The Yeast-Phase Virulence Requirement for α-Glucan Synthase Differs among Histoplasma capsulatum Chemotypes

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Histoplasma capsulatum strains can be classified into two chemotypes based on cell wall composition. The cell wall of chemotype II yeast contains a layer of α-(1,3)-glucan that masks immunostimulatory β-(1,3)-glucans from detection by the Dectin-1 receptor on host phagocytes. This α-(1,3)-glucan cell wall component is essential for chemotype II Histoplasma virulence. In contrast, chemotype I yeast cells lack α-(1,3)-glucan in vitro, yet they remain fully virulent in vivo. Analysis of the chemotype I α-glucan synthase (AGS1) locus revealed a 2.7-kb insertion in the promoter region that diminishes AGS1 expression. Nonetheless, AGS1 mRNA can be detected during respiratory infection with chemotype I yeast, suggesting that α-(1,3)-glucan could be produced during in vitro growth despite its absence in vivo. To directly test whether AGS1 contributes to chemotype I strain virulence, we prevented AGS1 function by RNA interference and by insertion mutation. Loss of AGS1 function in chemotype I does not impair the cytotoxicity of ags1Δ yeast to cultured macrophages, nor does it affect the intracellular growth of yeast. In a murine model of histoplasmosis, the ags1Δ- yeast mutant strains show no defect in lung infection or in extrapulmonary dissemination. Together, these studies demonstrate that AGS1 expression is dispensable for chemotype I yeast virulence, in contrast to the case for chemotype II yeast. Despite the absence of cell wall α-(1,3)-glucan, chemotype I yeast can avoid detection by Dectin-1 in a growth stage-dependent manner. This suggests the production of a unique Histoplasma chemotype I factor that, at least partially, circumvents the α-(1,3)-glucan requirement for yeast virulence.

Lung alveolar macrophages recognize and coordinate the mammalian immune response to inhaled pathogens. This immune recognition is based upon the interaction between highly conserved ligands on the microbial surface (pathogen-associated molecular patterns [PAMPs]) and host cell pattern recognition receptors (PRRs) (31). Successful engagement of PAMPs by PRRs results in rearrangements of the macrophage cytoskeleton to permit phagocytosis of the pathogen (16). The major PAMP on fungal cells is the common fungal cell wall polysaccharide β-(1,3)-glucan (4), which is recognized by the phagocyte PRR Dectin-1 (3). Dectin-1 recognition of β-(1,3)-glucans has been linked to enhanced phagocytosis (6, 20), reactive oxygen species production (39), and upregulation of cytokine production (14, 28). Therefore, successful infection by fungal invaders requires strategies to either conceal β-(1,3)-glucan or ameliorate the effects of its recognition. Indeed, the virulence of many human fungal pathogens, including Candida albicans and certain strains of Histoplasma capsulatum, involves masking of immunostimulatory β-(1,3)-glucans (11, 33, 40). Conversely, increased exposure of β-glucans has been shown to enhance antimicrobial responses by immune cells to Candida and Aspergillus (11, 38).

Histoplasma capsulatum is a dimorphic fungal pathogen that causes the respiratory and systemic disease histoplasmosis. Although immunocompromised patients (e.g., those with HIV infection or organ transplant recipients) have increased risk of invasive fungal disease (32), dimorphic fungal pathogens pose additional concern as they can infect immunocompetent individuals as well (36). Histoplasma is acquired by inhalation of conidia, which upon encountering mammalian body temperatures germinate into pathogenic yeast cells (25). Progression of respiratory disease depends upon the ability of Histoplasma yeast to survive and replicate within alveolar macrophages. Following infection of the lung, the yeast can disseminate to other organs of the body, including the spleen, liver, and heart, causing the most lethal form of histoplasmosis disease (9, 26).

Through gene sequencing, Histoplasma capsulatum has been classified into six primary phylogenetic groups that are associated with specific geographical locations: North America 1 (NAm 1), North America 2 (NAm 2), Panama (Pan), Latin America A (LAm A), Latin American B (LAm B), and Africa (23, 24). A second classification scheme categorizes Histoplasma strains into two groups, or chemotypes, based on whether the yeast cell wall contains the α-(1,3)-glucan polysaccharide (7). The cell wall of chemotype II strains contains α-(1,3)-glucan, and this class includes the vast majority of Histoplasma strains (five of the six phylogenetic groups), as well as other dimorphic fungal pathogens such as Blastomyces and Paracoccidioides (21, 22). Conversely, NAm 2 represents the sole chemotype I, or non-α-(1,3)-glucan-containing, group of Histoplasma strains.

Three genes have been identified that contribute to α-(1,3)-glucan synthesis in chemotype II yeast and have been shown to be necessary for full virulence. In chemotype II Histoplasma, deletion of the single α-(1,3)-glucan synthase gene (AGS1) results in yeast cells lacking cell wall α-(1,3)-glucan (34), as

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the mouse model of infection (29, 34). In chemotype II His-
G186A attenuates virulence in cultured macrophages and in
AMY1 function in the representative chemotype II strain
-glucan in their cell walls (7, 35), yet they are fully virulent in the
/H9251 cells grown in laboratory culture do not possess
tion by Dectin-1 (33).
/H9252 glucose-1-phosphate uridyltransferase (29). Loss of
does mutation of
-glucan synthase could
interference (RNAi) strains were determined by measurement of liquid culture
(1,3)-glucan virulence factor during
in vivo
-(1,3)-glucan lies exterior to the β-(1,3)-glucan, creating a physical mask that blocks β-(1,3)-glucan detection
by Dectin-1 (33).

