A Role for the Unfolded Protein Response (UPR) in Virulence and Antifungal Susceptibility in Aspergillus fumigatus

Daryl Richie, Lukas Hartl, Vishukumar Aimanianda, Michael Winters, Kevin Fuller, Michael Miley, Stephanie White, Jason McCarthy, Jean-Paul Latgé, Marta Feldmesser, et al.

To cite this version:
Daryl Richie, Lukas Hartl, Vishukumar Aimanianda, Michael Winters, Kevin Fuller, et al.. A Role for the Unfolded Protein Response (UPR) in Virulence and Antifungal Susceptibility in Aspergillus fumigatus. PLoS Pathogens, Public Library of Science, 2009, 5 (1), pp.e1000258. 10.1371/journal.ppat.1000258. pasteur-02944355

HAL Id: pasteur-02944355
https://hal-pasteur.archives-ouvertes.fr/pasteur-02944355
Submitted on 21 Sep 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
A Role for the Unfolded Protein Response (UPR) in Virulence and Antifungal Susceptibility in *Aspergillus fumigatus*

Daryl L. Richie¹, Lukas Hartl², Vishukumar Aimanianda², Michael S. Winters³, Kevin K. Fuller¹, Michael D. Miley¹, Stephanie White¹, Jason W. McCarthy⁴, Jean-Paul Latgé², Marta Feldmesser⁴,⁵,⁶, Judith C. Rhodes⁴, David S. Askew¹*

1 Department of Pathology & Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio, United States of America, 2 Unité des Aspergillus, Institut Pasteur, Paris, France, 3 Division of Infectious Diseases, Department of Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio, United States of America, 4 Division of Infectious Diseases, Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, United States of America, 5 Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, United States of America, 6 Department of Obstetrics & Gynecology and Women’s Health, Albert Einstein College of Medicine, Bronx, New York, United States of America

Abstract

Filamentous fungi rely heavily on the secretory pathway, both for the delivery of cell wall components to the hyphal tip and the production and secretion of extracellular hydrolytic enzymes needed to support growth on polymeric substrates. Increased demand on the secretory system exerts stress on the endoplasmic reticulum (ER), which is countered by the activation of a coordinated stress response pathway termed the unfolded protein response (UPR). To determine the contribution of the UPR to the growth and virulence of the filamentous fungal pathogen *Aspergillus fumigatus*, we disrupted the hacA gene, encoding the major transcriptional regulator of the UPR. The *hacA* mutant was unable to activate the UPR in response to ER stress and was hypersensitive to agents that disrupt ER homeostasis or the cell wall. Failure to induce the UPR did not affect radial growth on rich medium at 37°C, but cell wall integrity was disrupted at 45°C, resulting in a dramatic loss in viability. The *hacA* mutant displayed a reduced capacity for protease secretion and was growth-impaired when challenged to assimilate nutrients from complex substrates. In addition, the *hacA* mutant exhibited increased susceptibility to current antifungal agents that disrupt the membrane or cell wall and had attenuated virulence in multiple mouse models of invasive aspergillosis. These results demonstrate the importance of ER homeostasis to the growth and virulence of *A. fumigatus* and suggest that targeting the UPR, either alone or in combination with other antifungal drugs, would be an effective antifungal strategy.

Introduction

*Aspergillus fumigatus* is a soil-dwelling filamentous fungus that has become the predominant mold pathogen of the immunocompromised population [1,2,3]. The infection is acquired through the inhalation of aerosolized conidia (spores), which are small enough to reach the distal airways [4]. When the inhaled conidia germinate and develop into hyphae, secreted fungal hydrolases progressively damage the integrity of the pulmonary epithelium, allowing vascular invasion with subsequent hematogenous spread [4,5]. Despite the introduction and use of recently approved antifungals, invasive aspergillosis (IA) continues to be associated with a poor outcome [1,3,6,7]. Moreover, the incidence of IA is expected to rise with the expansion of the immunosuppressed population, making the search for novel treatments a high priority. Unfortunately, few effective drugs are identifiable in the late-stage development pipeline [8], emphasizing the need for increased understanding of the virulence of this organism to facilitate the rational design of novel therapeutic strategies.

The prevailing evidence suggests that the virulence of *A. fumigatus* involves gene products that have evolved to enhance the competitiveness of the fungus in the ecologically diverse environmental niche of decaying organic debris. The saprophytic nature of this lifestyle requires the secretion of abundant enzymes that enable the fungus to extract nutrients from complex polymeric material [5,9,10]. This high capacity secretory system has been exploited in other filamentous fungi for the industrial production of native and heterologous proteins and is a feature that distinguishes these organisms from the yeast *Saccharomyces cerevisiae* [11,12,13], in which secretion levels are sometimes too low for industrial application [14]. As in all eukaryotes, the endoplasmic reticulum (ER) of filamentous fungi is the major processing center for secreted and transmembrane proteins. The unique environ-
The pathogenic mold *Aspergillus fumigatus* is the leading cause of airborne fungal infections in immunocompromised patients. The fungus normally resides in compost, an environment that challenges the organism to obtain nutrients by degrading complex organic polymers. This is accomplished by secreted enzymes, some of which may also contribute to nutrient acquisition during infection. Extracellular enzymes are folded in the endoplasmic reticulum (ER) prior to secretion. If the folding capacity of the ER is overwhelmed by increased secretory demand, the resulting ER stress triggers an adaptive response termed the unfolded protein response (UPR). In this study, we uncover a previously unknown function for the master transcriptional regulator of the UPR, HacA, in fungal virulence. In the absence of HacA, *A. fumigatus* was unable to secrete high levels of proteins and had reduced virulence in mice. In addition, loss of HacA caused a cell wall defect and increased susceptibility to two major classes of antifungal drugs used for the treatment of aspergillosis. These findings demonstrate that *A. fumigatus* relies on HacA for growth in the host environment and suggest that therapeutic targeting of the UPR could have merit against *A. fumigatus*, as well as other eukaryotic pathogens with highly developed secretory systems.

**Author Summary**

The pathogenic mold *Aspergillus fumigatus* is the leading cause of airborne fungal infections in immunocompromised patients. The fungus normally resides in compost, an environment that challenges the organism to obtain nutrients by degrading complex organic polymers. This is accomplished by secreted enzymes, some of which may also contribute to nutrient acquisition during infection. Extracellular enzymes are folded in the endoplasmic reticulum (ER) prior to secretion. If the folding capacity of the ER is overwhelmed by increased secretory demand, the resulting ER stress triggers an adaptive response termed the unfolded protein response (UPR). In this study, we uncover a previously unknown function for the master transcriptional regulator of the UPR, HacA, in fungal virulence. In the absence of HacA, *A. fumigatus* was unable to secrete high levels of proteins and had reduced virulence in mice. In addition, loss of HacA caused a cell wall defect and increased susceptibility to two major classes of antifungal drugs used for the treatment of aspergillosis. These findings demonstrate that *A. fumigatus* relies on HacA for growth in the host environment and suggest that therapeutic targeting of the UPR could have merit against *A. fumigatus*, as well as other eukaryotic pathogens with highly developed secretory systems.

**Results**

**Disruption of the UPR by Deletion of hacA in *A. fumigatus***

The UPR-induced (hacAi) and uninduced (hacAu) forms of the *A. fumigatus* hacA mRNA were cloned by RT-PCR from RNA derived from cultures grown in the presence or absence of dithiothreitol (DTT)-induced ER stress, respectively. A comparison of the cDNA sequences with the *A. fumigatus* genome revealed a conventional intron with consensus border sequences that is excised in both the hacAi and hacAu mRNAs. In addition, an unconventional 20 nt intron is uniquely excised from the *A. fumigatus* hacA mRNA in response to ER stress (Figure 1A), similarly to what has been reported in other orthologs of this mRNA [26]. This atypical intron is much smaller than the corresponding 252 nt intron of *S. cerevisiae* HAC1 [24], but is similar in size to introns that are spliced by UPR activation in mammals (26 nt) [27], *Caenorhabditis elegans* (23 nt) [27], *Candida albicans* (19 nt) [28] and filamentous fungi (20 nt) [29,30]. The exact splicing sites of the unconventional intron in *A. fumigatus* could not be unambiguously identified by comparing cDNA and genomic sequences because of the presence of a CTGCCAG at each side of the intron, a feature that is also found in other filamentous fungi [29,30]. Although the size of the intron varies between genera, the border sequences are highly conserved (Figure 1A) and are located in a region of strong predicted RNA secondary structure (data not shown) [29,30].

