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SPHINGOLIPIDS MEDIATE FORMATION OF mRNA PROCESSING BODIES DURING THE HEAT STRESS RESPONSE OF *SACCHAROMYCES CEREVISIAE*

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Abstract

Recent work, especially in the yeast *Saccharomyces cerevisiae*, has demonstrated that mRNA movement from active translation to cytoplasmic granules, termed mRNA processing bodies (“p-bodies”), occurs in concert with the regulation of translation during cell stress. However, the factors regulating p-body formation are poorly defined. Recent data demonstrated a function for sphingolipids in regulating translation during heat stress, which led to the current hypothesis that p-bodies may form during heat stress in a sphingolipid-dependent manner. In this study, we demonstrate that heat stress induced formation of p-bodies as determined by localization of a GFP-tagged Dcp2p and RFP-tagged Edc3p to discrete cytoplasmic foci. Sphingoid base synthesis was required for this effect, in that inhibition of sphingoid base synthesis attenuated formation of these foci during heat stress. Moreover, treatment of yeast with exogenous sphingoid bases phyto- and dihydrosphingosine promoted formation of p-bodies in the absence of heat stress, and the *lcb4/5* double deletion yeast mutant defective in sphingoid base kinase activity demonstrated both highly elevated sphingoid bases and large, clearly defined p-bodies under non-stress conditions. Functionally, inhibition of sphingolipid synthesis during heat did not prevent translation arrest but extended translation arrest, supporting a role for sphingoid bases in the resumption of translation following heat stress. Together, these data demonstrate a critical and novel role for sphingolipids in mediating p-body formation during heat. While roles of p-bodies in translation are the subject of much current investigation, these data indicate a novel function for sphingoid bases in p-body formation during heat stress and may help to elucidate mechanisms by which sphingolipids regulate translation.

A central component of the cellular response to stress is the induction of large-scale changes in specific mRNA transcript levels (1,2). Though transcriptional changes in response to stress conditions are well established, an appreciation has recently developed for post-transcriptional mechanisms of regulating mRNA, such as mRNA sequestration to cytoplasmic granules including mRNA processing bodies (“p-bodies”)(3,4). In yeast, these cytoplasmic foci form during stress conditions including glucose depletion, salt treatment, and late stages of culture growth(5,6). P-bodies are comprised of mRNA, RNA-interacting proteins including the core P-body components involved in cap-dependent degradation (Dcp1p, Dcp2p, Edc3p, and others), and proteins involved in translation and/or its regulation (7,8). Recent data indicate that p-bodies function as sites of mRNA degradation (8). Moreover, though current models place translation arrest upstream from p-body formation (reviewed in X), several lines of evidence in yeast and mammalian cells indicate that mRNA

from p-bodies can return to translation, thus placing p-bodies themselves upstream from translation initiation (). This has led to the concept of an 'mRNA cycle' whereby mRNA moves dynamically through translation, p-bodies, and stress granules, though directionality has not been firmly established.

Recent decades have witnessed the emergence of sphingolipids including sphingoid bases and ceramide as key mediators of stress responses(9,10). Biosynthesis of sphingolipids occurs in response to cytokines, UV irradiation, DNA-damaging chemicals, and many other stressors including thermal stress(9,10). Thus, the heat stress response (HSR) of *S. cerevisiae* has been used as a model system for dissecting eukaryotic stress responses(11–13), including the role of sphingolipids in mediating HSR subprograms including transcriptional reprogramming(14), translational arrest(15), and protein degradation(16,17). These roles of sphingolipids in stress responses, along with recent studies implicating sphingolipid synthesis in translational regulation during heat stress suggested the hypothesis that sphingolipids may be linked mechanistically and functionally to p-body formation in yeast. Though p-body formation in response to heat has not been previously reported to occur in *S. cerevisiae*, since heat stress transiently decreases translation initiation(15), it seemed possible that a concomitant increase in p-bodies may occur in response to heat stress.

In this study, we show that heat stress induces p-body markers to discrete cytoplasmic foci. Mechanistically, the data demonstrate that this occurs in a sphingolipid-dependent manner. These data also provide insights on the roles of bioactive sphingolipids in mediating translational control during the HSR. Thus, these results provide important insights into the mechanisms of p-body formation as well as their functions, and they also underscore the fundamental and multiplex roles played by members of this lipid class in cellular adaptation to stress.

