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New Potential Cell Wall Glucanases of *Saccharomyces cerevisiae* and Their Involvement in Mating

CORINNA CAPPELLARO,† VLADIMIR MRSA, AND WIDMAR TANNER*

Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, 93040 Regensburg, Germany

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Biotinylation of intact *Saccharomyces cerevisiae* cells with a nonpermeant reagent (Sulfo-NHS-LC-Biotin) allowed the identification of seven cell wall proteins that were released from intact cells by dithiothreitol (DTT). By N-terminal sequencing, three of these proteins were identified as the known proteins β-exoglucanase 1 (Exg1p), β-endoglucanase (Bgl2p), and chitinase (Cts1p). One protein was related to the PIR protein family, whereas the remaining three (Scw3p, Scw4p, and Scw10p [for soluble cell wall proteins]) were found to be related to glucanases. Single knockouts of these three potential glucanases did not result in dramatic phenotypes. The double knockout of *SCW4* and the homologous gene *SCW10* resulted in slower growth, significantly increased release of proteins from intact cells by DTT, and highly decreased mating efficiency when these two genes were disrupted in both mating types. The synergistic behavior of the disruption of *SCW4* and *SCW10* was partly antagonized by the disruption of *BGL2*. The data are discussed in terms of a possible counterplay of transglucosidase and glucosidase activities.

Saccharomyces cerevisiae has become the best-known eucaryotic organism and serves as a model for the study of almost any problem in cell biology (2, 4). *S. cerevisiae* invests about 20% of its dry weight to build up the cell wall, but nevertheless, this extracellular organelle is not very well understood. Although great progress has recently been made in understanding the polysaccharide network of the wall (8), the proteins that contribute to this structure and amount to about 10% of the wall dry weight (7, 8, 16) have not been systematically and extensively studied. It has been shown that cell wall proteins can be subdivided into two classes: one class can be solubilized by sodium dodecyl sulfate (SDS) under reducing conditions; the other one is considered covalently linked to cell wall polysaccharides and is released only by β-glucanase treatment (12, 23).

A major problem in definitely identifying cell wall proteins has been contamination with intracellular material. Recently, labeling procedures using a nonpermeant biotinylation reagent have been described (3, 14). With one of these methods, about 20 cell wall (cell surface) proteins could be labeled in *S. cerevisiae* (14). Among the covalently attached cell wall proteins (Ccw = covalently linked cell wall proteins), the proteins of the PIR family (proteins with internal repeats [22]) have been identified as wall components (14).

Here we describe the identification of seven biotinylated, SDS-soluble proteins (Scw = soluble cell wall proteins) by N-terminal sequencing. Three of them (β-exoglucanase 1 [Exg1p], β-endoglucanase [Bgl2p], and chitinase [Cts1p]) were already known (6, 9, 24), while one was identical to the recently identified covalently linked cell wall protein Ccw5p/Ccw11p, which belongs to the PIR protein family (14). The three previously unidentified proteins (Scw3p, Scw4p, and Scw10p) all show significant homology to glucanases. These gene products had not previously been identified as cell wall components. Single and double knockouts were made, defining synergistic and antagonistic functions of the described proteins.

* Corresponding author. Mailing address: Lehrstuhl für Zellbiologie und Pflanzenphysiologie, Universität Regensburg, 93040 Regensburg, Germany. Phone: 49 941 943 3018. Fax: 49 941 943 3352. E-mail: widmar.tanner@biologie.uni-regensburg.de.

† Present address: Institut für molekulare Genetik, 37077 Göttingen, Germany.

MATERIALS AND METHODS

Chemicals, strains, and growth conditions. Calcofluor white was obtained from Sigma, Sulfo-NHS-LC Biotin was from Pierce, and streptavidin-peroxidase was from Amersham. All of the *S. cerevisiae* strains used in this work were derived from SEY6210 (*MAT*α *leu2-3,112 ura3-52 his3-Δ200 lys2-801 trp1-Δ901 suc2-Δ9 GAL*) or SEY6211 (*MAT***a** *ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901 ade2-101 suc2-Δ9 GAL*) (19). Disruption of *CCW5* was described by Mrsa et al. (14). All strains were grown in standard YPD medium (1% yeast extract, 2% peptone, 2% dextrose).

Isolation and purification of Scwps. Yeast cells (strain SEY6210) were harvested at an optical density at 578 nm (OD_{578}) of 6 to 8 (1 U of OD_{578} corresponds to 10^7 cells), washed with water and with 25 mM Tris/HCl (pH 8.5) and finally resuspended in this buffer containing 2 mM dithiothreitol (DTT). Cells were shaken vigorously for 2 h at 4°C and then pelleted. The supernatant was concentrated by lyophilization, resuspended in water, and dialyzed against 2 mM Tris/HCl (pH 8.0)–1 mM EDTA. The extract was adjusted to 20 mM sodium acetate (pH 7.3)–150 mM NaCl–1 mM KCl–1 mM $MnCl_2$–1 mM $MgCl_2$ and bound to concanavalin A (ConA)-agarose (Sigma). After washing, the material bound to ConA was eluted with 500 mM α-methylmannoside. An amount equivalent to 15 g of cells was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10), blotted onto polyvinylidene difluoride membranes (Millipore), and stained with Coomassie, and the protein bands were cut out for sequencing.

For the purification of Scw4p and Scw10p, DTT extracts of mutant cells lacking one or the other of the two proteins were applied to a MonoQ fast protein liquid chromatography (FPLC) column (1 ml). Chromatography was performed in 20 mM Tris-HCl buffer (pH 6.0), and proteins were eluted in a 0 to 500 mM NaCl gradient (total volume, 25 ml) at a flow rate of 0.5 ml/min. Fractions (1 ml) were analyzed electrophoretically.

