Inactivation of Transcription Factor Gene ACE2 in the Fungal Pathogen Candida glabrata Results in Hypervirulence

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During an infection, the coordinated orchestration of many factors by the invading organism is required for disease to be initiated and to progress. The elucidation of the processes involved is critical to the development of a clear understanding of host-pathogen interactions. For Candida species, the inactivation of many fungal attributes has been shown to result in attenuation. Here we demonstrate that the Candida glabrata homolog of the Saccharomyces cerevisiae transcription factor gene ACE2 encodes a function that mediates virulence in a novel way. Inactivation of C. glabrata ACE2 does not result in attenuation but, conversely, in a strain that is hypervirulent in a murine model of invasive candidiasis. C. glabrata ace2 null mutants cause systemic infections characterized by fungal escape from the vasculature, tissue penetration, proliferation in vivo, and considerable overstimulation of the proinflammatory arm of the innate immune response. Compared to the case with wild-type fungi, mortality occurs much earlier in mice infected with C. glabrata ace2 cells, and furthermore, 200-fold lower doses are required to induce uniformly fatal infections. These data demonstrate that C. glabrata ACE2 encodes a function that plays a critical role in mediating the host-Candida interaction. It is the first virulence-moderating gene to be described for a Candida species.

The number of invasive life-threatening infections caused by the members of the fungal genus Candida has increased dramatically over the past 20 years (1, 2). Candida species now cause more bloodstream infections than streptococci, Enterococcus faecalis, and all individual gram-negative bacterial species, including Escherichia coli (30). Indeed, candidemia now accounts for 10 to 15% of all bloodstream infections (18), one of the top 15 causes of mortality in the United States, resulting in over 200,000 deaths per year (24). Given the continuing use of broad-spectrum antibiotics and indwelling intravenous catheters, the two major risk factors for the development of candidemia, plus the inexorable rise of at-risk patients, it seems unlikely that this threat will diminish (6). Candida species thus exert a significant clinical and economic impact.

Candida albicans remains the most commonly isolated species, but Candida glabrata now accounts for almost 20% of all cases of systemic candidiasis (6) and 30% of urinary tract infections (3). This increase in the incidence of C. glabrata infections is a major cause for concern, as this species is often more resistant to antifungal agents and results in a higher overall mortality rate than other Candida species (11, 26).

Analyses of Candida virulence facilitate the elucidation of critical aspects of the host-pathogen interaction, which may result in the development of better options for therapeutic and/or diagnostic interventions. The majority of analyses have focused on C. albicans, although we have recently shown that the transcriptional regulator Ste12 plays a role in C. glabrata virulence (5). These investigations have demonstrated that genes which encode functions in many diverse areas of Candida biology, including environmental sensing, transcriptional responsiveness, adhesion, and morphogenesis, are crucial for the initiation and/or progression of candidiasis (14, 21). Like most microbial virulence studies, these experiments have focused on the identification of traits that are essential for virulence. The converse has not been explored, i.e., the identification of genes whose inactivation results in an augmented ability to cause disease. These so-called antivirulence genes have recently been described as crucial determinants of the host-pathogen interaction for several different microbial species (9). We prefer and will use hereafter the term “virulence moderating.” The inactivation of virulence-moderating genes can result in an enhanced capacity of the organism to cause tissue damage, to increase in vivo proliferation, and to require lower lethal doses, resulting in more severe disease and higher mortality rates (16, 29). These virulence-moderating genes represent a new and exciting aspect of microbial virulence. Their
identification and subsequent functional characterization in *Candida* species may have the power to reveal novel insights into host-fungus interactions.

