RNA Interference-Mediated Silencing of the YPS3 Gene of *Histoplasma capsulatum* Reveals Virulence Defects

Megan L. Bohse and Jon P. Woods*

Department of Medical Microbiology and Immunology, University of Wisconsin Medical School, Madison, Wisconsin 53706

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The YPS3 gene of *Histoplasma capsulatum* encodes a protein that is both surface localized in the cell wall of *H. capsulatum* and released into the culture medium. This protein is produced only during the pathogenic yeast phase of infection and is also expressed differentially in *H. capsulatum* strains of different virulence levels. In this study, we silenced the YPS3 transcript by using an interfering-RNA strategy and examined the silenced mutants for phenotypic differences in vitro and during infection. The mutants showed no growth defect during in vitro culture in a defined medium at 37°C and appeared to have normal virulence in a RAW 264.7 murine macrophage-like cell line. In a C57BL/6 mouse model of infection, however, the mutants caused significantly decreased fungal burdens, particularly in the peripheral phagocyte-rich tissues of livers and spleens. This defect in organ colonization was evident within 3 days of infection; however, it appeared to be exacerbated at later time points.

*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 per year in the United States alone. The regions where histoplasmosis is endemic include the U.S. Midwest as well as areas of South America. In the United States, the prevalence of infection reaches the 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 been exposed to the organism.

Environmentally, *H. capsulatum* is a soil-dwelling organism, often associated with the nitrogen-rich environments of bird or bat guano. Despite having no known requirement for the infection of a mammalian host as part of its life cycle, *H. capsulatum* is well adapted to cause respiratory and systemic diseases in mammals. It is a thermally dimorphic fungus and exists in the soil (or in the laboratory at 25°C) as a mold. After the inhalational infection of mammalian tissues (or transfer to 37°C in the laboratory), *H. capsulatum* transforms into its pathogenic budding-yeast phase. The mold-to-yeast transition is essential for virulence and is controlled by DRK1, the dimorphism-regulating histidine-yeast phase. In response to phase transition, *H. capsulatum* changes the mRNA levels corresponding to genes associated with nutrient acquisition, thermotolerance, cell structure, and stress response, as well as the apyrase named yeast phase-specific genes, many of which have no known function (5, 8–10, 12, 13, 15, 19, 20). A shotgun microarray study which analyzed approximately one-third of the genes in the *Histoplasma* genome found nearly 500 genes that are differentially expressed in the mold and yeast phases (11).

YPS3 is a yeast phase-specific gene originally identified in a differential hybridization screen (13). The encoded protein is found both on the *H. capsulatum* cell wall and is also secreted from cells (21). On the cell wall, it is surface exposed, and it gets to the surface via secretion from the cell and binding to the polysaccharide chitin (3). Beyond its yeast-phase specificity, it is also expressed only in a subset of *H. capsulatum* strains, and these strains are typically North American isolates that have the highest virulence levels (13). The Yps3 protein of strain G217B is 137 amino acids in length and is characterized principally by an N-terminal secretion signal sequence and a C-terminal epidermal growth factor-like domain (3).

To date, the function of Yps3 has not been determined, nor has its potential role in mammalian virulence. In this study, we silenced the YPS3 gene by using RNA interference (RNAi) and analyzed its effects on in vitro growth and mammalian infection. Our results indicate that YPS3-silenced mutants are defective in organ colonization in a mouse model of infection and that this deficiency is exacerbated in peripheral phagocyte-rich tissues.

**MATERIALS AND METHODS**

**Strains and culture conditions.** (i) Yeast strains. *H. capsulatum* strain G217B ura5-23 is a uracil auxotroph of the restriction fragment length polymorphism class 2 strain G217B (ATCC 26032) derived by UV radiation mutagenesis and has been described previously (18). *H. capsulatum* was grown in solid or broth Histoplasma-macrophage medium (HMM), a rich defined medium (23). Solid medium contained 0.5% agarose (SeaKem LE) and 10 μM supplemental FeSO₄. In the case of uracil auxotrophs, the medium included 0.1 mg of uracil/ml. All cells were grown in a 5% CO₂–95% air atmosphere. For growth curve analysis, yeast cells were taken from late-log-phase cultures and resuspended at a concentration of 3 × 10⁸ cells/ml in 20 ml of HMM (A₆₀₀ of 1 corresponds to 2.24 × 10⁹ CFU/ml) (data not shown). Culture turbidity was monitored with a photoelectric colorimeter (Manostat Corporation, New York, NY).