SDS-PAGE, blotting, and staining procedures. Cell surface labeling with Sulfo-NHS-LC-Biotin, SDS-PAGE, and blotting were carried out as previously described (14). For silver staining, the method of Morissey (13) was used, and the staining of gels for carbohydrates (with periodic acid-Schiff [PAS] stain) was done by the method of Zacharius et al. (25). Myosin (212 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), catalase (57.5 kDA), and aldolase (40 kDa) were used as molecular mass standards.

DNA amplification and disruption of *SCW* genes. *SCW* genes were amplified by standard PCR methods using *Taq* polymerase. The identity of a gene was verified by restriction analysis. Disruptions were made by fusing N- and C-terminal parts of the gene of interest with marker genes. In particular, *SCW4* (fragments −149 to −6 and 901 to 1166) was fused with *LEU2*, *SCW10* (−180 to 130 and 958 to 1338) was fused with *HIS3*, *SCW11* (−213 to 157 and 1435 to 1844) was fused with *TRP1*, and *SCW3* (−181 to 139 and 1147 to 1490) was fused with *TRP1* (numbers refer to the corresponding start ATG). The *BGL2* disruption was done as previously described (6). Disruptions were tested by PCR and by streptavidin-peroxidase blots of protein extracts from Sulfo-NHS-LC-Biotin-labeled cells.

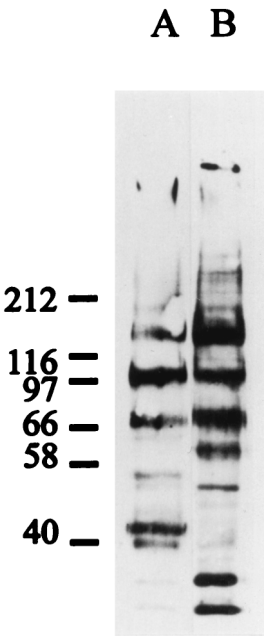

FIG. 1. Comparison of two extraction methods for Sulfo-NHS-LC-Biotin-labeled cell wall proteins. This blot was stained with a streptavidin-peroxidase conjugate. (A) Extraction of intact cells with DTT. An amount of extract equivalent to 4 ml of cells with an OD_{578} of 5 was subjected to SDS-PAGE. (B) Cell walls isolated from broken cells were extracted by heating in Laemmli sample buffer under reducing conditions (2% SDS, 5% β-mercaptoethanol). The equivalent of 200 μl of cells with an OD_{578} of 5 was applied. For the molecular weight standards used, see Materials and Methods. The values beside the gel are molecular sizes in kilodaltons.

Calcofluor white treatment. YPD agar plates with 0 to 50-μg/ml calcofluor white were prepared. Yeast cells were diluted to $2 \cdot 10^5$/ml, $2 \cdot 10^4$/ml, and $2 \cdot 10^3$/ml, and 5 μl of each suspension was dropped onto the plates.

Mating test. Minimal medium agar plates lacking lysine and adenine, thus allowing the growth of diploid cells only, were prepared. Stationary-phase **a** and α cells of the wild type and different mutants were mixed and diluted, and 5 μl of the suspension (corresponding to 10^4 cells) was dropped onto plates.

RESULTS

Isolation and identification of cell wall proteins soluble under reducing conditions. Previous attempts to identify SDS-solubilized cell wall proteins prepared from isolated cell walls repeatedly led to the identification of glycolytic enzymes like enolase, 3-phosphoglyceraldehyde dehydrogenase, triosephosphate isomerase, etc. (14a, 20a). The alleged occurrence of glycolytic enzymes as cell wall components has, indeed, been reported in the literature (1). To avoid this probable artifact, surface proteins were labeled. Intact *S. cerevisiae* cells were treated with the biotin reagent sulfosuccinimidyl-6-(biotinamido)hexanoate, which is unable to penetrate the cell membrane. Subsequent extraction of purified cell walls with SDS under reducing conditions gave rise to nine major biotinylated proteins, which were visualized with streptavidin-horseradish peroxidase (14). Since the biotinylation reagent blocks N-terminal amino groups, purification of individual biotinylated cell surface proteins for sequencing required copurification of the corresponding nonbiotinylated material. When this procedure was followed, the first N-terminal sequence finally obtained again corresponded to enolase (2a). Thus, it became clear that although it was possible to label cell wall proteins specifically, after breaking the cells and separating the cell walls, these proteins were contaminated with glycolytic enzymes, which were present, of course, per cell in large excess. It was necessary, therefore, to release the SDS-soluble cell wall proteins without breaking the cells. As has often been shown before, several cell wall proteins could be released from intact cells with SH reagents (3, 17, 18). A comparison of patterns of biotinylated proteins from SDS-extracted cell walls with that of DTT-released proteins from intact cells (Fig. 1) strongly suggested that several major bands of the SDS extract could also be released under these milder conditions from intact cells. It is not certain, of course, whether the proteins with the same apparent molecular weight obtained under the two conditions are, indeed, identical. However, for reasons given above, further work was concentrated on the biotinylated proteins released from intact cells by DTT. They were purified via a ConA column. As expected, all of the proteins released from the column by α-methylmannoside gave positive PAS staining, indicating that they were glycoproteins (Fig. 2).

When corresponding nonbiotinylated proteins obtained in an identical way were blotted onto Immobilon and N terminally sequenced, seven of them gave rise to informative sequences (Table 1). The 116-kD protein (Scw2p, according to Mrsa et al. [14]) turned out to be chitinase (9), and the 29-kD protein (Scw9p) and the 44-kD protein (Scw6p) were β-endoglucanase (Bgl2p) and exoglucanase 1 (Exg1p), respectively (6, 23). The finding that these three known soluble cell wall proteins were found among the biotinylated protein bands indicated once more the high specificity of the labeling method. The Scw8p sequence has previously been found among the covalently linked cell wall proteins (14), which could be solubilized with laminarinase (Ccw5p), as well as with 30 mM NaOH (Ccw11p). The gene coding for this protein belongs to the *PIR* gene family, although the typical conserved repetitive sequence of this gene family (protein with internal repeats) occurs only once within this protein. The other three N-terminal sequences, corresponding to Scw3p (95 kDa, open reading frame [ORF] YNL066w), Scw4p (66 kDa, ORF YGR279c), and Scw10p (66 kDa, ORF YMR305c), were completed with the information from the yeast genome project. These genes code for previously unknown proteins, which, however, all show homology to glucanases. Since Scw4p and Scw10p are