The first 213 amino acids encoded by the hacAi and hacAu mRNAs are identical. This region contains a leucine zipper dimerization motif adjacent to a basic DNA binding domain (Figure 1B), which is characteristic of bZIP-type family transcription factors. The atypical splicing of the *A. fumigatus* hacA mRNA changes the reading frame, resulting in an encoded protein that replaces 220 amino acids at the c-terminus of HacA with a unique c-terminal domain comprised of 129 amino acids (Figure 1B). The resulting HacA protein has 76% and 81% identity to the corresponding proteins in *A. nidulans* and *A. niger*, respectively. Alignment of the *A. fumigatus* HacA protein with orthologs from other filamentous fungi reveals extensive homology throughout the protein (Figure 1C). By contrast, most of the homology to *S. cerevisiae* Hac1p is concentrated in the DNA binding domain (data not shown).
Deletion of hacA was accomplished by replacing the hacA' open reading frame with the hygromycin resistance cassette (Figure 2). To determine whether loss of hacA was sufficient to disrupt UPR signaling in A. fumigatus, the expression of four known UPR target genes was examined by northern blot analysis, including bipA (ER chaperone), pdiA and figA (protein disulfide isomerases) and hacA itself. Each of these genes contains an unfolded protein response element (UPRE) in its promoter [31], and the abundance of each mRNA increases in response to UPR activation [30]. As expected, treatment of wt A. fumigatus with DTT increased hacA abundance and induced the conversion of hacA into hacAi, indicating activation of the UPR under these conditions (Figure 3A). The smaller size of the hacA' mRNA is consistent with a 5' mRNA truncation that has been reported following UPR induction in other filamentous fungi [29,30]. In contrast to wt A. fumigatus, the ΔhacA mutant was unable to increase the level of three other UPR target genes when treated with DTT, indicating a defect in UPR-regulated gene expression. Complementation of the ΔhacA mutant (C) restored UPR signaling to the ΔhacA mutant (Figure 3B).

The UPR Is Required under Conditions of ER Stress

To determine how loss of hacA impacts the growth of A. fumigatus under conditions of ER stress, the mutant was incubated in the presence of agents that disrupt ER homeostasis by different mechanisms, including DTT, tunicamycin (TM), and brefeldin A (BFA). DTT unfolds proteins directly by reducing disulfide bonds, TM impairs protein folding by inhibiting N-linked glycosylation, and BFA impairs anterograde protein transport from the ER to the Golgi [26]. The growth of the ΔhacA mutant was comparable to wt in the absence of ER stress, although condialation was decreased on solid medium (Figure 4, columns marked '0'). However, the ΔhacA mutant was unable to grow in the presence of concentrations of DTT, BFA or TM that could be tolerated by wt A. fumigatus, indicating heightened sensitivity to ER stress. The mutant was also hypersensitive to the superoxide-generating agent paraquat (Figure S1), consistent with the adverse effects of oxidative stress on protein folding and ER homeostasis [32].

The UPR Is Required for Thermotolerance in A. fumigatus

The radial growth rate of the ΔhacA mutant was almost indistinguishable from that of wt A. fumigatus at 37°C or 42°C (Figure 5A and Figure S2). However, at 45°C the ΔhacA mutant failed to grow beyond the site of the initial inoculum (Figure 5B). Microscopic analysis revealed that the ΔhacA conidia had germinated at 45°C, but subsequently arrested growth as young hyphae (data not shown). To determine whether this was due to
Figure 3. Loss of hacA disrupts UPR signaling in A. fumigatus. (A) Northern blot analysis of hacA expression and processing in the presence (+) or absence (−) of 1mM DTT for 1 h. Hybridization intensity was determined by phosphorimager analysis, and is presented graphically as fold expression relative to the levels of hacA\textsuperscript{u} in the absence of DTT. (B) Northern blot analysis of UPR target gene expression (bipA, pdiA, and tigA) in the presence (+) or absence (−) of DTT. Hybridization intensity is presented graphically as fold expression relative to the wt strain in the absence of DTT. doi:10.1371/journal.ppat.1000258.g003
The UPR Contributes to Cell Wall Integrity in *A. fumigatus*

The inability of the *ΔhacA* mutant to grow at 45°C could be reversed by osmotic stabilization of the medium with sorbitol (Figure 6A) or KCl (data not shown), suggesting that the impaired growth of *ΔhacA* at elevated temperature is due, in part, to loss of cell wall integrity. To test this more directly, conidia were inoculated onto solid medium (IMA) in a multi-well plate containing the indicated concentrations of BFA or TM and incubated at 37°C for 2–3 days. DMSO was used as the vehicle control for TM. The experiments were performed three times with similar results.

doi:10.1371/journal.ppat.1000258.g004

Figure 4. The *ΔhacA* mutant is hypersensitive to ER-stress. ER stress was induced by incubating in the presence of DTT, BFA, or TM. For analysis of DTT sensitivity, conidia from the indicated strains were inoculated into liquid AMM–glucose in a multi-well plate containing the indicated concentrations of DTT and incubated at 37°C for 4 days. For analysis of BFA or TM sensitivity, conidia were inoculated onto solid medium (IMA) in a multi-well plate containing the indicated concentrations of BFA or TM and incubated at 37°C for 2–3 days. DMSO was used as the vehicle control for TM. The experiments were performed three times with similar results.

Loss of viability, 200 conidia were evenly distributed onto an agar surface. After incubating at 45°C for 0, 12 and 24 h, the plates were shifted to 37°C, and surviving colony forming units (CFUs) were counted. As shown in Figure 5C, approximately 50% of the plated wt and complemented conidia survived 24 h of incubation at 45°C. This was in contrast to the *ΔhacA* mutant, where less than 1% of the plated conidia survived 24 h at 45°C.

*A. fumigatus* is a thermotolerant fungus that normally thrives at temperatures above 50°C [33], with an optimum for growth between 37°C and 42°C, depending on the medium. The *ΔhacA* mutant was unable to grow at a temperature that is only 5°C above the optimum range for this species. This phenotype was unexpected because temperature-induced lethality has not been previously reported in the corresponding *Δhac1* mutant of *S. cerevisiae* [20,34], suggesting a vulnerability in *A. fumigatus* that may not be present in yeast. *S. cerevisiae* grows optimally between 25°C and 30°C, and 37°C is considered thermal stress for this organism [35]. We found that the growth of a *S. cerevisiae* *Δhac1* mutant was indistinguishable from that of wt at either 30°C or 37°C, indicating that yeast differ from *A. fumigatus* in their ability to tolerate thermal stress in the absence of proper UPR signaling (Figure 5D).

**The UPR Contributes to Cell Wall Integrity in *A. fumigatus***

The inability of the *ΔhacA* mutant to grow at 45°C could be reversed by osmotic stabilization of the medium with sorbitol (Figure 6A) or KCl (data not shown), suggesting that the impaired growth of *ΔhacA* at elevated temperature is due, in part, to loss of cell wall integrity. To test this more directly, conidia were inoculated onto cover-slips in liquid medium and germinated overnight at 37°C. After shifting to 45°C, the hyphae were examined microscopically. Within 4 h of incubation at the elevated temperature, the hyphal tips began to swell, and tip lysis became apparent within 8 h (Figure 6B). Occasional areas of cytoplasmic leakage were also observed in subapical hyphae, possibly representing emerging branch points (data not shown). The *ΔhacA* mutant was severely growth impaired following the temperature shift, suggesting an important role for HacA in the maintenance of cell wall integrity at the hyphal tips during thermal stress.

Since thermal stress is likely to have pleiotropic effects on cell physiology, calcofluor white (CFW) was used as a more specific inhibitor of cell wall integrity. CFW is an anionic dye that weakens the wall by binding to nascent chitin chains [36]. Northern blot analysis revealed that treatment with CFW induces the hac1-dependent accumulation of *bip1* mRNA (Figure 7A), suggesting that the UPR is part of the normal adaptive response to CFW-induced cell wall stress. The *ΔhacA* mutant was unable to grow in the presence of concentrations of CFW that had minimal effect on the wt or complemented strains, consistent with a protective role for HacA under these conditions (Figure 7A). Normal growth could be restored to the mutant by osmotic stabilization of the medium with sorbitol, supporting the notion that the impaired growth of the mutant in the presence of CFW was a consequence of reduced cell wall integrity (Figure 7B). Microscopic analysis of the *ΔhacA* mutant in the presence of CFW revealed the same apical lysis that was observed under conditions of thermal stress (data not shown), suggesting that the mutant wall is particularly vulnerable to cell wall perturbation at the tips. Similar results were obtained using the related cell wall damaging compound Congo red (Figure S3). However, these findings in *A. fumigatus* were in contrast to *S. cerevisiae*, where the corresponding *Δhac1* mutant showed wt sensitivity to CFW (Figure 7C).

The increased vulnerability of the *ΔhacA* mutant to cell wall stress raises the possibility that HacA contributes to cell wall homeostasis in *A. fumigatus*. To test this, a biochemical analysis of the cell wall was performed. As shown in Table 1, the *ΔhacA* mutant revealed a significant decrease in glucose content in both the alkali insoluble (AI) and alkali soluble (AS) fractions of the cell wall relative to wt, suggesting a defect in both β(1–3) and α(1–3) glucan composition in the mutant cell wall.

