Sequential Fractionation and Two-dimensional Gel Analysis Unravels the Complexity of the Dimorphic Fungus *Candida albicans* Cell Wall Proteome*

Aida Pitarch‡§, Miguel Sánchez¶, César Nombela‡‖, and Concha Gil‡**

The cell wall proteins of *Candida albicans* play a key role in morphogenesis and pathogenesis and might be potential target sites for new specific antifungal drugs. However, these proteins are difficult to analyze because of their high heterogeneity, interconnections with wall polysaccharides (mannan, glucan, and chitin), low abundance, low solubility, and hydrophobic nature. Here we report a subproteomic approach for the study of the cell wall proteins (CWPs) from *C. albicans* yeast and hyphal forms. Most of the mannoproteins present in this compartment were extracted by cell wall fractionation according to the type of interactions that they establish with other structural components. CWPs were solubilized from isolated cell walls by hot SDS and dithiothreitol treatment followed by extraction either by mild alkali conditions or by enzymatic treatment with glucanases and chitinases. These highly enriched cell wall fractions were analyzed by two-dimensional PAGE, showing that a large number of proteins are involved in cell wall construction and that the wall remodeling that occurs during germ tube formation is related to changes in the composition of CWPs. We suggest that the CWP-chitin linkage is an important retention mechanism of CWPs in *C. albicans* mycelial forms. This article also highlights the usefulness of the combination of sequential fractionation and two-dimensional PAGE followed by Western blotting using specific antibodies against known CWPs in the characterization of incorporation mechanisms of such CWPs into the cell wall and of their interactions with other wall components. Mass spectrometry analyses have allowed the identification of several cell surface proteins classically associated with both the cell wall and other compartments. The physiological significance of the dual location of these moonlighting proteins is also discussed. This approach is therefore a powerful tool for obtaining a comprehensive and integrated view of the cell wall proteome. *Molecular & Cellular Proteomics 1:967–982, 2002.*

From the ‡Departamento de Microbiología II, Facultad de Farmacia, Universidad Complutense de Madrid, 28040 Madrid, Spain and the ¶Departamento de Microbiología y Genética IMB-CSIC, Universidad de Salamanca, 37007 Salamanca, Spain

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In the last 2 decades, the opportunistic fungus *Candida albicans* has been the center of considerable medical interest because it remains the cause of substantial morbidity and mortality in immunocompromised hosts, such as cancer patients, human immunodeficiency virus-infected individuals, or transplant recipients, among others (1, 2). Candidiasis can vary from superficial mucosal lesions to disseminated disease (3).

C. albicans is a polymorphic fungus that grows either in yeast form or as hyphae. Both types of morphology may be present in infected tissue, and it is therefore possible that both may play important roles in the pathogenesis of the microorganism (4, 5). Nevertheless, hyphal growth may be more critical for the pathogenesis of *Candida* since hyphae adhere more strongly to mammalian cells, promote tissue penetration, and provide a mechanism to escape the attack by macrophages. Its ability to switch between the budding and mycelial forms could be one of several factors involved in its virulence in systemic infections (6, 7). This yeast-to-hypha transition is favored by several environmental conditions such as growth at 37 °C and near neutral pH or exposure to an inductor (serum, *N*-acetylglucosamine, or proline) (8).

Since the cell wall, the outermost cellular structure, determines the shape of fungal cell, the morphogenetic conversion from the yeast to the filamentous form requires cell wall remodeling, which involves alterations in its composition and organization. This means that the cell wall is a plastic and dynamic structure that is constantly changing in response to environmental signals and the different stages of the fungal cell cycle (9, 10). Because of its privileged location within the cell, the cell wall is also the initial point of contact between the cell and its environment and thus contributes to host-fungus interactions (*e.g.* recognition, adhesion, etc.) (11). In addition, given that mammalian cells lack a cell wall, this cellular compartment could be a promising molecular target site to search for new specific antifungal drugs (12). In light of this, a better knowledge of *C. albicans* cell wall structure and composition may therefore contribute to the understanding of its involvement in fungal morphogenesis and pathogenesis as well as to the discovery of novel antifungal therapies.

The *C. albicans* cell wall is mainly composed of three components interconnected by covalent bonds: β-1,3- and β-1,6-

glucans (50–60%), mannoproteins (30–40%), and chitin (0.6–9%) (10). Cell wall structure has been studied most extensively in *Saccharomyces cerevisiae* (13–17). However, recent reports (18–21) concerning the cell wall organization of *C. albicans* have demonstrated that a similar model is also valid for this pathogenic fungus (10, 22, 23). The yeast cell wall, located outside the plasma membrane, seems to be a layered structure whose electron-dense outer layer consists of mannoproteins, whereas the electron-transparent inner layer is composed of β-1,3-glucan and chitin. Consistent with this, β-1,3-glucan and chitin form a microfibrillar network, providing rigidity to the cell wall, in which mannoproteins are embedded and determine the porosity of the cell wall (10). Chitin can be glycosidically linked to the non-reducing ends of β-1,3-glucan and/or β-1,6-glucan (24). In turn, cell wall proteins (CWPs)[1] can be coupled to cell wall components in different ways (22). Nevertheless, the total number and functions of CWPs are still poorly known. Several chemical and/or enzymatic strategies for their isolation, both from intact cells (25, 26) or from isolated cell walls after cell breakage (18, 19, 27–29), have been described. Alternatively, another approach for studying CWPs involves the analysis of proteins secreted into the medium when protoplasts are regenerating their cell walls (30–32).

In this study, in an attempt to obtain an overall view of different mannoproteins that make up the *C. albicans* wall structure, cell wall fractionation was carried out. We evaluated the protein profiles of different enriched cell wall fractions from yeast and hyphal forms using two-dimensional PAGE, allowing the establishment of reference 2-DE maps of *C. albicans* cell wall. Several cell envelope-associated proteins were analyzed by mass spectrometry. We report the identification of some non-classical CWPs previously described at the cell surface from other organisms, suggesting the presence of alternative secretory pathways hitherto undiscovered. We also highlight the usefulness of cell wall fractionation and two-dimensional PAGE followed by Western blotting using specific antibodies against *bona fide* CWPs in the characterization of their interactions with other structural cell wall components.

MATERIALS AND METHODS

Sample Preparation

Induction of the Yeast-to-hypha Transition

Cells of *C. albicans* strain SC5314 (33) were grown in YPD medium (1% Difco yeast extract, 2% peptone, 2% glucose, and 2% agar) at 28 °C up to an $A_{600\ nm}$ of 0.5–1 and washed with water. Cells were then resuspended at up to 10^5 cells/ml in Lee medium (34) at pH 4.3 or 6.7 and incubated for 6 h at 37 °C to obtain yeast or hyphal forms, respectively. Both assays were assessed by phase-contrast microscopy.

Isolation of Cell Wall Proteins

Cell wall fractionation was performed basically as described previously (35, 36) with some modifications. Yeast cells and hyphae were collected by centrifugation and filtration, respectively, and washed five times with lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride). Subsequently cells were resuspended in ice-cold lysis buffer and lysed mechanically with an equal volume of glass beads in a cell homogenizer (Braun, MSK). This procedure was carried out until complete cell breakage, verified beforehand by phase-contrast microscopic examinations and *a posteriori* by the failure of cells to grow on YPD-chloramphenicol plates. Lysed cells were separated by centrifugation at 3000 × *g* for 10 min in a cell wall fraction (pellet) and a soluble cytoplasmic fraction (supernatant). Following this, the cell wall fraction was washed five times with ice-cold water and rinsed another five times with each of the following ice-cold solutions: 5% NaCl, 2% NaCl, 1% NaCl, and 1 mM phenylmethylsulfonyl fluoride. Isolated cell walls were extracted by boiling with SDS extraction buffer (50 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 2% SDS, 10 mM DTT) for 10 min each time. This treatment was carried out once again, discarding the last extract. SDS-resistant walls were washed five times with ice-cold water and then a further 10 times with ice-cold 0.1 M NaAc, pH 5.5, 1 mM phenylmethylsulfonyl fluoride. The remaining pellet was divided into two fractions. One fraction was extracted with 30 mM NaOH overnight at 4 °C. The other fraction was digested at 37 °C for 17 h with Quantazyme *ylg* (Quantum Biotechnologies Inc., Montreal, Canada), a recombinant β-1,3-glucanase, in 50 mM Tris-HCl, pH 7.5, 10 mM DTT. Then Quantazyme-resistant cell walls were digested at 37 °C for 18 h with exochitinase (Sigma), isolated from *Serratia marcescens*, in 50 mM potassium phosphate buffer, pH 6.3. A flow chart of the experimental procedure is illustrated in Fig. 1. CWPs were precipitated with trichloroacetic acid/acetone. Protein concentration was measured with the Bradford assay (Bio-Rad).

Two-dimensional PAGE

Procedure

Samples containing 500 μg (analytical gels) or 5–10 mg (preparative gels) of protein were solubilized in a lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM dithioerythritol, 2% Pharmalyte pH 3–10 (Amersham Biosciences), bromphenol blue) (37) and were then applied onto Immobiline pH 3–10 non-linear DryStrips (18 cm long, Amersham Biosciences). Isoelectric focusing was performed on an IPGphor system (Amersham Biosciences) at 15 °C using the following program: (i) for analytical gels: passive rehydration for 16 h, 500 V for 1 h, 500–2000 V for 1 h, and 8000 V for 5.5 h, or alternatively (ii) for preparative gels: 30 V (active rehydration) for 13 h, 500 V for 1 h, 1000 V for 1 h, 2000 V for 1 h, 2000–5000 V for 3 h, and 8000 V for 11 h. After this, immobilized pH gradient strips were reduced (2% dithioerythritol) and then alkylated (2.5% iodoacetamide) in equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 6.8, 30% glycerol, 2% SDS) (38). The second dimension run was carried out on homogeneous 10% T, 1.6% C (piperazine diacrylamide) polyacrylamide gels (1.5 mm thick) at 40 mA per gel for 6 h using a Protean II gel tank (Bio-Rad). M_r and pI values were estimated using internal two-dimensional SDS-PAGE and external SDS-PAGE standards (Bio-Rad).

