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Identification of two cell cycle regulated genes affecting the β1,3-glucan content of cell walls in *Saccharomyces cerevisiae*

A.F.J. Ram[a,*], S.S.C. Brekelmans[a], L.J.W.M. Oehlen[b], F.M. Klis[a]

[a]*Institute for Molecular Cell Biology, University of Amsterdam, BioCentrum Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands*
[b]*The Rockefeller University, New York, NY 10021, USA*

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Abstract The Calcofluor white-hypersensitive mutants *cwh52* and *cwh53* are severely reduced in β1,3-glucan. *CWH52* was equivalent to *GAS1*. *CWH53* represented a new gene, located on the right arm of chromosone 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 G_1, 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; *GGP1*; 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 β1,3-linked glucose residues, and the remaining twenty percent are accounted for by β1,6-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-cyle 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.

*Corresponding author. Fax: (31) (20) 525 7934.

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

2. Materials and methods

2.1. Yeast strains, bacterial strains, and growth media

Saccharomyces cerevisiae AR27 (*MATα ura3-52*) and AR49 (*MAT*a *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 (*MAT*a *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 (*MAT*a *ade2-1 his4-15* p°[*KIL-k*] was obtained from Cold Spring Harbor Laboratory. *Escherichia coli* DH5α 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-^{35}S]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^{32}P]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 *Eco*RI fragment, and for *CWH52*

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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].

β1,3-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. Fluoresence microscopy

Cells were grown overnight in YPD in the presence of lethal concentrations of CFW (50 μg/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 β1,6-glucan [25,26] indicating that the levels of β1,6-glucan were unaffected (Table 1). *Hansenula mrakii* killer toxin HM-1 binds to both β-glucan and β1,6-glucan [27,28]. Table 1 shows that *cwh53-1* and *cwh52-2* had become more resistant to HM-1 toxin, whereas *chw52-3* remained sensitive; *cwh53-1*, *cwh53-2*, and *cwh53-3* were also resistant, whereas *cwh53-4*, the weakest allele, was only partially resistant. These observations indicate that in both *cwh52* and *chw53* the β-glucan partion 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.

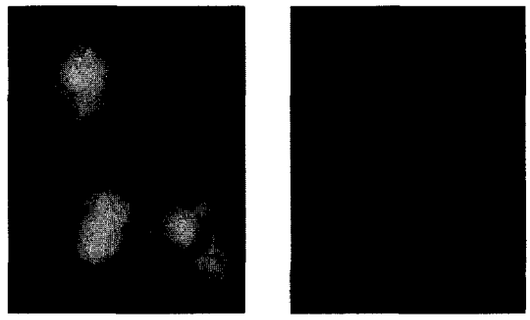

Fig. 1. Terminal phenotype of *cwh52* (left) and *cwh53* (right) grown in the presence of lethal concentrations CFW. Cells were stained with ConA-FITC and observed using a fluorescence microscope.

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 *CHW52* 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	K1 killer	HM-1 killer
	GN	Glc	Man	(Mean ± S.E.M.)*	resistance	resistance
AR27	1.1	49.8	49.1	1.014 ± 0.077 (n = 8)	S	S
cwh52-1	3.9	30.7	65.4	0.469 ± 0.031 (n = 2)	S	R
cwh52-2	2.7	32.7	64.6	0.506 ± 0.033 (n = 2)	S	± R
cwh52-3	1.8	45.0	53.2	0.846 ± 0.049 (n = 2)	S	S
cwh53-1	3.3	19.7	77.0	0.256 ± 0.001 (n = 2)	S	R
cwh53-2	2.9	20.4	76.7	0.266 ± 0.028 (n = 2)	S	R
cwh53-3	1.9	31.8	66.3	0.480 ± 0.024 (n = 2)	S	R
cwh53-4	1.6	36.4	62.0	0.587 ± 0.008 (n = 2)	S	± R

