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Oxidant-specific regulation of protein synthesis in *Candida albicans*

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ABSTRACT

Eukaryotic cells typically respond to stress conditions by inhibiting global protein synthesis. The initiation phase is the main target of regulation and represents a key control point for eukaryotic gene expression. In *Saccharomyces cerevisiae* and mammalian cells this is achieved by phosphorylation of eukaryotic initiation factor 2 (eIF2α). We have examined how the fungal pathogen *Candida albicans* responds to oxidative stress conditions and show that oxidants including hydrogen peroxide, the heavy metal cadmium and the thiol oxidant diamide inhibit translation initiation. The inhibition in response to hydrogen peroxide and cadmium largely depends on phosphorylation of eIF2α since minimal inhibition is observed in a *gcn2* mutant. In contrast, translation initiation is inhibited in a Gcn2-independent manner in response to diamide. Our data indicate that all three oxidants inhibit growth of *C. albicans* in a dose-dependent manner, however, loss of *GCN2* does not improve growth in the presence of hydrogen peroxide or cadmium. Examination of translational activity indicates that these oxidants inhibit translation at a post-initiation phase which may account for the growth inhibition in a *gcn2* mutant. As well as inhibiting global translation initiation, phosphorylation of eIF2α also enhances expression of the *GCN4* mRNA in yeast via a well-known translational control mechanism. We show that *C. albicans GCN4* is similarly induced in response to oxidative stress conditions and Gcn4 is specifically required for hydrogen peroxide tolerance. Thus, the response of *C. albicans* to oxidative stress is mediated by oxidant-specific regulation of translation initiation and we discuss our findings in comparison to other eukaryotes including the yeast *S. cerevisiae*.

1. Introduction

Candida albicans is a major human fungal pathogen which causes opportunistic infections (Pfaller and Diekema, 2007). Systemic *C. albicans* infections are life-threatening, particularly in immunocompromised patients undergoing organ transplantation and chemotherapy, and those with HIV/AIDS. This means that Candida infections are a leading cause of mortality arising from hospital-acquired bloodstream infections (Viudes et al., 2002; Wisplinghoff et al., 2004). Host cells mount important immunological defences against fungal pathogens including the generation of reactive oxygen species (ROS) by neutrophils and macrophages (Brown et al., 2009). However, increasing evidence suggests that a key virulence determinant of *C. albicans* is its ability to overcome the hosts ROS generation response. This is illustrated by findings showing that *C. albicans* mutants lacking antioxidants show increased susceptibility to macrophage attack and have attenuated virulence in mice models (da Silva Dantas et al., 2010; Hwang et al., 2002; Wysong et al., 1998). With the availability of the *C. albicans* genome sequence much attention has focused on analyzing the changes in gene expression/transcription during host infection. These studies have confirmed that the oxidative stress response is important since alterations in the expression profiles of antioxidants and other stress protective molecules are detected following exposure to macrophages and neutrophils (Fradin et al., 2005; Lorenz et al., 2004). However, many studies in other cell types including mammalian cells and the yeast *Saccharomyces cerevisiae* have shown that oxidative stress results in a rapid and reversible reduction in global protein synthesis meaning that it is often unknown how changes in the transcriptome are translated into the cellular proteome (Clemens, 2001; Proud, 2005; Shenton et al., 2006).

Most eukaryotic cells respond to stress conditions by invoking complex regulatory mechanisms including global inhibition of translation (Proud, 2005). Reducing protein synthesis prevents the continued burden of gene expression during potentially error-prone conditions as well as allowing for the turnover of

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existing mRNAs and proteins whilst gene expression is reprogrammed to deal with the stress. The initiation phase of translation is the main target of regulation and represents a key control point for eukaryotic gene expression. This inhibition is mainly dependent on kinases which phosphorylate the α subunit of translation initiation factor 2 (eIF2) (Donnelly et al., 2013). eIF2 is an essential GTP-binding protein which interacts with the initiator methionyl-tRNA (Met-tRNA$_i^{Met}$) to form a ternary complex (TC) that is competent for translation initiation. Phosphorylation of eIF2α at a conserved serine residue blocks GDP–GTP exchange resulting in reduced TC levels and reduced global translation (Harding et al., 2000; Pavitt et al., 1998). Four mammalian kinases have been identified which inhibit translation initiation by phosphorylating eIF2α. GCN2 (the amino acid control kinase), PKR (the double-stranded RNA-activated protein kinase), HRI (the heme-regulated inhibitor) and PERK (the PKR-like endoplasmic reticulum eIF2α kinase) which are regulated independently in response to various different cellular stresses (Dever, 2002; Proud, 2005). Additionally, eIF2α phosphorylation induces translation of specific mRNAs, such as that encoding the metazoan activating transcription factor 4 (*ATF4*). *ATF4* mediates the "integrated stress response" whose targets ultimately protect against the inducing stress conditions (Harding et al., 2003). Phosphorylation of eIF2α therefore pre-conditions cells to oxidative and ER stress conditions and increases oxidative stress tolerance via *ATF4* activation (Lewerenz and Maher, 2009; Lu et al., 2004).

