Yeast wall protein 1 of *Candida albicans*

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Yeast wall protein 1 (Ywp1, also called Pga24) of *Candida albicans* is predicted to be a 533 aa polypeptide with an N-terminal secretion signal, a C-terminal glycosylphosphatidylinositol anchor signal and a central region rich in serine and threonine. In yeast cultures, Ywp1p appeared to be linked covalently to glucans of the wall matrix, but, as cultures approached stationary phase, Ywp1p accumulated in the medium and was extractable from cells with disulfide-reducing agents. An 11 kDa propeptide of Ywp1p was also present in these soluble fractions; it possessed the sole N-glycan of Ywp1p and served as a useful marker for Ywp1p. DNA vaccines encoding all or part of Ywp1p generated analytically useful antisera in mice, but did not increase survival times for disseminated candidiasis. Replacement of the coding sequence of YWP1 with the fluorescent reporter GFP revealed that expression of YWP1 is greatest during yeast exponential-phase growth, but downregulated in stationary phase and upon filamentation. Expression was upregulated when the extracellular phosphate concentration was low. Disruption by homologous recombination of both YWP1 alleles resulted in no obvious change in growth, morphology or virulence, but the Ywp1p-deficient blastoconidia exhibited increased adhesiveness and biofilm formation, suggesting that Ywp1p may promote dispersal of yeast forms of *C. albicans*.

**INTRODUCTION**

*Candida albicans* is a commensal fungus of humans that can become pathogenic when host immune defences are compromised. It can invade most anatomical sites, resulting in morbidity when associated with epithelial surfaces and mortality at deep internal sites. *C. albicans* is capable of producing yeast and filamentous forms, and the presence of both forms typifies an infectious process; the role of each morphology in dissemination and pathogenesis continues to be investigated and debated (Gow et al., 2002; Saville et al., 2003).

Adhesive biofilms of *C. albicans* may form on tissue surfaces, bioprostheses and catheters; such biofilms exhibit increased resistance to antifungal agents and may be reservoirs for reinfection (Douglas, 2003). Adhesion of *C. albicans* is mediated by the cell wall, the primary structural basis of which is cross-linked glucans that make up 50–60 % of the wall weight (Kapteyn et al., 2000); mannoproteins with adhesive and other functions (Chaffin et al., 1998) make up another 30–40 %, and small amounts of chitin add tensile strength (Munro et al., 2003). Most wall proteins have structural features that result in replacement of their C termini with glycosylphosphatidylinositol (GPI) membrane anchors, which may themselves be removed prior to transfer of the polypeptide to β-1,6-glucans in the wall matrix (Kapteyn et al., 2000). Wall proteins are also typically N- and O-glycosylated, with the N-glycans including long, branched chains of mannose linked with glycosidic and occasional phosphodiester bonds. Adhesion of *C. albicans* may involve specific wall glycoproteins, such as Hwp1p and products of the ALS gene family (Hoyer, 2001; Sundstrom, 2002), or glycans alone (Masuoka, 2004); the regulation and mechanistic details of *in vitro* and *in vivo* cellular adhesion, however, remain poorly understood.

Thiol reagents, such as dithiothreitol (DTT) and...
2-mercaptoethanol, solubilize a subset of extracellular manno-proteins from live cells, presumably as a result of reduction of proteinaceous disulfide bonds in the wall (Chattaway et al., 1974; de Nobel & Barnett, 1991). Such extracts typically contain 5–10% protein and 90–95% carbohydrate and, when administered to mice in certain vaccine formulations, afford protection against disseminated candidiasis (Han & Cutler, 1995; Han et al., 1999). Little is known about the identity of potential protective epitopes in these thiol extracts, other than β1,2-di- and trimannose epitopes that are terminal elements of certain polysaccharide-chain branches (Han & Cutler, 1995; Han et al., 1997; Nitz et al., 2002). We continue to examine the polypeptide component of such extracts for additional protective epitopes, as well as possible carriers for vaccines. Here, we describe a wall mannoprotein termed Ywp1p that has a complex post-translational itinerary and appears to inhibit both adhesion and biofilm formation by blastoconidia. Experimentally, Ywp1p may be useful as a marker of the yeast form of C. albicans and thus complement the growing panel of markers of hyphae, such as Hwp1p (Staab et al., 2003).

**METHODS**

**Strains and culture media.** Strains are listed in Table 1. A defined, minimal culture medium (medium 13) was devised, consisting of sterile-filtered 100 mM glucose, 80 mM NH₄Cl, 5 mM NaCl, 5 mM KH₂PO₄, 1 mM MgSO₄, 1 mM MnCl₂, 0.2 mM CuCl₂, The pH was left unadjusted at ~4. The last six components were pre-mixed, sterile-filtered and stored at room temperature as a 1000 × stock, with the succinic acid serving to stabilize the cations. In experiments requiring low phosphate, a portion of the phosphate was replaced with chloride whilst maintaining the potassium at 5 mM (e.g. 0.2 mM KH₂PO₄ and 4.8 mM KCl). Supplements for auxotrophic strains included 1 mM arginine hydrochloride, 1 mM histidine hydrochloride and 0.5–5 mM uridine.

**Recombinant DNA methods.** Both alleles of YWP1 were disrupted by homologous recombination of transforming DNA cassettes as described previously (Wilson et al., 1999, 2000). Briefly, PCR amplicons were transfected into C. albicans strain BWP17 by use of lithium acetate (Gietz & Woods, 2002). YWP1 allele 1 was disrupted with an amplicon containing ARG4, and allele 2 with an amplicon containing a recyclable URA3-dpl cassette. In each case, flanking ~60 bp segments homologous to YWP1 facilitated in vivo replacement of YWP1 codons 22–271 with the selectable markers. Disruptions were confirmed by Southern blotting and numerous PCR assays using genomic DNA that had been purified according to the method of Hoffman & Winston (1987).

**Table 1. Strains of C. albicans used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3153A</td>
<td>Wild-type (standard laboratory strain)</td>
<td>Brawner &amp; Cutler (1989)</td>
</tr>
<tr>
<td>CA-1</td>
<td>Wild-type (clinical isolate)</td>
<td>Gillum et al. (1984)</td>
</tr>
<tr>
<td>SC5314</td>
<td>Wild-type (clinical isolate)</td>
<td>Wilson et al. (1999)</td>
</tr>
<tr>
<td>BWP17</td>
<td>ura3Δ::imm34/ura3Δ::imm34 arg4::hisG/arg4::hisG his1::hisG his1::hisG</td>
<td>Davis et al. (2000)</td>
</tr>
<tr>
<td>DAY185</td>
<td>arg4::hisG/arg4::hisG:ARG4-URA3 his1::hisG/ his1::hisG::HIS1</td>
<td>Newport &amp; Agabian (1997)</td>
</tr>
<tr>
<td>CNA3-1</td>
<td>ura3Δ::imm34/ura3Δ::imm34 kex2::hisG/kex2::hisG-URA3-hisG</td>
<td>This study</td>
</tr>
<tr>
<td>BJ3</td>
<td>ura3Δ::imm34/ura3Δ::imm34 arg3::hisG/arg3::hisG YWP1::ywp1::GFP-HIS1</td>
<td>This study</td>
</tr>
<tr>
<td>BJ3a1a</td>
<td>ura3Δ::imm34/ura3Δ::imm34 arg3::hisG/arg3::hisG ywp1::GFP-HIS1</td>
<td>This study</td>
</tr>
<tr>
<td>Ca#12</td>
<td>ywp1::ARG4/ywp1::URA3-dpl200 his1::hisG/his1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>_r1&amp;2</td>
<td>(Ca#12) his1::hisG/his1::HIS1-YWP1</td>
<td>This study</td>
</tr>
<tr>
<td>_s1&amp;2</td>
<td>(Ca#12) ywp1::(ARG4 or URA3-dpl200)::YWP1-HIS1/ywp1::(ARG4 or URA3-dpl200)</td>
<td>This study</td>
</tr>
</tbody>
</table>

