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Identification of a Mannoprotein Present in the Inner Layer of the Cell Wall of *Saccharomyces cerevisiae*

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Cell wall extracts from the double-mutant *mnn1 mnn9* strain were used as the immunogen to obtain a monoclonal antibody (MAb), SAC A6, that recognizes a specific mannoprotein—which we have named Icwp—in the walls of cells of *Saccharomyces cerevisiae*. Icwp runs as a polydisperse band of over 180 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of Zymolyase extracts of cell walls, although an analysis of the secretory pattern of the mannoprotein shows that at the level of secretory vesicles, it behaves like a discrete band of 140 kDa. Immunofluorescence analysis with the MAb showed that Icwp lies at the inner layer of the cell wall, being accessible to the antibody only after the outer layer of mannoproteins is disturbed by treatment with tunicamycin. The screening of a λgt11 expression library enabled us to identify the open reading frame (ORF) coding for Icwp. *ICWP* (EMBL accession number YLR391w, frame +3) codes for 238 amino acids, of which over 40% are serine or threonine, and contains a putative N-glycosylation site and a putative glycosylphosphatidylinositol attachment signal. Both disruption and overexpression of the ORF caused increased sensitivities to calcofluor white and Congo red, while the disruption caused an increased sensitivity to Zymolyase digestion, suggesting for Icwp a structural role in association with glucan.

The cell wall of *Saccharomyces cerevisiae* is made up of three components, namely, glucans, mannoproteins, and chitin, and represents some 20% of the dry weight of the cell. It consists of a layered structure, with an internal layer made up of β-1,3 and β-1,6 glucans, small amounts of chitin and mannoproteins, and an outer layer of mannoproteins (13, 23). The inner layer is responsible for the shape and mechanical strength of the wall (19, 24, 50), while the outer mannoprotein layer determines the surface properties of the cell, such as hydrophobicity, electrical charge, flocculence, and sexual agglutinability, as well as limiting the porosity of the cell wall (8–10, 50).

The mannoproteins can be divided into three groups according to the methods used for their extraction from the cell wall: sodium dodecyl sulfate (SDS)-extractable mannoproteins (44), glucanase-extractable mannoproteins, which can be released only after glucanase digestion of the glucan layer (31, 44, 47), and mannoproteins extractable by reducing agents (35). The glucanase-extractable mannoproteins identified so far have two common characteristics: one is a high serine/threonine content (up to 50% of the C-terminal half of the protein), and the other is the presence of a putative glycosylphosphatidylinositol (GPI) attachment site (23, 46). Several of the proteins characterized so far, such as the sexual α-agglutinin (27, 29, 30), the anchor subunit of the **a**-agglutinin (38), and the flocculin encoded by the *FLO1* gene, play a role on the surface of the cell (42). In these proteins, the highly O-glycosylated C-terminal half may endow them with a rod-like structure (22) that facilitates the exposure of their active sites on the outer surface of the wall.

The presence of a GPI anchor has been demonstrated in the intracellular precursor of α-agglutinin (49). This GPI-anchored precursor may be an intermediate in a process that ends with the transference of α-agglutinin from the GPI anchor to the cell wall glycans in a transglycosylation reaction (7). This hypothesis, proposed by De Nobel and Lipke as a general pathway for the anchoring of glucanase-extractable mannoproteins to the structure of the cell wall, is supported by experimental evidence involving the C-terminal part of α-agglutinin (29, 30, 41, 45, 49) and also by the recent discovery of carbohydrate side chains containing β-1,6-glucose residues attached to several glucanase-extractable mannoproteins (30, 31, 45, 47).

The total number of glucanase-extractable mannoproteins identified so far is relatively small (23, 46), but it is likely that this number will increase in the future, especially considering the existence of several cell wall-like open reading frames (ORFs) in the databases of the yeast genome. Van der Vaart et al. (46) have recently reported the identification of three glucanase-extractable mannoproteins and their corresponding ORFs. Deletion mutants in one of the ORFs identified, *CWP2*, showed a decrease in the thickness of the outer mannoprotein layer, indicating that Cwp2p forms part of this layer, and an increased sensitivity to Zymolyase.

In this paper we report the use of a monoclonal antibody (MAb) for the identification of a glucanase-extractable mannoprotein and the ORF encoding it. This ORF has a putative GPI anchor and a C-terminal rich in serine/threonine. The localization of the mannoprotein in the cell wall and the effect of the disruption of the ORF and of its overexpression have also been studied.

MATERIALS AND METHODS

Strains and media. *Escherichia coli* DH5α was used for the propagation of plasmids, and strain Y1090 was used in experiments involving λgt11. *E. coli* strains were grown in Luria broth supplemented with 100 μg of ampicillin per ml when necessary. The standard yeast strains X2180-1A (*MAT***a** *SUC2 mal mel gal2 cup1*) and BMA64-1A (*MAT***a** *ade2-1 can1-100 ura3-1 leu2-3,112 trp1-Δ2 his3-11*) were used. HMSF1 (*sec1-1*), HMSF6 (*sec7-1*), HMSF176 (*sec18-1*), HMSF331 (*sec53-6*), and the corresponding wild type, X2180-1A, were used for the secretory pattern experiments. All strains, apart from the *mnn1 mnn9* strain (used as

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source of antigen), were provided by the Spanish Type Culture Collection. The *mnn1 mnn9* strain was provided by Luis Miguel Hernandez (Universidad de Extremadura, Badajoz, Spain). Yeast strains were grown in YPD (1% yeast extract, 2% Bacto Peptone, 2% glucose) or synthetic minimal medium SD (0.7% yeast nitrogen base without amino acids, 2% glucose, and amino acids as required). To analyze the effect of tunicamycin on the accessibility of the epitope recognized by the MAb SAC A6, cells were grown in YPD to early logarithmic phase, the antibiotic was added to a final concentration of 20 μg/ml, and after 2 h of incubation, the cells were harvested and indirect immunofluorescence analysis (IIF) was performed.