**Loss of UPR Function Enhances Susceptibility to Antifungal Drugs**

All major classes of antifungal drugs that are currently in use against *A. fumigatus* attack the integrity of the membrane or cell wall. Fungi respond to these agents by upregulating cell wall and membrane repair systems [37,38,39], which may increase stress on the secretory system. To determine how loss of UPR function would affect growth in the presence of antifungal stress, susceptibility to amphoterocin B, caspofungin, itraconazole and fluconazole was compared using the Etest method. Conidia were grown optimally between 25°C and 30°C, and 37°C is considered thermal stress for this organism [35]. We found that the growth of a *S. cerevisiae* *Δhac1* mutant was indistinguishable from that of wt at either 30°C or 37°C, indicating that yeast differ from *A. fumigatus* in their ability to tolerate thermal stress in the absence of proper UPR signaling (Figure 5D).

Figure 8. Antifungal activity of caspofungin and fluconazole. Fungi were grown on plates containing different concentrations of caspofungin or fluconazole. Conidia were inoculated plates, and incubated at 37°C for 48 h. The *ΔhacA* mutant had larger areas of growth inhibition surrounding each strip, indicating heightened susceptibility to each of these drugs and a decrease in the minimal inhibitory concentration (Figure 8A). The incomplete clearing around the caspofungin strip on wt-inoculated plates, *ΔhacA*-inoculated plates, and wt-inoculated plates was able to grow when transferred to medium lacking any drug. However, no viable organism could be recovered from agar plugs taken from the cleared zone surrounding the caspofungin strip on *ΔhacA*-inoculated plates.
indicating that caspofungin becomes fungicidal in the absence of UPR function. Microscopic analysis of caspofungin-treated hyphae revealed normal morphology in the wt, but abnormal swelling and lysis in the ΔhacA mutant (Figure 8B). These defects were localized to hyphal tips and branch points, similar to what was observed under conditions of thermal stress and CFW treatment. This experiment was performed on RPMI agar in accordance with the manufacturer’s specifications, but comparable results were also obtained using IMA as the medium (Figure S4). Remarkably, the corresponding Δhac1 mutant in S. cerevisiae did not show increased sensitivity to either caspofungin, ketoconazole, amphotericin B or fluconazole (Figure S5).

The UPR Supports Protease Secretion and Growth on Complex Substrates
The ability of A. fumigatus to colonize the host begins with the germination of conidia in the lung followed by invasion of exploring hyphae into the surrounding tissue. The organism must acquire nutrients from host tissues at all steps of the infection, which requires continual secretion of a multitude of degradative enzymes. Since ER stress occurs when protein secretion is upregulated [41], we hypothesized that loss of UPR signaling would impair the secretory capacity of A. fumigatus. To test this prediction, secreted proteolytic activity was quantified with the Azocoll assay, using conditions previously described for A. fumigatus.
Azocoll is an insoluble collagen linked to an azo dye, and its hydrolysis releases soluble colored peptides that can be quantified colorimetrically [42]. As shown in Figure 9A, culture supernatants derived from the ΔhacA mutant were significantly less efficient at digesting Azocoll than wt cultures, indicating that protease secretion is abnormal in the mutant. This decrease in proteolytic activity was consistent with an overall reduction in secreted protein levels in the ΔhacA mutant, as revealed by SDS-PAGE analysis of culture supernatants (Figure 9A).

The reduced secretory capacity of the ΔhacA mutant predicts that this strain would have difficulty assimilating nutrients from a complex substrate. On IMA medium, the growth of the ΔhacA mutant was normal (Figure 5A). However, this rich medium contains a substantial amount of reduced carbon and nitrogen in the form of tryptone (pancreatic digest of casein), peptone (enzymatic digest of proteins), yeast extract, dextrose and starch. When challenged to use a more complex substrate such as skim milk, the ΔhacA mutant grew slower than the wt and complemented strains (Figure 9B). Osmotic stabilization with sorbitol was unable to rescue this phenotype, but the addition of a reduced nitrogen/carbon source completely restored growth to wt levels (Figure 9B, and data not shown). These findings argue that the impaired growth of ΔhacA on skim milk agar is due to inefficient nutrient acquisition rather than an indirect effect on cell wall stress. Similar observations were made when mouse lung tissue was used as a substrate. In contrast to the wt-inoculated lung tissue, which supported fungal growth within 24 h, the ΔhacA-inoculated lung showed no signs of fungal growth (Figure 9C). Collectively, these findings suggest that the UPR promotes the growth of A. fumigatus on complex polymeric material by facilitating the production of secreted hydrolases that are necessary to breakdown the substrate into usable nutrients.

The UPR Promotes Virulence of A. fumigatus

The ability to detect A. fumigatus proteases in vivo [43] implies that active secretion occurs in the host environment, suggesting that the UPR may contribute to virulence. To test this, we compared the virulence of the ΔhacA mutant to that of wt A. fumigatus. As shown in Figure 10A, the ΔhacA mutant was hypovirulent in an outbred mouse model of invasive aspergillosis that uses a single dose of triamcinolone acetonide (TA) to induce a period of transient immunosuppression. An increasing body of evidence suggests that the outcome of virulence testing in experimental models of aspergillosis is influenced by host strain and the type of immunosuppression [44,45,46]. Thus, virulence was also compared in two additional models that use inbred mice: a neutropenic model and a cortisone acetate model. The ΔhacA mutant had attenuated virulence in all three model systems, demonstrating that the UPR is an important stress signaling pathway in the host environment (Figure 10A, 10B, and 10C).

Discussion

All eukaryotes with an elevated capacity for protein production depend on the UPR to maintain ER homeostasis. ER stress is encountered under many adverse environmental conditions that cause protein unfolding, including high temperature [47,48], oxidative stress [49,50], hypoxia [51,52] or nutrient limitation.
However, it may also occur under normal physiological conditions in response to a change in the demand for secretion. For example, the UPR is induced in B cells when they are stimulated to secrete antibody [54]. Similarly, UPR-deficient mice fail to differentiate hepatocytes, pancreatic β cells or plasma cells, because the UPR protects against the ER stress that is generated when the intense secretory activity of these cells is activated [55,56,57].

A. fumigatus, like many other filamentous fungi, is well equipped for protein secretion [5,9,58], with over 1% of its genome dedicated to secreted proteases alone [59,60]. This robust secretory arsenal makes filamentous fungi excellent production hosts for proteins of biotechnology interest [11,12,13,61], and the ability of enforced HacA overexpression to further enhance protein secretion [62] illustrates the importance of the UPR to the maintenance of ER homeostasis under a high secretory load.

In this study, a UPR-deficient mutant of A. fumigatus was constructed in order to determine how the UPR impacts growth, secretion and virulence in A. fumigatus. The ΔhacA mutant was hypersensitive to agents that perturb ER homeostasis and was unable to increase the expression of four known UPR target genes in response to ER stress. Although a hacA-independent mechanism of bipA induction has been recently reported in A. niger strains that overproduce membrane proteins [63], the absence of bipA induction in the ΔhacA mutant treated with DTT indicates that bipA induction is hacA-dependent when DTT is used to induce ER stress.

In S. cerevisiae, Δhac1 mutants are inositol auxotrophs, a phenotype that is associated with defects in expression of INO1 [34]. However, inositol was dispensable for the growth of the Δhac1 mutant (data not shown), indicating that hac1 is not required for this pathway in A. fumigatus. On rich medium, the
growth of the ΔhacA mutant was comparable to that of wt. However, the ΔhacA mutant became growth impaired when it was forced to obtain nutrients from complex substrates such as skim milk or mouse lung tissue. These findings argue that a rapid growth rate per se does not constitute sufficient ER stress to require extensive support from the UPR, as long as an adequate supply of reduced nitrogen/carbon is present. By contrast, growth on more polymeric material would require an increase in secretory activity to breakdown the substrate, resulting in UPR activation. Communication from other stress response pathways may also influence the magnitude of this response, such as the ability of Gcn4p to control both amino acid starvation responses and UPR target gene expression [64]. Failure to trigger the UPR under situations that demand increased secretory capacity would be expected to impair secretion, thereby limiting nutrient availability and reducing growth. Furthermore, the unresolved ER stress caused by loss of UPR function may trigger a second feedback mechanism that is activated in response to impaired secretory protein folding or transport called repression under secretion stress (RESS) [65]. RESS involves the selective transcriptional down-regulation of genes encoding certain secreted proteins, and current evidence suggests that it is controlled differently from the UPR [66]. Our finding of reduced secreted collagenolytic activity and an alteration in the overall secretory profile of the ΔhacA mutant is consistent with this model (Figure 9A).