YWP1, HIS1 and URA3 were amplified by PCR from C. albicans strain 1535A genomic DNA. Amplicons included 999 bp upstream (′5′) and 852 bp downstream (′3′) from the coding sequence of YWP1, 231 bp ′5′ and 51 bp ′3′ from the coding sequence of HIS1, and 435 bp ′5′ and 141 bp ′3′ from the coding sequence of URA3. HIS1 and YWP1 alleles were cloned in the T vectors pGEM-T and pGEM-T Easy (Promega), respectively, in Escherichia coli TOP10 (Invitrogen). For combination of YWP1 and HIS1 in the same plasmid, a Nol restriction fragment containing YWP1 was cloned into a unique Nol site in the HIS1 plasmid after removal of a unique Nol site from pGEM-T; this resulted in 50 bp vector sequence between HIS1 and YWP1. Prior to transformation, plasmids were linearized at a unique Nra site in HIS1 (121 bp upstream from the start codon; clones designated ‘r’) or a unique Nol site in YWP1 (414 bp upstream from the start codon; clones designated ‘s’). The two alleles of YWP1 (YWP1-1 and YWP1-2) were distinguished readily by a HindIII site 920 bp upstream from the start codon of allele 1 only. Cloned C. albicans transformants had one of two independent HIS1 alleles (HIS1-3 or HIS1-4) and were named as follows, where ‘r>’ indicates the relative orientation of the transcript (′5′ >′3′) and protein (N>C): strain 3L1, HIS1-3; strain 4L1, HIS1-4; strains 2r and 2s, HIS1-3<--YWP1-2>; strains 7r and 7s, HIS1-3<--YWP1-1>; strains 13r and 13s, HIS1-4<--YWP1-2<; strains 16r and 16s, HIS1-4-->YWP1-1<.

For insertion of the green fluorescent protein (GFP) gene into C. albicans, yeast enhanced GFP (yEGFP; Cormack et al., 1997) was linked to the selectable markers HIS1 and URA3 to facilitate identification of transformants (Gerami-Nejad et al., 2001). The coding sequence of GFP was amplified by PCR from pyGFP3 and spliced by overlap extension (Horton et al., 1989) to the HIS1 and URA3 amplicons described above (GenBank accession nos AFY27707 and AUG65808, respectively); this presumably allows GFP to utilize the transcription-termination signals of the genes that are predicted to be immediately upstream and co-directional with HIS1 and URA3 in the C. albicans genome. Homologous recombination of transfected
PCR amplicons was used to replace the coding sequence of one allele of YWP1 with GFP-HIS1, such that the start codon of YWP1 became the start codon of GFP and expression of GFP was driven by the YWP1 promoter (generating a soluble, cytosolic form of GFP). PCR analyses of genomic DNA from fluorescent transformants confirmed that GFP had indeed replaced YWP1. One of these strains (BJ3) was subcloned and screened for increased fluorescence; several subclones, including BJ3a (and its successive subclones, BJ3a1 and BJ3a1a) were found to have undergone a presumptive gene conversion or mitotic recombination event (Enloe et al., 2000) that replaced the remaining YWP1 allele with GFP.

**DNA vaccines.** All vaccine plasmids were derivatives of pBSA, created by removal of a G-418 resistance cassette (Sall fragment) from the expression vector pBGSα (Uthayakumar & Granger, 1995; GenBank accession no. AY660671). Inserted coding sequences were assembled from PCR amplicons or synthetic oligonucleotides (codon-optimized) that encoded one or more of the following elements: (i) various segments of Ywp1p, as indicated below; (ii) entire (140 aa) mouse interleukin 4 (mIL4, from a cloned cDNA in p2A-E3, ATCC 37561; Maeczek et al., 1997); (iii) entire (406 aa) mouse lysosome-associated membrane protein 1 (mLAMP-1 or Igp-A; Granger et al., 1985); (iv) FISEAHHVLHSR, an H-2-restricted T-cell epitope of sperm-whale myoglobin (SWM; Rothbard & Taylor, 1988; Golzano et al., 1990); and (v) FDTGAFDPDWPA, a peptide mimotope (S9–24) of group B streptococci capsular polysaccharide recognized by mAb S9 (Pincus et al., 1998). For embedding of foreign coding sequences in the mLAMP-1 cDNA, the codon for proline 197 was changed to an alanine codon to create a unique Nhel site; the vector harbouring this construct lacked the terminator of pBSA, instead utilizing the 3′ untranslated region of the LAMP-1 cDNA. Sequences were confirmed by commercial, automated sequencing of plasmid DNA (Davis Sequencing).

Plasmids were delivered biolistically at 300 p.s.i. to the shaved abdomen of female BALB/c mice with a Helios gene gun (Bio-Rad) according to the manufacturer’s instructions. Each shot nominally contained 1 μg DNA that had been precipitated and dried onto 0.5 mg 1-0 μm gold spheres, giving approximately 4 × 10⁶ plasmids on each of 5 × 10⁵ spheres. Histological examination showed that most of the particles that penetrated the skin were delivered to the basal layers of the epidermis. Typically, single shots were administered with a spacing of at least 4 weeks and blood was collected from tail veins 8–9 days after each boost. Unfractionated sera were assayed by immunofluorescence microscopy of COS-1 cells that had been transfected with one of the vaccine plasmids (described below).

Administered DNA vaccines encoded all 533 aa of Ywp1p or one of the following six chimeric polypeptides (some with tandemly repeated segments of Ywp1p): (i) mIL4/Ywp1 aa 51–197/Ywp1 aa 51–197; (ii) mLAMP-1/Ywp1 aa 1–196/Ywp1 aa 51–197/mLAMP-1 aa 198–406; (iii) mL4/SWM/Ywp1 aa 105–161/S9–24/SWM; (iv) mIL4/SWM/Ywp1 aa 105–161/Ywp1 aa 105–161/S9–24/SWM; (v) mIL4/SWM/Ywp1 aa 21–116/S9–24/SWM; (vi) mIL4/SWM/Ywp1 aa 21–116/Ywp1 aa 21–116/S9–24/SWM. Up to six extraneous codons were present as linkers between the above segments and contained Nhel or AgeI sites that facilitated plasmid assembly (GenBank accession no. AY661302). Unexpectedly, segments encoding Ywp1p aa 21–169 and 1–334 could not be cloned in the desired orientation in the above expression plasmids, and most of the above YWP1-containing plasmids were difficult to create. Mice vaccinated with constructs (i), (iii), and the full-length YWP1 were challenged by intravenous injection of live C. albicans strain CA-1 in our standard protection assay (Han et al., 1999).