In contrast to chemotype II Histoplasma yeast, α-(1,3)-glucan lies exterior to the β-(1,3)-glucan, creating a physical mask that blocks β-(1,3)-glucan detection by Dectin-1 (33).

Materials and Methods

Yeast strains and growth conditions. Histoplasma capsulatum strains used in this study included the wild-type strains G217B (ATCC 26032) and G186A (ATCC 26029), representative of chemotype I and chemotype II, respectively. Mutant strains derived from these are described in Table 1. Yeast strains were grown in Histoplasma-macrophage medium (HMM) (42) at 37°C with shaking (200 rpm) until late log/early stationary phase (approximately 72 h) unless oth-
erwise noted. For growth of uracil auxotrophs, the medium was supplemented with uracil (100 µg/ml). The growth rates and stages of mutant and RNA interference (RNAi) strains were determined by measurement of liquid culture turbidity at 595 or 600 nm. For turbidity measurements of chemotype II strains,

1 M NaOH was added to reduce clumping of cells. For enumeration of CFU, 107 yeast cells were recovered by centrifugation, diluted 400-fold in 0.6% agarose and supplemented with 25 µM FeCl3.

Isolation of yeast RNA. Histoplasma yeast cells were collected from 10-ml HMM liquid cultures by centrifugation (2 min at 1,500 g) and resuspended in 1 ml TRIZol (Invitrogen). For in vivo RNA samples, male C57BL/6 mice (6 to 8 weeks old; Harlan Laboratories) were infected (as described below) with ap-
proximately 1 × 107 yeast cells. At 2 days postinfection, lungs were collected and processed for RNA isolation (E. D. Holbrook, J. A. Edwards, B. H. Youssef, and C. A. Rappleye, submitted for publication). Briefly, lungs were homogenized in hypotonic buffer (10 mM Tris, 1 mM EDTA), and passed through sterile gauze. Yeast cells were collected by centrifugation and resuspended in TRIZol. The yeast cells were then passed through a 21-gauge needle and washed in TRIZol four times. RNAs were liberated from in vitro– or in vivo-grown yeast by heating with 0.5-mm-diameter glass beads. Total RNA was purified by chloroform extraction and ethanol precipitation. Contaminating genomic DNA was removed by three sequential digestions with RQ1 RNase-free DNase (Promega). RNA concentration and purity were estimated by absorbance at 260 and 280 nm. The absence of DNA was verified by lack of PCR amplicon production using RNA as templates.

Gene expression analysis. Five micrograms of total RNA was reverse trans-
cribed using SuperScript III reverse transcriptase (Invitrogen) and 15-mer oligo(dT) primers. For endpoint PCR, reverse-transcribed templates were diluted 100-fold (liquid culture yeast) or 40-fold (infected lungs) in a PCR mix containing 0.5 µM each gene-specific primer and 150 µM deoxynucleotide triphosphate (dNTPs). PCR products were amplified for 35 cycles (liquid culture samples) or 45 cycles (lung samples) at 94°C for 10 s, 52 to 55°C for 15 s, and 72°C for 1 min. PCR products were examined for transcripts of interest by electrophoresis through 1% agarose containing ethidium bromide. For quanti-
tative PCR, templates were diluted 1:100 in a PCR mix containing 0.5 µM each gene-specific primer and 1 × SYBR green-containing amplification mix (Bio-
Rad). Real-time PCR was performed using a Mastercycler ep realplex2 thermal
cycler (Eppendorf) and transcripts quantified by the ΔΔCt method (1). Trans-
script levels were normalized to actin (ACT1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs. The specificity of amplification was deter-
mined by melting curve analysis and by confirming amplicon size by gel electro-
phoresis.

Transmission electron microscopy. Histoplasma wild-type yeast cells were grown to early stationary phase, fixed in 2% glutaraldehyde in cacodylate buffer (0.1 M cacodylic acid, pH 7.4), and postfixed in 1% osmium tetroxide in cac-
lylate buffer. Fixed cells were rinsed with buffer and placed in 2% warm, low-
temperature-gelling agarose. The agarose was solidified in an ice bath and cut into 1-mm3 blocks. Samples were rinsed and incubated in 1% uranyl acetate for 90 min prior to being dehydrated in a series of graded ethanol washes. Samples

