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The *Neurospora crassa* OS MAPK pathway-activated transcription factor ASL-1 contributes to circadian rhythms in pathway responsive clock-controlled genes

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ABSTRACT

The OS-pathway mitogen-activated protein kinase (MAPK) cascade of *Neurospora crassa* is responsible for adaptation to osmotic stress. Activation of the MAPK, OS-2, leads to the transcriptional induction of many genes involved in the osmotic stress response. We previously demonstrated that there is a circadian rhythm in the phosphorylation of OS-2 under constant non-stress inducing conditions. Additionally, several osmotic stress-induced genes are known to be regulated by the circadian clock. Therefore, we investigated if rhythms in activation of OS-2 lead to circadian rhythms in other known stress responsive targets. Here we identify three more osmotic stress induced genes as rhythmic: *cat-1*, *gcy-1*, and *gcy-3*. These genes encode a catalase and two predicted glycerol dehydrogenases thought to be involved in the production of glycerol. Rhythms in these genes depend upon the oscillator component FRQ. To investigate how the circadian signal is propagated to these stress induced genes, we examined the role of the OS-responsive transcription factor, ASL-1, in mediating circadian gene expression. We find that while the *asl-1* transcript is induced by several stresses including an osmotic shock, *asl-1* mRNA accumulation is not rhythmic. However, we show that ASL-1 is required for generating normal circadian rhythms of some OS-pathway responsive transcripts (*bli-3*, *ccg-1, cat-1, gcy-1 and gcy-3*) in the absence of an osmotic stress. These data are consistent with the possibility that post-transcriptional regulation of ASL-1 by the rhythmically activated OS-2 MAPK could play a role in generating rhythms in downstream targets.

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1. Introduction

The evolution of organisms in the cyclical environment of the earth has led to the development of internal biological clocks. Biological clocks provide a mechanism for organisms to anticipate cyclical daily stresses such as light, heat, and desiccation, and to mount appropriate physiological changes that will help the organism better adapt to their environment (Dodd et al., 2005; Ouyang et al., 1998; Woelfle et al., 2004; Yerushalmi and Green, 2009; Yerushalmi et al., 2011). We are beginning to understand that the circadian oscillator mechanism does this, at least in part, through the rhythmic activation of stress response pathways, even in the absence of obvious stress (de Paula et al., 2008). We previously showed that the *Neurospora crassa* clock transcription factor, the White Collar Complex (WCC), binds rhythmically to the promoter of the MAPK kinase (Smith et al., 2010) of the OS-pathway (*os-4*), and drives daily rhythms in *os-4* mRNA and protein (Lamb et al., 2011). This then leads to a rhythm in the phosphorylation of the terminal MAPK, OS-2 (Vitalini et al., 2007). Daily rhythms in OS-2 phosphorylation occur in the absence of stress, and in constant conditions (constant temperature, constant darkness) under control of the endogenous circadian clock mechanism.

OS-2 is a homolog of both the Hog1 MAPK from yeast and the p38 MAPK from mammals (Zhang et al., 2002). This MAPK is critical for mounting responses that can help organisms adapt to increased extra-cellular osmotic pressure, oxidative stress, heat and many other stresses (Hohmann, 2002; Sheikh-Hamad and Gustin, 2004). In fungi, the OS-pathway provides the target by which many anti-fungal drugs work to promote toxicity (Ochiai et al., 2002; Vetcher et al., 2007; Zhang et al., 2002). In mammals, the p38 MAPK is a target of the pyridinyl imidazole class of compounds that have anti-inflammatory activity (Schindler et al., 2007). Furthermore, there are links between the p38 MAPKs and cell death, cell cycle progression, and cancer (Cuenda and Rousseau, 2007; Fu and Lee, 2003; Yaakov et al., 2009). Thus, understanding how the p38 MAPK pathway is connected to the circadian clock and downstream rhythmic gene expression may be important for many aspects of human health.

Several targets of the OS-2 MAPK have been discovered in Neurospora, and these are similar to the targets in yeast and mammals (Irmler et al., 2006; Noguchi et al., 2007; Watanabe et al., 2007; Yamashita et al., 2007). In *Saccharomyces cerevisiae*, Hog1

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phosphorylates ion channels, kinases, phosphatases, and transcription factors that bring about the adaptive response and modulate pathway activity (Hohmann, 2002; Proft et al., 2006; Proft and Struhl, 2002, 2004; Rep et al., 2000). In mammals, a similar complement of p38 MAPK targets is also observed. One common target found in yeast and mammals is the bZIP transcription factor Sko1 or ATF/CREB. In yeast, Sko1 is phosphorylated by Hog1 (Proft et al., 2001; Proft and Struhl, 2002; Rep et al., 2001) and in mammals ATF/CREB is phosphorylated by p38 MAPK in response to various stresses (Hazzalin et al., 1996; Sen et al., 2005; Zayzafoon et al., 2002). Phosphorylation of Sko1 by the MAPK (Hog1) is known to convert it from a repressor to an activator, rapidly increasing the expression of many stress responsive genes (Proft and Struhl, 2002; Rep et al., 2001). Neurospora possesses a homolog of Sko1 designated ASL-1 for an ascospore lethal phenotype (Colot et al., 2006), and was subsequently referred to as ATF-1 for homology to the mammalian transcription factor (Yamashita et al., 2008). ASL-1 is required for the induction of some genes in response to the fungicide fludioxonil, and in response to osmotic stress (Yamashita et al., 2008).

