

Eng1 and Exg8 Are the Major β -Glucanases Secreted by the Fungal Pathogen *Histoplasma capsulatum**[§]

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Fungal cell walls contain β -glucan polysaccharides that stimulate immune responses when recognized by host immune cells. The fungal pathogen *Histoplasma capsulatum* minimizes detection of β -glucan by host cells through at least two mechanisms: concealment of β -glucans beneath α -glucans and enzymatic removal of any exposed β -glucan polysaccharides by the secreted glucanase Eng1. *Histoplasma* yeasts also secrete the putative glucanase Exg8, which may serve a similar role as Eng1 in removing exposed β -glucans from the yeast cell surface. Here, we characterize the enzymatic specificity of the Eng1 and Exg8 proteins and show that Exg8 is an exo- β 1,3-glucanase and Eng1 is an endo- β 1,3-glucanase. Together, Eng1 and Exg8 account for nearly all of the total secreted glucanase activity of *Histoplasma* yeasts. Both Eng1 and Exg8 proteins are secreted through a conventional secretion signal and are modified post-translationally by O-linked glycosylation. Both glucanases have near maximal activity at temperature and pH conditions experienced during infection of host cells, supporting roles in *Histoplasma* pathogenesis. Exg8 has a higher specific activity than Eng1 for β 1,3-glucans; yet despite this, Exg8 does not reduce detection of yeasts by the host β -glucan receptor Dectin-1. Exg8 is largely dispensable for virulence *in vivo*, in contrast to Eng1. These results show that *Histoplasma* yeasts secrete two β 1,3-glucanases and that Eng1 endoglucanase activity is the predominant factor responsible for removal of exposed cell wall β -glucans to minimize host detection of *Histoplasma* yeasts.

The dimorphic fungal pathogen *Histoplasma capsulatum* causes respiratory and systemic disease in mammals. Central to the pathobiology of *Histoplasma* is the ability of pathogenic yeasts to infect and parasitize host phagocytes (1). The cell wall is the first fungal structure encountered by host phagocytes,

and thus features of the cell wall contribute to the outcome of the interaction between pathogen and host. *Histoplasma*'s cell wall-localized Hsp60 protein drives internalization of the yeasts via CD11 family complement receptors (e.g. CR3 (2–4)) while limiting the host response (5–7). Even though this interaction is beneficial to yeasts in facilitating host cell infection, the close association of yeasts with the phagocyte plasma membrane can also activate host receptors that trigger immune responses. Given the polysaccharide nature of the fungal cell wall, many of these pathogen pattern-recognition receptors act as lectins. For example, Dectin-1 is the primary receptor for fungal β -glucans, and Dectin-1 recognition of this polysaccharide stimulates pro-inflammatory responses (8–11) leading to control and elimination of fungal cells. To reduce its detection by Dectin-1, *Histoplasma* yeasts produce an α -linked glucan polysaccharide that overlays the β -glucans, effectively masking the β -glucan from Dectin-1 (12–14). In addition, *Histoplasma* yeasts secrete a number of glycanases that can potentially remove exposed immunostimulatory polysaccharides from the yeast cell surface (15, 16).

Two of the glycanases abundantly secreted by *Histoplasma* are Eng1 and Exg8 (16). Both proteins are highly secreted by yeast cells, but not by avirulent mycelial phase cells, suggesting functional roles in pathogenesis. *Histoplasma* Eng1 belongs to the glycosyl hydrolase family 81 (GH81), which includes the Eng1 homologs of *Candida albicans* and *Saccharomyces cerevisiae*. In *C. albicans* and *S. cerevisiae*, Eng1 enzymes participate in cell separation during budding (17, 18). However, in *Histoplasma*, Eng1 does not contribute to cell separation and instead removes surface-exposed β -glucan molecules to reduce Dectin-1 detection of yeast cells (15). *Histoplasma* Exg8 is member of the GH55 family. The *S. cerevisiae* and *C. albicans* genomes do not contain any genes encoding GH55 proteins, but *Aspergillus fumigatus* encodes six GH55 proteins (19), including an Exg8 homolog. However, the cellular role for *A. fumigatus* Exg8 has not been defined. In this study, we provide biochemical characterization of the *Histoplasma* Exg8 and Eng1 proteins, defining their glucan substrate specificity and their endo- or exo-directed hydrolytic activity, and we investigate the role for Exg8 in Dectin-1 recognition and *Histoplasma* virulence.

Results

Eng1 and Exg8 Protein Features—Eng1 and Exg8 were identified initially as members of the secreted proteome of *Histoplasma* yeasts, the genes of which were transcriptionally up-

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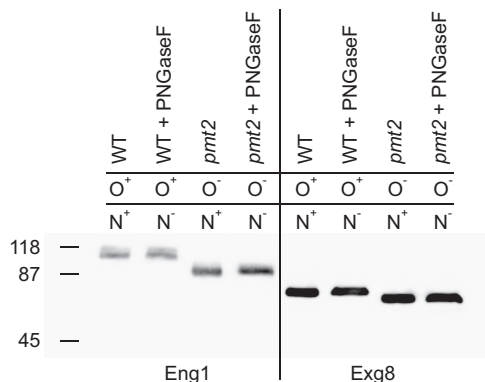


FIGURE 1. Eng1 and Exg8 have O-linked but not N-linked glycans. Glycan attachment to Eng1 and Exg8 proteins was determined by increased electrophoretic mobility of proteins when O-linked or N-linked glycans were absent. Culture filtrates from *Histoplasma* expressing Eng1-FLAG- or Exg8-FLAG-tagged proteins were prepared, and the Eng1 and Exg8 proteins were detected by immunoblotting for the FLAG epitope following electrophoretic separation. To prevent O-linked glycan attachment, fusion proteins were expressed in a mutant lacking O-linked mannosylation (*pmt2*) instead of wild type (WT). N-Linked glycans were removed by treatment of culture filtrate proteins with PNGaseF. The prevention of O-linked glycan attachment or removal of N-linked glycans are indicated by “–” in contrast to normal presence of the glycans (“+”). The molecular mass (kDa) of protein standards is indicated on the left of the immunoblot.

regulated in pathogenic phase yeasts compared with avirulent mycelia (16). The *ENG1* gene encodes a GH81 family protein with β -1,3-glucanase activity (supplemental Fig. 1A) (15). The *Histoplasma* Eng1 protein has high amino acid similarity with other fungal endoglucanases (60% similar to *A. fumigatus* Eng1), with a series of conserved aspartic acid and glutamic acid residues known to be important for activity (supplemental Fig. 1A) (20). The *EXG8* gene encodes a protein that is most similar to the *A. fumigatus* Exg8 protein (69% amino acid similarity) and is predicted to be in the GH55 family. Similar to other proteins in this family, the *Histoplasma* Exg8 amino acid sequence contains two pectate lyase domains (supplemental Fig. 1B), as well as a conserved aspartic acid and two conserved glutamic acid residues important for glucanase activity in this family (supplemental Fig. 1B) (21, 22). Both Eng1 and Exg8 have predicted N-terminal secretion signals and N-linked and O-linked mucin-like glycan attachment sites (supplemental Fig. 1, A and B) consistent with extracellular localization.