In order to perform a global analysis of *C. glabrata* virulence that would reveal potential virulence-moderating genes and genes encoding functions essential for virulence, we constructed a 9,600-member signature tag (15) library of insertional mutants (19). A preliminary analysis of this library revealed the presence of a strain (10G6) with an apparent increased ability to persist in vivo in a murine model of candidiasis (5). We hypothesized that this strain may contain a disruption of a *C. glabrata* virulence-moderating gene. Our objective for this study was to test this hypothesis. Specifically, we sought to determine the site of plasmid insertion in *C. glabrata* 10G6, to construct independent null and reconstituted mutants of any disrupted gene, and to determine the impact of these mutations on the ability of *C. glabrata* to cause disease. Our data show that *C. glabrata* 10G6 has an inactivated allele of the *C. glabrata* homolog of the *Saccharomyces cerevisiae* transcripational activator-encoding gene ACE2. Furthermore, we demonstrate that *C. glabrata* ace2 mutants are hypervirulent in a murine model of candidiasis. This hypervirulence is independent of the ace2 clumpy growth phenotype, at the time of infection, and is accompanied by a massive overstimulation of the proinflammatory arm of the innate immune response. *C. glabrata* ACE2 is thus the first virulence-moderating gene to be reported for a *Candida* species.

**MATERIALS AND METHODS**

*C. glabrata* strains and growth media. All strains used for this study are listed in Table 1. Fungal cells were routinely cultured in YAPD (2% [wt/vol] peptone, 2% [wt/vol] glucose, 1% [wt/vol] yeast extract, 0.01% [wt/vol] adenine) or SD (0.17% [wt/vol] yeast nitrogen base without amino acids [Difco], 2% [wt/vol] glucose, 1% [wt/vol] yeast extract, 0.01% [wt/vol] adenine) and an appropriate dropout mix [Clontech, Basingstoke, United Kingdom] (0.17% [wt/vol] yeast nitrogen base without amino acids [Difco], 2% [wt/vol] glucose, and an appropriate dropout mix [Clontech, Basingstoke, United Kingdom]) medium at 30 or 37°C. For solid media, 2% (wt/vol) agar was added prior to autoclaving. Other additives were filter sterilized and added at appropriate concentrations to the media after autoclaving.

Construction and preliminary analysis of a *C. glabrata* STM library. To undertake a functional analysis of virulence in *C. glabrata*, we constructed a 9,600-member STM insertional mutant library (19). Briefly, a pool of signature tags with the overall sequence CTAGTGTACCTAACACACTCAAGCTC (NC)19

| C. glabrata strain | Genotype or description | Source or reference |
|-------------------|------------------------|---------------------|
| ATCC 2001         | Type strain            | American Type Culture Collection |
| AH1               | his3::URA3 Δura3       | 28                   |
| AH76              | his3::URA3 Δura3 Δnap1 | 19                   |
| HLS120            | his3::URA3 Δura3 Δnap1 Δace2::HIS3 | This study |
| HLS122            | his3::URA3 Δura3 Δnap1 Δace2::HIS3(pCG ACT-14 [TRP1]) | This study |
| HLS121            | his3::URA3 Δura3 Δnap1 Δace2::HIS3(pKH355 [ACE2 TRP1]) | This study |

*pTW23 was inserted between nucleotides 452 and 453 of ACE2 in this strain.*

**TABLE 1. Fungal strains used for this study**

Virulence analysis of *C. glabrata* ace2 strains. Virulence analysis was performed essentially as previously described (5). Briefly, groups of up to 12 outbred CD1 mice were immunosuppressed with 200 mg of cyclophosphamide/kg of body weight on day −3 and every fourth day thereafter. Animals were infected with appropriate doses of *C. glabrata* blastospores in 20 μl of saline (as described in the legends to Fig. 1B and 2B) via tail vein injection.

For the preparation of *C. glabrata* cells for inoculation, fungi were cultured overnight in 50 ml of YAPD medium or YAPD medium containing 10% (vol/vol) glycerol in 1-liter flasks at 30°C and 180 rpm. Viability was determined for all cultures by trypan blue exclusion and was determined to be >99.9%. *C. glabrata* ATCC 2001 and HLS121 cells were enumerated with a hemocytometer. For determination of the concentration of *C. glabrata* HLS122 cells, cultures were vortexed with 0.45-mm-diameter glass beads for 45 s. This resulted in disruption of the large clumps of cells seen for the *C. glabrata* ace2 mutant (Fig. 1A and 2A) and gave groups of 10 to 20 cells that could be enumerated with a hemocytometer. The original cultures were washed and then diluted in saline to 100 micrometer plates such that each plate contained 96 *C. glabrata* transformants, with each carrying 1 of the 96 tags.