All expression plasmids were tested in cultured mammalian cells prior to administration to mice. Typically, transfection of COS-1 cells was followed 1 day later by formaldehyde or methanol fixation, then immunofluorescence microscopy utilizing the following antibodies specific for epitopes in the encoded polypeptides: mL4 (rat mAb 11B11; Pharmingen), mLAMP-1 (rat mAb 1D4B; Chen et al., 1985), S9–24 (mouse mAb S9; Pincus et al., 1998) or Ywp1p (the detection of antisera from vaccinated mice that developed during this study). Stability of the chimeric polypeptides was enhanced by adding the membrane-permeable protease inhibitor acetyl-leucyl-leucyl-norleucinal to the COS-1 cell medium at 20 μM for a few hours prior to fixation. The S9–24 peptide was included on the C-terminal side of some Ywp1p segments for two reasons: detection of the S9 epitope in transfected COS-1 cells demonstrated that the plasmid had directed synthesis of the chimeric polypeptide successfully and the presence of antibodies against S9–24 in vaccinated mice implied that their immune systems had also been exposed to the upstream Ywp1p segment. mL4 was included in most constructs to foster a humoral rather than a cell-mediated immune response, as part of a wider vaccine effort to elucidate the contribution of circulating antibodies to immunoprotection (Cutler et al., 2002). The mLAMP-1 chimeras tended to generate lower titres than the mL4 chimeras, but were useful when expressed in COS-1 cells because their membrane anchorage made them less likely to be lost before or during fixation, and because they represented an alternative carrier unable to bind antibodies that might have been generated against the other carriers. Antisera specificity was further demonstrated by Western blotting (described below), which showed that the anti-Ywp1p antisera gave strong signals that were absent from samples derived from Ywp1p/Ywp1 knockout strains; sera from unvaccinated mice gave no such signals.

**Virulence testing.** Prototrophic strains of C. albicans, with or without Ywp1p, were compared in a mouse model of disseminated candidiasis (Han et al., 1999). Cultures were grown in aerated limiting medium for 1–2 days at 30 °C; the cells were then washed and diluted in saline (155 mM NaCl) to an OD₆₀₀ of 0.060 (nominally 2.5 × 10⁶ cells ml⁻¹) and a 0.20 ml aliquot (~5 × 10⁵ cells) was injected into the tail vein of each mouse. Two trials utilized a single batch of female BALB/c mice from Charles River Laboratories.

**Protein analysis.** PAGE of SDS-denatured proteins was performed as described by Laemmli (1970) (Figs 1 and 6b) and Schägger & Von Jagow (1987); acrylamide/bis concentrations in the resolving gel were 15/0.1 and 10/0/3 %, respectively. Isoelectric focusing (IEF) was performed in immobilized pH gradients (13 cm pH 3–10 Immobiline DryStrips from Amersham Biosciences) in the presence of saturated urea, 50 mM DTT or hydroxyethyl disulfide (Olsson et al., 2002), and 1% pH 3–10 ampholytes. Western blotting involved electrophoretic transfer to nitrocellulose or PVDF filters in the presence of 20 ml Tris/acetate (pH 8:3) and 20% (v/v) methanol; antibody incubations and washes were performed in Tris-buffered saline (pH 7.5), 0.1% gelatin and 0.1% Tween 20. Secondary antibodies were conjugated to alkaline phosphatase and chromogenic detection utilized Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates. N-terminal sequencing by Edman degradation of polypeptides that had been alkylated with iodoacetamide, digested with peptide N-glycanase F (PNGase F; see below) and electroblotted from gels to PVDF filters was performed by Dr Laurey Steinke (Protein Structure Core Facility, University of Nebraska Medical Center, NE, USA). Mass-spectral analysis of in-gel-trypsinized Ywp1p peptide involved MALDI-TOF mass spectrometry (MS) and post-source decay analysis of a C-terminal fragment (Mann et al., 2001) and was performed by Dr John Leszky (University of Massachusetts Medical School, MA, USA).

Mannoproteins were precipitated from medium 13 culture supernatants upon mixing with an equal volume of ethanol. Supernatants were usually 0.45 μm-filtered prior to precipitation; adding 2 mM...
EDTA and neutralizing the pH prior to precipitation had no obvious effect on the yield of Ywp1p. Thiol extraction of washed, live cells was performed in 40 mM Tris, 15 mM EDTA, 100 mM NaCl (unadjusted pH, 8.5; TEN8-5 buffer) containing 20–50 mM DTT for 30 min at 0°C. Supernatants (usually 0.45 μm-filtered) were mixed with an equal volume of ethanol to precipitate the mannosproteins, which were then dissolved in water or 10 mM Tris/HCl, 1 mM EDTA, pH 8.

Digestion with PNGase F (New England Biolabs) removes N-glycans from polypeptides and converts the linking asparagines to aspartates, a fact that was taken into account when theoretical pl values were calculated (http://www.expasy.org). Digestions were typically performed for 2–3 h at 37°C in TEN8-5 buffer, with or without DTT at 20–50 mM. Samples for Fig. 1 were also heated with 0.1% SDS to first denature the proteins, and then mixed with 1% NP-40 prior to digestion. For quantification of the Ywp1 propeptide, stationary-phase culture supernatants were not precipitated or concentrated, but digested directly with PNGase F (after adding 0.2 vol. 5× TEN8-5 buffer), resolved by SDS-PAGE and silver-stained; densitometry of the stained bands was followed by normalization to an unidentified polypeptide, which was presumably liberated from the periplasm (de Nobel & DeWachter, 1976; de Nobel & DeWachter, 1977). The nucleotide sequence of YWP1 (orf6.3288 and its two 3′ flanks) from polypeptides and converts the linking asparagines to aspartates, was determined colorimetrically (Chen et al., 1956; Ames, 1966). Final concentrations of solutes in each complete mixture were 0.5 M sulfuric acid (diluted from a 1 M stock), 5 mM ammonium molybdate (diluted from a 100 mM stock), 0.01–0.2 mM phosphate (diluted from a culture supernatant or a calibration solution) and 50 mM ascorbic acid (diluted from a 1 M stock). After 1–6 h at 20–25°C, A200 was determined in polystyrene cuvettes.

Flow cytometry. Cultures of C. albicans were shaken at 200 r.p.m. in Erlenmeyer flasks that were 20% full of medium 13 at 30°C; such cultures were started with 100-fold dilutions of similar cultures that had an OD600 of 2–3. For analysis, aliquots of cell cultures were diluted in saline to an OD600 of ~0.02 and analysed immediately in a Becton Dickinson FACScan equipped with a 488 nm argon laser. Detectors were set at E-1 (FSC), 220 (SSC) and 600 (FL1) in logarithmic mode, with a threshold of FSC 220. Data were recorded for 104 events per sample and analysed with WinMDI 2.7 software (http://facs.scripps.edu/software.html); gates of FSC 70–167, SSC 50–157 and FL1 80–230 excluded no more than 0.55% of the events in any sample.