To determine whether these two proteins are glycosylated, culture filtrates from *Histoplasma* expressing Eng1-FLAG and Exg8-FLAG fusion proteins were treated with PNGaseF (for removal of N-linked glycans) and analyzed by anti-FLAG immunoblot for a reduction in molecular weight due to glycan removal (Fig. 1). The electrophoretic mobility of Eng1 and Exg8 did not change after PNGaseF treatment, suggesting the Eng1 and Exg8 proteins lack N-linked glycosylation. To test for O-linked glycosylation, Eng1 and Exg8 were expressed in a *Histoplasma* strain lacking an O-linked protein mannosyltransferase (*Pmt2*), which is required for the O-linked addition of the initial mannose onto proteins.⁴ When expressed in this strain, both Eng1 and Exg8 have reduced electrophoretic mobility consistent with a reduction in molecular weight due to loss of O-linked glycans. The 99-kDa Eng1 protein migration is

⁴ A. L. Garfoot, unpublished data.

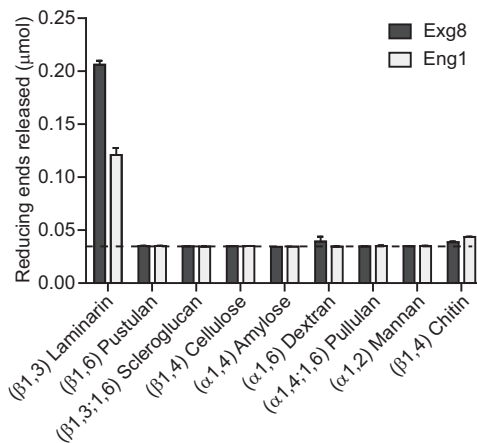


FIGURE 2. Eng1 and Exg8 are glucanases specific for β 1,3-glucosidic linkages. Glucan hydrolysis activity of purified Exg8 (dark bars) and Eng1 (light bars) is shown. Data represent the glucose or N-acetylglucosamine (for chitin substrate) equivalents of reducing sugars released after incubation of Eng1 or Exg8 with glycan substrates. Glycosidic linkages of the individual substrates are indicated in parentheses. Dashed line represents the limit of detection, and error bars represent the standard deviation among replicate reactions ($n = 3$).

reduced to 89 kDa when lacking O-glycans. Similarly, the apparent molecular mass of Exg8 is reduced from 77 to 73 kDa when *Pmt2*-dependent addition of O-glycans is lost. There was no further reduction in molecular weight when these O-linked glycan-deficient proteins were treated with PNGaseF. Together, these data indicate both Eng1 and Exg8 are O-glycosylated but not N-glycosylated (Fig. 1).

Eng1 and Exg8 are β 1,3-Glucanases—We previously showed *Histoplasma* Eng1 can hydrolyze β 1,3-glucan (15). To determine the specificity of Eng1 for β 1,3-linked glucan and to determine the activity of Exg8, we quantified the hydrolytic activities of Eng1 and Exg8 on polysaccharides with different glycosidic linkages (Fig. 2). The polysaccharides tested include β 1,3-glucan (laminarin), β 1,6-glucan (pustulan), β 1,3;1,6-glucan (scleroglucan), β 1,4-glucose (cellulose), α 1,4-glucan (amylose), α 1,6-glucan (dextran), α 1,4;1,6-glucan (pullulan), α 1,2-mannose (mannan), and β 1,4-N-acetylglucosamine (chitin). Substrates were incubated with purified *Histoplasma* Eng1 and Exg8 proteins, and the released reducing sugars were measured by reaction with dinitrosalicylic acid (23). Glycanase activity was below the limit of detection after 120 min of incubation for all substrates except laminarin (Fig. 2). Extending the reaction time to 24 h still did not reveal any significant glycanase activity for all substrates except laminarin (data not shown), indicating Eng1 and Exg8 enzymatic activities are specific to β 1,3-linked glucans. The specific activity of Exg8 was almost 2-fold higher than Eng1 (Table 1). Exg8 was also more efficient than Eng1, as the K_{cat} for Exg8 was 2.7-fold higher than Eng1, and the K_{cat}/K_m was 4.4-fold higher because of the greater substrate affinity of Exg8 for β 1,3-glucan.

Of the characterized GH81 family proteins, all are endo-acting glucanases; however, enzymes in the GH55 family can have either endo- or exoglucanase activity (24). To validate Eng1 as an endoglucanase and to determine the action of *Histoplasma* Exg8, laminarin was digested for 30 min with purified enzymes, and the mono- and oligosaccharide products were analyzed by

TABLE 1

Enzyme kinetics for *Histoplasma* Eng1 and Exg8

Steady-state kinetic parameters were determined for purified Eng1 and Exg8 hydrolyses of laminarin substrate. For each, two independent protein purifications were assayed by incubation with laminarin substrate and quantification of the reducing sugars released. V_{max} , K_m , and K_{cat} values were determined by curve fitting of the data obtained from two different enzyme concentrations while varying the laminarin substrate concentration.

	V_{max}	K_m	K_{cat}	K_{cat}/K_m
	units/mg	mg/ml	1/s	ml/(mg s)
Eng1	17.9 \pm 1.1	1.62 \pm 0.38	28.2 \pm 1.7	17.3 \pm 4.4
Exg8	48.1 \pm 2.5	1.00 \pm 0.24	75.8 \pm 3.9	75.8 \pm 16.4

mass spectrometry (Fig. 3, A and B). The major products identified were monosaccharides (203 m/z), disaccharides (365 m/z), trisaccharides (527 m/z), tetrasaccharides (689 m/z), and pentasaccharides (851 m/z). Eng1 produced predominantly disaccharides, as well as some tri-, tetra-, and pentasaccharides (Fig. 3A). Very little monosaccharide was produced, consistent with the enzymatic activity acting on internal glycosidic linkages. Extending the digestion time overnight reduced the tetrasaccharide product, leaving predominantly di- and trisaccharides with a very minor amount of mono-, tetra-, and pentasaccharide products (data not shown). This pattern was quite similar to digestion of laminarin with the known endoglucanase zymolyase (Fig. 3C). Conversely, the predominant product of Exg8 hydrolysis of laminarin was monosaccharides, with minor portions of di- and trisaccharide (Fig. 3B). This pattern is consistent with Exg8 being able to hydrolyze terminal glucose from a glucan chain, indicating exoglucanase activity. Extending digestion of Exg8 overnight results in a similar profile of predominantly monosaccharides with di- and trisaccharides still present, suggesting inefficient hydrolysis of glucose oligomers smaller than 4 units in length (data not shown). To quantify the Eng1 and Exg8 digestion products, the ion intensity of the $[M + Na]^+$ ion for each saccharide length was calculated relative to the sum of all $[M + Na]^+$ ion intensities (monosaccharide to octosaccharide; Fig. 3D). For Exg8, 49% of the saccharide ions were monosaccharides, compared with only 4% for Eng1 and zymolyase. No quantifiable saccharides longer than 5 units were detected for any enzyme digest after 30 min, except for zymolyase in which hexasaccharides were calculated at less than 1% of the total saccharide content. These data establish that Eng1 is an endoglucanase and Exg8 is an exoglucanase acting on β 1,3-glucan.