An initial screening of this library revealed a mutant that had an altered colony morphology and an apparent increased ability to persist in vivo (19). This mutant was designated *C. glabrata* 10G6.

Characterization of the site of insertion in *C. glabrata* 10G6. Plasmid rescue was used to identify the site of pTW23 insertion in *C. glabrata* 10G6. Genomic DNA was prepared from *C. glabrata* 10G6, and aliquots of 100 ng were digested to completion with Apal, NotI, SacI, Smal, and Xhol. These digested DNA populations were self-ligated and used to transform E. coli XL-10. Ampicillin-resistant colonies were selected, plasmids were recovered, and those that contained flanking DNA were identified. Flanking DNA was sequenced from the backbone M13 forward and reverse primer sites.
in small groups. Cells were extensively washed and then counted and diluted to appropriate concentrations in saline prior to inoculation. *C. glabrata* ATCC 2001 and HLS121 were treated in the same way.

**Analysis of immune modulators.** Groups of four neutropenic outbred CD1 mice were infected with $7 \times 10^7$ chitinase-treated *C. glabrata* wild-type (ATCC 2001) or ace2 (HLS122) cells in 200 μl of saline. Four mice were also injected with saline only. All mice were sacrificed at 18 h postinfection and their blood was collected. Sera were separated from the blood and were stored at $-20^\circ$C until assayed. Interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) levels were determined by use of a CBA mouse inflammation array (BD Biosciences) by the Cell Analysis Facility, Centre for Molecular Microbiology, Imperial College London.

**Nucleotide sequence accession number.** ACE2 sequence data have been submitted to the EMBL database under accession number AJ630371.

**RESULTS**

*C. glabrata* 10G6 has an inactivated ACE2 allele. A total of 327 nucleotides flanking the site of pTW23 insertion were identified in *C. glabrata* 10G6 by sequencing of plasmids rescued from this strain. A 258-bp fragment of this sequence was amplified from *C. glabrata* ATCC 2001 DNA with the primers GAGACTTGAATATGAAATGCG and TGTTTCAGAGACTTGCC. The resultant PCR product was used to screen a *C. glabrata* genomic library constructed in YEp24 (a kind gift from Dominique Sanglard, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). Three clones containing 5.4-kb inserts were obtained, and one was sequenced on both strands at the automated DNA sequencing facility at Imperial College London. This clone contained an uninterrupted 2,112-bp open reading frame, and a comparison with the sequence carried by plasmids rescued from *C. glabrata* ACE2 (HLS121); and viewed by differential interference microscopy. (B) Clumpy *C. glabrata* ace2 cells are hypervirulent. Three groups of 12 outbred male CD1 mice were immunosuppressed with cyclophosphamide (200 mg/kg every fourth day), and each mouse was infected with $7 \times 10^7$ *C. glabrata* cells in 200 μl of saline. ▲, *C. glabrata* ace2 (HLS122); ○, *C. glabrata* ACE2 (HLS121); ▪, *C. glabrata* wild type (ATCC 2001). Fungi were not digested with chitinase prior to inoculation.

FIG. 1. Phenotypes of *C. glabrata* ace2 cells. (A) *C. glabrata* ace2 cells fail to separate. All strains were cultured for 18 h in liquid SC medium without tryptophan at 37°C, vortexed vigorously for 2 min, and viewed by differential interference microscopy. (B) Clumpy *C. glabrata* ace2 cells are hypervirulent. Three groups of 12 outbred male CD1 mice were immunosuppressed with cyclophosphamide (200 mg/kg every fourth day), and each mouse was infected with $7 \times 10^7$ *C. glabrata* cells in 200 μl of saline. ▲, *C. glabrata* ace2 (HLS122); ○, *C. glabrata* ACE2 (HLS121); ▪, *C. glabrata* wild type (ATCC 2001). Fungi were not digested with chitinase prior to inoculation.