S. cerevisiae solely expresses the Gcn2 eIF2 kinase which was originally shown to phosphorylate eIF2α in response to amino acid starvation conditions (Dever, 2002; Hinnebusch, 2005). Yeast Gcn2 is now known to be activated in response to a variety of conditions including nutrient starvation (amino acids, purines, glucose) and exposure to sodium chloride, rapamycin, ethanol and volatile anesthetics (Hinnebusch, 2005; Palmer et al., 2005). Depletion of amino acids leads to an accumulation of uncharged tRNA which activates Gcn2 via its HisRS-related domain. It is likely that other stress conditions also ultimately affect the levels of uncharged tRNA in the cell (Palmer et al., 2005; Yang et al., 2000). Oxidative stress induced by exposure to H_2O_2 activates Gcn2-dependent phosphorylation of eIF2α and inhibits translation initiation in a dose-dependent manner (Shenton et al., 2006). This is a general response to ROS, since Gcn2 is also activated by exposure to an organic hydroperoxide (cumene hydroperoxide), a thiol oxidant (diamide) and a heavy metal (cadmium) (Mascarenhas et al., 2008). Phosphorylation of eIF2α by Gcn2 reduces global protein synthesis, but also induces translation of the *GCN4* mRNA in a translational control mechanism involving four short upstream open reading frames (uORFs) (Hinnebusch, 2005). Gcn4 is a transcription factor, which analogous to the mammalian integrated stress response, activates amino acid biosynthetic genes to overcome the imposed starvation which initially led to its translational control. As well as being required during amino acid starvation conditions, *S. cerevisiae* Gcn4 promotes tolerance to hydrogen peroxide stress (Mascarenhas et al., 2008).

Similar to *S. cerevisiae*, *C. albicans* only expresses the Gcn2 eIF2 kinase which can phosphorylate eIF2α in response to amino acid starvation conditions (Tournu et al., 2005). Amino acid starvation conditions can promote morphogenetic changes in *C. albicans*, including hyphal differentiation and biofilm formation, which are important determinants of virulence (Rubin-Bejerano et al., 2003; Tripathi et al., 2002). *C. albicans* can mount a GCN (general amino acid control) response; where it induces the expression of most amino acid biosynthetic pathways in response to amino acid starvation, dependent on the Gcn4 transcription factor (Tripathi et al., 2002). *GCN4* expression is regulated in response to amino acid starvation by a combination of transcriptional and translational control mechanisms (Sundaram and Grant, 2014; Tournu et al., 2005). We show here that *C. albicans* Gcn2 is also activated in response to diverse oxidative stress conditions. For both hydrogen peroxide and the heavy metal cadmium this results in a Gcn2-dependent inhibition of translation initiation, whereas, the thiol oxidant diamide inhibits translation initiation by a Gcn2-indepndent mechanism. Oxidative stress also induces the expression of *GCN4* by both transcriptional and translational control mechanisms. We show that *C. albicans* Gcn4 is particularly important for the response to hydrogen peroxide and mutants lacking *GCN4* are sensitive to hydrogen peroxide stress.

2. Materials and methods

2.1. Strains and growth conditions

The *C. albicans gcn2* and *gcn4* mutants are isogenic derivatives of CAI-4 (*ura3::λ imm434/ura3::λ imm434*) as described previously (Tournu et al., 2005; Tripathi et al., 2002). Strains were grown in complex YEPD (2% w/v glucose, 2% w/v bactopeptone, 1% w/v yeast extract) or synthetic complete (SC) medium (2% w/v glucose, 0.67% w/v yeast nitrogen base and 0.185% w/v complete amino acid supplement (Formedium) mixtures). Uridine was supplemented to a final concentration of 25 μg/ml. Strains were grown at 30 °C and 180 rev./min. Media were solidified by the addition of 2% (w/v) agar. Stress sensitivity was determined by growing cells to stationary phase and spotting diluted cultures (A_{600} = 1.0, 0.1, 0.01 and 0.001) onto agar plates containing various concentrations of oxidants.

2.2. mRNA analysis

Total RNA was extracted using the RNEasy mini kit (Qiagen, Germany). 200 ng of total RNA was reverse transcribed into cDNA using Oligo(dT)20 primer and iScript reverse transcriptase (Biorad, USA). RT-PCR was performed using the iTaq Universal SYBR Green Supermix (Biorad, USA) in a CFX Connect™ Real-Time PCR Detection System (Biorad, USA). Primers used for detection of *GAPDH* mRNA forward and reverse primer were 5′-CGAA GGTGCTCAAAAACACA-3′, 5′-TGTACCACCAACTGTTTGGC-3′, and *GCN4* mRNA forward and reverse primer were 5′-CCAGAAATG-CAAAAGGCTTC-3′, 5′-GACTTTGGC TCCGTCCATAA-3′.

2.3. Polysome and protein analysis

Extracts were prepared in the presence of cycloheximide for polysome analysis as described previously (Shenton et al., 2006). Monosome and polysome peaks were quantified using the National Institutes of Health Image J software (http://rsb.info.nih.gov/ij/). Immunoblots were probed using phosphospecific eIF2α antibodies (Shenton et al., 2006) and antibodies raised against yeast elongation factor 1 (Tef1) or phosphoglycerate kinase (Pgk1) as a loading control. The rate of protein synthesis was measured in exponential phase cells treated with various concentrations of oxidants. Cells were treated with oxidants for 1 h and pulse-labeled for the last 5 min of the treatment with 85 μM L-[^{35}S] cysteine/methionine as described previously (Shenton and Grant, 2003).

2.4. GCN4 reporter assays

The 600 bp immediately upstream of the *C. albicans GCN4* coding sequence was introduced immediately upstream of *RLUC* in the pCRW3N basal vector to create a *GCN4* translational reporter (Sundaram and Grant, 2014). Plasmids were integrated at the *ADE2* locus of wild-type and *gcn2* mutant strains and three independent colonies were used for each experiment. Luciferase analy-