ER stress response mechanisms in the pathogenic yeast Candida glabrata and their roles in virulence

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The maintenance of endoplasmatic reticulum (ER) homeostasis is critical for numerous aspects of cell physiology. Eukaryotic cells respond to the accumulation of misfolded proteins in the ER (ER stress) by activating the unfolded protein response (UPR), an intracellular signaling pathway that adjusts the folding capacity of the ER. Recent studies of several pathogenic fungi have revealed that the UPR is important for antifungal resistance and virulence; therefore, the pathway has attracted much attention as a potential therapeutic target. While the UPR is highly conserved among eukaryotes, our group recently discovered that the pathogenic yeast Candida glabrata lacks the typical fungal UPR, but possesses alternative mechanisms to cope with ER stress. This review summarizes how C. glabrata responds to ER stress and discusses the impacts of ER quality control systems on antifungal resistance and virulence.

Introduction

The endoplasmatic reticulum (ER) is involved in important cellular functions, including the processing of secreted proteins, membrane synthesis, and calcium storage. The vast majority of secretory and transmembrane proteins are folded and assembled in the ER lumen before being delivered to the cell surface or outside the cell.1 Increased demand of protein secretion overloads the ER with unfolded proteins, causing a condition termed ER stress. Unrelieved ER stress has detrimental consequences on eukaryotic cells. For instance, in humans, ER stress has been implicated in the pathology of various diseases, such as metabolic diseases, neurodegenerative disorders, and cancer.2,3

To maintain ER homeostasis, the cells activate a series of adaptive responses that are collectively termed the unfolded protein response (UPR). Many aspects of the UPR are broadly conserved across eukaryotes. The ER-resident transmembrane kinase/endoribonuclease Ire1 senses the accumulation of misfolded proteins in the ER and transmits a signal to the nucleus by splicing the mRNAs encoding basic-leucine zipper (bZIP) transcription factors (Hac1 in yeast, HacA in filamentous fungi, and Xbp1 in humans). After processing and translation of the mRNA, the transcription factor activates the UPR target genes, including those that function in the ER protein-folding machinery and ER-associated degradation.4,5 Recent studies on several pathogenic fungi have unveiled that the UPR plays important roles in fungal virulence and antifungal resistance; therefore, the pathway has been attracting attention as a potential target for antifungal therapy.6-11 While it is believed that the UPR is an evolutionarily conserved mechanism in eukaryotes, we recently discovered unexpected aspects of ER stress response mechanisms in the pathogenic yeast Candida glabrata.12 In this review, we describe the unique evolution of ER quality control systems in C. glabrata and its impact on antifungal resistance and virulence, through comparisons with other pathogenic fungi.

Pathogenicity of C. glabrata

C. glabrata has emerged as an important fungal pathogen in clinical practice, partly because of its decreased susceptibility to azole antifungals such as fluconazole.13,14 A recent study on the epidemiology and outcomes of candidemia in 3648 patients reported that C. glabrata is the second most common cause of candidemia after Candida albicans (26.7% vs. 42.1%), with a lower 90-d post-diagnosis survival rate, compared with C. albicans infection (57.6% vs. 62.1%).15 Echinocandin-class antifungals are the treatment of choice for candidemia with C. glabrata; however, recent surveillance data have revealed an increased number of C. glabrata clinical isolates that display resistance to not only azoles but also echinocandins.16,17

C. glabrata is phylogenetically close to Saccharomyces cerevisiae, yet it has evolved to survive within mammalian hosts. Several specific traits, including adhesion, high drug resistance, and enhanced ability to survive starvation, allow C. glabrata to exist as a human commensal and an opportunistic pathogen.18 Despite having the components of the mating machinery, C. glabrata does not appear to undergo a sexual cycle, and all clinical isolates of C. glabrata identified to date are haploid.19 Therefore, genomic plasticity seems to be important for the evolution of C. glabrata populations.20

