Expression of transglutaminase substrate activity on *Candida albicans* germ tubes through a coiled, disulfide-bonded N-terminal domain of Hwp1 requires C-terminal glycosylphosphatidylinositol modification

Janet F. Staab ‡1, Yong-Sun Bahn §1, Chia-Hui Tai 3#, Paul F. Cook 3, and Paula Sundstrom 1,2†*

1Department of Molecular Virology, Immunology, and Medical Genetics, and the 2Department of Microbiology, 333 West 10th Avenue, The Ohio State University, Columbus, Ohio 43201

3Department of Chemistry and Biochemistry, University of Oklahoma, 620 Parrington Oval, Norman, Oklahoma, 73019

*†Corresponding author:
Dartmouth Medical School
Department of Microbiology and Immunology
Vail Building HB7550
Hanover, NH 03755
Phone: 603-650-1629
FAX: 603-650-1318
Email: Paula.Sundstrom@Dartmouth.edu

‡Present address: Seattle Biomedical Research Institute
307 Westlake Avenue N. Suite 500
Seattle, WA 98109-5219

§Present address: Departments of Molecular Genetics and Microbiology (MGM),
Duke University Medical Center
315 CARL Building
Research Drive
Durham, NC 27710

Running title: Characterization of Hwp1 from *Candida albicans*
SUMMARY

By serving as a microbial substrate for epithelial cell transglutaminase, hyphal wall protein 1 (Hwp1) of Candida albicans participates in cross-links with proteins on the mammalian mucosa. To explore the molecular features of Hwp1 that enable the formation of cross-links between hyphae and mammalian cells, the N-terminal domain was expressed as a recombinant protein and subjected to biophysical characterization. The transglutaminase-reactive portion of Hwp1 has structural features similar to other transglutaminase substrates, the small proline-rich proteins of cornified cell envelopes found in stratified squamous epithelia. Recombinant Hwp1 lacks α and β structure by circular dichroism and likely exists as a disulfide crosslinked coiled coil. The transglutaminase substrate property prompted a unique approach for investigating the features of surface Hwp1 on germ tubes. A lysine analog, 5-(biotinamido)pentyamine, was cross-linked to germ tubes catalyzed by transglutaminase 2 prior to cell fractionation, immunoprecipitation and detection with Streptavidin conjugates. The majority of the transglutaminase-modifiable Hwp1 was covalently attached to the β-glucan of hyphae by the C-terminus of Hwp1 via a glycosylphosphatidylinositol remnant anchor. A putative precursor of cell wall forms of Hwp1 was identified in the cell extract and in the culture medium. Hwp1 was modified by relatively short N-linked glycans, and the protein’s molecular size was reduced by hypomannosylation when expressed in O-glycosylation mutant strains. Hwp1 combines features of mammalian transglutaminase substrate proteins with characteristics of fungal cell wall proteins to form an unconventional adhesin at the hyphal wall of C. albicans.
INTRODUCTION

*Candida albicans* is unique among oral pathogens in its ability to invade cornified layers of stratified squamous epithelium of the tongue, buccal surfaces, hard and soft palate and esophagus (1). Hyphal wall protein 1 (Hwp1), which mimicks host cell TGase substrates and forms tight attachments to stratified squamous epithelial cells with cell surface-exposed transglutaminase (TGase) activity, is required for oroesophageal candidiasis in mice (2). In mimicking host ligands, Hwp1 adds an example from the fungal kingdom to known microbial adhesins that imitate mammalian proteins (3). With a half mammalian-like, half-fungal hybrid primary structure, Hwp1 interacts with the cornified epithelium through the N-terminal domain, while being expressed on germ tube surfaces attached through the C-terminal domain. *HWP1* is highly induced during germ tube formation and absent during yeast growth (4). *HWP1* expression appears coordinately controlled by transcriptional activators and repressors that control morphology (4-9). High *HWP1* mRNA levels are reflected in high protein levels on germ tube surfaces. Recent experiments employing recombinant Hwp1 (rHwp1) have documented that the mature N-terminal third of Hwp1 serves as a substrate for mammalian TGases (10). The TGase activity on surfaces of buccal epithelial cells, attributed to TGase1 by others (11), utilizes rHwp1 as a substrate confirming that the similarity in primary structure of rHwp1 to mammalian small proline rich proteins extends to function. Furthermore, the requirement for Hwp1 in stabilized attachment of germ tubes to buccal epithelial cells (BEC) illustrates that Hwp1 participates in important interactions between *C. albicans* and the oral mucosa (10).

Insights into the structure-function relationships of the C-terminal region of Hwp1 are inferred by analogy to the primary amino acid sequences of known or putative cell wall proteins of *S. cerevisiae* and *C. albicans*. The last 50 amino acids have features consistent with glycosylphosphatidylinositol (GPI) anchor modification and anchorage to cell wall glucan. Elegant work on the α-agglutinin of *Saccharomyces cerevisiae* (12), a prototypical adhesin, has demonstrated that multiple processing events occur at the C-terminus that result in transient positioning of the adhesin in the cytoplasmic membrane and in the periplasm before becoming covalently attached to cell wall β-glucan.

However, very little information is available on how the structure of Hwp1 relates to its function. Here we delineate the similarities of Hwp1 to small proline rich (SPR) proteins that are likely to be responsible for the TGase substrate properties of Hwp1. SPR proteins are the major components of a specialized structure termed the cornified cell envelope of normal human buccal and gingival tissues that is essential for barrier function (11,13). The SPRs become cross-linked to themselves and other CE proteins by both disulfide bonds and isopeptide bonds formed by transglutaminase to form an insoluble macromolecular protein complex ideal for barrier function. Although both SPR proteins and Hwp1 are TGase substrates, Hwp1 lacks the tripartite domain structure that serves to cross-link the head and tail domains of SPR proteins to themselves or to other proteins. In addition, Hwp1 also lacks Lys residues that are usually found adjacent to reactive Gln residues in TGase
substrates. Very little information exists on the relatedness of C-terminal processing motifs in *C. albicans* to those studied in *S. cerevisiae*.

To understand the role of Hwp1 in the pathogenesis of candidiasis in the cornified layers of the oroesophageal epithelium, information on the structure of the TGase substrate domain that permits interaction with mammalian TGase is needed. The primary amino acid sequence is predictive of glycosylation and anchorage to the β-glucan, however the extent of glycosylation, the size of N-linked glycans, and C-terminal attributes that lead to cell wall incorporation are unknown. Determining the distribution of TGase-modifiable Hwp1 among the membrane, wall and cell free fractions is essential for delineating the mechanism of adherence of *C. albicans* to mammalian cells.

Few individual fungal cell wall proteins have been characterized at the molecular level. Here we have taken advantage of the TGase substrate property of the N-terminal domain of Hwp1 to selectively tag the molecule on germ tube surfaces using guinea pig liver TGase 2. Examination of subcellular germ tube fractions reveal that Hwp1 is a member of the GPI-anchor dependent family of cell wall proteins with the majority of the molecules being linked to the β-glucan as predicted. However, we also show that native Hwp1 is a complex mixture of forms that are cell free, membrane bound, as well as cell wall linked, and that the C terminus is required for retention of Hwp1 at the cell surface. The TGase substrate domain itself is a tight, disulfide linked coil without discernible secondary structure. These structural attributes provide a molecular explanation for a fungal adhesin that mediates a unique microbial-host parasite interaction mechanism.
EXPERIMENTAL PROCEDURES

C. albicans strains. C. albicans strains SC5314 (wt) (14), CAH7-1A (hwp1/hwp1 null mutant) and CAH7-1A1 (hwp1/hwp1 null mutant, Ura- strain) (10) were used in experiments to identify Hwp1. O-glycosylation mutant strains CAP1-312 (pmt1 null) (15) and NGY24 (mnt1 null) (16) were used to assess O-glycosylation of Hwp1. Organisms were stored at –80°C and cultured on yeast peptone dextrose agar at room temperature according to standard methods (17).