Experimental Methods

Yeast strains, culture, and treatment

Genotypes of yeast strains used in this study are listed in Table 1. Routine culture of yeast occurred in Yeast Proteose Dextrose media (1% yeast extract, 2% proteose peptone, and 2% dextrose) at 30°C with 200–250rpm shaking. Edc3p-mCherry was expressed from the pRP1574 vector(7) and selected on uracil drop-out media. Unless otherwise noted, strains were grown to mid-logarithmic phase (O.D.₆₀₀=0.4–0.6) prior to treatment. Phytosphingosine, sphingosine, and stearylamine were purchased from Sigma, and all other lipids were synthesized and purified at the Lipidomics Core Facility at the Medical University of South Carolina.

Microscopy

After treatment, cells were quickly pelleted in a bench-top microcentrifuge for 15–30 seconds. Media was decanted, and cell pellets were resuspended in residual media, and immediately spotted onto microscope slides. Since yeast under coverslips are subject to hypoxic stress(7), coverslips were applied to droplets only immediately prior to visualization. Cells were visualized by confocal microscopy using an LSM510 microscope (Carl Zeiss, NY). Images show a single plane of the sample.

Sphingoid base measurements

After treatment, cell pellets were harvested by centrifugation at 3500×g for 3 minutes. Pellets were immediately resuspended in 3 ml of 2:1 chloroform:methanol, and sphingolipids were extracted as previously described(18). The lipid fraction was subjected to

mild alkaline hydrolysis. Sphingoid bases were converted to fluorescent *o*-phthalaldehyde derivatives, and resolved by HPLC as described previously(19). Measurements were normalized to total phospholipid determined in an aliquot of the lipid extract as described(19).

Polyribosome Analysis

Yeast cytoplasmic extracts were prepared from log-phase cultures treated as described(20). Cells were treated as indicated, and then incubated for 15 minutes at room temperature with 0.1 mg/ml cyclohexamide added to the culture medium. Cells were collected by centrifugation and suspended in 1ml lysis buffer (10 mM Tris-Cl pH 7.5, 100mM NaCl, 30 mM MgCl₂, 0.1 mg/ml cycloheximide, and 0.2 mg/ml heparin). Cells were disrupted by vortexing with glass beads, and 20 O.D. units were loaded on a 15 to 50% sucrose gradient buffered with 50 mM Tris-acetate (pH 7.4), 50 mM NH₄Cl, 12 mM MgCl₂, 1mM DTT. Gradients were centrifuged in an SW41 rotor at 40,000 rpm for 120 min at 4°C. After ultracentrifugation, the tubes were punctured at the bottom, and the fractions were displaced upwards using Fluorinert FC-40 (Sigma). Absorbance was monitored continuously at 254 nm using ISCO-UA45 (model 640) density gradient fraction collector.

Results

Heat stress induces p-body formation

The protein comprising the mRNA decapping enzyme Dcp2p participates in mRNA degradation, and it localizes in p-bodies in response to several conditions including glucose deprivation and late growth stage(4,6). Thus, GFP-tagged Dcp2p has been used as a marker for p-bodies(7). In a strain containing a GFP tag sequence in the chromosomal DCP2 gene to permit p-body visualization by confocal microscopy in live cells, p-bodies were undetectable during log phase growth under normal conditions, but stress conditions including growth to saturation led to concentrations of GFP signal in several punctuate cytoplasmic foci(6). Consistent with these results, approximately 1–3 cytoplasmic foci were observed in yeast grown to saturation at 30°C in YPD media, but not in cells in logarithmic growth (Fig. 1A). In general, the intensity, duration, and type of stress regulates p-body size and number(6).

Because of the highly conserved nature of the stress response in *S. cerevisiae*, we sought to investigate whether heat stress would induce similar Dcp2p-GFP localization. Cells were grown in rich media to mid-log phase (an O.D. of 0.3–0.6), and then aliquots of live cells were quickly mounted and visualized by confocal microscopy over a time course. Indeed, shifting cultures from the normal growth temperature of 30°C to the heat stress temperature of 39°C caused formation of faint punctuate spots of a comparatively smaller size than those observed in saturated cultures (Figure 1B). These faint foci first appeared at 10 minutes of heat stress. Over 20 to 30 minutes these foci increased in apparent fluorescence intensity, and the number of cells displaying these foci increased such that by 20 minutes, virtually all cells showed some foci. In cells held at 39°C, dissolution of the signal occurred after 45–60 minutes (Figure 1B).