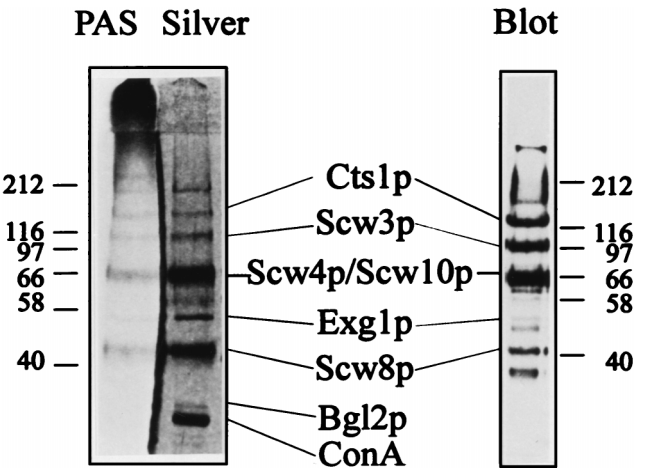

FIG. 2. Analysis of isolated proteins. (Left) Concentrated and ConA-purified DTT extract of intact cells stained for sugars (PAS) and protein (silver). (Right) Parallel extract isolated from biotinylated cells blotted and visualized with a streptavidin-peroxidase conjugate. The coincident patterns indicate that the purified materials are surface-exposed, glycosylated proteins. The names given resulted from N-terminal sequencing (Table 1). Cts1p, chitinase; Exg1p, exoglucanase 1; Bgl2p, β-endoglucanase 2; Scw8p, protein with an unknown function; Scw3p, Scw4p, and Scw10p, soluble cell wall proteins 3, 4, and 10 (see text). ConA is a contamination due to the purification procedure.

TABLE 1. N-terminal sequences of proteins shown in Fig. 2[a]

N-terminal sequence[b]	Gene
XXELAFNLXVK IGELAFNLGVK	BGL2 or SCW9
DVIXQIGDGQV DVISQIGDGQV	SCW8 or CCW5
QYDYDGGSXGE YYDYDHGSLGE	EXG1 or SCW6
AVVXAXVQKK AVVTTTVQKQ	SCW4
XVVXAXVXQE DVVTATVHAQ	SCW10
YAADIDTGXXX YAADIDTGCTT	SCW3
FDSSANTNIAVY FDSSANTNIAVY	CTS1 or SCW2

[a] Purified and concentrated extract of 15 g nonbiotinylated cells was separated by SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, and N terminally sequenced. The sequences obtained were compared to the N termini of ORFs from the Munich Information Center for Protein Sequences by the ALIGN function of the University of Wisconsin Genetics Computer Group package.

[b] The upper sequence of each pair was determined by sequencing, and the lower one is the corresponding sequence found by a database search.

practically the same size, Scw10p could only be purified and sequenced after an *scw4* mutant had been constructed (see below).

Three gene families of potential glucanases. The sequence of Scw4p was derived from the N-terminal sequence of the protein band with an apparent molecular mass of 66 kDa. This sequence was very similar to another one found in the yeast genome and designated Scw10p. Since both sequences have practically the same length and since the disruption of the *SCW4* gene resulted only in partial disappearance of the protein band (see later), it was assumed that the remaining band corresponded to Scw10p. Therefore, the protein was extracted from the *scw4* mutant cells, purified by MonoQ FPLC column chromatography, and sequenced. The sequence obtained, indeed, corresponded to that of Scw10p (Table 1). These two proteins are 63% identical; both contain a signal sequence, a Kex2p processing site, and no GPI anchoring sequence (Fig. 3). In addition, another ORF with significant homology to *SCW4* and *SCW10* has been found in the *S. cerevisiae* sequence and designated *SCW11* (ORF YGL028c). A comparison of the three protein sequences, together with the known cell wall glucanase Bgl2p, is given in Fig. 3.

The Scw3p sequence was found to be most closely related to a β-glucosidase of *Candida wickerhamii* (61% identity, 72% homology), which in this organism is required to ferment cellodextrins (21).

The four known *S. cerevisiae* glucanases are Exg1p, Exg2p, Bgl2p, and Ssg1p (6, 11, 20). They can be arranged according to the degree of homology, together with the new potential glucanases described in this work, as shown in Fig. 4. In Fig. 4, five additional ORFs, which may very well also code for glucanases or glucanase-related enzymes, were included according to their degrees of homology within the group of 13 genes. These putative glucanases can be subgrouped into three families as shown.

Phenotypes of *SCW3*, *SCW4*, *SCW10*, and *SCW11* disruptants. Disruptions of genes *SCW3*, *SCW4*, *SCW10*, *SCW11*, and *BGL2* were constructed and checked by PCR. As shown in Fig. 5, in the *scw3* and *scw8* mutants, the corresponding biotinylated protein bands were missing. The disruption of the *SCW4* gene brought about a significant decrease in the intensity of the corresponding electrophoretic band but did not lead to its complete disappearance. Only when the *SCW10* gene was also disrupted did the 66-kDa band seem to disappear; however, it was difficult to prove this since much more protein was extracted by DTT from the cell wall of the double mutant than from the wild type or single mutants (Fig. 5A).

Among the single mutants, only *scw11* showed a growth or morphological phenotype. This mutant did not separate well after division and grew clumpy. Not even sonication could change this property significantly (data not shown).