Recombinant Hwp1. rHwp1N13 consisting of AA 40 to 187 was produced in Pichia pastoris (Invitrogen, Carlsbad, CA) transformed with a recombinant expression plasmid (pPICN13). The DNA region encoding rHwp1N13 amino acids was amplified by the PCR with oligonucleotides 5’AACCGGGAAGCTTATTCAAAAGAGA and 5’CCCGGGTTATCTGGGATCCACAGTGGGAATATTTGG, engineered with SmaI sites (underlined nucleotides), and genomic DNA from the wild type strain SC5314 as template. The 3’ oligonucleotide introduced a stop codon (bold nucleotides) after the last HWP1 codon to avoid fusion of vector-encoded amino acids at the C-terminus of the recombinant protein. The PCR product was digested with SmaI (BRL/Life Technologies, Inc., Rockville, MD) and ligated into the SnaBI site of the expression vector pPIC9 (Invitrogen) to generate pPICN13. rHwp1N13 was secreted by P. pastoris and purified from culture media by anion exchange chromatography (Mono Q, Bio-Rad Laboratories, Hercules, CA) using a salt gradient (0-1 M NaCl) in column buffer (50 mM Tris-Cl pH 8, 2 mM mercaptoethanol, 1 mM EDTA) followed by gel-filtration chromatography over a 1.5 x 100 cm column packed with BioGel A fine gel (BioRad) equilibrated with PBS (10 mM NaPO₄, pH 7.4, 170 mM NaCl). Protein purity was determined by isoelectric focusing, and authenticity was verified by N-terminal sequencing (Louisiana State University Core Laboratories, New Orleans, LA).

Circular Dichroic Spectra. CD spectra of rHwp1N13 were recorded using an AVIV 62 DS spectropolarimeter and quartz cuvettes with 0.2 cm path length. The temperature of the cell compartment was maintained constant at 25°C with a RC-6 Lauda circulating water bath. All far UV spectra were scanned from 250 to 200 nm with a rHwp1N13 concentration of 100 µg/mL at intervals of 1 nm with 1.5 nm slit width and a 3 s dwell time. The buffer used for far UV spectra was 20 mM KH₂PO₄, pH 7. Each spectrum reflects an average of three scans. The sample spectra were corrected for the appropriate buffer blanks. The digital data for the corrected far UV spectra were converted to mean residue ellipticity according to equation 1.

\[ \theta = \theta_{obs}/10\{\text{MRC}\}l \]  

where \( \theta \) is the mean residue ellipticity in (deg)(cm²)/dmol, \( \theta_{obs} \) is ellipticity recorded by the instrument in millidegrees, MRC is the mean residue concentration of the enzyme estimated as the product of the number of amino acid residues and the protein concentration in dmol/mL, and l is the path length in cm.
Fluorescence Spectra. Fluorescence spectra were recorded on an SLM 8000 spectrofluorometer equipped with a water-jacketed sample compartment to maintain the temperature in the cuvette at 25°C. Excitation and emission slit widths were 4 nm. Spectra of blanks, i.e. of samples containing all components except rHwp1N13, were taken immediately prior to measurements of samples containing protein. Blank spectra were then subtracted from spectra of samples containing protein. Fluorescence spectra of rHwp1N13 (40 µg/mL) using the following buffers at 100 mM concentration for the pH ranges indicated: Homopipes, 5; Mes, 6; Hepes, 7-8; Ches, 9-10; Caps, 10.5-11; K2HPO4, 11.5-12. All buffers (Sigma-Aldrich) were titrated with KOH and the fluorescence data acquired at room temperature (25°C). Emission spectra from 300 – 500 nm were taken with the excitation monochromator fixed at 280, 260 and 298 nm. Excitation spectra from 250–310 nm were taken with the emission monochromator fixed at 330, 350 and 390 nm.

Free thiols. The presence of free thiols was investigated by measuring the absorbance at 324 nm in the presence of rHwp1N13 (11.3 µM) and 4,4'-dithiodipyridine (Aldrich, Milwaukee, WI) (0.4 mM) in 100 mM Hepes, pH 7. The 4,4'-dithiodipyridine solution was prepared in ethanol. All UV-visible spectra were collected at 25°C in quartz cuvettes of 1 cm path length in a volume of 1 mL. Absorbance spectra were measured at the times indicated in Fig. 2B using a Hewlett Packard 8453A diode array spectrophotometer. Protein concentration was determined as previously published (4).

Modification of Hwp1 on germ tube surfaces using the TGase reaction. Exposed Hwp1 available for acting as a substrate for exogenous TGases was modified by cross-linking the biotinylated lysine analog 5-(biotinamido)pentyamine (Pierce Chemical Co., Rockford, IL) to the surface of germ tubes catalyzed by guinea pig TGase 2 as described (10) except that germ tubes were induced in Lee’s pH 6.8 medium at 37°C from yeast cells grown in Lee’s pH 4.5 medium at 27°C to log phase (4). The TGase reaction was terminated by adding EDTA to 10 mM and washing the germ tubes twice with sterile dH2O before suspending in 1.5 mL of breakage buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 5 mM EDTA)(12) with protease inhibitors (1 µg per mL of leupeptin, antipain, pepstatin (Sigma-Aldrich, St. Louis, MO), and 0.1 mM Pefabloc (Boehringer Mannheim Co., Indianapolis, IN)) for cell fractionation and immunoprecipitations. A portion of the germ tubes were spotted onto slides, dried, and reacted with Z-avidin-fluorescein isothiocyanate (Zymed Laboratories, Inc., South San Francisco, CA) as before (10) to assess the TGase reaction prior to disrupting the cells.

Metabolic radiolabeling of germ tube proteins. Germ tubes were induced from log phase yeasts for 3 h at 37°C in Lee’s pH 6.8 medium with a low concentration of Met. The concentration of Met was reduced to 1 µg/mL to promote the uptake of Trans-35S-label metabolic labeling reagent ([35S]L-Met, [35S]L-Cys , >1000 Ci/mmol, ICN Biomedical Research Products, Cosa Mesa,CA) added at 5 µCi/mL. The average uptake of Trans-35S-label by germ tubes was between 50 to 60 %.

Radiolabeled germ tubes were washed twice with ice cold PBS and suspended in 1.5 mL of breakage buffer with protease inhibitors (see section above) for cell fractionation experiments detailed below.
**Preparation of cell extracts and cell walls.** Germ tubes in 1.5 mL of lysis buffer were broken with 1.2 gm of acid-washed glass beads (425-600 microns, Sigma-Aldrich) in a Mini-Beadbeater (Biospec Products) by ten 30 s bursts. The broken cells were centrifuged at 1000 x g for 10 min at 4°C, and the supernatants were transferred to new tubes. The glass beads were washed with 0.5 mL of lysis buffer and the wash was combined with the original supernatant. The crude cell extracts were centrifuged at 15,000 x g for 10 min at 4°C, and the supernatants transferred to new tubes. The supernatants (cell extracts/soluble fractions) were centrifuged a second time at 20,000 x g for 20 min at 4°C before use in immunoprecipitations. The pelleted cell walls from the initial 15,000 x g spin were washed in 25 mM Tris-Cl, pH 7.5, 1 M NaCl, 0.5 mM EDTA with protease inhibitors as above. The washed walls were boiled twice for 5 min in 100 µL of SDS lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 2% SDS, 10 mM DTT, 5 mM EDTA with protease inhibitors at 40 µg/mL, and 0.1 mM Pefabloc) to remove non-covalently-bound wall proteins.

**Quantzyme treatment of cell walls.** The SDS-boiled cell walls from 3-4 x 10⁸ organisms were washed twice in 25 mM Tris-Cl pH 7.5, 1 M NaCl, 0.5 mM EDTA with protease inhibitors, and suspended in 25 mM Tris-Cl pH 7.5, 40 mM 2-mercaptoethanol, 1 mM EDTA with protease inhibitors, and 75 units of Quantzyme ylg (Quantum Biotechnologies, InterSpex Products, Inc., Foster City, CA). The mixture was incubated at 37°C for 1 h with rocking. The residual undigested cell walls were removed by centrifugation at 15,000 x g at 4°C. The supernatants containing the cell wall material were centrifuged at 20,000 x g for 20 min at 4°C before being transferred to new tubes for immunoprecipitations.