In nature, A. fumigatus has evolved to thrive in compost, an environmental niche that generates heat from microbial activity. A. fumigatus has acquired unique mechanisms of thermotolerance to support its growth up to 60°C [33]. This study demonstrates that the UPR plays an essential role in thermotolerance. The ΔhacA mutant grew normally at 37°C but was unable to maintain cell wall integrity at 45°C. Cytoplasmic leakage was observed at hyphal tips and at various points along the hypha, possibly representing areas of weakness caused by dynamic remodeling of the cell wall at these sites. Reduced thermotolerance has also been reported in other cell wall mutants of filamentous fungi, suggesting that thermal stress has adverse effects on the cell wall [67,68]. Prolonged incubation of the ΔhacA mutant at 45°C was incompatible with viability. This finding is particularly notable in view of the extraordinary thermotolerance of A. fumigatus, but is also remarkable because the corresponding mutation in S. cerevisiae does not exhibit the same temperature-sensitive phenotype (Figure 5). This difference may reflect the need for more surface export functions in A. fumigatus to maintain the integrity of the apical cell wall during polarized growth, particularly at elevated temperature.

Direct perturbation of the cell wall by treatment with CFW increased apical lysis and reduced hyphal growth in the ΔhacA mutant. Sorbitol rescued this CFW sensitivity, suggesting that the phenotype is primarily a consequence of reduced cell wall integrity (Figure 7B). By contrast, neither the temperature sensitivity nor the reduced conidiation of the ΔhacA mutant could be fully rescued by sorbitol (Figure 6A), suggesting that the UPR has additional homeostatic functions under conditions of thermal stress that do not involve the cell wall. The components of the A. fumigatus cell

---

### Table 1. Monosaccharide composition of the alkali-insoluble and alkali-soluble fractions of the cell wall from the wt and ΔhacA strains

| Monosaccharide          | wt       | ΔhacA    | wt       | ΔhacA    |
|-------------------------|----------|----------|----------|----------|
| Mannose                 | 6.4±0.2  | 4.5±1.7  | 6.0±0.2  | 1.0±0.5  |
| Glucose                 | 47.0±3.0 | 33.0±3.5 | 66.0±1.0 | 52.0±4.0 |
| Galactose               | 6.6±1.0  | 6.5±0.5  | 1.7±0.7  | 4.5±0.7  |
| N-acetylglucosamine     | 24.0±2.0 | 21.0±1.8 | -        | -        |
| N-acetylgalactosamine   | 1.0±1.0  | 3.8±1.3  | 1.2±0.9  | 2.5±1.0  |

**Results expressed as percent – average of four replicates ± standard deviation.**

*Statistically significant (P<0.001).

The ratio of AI/AS for wt and ΔhacA was not significantly different (1.6±0.3 and 1.3±0.2 for wt and ΔhacA, respectively).

doi:10.1371/journal.ppat.1000258.t001

---

**Figure 8. The ΔhacA mutant is hypersensitive to antifungal drugs.** (A) Antifungal susceptibility using Etest: conidia were spread evenly onto a 150 mm plate of RPMI agar. Four Etest strips, each impregnated with a concentration gradient of caspofungin (CS), fluconazole (FL), amphotericin B (AmB), and itraconazole (IT) were applied to the surface, with the highest concentration oriented at the plate edge. (B) Microscopic analysis of caspofungin-treated hyphae. Equal numbers of conidia from the indicated strains were spread onto the surface of an IMA plate and allowed to germinate at 30°C for 24 h. A caspofungin Etest strip was then applied to each plate and incubated overnight at 37°C. The morphology of the hyphae surrounding the highest concentration on the strip was observed by DIC microscopy. Scale bar represents 50 μm.

doi:10.1371/journal.ppat.1000258.g008
wall can be divided into two main groups based on their alkali solubility [69]. The AI fraction is thought to provide the main structural rigidity of the wall and is composed of β(1–3) glucan, chitin and galactomannan. By contrast, the AS fraction contains predominantly α(1–3) glucan and galactomannan. The heightened sensitivity of the ΔhacA mutant to multiple types of cell wall stress, combined with the decreased glucose content in its cell wall, is consistent with a defect that reduces the overall glucan composition of the cell wall. The A. fumigatus wall is a highly dynamic structure, particularly at hyphal tips and branch points where the structural needs of the hypha must be balanced by the demand for new apical growth [69]. Since β(1,3) glucan synthase is transported to the growing tips as an inactive complex through the secretory pathway [70], it is intriguing to speculate that the predisposition of the ΔhacA mutant to apical lysis is due to inefficient delivery of the glucan synthase complex to the growing tips. The ΔhacA mutant had normal chitin levels however, suggesting that HacA is less important for chitin synthase activity.

Figure 9. The UPR supports protease secretion and growth on complex substrates. (A) Azocoll hydrolysis by A. fumigatus proteases: Equal numbers of conidia were inoculated in liquid AMM-FBS, and incubated at 37°C/150 rpm for 72 h. Culture supernatants were incubated in the presence of Azocoll for 3 h and the absorbance of the medium at 520 nm was determined. The experiment was performed in triplicate and values represent the mean A520/g dry weight±SD. Significance was assessed using a two-tailed t-test, and the asterisk indicates that the Azocoll hydrolysis is significantly less in the ΔhacA mutant than in the wt (p<0.05). 1D gel analysis of wt and ΔhacA secreted proteins: Equal numbers of wt and ΔhacA conidia were inoculated into liquid AMM and incubated for 3 days at 37°C without shaking. The hyphal mat was removed, and the supernatant was concentrated as described in Materials and Methods. A 5 μl aliquot of each sample was then fractionated by 1D SDS-PAGE and proteins were stained by SYPRO Ruby dye. The protein marker is shown in kD. (B) Growth on skim milk: Conidia were spotted onto skim milk agarose plates in the presence or absence of 1.2 M sorbitol and colony diameter was monitored daily at 37°C. The experiment was performed twice, with similar results. (C) Growth on mouse lung tissue: A freshly isolated piece of mouse lung tissue was inoculated with wt or ΔhacA conidia and incubated at 37°C for 24 h. Experiments in Figure 7 were repeated with similar results.

doi:10.1371/journal.ppat.1000258.g009
This may reflect the ability of multiple chitin synthases [71] to compensate for any reduction in chitin synthase delivery caused by loss of HacA. Interestingly, the *S. cerevisiae Δhac1* mutant had wt sensitivity to CFW, suggesting a fundamental difference between these two species in terms of their reliance on the HacA-dependent UPR for cell wall homeostasis.

Analysis of the antifungal susceptibility profile of the *ΔhacA* mutant revealed two important findings. First, the *ΔhacA* mutant showed a dramatic increase in susceptibility to antifungal drugs that are in use for the treatment of invasive aspergillosis. This suggests that targeting the UPR with novel therapy could act synergistically with currently approved antifungal drugs, as well as potentially increasing the susceptibility profile of other fungal pathogens that are intrinsically resistant to some antifungals. For example, *A. terreus* and most *Fusarium* and *Scedosporium* isolates are only moderately susceptible or resistant to amphotericin B [72,73,74,75]. Similarly, itraconazole has limited activity against *Fusarium* and *Scedosporium* species, and voriconazole and echinocandins are largely ineffective against zygomycetes [72,76]. Thus, targeting the UPR has the potential of expanding the number of therapeutic options for these emerging fungal pathogens. The second important observation is that the well known fungistatic effects of the β(1–3) glucan synthase inhibitor caspofungin became fungicidal to *A. fumigatus* in the absence of hacA function. This synergistic activity is likely to reflect the lethal effects of glucan synthase inhibition in a strain that is already deficient in glucan production. These observations also suggest that the UPR is an essential component of the adaptive response to antifungal stress, an idea that is supported by the upregulation of genes involved in ER and secretion functions, including *Hac1*, during caspofungin treatment of the dimorphic yeast *Candida albicans* [77]. This class of genes was not induced by caspofungin treatment of *S. cerevisiae*, suggesting an important difference between these yeasts [39,78]. In addition, loss of UPR function increases sensitivity to cell wall damage in *A. fumigatus* (Figure 5) and in *C. albicans* [79], but not in *S. cerevisiae* (Figure 5). One possible explanation for these differences is that the ability to form true hyphae in *A. fumigatus* and *C. albicans* increases the demand on the secretory system for cell wall repair, making these fungi more vulnerable to loss of UPR function.