Reagents. Agar, yeast extract, peptone, and yeast nitrogen base were purchased from Difco Laboratories (Detroit, Mich.); Zymolyase 20T was from Seikagaku Kogyo Co. (Tokyo, Japan); phenylmethylsulfonyl fluoride was from Boehringer Mannheim; DNA restriction and modification enzymes were from Boehringer Mannheim, New England Biolabs, Inc. (Beverly, Mass.), and Amersham (Little Chalfont, Buckinghamshire, United Kingdom). Common chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) and from Panreac (Barcelona, Spain). Electrophoresis reagents were from Bio-Rad Laboratories. Nitrocellulose membranes and the enhanced chemiluminiscence reagents for developing Western immunoblots were from Amersham. Goat anti-rabbit and anti-mouse immunoglobulin G (IgG)–peroxidase and fluorescein isothiocyanate-conjugated goat anti-mouse IgG for immunofluorescence labelling were from Bio-Rad.

Isolation of cell wall mannoproteins. Cell walls from *S. cerevisiae* cells were purified and extracted with Zymolyase 20T as described by Valentin et al. (44). Briefly, cells in the early logarithmic phase were harvested and washed twice in 10 mM Tris-HCl (pH 7.4)–1 mM phenylmethylsulfonyl fluoride (buffer A). The harvested biomass was resuspended in buffer A at a proportion of 2 ml per g (wet weight), glass beads (0.45 mm in diameter) were added up to 50% of the final volume, and the cells were broken by shaking them four times for 30 s, with 1-min intervals, in a CO_2-refrigerated MSK homogenizer (Braun Melsungen AG, Melsungen, Germany). Breakage was confirmed by phase-contrast microscopy, and the cell walls were washed six to eight times in buffer A. Removal of noncovalently bound proteins was achieved by boiling the cell walls in buffer A containing 2% SDS (10 ml per g of cell walls [wet weight]) for 5 min, followed by washing them six to eight times in buffer A. The purified cell walls were finally digested in buffer A containing 500 μg of Zymolyase 20T/ml (10 ml per g of walls [wet weight]) for 3 h at 30°C in an orbital incubator at 200 rpm. The extract was separated from the cell walls by centrifugation and concentrated 20-fold with a Centriprep-10 concentration device (Amicon). Protein concentration was determined by the method of Lowry et al. (28).

Spheroplast regeneration. Exponentially growing cells were harvested, washed, resuspended at a concentration of 8 mg of cells (wet weight) per ml in pretreatment buffer (100 mM Tris-HCl [pH 8], 5 mM EDTA, 5 mM dithiothreitol), and incubated for 30 min at 30°C with gentle shaking. After pretreatment, cells were washed and resuspended in the same volume of buffer A containing 150 μg of Zymolyase 20T per ml and incubated at 30°C with gentle shaking until spheroplast formation was evidenced by the osmotic sensitivity of the cells (20 to 40 min). Spheroplasts were washed in buffer A in 1 M sorbitol and resuspended in YPD that also contained 1 M sorbitol at a concentration of 2 mg (wet weight) per ml. Regeneration took place over a 24-h period, with samples being taken at 2, 4, 6, 8, and 24 h. After harvesting of the spheroplasts, the supernatant was concentrated 30-fold in a Centriprep-10 concentrator (Amicon) and processed for SDS-polyacrylamide gel electrophoresis (PAGE).

Generation of the MAb. A MAb was generated against the mannoprotein material released by Zymolyase from the walls of cells of the *S. cerevisiae mnn1 mnn9* strain. Female BALB/c mice (8 to 10 weeks old) were immunized with intraperitoneal (i.p.) injections of 100 μg of the antigen emulsified in Freund's complete adjuvant. Two and four weeks later, the mice received a booster i.p. injection with the same amount of antigen emulsified in Freund's incomplete adjuvant. One week following the last injection, the mice were tail bled and the sera were tested for anti-antigen antibody titer by enzyme-linked immunosorbent assay. After specific immunity was confirmed, a final soluble i.p. injection of 100 μg of antigen was given to a selected mouse; 3 to 4 days later, the animal was sacrificed and its spleen was used as the source of B lymphocytes for fusion.

Myeloma cells were cultured in high-concentration glucose Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 2 mM glutamine, nonessential amino acids, penicillin (100 U ml^{-1}), streptomycin (100 μg μl^{-1}), and 15% (wt/vol) fetal bovine serum (s-DMEM). Cell fusion and selection of hybrids were carried out essentially as described by Nowinsky et al. (33). Spleen lymphocytes from the immunized mouse were fused with P3-X63 (also called Ag 8.653) murine myeloma cells (American Type Culture Collection) at a 5:1 ratio with polyethylene glycol 1500 as the fusing agent. The fused cells were distributed to 96-well culture plates (Cell-Cult) at an approximate density of 4×10^5 cells in 100 μl of s-DMEM per well; 24 h after plating, 100 μl of HAT selection medium (s-DMEM supplemented with hypoxanthine-aminopterine-thymidine) was added to each well. On day 10 postfusion, the culture supernatants were screened for the presence of antibodies against the antigen. Positive hybridomas were cloned by limiting dilution on a feeder layer of BALB/c thymocytes (approximately 10^6 cells per well) and peritoneal macrophages (approximately 5,000 cells per well). Cells were grown in HAT selection medium for 2 weeks; this medium was then replaced by HT medium (HAT selection medium without aminopterine), and cells were maintained in this medium. Positive clones were expanded, and ascites were obtained by injecting hybridoma cells (approximately 5×10^6 per mouse) into the peritoneal cavity of pristane-treated BALB/c mice (18). MAbs were purified from clarified ascites by $(NH_4)_2SO_4$ precipitation followed by ion-exchange chromatography on DEAE-Sepharose. The titers of the antibodies during the immunization and purification processes were determined by enzyme-linked immunosorbent assay.