Gel Staining

Silver Staining—Preparative gels were silver-stained as described by Shevchenko *et al.* (39). Alternatively, analytical gels were fixed first

[1] The abbreviations used are: CWP, cell wall protein; 2-DE, two-dimensional electrophoresis; ACN, acetonitrile; AmBic, ammonium bicarbonate; DTT, dithiothreitol; GPI, glycosylphosphatidylinositol; Pir, protein with internal repeats; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; TOF/TOF, tandem TOF; MS, mass spectrometry; MS/MS, tandem MS.

in 40% methanol and 10% acetic acid for 1 h and then in 5% ethanol and 5% acetic acid overnight. The gels were rinsed with 7.5% acetic acid and incubated in 10% glutaraldehyde for 30 min. After this the gels were extensively washed with water and stained with an ammoniacal silver nitrate solution for 30 min. The gels were washed and then developed in 0.01% citric acid and 0.1% formaldehyde. Staining was halted with 5% Tris and 2% acetic acid.

Coomassie Brilliant Blue Staining—Preparative gels were stained as reported elsewhere (40).

Periodic Acid-Schiff Staining—Staining with the periodic acid-Schiff reagent was performed according to Zacharius *et al.* (41). Briefly, analytical gels were fixed in 12% trichloroacetic acid and washed with water. They were incubated in 1% periodic acid and 3% acetic acid for 50 min and rinsed with water for 18 h. Gels were incubated in Schiff's reagent (Merck) in the dark for 50 min and washed first with 0.5% sodium bisulfite and then with water.

Image Analysis

Two-dimensional images were captured by scanning the silver-stained gels using a GS-690 imaging densitometer (Bio-Rad) and digitalized with Multi-Analyst software (Bio-Rad). Different two-dimensional images were processed, including detection, volumetric quantification, matching, statistical analysis (Student's *t* test), and editing of molecular masses and pI of spots, using Melanie 3.0 software (Bio-Rad).

Western Blotting

Analytical two-dimensional PAGE and SDS-PAGE gels were electroblotted onto nitrocellulose membranes in Towbin buffer at 50 mA overnight (42). Blots were processed following standard protocols (43). Monoclonal antibody against *C. albicans* Enop (dilution 1:6000) and polyclonal antibodies against *C. albicans* Pgkp (dilution 1:5000) and *S. cerevisiae* Gapp (dilution 1:10000), Bgl2p (dilution 1:5000), Hsp150p/Pir2p (dilution 1:5000), Gas1p (dilution 1:3000), and Sec14p (dilution 1:3000) were used for immunodetection. Immunoreactive spots were detected using horseradish peroxidase-labeled anti-mouse or anti-rabbit IgGs (Amersham Biosciences), depending on the first antibody, and an enhanced chemiluminescence detection system (ECL, Amersham Biosciences).

Mass Spectrometric Analysis

In-gel Destaining, Reduction, Alkylation, Deglycosylation, and Digestion of Protein Samples

Spots of interest were manually excised from preparative silver- or Coomassie-stained 2-DE gels, depending on their intensity and relative volume. Silver-stained gel pieces were destained as described by Gharahdaghi *et al.* (44). Briefly, gel spots were incubated in 100 mM sodium thiosulfate and 30 mM potassium ferricyanide, rinsed twice in 25 mM ammonium bicarbonate (AmBic) and once in water, shrunk with 100% acetonitrile (ACN) for 15 min, and dried in a Savant SpeedVac for 20–30 min. Alternatively, Coomassie Blue-stained gel pieces were destained with ACN, washed twice with 50% ACN in 25 mM AmBic, and vacuum-dried. Both types of spots were reduced with 10 mM dithioerythritol in 25 mM AmBic for 30 min at 56 °C and subsequently alkylated with 55 mM iodoacetamide in 25 mM AmBic for 20 min in the dark. Gel pieces were alternately washed with 25 mM AmBic and ACN, and dried under vacuum. Following this, certain excised spots were also in-gel deglycosylated by treatment with 200 units/ml PNGase F (*N*-glycosidase F, Roche Molecular Biochemicals) in 25 mM AmBic overnight at 37 °C. Glycans were removed from the gel pieces using six changes of 25 mM AmBic with sonication for 30 min. Gel spots were subsequently shrunk with ACN and vacuum-dried. All gel pieces were incubated with 12.5 ng/μl sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mM AmBic overnight at 37 °C. After digestion, the supernatants (crude extracts) were separated. Peptides were extracted from the gel pieces first into 50% ACN, 1% trifluoroacetic acid and then into 100% ACN. All extracts were pooled, and the volume was reduced by SpeedVac.

MALDI-TOF MS and MALDI-TOF/TOF MS

One microliter of each sample (both crude extracts and extracted peptides) and then 0.4 μl of 3 mg/ml α-cyano-4-hydroxycinnamic acid matrix (Sigma) in 50% ACN, 0.01% trifluoroacetic acid were spotted onto a MALDI target. Samples for MS/MS sequencing were mixed 1:1 with α-cyano matrix (5 mg/ml in 50% ACN, 0.3% trifluoroacetic acid) and spotted on the stained steel plate. MALDI-TOF MS analyses were performed on a Voyager-DE STR instrument (PerSeptive Biosystems, Framingham, MA). Peptides were selected in the mass range of 700–4500 Da. All mass spectra were externally calibrated with the Sequazyme peptide mass standards kit (PerSeptive Biosystems) and internally with trypsin autolysis peaks. MS/MS sequencing analyses were carried out using the MALDI-tandem time-of-flight mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA).

Database Search

The peptide mass fingerprinting and peptide fragment-ion data obtained from MALDI-TOF and MS/MS analyses, respectively, were used to search for protein candidates in two sequence databases (*i.e.* SWISS-PROT/TrEMBL non-redundant protein database (www.expasy.ch/sprot) and a nearly complete *C. albicans* genomic database, namely CandidaDB (genolist.pasteur.fr/CandidaDB)) using MS-Fit and MS-Tag (prospector.ucsf.edu), ProFound (prowl.rockefeller.edu), and/or Mascot (www.matrixscience.com) software programs. Initial search parameters were as follows: Cys as *S*-carbamidomethyl derivative and Met in oxidized form, one missed cleavage site, peptide mass tolerance of 50 ppm, and MS/MS tolerance of $\pm$0.5 Da. Nucleotide sequence data for *C. albicans* were obtained from the Stanford Genome Technology Center website at www.sequence.stanford.edu/group/candida. Sequencing of *C. albicans* was accomplished with the support of the NIDCR, National Institutes of Health and the Burroughs Wellcome Fund. Information about coding sequences and proteins were obtained from CandidaDB available at www.pasteur.fr/Galar_Fungail/CandidaDB/, which has been developed by the Galar Fungail European Consortium (QLK2-2000-00795).

RESULTS

Enrichment of C. albicans CWPs Using Sequential Extraction According to the Type of Linkage with Other Cell Wall Components

Cell walls were obtained by mechanical disruption of *C. albicans* yeast and hyphal forms, verifying complete cell breakage by microscopic examinations and by lack of growth on YPD-agar plates. This confirmation was carried out to avoid cell lysis of putative intact cells by subsequent enzymatic extractions and thus contamination by intracellular material. In addition, cell walls were extensively washed with solutions of decreasing concentrations of NaCl to remove any extracellular or cytosolic protein contaminants that might be adhered to the cell walls through electrostatic forces.

Isolated cell walls from both *C. albicans* morphologies were treated with DTT and hot SDS, extracting cell surface-asso-