Adhesion assays. Microcultures were grown in polystyrene plates that were tissue-culture grade (Greiner or Costar) or bacteriological grade (Fisher). Cultures were started with an OD600 of ~0.1, using washed or unwashed cells from actively growing or stationary-phase cultures. After a period of growth, non-adherent cells were rinsed away by repeated flooding of the plate with water or other solution (as specified in Results) coupled with gentle rocking or swirling; adherent cells were stained for 10 min at 25°C with 0.1% crystal violet (CV) in water and destained with water. CV was extracted from stained cells with 50% ethanol/1% SDS and its absorbance measured. Stained cells on plates were imaged digitally with a flatbed scanner after drying. Confocal laser-scanning microscopy of GFP-expressing cells was performed by using a Leica TCS-SP2 AOBS microscope with a 63×, 0.9 NA, water-immersion objective and a 488 nm laser; image reconstruction was performed with Imaris software (Bitplane). Cells deep within a biofilm are imaged incompletely with this technique because of attenuation of laser excitation and fluorescence emission.

RESULTS

Identification of the Ywp1 propeptide

A phosphomannoprotein fraction was extracted from live C. albicans yeast cells with DTT. Digestion of this fraction with PNGase F liberated several polypeptides that appeared as sharp, new bands when analysed by SDS-PAGE (Fig. 1). N-terminal sequence analysis revealed the identity of two of the predominant bands: a mixture of Pho11p and Pho12p, constitutive acid phosphatases (Chattaway et al., 1974) that were presumably liberated from the periplasm (de Nobel & Barnett, 1991) and a putative cell-wall protein initially called Tep1 (for thiol-extractable peptide 1; Granger et al., 2001), but later named Ywp1 (for yeast-form wall protein 1; Sohn et al., 2003). In cultures with relatively low phosphate content, a third band at ~33 kDa was identified as Pho3p, an inducible acid phosphatase (see Fig. 4). Identifications were made by searching assemblies 4–6 of the C. albicans genome sequence (http://www-sequence.stanford.edu/group/candida; Jones et al., 2004).

The nucleotide sequence of YWP1 (orf6.3288 and its two identical alleles, orf19.3618 and orf19.11101) predicted a
533 aa polypeptide and a signal-peptide cleavage site (Nielsen et al., 1997) that coincided exactly with the N terminus of the identified peptide (Fig. 2). The discrepancy in size between the identified peptide (<15 kDa) and the full-length protein (>50 kDa) suggested that an additional cleavage must have taken place to generate the observed fragment. MS analysis of the peptide indeed revealed that cleavage had taken place at a triple-arginine site (-R121-R122-R123-), resulting in a propeptide of about 100 aa (11 kDa). This propeptide exhibited anomalous mobility during SDS-PAGE, migrating behind lactalbumin (14 kDa) in a Tris/glycine buffer system (Fig. 1), but ahead of it in a Tris/Tricine system (Figs 3, 4 and 6). In addition, mobility was enhanced significantly when the two cysteines of the propeptide formed an intrachain disulfide, causing it to co-migrate with the 11 kDa marker thioredoxin (see below).

The Ywp1 propeptide will hereafter be referred to as ‘11 kDa’, regardless of its relative mobility. IEF of the propeptide revealed one minor and two major isoelectric variants (Fig. 3a) that correlated with the MS data, showing that cleavage had left up to two arginines on the C terminus of the propeptide (.STAI/R/R/RLMG., with ‘/’ indicating cleavage sites). Prediction of the pl of these three

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\text{Fig. 2. Predicted amino acid sequence of Ywp1p. The empirical N-terminal sequence of the purified propeptide is underlined. The tribasic (RRR) and dibasic (KR) sites are double-underlined. The GPI anchor signal is designated by a dashed underline \((\text{hydrophobic C terminus})\), with an asterisk over the probable } \Longleftrightarrow \text{ glycosylation site. Repeating segments are aligned in a central region and a hash (\#) lies over each of the 11 cysteines. A dot lies over each of the 56 serines and 85 threonines; algorithms for reliable prediction of which of these might be O-glycosylated are not yet available. An arrow marks the sole } N\text{-glycan-addition site.}
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variants on the basis of their amino acid composition and sequence gave values of 4-4, 4-6 and 4-8; these values compare favourably, but not exactly, with the predicted pl of thioredoxin (4-7), probably because of imperfections in the prediction algorithms (Cargile et al., 2004) or unforeseen charge modifications.

Immediately downstream from the triple-arginine site of Ywp1p is a dibasic site (.IVKR.) with presumed sensitivity to cleavage by Kex2p, a pro-protein convertase (Newport & Agabian, 1997; Rockwell & Thorner, 2004). However, the 12 kDa species that would result from such cleavage was not evident in the MS analysis of the purified Ywp1 propeptide.

To investigate this further, Ywp1p was examined in CNA3-1, a \(kex2\) strain of \(C. albicans\) (Newport & Agabian, 1997; Newport et al., 2003). Surprisingly, this strain generated a Ywp1 propeptide that was about 12 kDa (Figs 3b and 4) and the 11 kDa form was not detected. The 12 kDa propeptide focused less discretely than the 11 kDa propeptide (Fig. 3b), but the two major and one of the minor variants seen on two-dimensional gels had pl values consistent with the predicted values of 4-9, 5-2 and 5-7 for three alternative C termini at the dibasic site (.STAI/RRLMGETPIV/K/R/DQ.). Heterogeneity of cleavage at dibasic sites in Kex2 strains has previously been noted (Newport & Agabian, 1997). The 12 kDa form was less abundant in \(kex2\) strains than the 11 kDa form in \(KEX2/KEX2\) lines; its quantity could be diminished slightly by culturing the cells in the presence of pepstatin (Fig. 4), but was undiminished in the presence of three other protease inhibitors (leupeptin, AEBSF or TLCK; data not shown).

**DNA vaccines and the utility of anti-Ywp1p**

Mice vaccinated with the full coding sequence of \(YWP1\) (aa 1–533) generated antibodies specific for Ywp1p, as did mice vaccinated with plasmids encoding shorter segments of Ywp1p (aa 21–116, 51–197 and 105–161) linked to one
PNGase F efficiently removes the reduction and both forms are recognized by the antisera; propeptide undergoes a mobility shift upon disulfide (Fig. 4), revealing or confirming that: (i) the 11 kDa removed was analysed by silver staining and Western blotting; (ii) PNGase F digestion for 4–6, but after digestion for 3). Sample unreduced, whereas samples 3–6 were reduced with DTT (prior to PNGase F digestion for 4–6, but after digestion for 3). Sample 1 was heated to 95°C prior to PNGase F digestion to test the effect of heat denaturation. The culture for sample 6 was supplemented periodically with fresh pepstatin (50 μM final). Three mobilities of the Ywp1 propeptide are denoted by arrows (non-reduced), arrowheads (reduced) and double arrowheads (reduced, from the kex2/kex2 line); the prominent band just above the double arrowhead in (a) has not been identified. The star indicates a possible propeptide dimer. The dot shows the position of PNGase F (36 kDa) and Pho3p (33 kDa), which co-migrate in the resolving gel; (iii) the HMM forms of Ywp1p migrate somewhat differently, depending on reduction and Kex2 status; and (vii) the kex2/kex2 samples show a small amount of mid-molecular-mass immunoreactivity that cannot be attributed to non-specific labelling (all other faintly labelled bands are also seen in samples from knockout lines that lack Ywp1p; data not shown). Interestingly, the antisera used in Fig. 4(c) recognize the 12 kDa propeptide but not the 11 kDa propeptide, even though the vaccines encoded all but the N-terminal 3 kDa of the propeptides; an immunogenic epitope that includes the segment between the tribasic and dibasic sites must therefore exist.

