaking at about START and, therefore, *CLN2* transcription serves as a indicator of late G₁. *CWH53* and *GAS1* were cell cycle regulated and showed similar expression patterns; maximal levels for both were observed in late-G₁/S-phase cells.

4. Discussion

Several lines of evidence indicate that *CWH53* and *CWH52* affect the β -glucan content of the cell wall. First, the cell walls of *cwh52* and *cwh53* mutants show a decrease in the amount of

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10      20      30      40      50      60
MNTDQPPYQG QTDYTGPGN GQSSEQDYDQ YGQPLYPQA DGYDPNVAA GTEADMYGQ

70      80      90      100     110     120
PPNESYDQDY TNGEYYPQP NMAAQDGENE SDFSSYGGP TPGYDSYGG YTASQMSYGE

130     140     150     160     170     180
PNSSGTSTPI YGNYDPNAJA MALPNEPYPA WTADSQSPVS IEQIEDIFID LTNRLGFRD

190     200     210     220     230     240
SMRNMFDHFM VLLDSRSSRN SPDQALLSLH ADYIGGDAN YKKWYFAQL DMDDEIGFRN

250     260     270     280     290     300
MSLGKLSRKA RKAKKKNKKA MEEANPEDE ETLNKIEGDN SLEAADFRNK AKMNQLSPLE

310     320     330     340     350     360
RVRHIALYLL CWGEANQVRP TAECLCFIYK CALDYLDSP CQQRQEPMP GDFLNRVITP

370     380     390     400     410     420
IYHFIRNQYV EIVDGRFVKR ERDHNKIVGY DDLNQLFWYP EGIKIVLED GTKLIELPLE

430     440     450     460     470     480
ERYLRLGDVW WDDVFFKTVL ETRTWLHLVT NENRIYVYHI SIFMYEAYN SPTFYTHNYQ

490     500     510     520     530     540
QLVDNQPLAA YKMWSCALGG TVASLTQIVA TLCEWSFVPR KWAGAQLSR REWELCTIEG

550     560     570     580     590     600
INLGIPIEYF AYDKDTIYFV AAHVVAAYFV EYAVATITFE SIMPLGGLET SYMKKSTRY

610     620     630     640     650     660
VASQTFTAFA APLHGLDRWM SYLVWMTVFA AKYSESYFL VLSLRDPIRI LSTTAMRCTG

670     680     690     700     710     720
EYMWGAVLCK VQPKIVLGLV IATDFILHVT DTYLWYTIYN TIESVGKSEY LGISILTPWR

730     740     750     760     770     780
NIFTRLPKRI YSKILATTDN EIKYKPKVLI SQVWNAIIS MYREHLAID HVQKLLYHQV

790     800     810     820     830     840
PSEIEGKRTL RAPTFFVSQD DNNFETEFFR RDSEAEARRIS FFAQLSTPI PEPLPVDNMP

850     860     870     880     890     900
TFTVLTPHYA ERILLSLREI IREDDQFSRV TLLEYLQLH PVWECEFKVD TKILAEEATA

910     920     930     940     950     960
YEGNEAEAEK EDALKSQIDD LPFYCIGFKS AAPEYTLRTR IWASLSRQTL YRTISGFMNY

970     980     990     1000    1010    1020
SRAIKLLYRV ENPEIVQMFG GNAEGLEREL EKMAARRKFK LVSMQRLAKF KPHELENAEF

1030    1040    1050    1060    1070    1080
LLRAYPDLQI AYLDDEEPLT EGEPEPIYSA LIDGHCEILD NGRRRPKFRV QLSGNPDLGD

1090    1100    1110    1120    1130    1140
GKSDNQNHAI IFYRGYIQL IDANQDNYLE ECLKIRSVLA EFEELNVEQV NPYAPGLRYE

1150    1160    1170    1180    1190    1200
EQTNHNPVAI VGAREYIFSE NSGVLGDVAA GKEQTFGLTF ARTLSQIGGK LHYGHPDFIN

1210    1220    1230    1240    1250    1260
ATFMTTRGGV SKAQKGLHLN EDIYAGNMAM LRGGRKHKCE YYQCGKGRDL GFGTILNETT

1270    1280    1290    1300    1310    1320
KIGAGMGEMQ LSREYVYLTG QLPVDRGLTF YYAHGFEHLN NLEIQLSLOM FMLTIVNLSS

1330    1340    1350    1360    1370    1380
LAHESIMCIY DRNPKPTDVL VPICGYNFOP AVDWVRRYTL SIFIVETIAE VPIVQDELIE

1390    1400    1410    1420    1430    1440
RGLMKATQRE ECHILLSLSPM EEVEAGQIYS SALLSOLAIG GARYISTGRG FATSRIPESI

1450    1460    1470    1480    1490    1500