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1  MRLSNLIASA SLLSAATLAA PANHEHKDKR AVVTTTVQKQ TTIIVNGAAS 50
2  MRFSNFLTVS ALLTGA.LGA PAVRHKHEKR DVVTATVHAQ VTVVVSGNSG
3  MISPISFLSS LLCLTYLTSA LPILP...KR EVVTRVHTAS TTNVVTDFYS
4  MRFSTTLATA ATALFFTASQ VS..... ..... ..... .....

1  T..... ..... ..... ..... ..... ..... 100
2  E..... ..... ..... ..... ..... .....
3  TTTEVVIAPT VEFLISDSVT FTTTLIPQGV NPTAEPTTTI TKVLLKKAEM
4  ..... ..... ..... ..... ..... .....

1  ..... ..... ..... ..... ..... ..... 150
2  ..... ..... ..... ..... ..... .....
3  STSSQPTSTL QPSTIPQSTS SFQAESTLQA VSTQQTAMSV SAGTSEDVQQ
4  ..... ..... ..... ..... ..... .....

1  ..... ..... ...PVAALEE NAVV...NSA P...AAATST 200
2  ..... ..... ...TIVPVNE NAVVATTSST A...VASQAT
3  LATTSTSISS SPSPTTTSTS TQNIVSGVGE ANVEGNTQSQ HHSYQAAATS
4  ..... ..... ..... ..... .....

1  TSSAASVATA AASSSENNS. ..QVSAAASP ASSSAATSTQ SSSSSQASSS 250
2  TSTLEPTTSA NVVTSQQQTS TLQSSEAAST VGSS..TSSS PSSSSSTSSS
3  TLNQQTSTSI ASQESTESTN TPTSSSTSSS TSSSTSSSTS SSTSSSTSSS
4  ..... ..... ..... ..... .....

1  SSSGEDVSSF ..... ..... ASGVRGITYT PYESSGACKS 300
2  ASSSASSSIS ..... ..... ASGAKGITYS PYNDDGSCKS
3  TSSSTSSSTS STQETAATTS EGSSSSSAAI TSSPKAIAYS PYNDDGSCKS
4  ..... ..... ..... AIGELAFNLG VKNNDGTCKS

1  ASEVASDLAQ L..TDFPVIR LYG.TDCNQV ENVFKAKAS. ....NQKVFL 350
2  TAQVASDLEQ L..TGFDNIR LYG.VDCSQV ENVLQAKTS. ....SQKLFL
3  ADAVSSDLTL IKSKGISKIR VYG.TDCNSF ETVQPAAVKL GIKINQGLYI
4  TSDYETELQA LKS.YTSTVK VYAASDCNTL QNLGPAAEAE GF....TIFV

1  GIYYVDQIQD GVNTIKSAVE SYGSWDDVTT VS...IGNEL VNGNQATPSQ 400
2  GIYYVDKIQD AVDTIKSAVE SYGSWDDITT VS...VGNEL VNGGSATTTQ
3  TSSGVDSIDD SVTTLIQYGQ TNG.WDVFDF IT...VGNEA INNGWCSVSD
4  GVWPTDDSHY AAE..KAALQ TYLPKIKEST VAGFLVGSEA LYRNDLTASQ

1  VGQYIDSGRS ALKAAGYTG. .....PVVSV DTFIAVINN PELCDYS..D 450
2  VGEYVSTAKS ALTSAGYTG. .....SVVSV DTFIAVINN PDLCNYS..D
3  LISKISSVKS KLSEAGYSG. .....QITTS EPPVSFENN PDLCKKSDID
4  LSDKINDVRS VV DISDSDG KSYSGKQVGTV DSWNVLVAG YNSAVIEASD

1  YMAVNAHAYF DKNTVAQDSG KWLLEQIQRV WTACDGKKNV VIT....ESG 500
2  YMAVNAHAYF DENTAAQDAG PWVLEQIERV YTACGGKKDV VIT....ETG
3  FVGINPHAYF DTSASAETAG TFVKGQVELI QGVC.GTSNV FVT....ETG
4  FVMANAFSYW QGQTMQNASY SFFDDIMQAL QVIQSTKGST DITFWVGETG

1  WPSKGETYGV AVPSKENQKD AVSAITSSC. ..GADTFLFT AFNDYWKAD. 550
2  WPSKGDTYGE AVPSKANQEA AISSIKSSC. ..GSSAYLFT AFNDLWKDD.
3  YPSSGIQNGG NIPSTANQIT AVQNILNEM. ..DLDVTILS TYNDYWKAP.
4  WPTDGTNFES SYPSVDNAKQ FWKEGICSMR AWGVNVIVFE AFDEDWKPNT

1  .GAYGVEKYWG ILSNE*.... ..... 575
2  .GQYGVEKYWG ILSSD*.... .....
3  .GDYGIEQSFG VIEYFP*... .....
4  SGTSDVEKHWG VFTSSDNLKY SLDCDFS*
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FIG. 3. Scw4p and three related gene products are potential glucanases. Amino acid sequences of Scw4p (sequence 1), Scw10p (sequence 2), Scw11p (sequence 3), and Bgl2p (sequence 4) are compared. The Munich Information Center for Protein Sequences database was screened with the sequence of Scw4p (by using FASTA). The resulting ORFs were aligned by using the pileup function of The University of Wisconsin Genetics Computer Group package. ORFs are available from the Munich Information Center for Protein Sequences under the following accession numbers (PIR code): Scw4p, S64614; Scw10p, S53975; Scw11p, S64030, Bgl2p, A33499. Boldface letters are used when at least two amino acids are identical.