**Immunoprecipitations, SDS-PAGE and Western blotting.** The cell extract and Quantzyme-released wall proteins received 20 µL of polyclonal rabbit serum raised to recombinant Hwp1 (rHwp1, coded by the partial cDNA (4)) or to rHwp1N13 (rabbit serum was generated as described for rHwp1 (4)) adsorbed to SC5314 whole yeast cells and to SC5314 yeast acetone powder (18). After the samples were incubated on ice overnight, 40 µL of a 50% slurry of Protein A-Sepharose (Sigma-Aldrich) in PBS was added, and incubation on ice continued for another 0.5-1 h. The Sepharose beads were washed as before (19), and suspended in 25 µL of sample buffer (Invitrogen/Novex Tris-Acetate PAGE system), heated to 70°C for 10 min, spun, and the supernatants transferred to new tubes. Samples (5-10 µL) were analyzed by SDS-PAGE (20) using 3-8% Tris-acetate gels as per the manufacturer’s specifications (Invitrogen). MultiMark protein standards (Invitrogen) served as molecular size markers. Following SDS-PAGE, the TGase 2-modified proteins were transferred to Immobilon P (Millipore Corp., Bedford, MA) membranes, and Hwp1-5- (biotinamido)pentylamine was detected by incubating the membranes with Streptavidin conjugated to horse radish peroxidase (Zymed) followed by chemiluminescence (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Immunoprecipitates from radiolabeled germ tubes were analyzed by SDS-PAGE as above followed by fluorography (21). The dried gels were exposed to X-ray film for several days at –80°C.
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Endoglycosidase H treatment of Hwp1 species. Immunoprecipitated 5-(biotinamido)pentylamine-cross-linked Hwp1 proteins were treated with Endo H (Boehringer Mannheim) as follows: after the last wash of the Protein A Sepharose, the Sepharose beads were boiled in 30 µL of Endo H buffer (50 mM potassium phosphate buffer, pH 6, 100 mM 2-mercaptoethanol, 0.02% SDS) for 3 min to denature and release the immunoprecipitated Hwp1 from the Protein A Sepharose followed by centrifugation for 30 s at 15,000 x g. The supernatants were split into two 15-µL portions. One sample received 5 mU (5 µL) of Endo H and the other additional Endo H buffer. The samples were incubated at 37°C overnight (18 h), and terminated by addition of sample buffer to 1X and heating for 10 min at 70°C. Portions of the reactions were analyzed by SDS-PAGE, followed by Western blotting as above.

Aqueous HF treatment of Hwp1. Quantazyme-released 5-(biotinamido)pentylamine-cross-linked Hwp1 was treated with ice cold aqueous HF (48%; Aldrich) for 18 h as described (22). After the HF was evaporated, the proteins were washed 3X with 90% cold methanol and dried. The samples were suspended in 30 µL of Endo H buffer, heated and split into two portions. Following overnight (18 h) incubation at 37°C with or without 5 mU of Endo H, the samples were electrophoresed in SDS-PAGs and transferred to Immobilon P membranes. Hwp1-5-(biotinamido)pentylamine species were detected with Streptavidin-conjugated horse radish peroxidase and chemiluminescence. In some experiments, isolated cell walls (boiled in SDS lysis buffer) from SC5314 were treated with aqueous HF for 18 h as above. The methanol-washed dried walls were taken up in 100 µL of RIPA buffer (18) and heated to 70°C for 10 min. The mixture was centrifuged (15,000 x g) for 5 min and Hwp1 was immunoprecipitated from the supernatant with anti-rHwp1 antiserum as above. The HF-released Hwp1 was also examined directly by adding 1X sample buffer to the methanol washed dried cell walls, heating to 70°C for 10 min and analyzing samples of the supernatants by SDS-PAGE and Western blotting.

Expression of a truncated form of Hwp1 in C. albicans. The C-terminal 26 amino acids of Hwp1 were removed by the introduction of a stop codon one amino acid upstream from the predicted GPI anchor addition site (Gly613) (23). Site-directed mutagenesis (GeneEditor, Promega) was used to insert a single bp (T/A) after bp 1825 (from the ATG) in HWP1 to shift the reading frame 1+ to a stop codon three amino acids post bp insertion. This created a truncated Hwp1 missing the predicted GPI anchor addition amino acid motifs and having three non-Hwp1 residues at its C-terminus (underlined): Ile608 Phe-Tyr-Ile. The mutagenized and a wild type HWP1 ORF were amplified by PCR and cloned downstream of the HWP1 promoter in pHWP1GFP3(24), a reporter plasmid constructed to analyze HWP1 promoter activity. The plasmid constructs containing a wild type (pHwp1WT) and truncated (pHwp1T) HWP1 ORFs were linearized at the unique Clal site and used to transform the Ura' hwp1 null strain CAH7-1A1 (25). Proper integrations of the plasmids at the chromosomal ENO1 locus were verified by Southern blot analysis. Stationary phase yeasts grown in YNB of two independent transformants from each construct were induced to form germ tubes in M199 at 37°C for 3 hr as described previously (26). The germ tubes were tested for surface
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Hwp1 by immunofluorescence assays (23,26) with rHwp1N13 antiserum used in immunoprecipitations described above. The germ tube culture media were filtered to remove any remaining cells, and total protein in a portion of the culture media precipitated in cold 50% trichloroacetic acid on ice for 1 hr. The protein pellets were washed with acetone, dried, suspended in 1X sample buffer, heated to 70°C, and analyzed by SDS-PAGE and Western blotting. Hwp1 was detected by anti-rHwp1N13 antiserum followed by incubation with goat anti rabbit Ig conjugated to horseradish peroxidase (Zymed). Hwp1 was visualized with chemiluminescence as above.

*Phosphoinositol-phospholipase C (PI-PLC) digestion of cell walls.* Unboiled cell walls from 1.5-2 x 10^8 organisms were washed once in 25 mM Tris-Cl pH 7.5, 1 M NaCl, 0.5 mM EDTA and once with 10 mM Tris-Cl pH 7.5, 0.2 mM EDTA (PI-PLC buffer) with protease inhibitors, and suspended 200 µl of PI-PLC buffer. One half of the cell wall suspension was treated with 0.1 U of PI-PLC (Sigma) and the other half was mock-treated. The samples were incubated at 37°C for 18 h followed by centrifugation (15,000 x g for 10 min at 4° C) to remove the cell walls. The supernatants were transferred to new tubes. The pelleted cell walls were boiled twice in SDS lysis buffer as above, and the supernatants moved to new tubes. The PI-PLC and SDS lysis buffer-released proteins from the cell walls were diluted 1:3 and 1:4, respectively, in RIPA buffer (18) before adding 20 µL of polyclonal rabbit anti-rHwp1N13 serum. The samples were processed as for immunoprecipitations, analyzed for Hwp1-5-(biotinamido)pentylamine species by SDS-PAGE and Western blotting as described above.

*Adhesion assays.* The ability of rHwp1N13 to compete or inhibit stabilized adhesion of germ tubes to buccal epithelial cells (BECs) was determined by pre-incubating rHwp1N13 at a concentration of 2 µM or salivary amylase at 4 µM (negative control, a gift from Dr. Frank Oppenheim at Boston University) with 1 x 10^5 BECs for 15 min. at 37°C in adhesion reaction buffer prior to the addition of 1 x 10^7 metabolically radiolabeled (^35S-L-Met) germ tubes for 1 h at 37°C as described for stabilized adhesion assays (10). The adhesion of germ tubes to BECs in the presence of rHwp1N13 or salivary amylase was set relative to assays in the absence of added proteins. The assays were performed at least twice in duplicate.
RESULTS

Role of the N-terminus of Hwp1 in adherence of C. albicans to buccal epithelial cells. The N-terminal domain of Hwp1 is composed of an acidic, degenerate amino acid repeat, rich in proline and glutamine amino acids, and is notably deficient in glycosylation sites (23). An important role for this domain in forming stabilized attachments to human BECs during oropharyngeal candidiasis is suggested by its TGase substrate activity (10). The function of the N-terminus of Hwp1 in stabilized adhesion to the surface of BECs was investigated by using rHwp1N13, comprising amino acids 1 through 148 of the mature protein as a competitor in adhesion assays (10). Pre-incubation with rHwp1N13 prior to the addition of germ tubes reduced relative adhesion by 60% compared to controls without added protein or in the presence of the control protein, salivary amylase (Fig. 1). Inhibition was 78% of the maximum expected amount based on adhesion of the hwp1/hwp1 null mutant which was 23% of control (10). Increasing amounts of rHwp1N13 above 2 µM did not increase the magnitude of inhibition suggesting that a concentration of 2 µM rHwp1N13 was sufficient to saturate BEC adhesion sites. The results show that the stabilized adhesion function of Hwp1 maps to the N-terminal portion of the protein.

