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Endoplasmic reticulum stress and fungal pathogenesis

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Abstract

The gateway to the secretory pathway is the endoplasmic reticulum (ER), an organelle that is responsible for the accurate folding, post-translational modification and final assembly of up to a third of the cellular proteome. When secretion levels are high, errors in protein biogenesis can lead to the accumulation of abnormally folded proteins, which threaten ER homeostasis. The unfolded protein response (UPR) is an adaptive signaling pathway that counters a buildup in misfolded and unfolded proteins by increasing the expression of genes that support ER protein folding capacity. Fungi, like other eukaryotic cells that are specialized for secretion, rely upon the UPR to buffer ER stress caused by fluctuations in secretory demand. However, emerging evidence is also implicating the UPR as a central regulator of fungal pathogenesis. In this review, we discuss how diverse fungal pathogens have adapted ER stress response pathways to support the expression of virulence-related traits that are necessary in the host environment.

Keywords

UPR; unfolded protein response; fungal pathogenesis; fungal virulence; ER stress; Ire1; Hac1; HacA; IreA

1. Introduction

The ability of fungi to sense environmental stress and mount an appropriate response is essential for survival in the diverse biological niches occupied by these organisms. The endoplasmic reticulum (ER) is important for many adaptive responses because of its role as the initial folding and processing center for proteins that are destined for delivery into, or across, the plasma membrane, or to other parts of the endomembrane system. Nascent polypeptides enter the ER in an unfolded state, but must be folded accurately before they can transit to their target organelles. The ER lumen provides an oxidizing environment that is conducive to protein folding, and contains an abundance of ER-resident chaperones, foldases and a variety of other enzymes to help proteins achieve their native conformation

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(Braakman and Hebert, 2013). However, the high concentration of proteins in this milieu increases the risk for illegitimate interactions during the folding process, which can lead to misfolding and aggregation events that are detrimental to cell physiology. Thus, when the demand for secretion is high, or when a fungus encounters environmental conditions that impair ER function beyond the folding capacity of the ER, aberrantly folded or unfolded proteins may accumulate in the ER lumen. The ensuing ER stress initiates a complex series of adaptive events that collectively form the unfolded protein response (UPR) (Moore and Hollien, 2012). The UPR restores protein folding homeostasis by increasing the folding capacity of the ER, in addition to regulating the disposal of irreparably damaged proteins by ER-associated degradation (ERAD).

2. The UPR in model fungi

The mammalian UPR is comprised of a tripartite signaling system, each of which is triggered by a separate ER stress sensor embedded in the ER membrane: Ire1, Atf6, and Perk (Moore and Hollien, 2012). When confronted by unfolded proteins, the first two proteins work together to reprogram the transcriptome into a state that bolsters the folding capacity of the ER. This is accomplished by transcription factors that induce the expression of genes that directly influence the secretory pathway at multiple levels. The transcriptional rewiring mediated by Ire1 and Atf6 works in conjunction with Perk, an ER transmembrane kinase that reduces the workload imposed on the ER by phosphorylating eukaryotic translation initiation factor 2 subunit α (eIF2 α), resulting in widespread translation attenuation (Harding et al., 1999). Current evidence suggests that fungi rely on Ire1 as the sole ER stress sensor, although accumulating data suggests that there is substantial divergence in pathway output within the fungal kingdom (Mori, 2009).