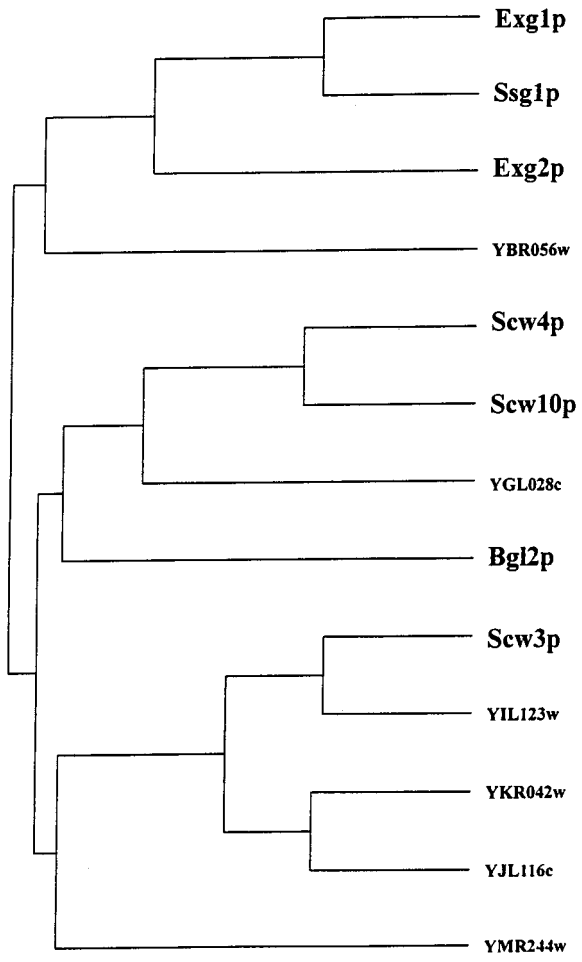

FIG. 4. Grouping of known and potential glucanases. ORFs of gene products related to known glucanases were obtained from the Munich Information Center for Protein Sequences based on a FASTA search with the sequences of Exg1p, Scw4p, and Scw3p (S53916). Sequences were processed with the lineup function of the University of Wisconsin Genetics Computer Group package.

In addition, double and triple disruptions were produced. Cells carrying the *scw4 scw10* double disruption displayed morphological changes: irregular and frequently larger cells were seen (Fig. 6). The growth rate of this double mutant was also decreased, such that the doubling time increased from about 70 min (wild type) to 100 min. Surprisingly, the increase in doubling time of the *scw4 scw10* double mutant was compensated if a third gene, *BGL2*, coding for a β-endoglucanase (6), was also deleted. Corresponding behavior was observed when the amount of proteins released from intact cells with DTT was compared. As shown in Fig. 7, a large amount of protein could be released from the *scw4 scw10* double mutant, which was not the case with the wild type or with any of the single mutants. Again, the additional deletion of the *BGL2* gene significantly compensated the effect. The same behavior was observed when not the total protein but rather the biotinylated cell wall proteins were visualized by streptavidin (Fig. 5A). For both phenomena, increased doubling time and enhanced release of cell wall proteins, it was clear that the two gene products, Scw4p and Scw10p, act synergistically. The double mutants containing *scw11* with *scw4*, *scw10*, or *bgl2* did not show any synergism; they did not differ from the corresponding single knockouts (Fig. 7).

The synergism of Scw4p and Scw10p was also seen when the cells were tested for increased sensitivity to the cell wall-weakening agent calcofluor white (Fig. 8). Of the single knockouts, only *scw10* showed somewhat increased sensitivity. Of the different double or multiple mutants tested, all combinations that included both *scw4* and *scw10* were found to be sensitive. The compensating effect of the *BGL2* disruption was not observed in this case. To test if the observed properties of the *scw4 scw10* double mutant were due to a change in the overall composition of the cell wall, the amounts of mannose, glucose, and *N*-acetylglucosamine were determined after a total wall hydrolysis with trifluoroacetic acid (4 M, 2 h). Cell walls of the wild type and *scw4* and *scw10* single mutants, the double mutant, and a triple mutant carrying an additional *bgl2* mutation were tested, but no significant change in the carbohydrate composition of the walls could be detected. The percentage of mannose ranged between 56 and 58% in the stationary-phase cells or between 54 and 57% in the logarithmically growing strains. Glucose was present in amounts between 39 and 42% in

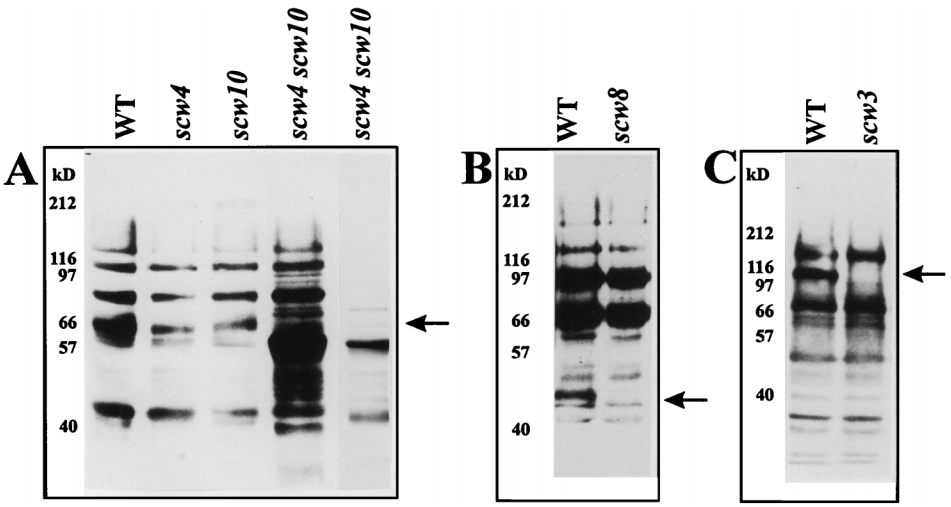

FIG. 5. *scw4*, *scw10*, *scw8* and *scw3* knockout mutants. The corresponding mutant cells were labeled with Sulfo-NHS-LC-biotin and extracted with DTT (*scw4*, *scw10*, and *scw8*), or whole cells were heated in Laemmli sample buffer (*scw3*). For the molecular size standards, see Materials and Methods. The arrows mark gene products missing in the mutants. Lane 5 in panel A contains 1/10 of the material in lane 4. WT, wild type.