Secondary structure features of the N-terminus of Hwp1. The far UV CD spectrum of rHwp1N13 is shown in Fig. 2A and consists of a single and well-defined minimum centered at 202 nm. The lack of any appreciable ellipticity in the 205-225 nm range indicates a protein that is comprised predominantly of a coiled structure. Since the sensitivity of rHwp1N13 to digestion by chymotrypsin increases in the presence of 5 mM CaCl2 and other divalent cations, we wanted to investigate the possibility that rHwp1N13 undergoes a conformational switch when Ca+2 was included in the solution. The CD spectrum in the presence of Ca2+ remained unchanged indicating that Ca2+ had little effect on the overall gross structure of rHwp1N13 (Fig. 2A).

To determine whether secondary structural elements might exist upon interaction of rHwp1N13 with a membrane, far UV CD spectra were measured in the presence of 2,2,2-trifluoroethanol. In the presence of 70% 2,2,2-trifluoroethanol, there are slight changes in the CD spectrum (Fig. 2A, inset) with a 10% decrease in ellipticity centered at 225 nm and an 11% increase in ellipticity centered at 208 nm, with an apparent isosbestic point at 215 nm.

Environment of aromatic amino acids. Excitation of rHwp1N13 at 280 nm gave a well-formed emission spectrum with a broad maximum centered at 340-350 nm and a low intensity shoulder at 390 nm, Fig. 2B, typical of Trp emission in proteins (27). Given the large number of Tyr residues (9), one might expect contribution to the emission from their excitation centered at 303 nm. The emission spectrum of Tyr overlaps the absorbance spectrum of Trp, and most of the Tyr emission is thus quenched as a result of singlet-singlet energy transfer. Quenching also occurs upon ionization of the tyrosine residue, but that is not a concern in the present case, since fluorescence spectra were obtained at pH 7, well below the pK of 10.2. A comparison of the emission contour for protein excited at 280 nm, where Trp and Tyr both absorb, with that for protein excited at 298 nm, where only Trp is expected to absorb, is shown in Fig. 2B with spectra corrected to the same maximum emission intensity. Note that
there is a significant difference in fluorescence on the blue side of the spectrum, indicative of a small contribution from Tyr fluorescence (28). The emission spectrum for Trp’s in proteins have maxima from 325 nm for those in a hydrophobic environment to about 350 nm for those exposed to solvent (29). The relatively long wavelength of the emission maximum of rHwp1N13 indicates that the side chain of at least one of the 2 Trp residues is exposed to solvent. Excitation at either 260\textsuperscript{3} or 298 nm exhibited an emission spectrum qualitatively identical to that shown in Fig. 2B but with lower intensity. Thus, it appears that at least one of the Trp residues is solvent exposed (emission at 355 nm). Addition of Ca\textsuperscript{2+} resulted in qualitatively similar emission spectra whatever the excitation wavelength, but with a slightly lower intensity, presumably as a result of collisional quenching of the solvent exposed Trp(s). Attempts to titrate the small change in relative fluorescence with Ca\textsuperscript{2+} gave no discernible binding isotherm. If Ca\textsuperscript{2+} has a specific binding site, it is not visible with the spectral probes used in these studies. The presence of 8 M urea had no effect on the fluorescence emission spectrum of rHwp1N13. Emission spectra obtained as a function of pH with excitation at 280 nm are identical in shape to those shown in Fig. 2B, and the relative fluorescence was independent of pH from 5 to 9\textsuperscript{3}.

The fluorescence spectrum of rHwp1N13 excited at 280 nm in the presence of 5 M guanidinium HCl and 200 µM reduced DTT was identical to that obtained in the absence of denaturant and reducing agent. On the other hand, excitation at 260 nm gave a 10% decrease in fluorescence intensity, while excitation at 298 nm gave a 22% increase in fluorescence intensity\textsuperscript{3}.

*Number of free thiols.* Addition of 0.4 mM 4,4’-dithiodipyridine to a solution of 11.3 µM rHwp1N13 resulted in a slow increase in absorbance at 324 nm due to the production of the chromophore 4-thiopyridine, Fig. 2C. The difference spectrum obtained by subtracting the spectrum at time zero from that at 125 h gives a single well-defined band at 324 nm, indicative of the chromophore\textsuperscript{3}. Given an extinction coefficient of 19,800 M\textsuperscript{-1}cm\textsuperscript{-1} for 4-thiopyridine (30), slightly more than 2 mols thiol/mol rHwp1N13 were titrated over the total time period. Modification is very slow, with the half-time of about 10 h for titration of the first thiol, and a half time of about 35 h for titration of the second thiol.

Less than 3% of the total rHwp1N13 protein formed what appeared to be dimers based on nonreducing-SDS-PAGE\textsuperscript{4}. The presence of reducing agent in the SDS-PAGE eliminated the small amount of dimer observed under nonreducing conditions. Although free thiol groups were titrated as indicated above, it is highly unlikely that the dimers were the result of intermolecular disulfide bond formation. First, the thiols were not readily accessible, and second, the Maldi-tof Mass Spectroscopy data revealed only monomers\textsuperscript{4} (Louisiana State University Core Laboratories, New Orleans, LA).

Hwp1 is attached to the cell wall β-glucan. Two species of TGase 2-modified Hwp1 of approximately 500 and 325 kDa were released from cell walls after treatment with the β-1,3 glucanase Quantazyme for one hour. The identity of the proteins as Hwp1 was established by their absence in the hwp1/hwp1 null strain and by the lack of precipitation by preimmune serum (Fig.
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In contrast, Hwp1 molecules from metabolically radiolabeled germ tubes released by Quantazyme were more polydisperse than the TGase2-labeled species and ranged in size from 325 to well over 500 kDa (Fig. 3B). Incubation of the cell walls in the absence of Quantazyme did not release Hwp1 (Fig. 3A). A cross-reacting protein of approximately 147 kDa was observed in different cell fractions of the wild type and *hwp1*/*hwp1* null strains (see Fig. 3A and 3C, and 4B). Because this protein was detected in fractions of the *hwp1*/*hwp1* null strain, it was discounted as a species of Hwp1. Phosphorimager analysis (Molecular Dynamics, ImageQuant software) of immunoprecipitated Hwp1 species from metabolically radiolabeled germ tubes revealed that the cell wall forms of Hwp1 comprise approximately 75% of the total Hwp1 (Table 1). Multiple radiolabeled soluble species of Hwp1 were detected that were not seen in TGase 2-modified germ tube fractions. The results are consistent with our previous studies (4,10,23) and with the amino acid sequence-based prediction that Hwp1 is a member of cell wall proteins covalently linked to the β-glucan of *C. albicans*.

**Soluble forms of Hwp1 exposed at the surface of germ tubes.** Two species of Hwp1 cross-linked with 5-(biotinamido)pentylamine were found in the cell extracts of mechanically disrupted wild type germ tubes (Fig. 3C, lane 2). The apparent sizes of the Hwp1 species (301 and 180 kDa) were much larger than the calculated size for the mature protein (61 kDa), an indication that Hwp1 is likely glycosylated as is typical of fungal wall proteins. The 180 kDa species did not fractionate, being found in both soluble and cell wall associated fractions upon extended periods of incubation (see below).

**Glycosylation of Hwp1.** The primary sequence of Hwp1 revealed three potential N-glycosylation sites (Asn202, Asn247 and Asn562) in addition to 184 Ser and Thr residues which can be O-mannosylated (23). N-glycosylation of Hwp1 was investigated by treating immunoprecipitated Hwp1 with Endo H followed by SDS-PAGE and Western blotting (Fig. 4A). Endo H digestion produced two abundant proteins of 289 and 258 kDa that were reduced in size by 12 and 43 kDa, respectively, compared to the undigested 301 kDa form. A minor form, 235 kDa in size, was likely derived from a 245.7 kDa breakdown product of membrane-bound Hwp1 (see below). N-linked glycans released from hyphal mannoproteins are shorter than those of yeasts, and range in size from 125 to 2500 mannose residues (20 k to 300 kDa) with an average size of 300 residues (50 kDa) (31).

One explanation for the results is that two of the three potential sites are glycosylated and the 289 kDa and the 258 kDa species have each lost one N-glycan chain (12 k and 43 kDa, respectively). Alternatively, the 258 kDa species could have resulted from the loss of two N-glycans of 12 and 31 kDa. The presence of more than a single Hwp1 species post Endo H digestion implies that N-linked glycan removal was not complete even though Endo H digests were performed on Hwp1 denatured by boiling in SDS with an excess of enzyme to ensure digestion. Repeated experiments gave the same results. Others have reported that N-glycans vary in their susceptibility to Endo H cleavage (32).