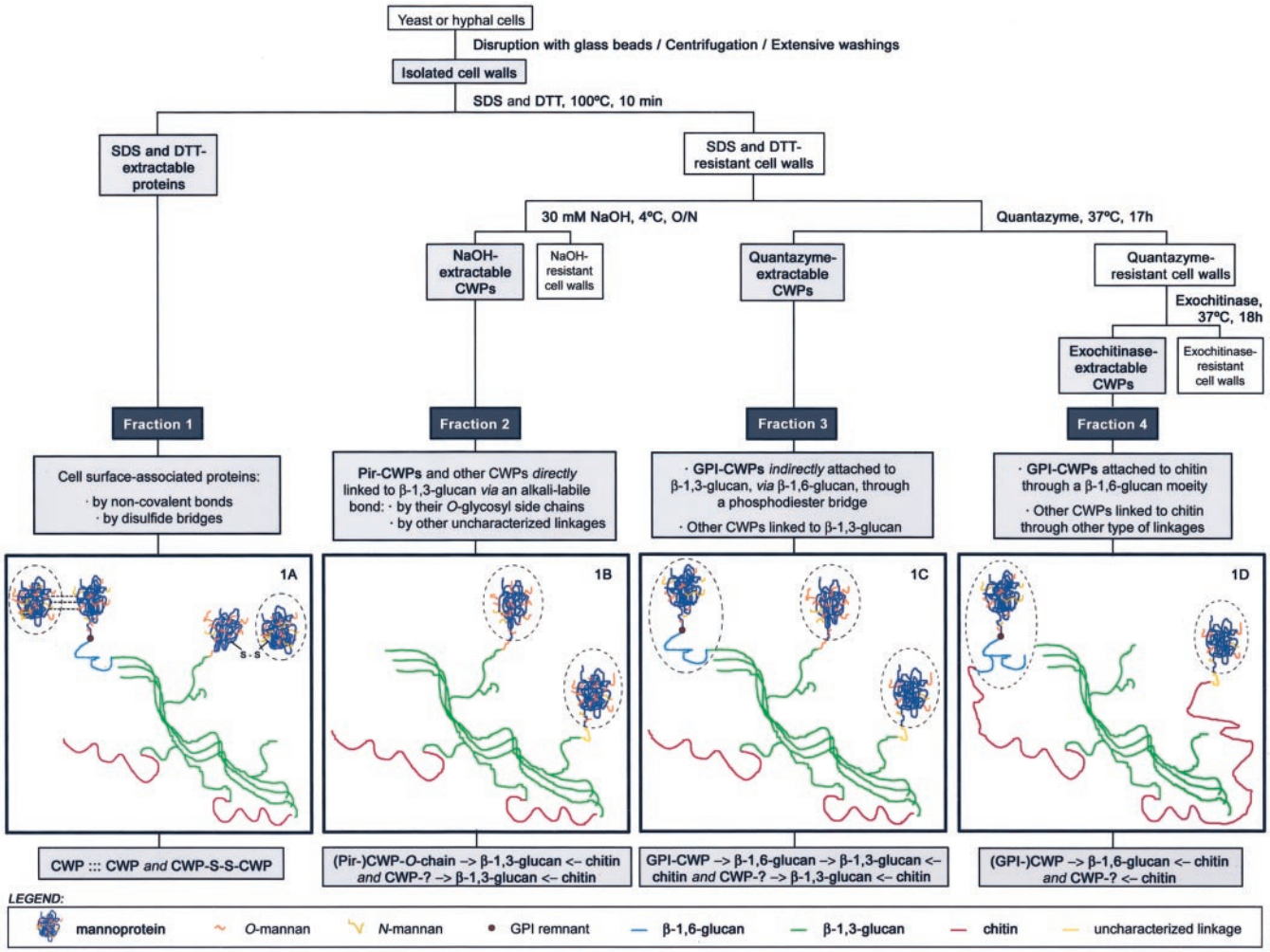

FIG. 1. **Schematic diagram showing the procedure used in cell wall fractionation in *C. albicans* yeast and hyphal forms.** CWPs were solubilized sequentially according to the type of linkages that they establish with other cell wall components. Four different cell wall fractions were obtained. At the *bottom*, a schematic representation of the macromolecular structural complex of these proteins is shown. *Circles* show the extracted proteins. *A*, proteins released by hot SDS under reducing agents are loosely associated with the cell surface through non-covalent interactions (*black broken lines*) or disulfide bridges (-*S*– *S*-). *B*, molecular module of CWPs released under mild alkali conditions (β-elimination process) based on data from Kapteyn *et al.* (107) and Mrsa *et al.* (108). They are covalently linked to the β-1,3-glucan network (*green*) either through their *O*-chains (*orange*), these CWPs (*e.g.* Pir-CWPs) being extracted by breakage of such chains, or by other uncharacterized linkages (*yellow*). *C*, characteristic supramolecular complex of GPI-CWPs solubilized by β-1,3-glucanases according to data from Kapteyn *et al.* (36) and Kollár *et al.* (57). GPI-CWPs are covalently linked to β-1,6-glucan (*blue*) through a phosphodiester bridge or a remnant of their GPI anchor (*brown*). In turn β-1,6-glucan is bound to β-1,3-glucan (*green*), which can be anchored to chitin (*red*). Other CWPs (*right*) attached to β-1,3-glucan through other linkages can be extracted by this treatment. *D*, module of β-1,3-glucanase-resistant proteins released by exochitinase treatment based on data from Kapteyn *et al.* (24). β-1,3-Glucanase-resistant CWPs linked to chitin (*red*) either through a β-1,6-glucan moiety (*blue*), such as some GPI-CWPs (*left*), or via some hitherto uncharacterized linkage (*yellow*) may be released by this procedure (*right*). *O/N*, overnight.

ciated proteins, *i.e.* proteins loosely associated with the cell wall and a potential small fraction of membrane proteins (Fig. 1, *Fraction 1*). This treatment was performed once again, discarding this second extract, to ensure the complete release of SDS-solubilized proteins. After various washings of SDS-resistant cell walls, these were divided into two aliquots to extract CWPs linked to the β-1,3-glucan network of the cell walls. (i) In the first aliquot, cell walls were treated with mild alkali, obtaining mainly an enriched fraction of proteins directly linked to β-1,3-glucan through their *O*-glycosyl side chains (such as Pir-CWPs) or by other alkali-sensitive linkages (Fig. 1, *Fraction 2*). (ii) In the second aliquot, a recombinant β-1,3-glucanase (Quantazyme *ylg*) was used to remove the β-1,3-glucan layer of the cell wall, releasing mainly an enriched fraction of mannoproteins indirectly attached to β-1,3-glucan, via β-1,6-glucan, by a phosphodiester bridge (GPI-CWPs) and other CWPs anchored to β-1,3-glucan (Fig. 1, *Fraction 3*). Finally, insoluble Quantazyme-extracted cell walls

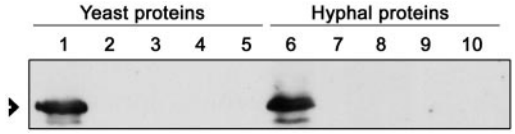

FIG. 2. **Western blot analysis of *C. albicans* cytoplasmic and cell wall extracts.** Soluble cytosolic protein (*lanes 1* and *6*) and SDS- and DTT- (*lanes 2* and *7*), NaOH- (*lanes 3* and *8*), Quantazyme- (*lanes 4* and *9*), and exochitinase (*lanes 5* and *10*)-extractable cell wall protein from yeast (*lanes 1–5*) and hyphal (*lanes 6–10*) forms were separated by SDS-PAGE, blotted, and immunodetected using antibodies against Sec14p. The *arrowhead* shows the position of Sec14p.

were digested with an exochitinase, allowing the extraction of an enriched fraction of β-1,3-glucanase-resistant proteins linked to chitin, such as some GPI-CWPs and other mannoproteins (Fig. 1, *Fraction 4*).

In an attempt to illustrate how specific the purification strategy utilized for the enrichment of CWPs is, protein extracts from both the soluble cytoplasmic fraction and the four cell wall fractions cited above were immunoblotted using antibodies against Sec14p, a soluble protein previously exploited as a marker for cytosolic contamination (45). No detectable signal of Sec14p was shown on the different cell wall extracts (Fig. 2), demonstrating that contamination with soluble intracellular proteins is very unlikely.

Visualization of a Large Number of Protein Spots on C. albicans Cell Wall Fractions

We analyzed the overall protein profiles of the different CWP fractions described above using two-dimensional PAGE. To this end, two experiments (yeast and hyphal CWPs) with four different kinds of samples (SDS-, NaOH-, Quantazyme-, and exochitinase-extracted CWPs) were performed in triplicate from a single preparation, visualizing identical and reproducible protein spot patterns for each fraction. In addition, similar protein patterns were also produced with different preparations obtained on different occasions, indicating their reproducibility. One gel of each yeast and hyphal CWP fraction was used as a reference 2-DE map (Figs. 3–6).

Using these four cell wall fractions, more than 1500 visible protein spots were developed on silver-stained 2-DE gels with a pH 3–10 non-linear gradient. About 1560 and 1660 spots were visualized on 2-DE gels from the yeast and filamentous walls, respectively. However, some individual proteins may be extracted under the different conditions described or correspond to processing or post-translational modifications of a single protein.

The distribution of all these CWP spots was not homogeneous in the four cell wall fractions, different protein percentages being obtained in each. For instance, (i) a large subpopulation of cell envelope proteins (~700 spots) was SDS- and reducing agent-extractable (Fig. 3). (ii) About 290 and 210 spots from SDS-resistant yeast and mycelial walls, respectively, were released under mild alkali conditions (Fig. 4). (iii)

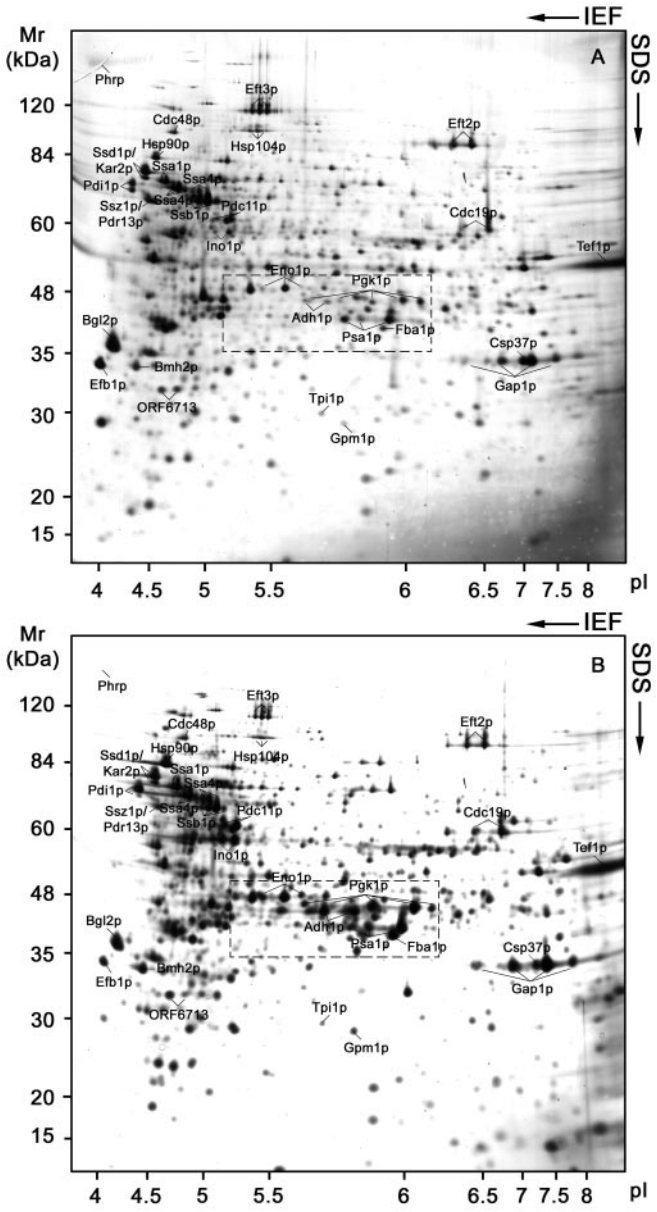

FIG. 3. **Silver-stained 2-DE gels of cell surface-associated proteins, SDS- and reducing agent-extractable proteins, from *C. albicans* yeast (*A*) and hyphal (*B*) forms.** Both gels were processed in parallel. Labeled spots were identified by mass spectrometry and/or immunodetected using specific antibodies against *bona fide* CWPs. The *broken rectangle* shows one of the most significant differences in protein expression between both morphological forms. *IEF*, isoelectric focusing.

