REVIEW

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The ER stress response and host temperature adaptation in the human fungal pathogen *Cryptococcus neoformans*

Virginia E Glazier and John C Panepinto

Department of Microbiology and Immunology; Witebsky Center for Microbial Pathogenesis and Immunology; University at Buffalo; The State University of New York; Buffalo, NY USA

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Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; ERAD, ER-associated degradation; UTR, untranslated region; RIDD, regulated Ire-1 dependent decay

In all eukaryotic cells, the ER stress response is pivotal to survival and adaptation under stress conditions. During temperature adaptation in the human fungal pathogen *Cryptococcus neoformans,* ER stress is engaged transiently. Studies of this response have demonstrated that both the engagement (turning on the response), as well as the resolution (turning off the response) are required for temperature adaptation and, therefore, pathogenesis. In this review, we synthesize our current understanding of ER stress response engagement and resolution in *C. neoformans* during host temperature adaptation with a focus on the posttranscriptional events that regulate it. Identification of fungal-specific and *Cryptococcus*-specific elements of the evolutionarily conserved ER stress response pathway could lead to identification of anti-fungal targets in this fundamental stress response.

Introduction

Fungi are a highly diverse group of organisms with the number of species projected to be in the millions.[1] Within the major human fungal pathogens, *Cryptococcus*, *Aspergillus*, *Candida*, *Blastomyces*, *Histoplasma*, and *Coccidioides*, there is diversity in morphology, virulence factors, cell wall components, encapsulation, and mechanisms of pathogenesis.[2-4] Despite these differences, a single unifying characteristic is possessed by all fungi capable of deep infection in humans: the ability to grow at 37 °C.[5] Indeed even within the *Cryptococcus* genus, there are species that produce the quintessential virulence factors, capsule, and melanin, yet only two species, *C. neoformans*, and *C. gattii* are able to cause lethal infections in humans, as these are also the only two species able to grow optimally above 30 °C.[6] In contrast, the fungal species that cause superficial skin and nail infections, and even lymphocutaneous infections, are rarely capable of thriving at 37 °C, thus restricting their growth to the temperature permissive extremities. A lack of thermotolerance limits their ability to cause invasive systemic disease.[7,8] Adaptation to growth at elevated temperatures is a complex process, involving the coordination of various stress responses and signaling pathways.[6] The components and pathways required for elevated temperature growth have been well studied in *Saccharomyces cerevisiae*, and for many pathways there is a conservation of function in *C. neoformans*.[6,9-16] Additional regulatory pathways that govern temperature adaptation will likely be identified by ongoing exploration of the full complement of signaling modules in the *C. neoformans* genome.

As mentioned previously, growth at 37 °C is a key virulence factor in *Cryptococcus neoformans*, and constitutes one of three classic virulence factors in *C. neoformans*, the other two being the production of melanin and the presence of a polysaccharide capsule.[17,18] One commonality between all three of these factors is their reliance on proper ER function for appropriate expression. Laccase is a secreted protein that traverses the ER secretory protein pathway, capsular material can be found in secreted exosomal vesicles suggesting packaging within the endomembrane system and growth at 37 °C requires ER protein chaperones and protein processing machinery.[16,19-22] The components of the ER stress signaling pathway, including the resident ER receptor kinase Ire1 and the ER stress-responsive transcription factor Hxl1, are important for temperature adaptation and virulence in *C. neoformans*, and will be reviewed elsewhere. In this review, we will focus on the regulation of ER stress during temperature adaptation, and highlight the role of posttranscriptional gene regulation in both in induction and resolution of ER stress during the host temperature adaptation in *C. neoformans.*

Host Temperature Adaptation and the ER Stress Response

It has been well established in the model yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* that global transcript reprogramming occurs under many different stress events.[9-11,23-26] This reprogramming event is followed by translation of the newly synthesized mRNAs resulting in a corresponding influx of peptides