The paradigm of fungal UPR signaling was elucidated in the model yeast *Saccharomyces cerevisiae* (Gardner et al., 2013). Like all Ire1 orthologs examined to-date, yeast Ire1 has a luminal ER stress-sensing domain and a cytosolic tail that contains both a kinase and an endoribonuclease (RNase) domain (Fig 1). The ER chaperone BiP/Kar2 binds to Ire1 in unstressed cells, but dissociates to assist with protein folding during ER stress conditions (Bertolotti et al., 2000; Okamura et al., 2000). Unfolded proteins activate Ire1 by direct interactions with the luminal sensing domain (Credle et al., 2005; Gardner et al., 2013); the Ire1-BiP/Kar2 interaction is thought to play a regulatory role by desensitizing Ire1 to low levels of ER stress, thereby ensuring that the level of activation is in proportion to the magnitude and duration of ER dysfunction (Pincus et al., 2010). Ire1 activation is associated with the formation of higher-order oligomeric Ire1 complexes in the ER membrane, resulting in trans-autophosphorylation and a conformational change that activates the RNase domain. Once active, the RNase catalyzes the spliceosome-independent removal of an unconventional intron from the cytoplasmic mRNA *HAC1*, which shifts the reading frame to allow translation of a bZIP transcription factor known as Hac1p. Hac1p then moves to the nucleus and increases the expression of UPR target genes (Travers et al., 2000). However, even with the intervention of the UPR, a substantial fraction of polypeptides inevitably fail to achieve the appropriate conformation (Hartl and Hayer-Hartl, 2009). These aberrant proteins are eliminated by ERAD, a signaling pathway that retrotranslocates misfolded proteins back into the cytosol, ubiquitinates them on the cytosolic face of the ER and

releases them for degradation by the proteasome (Ruggiano et al., 2014). The UPR is required for efficient ERAD, indicating a tight coordination between the protein folding and disposal machineries (Travers et al., 2000).

Although Ire1 is present in other fungal species, divergence exists in the mechanism it uses to mitigate ER stress. For example, although the fission yeast *Schizosaccharomyces pombe* has a clear Ire1 ortholog, bioinformatic analyses have failed to identify Hac1 orthologs in *S. pombe* or other yeasts of the same genus (Kimmig et al., 2012). Instead of using the canonical Ire1-Hac1 transcriptional program to modulate the abundance of mRNAs during ER stress, *S. pombe* exploits the Ire1 RNase to initiate the selective decay of a large subset of mRNAs encoding ER-localized proteins. This ‘regulated Ire1-dependent decay’ (RIDD) has been shown to relieve ER stress in human cells by reducing the load of proteins entering the ER, but it is not present in *S. cerevisiae* (Han et al., 2009) or *A. fumigatus* (Feng et al., 2011). *S. pombe* Ire1 has also been implicated in a novel mechanism to increase the levels of the mRNA encoding the ER-resident chaperone Bip/Kar2 (Jung et al., 2013; Morrow et al., 2011). In *S. pombe*, Ire1-dependent cleavage of the *Bip1* 3’UTR during ER stress stabilizes the mRNA, thereby increasing the levels of its encoded product (Kimmig et al., 2012). This is in striking contrast to the situation in *S. cerevisiae*, and many other species, where Bip/Kar2 upregulation is accomplished at the transcriptional level through the canonical Ire1-Hac1 pathway (Moore and Hollien 2012). These studies in model yeast, together with other eukaryotic systems, have established Ire1 as the most ancient branch of the ER stress response, but have also revealed divergence in Ire1 output that has likely evolved to suite the unique requirements of individual species (Hollien, 2013). Below, we discuss how diverse fungal pathogens have repurposed ER stress response pathways to support the expression of traits that protect the fungus from adverse environmental conditions that are encountered during infection.

3. Human fungal pathogens

Invasive fungal infections have a major impact on human health, with recent estimates showing as many deaths from the top 10 invasive fungal infections as from tuberculosis or malaria (Brown et al., 2012). Over 90% of fungal-related mortality is attributed to species within four genera: *Cryptococcus*, *Candida*, *Aspergillus* and *Pneumocystis*. As discussed below, current evidence indicates that ER stress responses in at least three of these genera are deeply entwined with fungal pathogenicity.