Table 1. Histoplasma strains

<table>
<thead>
<tr>
<th>Chemotype and strain*</th>
<th>Genotype</th>
<th>Other designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G217B</td>
<td>Wild type (ATCC 26032)</td>
<td>Wild type</td>
</tr>
<tr>
<td>WU15</td>
<td>ura5-Δ32</td>
<td>AGS1(+)</td>
</tr>
<tr>
<td>OSU4</td>
<td>ura5-Δ32 ags1-5::T-DNA [hph]</td>
<td>ags1(-)</td>
</tr>
<tr>
<td>OSU8</td>
<td>ura5-Δ32 cbp1-9::T-DNA [hph]</td>
<td>cbp1(-)</td>
</tr>
<tr>
<td>OSU25</td>
<td>ura5-Δ42 pCR473 [URA4, gfp-RNAi]</td>
<td>AGS1(+)</td>
</tr>
<tr>
<td>OSU26</td>
<td>ura5-Δ42 cbp1-9::T-DNA [hph] pCR473 [URA4, gfp-RNAi]</td>
<td>ags1(-)</td>
</tr>
<tr>
<td>OSU27</td>
<td>ura5-Δ42 ags1-5::T-DNA [hph] pCR473 [URA4, gfp-RNAi]</td>
<td>cbp1(-)</td>
</tr>
<tr>
<td>OSU32</td>
<td>ura5-Δ42 zzz::T-DNA [hph, PEIFα-gfp]</td>
<td>gfp-RNAi</td>
</tr>
<tr>
<td>OSU34</td>
<td>ura5-Δ42 zzz::T-DNA [hph, PEIFα-gfp] pCR473 [URA4, gfp-RNAi]</td>
<td>gfp-RNAi</td>
</tr>
<tr>
<td>OSU35</td>
<td>ura5-Δ42 zzz::T-DNA [hph, PEIFα-gfp] pCR474 [URA4, gfp:AGS1-RNAi]</td>
<td>gfp:AGS1-RNAi</td>
</tr>
</tbody>
</table>

II

| G186A                 | Wild type (ATCC 26027) | Wild type         |
| WU8                   | ura5-Δ32 | AGS1(+)          |
| OSU1                  | ura5-Δ32 ags1-Δ4::hph | ags1(Δ)          |
| OSU17                 | ura5-Δ32:pCR473 [URA45, gfp-RNAi] | AGS1(+)          |
| OSU18                 | ura5-Δ32 ags1-Δ4::hph:pCR473 [URA45, gfp-RNAi] | ags1(Δ)          |
| OSU22                 | ura5-Δ32 zzz::[hph, PEIFα-gfp] | gfp-RNAi         |
| OSU67                 | ura5-Δ32 zzz::[hph, PEIFα-gfp] pCR473 [URA45, gfp-RNAi] | gfp-RNAi         |
| OSU68                 | ura5-Δ32 zzz::[hph, PEIFα-gfp] pCR474 [URA45, gfp:AGS1-RNAi] | gfp:AGS1-RNAi    |

* Chemotype I strains are derived from G217B, and chemotype II strains are derived from G186A.
were incubated in propylene oxide for 20 min and infiltrated with Eponate 12 resin. Embedded samples were cut at 70 nm on a Leica EM 6 ultramicrotome. Sections were stained in 2% aqueous uranyl acetate and Reynolds lead citrate and observed in an FEI Tecnai Spirit transmission electron microscope at 80 kV. The cell wall thicknesses of 20 individual G186A and 17 individual G217B yeast cross sections were measured using ImageJ software. The average cell wall thickness was determined from three measurements of each cell.

Promoter analyses. Transcriptional fusions to the green fluorescent protein (GFP) reporter gene were constructed by amplification of regions immediately upstream of the AGS1 locus by PCR (primer sequences are available upon request) and insertion ahead of the GFP gene in plasmid pCR368. Cloning was facilitated by inclusion of BamHI and AscI sites in the 5′ tails of the PCR primers. Chimeric promoters were constructed by gene splicing by overlap extension (SOEing) (19). Promoter fusion vectors were linearized by PstI digestion and transformed into Histoplasma WUS or WU15 (29) yeast by electroporation (41). Nineteen or 20 random Ur′ transformants were spotted onto HMM plates. The GFP fluorescence of individual spots was visualized using an Alphamager UV transillumination system (Alphamager; Cell Biosciences) modified with a Trans-blue conversion screen and a 470-nm short-pass excitation filter (44). Fluorescence light was collected through a 530/15 band-pass filter and was quantified using AlphaEaseFC spot densitometry software (Cell Biosciences).

Generation of sentinel RNAi backgrounds and AGS1-RNAi lines. GFP-fluorescent sentinel background strains were constructed by transformation of Histoplasma with constructs in which the GFP gene was transcribed from the G217B EF1α promoter. WUS was transformed with NotI-linearized pCR482, and WU15 was transformed via Agrobacterium tumefaciens harboring pCR482, a plasmid carrying the EF1α promoter. EF1α-gfp constructs were linearized by PstI digestion and then transformed into Histoplasma WUS or WU15 (29) yeast by electroporation (41). Nineteen or 20 random Ur′ transformants were spotted onto HMM plates. The GFP fluorescence of individual spots was visualized using an Alphamager UV transillumination system (Alphamager; Cell Biosciences) modified with a Trans-blue conversion screen and a 470-nm short-pass excitation filter (44). Fluorescence light was collected through a 530/15 band-pass filter and was quantified using AlphaEaseFC spot densitometry software (Cell Biosciences).