LYSREAGSAT YMGARSMLML LFGTVAHMQA PLLWFWASLS SLTFAPEYFN PHQFAWEDFF

1510    1520    1530    1540    1550    1560
LDYRDYRWLW SRGNMQYHRN SWIGVVRMSR ARITGFKRKL VGDESEKAAG DASRAHRTNL

1570    1580    1590    1600    1610    1620
TMAETPCATL YAAGCEIAET ETNAQTGVKT TDDRVNSYVL RTIICTIAPT AVNLGVLEFC

1630    1640    1650    1660    1670    1680
MGMSCCSGPL FGMCKCKTGS VMAGIAHGVA VIVHIAFFIV MYVLESENFV RMLIGVVTCTI

1690    1700    1710    1720    1730    1740
QCQRLIFHCM TALMLTREFK NDHANTAFWT GKWYKGMGY MAWTPQSREL TAKVIELSEE

1750    1760    1770    1780    1790    1800
AADEVLGHVI LICQLPLIII PKIDKEHSIM LFWLKPSRQT RPPIYSLKQT RLRKRMVKKY

1810    1820    1830    1840    1850    1860
CSLYFLVLA FAGCIIGPAV ASAKIKHKIG DSDLGVVHNL FQPIINTINND TGSQMSTYQS

1870
HYTHTPSLK TWSTIK*

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Fig. 3. Predicted amino acid sequence of the CWH53 product, Cwh53p. The underlined residues represent putative transmembrane domains. Potential sites for asparagine-linked glycosylation are also underlined. The nucleotide sequence of CWH53 was deposited in the EMBL data bank with Accession Number X80817.

glucan relative to both mannan and chitin indicating that the β -glucan levels were reduced. This is in agreement with the observation that the more stronger *cwh53* and *cwh52* mutant

alleles are resistant to HM-1 killer toxin. Second, as only 20% of the total glucan is β 1,6-linked, the decrease in glucan of 75% in both *cwh53-1* and *cwh53-2*, and the 50% reduction in *cwh53-3*, *cwh53-1*, and *cwh52-2*, can not be explained by a reduction in the levels of β 1,6-glucan alone. In fact, it is unlikely that the levels of β 1,6-glucan were reduced because *cwh53* and *cwh52* mutants showed wild-type sensitivity to the K1 killer toxin, which specifically requires β 1,6-glucan for its action.

Although the GAS1/GPP1 protein has been studied extensively, no precise function for the gene product has been reported. Morphological analysis of the *gas1* null mutants showed no appreciable differences from wild type cells except that loss of *GAS1* function resulted in defective separation of the bud from the mother cell. It also led to increased resistance to Zymolyase during exponential growth [32]. Here we report a complete new phenotype for *GAS1/CWH52*, showing that it strongly affects the levels of β 1,3-glucan. This probably explains why *gas1/cwh52* mutants are hypersensitive to Calcofluor white. The strength of the cell wall depends on the integrity of the glucan-chitin complex in the wall. In *gas1/cwh52* mutants the strength of the cell wall is impaired by both a strong decrease in β 1,3-glucan and by the effect of Calcofluor on the formation of chitin fibrils.

The synthesis of β 1,3-glucan is carried out at the plasma membrane by an enzyme complex, which has its catalytic site at the cytosolic side of the plasma membrane and requires UDP-glucose as a substrate and GTP as activator. As Gas1p/Cwh52p is attached through a GPI-anchor to the external face of the plasma membrane [29], this seems to exclude a direct role for Gas1p in β 1,3-glucan synthesis. This is in agreement with the observation that the in vitro β 1,3-glucan synthase activity was comparable to wild type levels.