**GFP as a reporter of YWP1 expression**

The coding sequence of one or both alleles of YWPI was replaced with GFP to create strains BJ3 and BJ3a1a. We screened various growth media and conditions and found that low phosphate concentrations increased GFP fluorescence of these strains by upregulating YWPI expression. Quantitative analysis by flow cytometry revealed that, under normal phosphate conditions, GFP fluorescence reached a peak soon after the end of the exponential phase (OD₆₀₀ ~ 1), well before the culture reached stationary phase (Fig. 5a). This suggested that YWPI expression peaked during the exponential phase, as there is a lag of ~ 1 h between transcription of GFP and detection of GFP fluorescence under these growth conditions (based on our unpublished studies of GFP driven by the PHO3 promoter). Growth in low-phosphate medium resulted in greater fluorescence per cell, a delayed peak in fluorescence and greater fluorescence in stationary phase (Fig. 5b). Epifluorescence microscopy of different morphological forms of C. albicans revealed that YWPI expression was down-regulated upon filamentation. Hyphal and pseudohyphal forms devoid of detectable GFP fluorescence arose in liquid filamentation media, such as the medium of Lee et al. (1975), at 37°C, and in 1% yeast extract/2% peptone/0% Tween 80 at 23°C. Filaments that had invaded agar were seen to have little or no GFP fluorescence, but blastoconidia that arose at the distal tips of the filaments were fluorescent, having resumed GFP (YWPI) expression (data not shown).

**Effects of low phosphate on yeast growth and YWP1 expression**

Exploring the low-phosphate effect further, we found that cultures of wild-type C. albicans attained similar stationary-phase densities when the initial concentration of phosphate

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Fig. 4. Western blots of Ywp1p in culture supernatants. Mannoproteins were precipitated from supernatants of stationary-phase cultures of C. albicans SC5314 (lanes 1–4) and CNA3-1 (lanes 5 and 6) prior to digestion with PNGase F. Gels were silver-stained (a) or electroblotted onto PVDF filters prior to probing with mouse antisera against Ywp1p (b, c). Antisera were from mice vaccinated with DNA encoding aa 21–116 of Ywp1p (b) or aa 51–197 and 105–161 (pooled for (c)). Samples 1 and 2 were left unreduced, whereas samples 3–6 were reduced with DTT (prior to PNGase F digestion for 4–6, but after digestion for 3). Sample 1 was heated to 95°C prior to PNGase F digestion to test the effect of heat denaturation. The culture for sample 6 was supplemented periodically with fresh pepstatin (50 μM final). Three mobilities of the Ywp1 propeptide are denoted by arrows (non-reduced), arrowheads (reduced) and double arrowheads (reduced, from the kex2/kex2 line); the prominent band just above the double arrowhead in (a) has not been identified. The star indicates a possible propeptide dimer. The dot shows the position of PNGase F (36 kDa) and Pho3p (33 kDa), which co-migrate in this gel system. The bar marks the boundary between the stacking and resolving gels.

or more carriers to form hybrid proteins, as outlined in Methods. These antisera were useful for identifying various forms and fragments of Ywp1p on Western blots (described below); specific binding of these antibodies to Ywp1p in intact C. albicans cells under a variety of conditions, however, was not evident by immunofluorescence microscopy, presumably because of epitope inaccessibility. Perhaps accordingly, when groups of mice that had the highest anti-Ywp1p titres were challenged by intravenous injection of live C. albicans, survival times were not found to be changed significantly (data not shown).

Secreted Ywp1p from which the N-glycan had been removed was analysed by silver staining and Western blotting (Fig. 4), revealing or confirming that: (i) the 11 kDa propeptide undergoes a mobility shift upon disulfide reduction and both forms are recognized by the antisera; (ii) PNGase F efficiently removes the N-glycan from the propeptide, even if the propeptide is not first reduced or denatured; (iii) a minor fraction of the propeptide is associated with high-molecular-mass (HMM) manno-protein in the absence of reduction, but all of the propeptide immunoreactivity is present in the 11 kDa band upon reduction with DTT [note that the antibodies used in Fig. 4(b) are specific for the propeptide only]; (iv) a minor fraction of the propeptide may also exist as a dimer in the absence of reduction; (v) mature Ywp1p exists in an HMM form, remaining in the stacking gel or barely entering the resolving gel; (vi) the HMM forms of Ywp1p migrate somewhat differently, depending on reduction and Kex2 status; and (vii) the kex2/kex2 samples show a small amount of mid-molecular-mass immunoreactivity that cannot be attributed to non-specific labelling (all other faintly labelled bands are also seen in samples from knockout lines that lack Ywp1p; data not shown). Interestingly, the antisera used in Fig. 4(c) recognize the 12 kDa propeptide but not the 11 kDa propeptide, even though the vaccines encoded all but the N-terminal 3 kDa of the propeptides; an immunogenic epitope that includes the segment between the tribasic and dibasic sites must therefore exist.
in the medium was at least 0.2 mM, but less than half that density was reached when the starting concentration was 0.04 mM. A phosphate concentration of 0.2 mM was found to be low enough to upregulate inductive acid phosphatases strongly in C. albicans (PHO3; unpublished observations) and Saccharomyces cerevisiae (PHO5; Ogawa et al., 2000) and was therefore chosen as the standard concentration in our low-phosphate medium to upregulate YWP1 expression. The concentration of phosphate remaining in the medium became immeasurably low (<1 μM) when the initial concentration was <2 mM (data included as supplementary material with the online version of this paper).

**Fate of secreted Ywp1p**

Aliquots of liquid cultures were analysed at different stages of growth (Fig. 6). For both the culture-medium and DTT extracts, silver staining of the deglycosylated Ywp1p propeptide (Fig. 6a) showed that it was barely detectable just after exponential phase (14 h, OD_{600} ~ 1.8), but increased into early stationary phase (62 h, OD_{600} ~ 5.2). Similar increases were seen in the full complement of HMM mannoproteins, although not in the unidentified sharp bands in the DTT extracts. Western blotting of Ywp1p in these same fractions (without deglycosylation) showed a similar pattern (Fig. 6b), with the HMM form of Ywp1p undetectable in the earliest samples, but increasing toward stationary phase. Cultures grown in low-phosphate medium showed greater expression of Ywp1p and earlier detection with the antibodies. Electrophoretic mobility was retarded noticeably for HMM Ywp1p from supernatants of low-phosphate cultures, but not from the DTT extracts of those cells. Finally, β-1,3-glucanase digests of cells that had been exhaustively pre-extracted to remove disulfide bonded and non-covalently linked proteins showed immunoreactive Ywp1p at all time points (Fig. 6c). Attached β-1,6-glucans presumably contribute to the large size and exceptionally low mobility of these β-1,3-glucanase-digestion products.