(ii) Bacterial strain. Plasmids were cloned and propagated in the *Escherichia coli* strain JM109 [F’ traD36 proAB T1lacZAM15 (lac-proAB) glnV44 recA1 relA1 endA1 thi-1 hsdR17].

(iii) Mammalian cells. The mammalian cell line used in this study was RAW 264.7 (ATCC TIB-71), a murine macrophage-like cell line acquired from the American Type Culture Collection. RAW 264.7 cells were grown in RPMI medium (Cellgro, Herndon, VA) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA).

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* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, 420 SMI, University of Wisconsin Medical School, 1300 University Ave., Madison, WI 53706. Phone: (608) 265-6292. Fax: (608) 265-6132. E-mail: jppwoods@wisc.edu.

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The high-fidelity polymerase Triplemaster (Eppendorf, Westbury, NY). Constructs were verified by sequencing. All PCR products were amplified using (pYPS620) and moved into plasmid pWU45 digested with NheI and SphI.

Plasmid construction. The RNAi plasmids pYPS610 and pYPS620 (Fig. 1) were based on the previously described telomeric shuttle plasmid pWU45 (18). This vector contains the Podospora anserina URA5 gene and a telomeric cassette for selection and maintenance in H. capsulatum and the ampicillin resistance gene for selection in E. coli. &nbs...
of genes in *Histoplasma* (17). We utilized promoters from two different *H. capsulatum* genes, as heterologous expression in this organism is relatively uncharacterized and we wanted to increase the likelihood of effective silencing. pYPS610 uses the promoter region from *CBP1*, while pYPS620 utilizes the *H2B* promoter region. The plasmids are otherwise identical (Fig. 1).

We screened 36 pYPS610 and 36 pYPS620 transformants for reductions in the levels of secreted Yps3. Three of the 36 pYPS610 transformants and 3 of the 36 pYPS620 transformants showed considerably reduced levels of secreted protein. As observed previously for *H. capsulatum*, as well as other fungal systems, we noted variability in the degree of silencing achieved (17). We identified 6 out of 72 transformants with grossly reduced levels of secreted Yps3 and did not further examine the other transformants that showed more modest or no reduction. We colony purified three of these transformants for more detailed analyses. The RNAi 4 transformant strain was of pYPS610 origin, while the RNAi 8 and RNAi 11 strains were transformed with pYPS620, and each had low to undetectable levels of secreted Yps3 (Fig. 2). In all three strains, transforming DNA was detectable by Southern blotting (data not shown); however, the plasmids appeared to have integrated, rather than being maintained as episomal. Empty-vector control strains also appeared to have undergone chromosomal integration of the transforming DNA. As described below, we examined multiple RNAi and empty-vector control transformants in phenotypic assays in order to address the potential effects of different sites of integration or other transformant-specific variations. Levels of secreted Yps3 remained low to undetectable in the RNAi transformants used for further study for at least 6 months of in vitro culture with continuous passage in HMM broth.

**In vitro growth and infection of the murine macrophage-like cell line RAW 264.7.** Two RNAi mutants, RNAi 4 and RNAi 11, were assayed for growth at 37°C in HMM, a rich defined medium. Growth was measured by using culture turbidity (Fig. 3A). We tested the mutants for virulence in RAW 264.7 macrophage-like cell line RAW 264.7. The rate of survival of the RAW 264.7 host cell monolayer after infection was measured by BrdU uptake and expressed as the percentage of the value for uninfected control cells. MOI, multiplicity of infection; EV, *G217B ura5-23* strain transformed with pWU45, an empty vector. RNAi 4 and RNAi 11 are two independently generated *G217B ura5-23 YPS3*-silenced mutants. The assay was performed in triplicate, and results from a representative experiment are shown. Error bars indicate standard deviations.
phages by using a previously described BrdU uptake assay (18). There was no significant difference between the mutants and the empty-vector control cells in the level of destruction of RAW 264.7 macrophages after 4 days of infection at any multiplicity of infection tested (Fig. 3B). Similar assays using crystal violet to stain for viable macrophages after 4 days of infection (18) likewise showed no difference in macrophage virulence, nor did assays which allowed the infection to progress for shorter (2 days) or longer (5 days) time frames (data not shown).