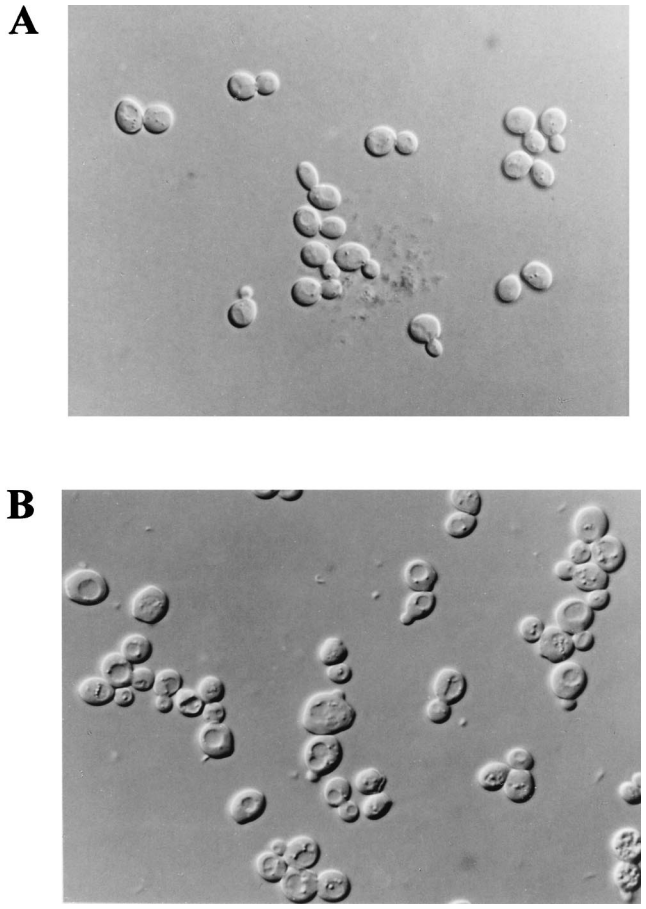

FIG. 6. Morphology of wild-type (A) and *scw4 scw10* double-mutant (B) cells.

stationary-phase cells and between 40 and 44% in logarithmic-phase cells, while the amount of *N*-acetylglucosamine was always between 2 and 3%. Such results indicate that the observed differences reflect finer structural changes in the wall rather than the overall carbohydrate composition.

The potential glucanases encoded by *SCW4* and *SCW10* also play a role in the mating process. When the various single and multiple disruptions were introduced into **a** and α cells and the cells were mixed for mating and incubated on agar plates selecting for diploids, the pattern shown in Fig. 9 was obtained. The double-mutant cross *scw4 scw10* × *scw4 scw10* showed poor mating. This phenotype was also partly compensated by deleting *BGL2* in addition (Fig. 9), an effect that was not due simply to the introduction of the *URA3* disruption marker, as shown by checking with a corresponding plasmid (data not shown). The synergism concerning the decrease in mating efficiency was again observed only with Scw4p and Scw10p, but not with Scw11p (data not shown). To study this effect in more detail, the mating of double mutant **a** and α strains was observed microscopically. It could be seen that shmoo formation, as well as contact between cells of opposite mating types and the formation of agglutinates, was not affected in the mutants. However, the formation of zygotes was much less frequent than in the case of the mating of wild-type cells.

Finally, the effect of *scw4* and *scw10* mutations on the amount of proteins covalently linked in the cell wall was also tested. As can be seen in Fig. 10, the *scw10* single mutant showed no difference, whereas *scw4* had some effect on the incorporation of the 55 to 70-kDa material extractable by laminarinase. The double mutation, however, decreased the amount of all glucanase-extractable protein bands significantly, again demonstrating the synergistic activity of the two proteins.

To investigate directly the potential exo- and/or endoglucosidase activity of Scw4p, the protein was extracted from the cell walls of the *scw10* mutant and purified to homogeneity by using a MonoQ FPLC column. However, no hydrolytic activity of the purified protein could be detected by using *p*-nitrophenyl-glucoside, laminarin, yeast glucan, chitin, or pustulan as the substrate, perhaps indicating either a more complex activity of Scw4p or an inactivation caused, for example, by reduction.

DISCUSSION

The use of nonpermeant biotinylation agents to label cell surface proteins (3, 14) most likely allows the identification of the main protein components of the cell wall. In a recent study, it was shown for *S. cerevisiae* that 20 proteins labeled by biotin could be distinguished in this way (14). These proteins were solubilized from intensively washed, isolated cell walls either by SDS or, following SDS extraction, by laminarinase treatment or by mild alkaline treatment. Thus, in principle, the procedure first suggested by Valentin et al. (23) to distinguish between extractable and covalently bound cell wall proteins has been followed. This procedure has been extended due to the observation that part of the covalently bound proteins could be released from the walls by 30 mM NaOH. The latter, after SDS-PAGE, were pure enough to be sequenced and identified. They turned out to be the *PIR* gene products (14), a gene family originally conspicuous because of internal repeats in the ORFs (22).

As pointed out here, the SDS-extracted cell wall proteins could not be purified enough to be sequenced. However, since release of proteins from intact cells by DTT resulted in a pattern comparable to that of biotinylated proteins after SDS-PAGE, such DTT-released cell wall proteins were used for further characterization, although the method required about 20 times more cells than the SDS extraction. Of the seven N-terminal sequences obtained from this group of proteins (Table 1), three were well-known cell wall proteins, showing that the labeling procedure was specific. One protein, Scw8p, was found to be the same as one of the previously identified Pir proteins (Ccw5p), while the remaining three were related, but