**YWP1 gene disruption and virulence testing**

The two alleles of YWP1 were disrupted sequentially (and partially deleted) by homologous recombination. The only phenotypic change that we noted was increased adhesion and biofilm formation, as described below. The histidine auxotrophy of the original ywp1Δ/ywp1Δ knockout strain (Ca#12) allowed YWP1 and prototrophy to be restored simultaneously by plasmid integration. For this purpose, YWP1 and HIS1 were combined in several plasmids that included both alleles of YWP1, as well as all possible orientations of YWP1 and HIS1 relative to each other. Half of these plasmids (see Methods) were then linearized at a unique restriction site in either HIS1 or YWP1 in order to create recombinogenic ends that would target integration to their respective loci. Two prototrophic clones from each transformation were analysed. All integrations that were targeted to the YWP1 locus resulted in lines that secreted amounts of Ywp1p similar to that of a heterozygous (YWP1/ ywp1Δ) line, whereas integrations that were targeted to the HIS1 locus resulted in lines that secreted variable amounts of Ywp1p, from none to normal (wild-type) amounts. We have not fully analysed the latter transformants genetically, but it seems likely that the 999 bp upstream from the coding sequence of YWP1 that was included in the transforming DNA has promoter activity, but does not necessarily restore normal patterns of expression. Combined with transformants in which just HIS1 was reintegrated, a panel of prototrophic lines with different amounts of Ywp1p was thus generated. Production of Ywp1p was estimated by examining the quantity of propeptide in supernatants of stationary-phase cultures and revealed four groups, referred to below as having normal (wild-type), half, trace amounts or no Ywp1p. The relative propeptide quantities were: 100 ± 5–8 % (ratio ± SD) for SC3514, DAY185 and 16r1; 42 ± 2–7 % for 2s1, 2s2, 7s1, 7s2, 13s1, 13s2, 16s1 and 16s2; 6–2 ± 0.8 % for 2r1, 2r2, 7r1 and 7r2; and 1.5 ± 1.5 % for 3L1, 4L1 and 16r2.

Prototrophic strains of C. albicans producing different amounts of Ywp1p (from none to normal) were tested in a mouse model of haematogenously disseminated candidiasis. We observed little or no difference in survival rates as a function of the presence or absence of Ywp1p (data included...
as supplementary material with the online version of this paper).

**YWP1 affects adhesion and biofilm formation**

Ywp1p-dependent differences in adhesion and biofilm formation were visualized most easily by comparative growth of microcultures in single polystyrene dishes. After removal of non-adherent cells, adherent cells were apparent upon oblique lighting against a dark background and after staining with CV (Fig. 7a). A strong correlation between adhesion and the lack of Ywp1p was evident under conditions in which all of the cells remained in the yeast form. Strains possessing little or no Ywp1p developed an adhesive blastoconidial biofilm that covered most of the plastic and was up to several cells thick, as shown in optical cross-section (Fig. 7b). Under the hemispherical droplets of culture medium, biofilm morphology varied across the diameter of the adhesive patch of cells, with thicker biofilms and discrete microcolonies more evident at the periphery (where conditions such as gas-exchange rates may be different). In contrast, cells with normal amounts of Ywp1p showed sparse, monolayer adhesion. Strains with half of the normal amount of Ywp1p showed intermediate amounts of adhesion, but were more similar to the Ywp1p-replete strains than Ywp1p-negative strains (Fig. 7a). Scraping and dispersal of the adherent Ywp1p-negative cells revealed that they were predominantly single cells without or with buds; filamentous forms were not observed.

Adherent cell mass was quantified for selected strains that were grown in quadruplicate in a 12-well tissue-culture plate, washed gently with water and stained. Extraction of bound CV and measurement of its absorbance gave the following relative proportions: strain 16r2 (no Ywp1p), 100 ± 1 % (SD); strain 7r1 (trace of Ywp1p), 96 ± 4 %; strain 16s1 (half Ywp1p), 28 ± 2 %; strain 16r1 (full Ywp1p), 10 ± 2 %. Strains possessing and lacking Ywp1p (DAY185 and 3L1) were found to bind the same quantity of CV per volume of packed cells, confirming visual and microscopic indications that CV measurements reflect cell numbers (Li et al., 2003). Under these conditions of blastoconidial growth (2–3 days at 30°C in medium 13), about half of the Ywp1p-negative cells present were adherent and most of those could be dislodged by a more forceful stream of water; in contrast, only a small percentage of the Ywp1p-replete cells remained adherent after gentle washing.

Assays were run routinely in polystyrene tissue-culture plates with a chemically modified surface that is hydrophilic. Similar Ywp1p-dependent adhesion patterns were seen on alternative substrates, such as unmodified polystyrene.

**Fig. 6.** Growth-phase dependence of detectable Ywp1p. Parallel cultures of *C. albicans* SC5314 were started in medium 13 containing either normal phosphate (N; 5 mM) or low phosphate (L; 0.2 mM); aliquots were taken at 14, 21, 38 and 62 h, as indicated; mannoproteins were precipitated from the culture medium (M) and from a DTT extract of the cells (D). (a) Samples were digested with PNGase F (run by itself in lane P), resolved by SDS-PAGE and silver-stained. Sample volumes were adjusted so that each lane represented the same number of cells, with 10 x more in the D lanes than the M lanes. The arrowhead denotes the Ywp1 propeptide. The culture that produced these samples was grown in low phosphate; qualitatively similar results were obtained for a parallel culture grown in normal phosphate, but the quantities of propeptide were lower (data not shown). (b) Samples were analysed by Western blotting with antisera specific for Ywp1p. Culture-medium lanes represent 0.3 x as many cells as DTT extract lanes. (c) Cells were extracted sequentially with DTT, hot SDS and urea, then digested with β-1,3-glucanase; material liberated by the glucanase was analysed by Western blotting for the presence of Ywp1p. Most of the immunoreactivity remained in the stacking gel in this Tris/Tricine system, but migrated slightly into the resolving gel in a Tris/glycine system (data not shown). Each lane represents the same number of cells. In all panels, the horizontal bar marks the boundary between the stacking and resolving gels.
(hydrophobic, bacteriological-grade plates), Tween 80-treated tissue-culture plates, glass coverslips that were untreated or cationized with Alcian Blue (Sommer, 1977), polyvinyl chloride coverslips and sheets of polyethylene and silicone elastomer. Non-adherent cells were routinely washed away with water, but similar adhesion patterns were seen upon washing with various concentrations of sodium chloride or with Tris-buffered saline containing 1 mM EDTA, 0.1% Tween 20, 0.1% Tween 80 or 0.1% gelatin. The patches of adherent cells on the various substrates persisted for days or weeks at room temperature in these solutions. Growth of the microcultures was routinely allowed to proceed for 2–3 days to early stationary phase, but biofilm patterns were similar from less than 1 day to more than 10 days growth. Similar adhesive biofilms were seen in the Ywp1p-negative microdroplet cultures when the plates were inverted during the growth period, which allowed non-adherent cells to fall away from the substrate.