Since recently published data have shown that the virulence of *A. fumigatus* is influenced by host strain and the type of immunosuppression, three distinct mouse models of invasive aspergillosis were used to assess virulence. The *ΔhacA* mutant was hypovirulent in all three models, emphasizing the importance of UPR signaling to the ability of the fungus to grow in the host.
environment. Metabolic evidence has suggested that A. fumigatus relies heavily on protein degradation as a major source of nutrients in vivo [80], which is consistent with the detection of secreted A. fumigatus proteases in vivo [43]. Although protease secretion has long been considered a virulence-related factor for A. fumigatus, single-gene disruptions have yet to demonstrate this because of the abundant secreted proteases encoded by the genome. Here, we provide the first genetic evidence to suggest that secretory activity is important to the virulence of this organism. The results are consistent with a model in which the UPR contributes to virulence by supporting the secretory activity that is necessary to degrade host tissues. In the absence of a functional UPR, this secretory capacity is impaired, which may lessen the ability of the organism to damage tissues and efficiently extract the nutrients required for growth. Failure to resolve ER stress could also contribute to the reduced virulence of theΔhacA mutant if unfolded proteins accumulate to toxic levels.

Taken together, the data from this study suggest that the high secretory capacity of A. fumigatus places it at considerable risk for ER stress and thus represents a vulnerability that could be exploited for therapeutic gain by disrupting the pathways that maintain ER homeostasis. Moreover, since secretory processes have prominent roles in the virulence of parasitic protozoa [01,02], the findings from this study may have relevance to other pathogenic eukaryotes.

Materials and Methods

Strains and Culture Conditions

The A. fumigatus and S. cerevisiae strains used in the study are listed in Table 2. Conidia were harvested from Aspergillus minimal medium (AMM) [83] containing 10 mM ammonium tartrate and osmotically stabilized with 1.2 M sorbitol. Unless otherwise specified, experiments involving theΔhacA mutant were performed on inhibitory mold agar (IMA, Fisher Scientific Cat. # 14-910-95) since the growth rate of theΔhacA mutant approximated that of wt on this medium. Radial growth rate was determined by spotting 5,000 conidia onto the center of a plate and monitoring colony diameter daily. For analysis of survival under thermal stress, 200–5,000 conidia were inoculated onto the center of a glass coverslip submersed in AMM and incubated at 37°C and 45°C after 24 h of growth. To demonstrate cytoplasmic leakage at 45°C, conidia were inoculated onto a glass coverslip submerged in AMM and incubated at 37°C for 24 h. After shifting to 45°C for 4 h and 8 h, the coverslip was inverted onto a glass slide and the hyphae were photographed by differential interference contrast (DIC) microscopy.

To monitor growth under ER or cell wall stress, 2,000 conidia were inoculated onto the center of a plate containing IMA supplemented with concentrations of BFA, TM, CFW, or Congo red specified in the Results section. The plates were incubated for 2–3 days at 37°C, and the extent of growth was used as a relative indicator of sensitivity. Since it is recommended that DTT-induced ER stress be performed in liquid rather than solid medium [26], analysis of DTT sensitivity was performed by inoculating 10,000 conidia in liquid AMM containing the indicated concentrations of DTT and incubating at 37°C for 4 days. Utilization of skim milk was determined by inoculating 5,000 conidia onto skim milk agarose plates (0.5% skim milk, 0.8% agarose), or skim milk agarose supplemented with 1.2 M sorbitol. The plates were incubated at 37°C and radial growth was monitored daily for 3 days.

For experiments involving S. cerevisiae, overnight cultures of the wt and theΔhac1 mutant (Invitrogen) were diluted to an OD600 of 0.1 and cultured at 30°C and 250 rpm until the OD600 reached 0.5. Serial 5-fold dilutions were then spotted onto YPD (1% yeast extract, 2% peptone, 2% glucose) plates containing TM (62.5 ng/ml) or CFW (25–50 μg/ml), and the plates were incubated at 30°C or at 37°C for 2 days.

Antifungal Susceptibility

Antifungal susceptibility of A. fumigatus strains was determined using the Etest diffusion assay (AB BIODISK) according to the manufacturer’s instructions. Briefly, conidial suspensions were prepared in sterile distilled water and adjusted to 1×10⁶ conidia/ml. One ml of the conidial suspension was then spread evenly onto the surface of a 150 mm plate of RPMI agar buffered with MOPS (Remel, Lenexa, Kansas), using a glass spreader. The inoculated agar surface was allowed to dry for 20 min before Etest strips containing amphoterixin B, caspofungin, itraconazole, fluconazole or ketoconazole were applied. The plates were incubated at 37°C for 48 h before being photographed. The MICs were read as the lowest drug concentrations at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip. For Etest experiments involving S. cerevisiae strains, overnight cultures in YPD were diluted to an OD600 of 0.1 and cultured at 30°C / 250 rpm until the OD600 reached 0.5. The cultures were diluted to an OD600 of 0.257 and the yeast were spread evenly onto the surface of duplicate YPD plates using a Q-tip. Etest strips were applied after allowing the plates to dry for 20 min, and the plates were incubated for 48 h at 30°C.

Deletion and Reconstitution of the A. fumigatus hacA Gene

All PCR primers used in the study are listed in Table 3. The A. fumigatus hacA gene (Genbank accession XM_743634) was disrupted using the split-marker approach [84]. The left arm of hacA was PCR amplified from genomic DNA using PFU turbo polymerase (Stratagene) with primers 522 and 523 creating PCR product #1. The first two thirds of the hygromycin resistance cassette was amplified from plasmid pAN7-1 using primers 395 and 398 to create PCR product #3. PCR products #1 and #3 were then combined in an overlap PCR reaction with primers 395 and 522 to generate PCR product #5. PCR product #5 was then cloned into pCR-Blunt II-TOPO (Invitrogen) to create p327. The right arm of the hacA gene was then PCR amplified from genomic DNA using primers 524 and 525 to generate PCR product #2. The second two-thirds of the hygromycin resistance cassette was amplified from pAN7-1 with primers 396 and 399 to make PCR product #4, and PCR products #2 and #4 were combined in an overlap PCR reaction with primers 396 and 525 to generate PCR

| Strain | Species | Genotype | Source |
|--------|---------|----------|--------|
| WT (ΔakuA) | A. f. | akuA-ptrA | S. Krappmann |
| ΔhacA | A. f. | akuA-ptrA, hacA-hph | This study |
| C' | A. f. | ΔhacA (hacA::lele) | This study |
| BY4741 S. c. | MATa:his3Δ1; leu2Δ10; met15Δ10; ura3Δ10, Invitrogen |
| BY4741 (Δhac1) S. c. | MATa:his3Δ1; leu2Δ10; met15Δ10; ura3Δ11, Δhac1::kan | Invitrogen |

doi:10.1371/journal.ppat.1000258.002
Table 3. PCR primers used in this study. M13-derived sequences used for overlap PCR are underlined

| Primer Gene Sequence (5'-3') | 395 | hph | CCTCATACAGCCACACCGG |
|-------------------------------|-----|-----|----------------------|
| 396                           | hph | CGTGGCAGCCGCTCGGAA |
| 398                           | hph | CGCAGGTTTCACGACGACAGAAGCGCGGTGAGC |
| 399                           | hph | AGCGGTAACATTTCCACACAGAATCGCCGAGCAAGAGC |
| 492                           | hacA | TGGCTAGACGCGCTGAAAG |
| 493                           | hacA | CATTCCGCTACAGACAGATGG |
| 494                           | bipA | GTGTGATGCGGAGGATGTC |
| 495                           | bipA | ATCTGGGAAAGACAGATGAC |
| 522                           | hacA | CCTCCTGACAGACAGACAG |
| 523                           | hacA | GTGCTGACTGGGAAAATCCCTGGCGGTAGACAGATCACAGG |
| 524                           | hacA | TCTGTGATGAAATATTGTACCCATTGAGCAGCTGGATGTTAGT |
| 525                           | hacA | CCTTATCGCTACAGACAGAT |
| 527                           | hacA | TGGCTGTACAGACAGATGAC |
| 602                           | pd2a | ATGGCTGGTACCGGCTAG CTG |
| 603                           | pd2a | TACCGGCGTAATATAGCTAG |
| 628                           | tigA | ATGCAGTGGTACGGTCCTG |
| 629                           | tigA | TAATAGCTGCTTGGCGACTT |

doi:10.1371/journal.ppat.1000258.t003

Product #6. PCR product #6 was then cloned into pCR-Blunt II-TOPO to create p528. The inserts from p527 and p528 were gel-purified following digestion with BstXI and Smal, and 10 µg of each was used to transform wt-ΔhacA protoplasts as previously described [85]. Inositol was included into the selection plates since S. cerevisiae UPR mutants are inositol auxotrophs [34]. Hygromycin-resistant colonies were screened by PCR, and loss of the hacA gene was confirmed on monomidal isolates by genomic Southern blot analysis as described in the results section. Probe A was PCR-amplified from wt genomic DNA using primers using primers 492 and 493 while probe B was amplified with primers 522 and 523. Genomic Southern blot genotyping confirmed single-copy deletion of the hacA gene in 2 transformants, and these clones were used for phenotypic analysis.