Aspergillus fumigatus

Filamentous fungi are specialized for secretion, a feature that has been exploited by the biotechnology industry for the synthesis of recombinant proteins (Nevalainen and Peterson, 2014). Initial studies on ER stress responses in these organisms focused on designing novel strategies to strengthen the secretory capacity of the fungus in order to alleviate ‘bottlenecks’ that limit production capacity (Arvas et al., 2006; Guillemette et al., 2011; Guillemette et al., 2007; Ohno et al., 2011). However, subsequent analyses have approached this high secretory capacity from a different angle, with the goal of identifying points of vulnerability in a pathogenic mold that could be targeted with novel antifungal therapy. *Aspergillus fumigatus* is a typical filamentous fungus, but it is unique among environmental

molds because of its status as the predominant species associated with systemic human infections (Binder and Lass-Flörl, 2013). This fungus utilizes a canonical UPR pathway to cope with ER stress, employing the ER transmembrane sensor, IreA (the ortholog of Ire1), that controls the splicing of the mRNA encoding the downstream transcription factor HacA (the ortholog of Hac1). IreA-induced splicing of HacA is always detectable under non-stressed conditions in *A. fumigatus*, suggesting that the UPR buffers minor fluctuations in ER stress that occur during normal filamentous growth (Feng et al., 2011). This is supported by evidence that Ire1 regulates the expression of over 10% of the genome under normal growth conditions (Feng et al., 2011).

In the absence of a functional UPR, *A. fumigatus* is rendered temperature sensitive, defective in hypoxia tolerance, defective in the secretion of hydrolases necessary for nutrient acquisition, growth impaired under low iron, and highly vulnerable to attack on membrane or cell wall integrity (Richie et al., 2009). Many of these traits, which undoubtedly evolved to support the growth of *A. fumigatus* in nature, are also known to contribute to virulence or antifungal drug resistance in this organism (Hartmann et al., 2011; Richie et al., 2009). Phenotypic and expression profile comparisons between *A. fumigatus* *ireA* and *hacA* mutants revealed that IreA has HacA-dependent and HacA-independent functions, both of which contribute to the expression of the virulence attributes described above (Feng 2011). Since the *S. cerevisiae* UPR is thought to be a simple linear pathway, this bifurcation in IreA signaling in *A. fumigatus* may reflect the need for expanded UPR functions in a mold pathogen relative to a non-pathogenic yeast.

Studies have shown that translational remodeling is also a major component of the ER stress response of *A. fumigatus* (Krishnan et al., 2014). The nature and scope of ER stress-induced translational regulation is strikingly different from the associated transcriptional response, which may provide the fungus with a rapid-response mechanism to cope with ER stress until the transcriptome can be modified appropriately. These transcriptional and translational reprogramming events adjust fungal physiology towards a more protein folding competent state, which is needed to support the growth of *A. fumigatus* in the host environment (Feng et al., 2011; Richie et al., 2009). The disposal of any proteins that ultimately fail to achieve an appropriate conformation is accomplished by ERAD, a pathway that is regulated in part by the UPR (Travers et al., 2000). The deletion of the ERAD genes *derA* or *hrdA* had no effect on the virulence of *A. fumigatus* (Krishnan et al., 2013; Richie et al., 2011). However, the combined loss of *derA* and *hacA* caused a more severe reduction in hyphal growth, antifungal drug resistance and protease secretion than the loss of either gene alone. Moreover, a *derA/ hacA* mutant was avirulent, demonstrating that the UPR and ERAD pathways act in parallel pathways to support the expression of multiple clinically relevant traits, and could therefore represent a point of vulnerability for therapeutic intervention.

Cryptococcus neoformans

C. neoformans is saprophytic basidiomycetous yeast that causes life-threatening pulmonary infections when inhaled, particularly in human immunodeficiency virus (HIV)-positive individuals (Gullo et al., 2013). The yeast are encapsulated, which helps them evade the immune system, and they have an unexplained tropism for invading the central nervous that