Evidence for N-glycosylation of the Quantazyme-released species of Hwp1 was only apparent for the smaller of the two species (325 kDa, Fig. 4A, lane 4). The decrease in size of only approximately 8 kDa relative to the untreated protein most
likely reflects poor resolution of small changes in molecular size of very large proteins. The lack of a discernable size difference of the larger species (500 kDa) is similarly explained. Lastly, there was no evidence for N-glycosylation of the common 180 kDa protein although we cannot rule out the possibility that this species has Endo H insensitive N-linked glycans.

_**O**-glycosylation mutant strains were used to determine the effect of hypo-mannosylation on Hwp1. In _C. albicans_, _O_-mannosylation is initiated by Pmt (O-D-mannosyltransferase) proteins that can be extended further by the function of the Mnt1 (α-1,2-mannosyltransferase) protein (15,16). The expression of Hwp1 at the surface of germ tubes in the _pmt/pmt1_ strain CAP1-312 appeared to be diminished by approximately one half relative to the wild type strain. In contrast, the amount of Hwp1 seemed unaffected in the _mnt1/mnt1_ strain as assessed by the amount of surface staining by avidin-fluorescein isothiocyanate^2 (see Experimental Procedures). The _pmt/pmt1_ and the _mnt1/mnt1_ null strains produced Hwp1 species with increased mobility in SDS-PAGs (Fig. 4B, _lanes 8 and 9_). An increase in gel mobility was more apparent in the 325 kDa cell wall species. The poorer resolution of the two cell wall species of Hwp1 from the _pmt/pmt1_ and the _mnt1/mnt1_ null strains probably reflects incomplete _O_-mannosylation. The results confirm the modification of Hwp1 protein species by _O_-glycosylation, a feature consistent with other fungal cell wall proteins.

**Glycosylphosphatidylinositol-dependent anchorage of native Hwp1 to cytoplasmic membranes.** Analysis of the amino acids sequences of _S. cerevisiae_ GPI-anchor dependent cell wall proteins reveals several common features: an N-terminal signal sequence, a hydrophobic C-terminal stretch of amino acids downstream of a putative GPI anchor addition signal, and Ser and Thr rich regions usually near the C-terminal region of the proteins (33,34). Cytoplasmic membrane intermediate forms of fungal cell wall proteins are frequently found as a consequence of GPI membrane anchor modification in the endoplasmic reticulum, a necessary prerequisite to covalent linkage to cell wall glucan. To investigate this maturation pathway for Hwp1, preliminary experiments were performed to determine if cytoplasmic membranes were associated with soluble extracts or broken cell walls. Antibodies to gel-purified native _S. cerevisiae_ H^+^-ATPase (35) were used to detect the _C. albicans_ H^+^-ATPase membrane protein (36) in Western blots of material released from cell walls by boiling in SDS. Cytoplasmic membrane H^+^-ATPase was found in the detergent-released cell wall fraction and not in soluble extracts^2_. This result is consistent with the observation by others (37) that plasma membrane fragments and membrane proteins are associated with the cell wall fraction and that the soluble fraction contains negligible amounts of membrane components.

Like cytoplasmic membrane H^+^-ATPase, a form of Hwp1 was also found to be associated with the SDS-boiled cell wall fraction (Fig. 5, _lane 4_). The amount of this apparent membrane-associated form of Hwp1 (245.7 kDa) was greatly reduced by first treating the cell wall/membrane fraction with PI-PLC before boiling the cell wall/membrane fraction in SDS lysis buffer (Fig. 5, _lane 3_), consistent with the release of GPI-anchored Hwp1 from membranes. Overnight incubation of cell walls/membranes in PI-PLC buffer was accompanied by spontaneous release of Hwp1 associated with membranes (325 kDa).
Cleavage with PI-PLC resulted in a decrease in mass of native Hwp1 from 325 to 301 kDa (Fig. 5, lanes 1 and 2). The absence of a 245.7 kDa species in supernatants of treated or untreated cell walls/membranes (Fig. 5A, lanes 1 and 2) suggested that membrane-anchored Hwp1 was unstable upon boiling in SDS, and that the 245.7 kDa species was a breakdown product. The small amounts of the 235 kDa form of Hwp1 seen in Endo H samples (Fig. 4A) likely originated from this 245.7 kDa species (a small amount of contaminating membranes likely released the 245.7 kDa species when the immunoprecipitated Hwp1 was boiled in Endo H buffer; see Experimental Procedures) as it was also present in Endoglycosidase H treatments of detergent treated walls. The results show that fractions containing cytoplasmic membrane H+ - ATPase also contain GPI-anchored Hwp1 that is susceptible to cleavage by PI-PLC.

Cell wall anchorage of Hwp1. Attachment of cell wall proteins to the β-1,6-glucan is through a GPI anchor remnant (38,39) that bridges the cell wall protein and the β-1,6-glucan through a phosphodiester bond which is susceptible to cleavage by treatment with cold aqueous HF (40) or by phosphodiesterases (22). Cold aqueous HF treatment of washed cell walls (Fig. 5B, lane 5) or of Quantazyme released cell wall material (Fig. 4A, lane 5) liberated Hwp1 species of 301 and 180 kDa from higher molecular weight material greater than 500 and 325 kDa. The presence of polydisperse Hwp1 species of high Mr, larger than 301 kDa suggests that release of the remaining β-glucan attached to Hwp1 was not complete even though HF treatment was allowed to proceed for 18 h. The fragments released by HF were identical in size to those detected in cell extracts with Mr of 301k and 180 k (Fig. 3C, lane 2). Direct examination of the HF-released Hwp1 before immunoprecipitation revealed a large amount of the common 180 kDa band (Fig. 5B, lane 6) indicating that the antiserum does not immunoprecipitate this form as readily as the other species of Hwp1 (Fig. 5B, lane 5 vs lane 6). The fact that the size of the Hwp1 species released by HF is identical to the size of a form identified in the cell extracts (301 kDa) suggests that this species becomes attached to the β-1,6 glucan.

Studies of GPI-dependent cell wall proteins of S. cerevisiae show that the C-terminal 30-40 amino acids contain the GPI anchor amino acid and the necessary information to direct localization of the mannoprotein to the cell wall (34,41). To determine if similar criteria exist for C. albicans GPI-dependent cell wall proteins, a truncated form of Hwp1 with a deletion of the 26 C-terminal amino acids was expressed in the hwp1/hwp1 null strain (See Experimental Procedures). Germ tubes of strains expressing the truncated form of Hwp1 (Hwp1T) did not stain positive for Hwp1 on the surfaces of germ tubes (Fig. 6A, panel d) unlike hwp1/hwp1 null strains expressing wild type Hwp1 (Hwp1WT) or a wild type C. albicans strain (SC5314). Analysis of the germ tube culture media showed large amounts of the 301 kDa Hwp1 species in the media of Hwp1T strains relative to the wild type (SC5314) and hwp1/hwp1 null strains expressing wild type Hwp1 (Fig. 6B). The data strongly suggest that the C-terminal amino acids of Hwp1 contain the necessary information to direct GPI-anchorage and localization of Hwp1 to the cell wall of germ tubes.
DISCUSSION

The 78% inhibition of germ tube stabilized adhesion by pretreatment of BEC’s with rHwp1N13 is consistent with the importance of the TGase substrate domain of native Hwp1 in attachment of *C. albicans* to the oral mucosa. The reasons that inhibition did not reach 100% are not known. The presence of additional TGase substrates is unlikely given that null *hwp1*/*hwp1* mutants do not possess surface TGase substrate activity (10). One possibility is that the physical interaction of the germ tube surface to a BEC exposes cross-linking sites that are not accessible to masking by soluble rHwp1N13 molecules, thereby leading to incomplete inhibition of stabilized adhesion.