Recently, *CWH53* has been independently cloned by three other groups. Sequence comparison has shown that *CWH53* is identical to *ETG1* (Echinocandin Target Gene), which was cloned as a gene that could complement the *etg1-1* mutation (C. Douglas, pers. comm.). The *etg1-1* mutant was isolated as a mutant resistant to L-733,560, a semisynthetic echinocandin [37], which inhibits β 1,3-glucan synthase in *Candida albicans* [38]. In vitro, the IC_{50} of β -glucan synthase activity to L-733,560 of *etg1-1* was found to be 50-fold higher compared to wild type, explaining the resistancy to L-733,560 in vivo, and indicating that *CWH53/ETG1* is involved in the formation of β 1,3-glucan. We have shown a 75% decrease in β 1,3-glucan content in *cwh53* cells walls, supporting the idea that *CWH53/ETG1* is involved in β 1,3-glucan formation.

The sequence of *CWH53* predicts a integral membrane protein with multiple transmembrane domains. As β 1,3-glucan synthase uses UDP-Glc as a substrate, one should expect a UDP-glucose binding site in Cwh53p if it were the catalytic subunit of β 1,3-glucan synthase. However, no UDP-Glc consensus sequence (R/KXGG) [39–41] was found in *CWH53*. Also, the in vitro β 1,3-glucan synthase activity in *cwh53-1* was comparable to wild type. This indicates that Cwh53p does not represent the catalytic subunit of the β 1,3-glucan synthase complex. The large number of transmembrane domains might mean that Cwh53p is capable of forming a channel-like structure in the membrane as seen in glucose transporters [42], myo-inositol transporters [43], and plasma membrane phosphate transporters [44]. One might therefore speculate that Cwh53p is involved in the transport of the intracellularly formed glucan

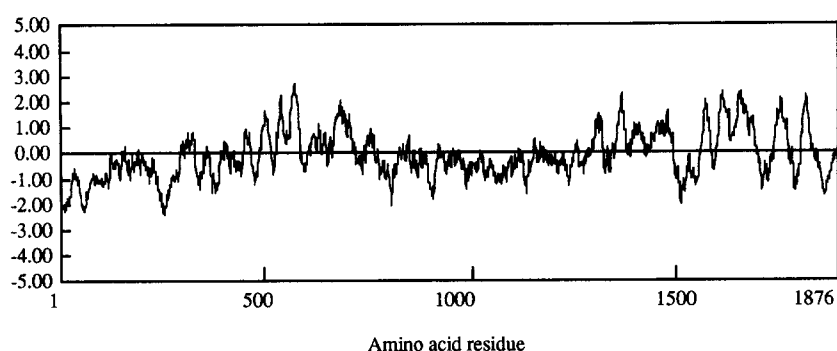


Fig. 4. Hydrophobicity plot of CWH53p according to Kyte and Doolittle [35].

chain through the plasma membrane to the outside of the cell. Interestingly, in heterozygote diploids from crosses between *cwh53* and *cwh52* mutant alleles the hypersensitivity to CFW was only partially complemented pointing to an interaction between the two proteins at the plasma membrane. Recently, a few other genes that are indirectly involved in the synthesis of β 1,3-glucan synthesis have been isolated by the use of the HM-1 killer toxin [45,46]. However, the precise role or function of these two genes in the synthesis of β 1,3-glucan is not yet clear.

Surprisingly, *CWH53* has also been cloned twice as a gene that is synthetically lethal with calcineurin mutants. Yeast contains three genes (*CNA1*, *CNA2*, *CNB1*) that encode subunits of the Ca^{2+} /calmodulin-dependent phosphoprotein phosphat-

ase or calcineurin, which is not required for vegetative growth under normal conditions [47–51]. Yeast calcineurin is inhibited by the immunosuppressant components FK506 and CsA [52]. Recently, an FK506- and CsA-hypersensitive mutant (*fks1*) was isolated, which was synthetically lethal with calcineurin mutants [53]. Cloning of *FKS1* showed that the gene was identical to *CWH53* (F. Foor, pers. comm.). Homology search in the GenEMBL data base revealed that *CWH53* was also cloned by the group of M. Cyert as *CND1*, which is, like *FKS1*, synthetically lethal with calcineurin mutants (unpublished data). Why would a gene involved in the formation of β 1,3-glucan be synthetically lethal with calcineurin mutants. The explanation is as follows. An *FKS1* homolog (*FKS2*) was cloned, which is 88% identical to *FKS1*. Simultaneous disruption of *FKS1* and