Eng1 and Exg8 Are the Major Secreted Histoplasma Glucanases—Examination of the *Histoplasma* genome identified additional glycosyl hydrolase family proteins in addition to Eng1 and Exg8 (data not shown) raising the possibility of additional secreted glucanases. To determine the relative contribution of Eng1 and Exg8 to the total extracellular glucanase activity of *Histoplasma* yeasts, we depleted Eng1 and Exg8 activity from *Histoplasma* yeasts either singly or combined using a *gfp* sentinel RNAi system (25); Eng1 and/or Exg8 knockdown was monitored by simultaneous knockdown of GFP fluorescence. Transformed yeasts showed at least 85% reduction in GFP fluorescence indicating efficient depletion of the co-targeted *ENG1* and/or *EXG8* transcripts (data not shown). With depletion lines established, the contribution of each enzyme to the extracellular glucanase activity on β 1,3-glucan was determined. Loss of

Eng1 function yielded culture filtrates that had 30% less β -glucanase activity compared with wild-type culture filtrates (derived from *gfp*-RNAi) (Fig. 4A), consistent with previous results (15). Loss of Exg8 function caused a greater reduction in extracellular glucanase activity (66% reduction; Fig. 4A). Simultaneous depletion of both Eng1 and Exg8 depletes 93% of extracellular β -glucanase activity, showing Eng1 and Exg8 constitute the major extracellular β -glucanases. Examination of the β -glucanase activity associated with the surface of *Histoplasma* yeasts showed slight activity above the limit of detection with wild-type yeasts, and this small amount of activity decreased a statistically significant amount when Exg8 but not Eng1 was depleted from *Histoplasma* (Fig. 4A). Thus, the major extracellular β -glucanase activity is in the soluble fraction with little associated with the surface of yeast cells. Consistent with this conclusion, abundant Exg8 protein was detected in the culture filtrate; however, only a minute amount of Exg8 protein was in the cell wall fraction (Fig. 4B). Together with the previous demonstration that Eng1 is not associated with the surface of yeast cells (15), these data indicate that Eng1 and Exg8 are responsible for the vast majority of extracellular β -glucanase activity and that this activity is secreted but not associated with the yeast cells themselves.

Despite the reduction in secreted glucanase activity, depletion of Exg8 or depletion of Exg8 and Eng1 together does not increase sensitivity to cell wall-disrupting agents (Table 2); *EXG8*-RNAi and *ENG1:EXG8*-RNAi *Histoplasma* yeasts had no significant change in the 50% inhibitory concentrations (IC_{50}) for cell wall-binding dyes (Congo Red, Uvitex, and Calcofluor White), detergent (SDS), and cell wall glycan-targeting antifungal drugs (Nikkomycin Z and Caspofungin, which impair chitin and β -glucan synthesis, respectively). These results suggest Exg8, like Eng1 (15), is not a glucanase required for major structural modifications or cell intrinsic function of the cell wall.

Eng1 and Exg8 Are Functional in Temperature and pH Conditions of the Phagosome—The pathogenic phase-specific expression of Eng1 and Exg8 suggests these glycans have a function related to pathogenesis. *Histoplasma* yeasts normally reside within the phagosomal compartment of host macrophages at mammalian temperatures. While in this compartment, the *Histoplasma* yeast prevents complete acidification and maintains a luminal pH around 5.5 to 6.0 (26–28). To determine whether Eng1 and Exg8 are enzymatically active under conditions that *Histoplasma* yeasts experience during infection of macrophages, we quantified the hydrolysis of laminarin at various pH and temperatures (Fig. 5, A and B). With respect to pH, Eng1 had maximal activity from pH 5 to pH 8, decreasing only at high alkalinity (pH > 10) and acidity (pH < 3) (Fig. 5A). Exg8 is active from pH 4 to pH 8 with optimal activity at pH 4 to pH 7 (Fig. 5A). Increasing temperature continues to increase activity up to 45 °C for both enzymes (Fig. 5B). At temperatures above 45 °C, Exg8 activity decreases significantly; however, Eng1 activity remains high up to 70 °C. The high glucanase activity at 37 °C and under moderately acidic conditions (pH 5 to 7) indicates Eng1 and Exg8 would be active in the environment within the phagosome.

Histoplasma Endo- and Exo- β -glucanases

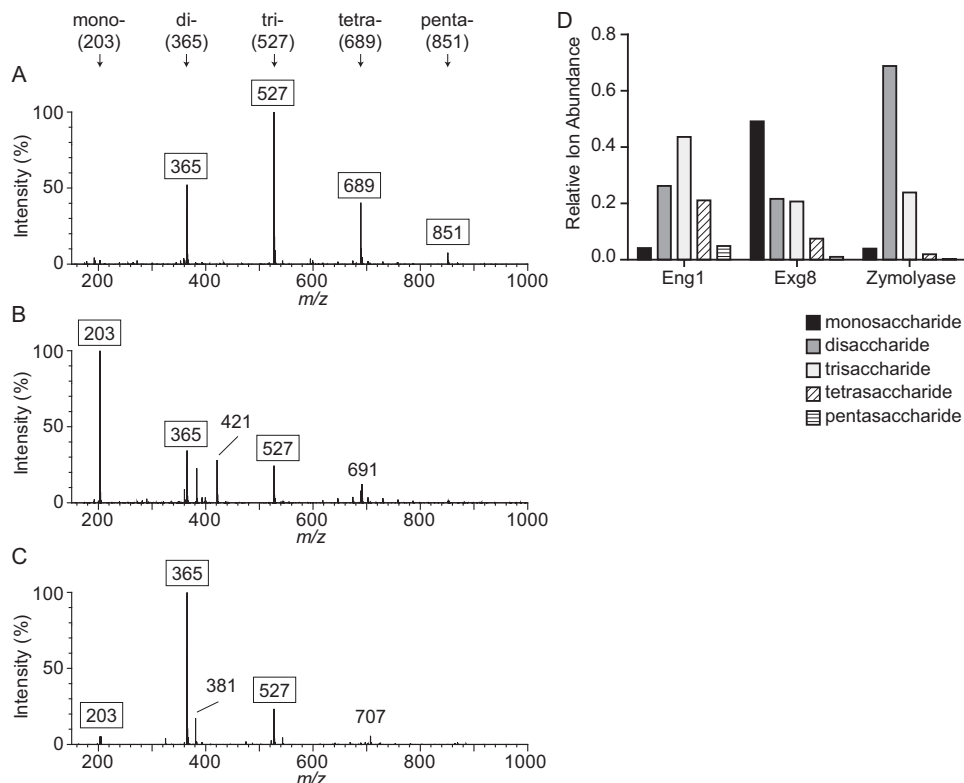


FIGURE 3. Eng1 is an endoglucanase and Exg8 is an exoglucanase. Hydrolysis products of Eng1 and Exg8 activity on laminarin (β 1,3-glucan) are shown. Laminarin was digested for 30 min with 30 ng of purified Eng1 (A), Exg8 (B), or the endo- β -glucanase zymolyase (C), and the products were detected by mass spectrometry. The relative ion intensities for ions between 150 and 1000 m/z are shown with peaks for monosaccharides (*mono*-, 203 m/z), disaccharides (*di*-, 365 m/z), trisaccharides (*tri*-, 527 m/z), tetrasaccharides (*tetra*-, 689 m/z), and pentasaccharides (*penta*-, 851 m/z) indicated with *arrows*. The m/z values for each saccharide peak represent sodiated glucose minus one water per each additional glucose unit. D, relative ion intensities for singly charged monosaccharide through pentasaccharide peaks. Data shown are representative of three independent experiments.