can result in life-threatening meningoencephalitis (O'Meara and Alspaugh, 2012; Sloan and Parris, 2014). *C. neoformans* responds to acute ER stress through an Ire1-dependent signaling UPR pathway that follows the *S. cerevisiae* paradigm, with the exception that the downstream transcription factor, Hx11 (Hac1 and XBP1-Like), is phylogenetically distant from the corresponding proteins in *S. cerevisiae* (Hac1) or humans (Xbp1) (Cheon et al., 2014; Cheon et al., 2011). The loss of either Ire1 or Hx11 is sufficient to render *C. neoformans* avirulent (Cheon et al., 2011). This is likely to be a consequence of reduced thermotolerance, since neither *ire1* nor *hx11* can grow at 37°C. However, distinct phenotypic differences were observed between *ire1* and *hx11* suggesting that *C. neoformans* Ire1 has functions outside of the canonical UPR, similar to what has been described in *A. fumigatus* (Feng 2011). For example, *ire1*, but not *hx11*, was defective in capsule biosynthesis. Since the capsule is a well-known host evasion mechanism for *C. neoformans* (O'meara and Alspaugh 2012), its absence in the *ire1* mutant may also contribute to the observed lack of virulence in that strain. Of course, the UPR is unlikely to function as the sole stress response pathway that operates under conditions of ER stress, and there is evidence that both the calcineurin and MAPK signaling pathways may work in parallel with the canonical UPR to relieve ER stress in this fungus (Cheon et al., 2011).

The ability of *C. neoformans* to adapt to the host temperature is accompanied by UPR activation, most likely because higher temperatures have adverse effects on protein folding (Matsumoto et al., 2005). A recent study showed that Ccr4, the major deadenylase responsible for the rate-limiting step in mRNA decay, plays a role in regulating the shutoff kinetics of this response through the degradation of ER stress mRNAs (Havel et al., 2011). Mutants that are defective in mRNA decay were unable to down-regulate the ER stress response and were rendered avirulent due to an inability to grow at host body temperature (Bloom et al., 2013; Havel et al., 2011). These studies establish mRNA decay as an important mechanism of ER stress resolution and reveal a tight coordination with host-temperature adaptation and pathogenicity.

Candida glabrata

C. glabrata and *C. albicans* are the two most common pathogenic yeasts of humans, collectively responsible for the majority of all systemic *Candida* infections (Brunke and Hube, 2013). *C. glabrata* is phylogenetically close to *S. cerevisiae*, but the mechanism by which it responds to ER stress is very different. Orthologs of Ire1 and Hac1 are present in the *C. glabrata* genome, with each predicted to encode the same transmembrane kinase/RNase and bZIP transcription factor proteins as *S. cerevisiae* Ire1 and Hac1, respectively. Surprisingly, deletion of these genes individually from *C. glabrata* showed that it is Ire1, but not Hac1, that primarily governs the ER stress response (Miyazaki and Kohno, 2014; Miyazaki et al., 2013). Moreover, *HAC1* mRNA splicing was not observed in *C. glabrata* under any condition tested, suggesting that Ire1 contributes to ER stress independently of Hac1 in this fungus. Complementation studies showed that *C. glabrata* Ire1 was unable to splice *S. cerevisiae* *HAC1* mRNA. However, *C. glabrata* Hac1 could rescue the growth of a *S. cerevisiae* *ire1* mutant under conditions of ER stress, suggesting that the transcription factor is sufficiently conserved to drive the expression of UPR target genes in *S. cerevisiae*. However, unlike *S. cerevisiae* *HAC1* mRNA, *C. glabrata* *HAC1* does not need to be spliced

by Ire1 to be translated into a functional protein. Interestingly, the RNase of *C. glabrata* Ire1 is required for Ire1-dependent downregulation of mRNAs encoding GPI-anchored cell wall and membrane proteins, involving a RIDD-like mechanism described in higher eukaryotes and *S. pombe* (Kimmig et al., 2012).