2A). We were concerned that the increased virulence phenotype of *C. glabrata* ace2 cells was therefore caused by vascular occlusion initiated at the time of infection. To test this hypothesis, we developed a chitinase digestion protocol that enables the separation of *C. glabrata* HLS122 cells (Fig. 2A). These separated *C. glabrata* ace2 yeast cells were compared with chitinase-digested wild-type *C. glabrata* ATCC 2001 and reconstituted ACE2 HLS121 cells for the ability to cause disease in a neutropenic murine model. These experiments revealed that the increased virulence of separated *C. glabrata* ace2 cells was even more marked than that of the untreated clumpy HLS122 cells (Fig. 2B). Infection with $7 \times 10^7$ chitinase-treated *C. glabrata* ace2 cells resulted in 100% mortality after 18 h, compared to 4 days with an equivalent dose of untreated clumpy ace2 cells ($P < 0.05$ by Kaplan-Meier log rank analysis); 100% mortality after 3 days with a dose of $1 \times 10^7$ cells; and 70% mortality within 5 days with a dose of $1 \times 10^6$ cells (Fig. 2B).

Surprisingly, chitinase-treated wild-type cells had an increased virulence phenotype compared to untreated *C. glabrata* ATCC 2001 cells. However, chitinase-treated separated *C. glabrata* ace2 cells were still more virulent than similarly treated wild-type or reconstituted ACE2 cells. A dose of $7 \times 10^7$ chitinase-treated wild-type or reconstituted ACE2 cells caused 100%
were infected with single or small groups of *C. glabrata* ace2 mutants are hypervirulent. Groups of eight outbred male CD1 mice were immunosuppressed with cyclophosphamide (200 mg/kg every fourth day). Mice in each group were infected with 7 \times 10^7 wild-type cells.

In all organs, the tissue parenchyma was invaded and fungi in infections with similarly treated wild-type cells (Fig. 3A to D). A histological examination of tissues (Fig. 3) revealed that organs from mice infected with separated *C. glabrata* ace2 cells were not restricted to the bloodstream but escaped into the tissue parenchyma. We also demonstrated that chitinase-digested wild-type cells resulted in 100% mortality within 18 h, compared to 5% mortality after 3 days (survival curves not shown), compared with 18 h for the *C. glabrata* ace2 strain HLS122 (*P* < 0.05 by Kaplan-Meier log rank analysis). Similarly, a dose of 10^8 chitinase-treated wild-type or reconstituted *ACE2* cells resulted in no mortality during the study period (Fig. 2B), compared with the 70% mortality for *C. glabrata* ace2 mutant-infected animals (*P* < 0.05 by Kaplan-Meier log rank analysis). Infection with 2 \times 10^6 wild-type *C. glabrata* ATCC 2001 cells is required to cause 100% mortality within 6 days (5). These data demonstrate that *C. glabrata* ace2 cells can cause a mortality rate similar to that caused by wild-type or reconstituted *ACE2* cells at almost 200-fold lower doses. *C. glabrata* ace2 mutants are therefore hypervirulent, and importantly, these data demonstrate that this enhanced virulence is separable at the time of infection, from the ace2 clumpy growth phenotype.