*Correspondence to: John C Panepinto; Email: jcp25@buffalo.edu
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requiring folding or glycosylation that are translocated into the ER lumen.[9,10] ER stress triggered by the accumulation of unfolded or misfolded proteins within the ER activates the UPR (unfolded protein response) and ERAD (ER-associated degradation). ER stress response mutants of the fungal pathogen *Aspergillus fumigatus*, a highly thermotolerant fungus capable of growing at up to 50 °C, exhibit increased temperature sensitivity (at 42 °C) as well as decreased virulence.[27-29] Likewise, *Candida* sp. display decreased virulence when ER components are deleted.[30] These results, taken with studies in *C. neoformans*, suggest that growth at elevated temperature and infection of a mammalian host are ER stress inducing events for pathogenic fungi.[21,22,27-34] In response to a shift from 30 °C to 37 °C, the core temperature of the human host, *C. neoformans* engages the ER stress response. Several ER stress responsive mRNAs are upregulated including; *KAR2*, which encodes an Hsp70 family member that is the ER sensor of unfolded proteins, *OST2*, a component of the ER resident oligosaccharyltransferase complex, and *ALG7*, involved in N-linked glycosylation and the target of the ER stress-inducing drug tunicamycin.[16] The induction of ER stress signaling when shifted to 37 °C indicates that for *C. neoformans*, mammalian core temperature is a stressor. When steady-state levels of *KAR2*, *ALG7*, and *OST2* were followed in a time course after a shift to 37 °C, expression levels peaked after one hour and return to pre-stress levels by three hours of incubation.[16] The transient nature of ER stress induction during growth at host temperature suggests that *C. neoformans* is able to sense the stress, respond to the stress and re-tool itself, and most importantly, adapt and recover.

Posttranscriptional Regulation of ER Stress Induction

The disruption of ER homeostasis can be caused by an increase in newly synthesized peptides into the ER lumen or by an increase in misfolded proteins, these unfolded or misfolded proteins have to be either properly folded or degraded to maintain proper ER function. It is hypothesized that during elevated temperature growth the change in gene expression in response to temperature stress may disrupt ER homeostasis triggering ER stress. Additionally, changes in protein folding dynamics between 30 °C and 37 °C may also place an increased demand on ER protein folding machinery. ER chaperones present within the ER lumen recognize and bind to the hydrophobic regions and truncated glycosylation residues present on unfolded and misfolded proteins.[35] Under normal growth conditions the ER chaperone Kar2 binds to Ire1 inhibiting Ire1 oligomerization. Ire1 is a transmembrane serine/threonine-protein kinase with a cytosolic endoribonuclease domain. Under stress conditions unfolded proteins accumulate within the ER lumen and interact with Kar2, causing it to be released from Ire1, allowing the oligomerization and transautophosphorylation of Ire1 to occur.[36] This oligomerization and transautophosphorylation changes the confirmation of Ire1, resulting in an active endonuclease domain that can bind and cleave mRNA.[37]

The primary mRNA target of Ire1 encodes a conserved basic region leucine zipper, or bZIP, transcription factor known as Hac1 in *S. cerevisiae*, Xbp1 in higher eukaryotes and Hxl1 in *C. neoformans*. Cleavage of this transcript occurs via the cytosolic endoribonuclease domain of activated, oligomerized Ire1.[37] Ire1-mediated cleavage of an unconventional intron in the *HAC1* mRNA occurs with both the 5′ and 3′ splice sites being utilized independent of any particular ordering. This is in contrast to conventional intron splicing by the spliceosome that requires splicing to occur in order with initial cleavage of the 5′ donor, lariat formation, and eventual cleavage and ligation of the 3′ splice acceptor.[38] The products of Ire1 mediated mRNA splicing are a 5′ exon with a 2',3'-cyclic phosphate and a 3′ exon with a free 5′ OH site. The two exons are then ligated back together by the tRNA ligase, Trl1.[39] The unconventional splicing of *HAC1* mRNA occurs at the ER cytosolic surface and results in the active form of the *HAC1* transcript which is then translated.[39]