Approximately one-third of all CWPs (nearly 450 spots) were solubilized with β-1,3-glucanases (Fig. 5). (iv) And finally, a small subpopulation of CWPs (about 65 and 250 spots from yeast and hyphal walls, respectively) was β-1,3-glucanase-resistant and exochitinase-extractable (Fig. 6).

A high molecular mass polydisperse smear was visualized by silver staining (Figs. 4–6) and by using the periodic acid-Schiff reagent (data not shown). Results indicated the glyco-

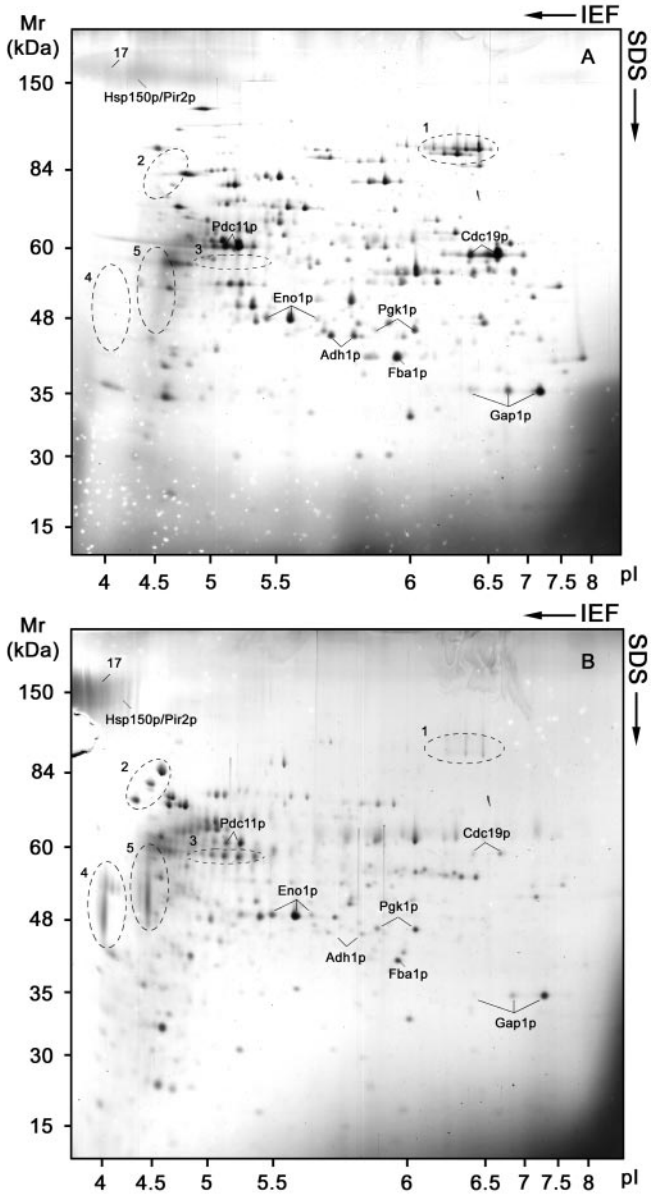

FIG. 4. **Silver-stained 2-DE protein expression profiles of mild alkali-solubilized CWPs from *C. albicans* yeast (*A*) and filamentous (*B*) forms.** Both gels were processed in parallel. Labeled spots were identified by mass spectrometry and/or immunodetected using specific antibodies against known CWPs. *Broken circles* indicate major differences between both types of growth. *IEF*, isoelectric focusing.

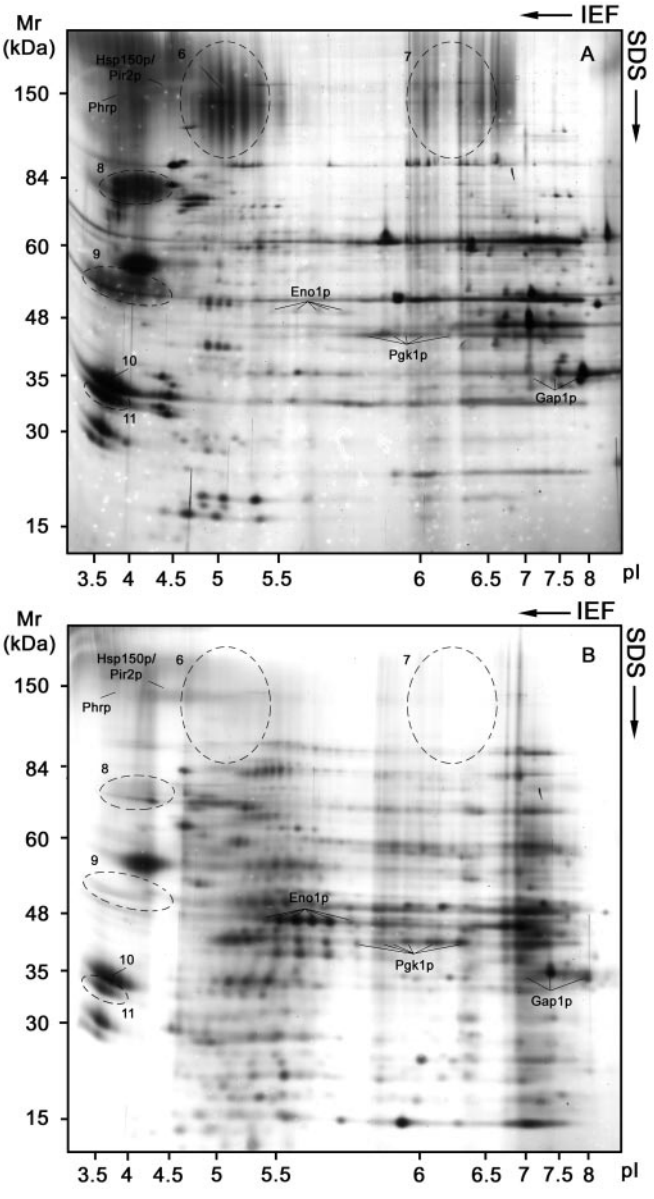

FIG. 5. **β-1,3-Glucanase-extractable CWPs from *C. albicans* yeasts (*A*) and hyphae (*B*) separated by two-dimensional PAGE and silver-stained.** Both gels were processed in parallel. Labeled spots were identified by mass spectrometry and/or immunodetected using specific antibodies against *bona fide* CWPs. *Broken circles* depict the main differences between both morphologies. *IEF*, isoelectric focusing.

sidic nature of the smear by its presence either at the top of 2-DE gels from Quantazyme-released CWPs or at the top left of 2-DE maps from both NaOH- and exochitinase-solubilized CWPs.

In parallel, a reference 2-DE pattern of soluble cell extracts was also obtained (Fig. 7) as a further control to evaluate the first step in the enrichment of cell surface proteins. Some of the most abundant spots were present in both whole cell extracts and DTT- and SDS-extracted fractions, both fractions displaying similar 2-DE profiles. However, various specific proteins were exclusively visualized in the SDS-released fraction (*e.g.* spot labeled *Bgl2p* in Fig. 3), and many other proteins present in the soluble cell extracts seemed to be absent from such cell envelope extracts. In addition, a noticeable enrichment of some DTT- and SDS-released proteins was observed on the whole (*e.g.* spots named as *Eft3p* and *Tef1p* in Figs. 3 and 7), indicating that these proteins cannot derive from mere intracellular contamination.