SDS-polyacrylamide gels and Western blot analysis. Proteins were separated by PAGE according to the method of Laemmli (26) in 10 or 12% polyacrylamide gels. The proteins separated by SDS-PAGE were transferred onto Hybond C nitrocellulose membranes as described by Towbin et al. (43) and Burnette (2). Membranes were blocked overnight in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% nonfat milk. The blocked membranes were washed three times in TBST and incubated for 1 h in TBST containing the MAb SAC A6 at a dilution of 1:1,000. After three washes in TBST, the membranes were incubated for 20 min in TBST containing goat anti-mouse IgG–peroxidase at a dilution of 1:6,000 and washed in TBST. Finally, antibody binding was visualized on X-ray film by the enhanced chemiluminescence method (Amersham).

Screening of λgt11 expression libraries. About 600,000 plaques containing *Eco*RI-*Not*I inserts from a *S. cerevisiae* cDNA library in λgt11 (provided by Juan Pedro Garcia Ballesta and obtained by German Bou, Centro de Biología Molecular, CSIC-Universidad Autonoma, Madrid, Spain) were screened with the MAb SAC A6. The screening of the library was done by the procedures described by Huyhn et al. (20). Two positive clones that later proved to be identical were isolated. The insert, 0.9 kbp in size, was recovered by PCR with universal primers, subcloned into the pGEMT vector (Promega) to give rise to plasmid pIMk1, and then sequenced.

Immunofluorescence microscopy. A small volume of exponentially growing culture was harvested and washed twice in phosphate-buffered saline (PBS). The cells were resuspended in 50 μl of PBS containing the MAb SAC A6 diluted 1:25 and incubated for 1 h at 37°C. After the incubation, cells were washed three times in PBS, resuspended in 50 μl of PBS containing fluorescein isothiocyanate-conjugated goat anti-mouse IgG diluted 1:20, and incubated at 37°C for a further 2 h. After the unreacted antibody was washed out, cells were mounted on glass slides and examined with a Zeiss Photomicroscope III.

Transformation of strains, DNA isolation, and sequencing. Basic DNA manipulation and transformation in *E. coli* was performed as described by Sambrook et al. (39). Yeast transformation was carried out by the lithium acetate method (21). Plasmid DNA from *E. coli* was prepared with a Flexi-Prep kit (Pharmacia), and DNA fragments were purified from agarose gels with a Sephaglass Band-Prep kit, also from Pharmacia. Sequencing of the cDNA was performed with Amplitaq polymerase and a Dye Terminator kit (Perkin-Elmer) in an Applied Biosystems 373A automatic sequencer.

Construction of the deletion cassette and confirmation of the deletion mutant by PCR. Replacement of the genomic copy of *ICWP* by the deletion cassette was performed by one-step transplacement (37). Plasmid pIMk1 was digested with *Hin*dIII, and the 132-bp fragment released was replaced by a 1.1-kbp *Hin*dIII fragment containing the yeast *URA3* gene. The resulting pIMk2 plasmid was digested with *Sac*I and *Spe*I, the 1.9-kbp fragment containing the *ICWP* cDNA interrupted by the *URA3* gene was purified, and about 1 μg was transformed into strain BMA64 in both the haploid (BMA641A) and the diploid (BMA64d) forms. To confirm the replacement in the *ICWP* locus, stable uracil-independent transformants of the viable haploid form were analyzed by PCR with the oligonucleotides CACCTACACCTCCTACTCCACCAC (located 350 bp upstream from the ATG codon) and GAAAAGAACAAGGAGCTGATCG (located 216 bp downstream from the TAA codon) as primers. The sequences of these oligonucleotides and the lengths of the predicted PCR products were derived from the yeast genome sequence.

Generation by PCR of the ORF and its regulatory sequences and subcloning in YEPlac112. The *ICWP* ORF was generated by PCR with the oligonucleotides described in the previous section as primers. A DNA polymerase with 3′-to-5′ proofreading activity (Vent polymerase; New England Biolabs) was used to improve fidelity. We obtained a 1.3-kbp fragment that included the complete *ICWP* ORF, which extends 350 bp upstream from the ATG codon and 216 bp downstream from the stop codon, and presumably contained the promoter and terminator sequences of *ICWP*. This blunt-ended fragment was ligated to *Sma*I-digested YEplac112 (15) to give rise to plasmid pIMkIII, which was transformed into the disrupted strain obtained as described in the previous section, and the resulting transformed strain was tested for Icwp overexpression.

Phenotypic analysis of the deletion mutant and the overexpressing strain. Calcofluor white and Congo red sensitivities were tested by streaking cells on plates containing different concentrations of calcofluor white or Congo red by following the method described by Van der Vaart et al. (46). Two-microliter samples of serial 1/10 dilutions of cells grown overnight in SD and adjusted to an optical density at 660 nm (OD_{660}) of 8 (6×10^7 cells/ml) were deposited on the surfaces of YPD plates containing different concentrations (0 to 30 μg/ml) of calcofluor white or Congo red, and growth was monitored after 3 days.

Zymolyase sensitivity was also tested according to the method of Van der Vaart et al. (46). Cells from exponentially growing cultures were adjusted to an OD_{660} of 0.4 (3×10^6 cells/ml) in 10 mM Tris-HCl (pH 7.5) containing 10 μg of Zymolyase 20T/ml, and the decreases in OD were monitored over a 2-h period.

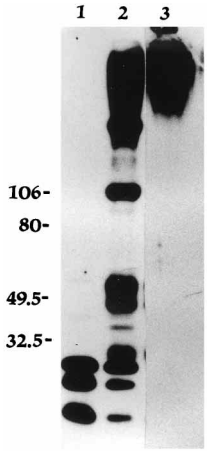

FIG. 1. Western immunoblot of Zymolyase extracts from the walls of cells of the *S. cerevisiae mnn1 mnn9* strain. Lane 1, Zymolyase; lanes 2 and 3, Zymolyase extract. Lanes 1 and 2 were incubated with the polyclonal antiserum raised against the Zymolyase extract. Lane 3 was incubated with the MAb SAC A6. Molecular mass markers (in kilodaltons) are noted at the left.