Though the Ire1-dependent splicing of the ER stress transcription factor is conserved from yeast to mammals (with the exception of *S. pombe*), the specific characteristics of the unconventional splicing event differ between species.[25,40] For instance, the length of the *HAC1* intron in *S. cerevisiae* is 252 nucleotides, in contrast, the length of the *HXL1* intron in *C. neoformans* is 56 nucleotides and is closer in length to the human *XBP1* intron (26 nucleotides).[22,40] A recent study on *HXL1* in *C. neoformans* demonstrated cleavage of *HXL1* by Ire1, suggesting this splicing event is conserved in *C. neoformans*.[22] In *S. cerevisiae* the primary tRNA ligase, Trl1, is responsible for ligating back together both tRNA and *HAC1* mRNA exons following endonuclease cleavage by either the tRNA splicing endonuclease complex or Ire1 (respectively).[41] A protein blast search of the annotated *C. neoformans* genome database at the Broad Institute, MIT, revealed a Trl1 homolog, CNAG_01250.2 which we hypothesize performs a similar *HXL1* mRNA ligase function to what has been reported in other species (*Cryptococcus neoformans* var *grubii* H99 Sequencing Project, Broad Institute of Harvard and MIT, http://www.broadinstitute.org/). The cleavage of *HAC1/XBP1* mRNA by Ire1 occurs at a conserved recognition site at both the 5′ and 3′ splice sites. This Ire1 recognition site contains a consensus sequence that forms a hairpin loop structure at the exon-intron junction where cleavage occurs.[40] Previous examination of the Ire1 splice sites in *HXL1* revealed a similar conserved hairpin loop at both the 5′ and 3′ exon-intron junction sites, as well as the Ire1 cleavage consensus sequence.[22]

The splicing of *HAC1/HXL1/XBP1* mRNA is a prerequisite for further ER stress signaling as the functional transcription factor can only be translated after this splicing event occurs. A major question that remains in the mechanistic understanding of ER stress induction in *C. neoformans* is how the unspliced *HXL1* mRNA is targeted to the ER surface from the cytosol for the Ire1-dependent splicing, and further, how the spliced mRNA is then selected for translation. In *S. cerevisiae* the comparably large *HAC1* intron (252 nt) plays a role in repressing translation by forming a base pairing interaction with the *HAC1* 5′UTR, thus stalling ribosomes and preventing translation. Splicing of *HAC1* mRNA removes this inhibitory structure allowing for Hac1p production.[42] However thus far, this inhibitory base pairing interaction has only been observed in *S. cerevisiae.* An analysis of

the *HXL1* transcript for sequence similarity between the unconventional intron and the 5′UTR did not reveal any structures indicative of a base pairing interaction that would inhibit the translation of Hxl1.[22]

The inhibition of translation through mRNA secondary structure in *S. cerevisiae* differs considerably from what is seen in higher eukaryotes, where both spliced and unspliced *XBP1* mRNAs are translated, with the unspliced protein product being targeted for rapid degradation. During translation of the unspliced Xbp1, the C-terminus of the nascent peptide chain binds to the ER membrane surface via a conserved hydrophobic residue region, acting to tether the *XBP1* mRNA/ribosome/nascent peptide complex to the ER surface where the *XBP1* mRNA can be spliced by Ire1.[43,44] However at this point the data does not suggest that an Hxl1u protein product is generated, and therefore there is no evidence to suggest a role for Hxl1u in *HXL1* mRNA localization and splicing.[22]

One of the most revealing observations to date is a novel mRNA localization component that has been implicated in *HAC1* mRNA targeting in *S. cerevisiae.* A study by Aragón et al. identified a *cis* element within the 3′ UTR of *HAC1* mRNA required for localization to Ire1 foci on the ER surface. *HAC1* mRNA targeting was also dependent on the unconventional intron, such that when Ire1 splicing occurs the *HAC1* mRNA is no longer localized to the ER surface, suggesting that both the cis element and intron cooperate in efficient *HAC1* mRNA targeting. This consensus element was found in *S. cerevisiae*, *Aspergillus nidulans*, *Candida glabrata*, *Coccidioides posadasii*, *Gibberella zeae*, *Kluyveromyces lactis*, *Magnaporthea grisea*, and *Neurospora crassa*, and is composed of two short sequences UGGCGCG and GCGAC that form a stem-loop structure.[45] However the study stopped short of identifying an mRNA binding protein that recognizes this consensus element. Despite the identification of several fungal species that possess this targeting element, there does not appear to be a targeting element in the *HXL1* transcript, suggesting that this mechanism of mRNA localization does not take place in *C. neoformans*.[22] Further studies are needed to determine the mechanism of *HXL1* mRNA localization to the ER surface in *C. neoformans.*

