Surface Localization of the Yps3p Protein of *Histoplasma capsulatum*

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**Histoplasma capsulatum** is a pathogenic fungus with worldwide distribution. It is the causative agent of histoplasmosis, one of the most common fungal respiratory infections in the world, with an estimated 500,000 cases in the United States alone per year. The regions where histoplasmosis is endemic include the midwestern and southwestern United States as well as areas of South America. In the United States it reaches its highest levels along the Ohio and Mississippi river valleys, where skin test reactivity to *H. capsulatum* antigens indicates that more than 90% of the population has had primary histoplasmosis (36).

A thermally dimorphic fungus, *Histoplasma capsulatum* exists in the soil as a mold, but after inhalational infection of mammalian tissues it transforms into its pathogenic yeast phase. This dimorphism is essential for virulence; chemically treated mycelial cultures that are unable to make the transition to yeasts are avirulent (26). Dimorphism is also the best-studied system of *H. capsulatum* gene regulation, and both mold-phase-specific and yeast-phase-specific genes have been identified (7, 13, 15–18, 28, 32, 33).

**YPS3** is a yeast-phase-specific gene originally identified in a differential hybridization screen (21). The encoded Yps3p protein is both found in the cell wall and secreted from cells (35). Beyond its phase specificity, Yps3p expression varies among *H. capsulatum* strains that differ in thermostolerance and virulence. Restriction fragment length polymorphism (RFLP) class 2 strains are the most virulent and thermostolerant and are predominantly North American isolates. In class 2 strains, **YPS3** transcription initiates between 2 h and 1 day after a temperature shift from ambient to 37°C and remains continuous during the yeast phase of growth (21, 25, 34). In RFLP class 3 strains, strains of intermediate virulence and thermostolerance found predominantly in Central and South America, **YPS3** transcription is initiated 3 days after a temperature shift, but expression drops off to become undetectable after approximately 12 days (21, 25, 34). **YPS3** is not expressed in RFLP class 1 strains, which are the least virulent and thermostolerant and are geographically widely distributed but have only been found as clinical isolates in severely immunocompromised patients (21, 31, 34).

In the present study, we sought to characterize the cellular localization of Yps3p based on predicted sequence homology that we noted with Bad1, an established virulence factor of another dimorphic fungus, * Blastomyces dermatitidis* (6). Our results indicate that Yps3p is surface localized on class 2 strains of *Histoplasma capsulatum* and that a mechanism of localization is the loading of secreted Yps3p on the surface via an interaction with the cell wall polysaccharide chitin.

**MATERIALS AND METHODS**

**Fungal strains and culture conditions.** *H. capsulatum* strains: G184AS, G184AR, G217B, and Downs have been described previously (1, 2, 14). Downs (ATCC 38904) and UCLA 531S are clinical isolates of RFLP class 1. G217B (ATCC 26032) and G222B (ATCC 26034) are clinical isolates of RFLP class 2. The clinical isolate G184AR (ATCC 26027) and its derivative G184AS are members of RFLP class 3. G186AS is a derivative of the class 3 clinical isolate G186AR (ATCC 26029). G184AS and G186AS are spontaneous smooth-colony morphology variants isolated from G184AR and G186AR, respectively. *H. capsulatum* was grown in *Histoplasma*-macrophage medium (HMM) broth, a rich defined medium (38), in a 5% CO2–95% air atmosphere. Most experiments were done with *H. capsulatum* grown as yeast cells at 37°C. Conversion to mycelial growth was achieved by incubating a culture at 28.5°C for 3 weeks. Under these conditions, yeast cells as well as mycelia are present.

**Cloning, expression, and purification of recombinant Yps3p.** We prepared *H. capsulatum* strain G217B genomic DNA as previously described (37). We PCR amplified the **YPS3** open reading frame from G217B genomic DNA. This open reading frame is different from that reported previously (20); see Results and Discussion for details. To allow nickel affinity purification, we included codons for six histidines prior to the stop codon at the C terminus of the **YPS3** open reading frame. The PCR-amplified **YPS3** was cloned into the pTYB2 expression vector (New England Biolabs, Beverly, Mass.) and sequenced. The recombinant Yps3p plasmid was transformed into *Escherichia coli* strain ER2566, and expression was induced with isopropylthiogalactopyranoside (IPTG) according to the supplier’s recommendations. Recombinant 6xHis-tagged Yps3p was purified with a Ni-nitrilotriacetic acid Superflow resin (Qiagen, Valencia, Calif.) under denaturing conditions and assessed for purity with silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels.
Antibodies. Rabbit antiserum was raised against nickel affinity-purified recombinant 6×His-tagged Yps3p by repeated subcutaneous immunizations at multiple sites, as described previously (10). Immune serum was tested by Western immunoblotting against strain G217B as a positive control and strain Downs as a negative control. In addition to recognizing Yps3p exclusively on the G217B blots, there was some cross-reactivity with a high-molecular-weight antigen in both G217B and Downs. We removed cross-reactive antibodies by adsorbing the final antiserum against Downs culture supernatant. Nitrocellulose strips were incubated in concentrated supernatant overnight at 4°C to allow antigen binding. Nonspecific protein binding sites were then blocked with phosphate-buffered saline (PBS; 1.9 mM NaH2PO4, 8.1 mM Na2HPO4, 154 mM NaCl, pH 7.2) containing 5% bovine serum albumin for 1 h at 4°C. Rabbit antiserum diluted 1:10 in PBS containing 5% bovine serum albumin for 1 h at 4°C. After incubation, the tubes were centrifuged for 1 min at 10,000 × g. The supernatant-PBS was removed from the polysaccharide pellet. The polysaccharide pellet was washed four times with PBS with centrifugation at 13,000 × g for 1 h. In some cases, cells were enzymatically digested or heat killed as described prior to the addition of supernatant.