Following the demonstration that the OS-2 MAPK is rhythmically phosphorylated during the course of a day in the absence of stress, it was of interest to determine if OS-2 phosphorylation rhythms would lead to rhythms in genes known to be stress-induced targets of the OS pathway. Indeed, some of the targets of the OS pathway, including *bli-3* (blue light induced-3, unknown function), *ccg-1* (clock controlled gene-1, unknown function), *ccg-9* (trehalose synthase), *ccg-13* (cell wall protein), and *ccg-14* (cell wall protein) are induced by an activated OS-2 pathway and are clock-controlled (Bell-Pedersen et al., 1996; Loros et al., 1989; Shinohara et al., 2002b; Watanabe et al., 2007; Zhu et al., 2001). However, there are several other genes induced by the OS-pathway that have not been specifically studied for rhythmicity. Furthermore, the role of the ASL-1 transcription factor in generating rhythmicity of OS-pathway output genes has not been examined. In this study we investigate the connection between the OS-pathway and circadian gene expression, with a focus on the role of the ASL-1 transcription factor.

2. Materials and methods

2.1. Strains and culture conditions

N. crassa strains utilized in this study were grown and maintained as previously described (Davis and de Serres, 1970). All strains are "clock strains" that contain the *ras-1^{bd}* allele, a mutation that reduces the growth rate, and clarifies the rhythmic asexual conidial banding pattern on racetubes (Belden et al., 2007; Sargent et al., 1966), but that does not alter the rhythm in phosphorylated OS-2 (Vitalini et al., 2007). Time course experiments were carried out as described (Lamb et al., 2011). The genotype of the circadian clock deficient ΔFRQ strain (*frq^{10}*) we analyzed was *ras-1^{bd}*, *Δfrq::hph* (Aronson et al., 1994), which was obtained from the Fungal Genetic Stock Center (FGSC 7490). Stress treatments were performed on mycelial disks grown in 1× Vogels, 2% glucose, 0.5% arginine pH 6.0 liquid media in constant light at 25 °C after 24 h of growth, except the 30 min light pulse was performed after growth in constant darkness for 24 h. The stress treatments were all for 30 min except the hydrogen peroxide treatment, which was for 15 min. The stress treatments consisted of 100% water, 4% NaCl, 1 M sorbitol, 1 μg/ml fludioxonil "Pestanal" (Sigma 46102), and 10 mM hydrogen peroxide. Tissue was harvested immediately following the stress treatments.

2.2. Deletion of the ASL-1 transcription factor

Deletion of the transcription factor ASL-1 causes an ascospore lethal phenotype (Colot et al., 2006), and thus a *ras-1^{bd} Δasl-1* strain could not be obtained via crossing of the available *Δasl-1* strain (FGSC 21336). Generation of the *Δasl-1* strain in the *ras-1^{bd}* background required new strain construction. Primers used in the construction of the new knockout are identical to those used in the gene knockout project (see http://www.dartmouth.edu/~neurosporagenome/primers.html), however, rather than using yeast to recombine the 5′ *asl-1* flank, the *hph* gene, and 3′ *asl-1* flank, a hybrid PCR was performed to fuse them together. This hybrid PCR product was then used to transform a *ras-1^{bd}* strain (DBP1258). Heterokaryons were selected for on plates containing 200 μg/ml hygromycin. Homokaryons of the *Δasl-1::hph* genotype were identified by PCR screening of single colonies derived from the heterokaryons. Three homokaryons derived from three independent transformants called DBP889, DBP1314, and DBP1315 were obtained, and we have observed no differences between these strains. Consistent with previous data (Colot et al., 2006; Yamashita et al., 2008), we find that the *ras-1^{bd} Δasl-1::hph* strain is ascospore lethal, has reduced conidial germination rates, and it is not sensitive to osmotic stress similar to the *Δasl-1::hph* strain (data not shown).

2.3. Gene expression analysis

RNA was extracted and purified from ground tissue and 10–15 μg were run on denaturing formaldehyde gels (Bell-Pedersen et al., 1996). RNA from control and test strains were always run side by side on the same gel so that changes in gene expression relative to the control could be determined. Gels were blotted to NitroPure membranes (GE, WP4HY00010) and hybridized to gene specific probes: *cat-1*, *dak-1*, *fbp-1*, *gyc-1*, *gcy-3*, and *pck-1* were detected with [α-^{32}P]-dATP-labeled DNA probes, while *asl-1*, *bli-3*, *ccg-1*, and *ccg-9* were detected with [α-^{32}P]-UTP labeled antisense RNA probes. As is standard in the field, gene expression was normalized to the ribosomal RNA visualized on the membrane with ethidium bromide. For time course experiments, the average normalized expression level of a strain was set to one and then to permit sine wave curve fitting, one was subtracted from the expression level at each time point.

2.4. Statistical analysis

Nonlinear regression to fit the rhythmic data to a sine wave (fitting period, phase, and amplitude) and a line (fitting slope and intercept), as well as Akaike's information criteria tests (Burnham and Anderson, 2002) to compare the fit of each data set to the two equations, were carried out using the Prism software package (GraphPad Software, San Diego, CA). The p values reflect the probability that, for instance, the sine wave fits the data better than a straight line.

3. Results

3.1. The OS-pathway stress induced genes, cat-1, gcy-1 and gcy-3 are circadianly regulated

Several Neurospora genes are induced after a salt shock or fungicide treatment, with induction being dependent on activation of the OS pathway. These include the catalase gene *cat-1*, the glycerol synthesis genes *gcy-1*, *gcy-3*, and *dak-1*, and the gluconeogenesis genes *fbp-1* and *pck-1*, amongst others (Noguchi et al., 2007; Watanabe et al., 2007; Yamashita et al., 2007). The OS-pathway is activated by the circadian clock in the absence of stress; therefore, we postulated that genes that are stress regulated by the OS pathway would also be circadianly regulated in the absence of stress. Thus, we examined if these mRNAs accumulated with a cir-

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