Adherence to host surfaces or medical devices is required for both initial colonization and persistence within the host. C. glabrata has gained a unique adhesion mechanism that is mediated largely by the EPA family genes located in subtelomeric regions.
and regulated by subtelomeric silencing.\textsuperscript{21,22} Although the \textit{C. glabrata} genome contains orthologs of most \textit{S. cerevisiae} genes and the two organisms have similar chromosomal structures in terms of gene order, whole genome sequencing of \textit{C. glabrata} has revealed an increased rate of loss of genes from their common ancestor.\textsuperscript{23} Specifically, genes involved in the metabolism of galactose, phosphate, nitrogen, and sulfur have been lost, and such reductive evolutionary processes make \textit{C. glabrata} rely on its host to overcome critical auxotrophies.\textsuperscript{18} Some \textit{Candida} species were found to release hydrolytic enzymes, such as proteinases, phospholipases, lipases, and hemolysins, in infected hosts,\textsuperscript{24} which facilitates not only the acquisition of nutrients in the host environment but also escape from host defense, allowing the colonization and invasion of \textit{Candida} cells. For instance, hemolysins play a role in degrading hemoglobin to acquire elemental iron, which is essential for \textit{Candida} cells to grow in the host. It has been reported that \textit{C. glabrata} was able to produce hemolysins in vitro;\textsuperscript{25} however, unlike \textit{C. albicans},\textsuperscript{26} \textit{C. glabrata} has no known receptors for heme.\textsuperscript{27} Siderophore-mediated iron acquisition through the sole siderophore-iron transporter Sit1 was shown to be critical for \textit{C. glabrata} cells to survive macrophage killing,\textsuperscript{27} indicating that distinct mechanisms of iron acquisition have been developed in \textit{C. glabrata}. Another important strategy to survive starvation is autophagy, which is a mechanism of intracellular nutrient mobilization by recycling cellular components and even organelles.\textsuperscript{28,29} To date, while the secretion of hydrolytic enzymes has no known role in \textit{C. glabrata} virulence, autophagy supports \textit{C. glabrata} survival during phagocytosis.\textsuperscript{30}

While \textit{C. albicans} and \textit{C. glabrata} are ubiquitous commensals and are the two most common pathogenic yeasts of humans, they have different strategies to survive in the hosts. In \textit{C. albicans}, the morphological flexibility between the yeast and filamentous forms plays an important role in escape from phagocytosis and the penetration into the host tissues; however extensive host cell damage concurrently elicits proinflammatory cytokine responses. On the other hand, \textit{C. glabrata} does not form hyphae and seems to follow a strategy of stealth and evasion to persist in the hosts without causing any strong immune response.\textsuperscript{31}

Together, these representative studies indicate that \textit{C. glabrata} has developed distinct strategies to become a successful commensal or pathogen of humans.

**ER Stress and Cell Death**

Tunicamycin and dithiothreitol are well-known ER-stressing agents that lead to the accumulation of unfolded protein in the ER thorough different mechanisms; tunicamycin inhibits N-linked glycosylation that is important for proper protein folding, and dithiothreitol unfolds ER-localized proteins by interfering with disulfide-bond formation. Cumulative evidence suggests that ER stress induces cell death. For instance, intense or prolonged ER stress induced by tunicamycin leads to the activation of apoptosis pathways in mammalian cells.\textsuperscript{32,33} However, because apoptotic pathways are not entirely conserved in fungi,\textsuperscript{34} ER stress-associated cell death is likely to be different. One possibility is that it involves an apoptotic-like process, but it is also possible that ER stress-induced cell death is simply due to the toxic accumulation of unfolded proteins that interfere with cell survival. Recently, Cunningham’s group demonstrated that tunicamycin led to cell death accompanied by vacuolar membrane permeabilization, which requires functional vacuolar H\(^+-\)ATPase, and that this dying process is not apoptotic in \textit{S. cerevisiae}.\textsuperscript{35,36} In contrast, tunicamycin induces apoptosis-like cell death in the fission yeast \textit{Schizosaccharomyces pombe}.\textsuperscript{37} To date, the mechanistic features and pathophysiological importance of ER stress-induced cell death in any of the \textit{Candida} species remain unclear.

**ER Stress Response Mechanisms in \textit{C. glabrata}**

Unfolded protein response (UPR) and regulated Ire1-dependent decay (RIDD)

Theoretically, ER-stressed cells must either increase the protein folding capacity of the ER or decrease the load of proteins entering the ER to maintain ER homeostasis. The \textit{ER} resident transmembrane stress transducer Ire1, which contains an ER luminal sensing domain and cytosolic protein kinase/endoribonuclease domains, directly interacts with accumulated unfolded proteins, resulting in the oligomerization of its luminal domain and subsequent signal transduction to the cytosolic domains.\textsuperscript{38,39} The protein kinase domain is required for auto-phosphorylation and concomitant activation of the nuclease domain that provides the endoribonuclease activity.\textsuperscript{40,42} Active Ire1 then cleaves the \textit{HAC1} mRNA to excise the intron, allowing translation of the bZIP transcription factor Hac1 that subsequently induces transcription of its target genes, including \textit{ER} chaperones.\textsuperscript{33,44} This cellular machinery is collectively called the UPR. It is believed that the UPR is an evolutionarily conserved mechanism and plays a central role in ER stress response in eukaryotes.