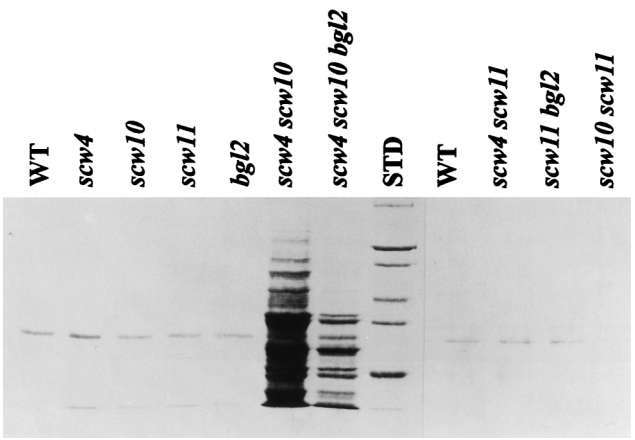

FIG. 7. Demonstration of cell wall lability. Log-phase cells were collected and extracted with DTT, and an aliquot was separated by SDS-PAGE and stained with Coomassie brilliant blue R-250. An amount corresponding to 6.6 ml of cells at an OD_{578} of 3.0 was applied to each lane of the gel. WT, wild type.

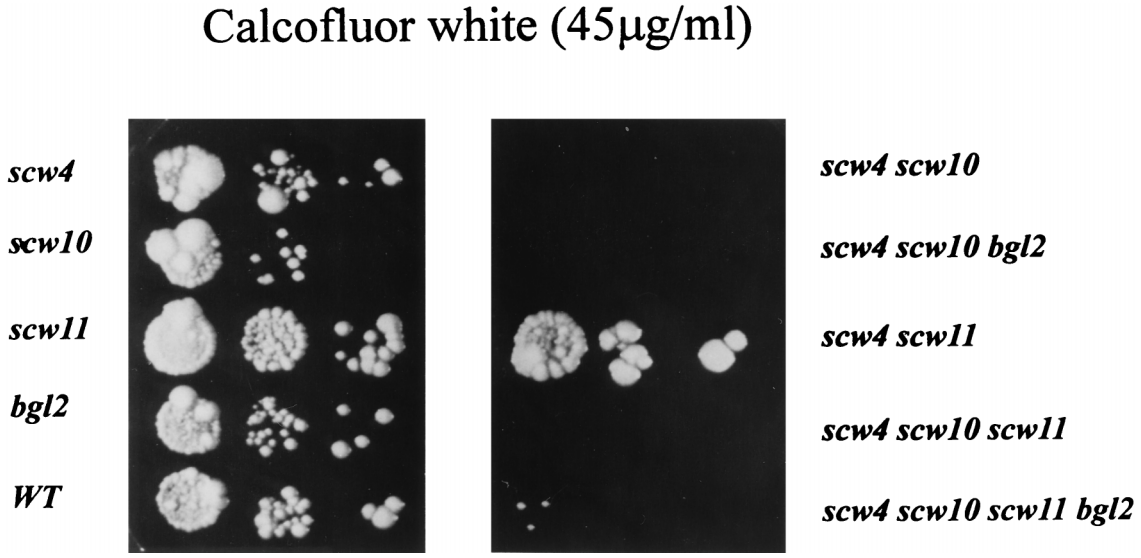

FIG. 8. Synergistic effect of the *scw4* and *scw10* mutations on calcofluor white sensitivity. Five-microliter volumes of different dilutions (2×10^5, 2×10^4, and 2×10^3 cells/ml) of stationary-phase cell cultures were dropped onto YPD plates containing 45 μg of calcofluor white and incubated for 5 days at 29°C. WT, wild type.

not identical, to previously described glucanases. Thus, the Scw4p and Scw10p proteins, as well as the related Scw11p protein, detected as a sequence contained in the yeast genome, were related to Bgl2p, an endo-β-glucanase, with 49 to 56% similarity and 27 to 29% identity. The Scw4p, Scw10p, and Scw11p proteins are 52 to 75% similar and 36 to 63% identical (Fig. 3 and 4). This group of cell wall proteins, therefore most likely represents potential endoglucanases or possibly transglucosidases (5). Scw3p, on the other hand, is not related to any known *S. cerevisiae* glucanase but rather to a β-glucosidase of *C. wickerhamii* (21) that degrades cellodextrins extracellularly. Since four additional *S. cerevisiae* ORFs detected in the yeast genome are related to the Scw3p protein identified here as a cell wall protein, this whole group of five gene products (Fig. 4) may constitute extracellular proteins involved in the degradation of polymeric glucans to make them available for fermentation. It seems possible that such proteins are substrate induced and therefore may not have been present on the cells used here.

Since processes like growth, budding, mating, ascospore formation and release, and polysaccharide fermentation most likely all require cell wall glucanases, it is not too surprising that as many as 13 genes seem to encode proteins with this or related enzymatic activities. In addition, not all of these enzymes need to be hydrolytic ones. As shown by Goldman et al. (5), the Bgl2p protein, under certain conditions, also shows