Exg8 Does Not Reduce Detection of Histoplasma Yeasts—To determine the functional and possible additive roles of Eng1 and Exg1 in virulence, we tested yeasts depleted of Eng1 and/or Exg1 in aspects of *Histoplasma* pathogenesis. Overall association of yeasts with primary macrophages was unchanged in the absence of either glucanase (Fig. 6A). The response of macrophages to yeasts, as measured by production of the pro-inflammatory cytokines TNF α and IL-6, was unaffected by loss of Exg8; however, loss of Eng1 singly or in combination with Exg8 significantly increased both TNF α and IL-6 (Fig. 7, A and B). This is consistent with Eng1 but not Exg8 affecting recognition of yeasts by signaling receptors but not binding of yeasts to phagocytic receptors on macrophages (*i.e.* CR3 (2–4)). We previously showed that the Eng1 glucanase activity decreases surface β 1,3-glucan exposure on yeasts, thereby enabling yeasts to avoid Dectin-1-dependent recognition by macrophages (15). As both Eng1 and Exg8 are active on β 1,3-glucan, we measured the Dectin-1 recognition of yeasts lacking single glucanases or both. To avoid the contribution of other macrophage receptors, yeasts were added to fibroblasts in which only Dectin-1 was expressed. Despite Exg8 having more activity than Eng1, loss of Exg8 had no significant effect on β 1,3-glucan recognition, whereas the loss of Eng1 resulted in a nearly 6-fold increase (Fig. 6B). No additional change was seen when both Eng1 and Exg8 were depleted. In the G186A background, in which α -glucan polysaccharides largely mask β -glucans (12, 14), loss of Exg8 similarly had negligible effects on Dectin-1 recognition of

yeasts (supplemental Fig. 2). In this same background, depletion of Eng1 results in a 4-fold increase. Again, no additional β 1,3-glucans are recognized when both Eng1 and Exg8 are depleted. Pre-incubation of Dectin-1-expressing fibroblasts with laminarin reduced the recognition of yeasts at least 10-fold, confirming the specificity as recognition of yeast β -glucan (Fig. 6B). Together, these data show that Eng1 but not Exg8 reduces β -glucan exposure on yeasts, which minimizes their detection by Dectin-1.

Eng1 Is the Primary Secreted Glucanase That Enhances Histoplasma Pathogenesis—Yeast survival against macrophages in culture was not affected by loss of either glucanase (Fig. 8A). In a murine model of respiratory histoplasmosis, loss of Exg8 function showed only a slight decrease (2-fold) in lung infection (Fig. 8B). Removal of Eng1, however, resulted in an 8-fold reduction compared with wild type (Fig. 8B). Similar to the *in vitro* effects on Dectin-1 binding, depletion of Exg8 did not further decrease the virulence of yeasts from that caused by single loss of Eng1 function. Thus, the Eng1 endoglucanase functionally contributes more than Exg8 to reducing the β 1,3-glucan exposure of yeasts and facilitating infection.

Discussion

The fungal cell wall features centrally in the pathogenesis of *H. capsulatum*. Unlike many of the opportunistic fungal pathogens, *Histoplasma* yeast cells have reduced detection of cell wall pathogen-associated molecular patterns (14, 15). Although the

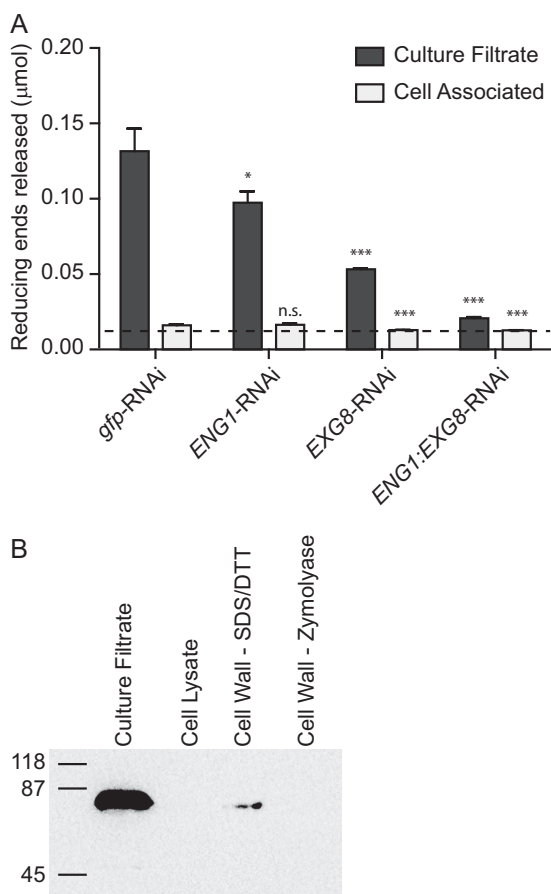


FIGURE 4. Eng1 and Exg8 are responsible for the majority of extracellular glucanase activity produced by yeasts. *A*, extracellular glucanase activity in the soluble fraction or associated with yeast cells. Culture supernatants were separated from yeast cells, and both fractions were tested for hydrolysis of laminarin to determine the activity in culture filtrates (dark bars) or activity associated with the yeast cells (light bars) of wild-type (*gfp-RNAi*) or glucanase-deficient strains (*ENG1-RNAi*, *EXG8-RNAi*, and *ENG1/EXG8-RNAi* strains). Data represent the glucose equivalents (micromoles) of reducing sugars released after 90 min of incubation with laminarin. Dashed line represents the limit of detection of the assay, and bars represent the mean reducing sugars released \pm S.D. among replicate assays ($n = 3$). *B*, localization of Exg8 in extracellular fractions. Soluble and cell-associated fractions were tested for Exg8-FLAG protein by immunoblot of the FLAG epitope. Proteins in the culture filtrate, the soluble yeast cell lysate, and proteins extracted by SDS + DTT or by zymolyase + chitinase digestion of the insoluble cell wall fraction were separated by electrophoresis and probed for the FLAG epitope. Protein samples were derived from equivalent numbers of yeast cells. Numbers represent the molecular mass (kDa) of protein standards. *n.s.*, not significant. *, $p < 0.05$; ***, $p < 0.001$.

cell wall is essential for fungal viability, *Histoplasma* yeasts effectively minimize cell wall β -glucans that are exposed to host phagocytes, either through masking or by removal from the exposed surface. This study characterizes two secreted glucanases (Eng1 and Exg8) important for this latter mechanism. Eng1 is important for pruning exposed β -glucans (15), and consistent with this role, we demonstrate that Eng1 is an endo-acting glucanase specific for β 1,3-glucans (EC 3.2.1.39). Exg8 is an exo-acting glucanase, also specific for β 1,3-glucans (EC 3.2.1.58). Together, the action of these enzymes can effectively hydrolyze β 1,3-glucans into monomeric glucose, and removal of both enzymes from *Histoplasma* effectively eliminates extracellular glucanase activity. The enzymes are highly active in conditions that *Histoplasma* yeasts experience during infec-

tion, 37 °C and pH between 5 and 6, which characterizes the lumen of the host cell phagosome. Thus, *Histoplasma* Eng1 and Exg8 are factors produced during infection, with the ability to alter the surface of the yeast cell, specifically in reducing immunostimulatory β -glucans of the cell wall.