HXL1 mRNA may not be the only target of Ire1 in *C. neoformans*. Previous studies in eukaryotic systems have revealed a new pathway for the ER stress response, RIDD (regulated Ire-1 dependent decay).[46-48] RIDD utilizes Ire1 nuclease activity to degrade select transcripts that encode proteins which pass through the secretory pathway. This process does not appear to be dependent on Ire1 kinase activity, as RIDD is still seen in a mouse cell line containing an Ire1 variant with an activated endonuclease domain and blocked kinase activity.[46] In *C. neoformans*, attempting to restore wild-type function in an *ire1*Δ mutant by expressing spliced *HXL1* mRNA does not rescue all *ire1*Δ phenotypes, suggesting Ire1 has some *HXL1* splicing independent roles. These include temperature sensitivity, capsule formation, diamide resistance, and virulence.[22] Given these phenotypes it is tempting to postulate a role for RIDD in host temperature adaptation in *C. neoformans*, although more research is needed.

Posttranscriptional Regulation of ER Stress Resolution

There are two opposing functions that make up overall RNA abundance: the transcription rate, and the decay rate. Changes in either of these two components can shift the relative RNA abundance. When looking at various stressors, a common pattern emerges that includes a decrease in ribosomal transcripts and an increase in stress response mRNAs. This is likely achieved through coordination between the transcription rate and decay rate, and will vary in any given species based on the stressor and the appropriate response.[9-11,23-26] During a stress event, important stress response proteins are synthesized, while unnecessary or abundant transcripts are either degraded or temporarily suspended within stress granules.[9,49-53] In both *S. cerevisiae* and *S. pombe*, in response to a stress event, there is a coupled decrease of highly abundant mRNAs such as ribosome biogenesis factors while stress response mRNAs are increased. These same studies have also shown an increase in ER chaperone transcripts following the addition of a stressor, this is hypothesized to reflect an influx of stress response proteins being translated and translocated into the ER for proper glycosylation and folding.[9-11,24,54]

In *C. neoformans*, there is a transient increase in the abundance of transcripts encoding ER stress proteins in response to host temperature. The transcript levels peak at 1 h after the stressor is applied, and return to pre-shift levels after 3 to 4 h.[16] Conversely, mRNAs encoding ribosomal proteins are transiently repressed during temperature adaptation, increasing back to pre-stress levels by 3 h. Investigation into the mechanism of these conversed patterns of gene expression has revealed mRNA degradation as a central regulator. In *C. neoformans* mutants lacking either Ccr4, the major cytoplasmic mRNA deadenylase, or Rbp4, an RNA polymerase II subunit, the transient repression of ribosomal protein transcripts seen upon a shift to 37 °C was absent or attenuated, respectively.[15,16] The regulation of the decay process by Rbp4 suggests that the transcription rate and decay rate are tightly regulated, and that mRNA synthesis and degradation are coupled processes in *C. neoformans*.

During unstressed growth, the *KAR2* mRNA is extremely stable, exhibiting identical decay kinetics in both mRNA degradation mutants and the wild type. Upon a shift to 37 °C, *KAR2* is destabilized by a Ccr4-dependent but Rpb4-independent mechanism. It is not until *KAR2* steady-state levels reach their peak at one hour after a shift to 37 °C that *KAR2* mRNA is further destabilized by an Rpb4-dependent mechanism.[15] Destabilization of highly expressed mRNAs has been reported previously, and may serve as a mechanism to limit the length of time during which a highly abundant stress-response transcript might persist and be translated.[54] This contention is supported by the phenotype of the *ccr4*Δ mutant, which exhibits hallmarks of chronic ER stress response induction including prolonged expression of ER stress mRNAs, temperature sensitivity, increased exposure of β-1,6 glucans, and resistance to tunicamycin.[16] Interestingly, the posttranscriptional regulation of *KAR2* mRNA during transcriptional induction is only seen at 37 °C, and not under other ER stress-inducing conditions such as tunicamycin treatment