**C. glabrata** ace2 hypervirulence is not due to vascular occlusion. A histological examination of tissues (Fig. 3) revealed that organs from mice infected with separated *C. glabrata* ace2 cells had much higher tissue burdens than those seen during infections with similarly treated wild-type cells (Fig. 3A to D). In all organs, the tissue parenchyma was invaded and fungi were not restricted to the vasculature (Fig. 3E). Histology also revealed that *C. glabrata* ace2 cells can proliferate in vivo. Mice were infected with single or small groups of *C. glabrata* ace2 cells, but microcolonies formed in the tissues (Fig. 3C to E). Interestingly, little or no cellular inflammatory response was seen around sites of either wild-type or *C. glabrata* ace2 infection (Fig. 3F). These data demonstrate that *C. glabrata* ace2 cells are not restricted to the bloodstream and therefore that hypervirulence does not result simply from vascular occlusion.

**C. glabrata** ace2 mutants overstimulate the proinflammatory arm of the innate immune response. Minimal IL-6, TNF-α, and IFN-γ levels were detected in sera collected after 18 h from neutropenic CD1 mice injected with saline (Table 2). Levels of the classic proinflammatory cytokines IL-6 and TNF-α were 16-fold (*P* < 0.05; t test) and 38-fold (*P* < 0.05; t test) higher, respectively, in mice infected with separated *C. glabrata* HLS122 ace2 mutant cells than in animals infected with chitinase-treated wild-type cells. The level of IFN-γ was 4.15-fold (*P* < 0.05; t test) higher in mice infected with wild-type *C. glabrata* than in animals infected with the ace2 mutant HLS122. This analysis suggests that *C. glabrata* ace2 cells can induce severe sepsis and that this may underpin the hypervirulence phenotype.

**DISCUSSION**

This study demonstrates that the inactivation of *C. glabrata* *ACE2* results in a strain that is hypervirulent in a murine model of candidiasis. Infection with *C. glabrata* ace2 cells results in an overwhelming, fatal, systemic disease. The infectious dose required to obtain 100% mortality is 200-fold lower for *C. glabrata* ace2 mutants than for wild-type cells. However, *C. glabrata* ace2 mutants do not undergo cell separation after cell division, instead forming large clumps of cells (Fig. 1A and 2A). We were concerned that the increased virulence phenotype of *C. glabrata* ace2 cells was therefore caused by vascular occlusion initiated at the time of infection. In *S. cerevisiae*, ACE2 regulates the expression of the chitinase gene *CTS1* (8). In the absence of ACE2, *CTS1* is not expressed, the chitin ring laid around the site of mother-daughter budding is not degraded after cell division, and daughters do not separate from their mothers (23). We were unable to detect a *CTS1* transcript in *C. glabrata* HLS122 cells by Northern blot analysis (data not shown), so it is likely that the failure of these cells to separate is also caused by a reduction in *CTS1* expression. We therefore developed a chitinase digestion protocol that enables the separation of *C. glabrata* HLS122 cells (Fig. 2A). These separated *C. glabrata* ace2 yeast cells were compared with similarly treated wild-type *C. glabrata* ATCC 2001 and reconstituted *ACE2* HLS121 cells for the ability to cause disease in a neutropenic murine model. The hypervirulence phenotype of separated *C. glabrata* ace2 cells was even more marked than that of untreated clumpy HLS122 cells (Fig. 1B and 2B). Separated cells resulted in 100% mortality within 18 h, compared to 5 days when an equivalent dose of clumpy *C. glabrata* ace2 cells was inoculated. Histological analysis revealed that *C. glabrata* cells were not restricted to the vasculature but escaped into the tissue parenchyma. We also demonstrated that chitinase-digested wild-type *C. glabrata* ATCC 2001 cells had an increased virulence phenotype compared with untreated cells. It is therefore possible that the increase in virulence of separated ace2 cells compared to clumpy ace2 cells was at least partially due to the chitinase treatment. This possibility notwithstanding, our
FIG. 3. Histological appearance of *C. glabrata* candidiasis. Representative appearances of the liver (A) and lungs (B) of mice infected with wild-type *C. glabrata* (ATCC 2001) cells demonstrate that these tissues are only sparsely colonized with fungi. Only a few single blastospores or very small microcolonies are visible (arrows). Representative appearances of the liver (C) and lungs (D) of mice infected with *C. glabrata ace2* (HLS122) cells demonstrate that these tissues have a substantial fungal burden. Many microcolonies are visible in each field, demonstrating the increased proliferation of *C. glabrata ace2* cells in vivo. (E) Representative appearance of *C. glabrata ace2* cells in the liver showing invasion of the tissue parenchyma and escape from the vasculature. (F) Representative appearance of *C. glabrata ace2* cells in the lungs demonstrating the lack of a cellular inflammatory reaction around *C. glabrata ace2* microcolonies (arrows). All tissues were recovered from neutropenic CD1 mice culled after 18 h, except for the tissue shown in panel B, which was from a mouse culled after 4 days. The tissues shown in panels A and B were from mice infected with $2 \times 10^8$ *C. glabrata* ATCC 2001 cells. Tissues shown in panels C to F were from mice infected with $7 \times 10^7$ *C. glabrata ace2* cells. All cells were treated with chitinase prior to infection. The sections in panels A to E were stained with Grocott and Light Green, and the section in panel F was stained with hematoxylin and eosin.
observations suggest that the increased virulence phenotype of C. glabrata ace2 mutants is not due simply to vascular occlusion but is a result of a change in an attribute of these cells and/or the host response to them. The elucidation of this attribute and the host changes that may be induced is a priority and has the potential to reveal novel insights into the host-C. glabrata interaction. Taken together, these data support the view that C. glabrata ACE2 encodes a virulence-modulating gene (13), the first to be described for a Candida species.