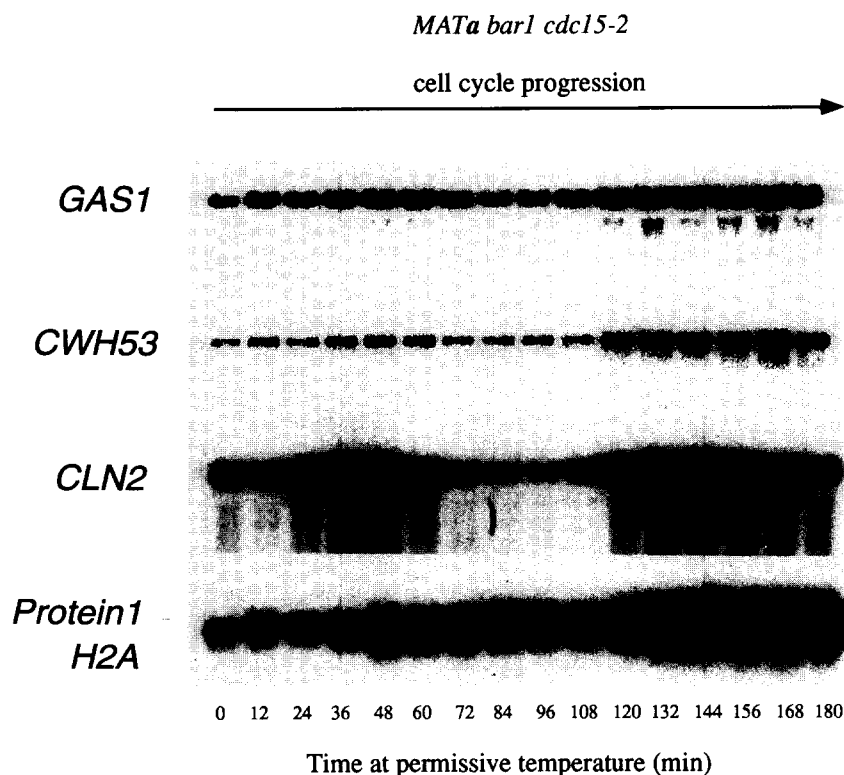


Fig. 5. Northern analysis of *GAS1/CWH52*, and *CWH53* during the cell cycle. Cells of strain K2944 (*MATa bar1::URA3 cdc15*) were arrested in the late M-phase by a temperature shift to 37°C. After 3 h of incubation, cells were shifted to 25°C, and RNA was extracted from samples taken every 12 min. To control the total amount of mRNA applied to each well, the transcript levels of Protein1, which is constitutively expressed, are also shown. Transcript levels of *CLN2* were monitored as an indicator of late G₁, and levels of *H2A* mRNA were used as an indicator for the S-phase.

FKS2 is lethal, indicating that *FKS1* and *FKS2* are partially redundant genes. Furthermore, the transcription of *FKS2* is dependent on calcineurin, and strongly inhibited by FK506 and CsA. In the presence of FK506 or in calcineurin mutants, no *FKS2* transcript is made, and the β 1,3-glucan formation is dependent on *FKS1*. Since β 1,3-glucan is required for viability, mutations in *FKS1* in the presence of FK506 or CsA or in an calcineurin mutant become lethal (F. Foor, pers. comm.)

The 5' upstream region of *GAS1* and *CWH53* contained several cell cycle regulatory elements. It has been shown for *GAS1* that after release from G₁ arrest, induced by mating pheromone, its mRNA level rapidly increases with a maximum at the onset of budding [34]. Using a different method for cell cycle synchronisation, we confirmed the observation that *GAS1* transcription is cell cycle regulated, and we found that, like *GAS1*, the *CWH53* transcript was expressed maximally in late-G₁/S-phase cells. Since both *GAS1/CWH52* and *CWH53* affect the levels of β 1,3-glucan, this indicates that the formation of this polymer is indeed cell cycle regulated as proposed before [7] and induced when a new bud is formed. The more severe *cwh53* and *gas1/cwh52* alleles showed a cell cycle specific growth arrest when the cells were grown in the presence of lethal concentrations CFW. This would imply that *CWH53* and *GAS1* are not involved in bud emergence, but rather involved in bud outgrowth.

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