Flow cytometric detection of surface Yps3p. We washed 10^7 H. capsulatum yeast cells from log-phase HMM broth cultures and resuspended them at a concentration of 10^6 cells/ml in PBS containing 5% bovine serum albumin. After a 5-min blocking incubation, the cells were stained directly with a 1:1,000 dilution of preimmune serum or a 1:10,000 dilution of Yps3p-specific rabbit antiserum followed by a 1:100 dilution goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate (FITC) (BD biosciences, San Jose, Calif.) for 30 min at 4°C. Stained cells were washed with PBS and fixed with 2% paraformaldehyde, pH 7.2, for 30 min at 4°C. Cells were analyzed with BD FACSCan or FACSCalibur flow cytometers (BD biosciences, San Jose, Calif.), and color analysis was performed with FlowJo analysis software (Treestar, Ashland, Oreg.). The middle line indicates the GenBank Yps3p sequence (accession no. AAA33384), which misreports the length of the open reading frame.

**FIG. 1.** Yps3p protein of *Histoplasma capsulatum* strain G217B and alignment with *Blastomyces dermatitidis* Bad1p. The predicted Yps3p protein product bears homology to the N and C termini of *Blastomyces dermatitidis* Bad1p. The white box indicates the internal 1,070-amino-acid tandem repeat region that constitutes the majority of Bad1p, which we have omitted for this alignment. The shaded boxes indicate six conserved cysteine residues thought to be important in disulfide bonding of EGF-like domains. The middle line indicates the GenBank Yps3p sequence (accession no. AAA33384), which misreports the length of the open reading frame.
Tris-Cl containing 2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, 10% glycerol, and 0.1% bromophenol blue for 5 min at 95°C, electrophoresed in an SDS–12% polyacrylamide gel, and electroblotted onto a nitrocellulose membrane. This membrane was blocked for 1 h with Tris-buffered saline (TBS; 25 mM Tris, 123 mM NaCl, 2.7 mM KCl) containing 0.01% SDS, 0.05% Tween 20, and 5% dried milk. The blot was then incubated with a 1:10,000 dilution of Yps3p-specific antiserum. Nonspecific antibody was removed by washing three times for 20 min in 0.01% SDS–0.05% Tween 20 in TBS. The washed nitrocellulose membrane was incubated in a 1:6,000 dilution of horseradish peroxidase-labeled goat anti-rabbit immunoglobulin antibody (Bio-Rad, Hercules, Calif.). Bound antibody was removed from this membrane by submersion in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50°C for 30 min, washed in TBS for 30 min, and rebotted with anti-G217B supernatant antibody (11) as a specificity control.

RESULTS

Yps3p bears homology to Bad1p from Blastomyces dermatitidis. The nucleotide sequence from our G217B YPS3 clones differed from the published sequence (20) but matched sequence that subsequently appeared in the Histoplasma capsulatum genome sequence database (http://www.genome.wustl.edu/projects/hcapsulatum/). When we queried GenBank with our own Yps3p predicted amino acid sequence, we detected homology (2e⁻²³) with the Blastomyces dermatitidis adhesin and virulence factor Bad1p (6). Alignments of the predicted protein products of these genes revealed homology in both an N-terminal signal sequence region and a cysteine-rich epidermal growth factor (EGF) domain similar to the EGF-like domain that characterizes the C terminus of Bad1 (Fig. 1). Additionally, our YPS3 clones and the genomic sequence of YPS3 revealed a shorter open reading frame than originally published (20). Specifically, there is an omission of a guanosine in the published sequence. The guanosine occurs after position 1687 of the YPS3 found in GenBank (accession number L16844). The published sequence predicts a 184-amino-acid protein, including a putative glycosyl-phosphatidylinositol (GPI) processing site near the C terminus (20). Our corrected protein sequence predicts a 137-amino-acid protein, truncated by 47 residues, including the putative GPI processing site.