Although both Eng1 and Exg8 are produced by pathogenic yeasts, Eng1 is the major β -glucanase that removes exposed β -glucans from the yeast cell wall. Functional tests show that loss of Eng1 activity significantly increases Dectin-1 recognition of yeast cell β -glucans, and this translates into reduced virulence in a murine model of respiratory histoplasmosis (15). By comparison, loss of Exg8 activity has only minor effects on Dectin-1 recognition of the yeast cells (Fig. 6*B*) and minimal impact on *Histoplasma* virulence (Fig. 8*B*). Although Eng1 reduces recognition of yeasts by Dectin-1, overall binding of yeasts to macrophages is unaffected by either Eng1 or Exg8. This is not surprising, as association and phagocytosis of *Histoplasma* yeasts are primarily mediated through binding of yeasts to the complement receptor (2–4). Thus, glucanase removal of β -glucans minimizes immune cell detection of infecting yeasts but preserves invasion mechanisms to bind and enter host macrophages.

In light of the altered Dectin-1 recognition due to Eng1 but not Exg8, and with the demonstrated endo- versus exo-activity of Eng1 and Exg8, respectively, we propose a model in which exposed β -glucans on the *Histoplasma* cell wall are not simply the exposed ends of glucan chains but instead are polysaccharide “loops” exposed on the surface of the cell wall. In this structure, only an endo-acting glucanase could hydrolyze the β -glucan molecule, whereas an exoglucanase would be ineffective as the exposed β -glucan lacks any terminal ends. Intriguingly, loss of both enzymes is no greater than loss of Eng1 alone with respect to both Dectin-1 recognition and *Histoplasma* virulence. This indicates hydrolysis of cell wall β -glucans by Eng1 is sufficient to render them undetectable by Dectin-1 without requiring the remaining mono- or disaccharide exposed termini to be hydrolyzed by Exg8. This model fits with the described ligand specificity of Dectin-1 being oligosaccharides of at least seven glucose units (29).

In the environment, secreted glucanases are positioned potentially to hydrolyze polysaccharides of other fungal cell walls, perhaps as a source of glucose that can serve metabolic needs. Although we cannot exclude such a role for *Histoplasma* Eng1 and/or Exg8, our data indicate that Eng1 and Exg8 have likely evolved to facilitate *Histoplasma* pathogenesis. First, Eng1 and Exg8 are expressed at high levels by pathogenic yeasts but are only minimally expressed by the environmental mycelial form (16) suggesting Eng1 and Exg8 function specifically in yeast cell biology (*i.e.* pathogenesis as opposed to the saprobic mycelia). Second, during infection, *Histoplasma* yeasts are found almost exclusively within the phagosome of host phagocytes in which *Histoplasma* is the only fungal cell present, making it unlikely that Eng1 and Exg8 act on other fungi. Third, Eng1 and Exg8 have evolved to be compatible with conditions within the host phagosome, whereas glucanase activity is reduced in acidic conditions, common to *Histoplasma*-containing soils. Finally, Eng1 in particular has been shown to be important for pathogenesis by reducing surface exposure of

TABLE 2

Sensitivity of glucanase-deficient *Histoplasma* yeasts to cell wall-destabilizing compounds

The mean concentration for 50% inhibition of yeast growth ($IC_{50} \pm S.D.$ among replicate assays ($n = 3$)) are listed. The following abbreviations are used: CFW, Calcofluor White; CR, Congo red; Uvtx; Uvitex 3BSA; Cfg, caspofungin; Flc, fluconazole; NikZ, nikkomycin Z.

	CFW	CR	Uvtx	SDS ^a	Cfn	Flc	NikZ
	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$m\%$	$m\%$	$\mu\text{g/ml}$	$\mu\text{g/ml}$	$\mu\text{g/ml}$
<i>gfp</i> -RNAi	6.1 ± 2.4	1.3 ± 0.1	11.0 ± 1.9	3.1 ± 1.5	10.9 ± 4.2	0.68 ± 0.07	4.3 ± 1.2
<i>EXG8</i> -RNAi	5.2 ± 2.0	1.1 ± 0.2	11.3 ± 0.3	4.8 ± 0.2	11.4 ± 4.5	0.52 ± 0.01	5.1 ± 0.4
<i>ENG1/EXG8</i> -RNAi	5.7 ± 1.8	1.1 ± 0.2	10.6 ± 1.5	4.4 ± 0.6	11.7 ± 4.7	0.59 ± 0.24	4.8 ± 1.6

^a Concentrations were expressed as milli-percent by volume (m %).

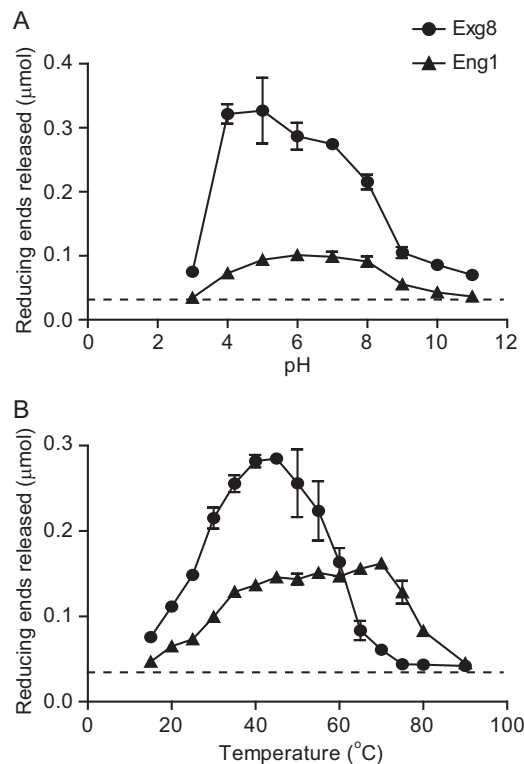


FIGURE 5. Eng1 and Exg8 are active under phagosome-like conditions. Glucanase activity is shown for Eng1 (circles) and Exg8 (triangles) at different ranges of pH (A) and temperature (B). Data represent reducing sugar equivalents of glucose released from laminarin after digestion with purified Eng1 or Exg8 for 2 h. Dashed line represents the limit of detection. Data points are the mean amount of reducing sugars released, and error bars represent the standard deviation among replicates ($n = 3$). Data are representative of two independently purified protein samples of Eng1 and Exg8.

yeast cell wall β -glucans (15). This does not preclude uptake of released mono- and oligosaccharides by yeasts, but together the biochemical and cellular characteristics support these β -glucanases functioning in *Histoplasma* pathogenesis, primarily by removing immunostimulatory β -glucans from the yeast cell surface to reduce host detection.

Experimental Procedures

Histoplasma Strains and Culture—*H. capsulatum* strains were derived from the wild-type clinical isolates G186A (ATCC 26029) and G217B (ATCC 26032) and are listed in Table 3. *Histoplasma* yeasts were grown in *Histoplasma*-macrophage medium (HMM)⁵ (30) and supplemented with 100 $\mu\text{g/ml}$ uracil

⁵ The abbreviations used are: HMM, *Histoplasma*-macrophage medium; GPI, glycosylphosphatidylinositol; m.o.i., multiplicity of infection; nt, nucleotide; PNGaseF, peptide:N-glycosidase F.