\textit{C. glabrata} is highly tolerant to ER stress relative to other fungi, such as \textit{S. cerevisiae} or \textit{Cryptococcus neoformans}.\textsuperscript{12} Intriguingly, our group recently uncovered that the canonical UPR mechanism regulated by Ire1–Hac1 signaling is not conserved in \textit{C. glabrata};\textsuperscript{12} to our surprise, Ire1 was found to be required for the ER stress response independently of Hac1. \textit{C. glabrata} retains a single ortholog of each of \textit{IRE1} and \textit{HAC1}; however, \textit{C. glabrata} Ire1 does not cleave mRNAs encoding Hac1 or other bZIP transcription factors identified in the \textit{C. glabrata} genome, even under severe ER stress conditions. These findings have raised the question as to how Ire1 is involved in the ER stress response in \textit{C. glabrata}.

In metazoans, ER stress triggers two distinct outputs of the nuclease activity from Ire1, namely \textit{XBPI} splicing and regulated Ire1-dependent decay (RIDD).\textsuperscript{1,45} The former activates the UPR pathway, whereas the latter is an Xbp1-independent pathway that selectively degrades a small subset of ER-associated mRNAs and remodels the repertoire of proteins translated in ER-stressed cells.\textsuperscript{46,47} Such a cellular response is predicted to reduce the ER load by limiting protein influx and unfolded protein load into the ER lumen. The RIDD pathway, first uncovered in \textit{Drosophila melanogaster}\textsuperscript{47} and later confirmed in mammalian cells,\textsuperscript{16,48} has not yet been fully determined in pathogenic fungi. Our recent
studies revealed that C. glabrata has lost the canonical UPR, but instead possesses RIDD that occurs in an Ire1 nuclease-dependent fashion. It is thought that RIDD targets are nicked by the Ire1 endoribonuclease at sites that do not display any identifiable consensus sequence, in contrast to Ire1-mediated splicing of HAC1/XBP1 mRNA at highly conserved splice junctions. Since excessive RIDD activation seems to be detrimental to cell integrity, reducing the load of proteins entering the ER must be balanced with the need to sustain the synthesis of essential proteins. It remains unclear as to how the RIDD activity is regulated in C. glabrata; however, unlike Ire1 in metazoans, C. glabrata Ire1 may not need to switch between specific and nonspecific modes of cleavage.

On the other hand, RIDD does not occur in S. cerevisiae; the classic Ire1-Hac1 UPR mediates the downregulation of some membrane protein-encoding mRNAs.47,49 Interestingly, a recent study by Kimmig et al.50 revealed that S. pombe lacks both a HAC1 ortholog and a UPR-dependent transcriptional program, but instead relies on RIDD as the sole means of alleviating ER stress. This study also estimated that RIDD may reduce the total protein influx into the ER by only 15%, even under severe ER stress conditions. It is unclear as to how such a minor reduction of bulk protein flux into the ER has a major impact on resolving lethal ER stress. As the authors suggested, one possible explanation is that the folding of RIDD targets may be difficult, thereby resulting in a disproportionate impact on the protein-folding load in the ER.

Other signaling pathways involved in ER stress response in C. glabrata

In C. glabrata, the transcriptional response to ER stress is not mediated by Ire1, but instead is dependent largely on calcineurin signaling and partially on the Slt2 MAPK pathway.12 The phenotypic analyses using single and double C. glabrata mutants of these signaling pathways have revealed that Ire1, calcineurin, and Slt2 function in parallel to cope with ER stress, similar to previous findings in S. cerevisiae.35-33 Calcineurin mediates the calcium cell survival pathway by regulating intracellular Ca\(^{2+}\) homeostasis. While tunicamycin increases Ca\(^{2+}\) uptake by stimulating the high-affinity Ca\(^{2+}\) channel Cch1–Mid1, calcineurin dephosphorylates the Cch1 subunit of the channel to inhibit Ca\(^{2+}\) influx and therefore prevents nonapoptotic cell death in S. cerevisiae.35-33 Crz1 is the only known transcription factor downstream of calcineurin in C. glabrata; however, compared with a calcineurin mutant, the phenotypes of the Δcrz1 mutant were found to be intermediate in terms of the sensitivity to ER stress-inducing agents and echinocandin antifungals, as well as virulence in mice, suggesting that calcineurin has an additional effector in C. glabrata.12,54