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

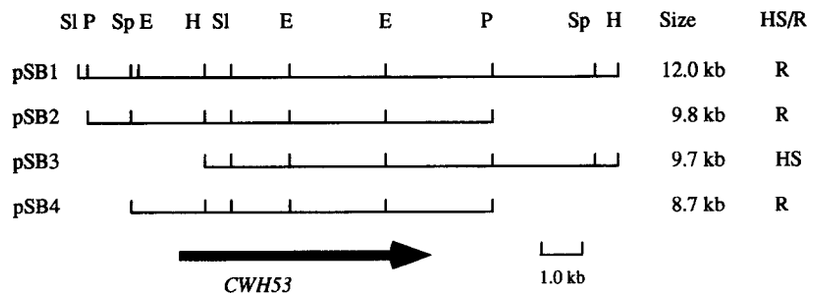

Fig. 2. Partial restriction map and subcloning of *CWH53*. The location of *CWH53* is indicated by the black line, and the direction is shown by the arrow. Subclones of the original genomic fragment were tested for their ability to complement the *cwh53* mutants. R, resistant to CFW; HS, hypersensitive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

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 *Xba*I 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 *Eco*RI 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 *CHW52* is identical to *GAS1/GPP1*, and we will refer to this gene as *GAS1* for the remainder of this paper. In the promotor 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 complemention 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 *Sph*I–*Pst*I subclone. *CWH53* was mapped onto chromosome XII by probing a chromosome blot with a 2.4-kb *Sal*I 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 *Hin*dIII site in the functional region of *CWH53* (Fig. 2), and the DNA sequence in this region was obtained. This revealed a continous open reading frame of 5,628 nucleotides, spanning the *Hin*dIII 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, *CHW53* 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 promotor region of *CWH53*, five SCBs were found at positions −1112, −743, −598, −500, and −395, respectively. Two *Mlu*I 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 G_1/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, *MAT*a *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 G_1. *CWH53* and *GAS1* were cell cycle regulated and showed similar expression patterns; maximal levels for both were observed in late-G_1/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
MNTDQQPYQG QTDYTQGPGN GQSQEQDYDQ YGQPLYPSQA DGYYDPNVAA GTEADMYGQQ

        70          80          90         100         110         120
PPNESYDQDY TNGEYYGQPP NMAAQDGENF SDFSSYGPPG TPGYDSYGGQ YTASQMSYGE

       130         140         150         160         170         180
PNSSGTSTPI YGNYDPNAIA MALPNEPYPA WTADSQSPVS IEQIEDIFID LTNRLGFQRD

       190         200         210         220         230         240
SMRNMFDHFM VLLDSRSSRM SPDQALLSLH ADYIGGDTAN YKKWYFAAQL DMDDEIGFRN

       250         260         270         280         290         300
MSLGKLSRKA RKAKKKNKKA MEEANPEDTE ETLNKIEGDN SLEAADFRWK AKMNQLSPLE

       310         320         330         340         350         360
RVRHIALYLL CWGEANQVRF TAECLCFIYK CALDYLDSPL CQQRQEPMPE GDFLNRVITP

       370         380         390         400         410         420
IYHFIRNQVY EIVDGRFVKR ERDHNKIVGY DDLNQLFWYP EGIAKIVLED GTKLIELPLE

       430         440         450         460         470         480
ERYLRLGDVV WDDVFFKTYK ETRTWLHLVT NFNRIWVMHI SIFWMYFAYN SPTFYTHNYQ

       490         500         510         520         530         540
QLVDNQPLAA YKWASCALGG TVASLIQIVA TLCEWSFVPR KWAGAQHLSR RFWFLCIIFG

       550         560         570         580         590         600
INLGPIIFVF AYDKDTVYST AAHVVAAVMF FVAVATIIFF SIMPLGGLFT SYMKKSTRRY

       610         620         630         640         650         660
VASQTFTAAF APLHGLDRWM SYLVWVTVFA AKYSESYYFL VLSLRDPIRI LSTTAMRCTG

       670         680         690         700         710         720
EYWWGAVLCK VQPKIVLGLV IATDFILFFL DTYLWYIIVN TIFSVGKSFY LGISILTPWR

       730         740         750         760         770         780
NIFTRLPKRI YSKILATTDM EIKYKPKVLI SQVWNAIIIS MYREHLLAID HVQKLLYHQV