RESULTS

In vitro cell wall ultrastructure. In liquid culture, α-glucan-negative chemotype I yeasts do not exhibit the clumping behavior of chemotype II yeasts, suggesting different surface characteristics. To discern any ultrastructural differences due to the lack of α-(1,3)-glucan, we compared the cell walls of the chemotype II strain G186A and the chemotype I strain G217B using transmission electron microscopy. The α-glucan-positive cell wall of chemotype II yeast cells appears as an electron-lucent region surrounding the yeast cell (Fig. 1A), consistent with other studies (8). In comparison, the α-glucan-negative cell wall of chemotype I yeast cells (Fig. 1B) is noticeably thinner than the cell wall cross section of chemotype II yeast. Quantification of the cell wall thickness in these two strains shows that the G217B cell wall is less than half the thickness of the G186A yeast cell wall (Fig. 1C), presumably due to the absence of the α-(1,3)-glucan polysaccharide layer around G217B yeast.

AGS1 expression in vitro and in vivo. Despite the difference in α-(1,3)-glucan content between chemotype II and chemotype I Histoplasma yeast cell walls, examination of the genome sequences from Histoplasma strains G186A and G217B indicates that both chemotypes possess the AGS1, AMY1, and UGP1 genes, whose products are necessary for α-(1,3)-glucan incorporation into the yeast cell wall (29, 34). These loci are highly conserved between the three sequenced Histoplasma strains, showing 98% to 99% amino acid identity in the translated products. This is equivalent to the nucleotide and amino acid identities of conserved “housekeeping” genes (e.g., ACT1 and the GAPDH gene, which show 100% and 99% identity, respectively, in amino acid sequence). In particular, the 7-kb AGS1 coding sequence shows only 2% nucleotide variation and 1% amino acid variation between the two chemotypes. Thus, the lack of genes known to be necessary for the biosynthesis of α-(1,3)-glucan does not explain the absence of α-(1,3)-glucan in chemotype I yeast.

To determine if the lack of α-(1,3)-glucan in chemotype I Histoplasma yeast results from deficient transcription of necessary genes, we examined the transcription of AGS1, AMY1, and UGP1 by reverse transcription-PCR (RT-PCR). As expected...
pected, the α-(1,3)-glucan-positive G186A yeasts express AGS1, AMY1, and UGP1 in vitro and in vivo (Fig. 2A). Surprisingly, the chemotype I G217B yeasts also express detectable levels of AGS1, AMY1, and UGP1 mRNAs during laboratory culture (Fig. 2A). More significantly, G217B expresses detectable levels of AGS1, AMY1, and UGP1 mRNAs during growth in the murine lung. Both chemotypes express the actin gene (ACT1) and the known virulence factor CBP1 (Fig. 2A). The expression of AGS1, AMY1, and UGP1 by chemotype I Histoplasma yeast during infection suggests that, although not manifest in vitro, α-(1,3)-glucan could contribute to the pathogenesis of chemotype I strains in vivo.

To determine if the transcription levels of key α-(1,3)-glucan synthesis enzymes differ between chemotype I and chemotype II strains, we analyzed AGS1, AMY1, and UGP1 transcription by quantitative RT-PCR. Template RNA was prepared from G186A and G217B grown in liquid culture. In order to compare transcript results across different strain backgrounds, we normalized results to two housekeeping genes, ACT1 and the GAPDH gene, which show the least variation when multiple genes are compared between species (data not shown). Comparison of mRNAs between strains shows that α-(1,3)-glucan-deficient chemotype I yeast expresses 20- to 23-fold less AGS1 mRNA than the α-(1,3)-glucan-producing chemotype II strain (Fig. 2B). The two chemotypes express AMY1 and UGP1 at similar levels (Fig. 2B). Together, these results suggest that chemotype I yeasts lack α-(1,3)-glucan due to diminished transcription of the AGS1 gene.

AGS1 promoter analysis. To determine why chemotype I yeasts have reduced transcription of AGS1 compared to chemotype II yeasts, we analyzed the AGS1 promoter regions from chemotype I (G217B) and chemotype II (G186A) Histoplasma strains. To delineate the full AGS1 promoter, we first created fusions of increasing lengths of the region upstream of the AGS1 coding sequence from the α-(1,3)-glucan-producing strain G186A to a GFP reporter gene. These promoter-GFP fusions were transformed into the chemotype II strain background, and AGS1 transcription was assessed by measuring GFP fluorescence (Fig. 3A). GFP fluorescence increases above background when the GFP gene is fused to a length of sequence 1,239 bp upstream from the translational start codon. A region encompassing 1,743 bp upstream of the AGS1 ATG codon provides maximal reporter gene expression, indicating that the full chemotype II AGS1 promoter requires approximately 1,700 bp upstream of the coding sequence.

Comparison of the AGS1 upstream genomic sequences in chemotype I and chemotype II strains reveals that the chemo-
Differences between chemotype II and the repaired promoter. There was no significant difference in GFP expression between the regions upstream and downstream of the insertion site by PCR. The mean fluorescence of double transfectants with transcriptional fusions was transformed into WU15, and fluorescence was quantified. Data points represent the fluorescence of GFP gene fusions and are distinguishable, and the fluorescence can be quantified to assess the level of RNAi-based gene silencing (Fig. 4A and B).