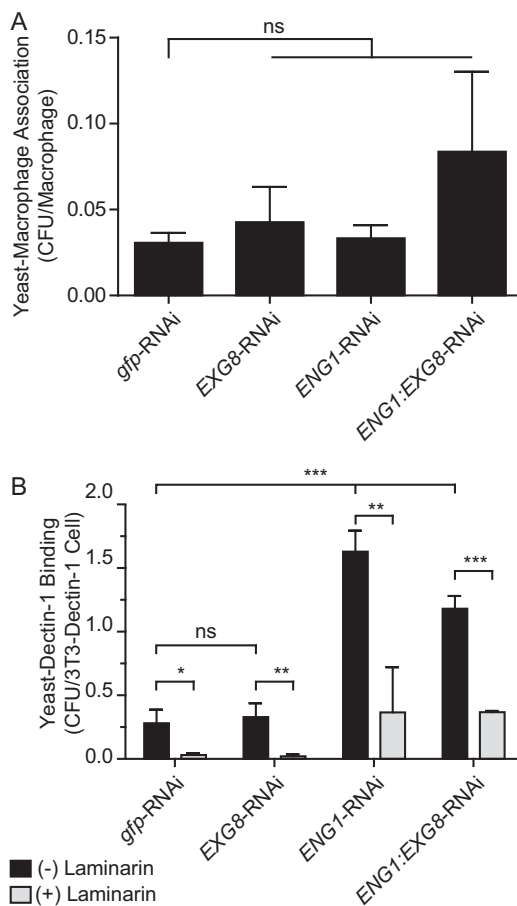


FIGURE 6. Eng1 and Exg8 do not affect yeast-macrophage association but Eng1 reduces yeast β -glucan recognition. A, association of yeast with macrophages. Yeasts lacking Exg8 (*EXG8*-RNAi), Eng1 (*ENG1*-RNAi), or both Exg8 and Eng1 (*ENG1:EXG8*-RNAi) were added to peritoneal macrophages (m.o.i. 0.5:1), and association after 2 h was determined by plating yeasts released from macrophages onto solid medium for cfu enumeration. B, relative recognition of glucanase-deficient *Histoplasma* yeasts by Dectin-1. Yeasts were added to Dectin-1-expressing 3T3 fibroblasts in the absence (black bars) or presence (gray bars) of the β -glucan competitor laminarin (1 mg/ml). Adherent yeasts were quantified by cfu determination. Data represent the mean cell-associated yeast per 2×10^5 macrophages (A) or 2×10^5 Dectin-1-expressing fibroblast (B) cells with error bars indicating standard deviation among replicate assays ($n = 3$). Asterisks indicate statistically significant differences in assay results between wild-type (*gfp*-RNAi) and glucanase-deficient yeasts as determined by Student's *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant).

when necessary. Hemocytometer counts were used for precise enumeration of yeasts for infection studies.

β -Glucanase Protein Sequence Analysis—Fungal homologs of the *Histoplasma* G186A Eng1 and Exg8 protein sequences were identified by BLAST search of *C. albicans*, *S. cerevisiae*, and *Aspergillus* genome databases and the derived protein sequences aligned using ClustalW with the BLOSUM62 matrix.

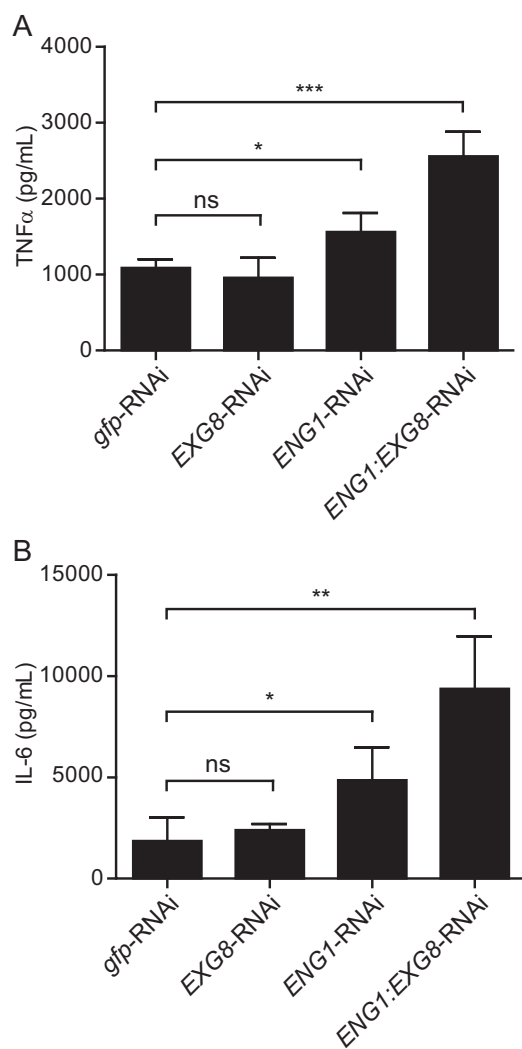


FIGURE 7. Eng1 but not Exg8 affects the macrophage proinflammatory cytokine response to *Histoplasma*. Macrophage production of TNF α (A) and IL-6 (B) 8 h after infection with wild-type (*gfp*-RNAi), *Exg8*-, *Eng1*-, or *Eng1-Exg8* glucanase-deficient yeasts (m.o.i. 0.5:1). Cytokines were measured by ELISA and levels determined by comparison with cytokine standards. Asterisks indicate statistically significant differences between wild-type and glucanase-deficient yeasts as determined by Student's *t* test (*ns*, not significant; *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001).

Protein features were predicted using SignalP (version 4.0) (31) and the GPI-SOM prediction server (32) for glycosylphosphatidylinositol (GPI) anchor signals. *N*-Linked glycosylation sites were predicted using the consensus motif (NX(S/T)). For prediction of *O*-linked mucin sites, protein sequences were scanned for regions with at least 40% serine or threonine density within a 40-amino acid window (33).

***Eng1* and *Exg8* Localization**—The *ENG1* and *EXG8* genes were amplified by high fidelity PCR and fused to the FLAG epitope (in plasmid pCR628 (34)) or a hexahistidine tag at the C terminus. Expression plasmids were transformed into *Histoplasma* uracil-auxotroph strains WU15 or WU8 (see Table 3) via *Agrobacterium tumefaciens*-mediated transformation (35). Expression, electrophoretic mobility, and subcellular localization of proteins was monitored by epitope tag immunoblotting (FLAG: M2 clone (Sigma), HIS₆: 6G2A9 clone (GenScript)) of culture filtrates following electrophoretic separation of pro-