Slt2 plays a role in the ER stress surveillance (ERSU) pathway that ensures the transmission of only functional ER to daughter cells during cell division.55 Upon ER stress, the ERSU pathway delays ER inheritance and cytokinesis to prevent the death of both mother and daughter cells. Indeed, many genes involved in the ribosome activity and cytoplasmic translation are downregulated in a Slt2-dependent manner during the late phase of the ER stress response in C. glabrata, in contrast to RIDD, which is a relatively rapid response.12 Slt2 regulates two downstream transcription factors, Rlm1 and the SBF (Swi4/Swi6) complex. Contrary to the Δslt2 mutant, Δrlm1 and Δswi6 mutants did not display increased susceptibility to either tunicamycin or dithiothreitol (Miyazaki et al., unpublished data), suggesting that Slt2 plays a role in the ER stress response independently of Rlm1 and SBF. The signaling pathways implicated in the ER stress response in C. glabrata are schematically represented in Figure 1.

Figure 1. Signaling pathways involved in the ER stress response in C. glabrata. The classic UPR pathway mediated by Ire1-Hac1 signaling (dotted lines) is not conserved in C. glabrata. In response to ER stress, Ire1 induces RIDD to degrade ER-localized mRNAs in an Ire1 nuclease-dependent fashion, whereas calcineurin regulates both Crz1-dependent and Crz1-independent pathways. In addition, Slt2 is required for the ER stress response independently of its downstream transcription factors Rlm1 and SBF (Swi4/Swi6). Ire1, calcineurin, and Slt2 have also been implicated indirectly in antifungal resistance and virulence in C. glabrata. HAC1\(^{\mu}\), uninduced form of HAC1; HAC1\(^{\iota}\), induced form of HAC1; SBF, Swi4/Swi6 cell cycle box-binding factor; UPR, unfolded protein response; RIDD, regulated Ire1-dependent decay; CCS, calcium cell survival; ERSU, ER stress surveillance.
Diversity in Ire1-Dependent Stress Response Mechanisms between C. glabrata and Other Fungi

The term UPR is originally derived from a cellular response to chemicals that interfere with proper protein folding (e.g., tunicamycin and dithiothreitol); to date, the UPR signaling pathway is known to be involved in various stress responses from yeast to humans.\(^\text{56,57}\) Indeed, in \textit{S. cerevisiae}, downstream targets of the UPR include not only ER chaperones but also genes involved in diverse functions, including protein trafficking and quality control, lipid and sterol metabolism, heme biosynthesis, and cell wall biogenesis.\(^\text{58}\)

Importantly, the involvement of the UPR in antifungal resistance has been revealed in several pathogenic fungi. In \textit{Aspergillus fumigatus}, the loss of HacA led to increased susceptibility to azole antifungals and an echinocandin-class antifungal caspofungin.\(^\text{10}\) Increased susceptibilities to azoles and caspofungin have also been reported in \textit{C. neoformans} \textit{Δire1} and \textit{C. albicans} \textit{Δhac1} mutants.\(^\text{6-8,10,11,59}\) There seems to be no role for \textit{IRE1} in susceptibility of \textit{C. glabrata} to echinocandins.\(^\text{12}\) Concerning azoles, the deletion of \textit{IRE1} increases the susceptibility of calcineurin mutants, but not of wild-type cells. This possibly suggests redundancy of azole stress signaling or, alternatively, that azoles induce a more severe stress in calcineurin-defective \textit{C. glabrata} mutants, and that Ire1 function is required to survive this severe stress.

Previous studies on several yeasts and filamentous fungi have demonstrated that the UPR plays a role in maintaining cell wall integrity and that mutants lacking Ire1 or Hac1 exhibited decreased tolerance to a variety of cell wall-perturbing agents.\(^\text{6-8,10,11,59}\) Tunicamycin also induces cell wall stress, which may provide an explanation for why certain genes implicated in cell wall biogenesis were found to be upregulated during the UPR in tunicamycin-treated \textit{S. cerevisiae} and \textit{C. albicans} cells.\(^\text{10,58,60}\) In contrast, the loss of Ire1 alone in \textit{C. glabrata} did not confer a cell wall-defective phenotype, and the upregulation of some cell wall-related genes upon tunicamycin exposure was mainly confined to impaired ER stress responses, whereas the pathway is dispensable for the induction of the Kar2 homolog GRP78/BiP in mammalian cells.\(^\text{33,64}\) In \textit{C. glabrata}, the majority of ER stress-induced genes, including \textit{KAR2}, are dependent on the calcineurin–Crz1 pathway, but not Ire1 signaling.\(^\text{12}\) Although Ire1 seems to play a limited role in environmental stress responses, it is required for \textit{C. glabrata} virulence. To our surprise, \textit{C. glabrata}, unlike \textit{S. cerevisiae}, lacks the canonical Ire1–Hac1 UPR and possesses a metazoan-like RIDD mechanism. Future studies should aim to focus on understanding how and why this unique property has been developed in \textit{C. glabrata}, as well as which virulence trait is affected by such an evolutionary process.\(^\text{60}\) It is important to seek a potential interaction between Ire1 and other signaling pathways, since whether the Ire1 kinase domain has another effector other than Ire1 itself remains a major unsolved question in this field.