Using these sentinel backgrounds, we depleted any Ags1 function using an RNAi plasmid that targets both AGS1 and the GFP gene. Cotargeting AGS1 and the GFP gene allows depletion of Ags1 to be monitored by loss of cotargeted GFP fluorescence. In the α-(1,3)-glucan-expressing chemotype II sentinel background, yeast transformed with an RNAi plasmid by at least 6-fold is observable (Fig. 4A). When the GFP gene and AGS1 are simultaneously targeted, loss of GFP fluorescence mirrors the depletion of AGS1, which is confirmed by the loss of rough colony morphology (Fig. 4A). Thus, silencing of GFP fluorescence with the gfp::AGS1 RNAi plasmid by at least 6-fold is sufficient to block Ags1 function and prevent α-(1,3)-glucan production (34). Similar to the case for chemotype II, when the GFP gene is targeted alone or in combination with AGS1 in type I promoter is disrupted. The AGS1 promoter of chemotype II strain G186A closely aligns with another chemotype II isolate (the NAm 1 phylogenetic group type strain WU24) throughout the upstream 2 kbp (see Fig. S1 in the supplemental material). The G217B (chemotype I) AGS1 promoter has a 2.7-kb insertion of repetitive DNA (composed primarily of retrotransposon remnants), that interrupts the promoter at bp 1495 upstream of the AGS1 start codon (Fig. 3B). To determine if this large insertion is responsible for the decreased promoter activity, we used PCR to construct a synthetic G217B AGS1 promoter-GFP gene fusion in which sequences homologous to the chemotype II promoters upstream and downstream of the insertion site were spliced together, effectively removing the insertion. This “repaired” chemotype I AGS1 promoter restores expression of the reporter gene to levels similar to those for the chemotype II promoter (Fig. 3C).

Depletion of AGS1 by RNA interference in chemotype I Histoplasma. To determine if α-(1,3)-glucan is required in vivo for the pathogenesis of chemotype I Histoplasma yeast, we used RNAi to block the function of Ags1, a central enzyme in α-(1,3)-glucan synthesis. Wild-type chemotype II yeasts exhibit a rough morphology when grown on solid media. Depletion of Ags1 function by RNAi in this background prevents production of cell wall α-(1,3)-glucan, resulting in a smooth instead of a rough colony morphology (34). This allows for easy identification of chemotype II strains that lack Ags1 function. However, AGSI(+) chemotype I yeasts grown on solid media exhibit a smooth morphology, preventing validation of RNAi-based depletion of Ags1 by a visible switch in morphology. Thus, monitoring the depletion of Ags1 in the chemotype I strain requires the use of a sentinel system in which depletion of a marker cotargeted by RNAi yields an observable phenotype. To accomplish this, we engineered strains in the chemotype II and chemotype I backgrounds that stably express the GFP gene from a constitutive promoter (OSU22 and OSU32, respectively) (Table 1). The fluorescence of these strains above that of the respective GFP-negative parents is clearly distinguishable, and the fluorescence can be quantified to assess the level of RNAi-based gene silencing (Fig. 4A and B).

FIG. 3. The AGS1 promoter is disrupted in chemotype I yeast. (A) Delineation of the upstream sequence necessary for full AGS1 promoter activity in chemotype II yeast. The scatter plot depicts the GFP fluorescence of individual Histoplasma transformants with transcriptional fusions of the GFP gene to regions upstream of the AGS1 coding sequence (CDS). The amount of sequence fused to the GFP gene is indicated (horizontal axis) as the beginning nucleotide of the region relative to the start codon of AGS1. Plasmids with transcriptional fusions were transformed into WU8 Histoplasma yeast (chemotype II). Horizontal bars represent the mean fluorescence (n = 18); asterisks represent significant differences (P < 0.05) by one-tailed t tests. (B) Schematic representation of the region upstream of the AGS1 CDS in chemotype II and chemotype I strains. Horizontal shaded bars show regions of >90% nucleotide identity between chemotypes, which is interrupted in chemotype I yeast by a 2.7-kb insertion at bp 1495 upstream of the AGS1 start codon. (C) Removal of the 2.7-kb insertion in the chemotype I AGS1 promoter restores normal levels of transcription. Chemotype II and chemotype I promoter-GFP gene fusions were introduced into chemotype I yeast (WU15), and fluorescence was quantified. Data points represent the fluorescence of individual transformants (n > 19). The repaired 1,896-bp chemotype I promoter (~1896 bp) was constructed by fusing homologous regions upstream and downstream of the insertion site by PCR. There was no significant difference in GFP expression between the 1,898-bp chemotype II and the repaired 1,896-bp base-pair chemotype I promoters as determined by one-tailed Student’s t test (ns; P < 0.05).
the chemotype I background, relative GFP fluorescence drops nearly to background levels (Fig. 4B). The 8-fold decrease in GFP fluorescence caused by the gfp:AGS1 RNAi plasmid signifies suppression of any Ags1 function in the chemotype I background even though no visible change in morphology can be detected in this strain (Fig. 4B). These results verify the successful creation of chemotype II and chemotype I strains in which Ags1 function is effectively silenced.