Biophysical and biochemical analyses of Hwp1 elucidate structural features that determine function in stabilized adhesion to the oroesophageal mucosa. The far UV CD spectra of rHwp1N13 suggest that the N-terminal domain exists completely as a coil. The ability to modify thiols only very slowly even in the presence of urea suggests that most of the cysteine side chains are present as disulfides, and the remainder are inaccessible to reagent. Therefore, the N-terminal domain of Hwp1 is likely a rigid coiled structure. The overall coiled structure is similar to the human SPR protein family of cornified cell envelope proteins (13,42,43). A comparison of the sequence of the N-terminal domain of Hwp1 and three members of the SPR family are shown in Table 2. The predicted mature N-terminus of Hwp1 resembles those of all three SPR in having Ser as the first amino acid. The presence of Tyr residues close to the amino terminus as well as immediately prior to the first string of glutamine residues is similar to SPR2 and SPR3. The Hwp1 repeats (10 amino acids) are similar in size to SPR internal domain repeats (8 amino acids) and are also similar in the presence of Cys, Pro and Glu residues. The Hwp1 repeats differ from those of the SPR family in the absence of Lys residues indicating that, unlike the SPR family, Hwp1 participates in cross-linking reactions solely as the Gln donor. The presence of 31 acidic residues characterizes rHwp1N13 as a very soluble protein, as is also observed for the other proteins in this family. There are no significant changes in the CD spectrum in the presence of denaturant, but there are slight changes in the presence of 2,2,2-trifluoroethanol up to a concentration of 70%. The latter changes are similar to those observed by Steinert et al. for SPR3 (43), but to a lesser extent. The changes in ellipticity in the case of SPR3 were suggested to reflect β-turns that exist between the repeating amino acid units. Although there is evidence for similar organized structure in Hwp1, the amount is much lower than that found in SPR3.

Fluorescence spectra provide information on the environment around Trp residues in rHwp1N13. Emission spectra obtained as a function of the excitation wavelength differ with those excited at wavelengths up to 280 nm, where Tyr and Trp residues will both absorb, giving a broad unresolved spectrum with a maximum at 350 nm and a shoulder on the red side of the maximum. Qualitatively identical spectra are obtained upon excitation at 260 and 298 nm. Some of the Trp fluorescence is quenched in the presence of 10 mM Ca$^{2+}$, in agreement with its accessibility to at least one of the Trp residues. Not only is Trp accessible in the truncated form of the protein, rHwp1N13, but it is probably accessible in the native protein in its entirety.
based on the chymotrypsin sensitivity of native Hwp1 on germ tube surfaces\(^2\), further strengthening the argument for the separation of the N-terminal domain from the rest of the protein. Urea, on the other hand, has no effect on fluorescence, in agreement with a rigid disulfide-stabilized polypeptide. The number of disulfides is in question, but there appear to be at least 3 thiols in the reduced form, with the other 8 likely in disulfide linkage. The very slow titration, and the low abundance of dimers, indicates all of the thiols are in the interior of the protein with only limited accessibility to the thiol reagent and other thiols in separate rHwp1N13 molecules.

Whereas the N-terminal domain of Hwp1 functions as a TGase substrate, the function of the C-terminal domain is to anchor the protein to the β-glucan by a covalent attachment via a GPI anchor remnant. Hwp1’s primary sequence has features consistent with other GPI-anchor dependent wall proteins described in \textit{S. cerevisiae} and \textit{C. albicans} (12,23,34,44-46): an N-terminal signal sequence, a stretch of 10-15 hydrophobic amino acids at the C-terminus, and a GPI attachment site that excludes basic amino acids just upstream of the GPI anchor residue (41). Although Hwp1 has all the features of a GPI-anchor dependent wall protein, some non-cell wall intermediates of Hwp1 were detected. The finding of a membrane-bound species of Hwp1 is consistent with the presence of membrane-bound forms of α-agglutinin in \textit{S. cerevisiae} (47) that were found to be intermediates of the mature cell wall anchored protein in pulse-chase experiments (12). Additional evidence for the common occurrence of both membrane and cell wall-anchored forms of cell wall localized proteins comes from studies of fusion proteins consisting of α-galactosidase fused to C-terminal sequences from GPI-CW proteins. Both membrane localization and PI-PLC susceptibility of fusion proteins were found (48). The results are consistent with the presence of a 325 kDa GPI anchored membrane species which may be a precursor of a periplasmic 301 kDa intermediate destined to become covalently attached to glucan. The idea that the soluble 301 kDa Hwp1 may represent a precursor population of Hwp1 in transition between a membrane protein and the wall anchored mature form stems from the observation that HF treatment of cell walls releases a 301 kDa species. Small amounts of the 301 kDa species are present in the culture media of germ tubes indicating that this form, if not bound to the cell wall, can diffuse out into the medium. Furthermore, expressing a C-terminal truncated form of Hwp1 produced a protein of 301 kDa that was not bound to germ tube walls but instead found in the culture medium. The latter results also showed that the C-terminal 26 amino acids of Hwp1 contain the necessary information to guide and link the protein to the β-glucan. The predicted function of the omega site in cell wall anchorage was recently confirmed in studies using a green fluorescent protein reporter to study localization properties of the C-terminus of Hwp1 (49).

See Supplemental Material for a diagram summarizing the proposed cellular localizations of the Hwp1 species.

A notable finding relative to the question of cell wall porosity was the detection of a membrane form of Hwp1. Other studies have suggested that hyphal walls are more porous than yeast walls in \textit{C. albicans}. For example, loss of the periplasmic enzyme N-acetylglucosaminidase into the medium was increased by four-fold upon germ tube formation (50,51). In the
present study, the accessibility of the 325 kDa species to cross-linking with 5-(biotinamido)pentylamine by TGase 2 indicates that membrane bound Hwp1 is exposed to the environment. The results indicate that the relatively porous hyphal cell wall and the presence of a reducing agent (51) (see Experimental Procedures) permit access of TGase 2 ($M_r$ of 72 k) to the plasma membrane where it cross-links 5-(biotinamido)pentylamine to the 325 kDa species and to any other Hwp1 molecules residing in the periplasm. This may also explain how the soluble 301kDa species was detected by our assay.

Although non-covalently bound forms of Hwp1 exist at the surface of germ tubes, it is likely that TGase associated with host buccal epithelial cell surfaces interact only with the mature cell wall attached Hwp1 during adhesion. TGase activity of the surfaces of BEC has been attributed to membrane-bound TGase 1 (11) where it functions to cross-link proteins in the formation of the cornified cell envelope. During stabilized adhesion of germ tubes to the surface of BEC, the insoluble TGase 1 probably contacts the most surface exposed Hwp1 in the cell wall and cross-links the protein (and the organism) to yet unidentified host surface protein(s). Physical constraints would prevent BEC TGase 1 from penetrating the hyphal wall to interact with non-wall species of Hwp1.

The location of the common 180 kDa species and cannot be inferred from our results. The two main features of this species were the apparent lack of $N$-linked glycans and a decreased avidity of the antiserum towards the 180 kDa protein. Mechanical disruption disperses the 180 kDa form of Hwp1 into all the subcellular fractions suggestive of a loose association with the cell surface. Detection of the 180 kDa species is detected in Quantazyme digestions allowed to proceed for 18 h$^2$ and in HF treatment of walls that were incubated for 18 h, see Experimental Procedures, suggests that perhaps this species of Hwp1 is a degradation product. Alternative possibilities include improper maturation through the secretory pathway possibly as a consequence of the high abundance of $HWP1$ mRNA and protein and saturation of secretory factors and glycosylation enzymes.

Reduced $O$-glycosylation of Hwp1 did not affect the eventual maturation into the cell wall bound form. Although the amount of surface Hwp1 produced in the $pmt1/pmt1$ mutant was reduced, mature Hwp1 was still generated at the cell wall consistent with the presence of multiple genes that encode mannosyltransferases. The changes in molecular sizes of the (partially) deglycosylated proteins were small but consistent with the report of shorter $N$-glycan chains found in hyphal versus yeast cell wall mannoproteins (31,52).