RESULTS

Raising of the MAb SAC A6 and characterization of the epitope recognized. To study the glucanase-extractable mannoproteins present in the cell wall of *S. cerevisiae*, we undertook the raising of MAbs against Zymolyase extracts of the double-mutant *mnn1 mnn9* strain. The use of this strain as the antigen source was preferred, because the low degree of glycosylation of its mannoproteins (1, 17) diminishes the risk of obtaining MAbs that recognize the glycosidic moiety. The only MAb obtained, SAC A6, was tested on immunoblots of the Zymolyase extracts used as the antigen (Fig. 1) by comparing its reactivity with that of a polyclonal antibody against the same antigen. The MAb reacts with a very polydisperse band with an average molecular mass of 200 kDa that is also recognized by the polyclonal antibody. This polydispersity may be caused either by glycosylation or by the association of the protein with the glucan network of the cell wall, which is degraded by Zymolyase.

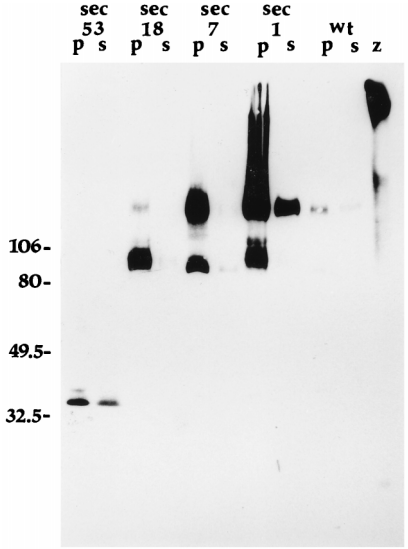

FIG. 2. Icwp forms accumulated in *sec* mutants. Exponentially growing cells were incubated at 37°C for 90 min to induce blockage in the secretory pathway. After lysis and elimination of cell walls and cell debris, the supernatant was centrifuged at 12,000 × *g* to obtain a pellet (p) and a supernatant (s), which were analyzed by Western immunoblotting with the MAb SAC A6. wt, wild-type *S. cerevisiae* X2180-1A; z, Zymolyase extract of the walls of S. cerevisiae X2180-1A cells. Molecular mass markers (in kilodaltons) are noted at the left.

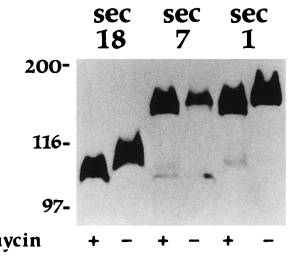

FIG. 3. Analysis of the N glycosylation of Icwp. Exponentially growing cells of the *sec* mutants were incubated for 2 h at 24°C and then for a further hour at 37°C in the presence (+) or in the absence (−) of 20 μg of tunicamycin per ml. The lysates were analyzed by Western immunoblotting with the MAb SAC A6. Molecular mass markers (in kilodaltons) are noted at the left.

Secretory pattern of Icwp and detection of N glycosylation. The secretory pattern of the protein recognized by the MAb SAC A6 was studied with temperature-sensitive secretory mutants that block secretory protein transport at specific steps at the restrictive temperature. Four different temperature-sensitive *sec* mutants were used: *sec53*, a mutant which does not produce GDP-mannose and cannot incorporate N- or O-linked oligosaccharides or GPI anchors into glycoproteins at 37°C (6); *sec18*, a mutant that is affected in vesicle fusion and blocks protein transport in a pre-Golgi compartment (11, 16); *sec7*, which blocks transport from the Golgi apparatus (14, 32, 40); and *sec1*, which blocks the fusion of the secretory vesicles with the plasma membrane (32, 40). In all cases, the presence of intermediate forms of Icwp was studied in pellet and supernatant fractions after elimination of cell walls and heavy cell debris by centrifugation at 12,000 × *g*.

A 35-kDa form was detected by the MAb in the extracts of *sec53* at the restrictive temperature (Fig. 2). This size should correspond to the protein after cleavage of the signal peptide but before any glycosylation or addition of GPI. *sec18* represents a step further in that the protein remains in the endoplasmic reticulum, but the addition of the inner cores of the N-glycosidically bound sugar moiety, of the first O-glycosidically bound mannose, and of GPI anchors has already taken place. The size of the form recognized by the MAb in *sec18* extracts, 90 kDa, shows a strong increase in size with respect to that seen in the previous step, reflecting the addition of mannose and the possible addition of a GPI anchor. Finally, the increase in size of the form detected in *sec7* extracts, 140 kDa, may reflect the modifications that take place at the Golgi apparatus: the addition of outer chains to the inner core and the addition of up to three additional mannoses to the O-

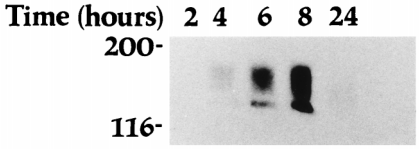

FIG. 4. Icwp forms accumulated in the supernatant of regenerating spheroplasts. Spheroplasts after different periods of regeneration were separated by centrifugation, and the supernatants were concentrated and analyzed by Western immunoblot analysis with the MAb SAC A6. Molecular mass markers (in kilodaltons) are noted at the left.