**Macrophage cytotoxicity of Histoplasma strains lacking Ags1 function.** To determine the contribution of AGS1 expression in chemotype II virulence, we examined the ability of Ags1-depleted yeast cells to replicate within macrophages. In addition to using the RNAi strains we generated, we also employed an insertional mutant that disrupts the AGS1 locus in the chemotype I background (43). These strains, representing two independent methods to block Ags1 function, were used to infect P388D1 macrophages. For chemotype II yeasts, loss of Ags1 function markedly reduces their ability to lyse the macrophages (34). In contrast, neither the depletion of AGS1 by RNAi nor that by genetic disruption in chemotype I yeasts impairs the ability of yeast to lyse macrophages (Fig. 5A). Examination of the intracellular replication rate of chemotype I yeasts shows that yeast cells replicate equivalently in macrophages regardless of whether AGS1 is intact or disrupted (Fig. 5B). For comparison, we also tested a chemotype I insertional mutant with a mutation in the known virulence gene CBP1 (43). As expected, this mutant has decreased cytotoxicity to macrophages (Fig. 5A) and replicates more slowly within phagocytes (Fig. 5B).

**In vivo virulence of strains lacking Ags1 function.** To determine the contribution of AGS1 expression to chemotype II and chemotype I yeast virulence in vivo, we measured the ability of AGS1-depleted and AGS1-disrupted strains to infect murine tissues. *Histoplasma* yeast cells were introduced into wild-type C57BL/6 mice intranasally, and the fungal burdens in lungs and spleens were determined at incremental time points following infection as an indicator of respiratory and disseminated disease, respectively. The Ags1+ chemotype II strain impairs and even decreases lung infection through day 9 postinfection. In the spleen, AGS1-disrupted strains to infect mice. With no depletion of AGS1 function (i.e., gfp-RNAi), chemotype I yeasts replicate within the lung, showing a 2-log increase in lung infection over the 9-day infection period and disseminate to the spleen by day 6 (Fig. 6A). Depletion of AGS1 by RNAi in the sentinel chemotype II strain impairs and even decreases lung infection through day 9 postinfection. In the spleen, AGS1-RNAi yeast cells were detected in only one of the three mice infected, indicating minimal extrapulmonary dissemination (Fig. 6A). These results show that, similar to the case for the previously described ags1Δ strain (34), the AGS1-RNAi in sentinel chemotype II yeast is less virulent in the murine model of infection. In the chemotype I background, the requirement for AGS1 was assessed using both the AGS1-depleted and AGS1-disrupted strains to infect mice. With no depletion of AGS1 function (i.e., gfp-RNAi), chemotype I yeasts replicate within the lung, showing a 2-log increase in lung infection over the 9-day infection, and disseminate to the spleen by day 6 (Fig. 6B). Depletion of Ags1 function by RNAi in chemotype I yeast results in near-identical infection kinetics in both the lung and spleen (Fig. 6B). Similarly, disruption of the AGS1 locus by T-DNA insertion does not compromise the virulence of chemotype I yeast, as the ags1 mutant yeasts infect both organs with comparable fungal burdens (Fig. 6C). In contrast, the chemotype I strain in which the CBP1 gene is disrupted shows impaired infection in the lung and barely detectable dissemination to the spleen (Fig. 6C). Altogether, these results verify
in vivo silencing that is preserved served decrease in the mean GFP fluorescence, resembling that observed in infected lungs, yeast cells from liquid culture (Fig. 7). For chemotype II yeasts, lungs closely matched the GFP fluorescence distribution of the GFP fluorescence of individual yeast cells from infected chemotype II and chemotype I RNAi lines, quantification of infection the mice, and the lungs were harvested after 24 h. For all 6A), 100-fold more yeast from this RNAi line were used to indicate RNAi-based suppression of Ags1 function. Lungs from mice infected for 7 to 8 days were homogenized and the sentinel GFP fluorescence of individual yeast cells to infect the lungs closely matched the GFP fluorescence distribution of yeast cells from liquid culture (Fig. 7). For chemotype II and chemotype I RNAi lines, quantification of the GFP fluorescence of individual yeast cells from infected lungs closely matched the GFP fluorescence distribution of yeast cells from liquid culture (Fig. 7). For chemotype II yeasts in infected lungs, gfp:AGS1-RNAi yeast decreases over time (Fig. 6A), 100-fold more yeast from this RNAi line were used to infect the mice, and the lungs were harvested after 24 h. For all chemotype II and chemotype I RNAi lines, quantification of the GFP fluorescence of individual yeast cells and the yeasts labeled with Uvitex to provide identification of yeast cells independently of their GFP fluorescence. Since lung colonization by gfp:AGS1-RNAi yeast decreases over time (Fig. 6A), 100-fold more yeast from this RNAi line were used to infect the lungs, and the lungs were harvested after 24 h. For all yeast cells transition to stationary-phase growth, β-glucan exposure decreases considerably, with very few stationary-phase yeast cells binding to Dectin-1 expressing 3T3 cells (Fig. 8C). These results indicate that chemotype I yeasts, despite the lack of any α-(1,3)-glucan mask, are able to prevent Dectin-1 recognition, and thus this α-(1,3)-glucan-negative chemotype I yeast cells are unable to avoid Dectin-1-mediated detection. To determine β-(1,3)-glucan exposure, binding of yeast cells to Dectin-1-expressing 3T3 fibroblasts was assayed. Chemotype II AGS1 (+) yeasts grown to late log/early stationary phase do not bind Dectin-1, as a consequence of β-glucan concealment by α-(1,3)-glucan, whereas the ags1Δ strain readily binds to Dectin-1 (33). A more thorough examination of binding at different phases of growth shows that the presence of α-(1,3)-glucan in chemotype II blocks the interaction between β-(1,3)-glucans and Dectin-1, irrespective of the yeast growth phase; AGS1 (+) yeasts do not bind to 3T3 cells expressing Dectin-1 (Fig. 8A) whereas ags1Δ yeasts consistently bind during all phases of growth (Fig. 8B). Interestingly, Dectin-1 recognition of chemotype I yeast cells is dependent upon the growth phase of the yeast (Fig. 8C). Chemotype I yeast in exponential growth binds to Dectin-1, indicating some exposure of β-glucans, although not to levels observed with the α-(1,3)-glucan-lacking chemotype II ags1Δ yeast. As yeast cells transition to stationary-phase growth, β-glucan exposure decreases considerably, with very few stationary-phase yeast cells binding to Dectin-1 expressing 3T3 cells (Fig. 8C). These results indicate that chemotype I yeasts, despite the lack of any α-(1,3)-glucan mask, are able to prevent Dectin-1 recognition, and this α-(1,3)-glucan exposure minimization mechanism is dependent on the growth phase of the yeast cells.