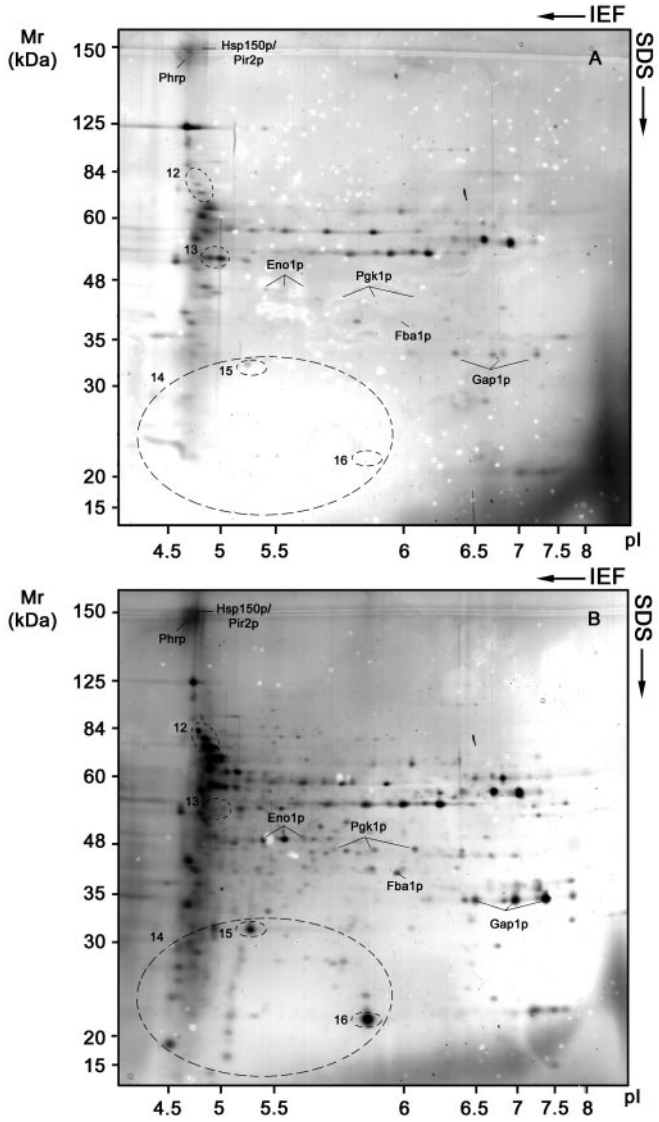

FIG. 6. **Silver-stained 2-DE protein patterns of exochitinase-released CWPs from *C. albicans* yeasts (*A*) and hyphae (*B*).** Both gels were processed in parallel. Labeled spots were identified by mass spectrometry and/or immunodetected using specific antibodies against known CWPs. *Broken circles* depict the major differences between both types of growth. *IEF*, isoelectric focusing.

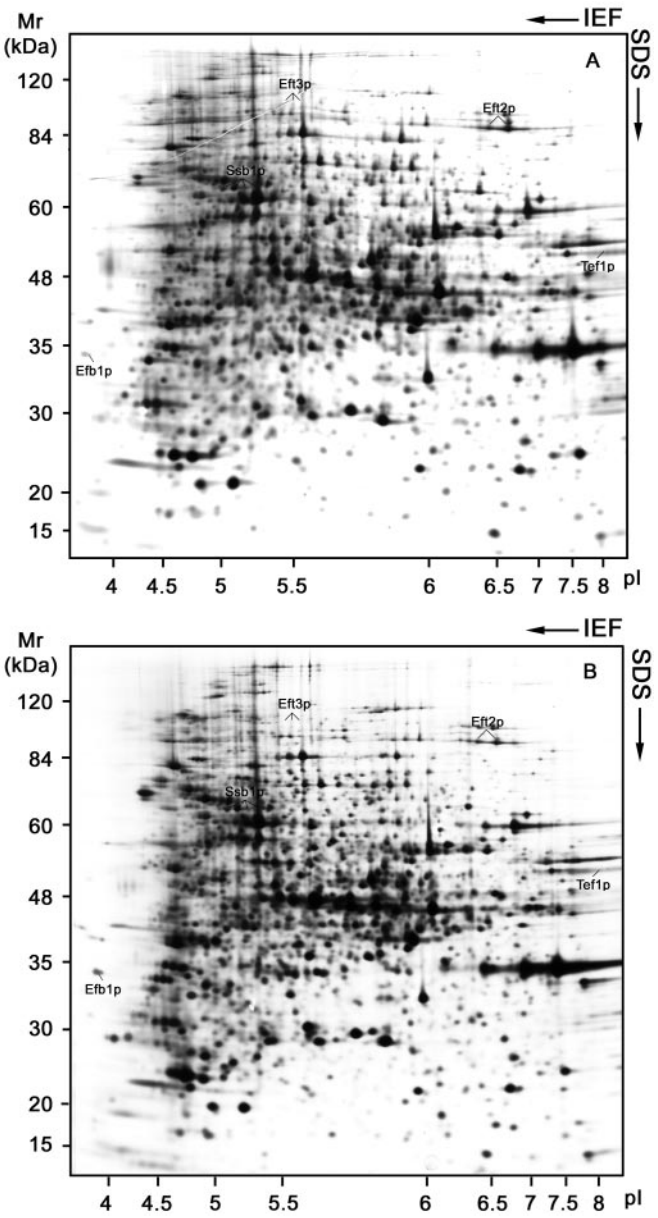

FIG. 7. **Silver-stained 2-DE protein patterns of soluble cytoplasmic proteins from *C. albicans* yeast (*A*) and hyphal (*B*) forms.** Both gels were processed in parallel. *Labeled spots* depict some examples of proteins whose SDS- and DTT-extracted counterparts (Fig. 3) show a noticeable enrichment. *IEF*, isoelectric focusing.

Detection of Some Bona Fide CWPs in the Different Cell Wall Fractions

To characterize the mechanisms of retention of some well known CWPs in the cell wall, yeast and hyphal CWP extracts were separated by two-dimensional PAGE, blotted onto nitrocellulose membranes, and immunodetected by specific antibodies (against Bgl2, Hsp150/Pir2, Gas1, Eno, Pgk, and Gap proteins). Bgl2p was detected only among SDS-solubilized proteins (Fig. 3). A Hsp150p/Pir2p-related protein was visualized on NaOH-, β-1,3-glucanase-, and exochitinase-extractable CWP two-dimensional gels (Figs. 4–6). A Gas1p homologue protein (Phrp) was found in SDS-, β-1,3-glucanase-, and exochitinase-released protein fractions (Figs. 3, 5, and 6). Intriguingly, three glycolytic enzymes, Enop, Pgkp, and Gapp, were immunodetected in all CWP fractions (Figs. 3–6).

All the protein spots were subsequently confirmed by mass spectrometry analysis. These were successfully identified (Table I) with the exception of Hsp150p and Phrp (two highly glycosylated CWPs), whose MS/MS spectra yielded short amino acid sequences that did not permit their identification.

TABLE I
Summary of confirmed identifications of cell surface-associated protein spots

Spot name[a,b]	Protein function	Cell wall fraction[c]	Mr (kDa)[d]	pI[d]	Hyphal induction[e]	No. matched peptides[f]	Percentage of sequence coverage[g]	Accession number[b]
I. Classical CWPs								
Bgl2p	β-1,3-Glucosyltransferase	1	35–37	4.3		8	20	CA1541
Hsp150p/Pir2p	150-kDa heat shock glycoprotein, member of the Pir-CWP family	2,3,4	150	3.5–5.1		MS/MS[h,i,j]		
Phrp	GPI-anchored pH-responsive glycosyl transferase (homologue of *S. cerevisiae* Gas1p)	1,3,4	130–145	3.5–5.1		MS/MS[h,i,j]		
10–11	Putative β-1,3-glucanase[k]	3	35–37	3.4–4.2	−	MS/MS[h,i,k]		
II. Heat shock and chaperone proteins								
Hsp90p	90-kDa heat shock protein	1	82	4.7	+	26	39	CA4959
Ssa1p	Member of the HSP70 family	1	74	4.7–4.8		6–14	13–37	CA2857
Ssa4p	Member of the HSP70 family	1	71	4.8–4.9		8–17	14–30	CA1230
		1	69	4.9–5.1	+	9–21	19–39	
Ssb1p	Member of the HSP70 family	1	66	4.9–5.1		11–26	25–36	CA3534
Ssd1p/Kar2p	Member of the HSP70 family	1	74–78	4.6		13–16	23–32	CA0915
Ssz1p/Pdr13p	Member of the HSP70 family	1	66	4.6	−	8	22	CA4844
Hsp104p	104-kDa heat shock protein	1	99	5.3–5.5		16–27	21–33	CA5135
III. Folding proteins								
Pdi1p	Protein disulfide isomerase	1	73	4.4	+	14	23	CA1755
		1	70	4.4	−	12	22	
IV. Elongation factors								
Eft3p	Translation elongation factor 3	1	116	5.3–5.5		16–31	14–28	CA3081
Eft2p	Translation elongation factor 2	1	93	6.3–6.5		12–15	16–24	CA2810
Tef1p	Translation elongation factor 1α	1	51	7.6–8.9		11	30	CA0362
Efb1p	Translation elongation factor 1β	1	35	4.1		MS/MS[h,l]		CA4862
V. Glycolytic and fermentation enzymes								
Fba1p	Fructose-bisphosphate aldolase	1	39	5.8	+	17	45	CA5180
		2	40	5.9	−	7	25	
		4	40	5.9	+	9	30	
Tpi1p	Triose phosphate isomerase	1	30	5.6		7	29	CA5950
Gap1p	Glyceraldehyde-3-phosphate dehydrogenase	1	35	6.4–7.7	+	4–13	13–50	CA5892
		2	35	6.9–7.5	−	6–11	22–42	
		3	35	7.1–7.7		5–9	16–31	
		4	35	7.0–7.4	+	4–6	17–31	
Pgk1p	Phosphoglycerate kinase	1	46	5.8–6.0	+	4–9	15–22	CA1691
		2	46	5.8–6.0		6–7	17–19	
		3	46	5.9–6.1	+	5–8	13–20	
		4	46	5.8–6.0	+	4–6	15–17	
Gpm1p	Phosphoglycerate mutase	1	30	5.7		9	35	CA4671
Eno1p	Enolase	1	48	5.4–5.6	+	6–13	10–37	CA3874
		2	48	5.4–5.5		6–11	18–28	
		3	48	5.4–5.5	+	9–12	24–30	
		4	48	5.3–5.4	+	7–20	24–52	
Cdc19p	Pyruvate kinase	1	60	6.4–6.7	+	13–15	28–39	CA3483
		2	60	6.4–6.7	−	10–12	18–38	
Pdc11p	Pyruvate decarboxylase	1	62	5.1–5.2	+	10–12	17–20	CA2474
		2	63	5.0–5.2	−	7–9	15–17	
		4	63	5.0–5.1	+	11–13	20–33	
Adh1p	Alcohol dehydrogenase	1	45	5.6–5.7	+	4–8	9–14	CA4765
		2	45	5.7–5.8	−	4–6	7–13	
VI. Miscellaneous								
Csp37p	37-kDa cell surface protein	1	35	7.1		9	23	CA1075
Psa1p	GDP-mannose pyrophosphorylase	1	41	5.7–5.9	+	13–17	35–47	CA3208