Computer modeling predicts that the N-terminal domain of Hwp1 is hydrophilic (23) and a predominantly coiled structure. Searches for other proteins with similarities to rHwp1N13 were not fruitful. In contrast, searches for other proteins with similarities at the Hwp1 C-terminus in GenBank and at Stanford University’s final assembly (Assembly 6) of the Candida albicans genome sequencing project (http://sequence-www.stanford.edu/group/candida/index.html) identified two gene products. One of these is Rbt1p (repressed by $TUP1$ protein) previously characterized by Braun et al. (6) as a putative wall
Characterization of Hwp1 from *Candida albicans*.

protein. The other is to an unnamed gene, ORF 2929 on Contig 6-2182. The identity between the C-terminal sixty amino acids of Hwp1 to the same sixty amino acids of ORF 2929 is 50%. ORF 2929 has other features of cell wall proteins (N-terminal secretion signal, Ser/Thr rich middle region and a hydrophobic C-terminus with the same characteristics as GPI-anchored wall proteins) and, as such, is likely to be another wall protein. The open reading frame codes for a protein with 908 amino acids (Hwp1 is 634 amino acids in length) and has very poor similarity to Hwp1 outside of the terminal sixty amino acids, thus it is unlikely that ORF 2929 is an allele of *HWP1*. Taken together, the data show that *HWP1* encodes for a unique protein with combined mammalian and fungal functional domains.
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FOOTNOTES

1 The abbreviations used are: Hwp1, Hyphal wall protein 1; TGase(s), transglutaminase(s); BECs, buccal epithelial cells; SPR, small proline rich; GPI, glycosylphosphatidylinositol; AA, amino acid; CD, circular dichroic; PCR, polymerase chain reaction; rHwp1N13, recombinant Hwp1 produced in *Pichia pastoris*; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HRP, horse radish peroxidase; HF, hydrofluoric acid; Endo H, Endo *N*-acetylglicosaminidase H.

2 J.F. Staab and P. Sundstrom, unpublished observations.

3 C-H. Tai and P.F. Cook, unpublished observations.

4 J.F. Staab, P. Sundstrom and P.F. Cook, unpublished observations.
FIGURE LEGENDS

FIG. 1. Inhibition of germ tube adhesion by rHwp1N13. Buccal epithelial cells (BECs) were incubated with rHwp1N13 or salivary amylase at 2 or 4 µM, respectively, prior to adding radiolabeled wild type (SC5314) germ tubes (see Experimental Procedures) to measure the ability of rHwp1N13 to interfere with germ tube adhesion. Germ tube adhesion to BECs was determined relative to assays performed in the absence of added protein (No protein, set at 100% adhesion). Increasing the concentration of rHwp1N13 to 8 µM did not increase the magnitude of inhibition (data not shown). The germ tube adhesion inhibition by rHwp1N13 was significant relative to no protein (P < 0.0002) as determined by the Student’s t test. Salivary amylase did not have an appreciable affect on germ tube adhesion (P > 0.05 relative to no protein control).

FIG. 2. Spectral studies of rHwp1N13. A. Far UV CD spectra. The CD spectra of rHwp1N13 was measured at pH 7 in 10 mM phosphate and at 25°C in the absence (____) and presence (----) of 10 mM Ca²⁺ at protein concentration of 100 µg/mL. Each spectrum is the average of triplicate determinations. Inset, Far UV CD spectra in the absence (____) and presence (----) of 70% 2,2,2-trifluoroethanol. B. Fluorescence spectra. The spectra were obtained for the protein at 40 µg/mL in 10 mM phosphate, pH 7 at 25°C. The excitation monochromator was fixed at 280 (thin line —), or 298 (heavy line ▬), and the emission monochromator was scanned from 300 to 450 nm. The bandwidths for excitation and emission were both fixed at 4 nm. The dotted line (----) shows the spectrum for the 298 nm emission spectrum corrected (based on maximum intensity) to that obtained upon excitation at 280 nm. C. Thiol titration of with 4,4'-dithiodipyridine. The presence of free thiols in rHwp1N13 was measured in a solution of 0.4 mM 4,4'-dithiodipyridine in Hepes pH 7. The increase in absorbance at 324 nm indicates the production of the chromophore 4-thiopyridine.

FIG. 3. Identification of Hwp1 covalently attached to the cell walls of germ tubes. A. Isolated cell walls from germ tubes treated with TGase 2 and 5-(biotinamido)pentylamine were digested with the β-1,3-glucanase, Quantazyme, and the released Hwp1 proteins immunoprecipitated and analyzed by SDS-PAGE and Western blotting. Immunoprecipitated Hwp1-5-(biotinamido)pentylamine was visualized with Streptavidin-HRP and chemiluminescence. Germ tubes of the hwp1/hwp1 null mutant (lanes 3, 4, 7, and 8) served as negative controls for wild type cells (lanes 1, 2, 5, and 6). Samples in lanes 1, 3, 5, and 7 were incubated with preimmune serum, and lanes 2, 4, 6, and 8 were incubated with immune serum prior to SDS-PAGE and Western blotting. B. Hwp1 immunoprecipitated from metabolically radiolabeled wild type strain germ tubes. Isolated cell walls were digested with Quantazyme, and the released wall proteins reacted with preimmune serum (lane 9) and immune serum (lane 10) prior to SDS-PAGE and fluorography. C. Soluble forms of 5-(biotinamido)pentylamine-modified Hwp1 species. Soluble forms of Hwp1 exposed to the environment were found in cell extracts of wild type (lanes 1 and 2) but not in the hwp1/hwp1 null mutant (lanes 3 and 4) germ tubes. Hwp1 species were immunoprecipitated with preimmune (lanes 1 and
Characterization of Hwp1 from *Candida albicans.*

3) or immune serum (*lanes 2 and 4*) and analyzed by SDS-PAGE and Western blotting as in *A.* Arrows point to the relative molecular sizes of Hwp1 species in kDa. Molecular size standards are shown as black bars in kDa.

FIG. 4. **Glycosylation of Hwp1 proteins.** *A.* Hwp1-5-(biotinamido)pentylamine was immunoprecipitated with immune serum from wild type cell fractions and digested with Endo H (“+” above lanes) before SDS-PAGE and Western blotting. Deglycosylated Hwp1 species (289 and 258 kDa in descending order) in cell extracts are indicated by asterisks. Endo H appears not to affect the 180 kDa species. The 235 kDa form reflecting the presence of membranes is marked by a dot. Quantazyme-released Hwp1 from cell walls (*lanes 3 through 6*) in *lanes 5* and *6* were subsequently treated with aqueous HF before digesting with Endo H (see text, *Cell wall anchorage of Hwp1*). *B.* Hypomannosylation of Hwp1. Hwp1-5-(biotinamido)pentylamine proteins were immunoprecipitated with immune serum from cell wall fractions treated with Quantazyme of wild type and *O*-mannosylation mutant strains prior to analysis by SDS-PAGE and Western blotting. The genotype of each strain is shown above the corresponding lanes. The sample volume analyzed by SDS-PAGE for the pmt1/pmt1 mutant strain was doubled to ensure close to equal visualization of Hwp1 proteins (see *Glycosylation of Hwp1*).

The location of wild type Hwp1 species and molecular sizes in kDa are shown by open arrows. Under manniosylated Hwp1 species are shown by a bracket. Hwp1-5-(biotinamido)pentylamine was visualized by Streptavidin-HRP followed by chemiluminescence. Molecular size standards in kDa are shown at right.

FIG. 5. **Hwp1 is anchored to the cell wall by a GPI remnant.** *A.* Release of a membrane-bound form of Hwp1 by treatment of cell walls/plasma membrane fractions with phosphoinositol-phospholipase C (PI-PLC). Isolated cell walls (not boiled in SDS) from TGase 2 and 5-(biotinamido)pentylamine treated wild type strain germ tubes were incubated with (“+” above lanes) or without (“-” above lanes) PI-PLC (see Experimental Methods). After an 18 h incubation with PI-PLC, the cell walls were removed by centrifugation, and the supernatants analyzed for Hwp1-5-(biotinamido)pentylamine proteins (*lanes 1* and *2*; see Experimental Methods). The pelleted cell walls were boiled in SDS to release any remaining membrane-associated Hwp1, centrifuged, and the supernatants analyzed for Hwp1-5-(biotinamido)pentylamine species (*lanes 3* and *4*). Immunoprecipitated Hwp1-5-(biotinamido)pentylamine proteins from the PI-PLC and SDS fractions were visualized by SDS-PAGE and Western blotting. Hwp1 species are shown by open arrows. Hwp1 released from cell walls by boiling in detergent is shown by a closed arrow. *B.* TGase 2 and 5-(biotinamido)pentylamine treated wild type strain germ tubes were fractionated, and the detergent boiled cell walls treated with Quantazyme or aqueous HF (“+” above the appropriate lanes). Hwp1-5-(biotinamido)pentylamine released from the cell walls was analyzed directly (*lane 6*) or immunoprecipitated with immune serum (*lanes 5* and *7*) prior to SDS-PAGE and Western blotting. Quantazyme-released Hwp1 species are shown by open arrows at right and HF released Hwp1 species are shown by open arrows at left. Hwp1-5-(biotinamido)pentylamine was visualized by Streptavidin-HRP followed by chemiluminescence. Molecular size standards in kDa are shown at right.
FIG. 6. **Anchorage of Hwp1 to the cell wall is through its C-terminus.**  

**A.** Localization of Hwp1 in germ tubes expressing a truncated form of Hwp1. Immunofluorescence assays were performed to detect Hwp1 on the surface of germ tubes expressing a wild type (panel c, Hwp1WT) or a truncated form of Hwp1 (panel d, Hwp1T). The Hwp1 constructs were expressed in the hwp1/hwp1 null strain (CAH7-1A1, panel b). The wild type strain, SC5314 (panel a) served as control for detection of Hwp1 at the surfaces of germ tubes. Antibodies to Hwp1 were detected with goat anti-rabbit IgGs conjugated to FITC. Non-reactive cell surfaces were counter-stained with BSA conjugated to rhodamine.  