To construct the complementation plasmid, a phleomycin resistance cassette was excised from plasmid pBChplo as a SalI/ HindIII fragment and ligated into the XhoI/HindIII sites of plasmid pSL1180. The ttpC terminator was PCR-amplified from plasmid pAN7-1 and cloned into the vector as a SalI/SalI fragment to create plasmid pTTP. The hacA gene containing 1128 bp upstream of the predicted translational start site was PCR-amplified from wt genomic DNA using primers 522 and 523 and cloned into pCR-Blunt II-TOPO. The hacA gene fragment was excised from pCR-Blunt II-TOPO with a BamHI and NotI restriction digest and inserted into the BglII/NotI sites of pTTP. Ten µg of the plasmid was linearized with Apal and transformed into ΔhacA mutant protoplasts as previously described [85]. Southern blot analysis of phleomycin-resistant colonies revealed that the complementation plasmid integrated homologously at the hacA locus, and at least one ectopic site (data not shown).

Northern Blot Analysis and cDNA Cloning

Total RNA was extracted from overnight cultures by crushing the mycelium in liquid nitrogen and resuspending in TRI reagent LS (Molecular Research Center, Cincinnati, OH). The RNA was fractionated by formaldehyde gel electrophoresis and ribosomal RNA (rRNA) loading was visualized by SYBR-Green II staining and quantified using a STORM phosphorimagere (Molecular Dynamics). The RNA was transferred to BioBond nylon membranes (Sigma), and hybridized to a 32P-labeled DNA probe for A. fumigatus bipl, pdi, tigA or hacA. The bipl fragment was PCR-amplified from wt A. fumigatus genomic DNA using primers 494 and 495, the pdi fragment was amplified using primers 602 and 603, the tigA fragment was amplified using primers 628 and 629, and the hacA fragment was amplified with primers 492 and 493. Hybridization intensities were quantified by Phosphorimagere analysis and normalized against SYBR®-Green II-stained rRNA intensity.

The uninduced form of the hacA mRNA (hacA+) was obtained by extracting RNA from overnight cultures of A. fumigatus, and the induced form of the hacA mRNA (hacA-6) was obtained by extracting RNA from overnight cultures that were treated for 1 h with 1 mM dihydrothiouracil (DTT). Confirmation that these conditions differentially modulated the conversion of hacA+ to hacA- was obtained by Northern blot analysis prior to reverse transcription (Figure 3A). The RNA was then reverse-transcribed using the Superscript III reverse transcriptase first-strand synthesis system (Invitrogen) and primers 493 and 572. The resulting cDNAs were cloned into pCR-Blunt II-TOPO® and sequenced.

Analysis of Protein Secretion

Protease secretion in A. fumigatus was quantified using Azocoll (Calbiochem) hydrolysis as previously described [10]. Azocoll is an insoluble collagen linked to a red azo dye, and the release of the dye is indicative of collagen hydrolysis. Conidia were inoculated at a concentration of 1 x 10^7/ml in 50 ml of AMM-FBS (Aspergillus minimal medium containing 10% heat-inactivated fetal bovine serum (FBS) as the nitrogen and carbon source) [10]. The cultures were incubated at 37°C with gentle shaking at 150 rpm. After 72 h, a 1 ml aliquot of the culture was microfiltered at 15,000g for 5 min, and a 15 µl aliquot of the supernatant was added to 2.5 ml of a 5 mg/ml suspension of pre-washed Azocoll (prepared by washing and resuspending the collagen particles in buffer containing 50 mM Tris (pH 7.5), 1 mM CaCl2, and 0.01% sodium azide). The collagen/supernatant mixture was incubated at 37°C for 3 h, with constant shaking at 350 rpm. The Azocoll/supernatant mixture was centrifuged at 13,000 g for 5 min and the release of the azo dye was determined by measuring the absorbance at 520 nm. Values were normalized to the lyophilized weight of the 72 h biomass.

For analysis of total protein secretion, 2.5 x 10^7 conidia were inoculated into a 500 ml flask containing 100 ml of AMM and incubated for 3 d at 37°C without shaking. Under these conditions, the wt and ΔhacA strains generated a similar amount of dried biomass. The supernatant was removed from both cultures and concentrated to approximately 500 µl using the Amicon 8050 ultrafiltration system with a membrane cut-off of 10 kD. An equal volume of water was added to each sample and further concentrated to 100 µl using an Amicon Ultra Centrifugal Filter Device. Protein concentrations were determined using the Bradford assay and samples were stored at −80°C prior to analysis. For 1D gel analysis, each sample was mixed with sample buffer (200 mM Tris-HCl pH 6.8, 50% glycerol, 5% sodium dodecyl sulfate (SDS), 0.5% bromophenol blue and 5% (v/v) β-mercaptoethanol), heated to 95°C and 5 µl of each sample was loaded onto a 12% SDS PAGE gel. Gels were run at 150 V for ~2 h. After fixing for 30 min in 10% methanol and 7% acetic acid, gels were stained overnight with SYPRO Ruby fluorescent dye, destained for 30 min in fixing solution, then washed with water for 5–10 min prior to imaging with a GE Healthcare Typhoon scanner.
Cell Wall Analysis

Mycelial cell wall fractionation was performed according to the method described by Fontaine et al. [86] with slight modification. Briefly, wt and Δhac1 strains were grown in a 1.2-liter fermenter in liquid Sabouraud medium. After 24 h of cultivation (linear growth phase), the mycelia were collected by filtration, washed extensively with water and disrupted in a Dyno-mill (W. A. Bachofen AG, Basel, Switzerland) cell homogenizer using 0.5-mm glass beads at 4°C. The disrupted mycelial suspension was centrifuged (3,000 × g for 10 min), and the cell wall fraction (pellet) obtained was washed three times with water, subsequently boiled in 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM EDTA, 2% SDS and 40 mM β-mercaptoethanol (β-ME) for 15 min, twice. The sediment obtained after centrifugation (3,000 × g, 10 min) was washed five times with water and then incubated in 1 M NaOH containing 0.5 M NaBH₄ at 65°C for 1 h, twice. The insoluble pellet obtained upon centrifugation of this alkali treated sample (3,000 × g, 10 min, Al-fraction) was washed with water to neutrality, while the supernatant (AS-fraction) was neutralized and dialyzed against water. Both fractions were freeze-dried and stored at −80°C until further use. Hexose composition in the samples was estimated by gas-liquid chromatography using a Perichrom PR2100 Instrument (Perichrom, Saulx-les-Chartreux, France) equipped with an internal standard. Derivatized hexoses (alditol acetates) were obtained after hydrolysis (4N trifluoroacetic acid/8N hydrochloric acid, 100°C, 4 h), reduction and peracytlation. Monosaccharide composition (percent) was calculated from the peak areas with respect to that of the internal standard.

Mouse Models of Invasive Aspergillosis

For the cortisone acetate (CA) immunosuppression models, cultures were grown for 14 d on IMA agar (Difco) at 25°C. Conidia were collected by washing the agar surface with phosphate buffered saline (PBS) containing 0.05% Tween 20. The conidial suspension was filtered first through sterile gauze and then through a 12 μm filter (Millipore) before washing twice with PBS/Tween. Female 4–6-week old C57BL/6 mice were used in all experiments, with the exception of the triamcinolone model. Inoculum sizes were selected on the basis of pilot experiments with the different immunosuppression methods to determine the minimum number of wt (Δhac1) conidia that resulted in 100% mortality (not shown). Mice were immunosuppressed with CA (2 mg subcutaneously) administered on days −4, −2, 0 +2 and +4 in relation to infection, anesthetized with ketamine and xylazine and infected intratracheally with a target inoculum of 10⁶ conidia for wt or Δhac1 (n = 13) in PBS with 0.05% Tween 20. Five sham-infected mice were immune suppressed and then inoculated intratracheally with PBS containing 0.05% Tween 20. Based upon plating efficiencies, mice received 1.1 × 10⁷ of the wt or 1.2 × 10⁶ conidia of the Δhac1 strains. Statistical significance was assessed by the log rank test using Sigma Stat 3.5.

For the neutropenic model, mice were immunosuppressed with CPS (150 mg/kg intraperitoneally) and monoclonal antibody RB6-4C5 (25 μg intraperitoneally) one day before infection. CPS was then readministered three days after infection. Mice were infected intratracheally with a target inoculum of 5 × 10⁵ conidia of wt or Δhac1 (n = 13 or 12 per group, respectively) in PBS with 0.05% Tween 20. Five sham-infected mice were immunosuppressed and then inoculated intratracheally with PBS containing 0.05% Tween 20. Based upon plating efficiencies using the infecting inoculum, mice received 4.3 × 10⁵ of the wt or 5.2 × 10⁵ conidia of the Δhac1 strains. Statistical significance was assessed by the log rank test using Sigma Stat 3.5.