TABLE I—*continued*

Spot name[a,b]	Protein function	Cell wall fraction[c]	Mr (kDa)[d]	pI[d]	Hyphal induction[e]	No. matched peptides[f]	Percentage of sequence coverage[g]	Accession number[b]
Ino1p	*myo*-Inositol-1-phosphate synthase	1	58	5.1–5.2	+	6–12	18–30	CA5986
Cdc48p	Member of the ATPases associated with diverse cellular activities (AAA) superfamily	1	97	4.8–4.8		14	18	CA3333
Bmh2p	Homolog of mammalian 14-3-3 protein	1	34	4.5		8	30	CA5050
VII. Unknown function proteins and others								
ORF6713	Unknown function	1	33	4.7	+	6	30	CA4220
		1	33	4.8	−	7	34	
4–5	ND[m]	2	45–57	3.7–4.3	s	MS/MS[h,i,j]		
17	ND	2	160	4.2		MS/MS[h,i,j]		
6	ND	3	100–175	5.7	−	MS/MS[h,i,j]		
8	ND	3	76	4.1	−	MS/MS[h,i,j]		
15	ND	4	32	5.2	s	MS/MS[h,i,j,l]		
16	ND	4	21	5.8	s	MS/MS[h,i,j,l]		

[a] Spot name as given on the Fig. 3–6.

[b] Protein name and accession number according to CandidaDB (genolist.pasteur.fr/CandidaDB).

[c] Cell wall fractions obtained from isolated walls by SDS and DTT (fraction 1), NaOH (fraction 2), Quantazyme (fraction 3), and exochitinase (fraction 4) treatment.

[d] Experimental M_r and pI (Melanie 3.0).

[e] Specific (s), up-regulated (+), or down-regulated (−) proteins after germ tube induction.

[f] Number of peptide masses matching the top hit from MS-Fit peptide mass fingerprinting. For several isoforms of a single protein, the minimum and maximum numbers of peptides matched are only given.

[g] Amino acid sequence coverage for the identified protein. For several isoforms of a single protein, the minimum and maximum percentages of sequence coverage are only given.

[h] Proteins analyzed by tandem mass spectrometry using a MALDI-TOF/TOF mass spectrometer.

[i] Spots in-gel deglycosylated with PNGase F before trypsin digestion.

[j] Peptide sequences that failed to match against *C. albicans* databases or partial short amino acid sequences that did not enable their positive identification.

[k] Two of its peptides were identified as the sequences ESTVAGFLVGSEALYR and NDLTASQLSDKINDVR from *S. cerevisiae* Bgl2p, but not from *C. albicans* Bgl2p, notwithstanding the fact that *C. albicans* Bgl2p is present in databases and we have identified it here by peptide mass fingerprinting. It might be a protein of the same family hitherto unannotated in *C. albicans* databases.

[l] Low molecular weight proteins with only few tryptic fragments present in their peptide mass fingerprinting.

[m] ND, not determined.

Comparison of Protein Profiles from Yeast and Hyphal CWPs: Identification of Cell Surface Proteins by Mass Spectrometry

The different fractions obtained were subjected to a more stringent comparative analysis using Melanie 3.0 software. We only accepted those dimorphic transition-associated changes in the protein pattern of a cell wall fraction if such differences were repeated in all preparations obtained from that fraction.

After automated spot detection, spots were checked manually (adding, modifying, or deleting spots) to eliminate any possible artifacts such as background noise or streaks. The yeast and mycelial CWP patterns of each fraction were overlapped and matched, using the selection of 30–40 common spots present in both images as landmarks, to detect potential differential or specific proteins from each other. Silver-stained spot intensity was normalized as a relative volume corresponding to the volume of each spot divided by the total volume of the all spots in the gel, and in parallel, this value was adjusted subtracting a background relative volume of identical area in an attempt to correct differences in staining intensity among different gels. The normalized volume of each yeast protein spot was compared with its hyphal counterpart to determine whether that ratio indicated a statistically significant difference ($p < 0.01$) occurring during the morphogenic switch.

A large number of protein spots from the different CWP fractions, mainly those that were differentially expressed, were identified by MS analysis (Table I). Around ninety unambiguous *C. albicans* proteins were identified by MALDI-TOF MS, representing a total of 27 different proteins. MALDI-TOF spectra of some cell surface-associated proteins are illustrated in Fig. 8. Three low molecular weight proteins with few tryptic fragments were sequenced by tandem mass spectrometry using the MALDI-TOF/TOF work station (Table I and Fig. 8*B*). Twelve glycosylated spots were in-gel deglycosy-

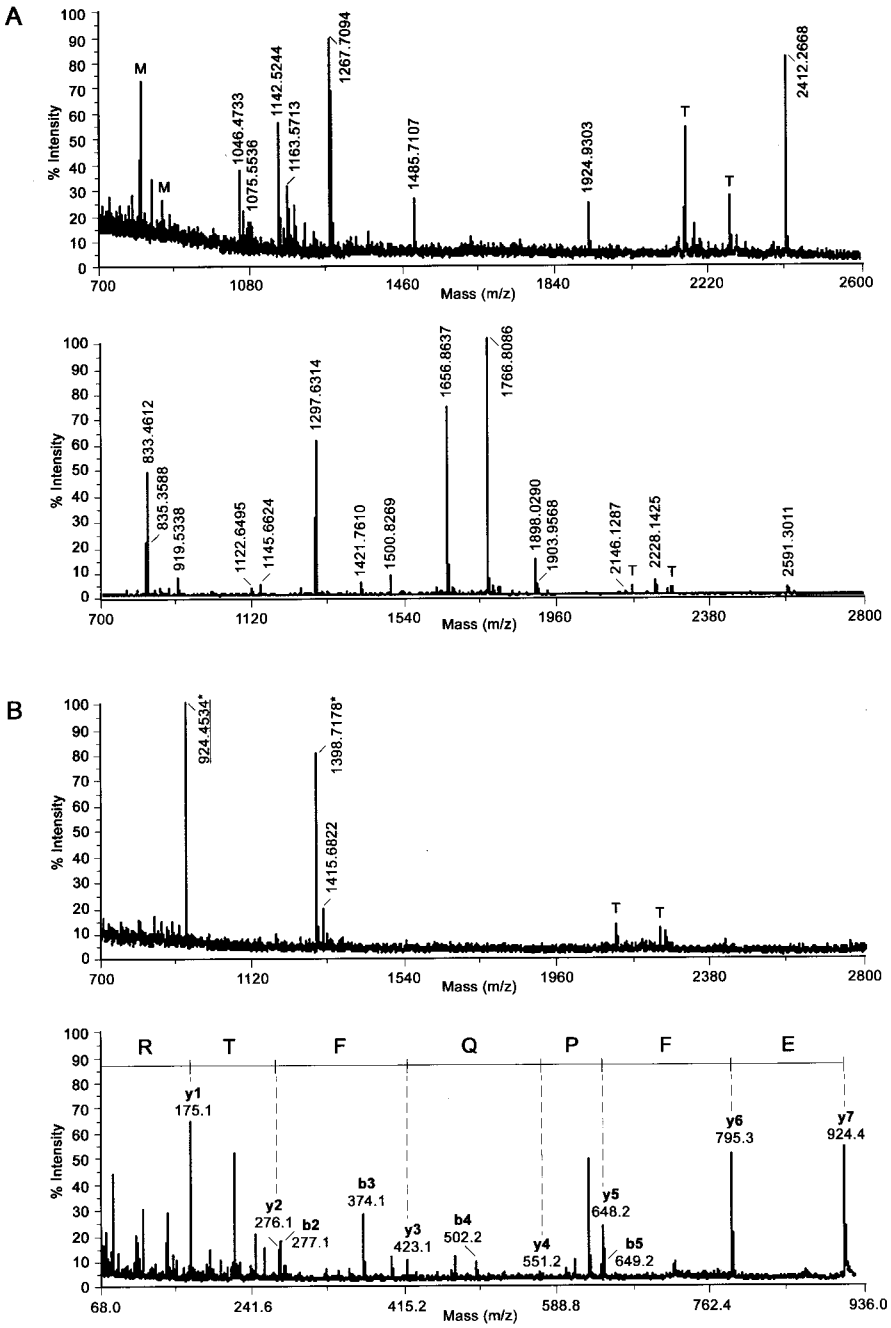

FIG. 8. *A*, MALDI-TOF spectra of the tryptic digests of two cell surface-associated proteins (Fig. 3). *Labeled peaks* represent the matched peptides to *C. albicans* Bgl2p (*top*) and Gap1p (*bottom*). Peaks of matrix and autolysis products of the trypsin are labeled with "*M*" and "*T*", respectively. *B*, characterization of Efb1p by MS. Peptide mass fingerprinting of its tryptic digest is shown at *top*. *Asterisks* show the peptides analyzed by MALDI-TOF/TOF MS. The MS/MS spectrum of the 924.45-Da peptide is shown at *bottom*. The 924.45- and 1398.71-Da peptides were matched to the sequences EFPQFTR and AFQKEFPQFTR, respectively, their identity being *C. albicans* Efb1p.

lated with PNGase F prior to trypsin digestion and analyzed by MS/MS (see Table I).