The potent activation of pattern recognition receptors that mediate innate and adaptive immune responses can lead to overwhelming septicemia; rising levels of the proinflammatory cytokines IL-6 and TNF-α can reflect their activation (7, 17). S. cerevisiae strains with inactivated SSD1 alleles induce threefold higher levels of TNF-α and IL-6 from peritoneal macrophages than do wild-type cells. This is accompanied by major alterations in the cell wall composition and by increased virulence in DBA/2 mice (29). Similarly, a proteomic analysis of C. glabrata revealed >200 differences between wild-type and ace2 cells (A. J. Brown, B. Dujon, and K. Haynes, unpublished data). These data demonstrate that C. glabrata ACE2 plays a role in the regulation of (among other things) protein folding and degradation and cell wall biogenesis. We therefore speculated that the inactivation of C. glabrata ACE2 may result in the appearance (or exposure) of a fungal component that can potently and inappropriately activate pattern recognition receptors. To investigate this hypothesis, we determined the levels of circulating IL-6, TNF-α, and IFN-γ at 18 h postinfection with chitinase-treated ace2 or wild-type C. glabrata cells (Table 2). Both IL-6 and TNF-α are known to be critical for the host defense against Candida species (22). The acute induction of IL-6 and TNF-α is known to reflect the severity of infection in humans (10, 13). It is also a typical consequence of severe sepsis (23). The lack of IFN-γ induction is also consistent with severe sepsis but suggests lymphocyte apoptosis rather than necrosis (27). Our observations are consistent with the hypothesis that C. glabrata ace2 cells induce severe sepsis. This provides a testable mechanistic explanation for the hypervirulence phenotype of C. glabrata ace2 cells.

Why has C. glabrata ACE2 been retained when its deletion results in such a dramatic increase in virulence and concomitant ability to proliferate in vivo? Ace2 is not an essential protein, but it does play a crucial role in cell cycle progression and is required for cell separation (23, 25). Many fungal mother and daughter cells, including those of the pathogens Aspergillus fumigatus and hyphal C. albicans, do not separate after cell division. However, these species do have efficient methods to ensure successful dispersal. A. fumigatus produces asexual conidia in prodigious numbers, while C. albicans hyphae can give rise to yeast cells that are able to separate from their progenitor (12). The only known method for C. glabrata to effect dispersal is via the separation of mother and daughter cells. This is prevented in ace2 mutants and provides, in addition to the many other advantageous functions of Ace2, a compelling evolutionary rationale for the retention of the ACE2 gene.

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