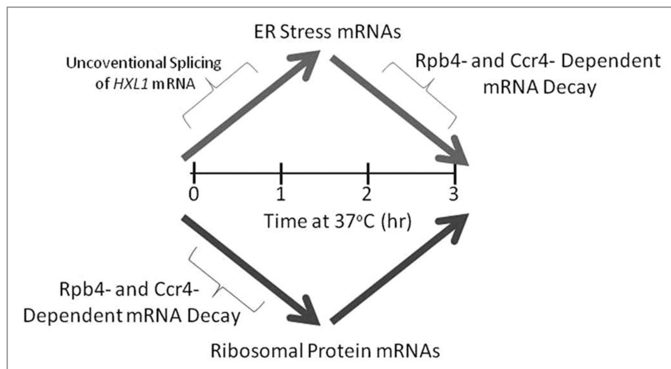

Figure 1. A model depicting the role of posttranscriptional processes in host temperature adaptation of *C. neoformans*. Upon a shift to 37 °C, the ER stress response is initiated by *HXL1* mRNA splicing to generate the active transcription factor Hxl1, which goes on to promote transcription of ER stress genes. Concurrently, ribosomal encoding transcripts levels decrease due to Rbp4 and Ccr4 dependent mRNA decay. During the shutoff phase of the ER stress response Ccr4 and Rbp4 play a role in the degradation and decrease of ER stress transcripts.

(Glazier and Panepinto, unpublished data). This suggests that the accelerated Rpb4- and Ccr4-dependent degradation of *KAR2* may be under the control of a temperature-responsive signaling pathway. The Pkh2 pathway was found to regulate ribosomal protein transcript decay kinetics in *C. neoformans*, but did not impact the decay kinetics of *KAR2*, suggesting regulation by a different pathway.[15] Persistent expression of ER stress mRNAs in mRNA decay mutants, combined with the observation that these mutants exhibit temperature sensitivity and decreased virulence suggest that appropriate resolution, or turning off the ER stress response may be essential to the ability of *C. neoformans* to adapt to host temperature and cause disease.

Final Remarks

C. neoformans is a basidiomycete pathogen capable of causing life threatening meningeoencephalitis in immunocompromised patients. In order to cause a systemic infection *C. neoformans* has adapted to grow at 37 °C. Contrast this adaptation with the two other major opportunistic fungal pathogens, *C. albicans* and *A. fumigatus*: *C. albicans* is a commensal and part of the normal oral microbiota in humans; thus, mammalian body temperature may not be perceived as a severe stress. *A. fumigatus* is one of the few microorganisms able to participate in high-temperature composting. Thus, for *A. fumigatus*, host temperature is also a minimal stress. However *C. neoformans* is found in the environment and therefore a shift from environment temperature to host temperature may be perceived by *C. neoformans* as a considerable stress. In response to this stress, transcriptional reprogramming upregulates stress response transcripts necessary for adaptation. Concurrently, mRNA degradation machinery is engaged to coordinate the time-frame for which a particular transcript is available to be translated. This coordinated transcriptional reprogramming coupled with mRNA decay is thought to succinctly control the engagement and duration of the stress response, allowing the cell to respond to, adapt to and recover from a particular stress event. A model of the impact of posttranscriptional gene regulation in *C. neoformans* temperature adaptation is presented in **Figure 1**. Although we have begun to define the signaling pathways responsible for ribosomal transcript decay during host temperature adaptation, the pathways responsible for ER transcript decay remain to be defined.

When we compare the ER stress response in *C. neoformans* to other species we see both conserved and unique attributes. The basic mechanism of *HAC1*/*HXL1* splicing appears similar to what has been reported for *S. cerevisiae* with the transcript being spliced by Ire1 via recognition of a conserved hairpin loop. However *HXL1* lacks the RNA structure and consensus element required for translational repression and localization to Ire1 foci (respectively), this suggests other mechanisms may be responsible for these components in *C. neoformans*. The presence of *HXL1* independent Ire1 function in *C. neoformans* also suggests RIDD may play a role in the ER stress response. Future investigation into the regulation of *HXL1* mRNA localization, splicing, and translation will likely uncover additional components and processes unique to fungi in general or to *C. neoformans*. Within these unique components and processes, we may find prime candidates for novel antifungal therapies to reduce the global burden of cryptococcosis and other invasive fungal infections.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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