**DISCUSSION**

To prevent infection by fungal invaders, phagocytes recognize and respond to β-glucans, which are one of the main components of the fungal cell wall. Chemotype II Histoplasma capsulatum yeasts utilize α-(1,3)-glucan to block β-(1,3)-glucan from detection by the phagocyte pathogen recognition receptor (PRR) Dectin-1. In the absence of α-(1,3)-glucan, the host
controls chemotype II infection in the lung and limits fungal dissemination (33, 34). In contrast, chemotype I Histoplasma isolates lack the α-(1,3)-glucan masking of α-(1,3)-glucan polysaccharide, at least during in vitro growth. Despite this, chemotype I Histoplasma yeasts remain fully virulent in vivo.

To define the basis for the phenotypic differences between chemotype II and chemotype I strains, we examined the gene products identified to date that are necessary for α-(1,3)-glucan synthesis. We show that lack of α-(1,3)-glucan production in chemotype I correlates with significantly decreased transcription of AGS1, which is necessary for α-(1,3)-glucan production (29, 34). For the AGS1 gene, reduced transcription in chemotype I results from a disruption of the AGS1 promoter by a segment of repetitive DNA that is common to the chemotype I genome. This large insertion contains sequence remnants of mobile DNA (e.g., partial retrotransposon regions and shorter direct and inverted repeat sequences). These retroelement sequences are not similar to the crypton elements described for many pathogenic fungi (15). As the interruption of the AGS1 promoter is not found in the sequenced genomes of

FIG. 6. Blocking Ags1 function of chemotype I yeast does not impair lung infection or extrapulmonary dissemination in mice. The fungal burdens in murine lungs (left panels) and spleens (right panels) were quantified at incremental time points following respiratory infection with chemotype II (A) or chemotype I (B and C) yeast cells. (A) Infection of mice with chemotype II RNAi sentinel strains. Wild-type mice were intranasally infected with AGS1 (+) yeast (gfp-RNAi; OSU67) or AGS1-depleted yeast (gfp:AGS1-RNAi; OSU68), and the fungal burden in murine organs was determined by enumeration of CFU. (B) Infection of mice with chemotype I RNAi sentinel strains. Wild-type mice were intranasally infected with AGS1 (+) yeast (gfp-RNAi; OSU34) or AGS1-depleted yeast (gfp:AGS1-RNAi; OSU35), and the fungal burden in murine organs was determined by enumeration of CFU. (C) Infection of mice with chemotype I insertional mutants. Wild-type mice were intranasally infected with AGS1 (+) yeast (OSU25), yeast lacking Ags1 function (ags1:T-DNA; OSU27), or yeast lacking the Cbp1 virulence factor (cbp1:T-DNA; OSU26), and the fungal burden in murine organs was determined by enumeration of CFU. Data points represent the CFU counts per organ for each mouse (n = 3) at each time point postinfection (x axis). Mice were infected with approximately 5 × 10⁴ yeast cells, and the actual inoculum dose was determined by CFU plating and is shown in the lung graphs at day 0.
the other Histoplasma phylogenetic groups and its removal restores full promoter activity, we suspect that this mutational event arose by retrotransposon insertion in the chemotype I lineage sometime during the divergence of the two North American Histoplasma phylogenetic groups (between 3.2 and 13 million years ago [24]). Nearly 30% of the NAm 2 (chemotype I) genome is characterized as repetitive DNA, which is largely interspersed between uninterrupted genes that are highly similar and syntenic between the chemotype I and II strains. Thus, additional promoters in NAm 2 strains may have altered transcriptional activity due to insertion of repetitive sequences and thereby may contribute to phenotypic differences between Histoplasma groups.

Although the disrupted AGS1 promoter impairs production of α-(1,3)-glucan by chemotype I strains in vitro, it remained possible that expression of AGS1 is enhanced in vivo and that α-(1,3)-glucan production is necessary for the chemotype I yeast pathogenesis as it is for chemotype II. To definitively address this question, we prevented AGS1 expression, and thus α-(1,3)-glucan production, by genetic means in chemotype I Histoplasma yeast. We utilized RNAi to deplete Ags1 from yeast and employed an RNAi sentinel to ascertain RNAi-based knockdown of the AGS1 gene product both in vitro and in vivo. The GFP sentinel RNAi system was used to assess target gene silencing during lung infection by chemotype II (A) and chemotype I (B) Histoplasma RNAi strains. The GFP fluorescence of individual yeast cells was determined by microscopy of aliquots from homogenized lung homogenates (“in vivo”) or of yeast collected from liquid culture (“in vitro”). Data points represent the mean GFP fluorescence of individual yeast cells (n > 50 for each strain), and horizontal bars indicate the mean population fluorescence. Yeast cells were identified independently of their GFP fluorescence by labeling lung homogenates and liquid culture samples with Uvitex.