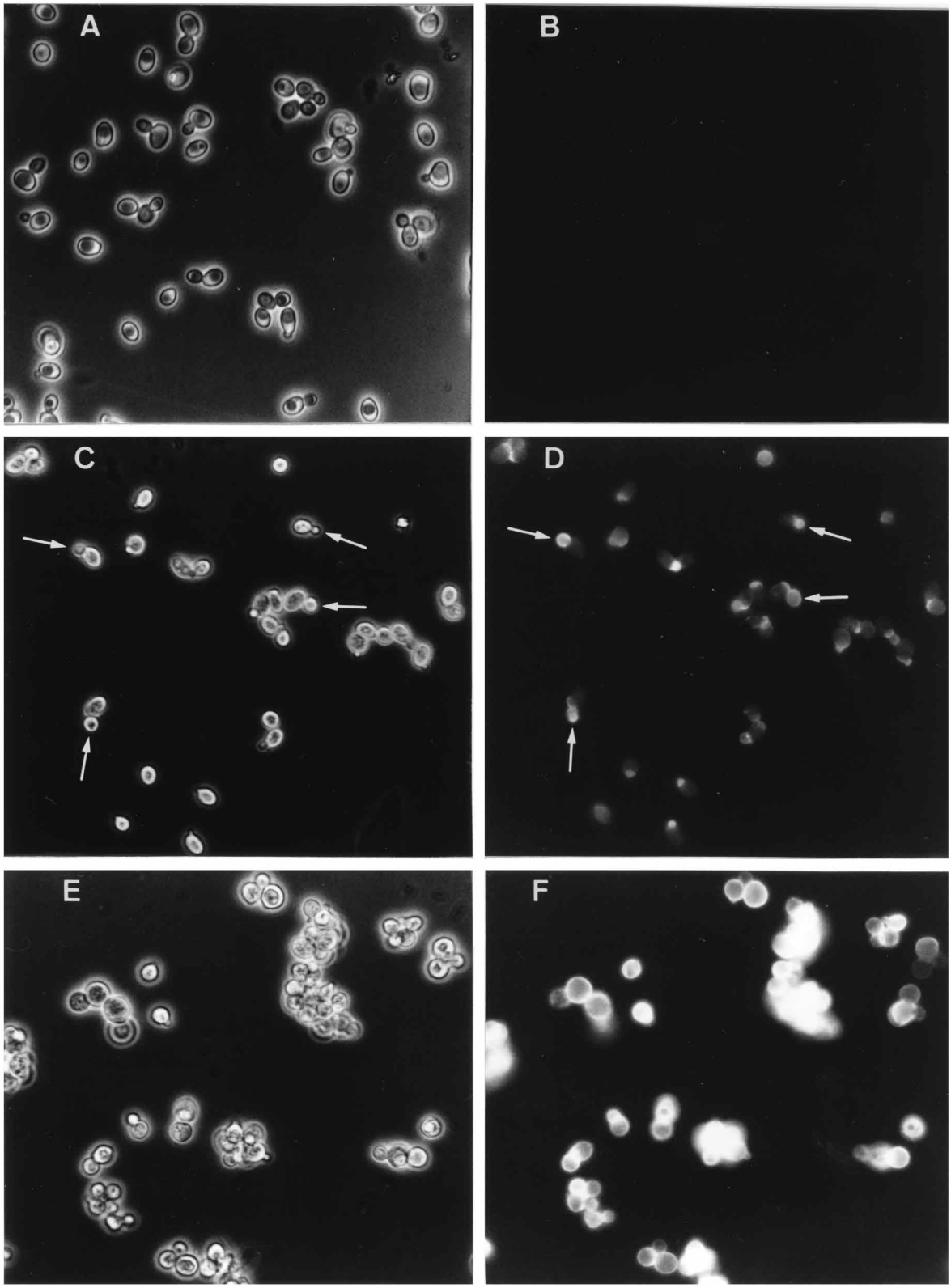

FIG. 5. IIF of *S. cerevisiae* X2180-1A cells (A to D) and *S. cerevisiae mnn1 mnn9* cells (E and F) with the MAb SAC A6. Panels C and D show cells that had been incubated for 2 h in the presence of 20 μg of tunicamycin/ml. Arrows point to the labelled surfaces of the daughter cells or growing buds. Panels A, C, and E are phase-contrast micrographs; panels B, D, and F were taken after IIF.