**B.** Fate of Hwp1 missing its C-terminal 26 amino acids. The culture media of strains induced to form germ tubes was analyzed for Hwp1 by Western blotting. The wild type strain (wt) releases a detectable amount of the 301 kDa form of Hwp1 (arrow) into the medium which is missing in the medium of the hwp1/hwp1 null strain (∆). Two independent hwp1 null transformants (∆ in upper row) expressing the wild type (wt, lower row) or the truncated (T, lower row) forms of Hwp1 were analyzed for loss of Hwp1 into the medium.
TABLE 1. Relative cellular distribution of radiolabeled Hwp1 species.

| Subcellular fraction         | Average total cpm +/- SD* |
|-----------------------------|--------------------------|
| Extracts                    | 10.5% +/- 3.0 %          |
| SDS boiled cell walls       | 14.5% +/- 1.8%           |
| Quantazyme digested cell walls | 75.0% +/- 4.0%          |

*ImageQuant software (Molecular Dynamics) was used to quantify the relative amounts of Hwp1 from metabolically radiolabeled germ tubes in three separate immunoprecipitations performed with two different rabbit antisera to rHwp1 (326 antiserum (4) and R1 antiserum raised to rHwp1N13). Quantazyme digests were performed for 18 h to release the maximal amount of Hwp1 from SDS-boiled and washed germ tube cell walls.
TABLE 2. Similarities between Hwp1 and SPR proteins.

|                  | SPR1 | SPR2 | SPR3 | Hwp1 |
|------------------|------|------|------|------|
| **Hwp1 aligned with head domains of SPR proteins:** |      |      |      |      |
| SPR1             | ---SSQQQKQPCIPPPPQLQQQQVKPCQ2  |      |      |      |
| SPR2             | ---SYQQQCKQPCQPPPVCPT19       |      |      |      |
| SPR3             | ---SSYQQKQTFT-PPPQLQQQQVKQPSQ25 |      |      |      |
| **Hwp1**         | SYDYYQEPCCD—YPQQQQQQEPCDYPQQQQQQEPCDYPQQQ40 |      |      |      |

**Hwp1 repeats compared to repeats in the internal domains of SPR proteins:**

|      | SPR1 | SPR2 | SPR3 | Hwp1 |
|------|------|------|------|------|
| 20PPQPEPCI | 20PKCPEPCPP | 38EPCHSKVP | 20EPCDYPQQQQQEQE |
| 34PKTKEPCHP | 29PKCPEPCPP | 46QPGNTKIP | 32EPCDYPQQQQQEQ |
| 42PKVEPCH | 38PKCPQPSPP | 54EPCGTKVP | 43EPCDYPQQQQQEQ|
| 50PKVEPCCQ |                     | 62EPCGTKVP | 53EPCDYPQQQQQEQ|
| 58PKVEPCCQ |                     | 70EPCGTKVP | 63EPCDYPQQQQQEQ|
| 66PKVEPCCP |                     | 78EPCGTKVP | 73EPCDNPPQPD |
|           |                     | 86EPCGTKVP | 83VPCDNPPQPD |
|           |                     | 94EPCGTKVP | 93VPCDNPPQPD |
|           |                     | 102EPGYTKVP | 103IPCDNPPQPD |
|           |                     | 110EPGSIKVP | 113IPCDNPPQPD |
|           |                     | 118DQGFIKFP | 123QPDDNPPIPN |
|           |                     | 126EPGAIKVP | 133IPTD-W---IPN |
|           |                     | 134EPGYTKVP | 141IPTD-W---IPD |
|           |                     | 142VPGYTKLP |                     |

Residues in bold become cross-linked in the presence of epithelial cell TGases *in vitro* (13,42,43) Glns in Hwp1 that are predicted to become cross-linked are shown underlined in bold. Trp residues monitored in the fluorescence spectra of Hwp1 are shown in *bold italic.*
FIGURE 1

![Graph showing enzyme activity](image-url)
FIGURE 2

A

[Θ] Molar Ellipticity

Wavelength (nm)

B

Relative Fluorescence

Emission (nm)

C

delta OD$_{324}$ nm

Time (Hour)
FIGURE 3

A.  TGase-labeled  

| Quantzyme | HWP1 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-----------|------|---|---|---|---|---|---|---|---|
| wt | wt | wt | wt | wt | wt | wt | wt | wt |

B.  35S-Protein  

| Extracts | HWP1 |
|----------|------|
| wt | wt | wt | wt |

C.  Extracts  

- 500 → 325 → 250 → 148

- 301 → 180 → 250 → 148
FIGURE 4

A. Cell Ext. Quant. walls
Aq. HF - - + +
Endo H - + - + - +
1 2 3 4 5 6

B. Quant. walls
wt pmtl mntl
7 8 9

~500 325 250 148
FIGURE 5

A.

| PI-PLC | 1 | 2 | 3 | 4 |
|--------|---|---|---|---|
| +      | - | + | - |

B.

| Quant. | - | - | + |
|--------|---|---|---|
| Aq. HF | - | - | + |
| +      | + | - | + |
| antiserum | 5 | 6 | 7 |

Assays: X46/X49/X47/X55/X52/X45/X20/X35
FIGURE 6

A. SC5314 CAH7-1A1

Hwp1WT Hwp1T

B. Strain Construct wt Δ Δ Δ Δ Δ

250

301

148
Supplemental Figure. Anatomy and processing of Hwp1. A. Features of the primary amino acid sequence of the Hwp1 proprotein consisting of: a signal peptide followed by a Kex2p cleavage site; TGase substrate domain devoid of sites for glycosylation with solvent-exposed tryptophan(s) at the COOH-terminal end; Ser/Thr-rich regions that become extensively O-mannosylated; three motifs specifying N-linked glycosylation (bow tie shapes); ω site for GPI modification followed by hydrophobic COOH-terminal residues which are proteolytically cleaved prior to GPI anchor addition via transamidation in the ER; informational determinants (isoleucine at -5 residues upstream from the ω site) for cleavage and transglycosylation by an unknown transglycosidase just upstream of the ω site as indicated by the red oval. B. GPI-anchored membrane form. This form is released from membranes by treatment with PI-PLC or cold aqueous HF which attacks the phosphodiester bonds in the GPI anchor. The site of action of a predicted transglycosylase (gray arrow) and sites of action of PI-PLC and aqueous HF are indicated (purple arrows). The S. cerevisiae GPI anchor structure (1) is shown since the C. albicans GPI anchor structure is unknown. The pink circle is inositol, blue and green circles are mannoses, gray square is glucosamine, brown diamond is ethanolamine. C. Intermediate form of Hwp1 predicted to be attached to a GPI remnant prior to being transferred to β(1,6)-glucan. This form is found soluble in cytoplasmic extracts and in small amounts in culture supernatants. A species of the same size is released from membranes by PI-PLC and from cell walls by aqueous HF. D. Hwp1 cross-linked to β(1,3)-glucan via β(1,6)-glucan in the cell wall. This form of Hwp1 is released from cell walls by digestion with β(1,3)-glucanase or by aqueous HF which attacks the phosphodiester bond in the GPI remnant.

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Expression of transglutaminase substrate activity on Candida albicans germ tubes through a coiled, disulfide-bonded N-terminal domain of Hwp1 requires C-terminal glycosylphosphatidylinositol modification

Janet F. Staab, Yong-Sun Bahn, Chia-Hui Thai, Paul F. Cook and Paula Sundstrom

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