       790         800         810         820         830         840
PSEIEGKRTL RAPTFFVSQD DNNFETEFFP RDSEAERRIS FFAQSLSTPI PEPLPVDNMP

       850         860         870         880         890         900
TFTVLTPHYA ERILLSLREI IREDDQFSRV TLLEYLKQLH PVEWECFVKD TKILAEETAA

       910         920         930         940         950         960
YEGNENEAEK EDALKSQIDD LPFYCIGFKS AAPEYTLRTR IWASLRSQTL YRTISGFMNY

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

      1030        1040        1050        1060        1070        1080
LLRAYPDLQI AYLDEEPPLT EGEEPRIYSA LIDGHCEILD NGRRRPKFRV QLSGNPILGD

      1090        1100        1110        1120        1130        1140
GKSDNQNHAL IFYRGEYIQL IDANQDNYLE ECLKIRSVLA EFEELNVEQV NPYAPGLRYE

      1150        1160        1170        1180        1190        1200
EQTTNHPVAI VGAREYIFSE NSGVLGDVAA GKEQTFGTLF ARTLSQIGGK LHYGHPDFIN

      1210        1220        1230        1240        1250        1260
AIFMTTRGGV SKAQKGLHLN EDIYAGMNAM LRGGRIKHCE YYQCGKGRDL GFGTILNFIT

      1270        1280        1290        1300        1310        1320
KIGAGMGEQM LSREYYYLGT QLPVDRFLTF YYAHPGFHLN NLFIQLSLQM FMLTLVNLSS

      1330        1340        1350        1360        1370        1380
LAHESIMCIY DRNKPKTDVL VPIGCYNFQP AVDWVRRYTL SIFIVFWIAF VPIVVQELIF

      1390        1400        1410        1420        1430        1440
RGLWKATQRE FCHLLSLSPM FEVFAGQIYS SALLSDLAIG GARYISTGRG FATSRIPFSI

      1450        1460        1470        1480        1490        1500
LYSRFAGSAI YMGARSMLML LFGTVAHMQA PLLWFMASLS SLIFAPFVFN PHQFAWEDFF

      1510        1520        1530        1540        1550        1560
LDYRDYIRWL SRGNNQYHRN SWIGYVRMSR ARITGFKRKL VGDESEKAAG DASRAHRTNL

      1570        1580        1590        1600        1610        1620
IMAEIIPCAI YAAGCFIAFT FINAQTGVKT TDDDRVNSVL RIIICTLAPI AVNLGVLFFC

      1630        1640        1650        1660        1670        1680
MGMSCCSGPL FGMCCKKTGS VMAGIAHGVA VIVHIAFFIV MWVLESFNFV RMLIGVVTCI

      1690        1700        1710        1720        1730        1740
QCQRLIFHCM TALMLTREFK NDHANTAFWT GKWYGKGMGY MAWTQPSREL TAKVIELSEF

      1750        1760        1770        1780        1790        1800
AADFVLGHVI LICQLPLIII PKIDKFHSIM LFWLKPSRQI RPPIYSLKQT RLRKRMVKKY

      1810        1820        1830        1840        1850        1860
CSLYFLVLAI FAGCIIGPAV ASAKIHKHIG DSLDGVVHNL FQPINTINND IGSQMSTYQS

      1870        1876
HYYTHTPSLK 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 β1,6-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 β1,6-glucan alone. In fact, it is unlikely that the levels of β1,6-glucan were reduced because *cwh53* and *cwh52* mutants showed wild-type sensitivity to the K1 killer toxin, which specifically requires β1,6-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 β1,3-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 β1,3-glucan and by the effect of Calcofluor on the formation of chitin fibrils.

The synthesis of β1,3-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 β1,3-glucan synthesis. This is in agreement with the observation that the in vitro β1,3-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 β1,3-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 β1,3-glucan. We have shown a 75% decrease in β1,3-glucan content in *cwh53* cells walls, supporting the idea that *CWH53/ETG1* is involved in β1,3-glucan formation.