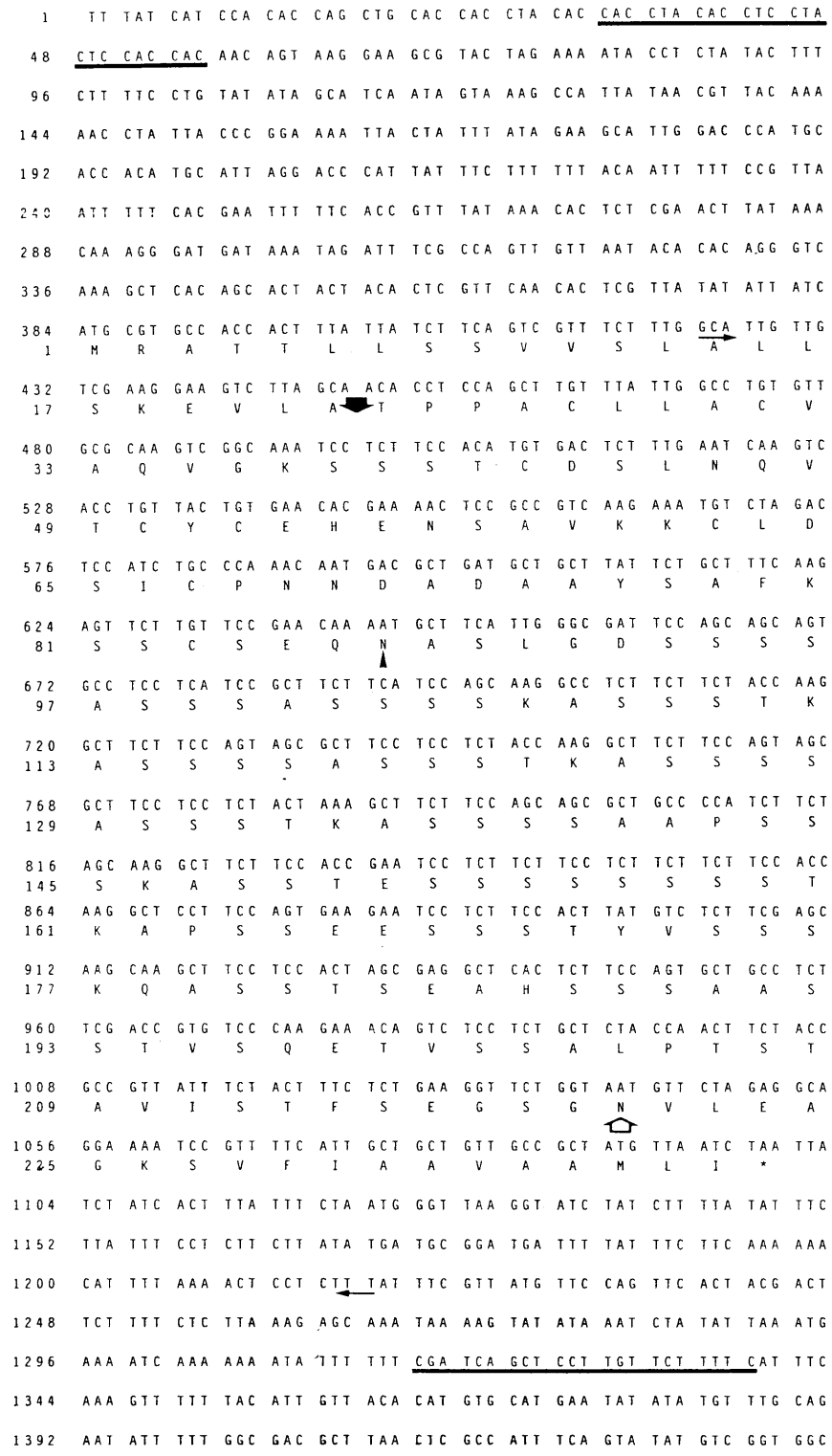

FIG. 6. DNA sequence and deduced amino acid sequence of *ICWP*. →, limits of the cDNA contained in the λgt11 clones recognized by the MAb SAC A6; ▼, putative signal peptidase recognition site; ▲, putative N-glycosylation site; ⌓, putative GPI attachment site. The sequences recognized by the oligonucleotides used to generate the complete *ICWP* ORF are underlined.

glycosidic moiety. No further increase in size was detected in the *sec1* extracts.

To determine the percentage of increase in the size of the protein that corresponded to N-glycosydically bound sugars, *sec18*, *sec7*, and *sec1* mutants were treated with tunicamycin at the restrictive temperature. The treatment resulted in a shift from the 90- to an 86-kDa form in *sec18* (Fig. 3) and a shift from the 140- to a 130-kDa form in *sec7* and *sec1*. This behav-

ior is compatible with the presence of a single N-linked carbohydrate chain and still leaves a large proportion of the molecular mass to be accounted for by O-linked carbohydrate or GPI modification.

Analysis of Icwp in the supernatant of regenerating spheroplasts. To complete the analysis of the secretory pattern, the MAb SAC A6 was used to detect the mannoprotein in the supernatant of spheroplasts after 2, 4, 6, 8, and 24 h of regeneration time. Two forms were detected after approximately 4 to 6 h (Fig. 4), a discrete band that corresponded in size to those detected in *sec7* and *sec1*, and a polydisperse band with an average molecular mass of 160 kDa. This band may represent an intermediate in the association of the mannoprotein with the incipient glucan network of the regenerating cell wall. No traces of either of the two forms could be detected after 24 h of regeneration, possibly indicating degradation of the material that had not been incorporated into the cell wall.

Immunolocalization of Icwp by IIF. The localization of the mannoprotein recognized by SAC A6 within the structures of the cell walls of wild-type and *mnn1 mnn9* cells was studied by IIF. The results show that the antibody does not label the surfaces of wild-type cells (Fig. 5A and B), indicating that the epitope is not exposed to the outside of the cell wall. However, when the cells had been treated with tunicamycin, the antibody did clearly label the daughter cells and the growing buds (Fig. 5C and D), that is, the surfaces of the cell walls that had been synthesized during the 2 h of incubation with tunicamycin. Finally, IIF of *mnn1 mnn9* cells showed complete labelling of all surfaces of the cell walls (Fig. 5E and F). Taken as a whole, these results suggest that the mannoprotein recognized by the MAb SAC A6 is not localized on the surfaces of the cell walls and becomes accessible only when the outer, porosity-limiting layer of mannoproteins becomes disturbed by the effect of tunicamycin or by the *mnn1 mnn9* mutation; accordingly, we named this mannoprotein the inner cell wall protein (Icwp).

Cloning of a cDNA coding for Icwp by screening of λgt11 expression libraries with SAC A6 MAb. To clone the gene coding for Icwp, we proceeded to screen a λgt11 cDNA expression library using the SAC A6 MAb. Two positive clones were isolated from 600,000 plaques (average size of the insert, 1.5 kb). The sizes of the inserts, the results of their restriction analysis, and their sequences showed that the two clones were identical. Comparison of the sequence obtained with those present in GenBank revealed that the insert corresponded to a sequence of chromosome XII (EMBL accession number U19729; that for the ORF was YLR391w). However, the ORF described in GenBank as the correct one of two possible ORFs for this locus (L8084.9) did not correspond to our cDNA, which extended well beyond the ATG for this ORF (frame +1), and accordingly we concluded that it corresponded to the larger, second ORF (frame +3) present in this locus.

Structural analysis of the amino acid sequence encoded by *ICWP*. The sequence of the ORF, which we termed *ICWP*, is shown in Fig. 6; it has a length of 714 bp, codes for 238 amino acids, and includes a putative signal peptide (48) with a positively charged N terminus (R at position 2), a hydrophobic core (LLSSVVSLALL, positions 6 to 16), and a more polar C terminus (SKVLATPP, positions 17 to 24) with a possible signal peptidase site (between A and T at positions 22 and 23). One hundred one of the 238 amino acids are serine or threonine (42.5%), indicating that Icwp is a highly O-glycosylated protein; the sequence also shows a unique N-glycosylation site (NAS positions 87 to 89) and, finally, a putative GPI attachment site. No specific sequence seems to be required for peptide cleavage and glycolipid addition (3), although Nuoffer et al. (34) showed that in yeast, an asparagine followed by two amino acids with relatively short side chains is the most efficient anchor attachment site. According to this, the possible GPI attachment site in our sequence would be represented by asparagine in position 220. The polar (amino acids 215 to 218) and hydrophobic (amino acids 227 to 238) regions described as necessary for GPI attachment by Caras et al. (4, 5) are also present in the sequence, as shown in the Kyte and Doolittle hydrophilicity plot (Fig. 7).