Yps3p is surface exposed. Although Yps3p has been described as cell wall associated (35), it had not been determined whether this cell wall-associated fraction is surface localized. Flow cytometric analysis of representatives from each of the three RFLP classes revealed that Yps3p is surface localized.

FIG. 2. Flow cytometric determination of Yps3p surface localization. The top left histogram is a representative class 1 strain, UCLA 531S, which never expresses YPS3. The top right histogram is a representative class 2 strain, G217B, which both secretes and surface localizes Yps3p. The bottom left histogram is a class 3 strain, G184AS, which has turned off its transient Yps3p expression during continuous culture in the yeast phase. On all histograms, preimmune serum was included to demonstrate a lack of any autofluorescence of yeasts or cross-reactivity of antibody. Yps3p is only expressed in class 2 strains, where it is also surface localized.
on class 2 strains G217B (Fig. 2) and G222B (data not shown). As expected, Yps3p could not be detected on the surface of the class 1 strains UCLA 531S (Fig. 2) and Downs (data not shown). We also detected no surface-exposed Yps3p on class 3 strains G184AS (Fig. 2), G186AS, and G184AR (data not shown) that had been grown continuously as yeasts.

Interestingly, in some instances strain G217B yeasts appeared to have two different populations of Yps3p binding cells, detectable as a bimodal peak in flow cytometry (Fig. 3A). The cells that stained with a lower fluorescence were also the smallest and least granular cells on the flow cytometric scatter plots, so we hypothesize that this low portion of the bimodal population represents yeasts which have recently budded and therefore have a smaller surface area to coat with Yps3p. We also detected surface-localized protein on class 2 strains via immunofluorescence microscopy (Fig. 3B, top panel). These

FIG. 3. Yps3p secreted from the class 3 strain G217B binds to the surface of the class 1 strain UCLA 531S. Flow cytometry (A) and immunofluorescence microscopy (B) revealed that incubation of UCLA 531S with filtered, concentrated G217B supernatant resulted in accumulation of Yps3p on the yeast surface. The bottom half of the UCLA 531S immunofluorescence panel is data captured from a 10-fold-longer exposure, revealing the very low level of background fluorescence on the yeasts in the field. This interaction did not require live UCLA 531S cells (C) and happened within minutes of exposure (D).
differences in strain expression and localization to the cell wall fraction of expressing strains were confirmed by Western immunoblotting (data not shown).

**Yps3p released into the culture supernatant binds to the yeast cell surface.** As Yps3p is also secreted from the cell and, except for the putative signal sequence, has no obvious transmembrane or GPI anchor domain, we examined whether the secreted protein could adhere to the surface of *Histoplasma capsulatum* yeasts, a mechanism of localization employed by Bad1p (4). The RFLP class 1 strain UCLA 531S never expresses Yps3p. After exposure to filtered concentrated supernatant from the class 2 strain G217B, which contains Yps3p, UCLA 531S yeasts bound Yps3p to their surface, as detected by flow cytometry (Fig. 3A). Immunofluorescence microscopy revealed this bound Yps3p to be uniformly distributed, similar to the surface-localized Yps3p of class 2 strains (Fig. 3B). The viability of “recipient” yeasts was not necessary for binding of Yps3p (Fig. 3C). Binding occurred rapidly after exposure (Fig. 3D) and was saturable (data not shown). Yps3p from filtered concentrated G217B supernatant bound to all other class 1 and 3 strains tested, including Downs, G184AS, and G184AR (Fig. 4A). We also tested whether the Yps3p could bind to the mycelial form of *H. capsulatum*, which does not express the *YPS3* gene (21). When strain G217B was grown under conditions in which both yeast and mycelial cells were present, we found Yps3p coating both forms (Fig. 4B), although yeasts showed more immunofluorescence than the mycelial filaments. When we examined chitin exposure in a mixed culture via
fluorescence microscopy with FITC-labeled wheat germ agglutinin, we found a similar distribution of chitin (data not shown).

**Chitinase digestion reduces Yps3p surface binding.** To identify a ligand for Yps3p on the surface of *H. capsulatum*, we performed enzymatic digestions of yeast cell surface components prior to exposure to Yps3p. Proteinase, lipase, and PIPLC treatment of heat-killed UCLA 531S cells prior to filtered concentrated G217B supernatant exposure did not inhibit Yps3p binding (data not shown). Chitinase treatment degrades the cell wall polysaccharide chitin, which is surface exposed in *H. capsulatum*, as shown by fluorescently labeled wheat germ agglutinin (WGA) binding (Fig. 5A). Chitinase treatment removed Yps3p from the surface of G217B cells as well as reduced binding of Yps3p from G217B to the surface of UCLA 531S cells (Fig. 5B). As expected, chitinase treatment reduced the binding of FITC-WGA, but enzymatic digestion did not completely abolish FITC-WGA binding (data not shown).