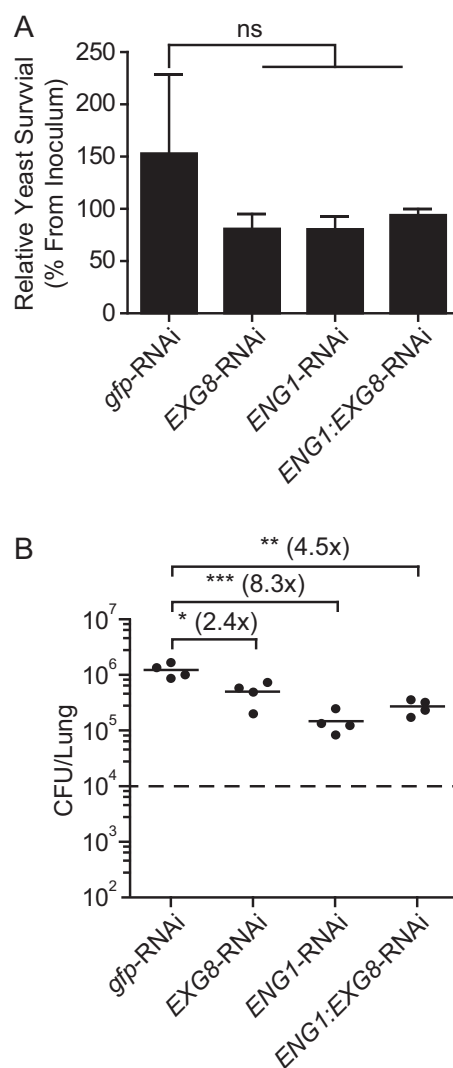


FIGURE 8. Eng1 but not Exg8 is the major glucanase facilitating *Histoplasma* virulence. A, survival of yeast following infection of peritoneal macrophages. Total viable yeasts (cfu) were quantified 8 h after infection with wild-type (*gfp*-RNAi), *Exg8*-, *Eng1*-, or *Eng1-Exg8* glucanase-deficient yeasts and compared with the cfu of yeasts in the absence of macrophages. B, *Histoplasma* burden in the lungs 8 days following respiratory infection with glucanase-producing (*gfp*-RNAi) and single glucanase-deficient yeasts (*EXG8*-RNAi and *ENG1*-RNAi) or yeasts lacking both glucanases (*ENG1:EXG8*-RNAi). Data points represent the cfu counts from individual mouse lungs (*n* = 4), and horizontal bars indicate the mean fungal burden. The dashed horizontal line indicates the inoculum given. Fold change of the mean cfu value from wild type is indicated. Asterisks indicate statistically significant differences between wild-type and glucanase-deficient yeasts as determined by Student's *t* test (*ns*, not significant; *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001).

teins through 10% polyacrylamide with SDS (SDS-PAGE). Potential *N*-linked glycans were removed by PNGaseF (New England Biolabs) prior to SDS-PAGE. *O*-Linked mannosylation was tested by expression of proteins in a *Histoplasma* strain that lacks the *Pmt2* protein mannosyltransferase, which is required for *O*-linked glycosylation.⁵

For subcellular fractionation, 5×10^8 yeast cells expressing FLAG-tagged *Eng1* or *Exg8* were separated from culture filtrates by centrifugation and filtration. Cellular lysates were prepared from the yeast by mechanical disruption with 0.5-mm diameter glass beads. The cytosolic fraction was separated from cellular debris by centrifugation (10 min at $14,000 \times g$). Insol-

TABLE 3
Histoplasma strains

Strain	Genotype ^a	Other designation
WU8	(G186A) <i>ura5-32Δ</i>	WT
OSU22	(G186A) <i>ura5-32Δ zzz::pCR482 [hph, gfp]</i>	RNAi sentinel
OSU244	(G186A) <i>ura5-32Δ zzz::pCR482 [hph, gfp] zzz::pED02 [URA5, gfp-RNAi]</i>	<i>gfp</i> -RNAi
OSU245	(G186A) <i>ura5-32Δ zzz::pCR482 [hph, gfp] zzz::pED06 [URA5, gfp:ENG1-RNAi]</i>	<i>ENG1</i> -RNAi
OSU205	(G186A) <i>ura5-32Δ zzz::pCR482 [hph, gfp] zzz::pAG26 [URA5, gfp:EXG8-RNAi]</i>	<i>EXG8</i> -RNAi
OSU214	(G186A) <i>ura5-32Δ zzz::pCR482 [hph, gfp] zzz::pAG37 [URA5, gfp:ENG1:EXG8-RNAi]</i>	<i>ENG1:EXG8</i> -RNAi
WU15	(G217B) <i>ura5-42Δ</i>	WT
OSU194	(G217B) <i>ura5-42Δ zzz::pAG21 [G418^R, gfp]</i>	RNAi sentinel
OSU247	(G217B) <i>ura5-42Δ zzz::pAG21 [G418^R, gfp] zzz::pED02 [URA5, gfp-RNAi]</i>	<i>gfp</i> -RNAi
OSU248	(G217B) <i>ura5-42Δ zzz::pAG21 [G418^R, gfp] zzz::pED06 [URA5, gfp:ENG1-RNAi]</i>	<i>ENG1</i> -RNAi
OSU273	(G217B) <i>ura5-42Δ zzz::pAG21 [G418^R, gfp] zzz::pAG26 [URA5, gfp:EXG8-RNAi]</i>	<i>EXG8</i> -RNAi
OSU275	(G217B) <i>ura5-42Δ zzz::pAG21 [G418^R, gfp] zzz::pAG37 [URA5, gfp:ENG1:EXG8-RNAi]</i>	<i>ENG1:EXG8</i> -RNAi
OSU265	(G217B) <i>ura5-42Δ zzz::pQS14 [URA5, P_{H2B}-ENG1:FLAG]</i>	<i>ENG1:FLAG</i>
OSU266	(G217B) <i>ura5-42Δ zzz::pAG39 [URA5, P_{H2B}-ENG1:6xHIS]</i>	<i>ENG1:6xHIS</i>
OSU313	(G217B) <i>ura5-42Δ zzz::pKD02 [URA5, P_{H2B}-EXG8:FLAG]</i>	<i>EXG8:FLAG</i>
OSU314	(G186A) <i>ura5-32Δ zzz::pKD01 [URA5, P_{H2B}-EXG8:6xHIS]</i>	<i>EXG8: HIS₆</i>
OSU328	(G217B) <i>ura5-42Δ pmt2::T-DNA [hph] zzz::pQS14 [URA5, P_{H2B}-ENG1:FLAG]</i>	<i>pmt2:ENG1:FLAG</i>
OSU329	(G217B) <i>ura5-42Δ pmt2::T-DNA [hph] zzz::pKD02 [URA5, P_{H2B}-EXG8:FLAG]</i>	<i>pmt2:EXG8:FLAG</i>

^a Strains were constructed in the G186A (ATCC no. 26029) or G217B (ATCC no. 26032) *Histoplasma* strain backgrounds.

uble cellular material was treated with 1% SDS and 0.1 M dithiothreitol (DTT) to extract cell wall-associated proteins or incubated with 3 milliunits/μl zymolyase (GBiosciences) to release glycan-embedded cell wall proteins. Subcellular fractions representing secreted or cellular material from 2 × 10⁷ yeasts were probed by immunoblotting.

Eng1 and Exg8 Protein Purification—*Histoplasma* yeasts expressing Eng1 or Exg8 with the C-terminal hexahistidine tag were grown in liquid HMM, and tagged proteins were purified by affinity chromatography (HisPur Co²⁺ Resin, Thermo Fisher Scientific). Protein homogeneity of the eluted fraction was determined by SDS-PAGE followed by silver staining of the protein gel. Purified proteins were stored at -20 °C in 50% glycerol. No significant loss of activity of the protein preparations was observed over at least 6 months of storage.