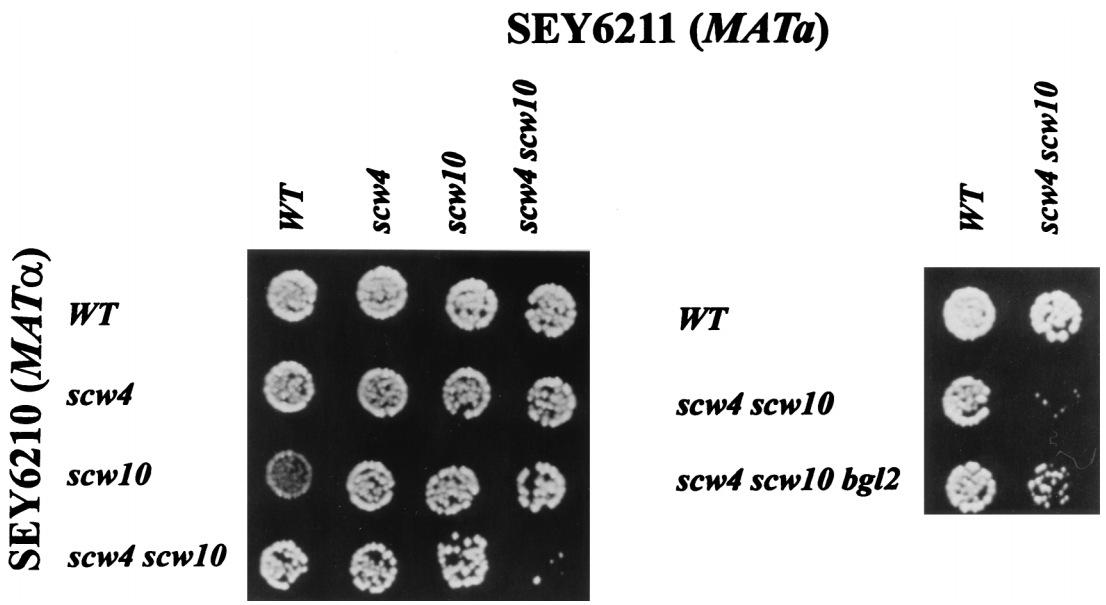

FIG. 9. Requirement of Scw4p and Scw10p for mating. The mutants indicated in a Lys^- background (SEY 6210, *MAT*α) were mated with mutants in an Ade^- background (SEY6211, *MAT***a**). Portions (10^4 cells) of stationary-phase cell cultures were mixed and dropped onto Lys^- Ade^- minimal-medium plates for detection of diploid (Lys^+ Ade^+) cells. WT, wild type.

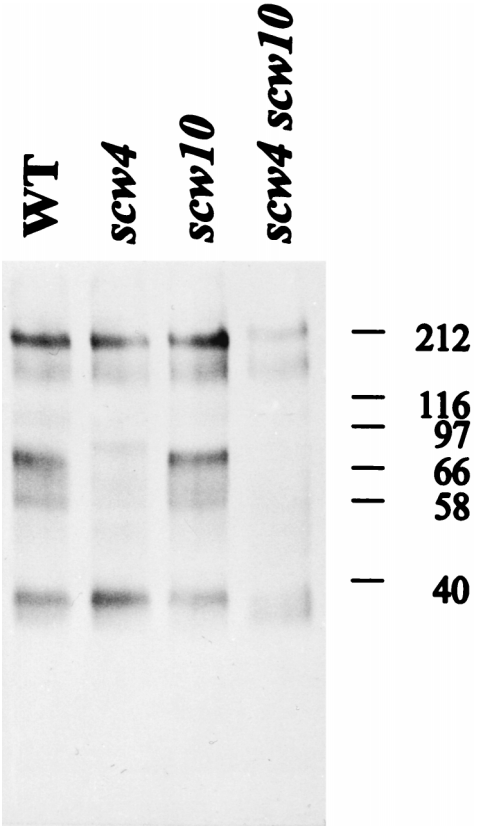

FIG. 10. Influence of Scw4p and Scw10p on the incorporation of covalently bound cell wall proteins. Cell wall proteins of the wild type (WT) and mutants were labeled by biotinylation (see Materials and Methods). Walls were purified, and the SDS-extractable proteins were extracted by Laemmli sample buffer. The remaining covalently attached proteins were then solubilized by laminarinase, subjected to electrophoresis, and blotted, and the blot was visualized by using a streptavidin-peroxidase conjugate. Each lane contains proteins obtained from cells corresponding to 25 U of OD_{600}. The values on the right are molecular sizes in kilodaltons.

transglucosylating activity in vitro. On the other hand, this cannot be taken as evidence that in vivo, this enzyme really acts as a transglucosidase. Furthermore, our attempts to demonstrate exo- or endoglucanase activity of Scw4p failed, suggesting that this protein might preferentially catalyze a transglucosylation reaction. However, the possibility that the enzyme was inactivated during the extraction with DTT cannot be excluded, either. The observation that in the *scw4 scw10* double mutant, the amount of laminarinase-extractable proteins is significantly decreased suggests that the two proteins may play a direct or indirect role in the anchoring of proteins to β-1,6-glucan, as has been suggested (8, 12). However, since some protein could still be released from the wall by glucanases even in the double mutant, a corresponding low activity must still be present in the cell wall. The fact that the mannose content of the mutant cell wall did not differ significantly from that of the wild type could be explained if proteins were attached to, or associated with, the wall by an alternative mechanism in a glucanase-insoluble form or if they could even become members of the cell wall SDS- or DTT-soluble proteins. The latter assumption would partially explain the increased amount of cell wall proteins released by DTT from the *scw4 scw10* cells (Fig. 7). It is interesting that in a number of phenomena, the potential glycosidases Scw4p and Scw10p show synergistic effects, whereas Bgl2p acts antagonistically in relation to the same phenomena (growth rate, release of proteins, mating). The observation that much more cell wall proteins are released by DTT in an *scw4 scw10* double mutant and significantly fewer are released when *BGL2* is disrupted in addition might suggest that Scw4p and Scw10p are transglucosidases. Their disruption leads to a less stable cell wall, especially if the *BGL2* gene product acts as an endoglucosidase and degrades nontransglucosylated glucan chains (see the effect of calcofluor white, Fig. 7). Disruption of *BGL2* under these conditions would give rise to a less destabilized glucan layer. The decrease in mating efficiency in *scw4 scw10* double mutants could be interpreted in an analogous way: transglucosylation of glucan polymers may be a prerequisite for stabilization of the fusing stage of the two haploid mating partners. Again, decreased glucan transglucosylation may favor the degradation of glucan chains by Bgl2p, thus further preventing successful mating. In any event, the cell wall proteins coded for by *SCW4* and its homologue *SCW10*, which most likely represent glucanases or transglucosidases, are the first such proteins shown to be involved in mating. The only glucanase known that has a similar specific function for yeast cells is that encoded by the sporulation-specific glucanase gene *SSG1* (15, 20). The precise role of the Scw4p-Scw10p-Bgl2p glucanase family, especially in mating, needs to be elucidated in future work.

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