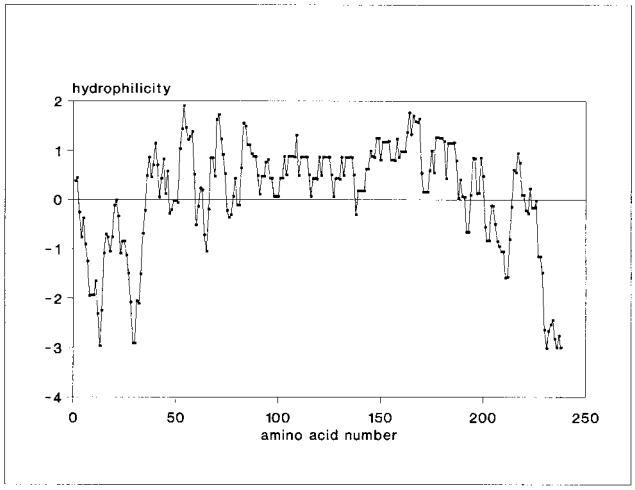

FIG. 7. Hydrophilicity plot of the predicted *ICWP* amino acid sequence by the method of Kyte and Doolittle with a window value of 7.

All the above-described features are common to other cell wall proteins (27, 29, 38, 42, 46) and are also consistent with the results shown in the previous sections. The difference between the predicted size of the polypeptide (24 kDa) and that of the mature protein at the level of secretory vesicles (140 kDa) can be accounted for by O glycosylation, a single N-glycosydic residue, and GPI modification.

Disruption of *ICWP* and characterization of the deletion mutants. Confirmation of the fact that *ICWP* codes for the protein recognized by the SAC A6 MAb was obtained after the disruption of the *ICWP* ORF and comparison of the Zymolyase extracts from the walls of cells of the parent strain and the deletion mutant by Western analysis with the SAC A6 antibody. As shown in Fig. 8, the MAb did not recognize any band in the extract corresponding to the deletion mutant. The effect of Icwp depletion on the cells was also investigated. No differences in the morphology or growth rates between the parent strain and the deletion mutant were found (data not shown). Changes in the cell wall were studied by testing the sensitivities of the deletion mutant to calcofluor white, Congo red, and Zymolyase, as described by Van der Vaart et al. (46). Sensitivities to calcofluor white and to Congo red did increase with respect to those of the parental strain (Fig. 9), and the sensitivity to Zymolyase was also markedly increased in exponentially growing cells (Fig. 10). The depletion of Icwp permitted a faster start to the digestion of the glucan by the glucanases contained in the Zymolyase, in a fashion similar to that described for the depletion of Cwp2p (46). These results are consistent with the hypothesis regarding the association of Icwp with the inner, glucan-rich layer of the cell wall.

Overexpression of *ICWP* and characterization of the overexpressing strain. The overexpression of Icwp was achieved by subcloning a PCR-generated fragment containing the *ICWP* ORF, which extends approximately 350 bp upstream from the ATG codon and approximately 216 bp downstream from the

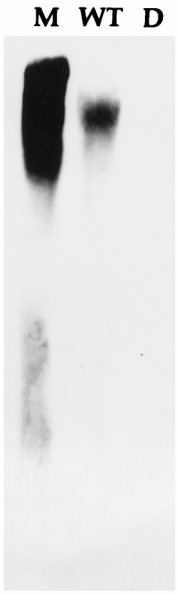

FIG. 8. Western immunoblot of Zymolyase extracts from the walls of the cells of the overexpressing strain harboring *ICWP* in a multicopy plasmid (lane M), the parental wild-type strain (lane WT), and the deletion mutant *icwp*Δ (lane D), all of which were incubated with the MAb SAC A6.

stop codon in a YEplac112 episomal vector (15). Overexpression of Icwp was confirmed by Western blot analysis of Zymolyase extracts of the deletion mutant transformed with the construction (Fig. 8). Overexpression of Icwp did not induce changes in the morphology or growth rate of the cells, but it did increase the sensitivities of the overexpressing strain to calcofluor white and Congo red (Fig. 9), so that the behavior of the disrupted strain and that of the overexpressing strain in the presence of these drugs were very similar. These results seem to indicate that any deviation from the optimal proportion of Icwp in the cell wall increases the sensitivities of the cells to drugs that interfere with the assembly of the cell wall. Overabundance of Icwp, however, neither decreased nor increased the sensitivity of the cells to Zymolyase digestion with respect to that of the parental strain (data not shown).

DISCUSSION

***ICWP* codes for Icwp.** There is clear evidence that Icwp is encoded by *ICWP*, first, because the disruption of this ORF leads to the depletion of Icwp and second, because the corresponding increase in the gene dosage leads to an increase in the amount of Icwp, both of which were detected by Western immunoblotting. Furthermore, the data that can be deduced from the sequence match the characteristics of the protein, as deduced from its secretory pattern: it is a secretory protein, it has a single N-glycosylation site, it is heavily O glycosylated, and most likely, it is modified by GPI addition. The only discordant datum is the apparent size of the protein, as determined by SDS-PAGE, both in *sec53* and in *sec1*; in *sec53*, Icwp's size is higher than the theoretical size deduced from the sequence, and in *sec1*, the total size is substantially higher than expected after glycosylation and addition of GPI. However, this anomalous behavior in SDS-PAGE analysis has also been reported for other serine- and threonine-rich, highly glycosylated cell wall proteins (29, 46).