Evaluating the RNAi mutants in a C57BL/6 mouse model of infection. We tested the RNAi mutants in a C57BL/6 mouse model of either intranasal or intraperitoneal infection. For intranasal infection, mice were given $2 \times 10^6$ CFU of an empty-vector control strain or the mutant RNAi 4 or RNAi 11 strain. After 3, 7, 10, or 14 days, the mice were sacrificed and their lungs, livers, and spleens were examined for the presence of *H. capsulatum* via plate assays for the enumeration of CFU. For intraperitoneal infection, mice received $6 \times 10^7$ CFU of an empty-vector control strain or the mutant RNAi 4 or RNAi 11 strain. After 14 days of infection, the mice were sacrificed and their livers and spleens were analyzed for fungal burdens. During the 7-day intranasal infection (Fig. 4), eight mice were infected with an empty-vector control strain, eight mice were infected with the RNAi 4 strain, and seven mice were infected with the RNAi 11 strain. RNAi 4- and RNAi 11-infected lungs showed numbers of CFU lower by log$_{10}$ of 1.28 and 1.66, respectively, than the numbers of CFU in lungs infected with the empty-vector control strain. The livers of RNAi 4- and RNAi 11-infected mice had numbers of CFU lower by log$_{10}$ of 2.48 and 2.20 than the numbers in the livers of mice infected with the control strain. In the spleens of mutant-infected mice, the reductions in the fungal burdens were log$_{10}$ of 2.06 CFU for RNAi 4 and log$_{10}$ of 1.97 CFU for RNAi 11 compared to the fungal burdens in the spleens of control strain-infected mice (Fig. 4). Similar results were obtained in four independent experiments. Intranasal infections were also conducted with the mutant RNAi 8 strain and two other RNAi mutant strains derived from an additional independent transformation, all of which yielded results similar to those obtained with RNAi 4 and RNAi 11 strains, as well as an additional, independently derived, empty-vector control strain, which yielded results similar to those obtained with the original empty-vector control strain (data not shown). When the mice were infected intraperitoneally (4, four mice per strain tested), the RNAi 4 and RNAi 11 mutants yielded reductions in CFU of log$_{10}$ of 1.83 and 1.73 in the livers and log$_{10}$ of 1.28 and 1.5 in the spleens after 14 days of infection, respectively (Fig. 5). To assess colonization deficiency over time after intranasal infection with either an empty-vector control strain or the RNAi 11 strain, we allowed the infection to progress for 3, 10, and 14 days prior to sacrifice. The colonization deficiency of the RNAi mutant was detectable by 3 days but appeared to become amplified over time, particularly in the sites of dissemination. In the lungs, the reductions in CFU in the 3-, 10-, and 14-day samples were, respectively, log$_{10}$ of 1.08, 0.73, and 1.68. In the livers, the reductions in CFU were log$_{10}$ of 0.81, 1.03, and 2.35 as the infection progressed, while in the spleens, differences in CFU of log$_{10}$ of 0.38, 2.05, and 2.89 were identified on days 3, 10, and 14 (Fig. 6). Several random isolates of empty-vector and RNAi strains from lungs, livers, and spleens of infected mice were transferred into HMM broth at 37°C and tested for Yps3 protein expression. All strains tested maintained a consistent control or silenced phenotype before and after mouse infection (data not shown).
DISCUSSION

The role of Yps3 in virulence has remained speculative since the identification of this protein in 1989. There is a correlation of YPS3 expression with pathogenicity, since only the most virulent strains of Histoplasma express YPS3 and only in the host-adapted yeast morphotype (2, 13). These observations were made with nonisogenic H. capsulatum strains, and the levels of Yps3 production were only one of many differences among the strains. In this study, we provide the first genetic evidence that the YPS3 gene of the restriction fragment length polymorphism class 2 strains of H. capsulatum influences virulence. We demonstrate that RNAi-silenced YPS3 mutants have a virulence defect in a mouse model of infection, although they grow normally as yeasts in vitro, can transition to the...

FIG. 5. Silencing of YPS3 reduces the colonization of livers and spleens in C57BL/6 mice following intraperitoneal infection. Graphs represent numbers of CFU (expressed as log_{10} values) recovered from homogenates of organs 14 days after intraperitoneal infection with 6 × 10^7 CFU of Histoplasma capsulatum. Four mice per fungal strain were used, and error bars indicate standard deviations. P values represent significant differences in comparison to the control empty-vector strain (EV).

FIG. 6. The YPS3-silenced mutants show an initial colonization defect in lungs, livers, and spleens. This defect is amplified at the later time points in the peripheral tissues of livers and spleens. Graphs represent numbers of CFU (expressed as log_{10} values) recovered from homogenates of organ tissues 3, 10, or 14 days after intranasal infection with 2 × 10^6 CFU of H. capsulatum. Each time point is represented by the mean of results for four to five mice, and error bars indicate standard deviations. EV, empty-vector control strain.
mycelial morphotype when the growth temperature is shifted to 25°C (data not shown), and appear normally virulent during the in vitro infection of RAW 264.7 macrophages.