The sequence of *CWH53* predicts a integral membrane protein with multiple transmembrane domains. As β1,3-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 β1,3-glucan synthase. However, no UDP-Glc consensus sequence (R/KXGG) [39–41] was found in *CWH53*. Also, the in vitro β1,3-glucan synthase activity in *cwh53-1* was comparable to wild type. This indicates that Cwh53p does not represent the catalytic subunit of the β1,3-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

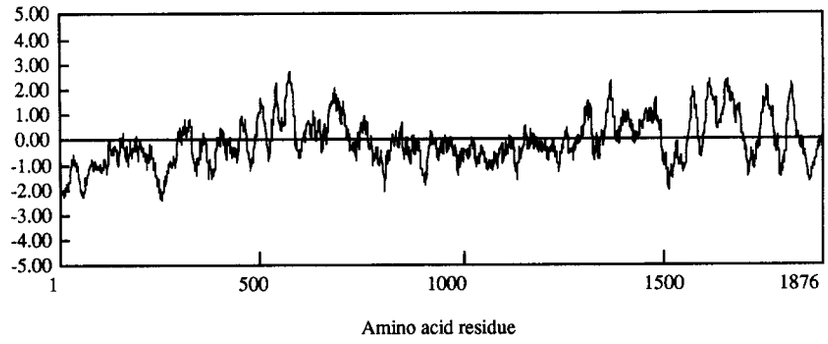

Fig. 4. Hydrophobicity plot of CWH53p according to Kyte and Doolittle [35].

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 β1,3-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 β1,3-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 phosphatase 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 β1,3-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

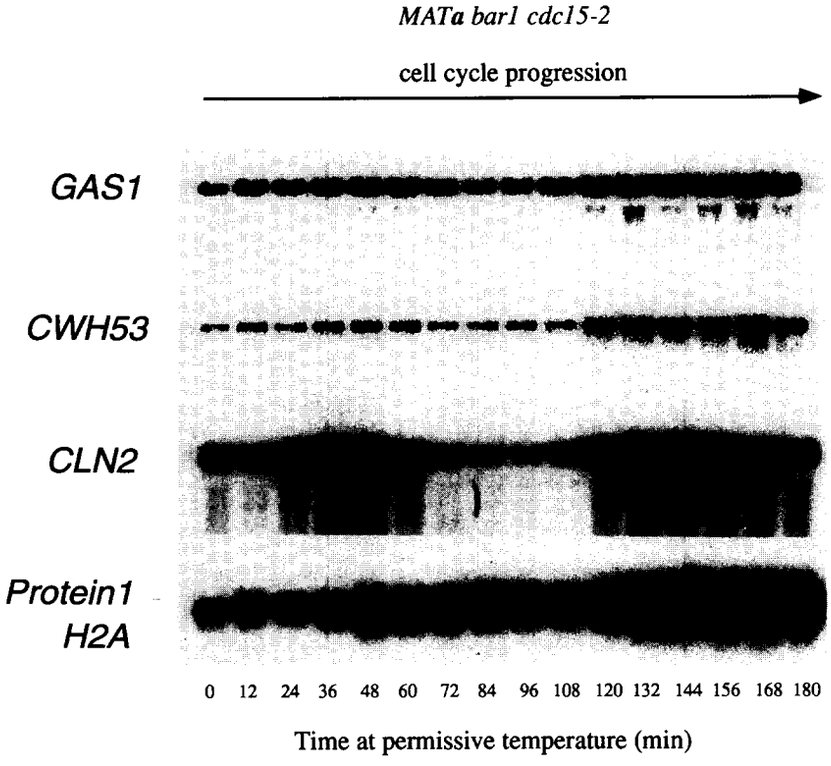

Fig. 5. Northern analysis of *GAS1*/*CWH52*, and *CWH53* during the cell cycle. Cells of strain K2944 (MATa *bar1*::*URA3 cdc15*) were arrested in the late M-phase by a temperature shift to 37°C. After 3 h of incubation, cells were shifted to 25°C, and RNA was extracted from samples taken every 12 min. 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 G_1, 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 β1,3-glucan formation is dependent on *FKS1*. Since β1,3-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 G_1 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-G_1/S-phase cells. Since both *GAS1/CWH52* and *CWH53* affect the levels of β1,3-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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