For the triamcinolone (TA) immunosuppression model, conidia were harvested from plates of AMM supplemented with 1.2 M sorbitol and resuspended in sterile saline. Groups of 10 CF-1 outbred female mice (20–28 g) were immunosuppressed with a single dose of TA (40 mg kg⁻¹ of body weight injected subcutaneously) on day −1. The mice were anesthetized with 3.5% isofluorane and inoculated intranasally with 2 × 10⁵ conidia from the wt, Δhac1 mutant, or the complemented strain on day 0 in a 20 μl suspension. Mortality was monitored for 15 days, and statistical significance was assessed by ANOVA using Sigma Stat 3.5.

Supporting Information

Figure S1 The Δhac1 mutant is hypersensitive to oxidative stress. 1 × 10⁶ conidia were spread evenly onto the surface of a 100 mm plate of IMA and a filter paper disk containing 15 μl of a 100 μg/ml solution of the superoxide-generating agent paraquat (methyl viologen) was placed onto the center. The plates were then incubated for 24 h at 37°C, and the zone of inhibition around the disk was used as an estimate of paraquat sensitivity. Found at: doi:10.1371/journal.ppat.1000258.s001 (2.72 MB TIF)

Figure S2 Growth rate of Δhac1 at 42°C. A 5 μl suspension of conidia was spotted onto the center of an IMA plate and colony diameter was monitored for 3 days at 42°C. Values represent the mean of triplicate plates ± SD. Colony morphology is shown after 3 days 42°C. Found at: doi:10.1371/journal.ppat.1000258.s002 (0.81 MB TIF)

Figure S3 Hypersensitivity of Δhac1 to Congo red. A 5 μl suspension of conidia was spotted into the center of each well in a multi-well plate containing IMA and the indicated concentrations of Congo red and incubated for 3 days at 37°C. The experiment was repeated with similar results. Found at: doi:10.1371/journal.ppat.1000258.s003 (5.62 MB TIF)

Figure S4 The experiment was performed as described in Fig. 8, except that IMA medium was used instead of RPMI agar, and a ketoconazole strip was used instead of a fluconazole strip. Amphotericin B (Amb), caspofungin (CS), itraconazole (IT), and ketoconazole (KE). Found at: doi:10.1371/journal.ppat.1000258.s004 (1.79 MB TIF)

Figure S5 Antifungal susceptibility of S. cerevisiae Δhac1. Yeast cultures were prepared as described in materials and methods prior to spread-plating onto YPD and overlaying with Etest strips containing caspofungin (A), ketoconazole (B), amphotericin B (C) or fluconazole (D). The plates were incubated for 48 h at 30°C. Found at: doi:10.1371/journal.ppat.1000258.s005 (9.30 MB TIF)

Acknowledgments

We thank Jay Card for photography assistance.

Author Contributions

Conceived and designed the experiments: DLR JPL MF JCR DSA. Performed the experiments: DLR LH VA MSW KKF MDM SW JWM JPL MF JCR DSA. Contributed reagents/materials/analysis tools: JPL MF JCR. Wrote the paper: DLR DSA.
References

1. Maschmeyer G, Haas A, Cornely OA (2007) Invasive aspergillosis: epidemiology, diagnosis and management in immunocompromised patients. Drugs 67: 1363–1401.

2. Morgan J, Wannemuehler KA, Ott SM, Saffitz JE, Cookson LV, et al. (2005) Incidence of invasive aspergillosis following hematopoietic stem cell and solid organ transplantation: interim results of a prospective multicenter surveillance program. Med Mycol 43 Suppl 1: S11-S17.

3. Upton A, Kirby KA, Carbone P, Bocchi M, Kerr MA (2009) Invasive aspergillosis following hematopoietic cell transplantation: outcomes and prognostic factors associated with mortality. Clin Infect Dis 49: 531–540.

4. Filler SG, Sheppard DC (2006) Fungal infection of normally non-phagocytic host cells. PLoS Pathog 2: e129. doi:10.1371/journal.ppat.0020129.

5. Tekaiha F, Latge JP (2005) *Aspergillus fumigatus*: saprophyte or pathogen? Curr Opin Microbiol 8: 385–392.

6. Williams LS, Reyes CM, Stolpman M, Speckman J, Allen K, et al. (2002) The Streptomyces virulence factor successine is a virulence determinant that regulates expression of cell wall proteins. Infect Immun 70: 19–26.

7. Lin SJ, Wilson LS, Reyes CM, Speckman J, Allen K, et al. (2006) Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clin Infect Dis 42: 657–668.

8. Robson GD, Huang J, Wiesman J, Archer DB (2005) A preliminary analysis of the process of protein secretion and the diversity of putative secreted hydrolyses encoded in *Aspergillus fumigatus*: insights from the genome. Med Mycol 43 Suppl 1: S41–S47.

9. Gifford AH, Klippenstein JR, Moore MM (2002) Serum stimulates growth of an *Aspergillus* protease secreted by *Aspergillus fumigatus*. Infect Immun 70: 681–685.

10. Berka RM, Kodama KH, Rey MW, Wilson LJ, Ward M (1991) The development of *Aspergillus* var. awamori as a host for the expression and secretion of heterologous gene products. Biochem Soc Trans 19: 591–595.

11. Berka RM, Kodama KH, Rey MW, Wilson LJ, Ward M (1991) The development of *Aspergillus* var. awamori as a host for the expression and secretion of heterologous gene products. Biochem Soc Trans 19: 591–595.

12. Nyyssonen E, Penttila M, Harkki A, Saloheimo A, Knowles JK, et al. (1993) Efficient production of antibody fragments by the filamentous fungus *Aspergillus fumigatus*. J Biotechnol 1: 63–71.

13. Ron D, Walter P (2007) Signal integration in the endoplasmic reticulum. Science 318: 1242–1247.

14. Romanos MA, Scorer CA, Clare JJ (1992) Foreign gene expression in yeast: a review. Yeast 8: 391–404.

15. Muller HJ, Saloheimo M, Madrid SM (2004) The transcription factor HACA mediates the unfolded protein response in *Aspergillus fumigatus*, and upregulates its own transcription. Fungal Genet Biol 41: 130–140.

16. Muller HJ, Nikelaev I, Madrid SM (2006) HACA, the transcriptional activator of the unfolded protein response (UPR) in *Aspergillus fumigatus*, binds to partly palindromic UPR elements of the consensus sequence 5'-CAN/G-AANTG/T-3'. Fungal Genet Biol 43: 560–572.

17. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277–2293.

18. Agarwal AK, Rogers PD, Barson SR, Jacob MR, Barker KS, et al. (2005) Genome-wide expression profiling of the response to azole, pyrrole and pyrimidine antifungal agents in *Candida albicans*. Antimicrob Agents Chemother 49: 2226–2236.

19. Douglas CM (2006) Understanding the microbiology of the *Aspergillus* cell wall and the efficacy of caspofungin. Med Mycol 44 Suppl 95: 99–107.

20. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, et al. (2000) The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. Semin Cell Dev Biol 11: 59–69.

21. Sidrauski C, Walter P (1997) The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. Cell 132: 89–100.

22. Rementeria A, Lopez-Molina N, Ludwig A, Vivanco AB, Bikandi J, et al. (2005) Genes and molecules involved in *Aspergillus fumigatus* fungivorous behaviour. Rev Iberoam Micr 22: 1–23.

23. Sikes S, Xu R, Nguyen CK, Chamulis G, Koutsioupiannis DP, et al. (2008) Gliotoxin production in *Aspergillus fumigatus* contributes to host-specific differences in virulence. J Infect Dis 197: 479–486.

24. Sugui JA, Pardo J, Chiang YC, Zarember KA, Nardone G, et al. (2007) Gliotoxin is a virulence factor of *Aspergillus fumigatus*: gliP deletion attenuates virulence in mice immunosuppressed with hydrocortisone. Eukaryot Cell 6: 1562–1569.

25. Matsumoto R, Akama K, Rakaval R, Iwahashi H (2007) The stress response against denatured proteins in the deletion of cytosolic chaperones SSA1/2 is different from heat-shock response in *Saccharomyces cerevisiae*. BMC Genomics 6: 141.

26. Agarwal AK, Rogers PD, Barson SR, Jacob MR, Barker KS, et al. (2005) Genome-wide expression profiling of the response to azole, pyrrole and pyrimidine antifungal agents in *Candida albicans*. Antimicrob Agents Chemother 49: 2226–2236.

27. Brewer JW, Enderstrom LM (2005) Building an antibiotic factory: a job for the unfolded protein response. Nat Immunol 6: 23–29.

28. Chavira JR, S Domestico M, Hagerman JH (1984) Assaying proteinases with ascomycetous fungi. Curr Microbiol 10: 155–159.