Quantitative Differences in SDS-extractable Protein Patterns—Although no major differences in the overall SDS-released protein profile of either morphology were observed, significant quantitative changes were shown (Fig. 3 and Table I). At least 94 matched spots were up-regulated after germ tube induction, whereas only 38 spots were down-regulated. One of the most significant increases in hyphal protein expression was displayed in a group of proteins with molecular masses ranging between 35 and 50 kDa (indicated with a *broken rectangle* in Fig. 3 and enlarged in Fig. 9) and from 58 to 62 kDa (Fig. 3, *Pdc11p* and *Ino1p*). In contrast, some down-regulated SDS-extractable proteins were also visualized during hyphal formation, such as one 70-kDa acidic protein (Fig. 3, *Pdi1p*).

Different 2-DE Profiles of NaOH-extractable CWPs—A moderate decrease was observed in the number of visible protein spots from hyphal forms compared with the number from yeast forms (from 290 to 210) in the 2-DE mild alkali-released CWP patterns (Fig. 4). Volumetric analysis revealed at least 28 decreases and 13 increases in spot intensity after

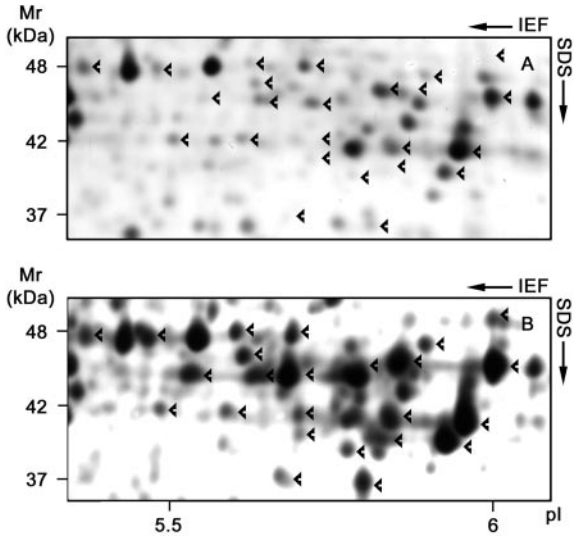

FIG. 9. **Enlargements of a section of SDS- and reducing agent-extractable protein gels from *C. albicans* yeasts (*A*) and hyphae (*B*), corresponding to the *broken rectangle* in Fig. 3.** *Arrowheads* show protein spots differentially expressed during germ tube formation. *IEF*, isoelectric focusing.

mycelial formation. For instance, a cluster of 94–110-kDa and two groups of 63- and 60-kDa spots (Fig. 4, *cluster 1*, *Pdc11p* and *Cdc19p*, respectively) were down-regulated, while another group of 59-kDa proteins (Fig. 4, *cluster 3*) was increased during the *C. albicans* transition to filamentous forms. Despite this, some qualitative differences between both profiles were also visualized. For example, three acidic spots with M_r 74,000–91,000 and two rod-like spots with M_r 45,000–57,000 (Fig. 4, *cluster 2* and *spots 4–5*, respectively) were detected only in hyphal forms.

Different 2-DE Patterns of β-1,3-Glucanase-extractable CWPs—A general decreasing trend in the number and level of rod-like spots was shown during the conversion to the mycelial form. Examples of this trend are two groups of high molecular weight mannoproteins and three acidic clusters of rod-like spots with molecular masses of 76, 54, and 35 kDa (Fig. 5, *groups 6–7* and *clusters 8–10*, respectively). The latter cluster gave an exact match to *S. cerevisiae* Bgl2p but not to *C. albicans* Bgl2p (see Table I). Alternatively, some up-regulated hyphal proteins were observed (such as *Eno1p* and *Pgk1p*, in Fig. 5). Qualitative changes between both morphologies were also found.

Exochitinase-extractable CWPs from Hyphal Forms Are Highly Expressed—2-DE exochitinase-soluble CWP profiles revealed a dramatic increase in the number of mycelial spots when compared with the number from yeasts (from 65 to 250). About 24 significant protein spots were enhanced (such as *cluster 12*, *Eno1p*, *Pgk1p*, *Fba1p*, and *Gap1p*, in Fig. 6), whereas two relevant spots decreased in intensity after hyphal growth (Fig. 6, *spots 13*). About 185 protein spots were detected only in mycelial forms. Acidic proteins with M_r from 32,000 to 15,000 were expressed specifically in hyphal CWP gels (Fig. 6, *cluster 14*). Two major protein spots of 32 and 21 kDa (Fig. 6, *spots 15* and *16*, respectively) were highly expressed and were only stained in filamentous protein gels.

DISCUSSION

C. albicans Cell Wall Proteome Consists of Highly Heterogeneous Proteins—CWP samples are too complex to analyze by two-dimensional PAGE due to their high heterogeneity, low abundance, low solubility, hydrophobic nature, and interconnections with mannan, glucan, and/or chitin side chains hampering their resolution (46, 47). However, by sequential solubilization of CWPs according to the type of linkages that they establish with other cell wall components, involving both loosening the cell wall polysaccharide network and breaking covalent protein-sugar bonds, it has been possible, for the most part, to solve these problems. Furthermore, it has permitted, for the first time, the definition of reference 2-DE protein patterns of highly enriched cell wall fractions from *C. albicans* and, ultimately, the elaboration of a comprehensive and integrated view of the great complexity of its CWP composition.

Our results suggest that a heterogeneous population of proteins is present in the fungal cell envelope. A high percentage is loosely associated with the cell surface. This is consistent with the observation that over 70% of the radioactivity in *C. albicans* ^{14}C-labeled walls is released when they are treated with SDS (27). Conversely, many CWPs are covalently linked or tightly entrapped within the structural β-1,3-glucan and/or chitin network. Apparently, around one-third of all CWPs are attached to or enmeshed in the β-1,3-glucan framework, although such percentage might be higher since the glycosidic nature of these proteins (*i.e.* β-1,6-glycosylated, *N*- and/or *O*-mannosylated CWPs (15)) hampers their resolution by two-dimensional PAGE and their subsequent visualization. A small number of CPWs are linked to β-1,3-glucan through an alkali-sensitive bond. Similarly, as previously estimated (48), 5–13% of all CWPs and 13–26% of structural CWPs from *C. albicans* can be extracted under β-elimination process. Thus this polymer certainly plays a key role in the retention of CWPs. Alternatively, a varying population of CWPs, typical of the *C. albicans* morphological form, is connected to or trapped inside the chitin skeleton. The smear detected by periodic acid-Schiff staining might correspond to β-1,6-glycosylated CWPs anchored to chitin (Fig. 1*D*).

Different Mechanisms of Retention of Some CWPs into the Cell Wall—The combination of sequential cell wall fractionation and two-dimensional PAGE followed by immunoblotting with specific antibodies against well known CWPs provides a powerful tool to obtain better knowledge of the mechanisms of incorporation, assembly, and retention of such mannoproteins into the cell wall and of their interactions with wall polysaccharides. Recent studies have shown that some CWPs, such as *S. cerevisiae* α-agglutinin (49), Flo1p (50), or Cwp1p (51), can become attached to the cell wall through different mechanisms. Our findings suggest that a Pir2p-re-

lated protein could be double-anchored either to β-1,3-glucan through an alkali-labile linkage (*i.e.* via their *O*-chains) as reported in *C. albicans* walls (21, 52) or to chitin by a β-1,3-glucanase-resistant bond. However, Pir2p has neither *N*-chains nor a GPI anchor (53, 54), making clear that these moieties cannot serve as an anchor to chitin. Consequently, further biochemical studies will certainly be required to determine the exact linkage between Pir2p and chitin.

Similarly, a Gas1p homologue protein (Phrp) could be retained in the *C. albicans* cell wall by non-covalent and covalent linkages. This protein could be loosely associated with the cell surface, maybe with the plasma membrane, as described in its *S. cerevisiae* homolog (55). On the other hand, Phrp could also be cross-linked either to the glucan framework, probably through a remnant of its GPI anchor-linked β-1,6-glucan-β-1,3-glucan heteropolymer as proposed in *S. cerevisiae* Gas1p (56, 57), or to chitin polymer possibly through its β-1,6-glucan side chains. This is in agreement with the estimate that about 90% of *C. albicans* GPI-CWPs are linked to β-1,3-glucan through β-1,6-glucan, whereas the remaining 10% are anchored to chitin via β-1,6-glucan (21).

Finally, our results allow us to speculate that one portion of some glycolytic enzymes could be tightly entrapped within the glucan-chitin network and thus can only be liberated after treatment with glucanases and chitinases. This is consistent with both the identification of *C. albicans* enolase as a major glucan-associated CWP (58, 59) and the requirement of either inhibition of glucan synthesis or digestion of cell walls with glucanases to release *C. albicans* enolase into the supernatant (60). In addition, it could also suggest that these enzymes might be therefore present throughout the cell wall. Supporting this idea, immunogold labeling recently localized *S. cerevisiae* Enop and Gpmp as well as *C. albicans* Pgk and Gap enzymes both at the cell surface and in the inner layers of the cell wall (61–64).

Identification of Classical and Noncanonical Proteins in the C. albicans Cell Envelope—Proteomic analysis of these enriched *C. albicans* cell envelope fractions has allowed the identification of 31 different proteins, most of them derived from the DTT- and SDS-extractable protein fraction (Table I). Various differentially expressed proteins from the rest of CWP fractions (highly glycosylated CWPs) could not be identified by peptide mass fingerprinting as a result of either spectra with weak signals of glycopeptides or no spectra since glycosylation probably hinders tryptic digestion. In-gel deglycosylation with PNGase F (or *N*-glycosidase F, which cleaves all types of asparagine-bound *N*-glycans) before trypsin digestion can circumvent this obstacle. We have set up such an approach with a small number of CWPs. However, most of the MS/MS analyses rendered either peptide sequences that failed to match *C. albicans* databases (because these proteins could be hitherto unannotated in the public *C. albicans* databases) or short peptide sequences that did not reveal any identity. The identification of further glycosylated CWPs is currently being addressed at our laboratory.