Determination of Glucanase Activity—Proteins were incubated with polysaccharide substrates in a 30-μl reaction volume (20 μl of substrate and 10 μl of protein solution). Substrates were prepared at 5 mg/ml in PBS, which included laminarin (Sigma), pustulan (Invivogen), carboxymethylcellulose (Sigma), amylose (Sigma), mannan (Sigma), swollen chitin (Sigma), scleroglucan (Invivogen), dextran (Spectrum Chemical), and pullulan (Tokyo Chemical Industry). Polysaccharide hydrolysis was measured by the addition of 3 volumes of dinitrosalicylic acid solution (0.687% (w/v) 3,5-dinitrosalicylic acid, 1.28% (v/v) phenol, 19.92% (w/v) potassium/sodium/tartrate, 1.226% (w/v) sodium hydroxide) and incubation at 95 °C for 5 min (23, 36). Saccharide-dependent reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid was quantified by absorbance at 540 nm and compared with a standard curve created from glucose or *N*-acetylglucosamine. Total extracellular and cell-associated glucanase activity was determined using culture filtrate equivalent to media from 2 × 10⁷ cells or whole yeasts (2 × 10⁷ cells), respectively.

Optimal temperatures for glucanase activities were determined by enzyme reactions carried out between 15 and 90 °C in 5 °C intervals. 30 ng of Eng1 or Exg8 protein was added to 5 mg/ml laminarin for 120 min, and reducing sugars were quantified. Similarly, the pH optimum was carried out in reactions incubated for 120 min at 37 °C using 25 mM buffer to control

pH: sodium citrate (pH 3.0–5.0), MES (pH 6.0), HEPES (pH 7.0–9.0), and glycine (pH 10.0–11.0).

Kinetic Analysis of Eng1 and Exg8 Activity—Steady-state kinetic values were estimated using increasing concentrations (0, 0.5, 1, 2.5, 5, 10, 15, and 20 mg/ml) of laminarin. Reactions were carried out in a 30-μl reaction volume in 25 mM MES buffer, pH 6.0. Reducing sugars were quantified after 0, 1, 2, 5, and 15 min, and the slope of the linear region of the regression curve was calculated for each substrate concentration. Kinetic values were calculated using nonlinear least squares fitting to the expression $v = V_{max} [S]/(K_m + [S])$ using Prism 5.03 software (GraphPad).

Mass Spectrometry of Digestion Products—Digests of the laminarin samples were subjected to nano-electrospray mass spectrometry analysis using a Waters Synapt G2 or Synapt G2-S instrument (Waters). 30 ng of protein was incubated with 5 mg/ml laminarin in water for 30 min at 37 °C and then diluted 20-fold with 50:50 water/LC-MS grade acetonitrile (Fisher). Samples were sprayed into the mass spectrometer using a pulled fused silica capillary fitted around a platinum electrode posed at the capillary voltage from the MassLynx 4.1 software (Waters). Acquisitions were from 50 to 5000 *m/z*. Typical voltages and amplitudes are as follows: capillary, 1.1 kV; source temperature, 30 °C; cone, 20 V; source offset, 5 V; trap collision energy, 4 V; transfer collision energy, 2 V. Ion mobility parameters as follows: helium gas flow, 180 ml/min; ion mobility gas flow, 45 ml/min; wave height, 7 V; wave velocity, 450 m/s.

After collection of the mass spectra, relative quantitation of the [M + Na]⁺ versions of glucose oligomers (mono- to octomers) was performed by using ion mobility drift times of the first four isotopic peaks of the ion of interest using the DriftScope 2.0 software (Waters). The ratios of the individual saccharides to the sum intensity of all saccharides (mono- through octosaccharides) were then determined for the specific enzymatic digestions.

Depletion of Glucanase Function(s)—Eng1 and/or Exg8 function was depleted from *Histoplasma* yeasts by RNA interference (RNAi) (25) using coding regions as follows: *ENG1* = nucleotides (nts) 445-2091; *EXG8* = nts 1744–2561; *ENG1:EXG8* double knockdown fused *ENG1* (nts 445–1521) and

EXG8 (nts 1744–2561). RNAi plasmids were transformed into the RNAi sentinel strains OSU22 and OSU194 using *A. tumefaciens*-mediated transformation (35, 37), and Ura⁺ transformants were selected. GFP fluorescence of individual transformants was quantified using a modified gel documentation system (25) and ImageJ software (version 1.44p).

Cell Wall Sensitivity Assays—Yeast cells were grown in 96-well microtiter plates (38) with graded concentrations of antifungal compounds (fluconazole (Glenmark Generics); caspofungin (Merck); and nikkomycin Z (gift from John Galgiani)) and cell wall-destabilizing compounds (Calcofluor White (Sigma); Congo red (MP Biomedicals); SDS; and Uvitex) (39). Growth after 4 days was measured by optical density at 595 nm and compared with the turbidity of wells lacking inhibitors. 50% inhibitory concentration (IC₅₀) values were computed by nonlinear regression of the dose-response data.

Yeast-Macrophage Interaction Assays—Peritoneal macrophages were harvested from C57BL/6 mice (Charles River) by peritoneal lavage and infected with yeast at 0.5:1 m.o.i. (yeast/macrophage). For association, nonadherent yeast were removed after 2 h. Yeasts were quantified by lysis of mammalian cells in water and plating of the lysate on solid HMM for colony-forming units (cfu). For survival, yeast cfu were determined after 8 h.

Cytokine Analysis—Peritoneal macrophages were infected at 0.5:1 m.o.i. with yeast, and supernatant was collected after 8 h. Cytokines (TNF α and IL-6) were quantified by ELISA (R&D Systems) and compared with standard curves.

Murine Model of Respiratory and Disseminated Histoplasmosis—C57BL/6 mice (Charles River) were infected with *Histoplasma* yeast by intranasal delivery of $\sim 1 \times 10^4$ yeast cells (15). At 8 days post-infection, lungs and spleens were collected and homogenized. Serial dilutions of the homogenates were plated on solid HMM to determine the fungal burden (cfu) in each organ.

Dectin-1 Binding Assay—Dectin-1-expressing 3T3 fibroblasts (3T3-D1) (8, 12) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO₂, 95% air. 3T3-D1 cells were adhered to wells of a 24-well plate (3×10^4 cells/well). Yeast cells were added to the 3T3-D1 cells for 2 h at 37 °C at an m.o.i. of 50:1 (yeast/3T3 cells) in the absence or presence of 1 mg/ml laminarin. Unbound yeasts were removed by washing with PBS. Fibroblast-associated yeasts were released by lysing the 3T3-D1 cells with water and the yeasts quantified by plating of the lysate to enumerate cfu.

Statistical Analyses—All assays were performed with independent biological replicates ($n = 3$ unless otherwise noted) and statistically significant differences from wild type or other parental backgrounds ($p < 0.05$) as determined by one-tailed Student's *t* test.

Ethics Statement—All animal experiments were performed in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Ohio State University (protocol 2007A0241). Animal infections were performed under anesthesia, and all efforts were made to minimize suffering.

Author Contributions—A. L. G. and C. A. R. conceived of the study and were responsible for overall design of the experiments. A. L. G. performed and analyzed data for Figs. 1, 2, 4, and 6–8, supplemental Figs. S1 and S2, and Table 1. K. L. D. produced data for Figs. 2 and 5 and Table 2. A. D. V. and V. H. W. designed, produced, and analyzed experiments for the data in Fig. 3. A. L. G. and C. A. R. wrote the manuscript. All authors reviewed the results and approved the final version.

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