Mutant strains of *C. glabrata* that are deficient in calcineurin (*cnb1*) and PKC1-MAPK stress response pathways (*slt1*) were also hypersensitive to ER stress, and deletion of *C. glabrata* *IRE1* in the background of these mutants rendered the organism even more sensitive to ER stress (Miyazaki et al., 2013). Importantly, the loss of any of these three pathways attenuates the virulence of *C. glabrata* (Miyazaki et al., 2010a; Miyazaki et al., 2013; Miyazaki et al., 2010b), illustrating the crucial importance of ER stress responses to the pathogenicity of this organism. UPR mutants lacking Ire1 or Hac1 in several pathogenic fungi, including *A. fumigatus*, *C. neoformans* and *Candida albicans*, show a striking increase in susceptibility to antifungal drugs that target the cell wall or membrane (Blankenship et al., 2010; Cheon et al., 2011; Feng et al., 2011; Richie et al., 2009; Xu et al., 2007). By contrast, the corresponding mutants in *C. glabrata* mutants did not exhibit alterations in antifungal drug resistance. However, a *cnb1/ ire1* double mutant of *C. glabrata* was more susceptible to azole antifungals than either single mutant, suggesting that the Ire1 and calcineurin pathways serve redundant roles in ER stress signaling that impact antifungal drug resistance (Miyazaki et al., 2013). Together, these findings indicate that *C. glabrata* has lost the unconventional splicing mechanism that is prevalent in many other eukaryotic species, but employs an Ire1-dependent RIDD mechanism that acts in parallel with calcineurin and MAPK pathways to relieve ER stress. These adaptations may have evolved to support the survival of *C. glabrata* as a commensal organism following its divergence from *S. cerevisiae*.

Candida albicans

C. albicans is a pathogenic yeast that is a member of the normal human microbiota and, like *C. glabrata*, is responsible for mucosal and systemic infections when the commensal balance is breached or when the immune system is suppressed (Mayer et al., 2013). *C. albicans* *HAC1* mRNA undergoes ER stress-responsive splicing, similar to the canonical UPR of other species. However, in contrast to *C. glabrata*, a *hac1*^{−/−} mutant of *C. albicans* was hypersensitive to ER stress, indicating that the canonical Ire1-Hac1 pathway operates in this organism (Wimalasena et al., 2008). Although the virulence of UPR mutants of *C. albicans* has yet to be published, three lines of evidence suggest that UPR function influences the expression of clinically relevant traits in this organism. First, *C. albicans* can switch between yeast and hyphal forms, and the ability to do so is implicated in virulence (Mayer et al., 2013). Several UPR target genes are induced during the yeast-to-hyphae transition (Monteoliva et al., 2011), and the loss of either *HAC1* (Wimalasena et al., 2008) or *IRE1* (Blankenship et al., 2010) impairs the ability of *C. albicans* to switch to the hyphal form. Interestingly, a *hac1* mutant of the non-pathogenic dimorphic yeast *Yarrowia lipolytica* was also impaired in hyphal switching (Oh et al., 2010), suggesting that the transition from isotropic growth (yeast) to polarized growth (hyphae) may exert sufficient ER stress on the secretory pathway to require UPR intervention. Secondly, the UPR regulates the expression of adhesive and cell wall synthesis proteins, and the loss of UPR

function impairs adhesion and sensitizes the fungus to cell wall- and membrane-targeting antifungal drugs (Blankenship et al., 2010; Wimalasena et al., 2008; Xu et al., 2007). Finally, systemic infections with *C. albicans* can be seeded from biofilms that develop on implanted medical devices (Finkel and Mitchell, 2011). These biofilms, which are comprised of yeast and hyphal forms embedded in an extracellular matrix, are defective in a *C. albicans ire1*–/– mutant (Blankenship et al., 2010; Finkel and Mitchell, 2011), most likely due to Ire1's ability to support filamentation (Blankenship et al., 2010; Wimalasena et al., 2008), adherence and attachment (Wimalasena et al., 2008), and the secretion of cell wall and matrix components (Blankenship et al., 2010).