29. Agarwal AK, Rogers PD, Barson SR, Jacob MR, Barker KS, et al. (2005) Genome-wide expression profiling of the response to azole, pyrrole and pyrimidine antifungal agents in *Candida albicans*. Antimicrob Agents Chemother 49: 2226–2236.

30. Agarwal AK, Rogers PD, Barson SR, Jacob MR, Barker KS, et al. (2005) Genome-wide expression profiling of the response to azole, pyrrole and pyrimidine antifungal agents in *Candida albicans*. Antimicrob Agents Chemother 49: 2226–2236.

31. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277–2293.

32. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277–2293.

33. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277–2293.

34. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277–2293.

35. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277–2293.

36. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277–2293.

37. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277–2293.

38. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277–2293.

39. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9: 2277–2293.
56. Reimold AM, Ekin A, Claus I, Perkins A, Friend DS, et al. (2000) An essential role in liver development for transcription factor XBP-1. Genes Dev 14: 152–157.

57. Iwakoshi NN, Lee AH, Vallabhajosyula P, Orjolo KI, Rajewsky K, et al. (2003) Plasma cell differentiation and the unfolded protein response interact at the transcription factor XBP-1. Nat Immunol 4: 321–329.

58. Schwienbacher M, Weig M, Thies S, Regula JT, Heinemann J, et al. (2005) Analysis of the major proteins secreted by the human opportunistic pathogen Aspergillus fumigatus under in vitro conditions. Med Mycol 43: 623–630.

59. Galagan JE, Calvo SE, Cuomo C, Ma LJ, Wortman JR, et al. (2005) Sequencing and analysis of Aspergillus oryzae genome. Nature 438: 1105–1115.

60. Machida M, Asai K, Sano M, Tanaka T, Kumaqai T, et al. (2005) Genome sequencing and analysis of Aspergillus oryzae. Nature 438: 1157–1161.

61. Durand H (1988) Classical and molecular genetics applied to Trichoderma reesei for selection of improved cellulolytic industrial strains. In: Aubert J-P, Beguin P, Millot P, eds. Biochemistry and genetics of cellulase degradation. London: Academic Press. pp 135–152.

62. Valkonen M, Ward M, Wang H, Penttila M, Saloheimo M (2003) Improvement of foreign-protein production in Aspergillus niger and comparative analysis with A. fumigatus and A. oryzae. Nature 438: 1105–1115.

63. Fujioka T, Mizutani O, Furukawa K, Sato N, Yoshimi A, et al. (2007) MpkA controls the transcription factor XBP-1. Nat Immunol 4: 321–329.

64. Patil CK, Li H, Walter P (2004) Gcn4p and novel upstream activating sequences of the human unfolded-protein response. J Biol Chem 279: 30333–30342.

65. Pakula TM, Laxell M, Huuskonen A, Uusitalo J, Saloheimo M, et al. (2003) The effect of the unfolded protein response upon genome-wide expression patterns, and the role of Hac1 in the polarized growth, of Aspergillus fumigatus. Fungal Genet Biol 38: 98–109.

66. Al-Sheikh H, Watson AJ, Lacey GA, Punt PJ, MacKenzie DA, et al. (2004) Evidence for down-regulation of genes that encode secreted proteins in the filamentous fungus Aspergillus niger var. awamori by constitutive induction of the unfolded-protein response. Appl Environ Microbiol 70: 6979–6986.

67. Wimalasena TT, Enjalbert B, Guillemette T, Plumridge A, Budge S, et al. (2008) Analysis of the major proteins secreted by the human opportunistic pathogen Aspergillus fumigatus under in vitro conditions. Med Mycol 43: 623–630.

68. Catlett NL, Lee B-N, Yoder OC, Turgeon BG (2002) Split-marker recombination for efficient targeted deletion of fungal genes. Fungal Genet Biol 38: 98–109.

69.(Fontaine T, Simenel C, Dubreucq G, Adam O, Delepierre M, et al. (2000) Secretory organelles of pathogenic protozoa. An Acad Bras Cienc 78: 271–291.

70. Reinoso-Martin C, Schuller C, Schutzer-Mollabauer M, Kuchler K (2003) The yeast protein kinase C cell integrity pathway mediates tolerance to the antifungal drug caspofungin through activation of Slt2p mitogen-activated protein kinase signaling. Environ Cell Biol 2: 1268–1277.

71. Reimold AM, Ekin A, Claus I, Perkins A, Friend DS, et al. (2000) An essential role in liver development for transcription factor XBP-1. Genes Dev 14: 152–157.

72. Pfaffer MA, Messer SA, Hollis RJ, Jones RN (2002) Antifungal activities of posaconazole, ravuconazole, and voriconazole compared to those of itraconazole and amphotericin B against 239 clinical isolates of Aspergillus spp. and other filamentous fungi: report from SENTRY Antimicrobial Surveillance Program, 2000. Antimicrob Agents Chemother 46: 1032–1037.

73. Singh J, Rinek D, Kappe R (2006) Intrinsinc in vitro susceptibility of primary clinical isolates of Aspergillus fumagatus, Aspergillus terreus, Aspergillus nidulans, Candida albicans and Candida lusitaniae against amphotericin B. Mycoses 49: 96–103.

74. Araus R, Pinz-Vaz C, Rodrigues AG (2007) Susceptibility of environmental versus clinical strains of pathogenic Aspergillus. Int J Antimicrob Agents 29: 101–111.

75. Lass-Floerl C, Griff K, Mayr A, Petrez A, Gasti G, et al. (2005) Epidemiology and outcome of infections due to Aspergillus terreus: 10-year single centre experience. Br J Haematol 131: 201–207.

76. Spanakos EK, Ageris, G, Mylonakis E (2006) New agents for the treatment of fungal infections: clinical efficacy and gaps in coverage. Clin Infect Dis 43: 1069–1080.

77. Braun VM, Kalachakov S, Sobaran R, Nobile GJ, Kyrsouso C, et al. (2006) Control of the C. albicans cell wall damage response by transcriptional regulator Cas5. PLoS Pathog 2: e21. doi:10.1371/journal.ppat.0020021.

78. Reimoso-Martín C, Schüller C, Schutzer-Mollabauer M, Kuchler K (2003) The yeast protein kinase C cell integrity pathway mediates tolerance to the antifungal drug caspofungin through activation of Slt2p mitogen-activated protein kinase signaling. Environ Cell Biol 2: 1268–1277.

79. Bruno VM, Kalachakov S, Sobaran R, Nobile GJ, Kyrsouso C, et al. (2006) Control of the C. albicans cell wall damage response by transcriptional regulator Cas5. PLoS Pathog 2: e21. doi:10.1371/journal.ppat.0020021.

80. Reimoso-Martín C, Schüller C, Schutzer-Mollabauer M, Kuchler K (2003) The yeast protein kinase C cell integrity pathway mediates tolerance to the antifungal drug caspofungin through activation of Slt2p mitogen-activated protein kinase signaling. Environ Cell Biol 2: 1268–1277.

81. Souza W (2006) Secretory organelles of pathogenic protozoa. An Acad Bras Cienc 78: 271–291.

82. Armstrong PB (2006) Proteases and protease inhibitors: a balance of activities in host-pathogen interaction. Immunobiology 211: 263–281.

83. Cove DJ (1966) The induction and repression of nitrate reductase in the fungus Aspergillus nidulans. Biochim Biophys Acta 113: 51–56.

84. Catlett NL, Lee B-N, Yoder OC, Turgeon BG (2002) Split-marker recombination for efficient targeted deletion of fungal genes. Fungal Genet Biol 38: 98–109.

85. Bhabhra R, Miley MD, Mylonakis E, Boettner D, Fortwendel J, et al. (2004) Methylcitrate synthase from Trichoderma reesei is essential for manifestation of invasive aspergillosis. Cell Microbiol 10: 134–148.

86. Fontaine T, Simenel C, Dubreucq G, Adam O, Delepierre M, et al. (2000) Secretory organelles of pathogenic protozoa. An Acad Bras Cienc 78: 271–291.

87. Brunet J (1966) The induction and repression of nitrate reductase in the fungus Aspergillus nidulans. Biochim Biophys Acta 113: 51–56.

88. Carlett NL, Lee B-N, Yoder OC, Turgeon BG (2002) Split-marker recombination for efficient targeted deletion of fungal genes. Fungal Genet News 50: 9–11.

89. Bhaktha R, Miley MD, Mylonakis E, Boettner D, Fortwendel J, et al. (2004) Disruption of the Aspergillus fumigatus gene encoding nucleolar protein GisA impairs thermotolerant growth and reduces virulence. Infect Immun 72: 4731–4740.

90. Fontaine T, Šimunec C, Dubreucq G, Adam O, Delpeyrre M, et al. (2000) Molecular organization of the alkaline-insoluble fraction of Aspergillus fumigatus cell wall. J Biol Chem 275: 27094–27097.