In mice, Yps3 RNAi mutants were able to infect the lungs and disseminate to livers and spleens and showed initial proliferation, followed by clearance. But the RNAi downregulation of Yps3 resulted in significant quantitative defects relative to the wild type early in infection that expanded over time and defects at mononuclear phagocytic system sites of dissemination that were greater than the defect at the primary site of infection in the lungs. There are many possible mechanisms for this attenuation of virulence, which are not mutually exclusive. For instance, intranasally administered Yps3 mutants may not efficiently get to the lungs or establish or maintain infection once there. Alternatively, Yps3 RNAi mutants may infect the lungs normally after intranasal infection but become defective for dissemination to the liver or spleen or for proliferation or the avoidance of clearance at these sites once dissemination is achieved. The mutants' more rapid clearance from organs correlates with the onset of acquired immunity and may suggest that Yps3 interacts with the host immune system to perpetuate infection.

The few *H. capsulatum* genes so far demonstrated to influence infection have generally shown concordant results in mouse and in vitro macrophage infection models. For example, *ura5* (18) and *ags1* (17) mutants are relatively defective both for mouse infection and for in vitro infection of RAW 264.7 and U937 cells or P388D1 cells, respectively. In contrast, Yps3 RNAi mutants displayed normal virulence in RAW 264.7 macrophages in vitro, although their virulence was compromised during mouse infection. This finding is consistent with a role for Yps3 specifically in the in vivo animal infection environment or one involving host mechanisms or responses that are present or fully manifested only in the animal. Of course there are many differences between these models, and the elucidation of the mechanistic basis awaits further work.

The Yps3 mutants displayed lower fungal burdens in the lungs after intranasal infection, and this defect became amplified as the infection progressed. A defect in initial lung colonization or growth in the lungs may exist but is probably not adequate to explain all our findings. While the levels of lung colonization were lower for the RNAi mutants, these differences were not as great as those in livers and spleens, and the mutants maintained higher fungal burdens in the lungs than in the livers or spleens throughout the course of infection.

The empty-vector control strains of *H. capsulatum* were detectable in the livers and spleens of mice by 3 days, the earliest time point sampled, after intranasal infection, and increasing levels of fungi were present through 14 days postinfection. The Yps3 RNAi mutants disseminated to these peripheral tissues, but there were consistently at least 10-fold fewer cells per organ in tissues from mutant-infected mice than in those from control strain-infected mice. The mechanism for the impaired dissemination or reduced growth or faster clearance after dissemination remains to be determined. The mutant yeast cells may be defective at leaving the lungs or entering the bloodstream, extracellularly or within migratory host cells, perhaps due to an altered cellular or subcellular localization pattern, or perhaps they are cleared from the bloodstream more easily. In the related fungus *Blastomyces dermatitidis*, surface-localized Bad1, which is a Yps3 homolog, prevents complement molecule C₃ deposition onto yeast cells (24). We have noticed a similar defect in complement deposition after coating strains of *H. capsulatum* that do not normally produce Yps3 with exogenous protein (our unpublished results). Increased opsonization due to reduced Yps3 production may be a potential mechanism of clearance.

Intraperitoneal infection bypasses the issues of lung infection and exit and directly addresses the question of whether the RNAi mutants can infect peripheral tissues as well as control transformants. When *H. capsulatum* cells were injected intraperitoneally, the RNAi mutants did not achieve wild-type levels of infection in the livers or spleens. This result implies that in addition to showing reduced levels in the lungs and a potential defect in dissemination from the lungs after intranasal infection, the RNAi mutant does not survive, proliferate, or resist clearance as well as the control strain in the peripheral tissues.

Time course experiments revealed that the control strain increased or sustained fungal burdens in lungs, livers, and spleens during the progression of infection over 2 weeks. With the Yps3 RNAi mutants, infection levels in all organs dropped between the 10- and 14-day time points, which correlates with the onset of acquired immunity and may be consistent with Yps3 involvement in some aspect of this process. Acquired immunity, particularly a potent T-cell response and the production of the cytokines gamma interferon and tumor necrosis factor alpha, is critical for reducing fungal burdens and promoting organ clearance (1, 4, 22). In *Blastomyces dermatitidis*, the Bad1 protein imparts virulence through the modulation of host tumor necrosis factor alpha (6, 7). This immunomodulation has been ascribed to an intracellular domain not encoded by the expressed YPS3 genes (2, 3), however, suggesting that if Yps3 is modulating virulence or host cytokine responses, it is doing so via a different mechanism.

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