**Wheat germ agglutinin incubation reduces Yps3p binding.** We incubated UCLA 531S cells with the chitin binding lectin WGA prior to incubation with filtered concentrated G217B supernatant. After an hour of incubation with WGA, we added identical amounts of G217B supernatant to WGA-treated and untreated cells. Yps3p binding was reduced by more than 50% in cells that had been incubated with wheat germ agglutinin, as assessed by flow cytometry (Fig. 6).

**Yps3p from G217B supernatant binds to purified chitin.** We incubated filtered concentrated G217B supernatant with either crab shell chitin or cellulose as a polysaccharide control for 1 h and then separated the insoluble polysaccharide fraction from the remaining supernatant. We then refiltered the supernatant, extensively washed the polysaccharide, and examined the fractions by Western immunoblotting. The polysaccharide chitin bound and extracted all of the Yps3p from the supernatant (Fig. 7, top panel). To assay the specificity of this binding, we then stripped the membrane and reblotted with an antibody raised against total G217B supernatant (11) (Fig. 7, bottom panel). Among the supernatant components detected by this technique, chitin specifically bound Yps3p. Although a few higher-molecular-weight bands were visible, these also appeared in the cellulose control column and presumably represent nonspecific polysaccharide-binding proteins. When polysaccharide was incubated with PBS alone, neither chitin nor cellulose demonstrated any components or contaminants cross-reactive with these antibodies (Fig. 7).
DISCUSSION

We demonstrated that cell wall-localized Yps3p is surface exposed and characterized H. capsulatum strains of three RFLP classes for natural surface localization and the ability for surface localization in expressing and nonexpressing strains. We demonstrated that secreted Yps3p has the ability to bind to the surface of Histoplasma capsulatum. This surface binding occurs quickly upon exposure to the protein. Enzymatic digestions and in vitro polysaccharide binding assays indicate that Yps3p binds to chitin, a component of fungal cell walls.

The binding of Yps3p to chitin is unusual. Saturable and specific binding often suggests a protein, but proteinase treatment did not decrease binding. Our results demonstrate binding to chitin both in its purified form and in yeast cell walls. The residual Yps3p binding after chitinase digestion could be due to incomplete chitin digestion or to some contribution from other cell wall constituents resistant to the degradative enzymes used in this study. We also cannot completely exclude the possibility that Yps3p interacts with another H. capsulatum supernatant component and this interaction contributes to surface binding, but two lines of evidence are inconsistent with this. First, although we mainly used supernatant from strain G217B for binding experiments, recombinant Yps3p purified from E. coli does bind to yeast cell surfaces and purified chitin (data not shown). Second, chitin affinity purification from strain G217B supernatant revealed Yps3p as the only specific product (Fig. 7). Strain G184AR, which possess the cell wall polysaccharide α-(1,3)-glucan, bound Yps3p severalfold less well than G184AS, the related variant strain that lacks α-(1,3)-glucan (22, 23). This polymer has been reported to make up the outermost layer of the Histoplasma cell wall (19), and it may shield chitin and therefore limit Yps3p binding. Immunofluorescence microscopy with FITC-WGA supported this hypothesis; we detected severalfold less surface-exposed chitin in strain G184AR than in strain G184AS (data not shown).

Although a function of Yps3p has not been determined, the release and surface attachment mechanism that we have demonstrated are features shared with other important microbial products that play important roles in pathogenesis. Secretion followed by surface localization has been described for surface entities from a range of microbes, both prokaryotes and eukaryotes (3, 4, 12). Interestingly, some secreted and reattached proteins, like Blastomyces dermatitidis Bad1p and MIC3 of Toxoplasma gondii, contain EGF-like or chitin binding domains (5, 12). This mechanism of localization has also been described for nonproteinaceous surface components. The extensive capsule of the pathogenic fungus Cryptococcus neoformans is attached to the cell wall polysaccharide α-1,3-glucan in a similar manner (29). In some cases, the receptor for surface binding has been identified, as is the case for chitin for Bad1p and Yps3p, but even in many of the cases where the receptor has been identified, detailed characterization of surface molecules involved in binding has not been accomplished. Intermo-
molecular interactions and structural features involved in binding have also generally not been fully described.

It is not yet clear whether the secreted form, the surface-localized form, either, or both are important during Histoplasma capsulatum infection. Based on homology with Bad1, we are tempted to speculate that Yps3p could function as an adhesin, mediating interaction between Histoplasma cells and the mammalian host. The identification of Hsp60 as the ligand adhesin, mediating interaction between...1211–1123. 

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