### Impact of the ER Stress Response on \textit{C. glabrata} Virulence

The importance of the UPR for the virulence of pathogenic fungi, including \textit{C. neoformans} and \textit{A. fumigatus}, is reviewed in this issue. Briefly, \textit{C. neoformans} mutants lacking Ire1 and its downstream bZIP transcription factor encoded by \textit{HXL1} (\textit{HAC1} and \textit{XBP1-Like gene 1}) are unable to grow at 37 °C and are avirulent in a mouse model of systemic cryptococcosis.\(^\text{7}\) In \textit{A. fumigatus}, attenuated virulence of the \textit{Δhac1} mutant has been demonstrated using murine models of invasive aspergillosis.\(^\text{10}\) These observations may be explained by the fact that the UPR plays a role in the expression of several virulence-related traits, including protein secretion, rapid apical growth, and maintenance of both membrane and cell wall homeostasis.\(^\text{61}\) Interestingly, the \textit{A. fumigatus Δire1} mutant is avirulent, in contrast to the partially attenuated \textit{Δhac1} mutant, indicating that Ire1 has hacA-independent functions that are important for the virulence of \textit{A. fumigatus}. Consistently, several phenotypic differences have been observed between these mutants. For example, the \textit{Δire1} mutant is more sensitive to elevated growth temperature, hypoxia, and nutritional limitation such as iron starvation, all of which are conditions commonly encountered by pathogenic fungi in infected hosts.\(^\text{8}\) In \textit{C. albicans}, while the role of the UPR in the pathogenicity has not yet been directly investigated in vivo, Hac1 is known to be required for hyphal development, which is an important factor for biofilm formation and virulence.\(^\text{11}\)

In \textit{C. glabrata}, Hac1 is dispensable for both the ER stress response and virulence, whereas loss of Ire1 resulted in hypovirulence in both immunocompromised and immunocompetent mice.\(^\text{12}\) The phenotype of the \textit{C. glabrata Δire1} mutant is confined almost exclusively to impaired ER stress responses, in contrast to UPR-defective mutants of other fungi where the UPR is involved in various stress responses. These findings imply that \textit{C. glabrata} cells experience ER stress during the process of infection. Inhibition of either calcineurin or Slt2 also resulted in attenuated virulence in \textit{C. glabrata},\(^\text{34,62}\) which may be attributed to multifactorial causes, since these signaling pathways have various physiological functions, including the maintenance of intracellular pH and cell wall homeostasis. However, it is very likely that attenuated virulence of the calcineurin and Slt2 mutants was caused at least in part by impaired ER homeostasis.

### Concluding Remarks and Future Perspectives

While the basic aspects of the UPR are highly conserved throughout eukaryotes, diversities in the transcriptional induction system in response to ER stress have also been developed during evolution.\(^\text{30,65}\) For example, the Ire1–Hac1 signaling pathway is required for the upregulation of the ER-resident chaperone Kar2 in \textit{S. cerevisiae}, whereas the pathway is dispensable for the induction of the Kar2 homolog GRP78/BiP in mammalian cells.\(^\text{33,64}\) In \textit{C. glabrata}, the majority of ER stress-induced genes, including \textit{KAR2}, are dependent on the calcineurin–Crz1 pathway, but not Ire1 signaling.\(^\text{12}\) Although Ire1 seems to play a limited role in environmental stress responses, it is required for \textit{C. glabrata} virulence. To our surprise, \textit{C. glabrata}, unlike \textit{S. cerevisiae}, lacks the canonical Ire1–Hac1 UPR and possesses a metazoan-like RIDD mechanism. Future studies should aim to focus on understanding how and why this unique property has been developed in \textit{C. glabrata}, as well as which virulence trait is affected by such an evolutionary process. It is important to seek for a potential interaction between Ire1 and other signaling pathways, since whether the Ire1 kinase domain has another effector other than Ire1 itself remains a major unsolved question in this field. In addition, given the importance of the ER stress response to \textit{C. glabrata} virulence, future targeting of Ire1 in conjunction
with calcineurin might be a promising approach to improve the treatment options for *C. glabrata* infections.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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