When grown at 24°C, the adhesion patterns were qualitatively similar to those at 30°C, but quantitatively, there was slightly less difference between the Ywp1p-containing and Ywp1p-lacking strains. At 30°C, adhesion patterns were similar in a variety of growth media, such as yeast nitrogen base with 50 mM glucose, the medium of Lee et al. (1975) and medium 13, which had galactose substituted for glucose, low (0.2 mM) phosphate or the additives 0.1% BSA or 100 mM NaMOPS at pH 7.4 (conditions under which the cultures remained blastoconidial). At 37°C, however, the Ywp1p correlation with adhesion was usually much less or not evident when cultures became filamentous (data not shown).

When actively growing or stationary-phase blastoconidia were washed free of culture medium, suspended in 5 mM NaCl and allowed to settle onto tissue-culture plates at 0 or 30°C (conditions that do not support growth), the same patterns of adhesion were observed (more adhesion of Ywp1p-negative strains). Furthermore, secretions of strains that possessed or lacked Ywp1p were found to be capable of inhibiting adhesion and biofilm formation, even after fractionation of the secretions by ethanol precipitation. Fig. 7(c) shows that such fractions, when added at their original concentration to fresh culture medium, inhibited adhesion of all strains in our microdroplet assay; fractions from cultures that contained Ywp1p, however, were more inhibitory than fractions from cultures that lacked Ywp1p.

**DISCUSSION**

Ywp1p is a yeast-specific wall protein that inhibits adhesion

Ywp1p has a structure that is typical of many wall glycoproteins in *C. albicans* (Kapteyn et al., 2000). The amino acid sequence predicts an N-terminal secretion signal, a site for N-glycan addition (a single occurrence of the consensus, -N-X-S/T-), a C-terminal signal for GPI anchorage and subsequent wall attachment (Sundstrom, 2002; de Groot et al., 2003; Lee et al., 2003) and a repetitive central domain rich in serine and threonine that may become heavily O-glycosylated. Heterogeneous glycosylation in this domain may be responsible for the observed low mobility and broad distribution of HMM Ywp1p in our Western blots. A
central motif with the sequence T-I/V-T-S-C-X-X-X-C, where one to four of the amino acids represented by X have polar or charged side chains, is present twice in Ywp1p, as well as once or twice in at least 17 other proteins in C. albicans; most of these proteins are predicted to be GPI-anchored and include Hwp1p, Hyr1p, Chl2p and Rbt1p. Slight variations of this cysteine-containing motif are considerably more common, but the function of this sequence remains unknown. Otherwise, there is little sequence similarity between Ywp1p and other known proteins. A search of available genomic sequences reveals an apparent orthologue in Candida dubliniensis (http://www.sanger.ac.uk) and a tandem pair of homologues in Debaryomyces hansenii (Dujon et al., 2004). Nevertheless, neither homologies nor sequence features of Ywp1p have revealed an unequivocal function. Six kb upstream from YWPI in the C. albicans SC5314 genome is a homologue with a stop codon in its putative signal peptide-coding sequence. We confirmed this sequence in C. albicans strains 3153A and BWP17. Considering the possibility of nonsense suppression in C. albicans (Resende et al., 2002), we attempted to detect expression of this homologue under various conditions, utilizing GFP and HIS1 reporters of gene expression, antibodies generated by DNA vaccines and phenotypic changes resulting from gene disruption. No evidence of expression was found, supporting the possibility that it is a pseudogene. Interestingly, the C. dubliniensis genome also appears to possess this homologue, but without the stop codon in its putative signal peptide.

Upon disruption of both alleles of YWPI, the only phenotypic change that we noted was slightly increased adhesiveness of yeast cells, but no obvious tendency to flocculate in liquid suspension. We developed a simple method to demonstrate that cells lacking Ywp1p were intrinsically more adhesive toward plastic surfaces and formed thicker biofilms when allowed to grow at 30°C on these surfaces. This tendency was less evident when cells were grown at 37°C, a temperature that promotes filamentation in these strains, and rarely evident in filament-inducing media at 37°C; this is presumably because YWPI is downregulated under these conditions or because additional mechanisms of adhesion regulation become operative. A large number of independently engineered strains of C. albicans were compared in our studies, to help rule out the possibility that unanticipated genetic modifications or differences in the expression of selectable markers were responsible for the observed differences in adhesion (Bain et al., 2001). We were able to correlate adhesice biofilm formation inversely with the amount of Ywp1p that each strain possessed, as measured by the quantity of Ywp1p prepeptide secreted. Micro-manipulation of exclusively blastoconidial biofilms formed by strains lacking Ywp1p revealed the presence of an extracellular polymeric material holding the cells together (data not shown; Chandra et al., 2001; Douglas, 2003); such biofilms were dispersed readily to predominantly single cells and single cells with buds by repetitive pipetting, indicating that the extracellular material was relatively fragile and that the thickness of the biofilm was not a result of the failure of mother–daughter yeast pairs to separate physically from one another. Yeast cells appeared to be anchored more firmly to the substrate than to each other, defining at least two layers or types of adhesion, as has been described for other types of C. albicans biofilms (Chandra et al., 2001; Douglas, 2003). The blastoconidial nature of these biofilms has some resemblance to certain wild-type strains of Candida parapsilosis (Kuhn et al., 2002). Only sparse adhesive monolayers were evident for strains possessing normal amounts of Ywp1p, indicating that Ywp1p must interfere with both adhesion to plastic and subsequent biofilm formation in the absence of filamentation. The nature of this interference is currently being explored, but potential clues have emerged from the current work. For example, strains with half of the normal amount of Ywp1p show less than half of the blastoconidial-biofilm accumulation of strains with no Ywp1p, suggesting a non-linear relationship between Ywp1p content and biofilm quantity. Secretions containing Ywp1p inhibit the adhesion of cells to plastic, as well as the subsequent development of biofilms, even after such secretions have been through an ethanol-preservation cycle. Washed cells that have Ywp1p in their walls are intrinsically less adhesive to plastic. Whether these are direct or indirect effects of Ywp1p remains to be determined; a direct effect might be binding of Ywp1p to, and inactivation or blockage of, adhesins, whereas an indirect effect might be catalytic modification of the cell wall or extracellular polymeric material. Additional clues are starting to emerge from biochemical and structural descriptions of Ywp1p, as discussed below.

Transcription of YWPI (also termed IPF5185 and CA1678) was recently reported to be greater in C. albicans biofilms than in planktonic cells (García-Sánchez et al., 2004). The structural similarity of Ywp1p to known adhesins suggested that this might ‘facilitate the cohesion of cells within the biofilm’. Our data support the opposite interpretation, i.e. that overexpression of YWP1 may result in detachment and dissemination of blastoconidial daughter cells from mature biofilms. Comprehensive transcript profiling has indicated that YWP1 transcripts do not show a change of more than twofold during the white/opaque phenotypic switch (Lan et al., 2002), or more than threefold when receptive cells are exposed to α-factor mating pheromone (Bennett et al., 2003); however, such changes in Ywp1p levels can have significant effects on adhesion and biofilm formation, leaving open the possibility that Ywp1p plays some role in phenotypic switching and/or mating.