Icwp forms part of the inner layer of the cell wall. Icwp appears as a polydisperse band in extracts obtained by digestion with glucanases. This polydispersity can be conferred only by the presence of glucan side chains attached to the mannoprotein moiety, which itself has a lower and well-defined molecular mass of approximately 140 kDa, both in secretory vesicles (*sec1*), immediately before secretion, and in the supernatant of spheroplasts, just after secretion. The existence of glucan side chains has been exhaustively reported for other

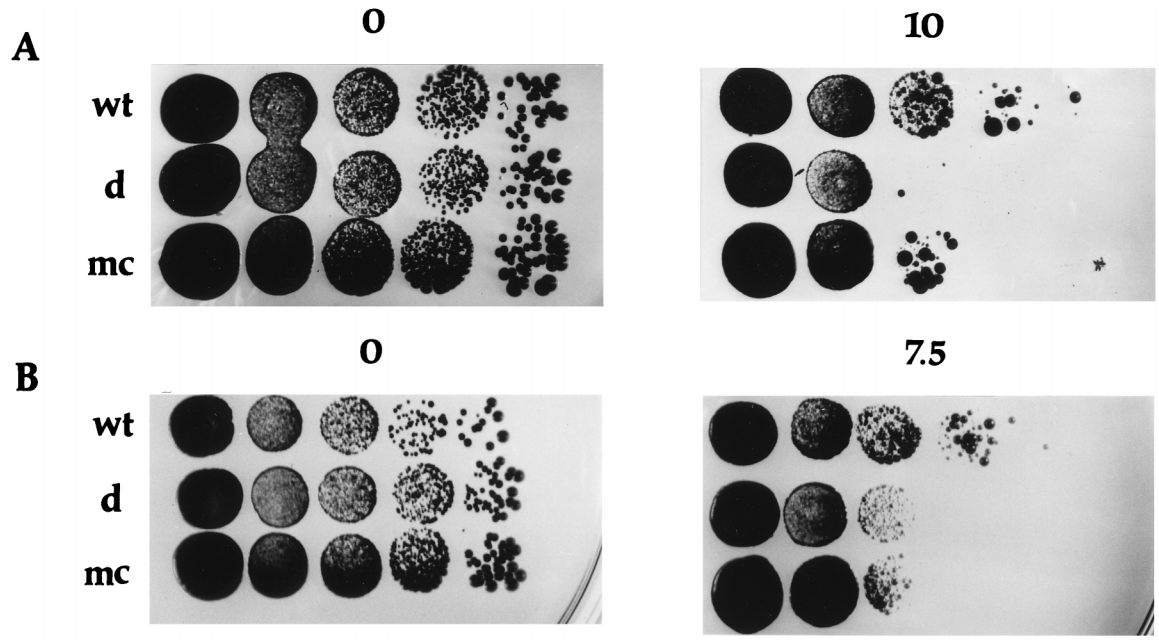

FIG. 9. Hypersensitivities to Congo red (A) and calcofluor white (B) of the disruption mutant *icwp*Δ (d), the overexpressing strain harboring *ICWP* in a multicopy plasmid (mc), and the parental wild-type strain (wt). Cells were grown in YPD, and a 1/10 dilution series of each strain was inoculated on YPD plates containing the indicated amounts (in micrograms per milliliter) of Congo red or calcofluor white.

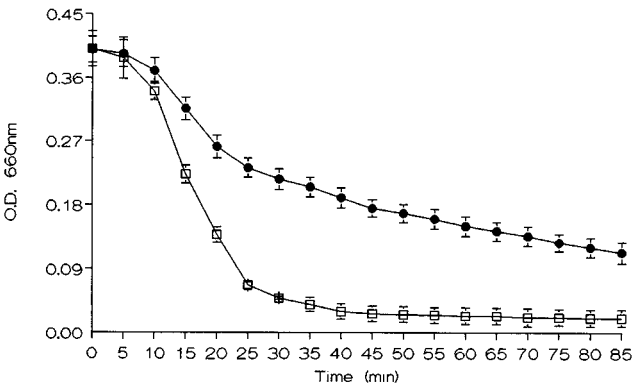

FIG. 10. Sensitivities to Zymolyase of the disruption mutant *icwp*Δ (□) and the parental strain (●). Exponentially growing cells were incubated in 10 μg of Zymolyase 20T/ml, and the decreases in the OD were monitored.

glucanase-extractable cell wall proteins (31, 45–47) with which Icwp shares features, such as a high degree of O glycosylation and a putative GPI attachment site, which may play a role in the covalent linkage to the glucan network. However, the localization of Icwp within the cell wall differs from the localizations of cell wall proteins reported so far. The immunofluorescence results show that the protein is not normally exposed to the exterior, being accessible only after the outer layer of mannoproteins has been disturbed. This result reinforces the hypothesis regarding the association of Icwp with the glucan network, since both have the same localization. Also, the presence of the free form of Icwp in the supernatant of spheroplasts indicates that this association to glucan takes place after the protein is secreted, as has been reported by Lu et al. (30) for the α-agglutinin.

Depletion and overabundance of Icwp increase the sensitivities of the cells to drugs that interfere with the assembly of the cell wall. Neither the depletion nor the overexpression of Icwp seems to affect the morphology or growth rate of the cells, indicating that Icwp is not necessary for the viability of the cells when they grow under normal conditions. However, when either the deletion mutant or the overexpressing strain was grown in medium containing calcofluor white or Congo red, an increased sensitivity was found in both cases. These compounds interfere with the assembly of the cell wall (12, 25) and have been used for the screening of cell wall mutants (36, 46) because of their ability to aggravate the effects of mutations affecting the cell wall. The fact that both the depletion of Icwp and its overabundance induced increased sensitivities to these compounds suggests that the role of Icwp requires the protein to be present in a proportional amount and that deviation from this causes a change in the structure of the cell wall.

Sensitivity to Zymolyase has also been used to highlight cell wall defects. Van der Vaart et al. (46) identified a cell wall mannoprotein (Cwp2p) that forms part of the outer layer of the cell wall and showed that its depletion induces an increased sensitivity to Zymolyase digestion. These authors related the decrease in thickness of the outer layer caused by the depletion of Cwp2p to the increased accessibility of the structural glucan to the glucanases in the Zymolyase. In our study, depletion of Icwp caused an increased sensitivity to Zymolyase. However, since Icwp does not form part of the outer layer of the cell wall, the mechanism that induces this increased sensitivity should relate to changes in the structure of the glucan network caused by the lack of Icwp.

By using a MAb, we have identified a yeast cell wall protein and the ORF that encodes it. Furthermore, we have shown that this protein lies in the inner layer of the cell wall, where it may associate with the structural glucan.

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