4. Plant fungal pathogens

Alternaria brassicicola

A. brassicicola is a filamentous ascomycete that causes black spot disease on various *Brassicaceae* such as cabbage and broccoli (Pochon et al., 2012). As a typical necrotrophic fungus, *A. brassicicola* uses a destructive pathogenesis strategy that involves the secretion of an armamentarium of hydrolytic enzymes and toxins to directly kill host cells, resulting in the decomposition of plant tissue into reduced forms that are suitable for uptake (Oliver and Solomon, 2010). A UPR mutant of *A. brassicicola* that lacks the *hacA* gene was unable to penetrate healthy leaves and cause disease in *Arabidopsis thaliana* and *Brassica oleracea* (Guillemette et al., 2014; Joubert et al., 2011). This lack of virulence for intact leaves was also observed on mechanically wounded leaves, indicating that the UPR is required at multiple stages of this infection. The *hacA* mutant had a reduced secretory capacity, suggesting that an intact UPR is necessary for the fungus to secrete sufficient quantities of cell wall-degrading enzymes, proteases, pectinases and other enzymes needed to penetrate the plant surface and cause necrosis. The heightened sensitivity of the *hacA* mutant to antifungal plant metabolites may also contribute to the inability of this mutant to establish infection (Joubert et al., 2011).

Magnaporthe oryzae

Magnaporthe oryzae is one of the most destructive fungal pathogens of rice crops, responsible for rice blast disease. The pathogenicity of this fungus involves the secretion of effector proteins at the host-pathogen interface, the purpose of which is to breach the plant surface and manipulate plant defenses and cell physiology (Liu et al., 2010; Zhang and Xu, 2014). Lhs1 is an Hsp70 family chaperone in the ER lumen that is a well-established UPR target that is induced by ER stress (Craven et al., 1996). Disruption of the *LHS1* gene in *M. oryzae* triggered the UPR, consistent with the central role of this protein in protein folding homeostasis (Yi et al., 2009). The *lhs1* mutation reduced the level of secreted enzymes and effector proteins, and was associated with severely reduced pathogenicity, affecting both appressorial penetration and subsequent biotrophic invasion of susceptible rice. These findings demonstrate the importance of the proper processing of secreted proteins by a UPR-regulated chaperone to fungal pathogenesis, raising the possibility that other UPR targets could serve as novel targets for therapeutic intervention (Yi et al., 2009).

5. Conclusions

Fungi represent emerging infectious threats to human, animal and plant populations worldwide. The mechanism by which they cause disease is complex, reflecting adaptation to the diverse habits occupied by each species in nature. However, a common thread among each of these pathogens is their reliance upon ER stress responses for virulence. This review highlights current evidence that stress response pathways emanating from the ER endow pathogenic fungi with the necessary physiologic attributes to protect the fungus from the adverse conditions encountered in the host environment, including attack from the current armamentarium of antifungal drugs. Although these traits do not constitute virulence factors in the traditional sense, their importance for fungal pathogenesis may constitute a point of vulnerability for future therapeutic intervention.

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Fig 1.

The canonical UPR pathway established in *S. cerevisiae*. The chaperone BiP/Kar2 is bound to the ER stress sensor Ire1 in unstressed cells, but dissociates during ER stress to assist with protein folding. Yeast Ire1 is activated by direct interactions with unfolded proteins, and the binding to BiP/Kar2 plays a regulatory role by fine-tuning the sensitivity and shutoff kinetics for Ire1 activation. Activated Ire1 forms oligomeric complexes in the ER membrane, resulting in trans-autophosphorylation by the kinase (K) domain and a conformational change that activates the RNase domain (R). The RNase mediates the splicing of an intron from the cytosolic *HAC1* mRNA, causing a frame-shift that is a prerequisite for translation of *HAC1* mRNA. *Hac1p* translocates to the nucleus and increases the expression of UPR target genes that boost ER protein folding capacity. Proteins that fail to achieve the appropriate conformation are disposed of by ERAD, a UPR-linked pathway that retrotranslocates aberrant proteins back into the cytoplasm for proteasomal degradation. In some fungal species, Ire1 may participate in 'regulated Ire1' dependent decay (RIDD)', a process in which mRNAs encoding ER-targeted proteins are degraded as a way to reduce the workload of the ER. RIDD is not present in *S. cerevisiae*, but has been reported in *S. pombe* and *C. glabrata*.