Low phosphate upregulates YWP1

YWPI expression is upregulated when the extracellular phosphate concentration is low, suggesting that Candida may avoid adhesion and biofilm formation in environments where phosphate is sensed as limiting. This does not appear to be because biofilms require more phosphate than do planktonic cells, however, as is the case for Pseudomonas fluorescens (Kemner et al., 2004); our preliminary
comparative measurements of total phosphate by chemical methods (Ames, 1966) and total phosphorus by scanning electron microscopy/energy-dispersive X-ray spectroscopy have shown no enrichment in biofilm blastoconidia (data not shown). Our suspension cultures of *C. albicans* showed uniform uptake of phosphate from the culture medium (independent of the external concentration, as long as there was a surplus) prior to stationary phase and no uptake thereafter, indicating a limited capacity for phosphate storage. In the absence of measurable external phosphate, one to three additional doublings occurred, presumably through utilization of stored phosphate (cf. Lillie & Pringle, 1980). Upregulation of *YWP1* by low phosphate does not appear to be part of a general starvation response, as no upregulation was noted when essential arginine or uridine was limited in cultures of the auxotrophic BJ3a1a, and only a slight upregulation (~10%) was observed upon glucose limitation (data not shown). By using whole-genome expression analysis, many *S. cerevisiae* genes involved in the acquisition and storage of phosphate were found by Ogawa et al. (2000) to be upregulated by low phosphate, but no gene that encodes a GPI protein was upregulated consistently under the conditions of their experiment (three to four exponential-phase doublings in up to approx. 0.1 mM inorganic phosphate). As *S. cerevisiae* has no obvious sequence homologue of Ywp1p, there is currently no indication that *S. cerevisiae* has any protein with an analogous role.

**YWP1 expression patterns and virulence**

As reported by GFP fluorescence, expression of *YWP1* in phosphate-replete, batch cultures of blastoconidia appears to peak soon after the exponential phase of growth, when the OD<sub>600</sub> is 30–50% of its ultimate stationary-phase value. The decline in fluorescence per cell thereafter is presumably due to dilution of GFP into daughter cells, followed by slow degradation without replenishment in non-dividing cells. This suggests that the expression of *YWP1* is greatest during maximal growth rates of yeast, but declines or shuts off as the growth rate declines, and is low or non-existent in stationary-phase cells. Ywp1p may thus foster dispersal of blastoconidia when growth conditions are most favourable. Expression of *YWP1* shuts down upon filamentation, as shown by GFP fluorescence (this study) and transcript analysis (Nantel et al., 2002; Sohn et al., 2003), perhaps to promote initial adhesion and biofilm formation. *YWP1* expression appears to be under complex regulatory control (Sohn et al., 2003; Doedt et al., 2004), but our studies of protein levels have not indicated that disruption of one allele of *YWP1* results in a compensatory upregulation of the other allele or restoration of normal amounts of Ywp1p.

Several of our engineered prototrophic strains, with or without Ywp1p, were compared in a mouse model of disseminated candidiasis. Little or no correlation between Ywp1p levels and survival times was evident. In this model, the infecting cells grew at 37°C in a filamentation-promoting environment, conditions under which the expression of *YWP1* and effects of Ywp1p on adhesion may have been minimal. Thus, alternative pathogenesis models, such as infection of epithelia at lower temperatures or dissemination from one site of infection to another, might be more likely to reveal a link between *YWP1* and virulence.

**Post-translational itinerary of Ywp1p**

Our data suggest that Ywp1p has a role in inhibiting adhesion as an integral cell-wall component, as well as after being shed or secreted into the extracellular milieu. We observed a considerable lag between the period of peak *YWP1* expression and the phases at which Ywp1p could be extracted from cells with DTT or found free in the surrounding medium. The expected fate of GPI proteins with structures similar to Ywp1p is covalent incorporation into the cell wall through β1,6-glucan (Kapteyn et al., 2000); indeed, covalent attachment of Ywp1p to glucan is supported by our antibody detection of Ywp1p that was liberated from wall residues of proliferating and stationary-phase cells by β1,3-glucanase. Similarly, de Groot et al. (2004) demonstrated the covalent linkage of Ywp1p (termed Pga24p) to this matrix by MS analysis of tryptic peptides after digestion of wall residues with glucanase or HF-pyridine, which cleaves phosphodiester bonds of GPI anchors. Conceivably, the Ywp1p that we found in the surrounding medium was liberated from the wall during early stationary-phase remodelling or was never incorporated covalently into the wall; perhaps an intermediary form that was liberated from its GPI anchor, making it effectively soluble in the periplasm or wall itself (de Nobel & Lipke, 1994), diffused (or was released) slowly into the medium. Formation of intermolecular disulfide bonds might slow the egress of such forms through the cell wall and allow a fraction of the Ywp1p to accumulate in a DTT-extractable form as the culture matures; this would parallel the observed six- to sevenfold increase in disulfide-bond content of *S. cerevisiae* cell walls during the stationary phase (de Nobel et al., 1990). Accurate quantification of the relative proportion of each form of Ywp1p at each destination and at each growth stage may eventually reveal functional correlations.

**The propeptide of Ywp1**

The large, persistent propeptide of Ywp1p was the first form of Ywp1 to be identified, and it subsequently proved to be a useful surrogate marker for the presence and abundance of Ywp1. We have not noted any extracellular form of Ywp1p with uncleaved propeptide, consistent with the usual cleavage of propeptides from pro-proteins in a late Golgi compartment prior to secretion (Redding et al., 1991). Propeptides often act as intramolecular chaperones, specifically catalysing the proper folding and, in some cases, proper disulfide-bond formation of the collinear polypeptide (Eder & Fersht, 1995). Cleaved propeptides may be degraded after this function or they may persist to serve as inhibitors, anchors or independent signalling molecules.
(e.g. Annes et al., 2003). Our data show that the Ywp1 propeptide remains associated with the cell wall and appears in the culture medium only when the HMM form of Ywp1p also appears there. The contribution of the Ywp1 propeptide to early protein maturational events, as well as later adhesion-related events, remains to be determined.

Variability in the C terminus of the Ywp1p propeptide suggested that more than one proteinase could be involved in its cleavage and maturation. Cleavage at the trispecific site of Ywp1p generated the 11 kDa form seen in Kex2p strains, whereas cleavage at the dibasic site generated the 12 kDa form seen in Kex2p strains. This was surprising, because Kex2p is thought to be primarily responsible for cleavage at consensus dibasic (-KR-) sites; however, in C. albicans, dibasic sites may be cleaved by proteinases other than Kex2p, albeit less efficiently (Newport & Agabian, 1997). Perhaps Kex2p is essential for activation of proteinase(s) that trim the Ywp1 propeptide from 12 to 11 kDa, or Kex2p is itself responsible for cleavage at the trispecific site. Regardless of which proteinases cleave Ywp1p in the trispecific-dibasic region, the N terminus of mature HMM Ywp1p is conceivably the same in wild-type and Kex2p strains. In our assay of blastoconidial adhesion, Kex2p strain CNA3-1 showed no increase in adhesion or biofilm formation over Ywp1p-replete strains (data not shown). Additional Kex2p substrates, which include Hwp1p and other adhesion-like proteins, have been tentatively identified in C. albicans (Newport et al., 2003), raising the possibility of a more expansive role for propeptides in regulating cellular adhesion.

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REFERENCES