The GFP gene sentinel system employed is ideally suited for these experiments, as (i) it provides an easily observable indicator of depletion of the cotargeted transcript and (ii) the silencing of GFP fluorescence can be used for in vivo studies, as the GFP gene is not essential for growth or pathogenesis of Histoplasma yeast. The efficacy of gfp:AGS1-RNAi in blocking α-(1,3)-glucan synthesis in chemotype I yeast by multiple means has no detrimental effect on four different measures of yeast virulence: (i) lysis of cultured macrophages, (ii) replication in cultured macrophages, (iii) infection of lungs after intranasal instillation, and (iv) growth in liquid culture. The GFP sentinel RNAi system is ideally suited for these experiments, as (i) it provides an easily observable indicator of depletion of the cotargeted transcript and (ii) the silencing of GFP fluorescence can be used for in vivo studies, as the GFP gene is not essential for growth or pathogenesis of Histoplasma yeast.
tion of the murine respiratory tract, and (iv) extrapulmonary dissemination. Thus, chemotype I yeast virulence does not require α-(1,3)-glucan production, and the yeast remain as virulent, if not more so, than chemotype II yeast in murine models of histoplasmosis (Fig. 6) (30).

These results stand in contrast to the essential role of α-(1,3)-glucan in chemotype II Histoplasma yeast pathogenesis (34) as well as the suspected requirement for α-(1,3)-glucan by other dimorphic fungal pathogens (17, 37). For α-(1,3)-glucan-positive, chemotype II Histoplasma, the α-linked polysaccharide prevents detection of yeast by host Dectin-1 (33), which would normally occur during interaction of Histoplasma yeast with macrophages. The lack of α-(1,3)-glucan in the chemotype I strain suggests that these yeasts cannot shield cell wall β-glucans from Dectin-1. However, our results show that β-glucans on chemotype I yeast are not completely exposed. During exponential growth of chemotype I yeast in vitro, some interaction of yeasts and Dectin-1 occurred. However, stationary-phase chemotype I yeasts were largely undetected.

The growth phase dependence on β-glucan exposure is not characteristic of α-(1,3)-glucan-positive and -negative chemotype II yeast, suggesting that chemotype I yeasts utilize a unique mechanism for blocking β-glucan detection. In what growth phase Histoplasma exists during infection remains unknown, but studies of the intracellular pathogen Mycobacterium tuberculosis have shown that Mycobacterium virulence in vitro requires expression of stationary-phase factors (5, 12). We hypothesize that, in vitro, Histoplasma yeasts in exponential-phase-like growth have some exposure of β-glucans, possibly at bud sites that are more abundant during rapid growth in vitro. In support of this, the bud scars of Candida albicans and the swelling of conidia of Aspergillus fumigatus are primary sites of β-glucan exposure that allow for recognition by host macrophages (11, 13, 38). However, in vivo, and when in vitro chemotype I yeast cultures reach stationary phase, an alternative β-glucan-masking mechanism circumvents the need for α-(1,3)-glucan in chemotype I Histoplasma yeast. The nature of this mechanism could include production of a different masking component that physically blocks detection of yeast β-glucans, modification of cell wall β-glucans that makes them unrecognizable to Dectin-1, or production of a factor that obscures the β-glucan binding site on Dectin-1. Experiments to uncover molecular clues to this mechanism in chemotype I yeast are under way.

The results of this study highlight an important difference in the pathogenic mechanisms employed by separate phylogenetic lineages of Histoplasma capsulatum. Chemotype I Histoplasma yeast strains have overcome reliance upon cell wall α-(1,3)-glucan for virulence and do so in a manner that mirrors chemotype II avoidance of Dectin-1 detection. This may have arisen as a consequence of losing sufficient α-(1,3)-glucan synthase activity that resulted from disruption of the AGSI promoter. Alternatively, the evolution of a non-Ags1-dependent β-glucan-masking mechanism may have permitted retroelement insertion in the AGSI promoter, and this may be a snapshot in the evolution of chemotype I yeast. While chemotype I and chemotype II Histoplasma strains share a pathogenic lifestyle (i.e., infection and replication within mammalian phagocytes), they are now sufficiently diverged that they employ different means to accomplish this. Consistent with this is the fact that representatives of the Histoplasma phylogenetic groups also differ in the extracellular molecules produced (18). These phenotypic differences, especially as they pertain to virulence mechanisms, might argue that the various Histoplasma groups should be recognized as separate species, as was established for Coccidiodium immitis and Coccidiodium posadasii (10) and for Cryptococcus neoformans and Cryptococcus gattii (2, 27). Regardless, caution is recommended when extrapolating experimental results across different strains of Histoplasma, as the mechanisms of pathogenesis seem more dissimilar than alike.

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