Several well known CWPs, such as Bgl2p, Hsp150p/Pir2p, and Phrp, which contain a predictable signal sequence and are involved in cell wall construction and maintenance, have been characterized herein. We have also identified a protein homologous to Bgl2p of *S. cerevisiae* that did not correspond to the counterpart of *C. albicans*. It could possibly be a protein of the same family (β-1,3-glucanases) that is not yet annotated in *C. albicans* databases.

On the other hand, some identified proteins fail to possess a classical secretory signal sequence, which hints at the existence of a signal peptide hitherto unreported, gene splice variants, alternative secretory pathways, or potential contamination. Although cytosolic protein contamination is very unlikely, the unambiguous evidence of cell surface location of such non-classical proteins will only be yielded by *in situ* immunolocalization or by using tagged fusion proteins (14). Intriguingly, different groups have already demonstrated the presence of most of the proteins identified in this work using these strategies (see below), strongly suggesting that they are genuine cell envelope components and not experimental artifacts. Hence, the dual location of these proteins "classically" considered to be confined to the intracellular compartment might imply the presence of alternative secretory pathways hitherto undiscovered (45), "moonlighting proteins" with different functions depending on whether they are located inside or outside the cell (65, 66) and/or proteins without any catalytic function recruited as structural components into the cell wall during the evolution of this compartment (65, 67).

As stated above, two members of the Hsp70 family and a fragment of Hsp90 have previously been immunolocalized on the *C. albicans* cell wall (26, 68). Other Hsp70p have been found on the surface of *S. cerevisiae* (69, 70), *Histoplasma capsulatum* (71), *Coxiella burnetii* (72), *Plasmodium falciparum* merozoites (73), and *Chlamydia trachomatis* elementary bodies (74). These molecular chaperones, involved in protein folding and translocation (75, 76), might play a key role in the biosynthesis, secretion, and assembly of other cell wall components as well as in the wall structure (70). In addition, due to their extracellular location, some of them exhibit antigenic properties (77, 78).

Likewise, Pdip, which catalyze the folding of proteins containing S–S bonds, have been described in the cell envelope of *Trichoderma reesei* (79), *Giardia lamblia* (80), and *Arabidopsis thaliana* (81). Since Pdip co-immunolocalizes with the CWPs on the cell envelope, it may participate in the CWP maturation on the cell surface (80). Because of its transglutaminase activity (82), Pdip might also cross-link CWPs in the inside of the wall (83).

Elongation factor-1α has been located in tobacco cell walls by immunogold labeling (84). Extracellular or cell envelope-associated elongation factors have also been reported in *C. albicans* (85), *T. reesei* (79), *Mycobacterium leprae* (86), *Bacillus subtilis* (87), *Staphylococcus aureus* (88), and *A. thaliana* (81) among others. A role as an adhesin has been

suggested as an explanation for their cell surface location (84). In parallel, by mimicking their traditional biological role they might have a chaperone activity and promote translocation and processing mechanisms of cell wall components inside the wall network.

Immunogold electron microscopy has revealed the presence of some glycolytic enzymes on the cell surface (61–64). The unambiguous cell wall location of Enop and Fbap has been genetically demonstrated (69). As components of the cell surface, some glycolytic enzymes can act as binding receptors to extracellular matrix proteins (64, 89–91), immunodominant antigens (32, 78), constitutive proteins of the cell wall (92), or enzymatically active proteins (62, 93).

Among miscellaneous proteins identified, *C. albicans* Csp37p could play a role in cell adhesion (94). Psa1p, involved in cell wall maintenance and biogenesis (95, 96), might participate in CWP mannose outer chain elongation at the cell surface. Ino1p is required for the biosynthesis of *myo*-inositol and phosphatidylinositol. Recent studies (97, 98) have shown that *myo*-inositol hexakisphosphate is a major component of the hydatid cyst wall, and the *Mycobacterium smegmatis* cell wall possesses phosphatidylinositol synthase activity. *A. thaliana* Cdc48p has been immunolocalized in a region where membrane vesicles fuse to form new plasma membrane and cell wall during cytokinesis (99). And *S. cerevisiae* Bmh1p, exhibiting a strong similarity to Bmh2p, has been found among the proteins secreted by regenerating protoplasts, suggesting a possible function in the secretion of proteins bound for wall regeneration (100). Its presence on the cell surface might thus be considered a result of protein externalization.

The Morphological Transition Is Correlated with Changes in the Composition of CWPs—The morphogenetic conversion from the yeast form to the filamentous form involves changes in *C. albicans* cell wall composition, organization, and structure since the cell wall is a plastic organelle (9, 10, 16). Nevertheless, the information about the differential expression of CWPs in both morphologies is limited. In the present work, a comparative cell wall proteome analysis revealed the differential expression of some CWPs and the expression of specific proteins in yeasts and hyphae (Figs. 3–6 and Table I), indicating that there is a different molecular organization in both morphological types of cell walls. As already reported (18, 27, 101), we did not observe any significant qualitative changes in cell surface-associated protein patterns either, suggesting that proteins of this type are similar but differentially regulated in both morphologies.

We detected a slightly higher number of CWPs extracted under β-elimination process in yeast walls. Correspondingly, twice the amount of radioactivity was released from *C. albicans* SDS-resistant and mild alkali-treated yeast walls than from the hyphal walls (48). There are two potential explanations for these observations. It is possible that some of these hyphal CWPs might be retained inside the wall skeleton by other means. Alternatively, since the mycelial walls contain less mannan than do the yeast walls (30), it could be appealing to speculate that the former might have fewer *O*-glycosylated mannoproteins that can be released using this treatment.

Previously, a higher number of SDS-PAGE bands were detected in yeast walls solubilized by β-1,3-glucanases with regard to mycelial walls (27). Similarly, we also observed that some rod-like spots from hyphal forms were poorly extracted by Quantazyme treatment, suggesting that some β-1,6-glycosylated GPI-CWPs from hyphal forms may be retained in a larger proportion than in their yeast counterparts by chitin polymer (see below) and thus be resistant to extraction with β-1,3-glucanases. It follows that the assembly of such CWPs differs in both morphologies.

C. albicans ^{14}C-labeled and chitinase-digested cell walls released 4 times more radioactivity from hyphal walls than from yeast walls (48). In addition, a noteworthy enhancement in the percentage of exochitinase-released CWPs (from 1–2% to 40% of the covalently linked CWPs) has recently been reported in *S. cerevisiae* cell wall mutants with less β-1,3-glucan and more chitin (24). This might explain the higher amount (from 65 to 250 spots, *i.e.* 4 times greater) of this type of hyphal CWPs contemplated in our results since chitin is enhanced 4-fold in hyphal walls (5%) with respect to yeast walls (1.4%) (102). It is therefore probable that hyphal chitin might be able to establish more connections with other cell wall components and retain larger amounts of mannoproteins. Accordingly, we believe that this particular retention mechanism of *C. albicans* CWPs is especially important in hyphal forms. In a nutshell, some specific mannoproteins might form part of a "morphogenetic code" that could determine the construction of both morphologies and modulate the molecular organization of the cell wall (103) and/or play a role in pathogenesis.

The strong expression of some glycolytic enzymes in hyphal walls is not unprecedented. As estimated previously (104), enolase represents 0.7 and 2% of the total protein of *C. albicans* yeast and mycelial cells, respectively. A stronger expression of enolase mRNA is also observed under filamentous growth (105). This might reflect a different metabolism for both morphologies. The cell wall remodeling occurring during the hyphal transition might hypothetically need an extra contribution of energy, perhaps provided by the ATP- and NADH-generating part of the pathway present in the wall, for the incorporation of new building blocks into the cell envelope or the local biosynthesis of covalent linkages among its components. In any case, the enzymatic activity of glycolytic enzymes in the wall remains to be determined, although Gapp and Gpmp catalytic activity has recently been reported in the yeast wall (62, 93). Nevertheless, further assays to shed light on the genuine function(s) of all these enzymes together with the possibility of a putative functional glycolytic pathway in the cell wall are in progress.

New Perspectives—Because of the therapeutic problems

currently encountered with these fungal infections, any insight into the CWPs, with no mammalian counterpart, could be particularly valuable to search for novel specific targets for antifungal drugs (12). For this reason, the *C. albicans* cell wall proteome promises new perspectives for the development of innovative therapeutic strategies for candidiasis. To our knowledge the present study is the first to report 2-DE reference maps using immobilized pH gradients of different *C. albicans* yeast and hyphal CWP fractions, and this is found to be a useful approach for proteomic analysis of the *C. albicans* cell wall and for both basic and applied research. All these 2-DE maps and identified proteins are now available on an electronic yeast two-dimensional PAGE database linked to the SWISS-PROT and TrEMBL databases and designated COMPLUYEAST-2DPAGE, whose World Wide Web server is www.expasy.ch/ch2d/2d-index.html or babbage.csc.ucm.es/2d/2d.html (106). It is our hope that this data could be a helpful contribution to subsequent studies of the *C. albicans* cell wall proteome.

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** To whom correspondence should be addressed: Dept. de Microbiología II, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain. Tel.: 34-91-394-1744; Fax: 34-91-394-1745; E-mail: conchagil@farm.ucm.es.

‖ Director of the Merck Sharp & Dohme Special Chair in Genomics and Proteomics.

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