This yeast strain provided a distinct advantage over heterologous expression of p-body markers, in that this strain did not require growth on selection media, which, in our hands, caused a small amount of p-body formation under conditions of logarithmic growth in some genetic backgrounds. On the other hand, it was important to employ another independent p-body marker. Thus, we utilized Edc3, which activates mRNA decapping and along with enzymes required for decapping and mRNA degradation serves as a core component of p-bodies. An Edc3p construct tagged with the red-fluorescent protein mCherry was expressed

on a low-copy plasmid selectable on uracil drop-out media. Yeast transformed with this construct showed very few p-bodies during log-phase growth at 30°C. In contrast, a shift in culture temperature to 39°C for 25 minutes precipitated a clear-cut induction of p-bodies (Figure 1C) similar in size and number to those observed using the Dcp2p-GFP expressing strain (Figure 1B). The observation of p-bodies using two different markers and two different background strains supported the hypothesis that heat stress induces p-body formation in yeast, albeit both greater in number and smaller in size than those observed in saturated yeast cultures.

Heat-induced p-body formation requires *de novo* sphingolipid synthesis

We and others previously demonstrated the acute flux through *de novo* sphingolipid synthesis at early time points of heat stress(21,22). This flux induces transient increases in sphingolipids over a time course of heat stress through increasing serine supply to serine palmitoyltransferase(23). Initially, increases occur in the sphingoid bases dihydro- and phytosphingosine, with subsequent elevation in sphingoid base phosphates and ceramides(21,22,24). While this phenomenon is observed among a wide variety of yeast strains, we previously reported significant variation between parental wild-type strains in the magnitude of this aspect of the heat stress response(23). Thus, we tested the Dcp2-GFP expressing strain for its ability to increase sphingoid bases in response to heat stress. Indeed, this strain demonstrated a robust 2–5 fold increase in sphingoid base species by 10 minutes of heat stress, which was completely attenuated by a 5-minute pre-treatment with the serine palmitoyltransferase inhibitor, myriocin (Figure 2A). Thus, we used these conditions to test whether sphingoid base production during heat was a requirement for p-body formation. Pre-treatment with myriocin significantly blunted formation of p-bodies at 20 and 30 minutes following heat stress (Figure 2B). These results indicate that *de novo* sphingolipids are critical for p-body formation during heat stress.

Treatment with sphingoid bases induces p-body formation

Though heat stress initiates a cascade of sphingolipid synthesis, the sphingoid bases phyto- and dihydrosphingosine are well established signaling sphingolipids in yeast and their levels increase in a time frame commensurate with the formation of p-bodies(21,22). In an attempt to identify the active species for heat-mediated p-body formation, cells at the non-stress temperature of 30°C were treated with a panel of sphingoid bases or similar compounds at 10µM. Treatment of cells with yeast sphingoid base species initiated the localization of Dcp2-GFP to cytoplasmic foci, with phytosphingosine being the most active, followed by D-erythro-dihydrosphingosine, and then its unnatural stereoisomer, L-threo-dihydrosphingosine (Figure 3). In contrast, sphingosine, the major sphingoid base in mammalian cells, was unable to induce p-body formation. Likewise, neither ceramide nor stearylamine caused p-bodies to form (Figure 3). Thus, yeast sphingoid bases, and particularly, phytosphingosine, seem the likely candidates for p-body formation during heat stress.

Sphingoid bases undergo conversion to phosphorylated derivatives that may play roles in growth and mitochondrial function(25,26). Moreover, phosphorylation of sphingoid bases serves to regulate their levels, as deletion of the sphingoid base kinases LCB4 and LCB5, increases sphingoid base levels several fold(27). Indeed, during logarithmic growth at 30°C, cells deleted for these kinases demonstrated well over 10-fold increases in both C18 and C20 sphingoid base species (Figure 4A). Thus, this strain provided a useful tool in which to test whether elevation of endogenous sphingoid bases is sufficient to induce p-bodies even under normal growth conditions, and, moreover, whether their phosphorylation is required for p-body formation. The *lcb4* *lcb5* deletion strain and its parental wild type strain, JK93d were both transformed with the Edc3-mCherry construct. Transformed strains were

grown to an O.D. of 0.7 in selective media at 30°C and visualized with confocal microscopy. Indeed, under these conditions, fluorescent foci were virtually undetectable in the parental background strain. In contrast, the *lcb4 /lcb5* double deletion strain showed 1–2 relatively large p-bodies in most cells (Figure 4B). These data further connect sphingolipid metabolism to p-body formation and indicate that elevating sphingoid bases increases p-bodies, even in the absence of heat stress. Additionally, these data confirm that further metabolism of sphingoid bases to their phosphorylated derivatives is not required for sphingolipid-mediated p-body formation.

Myriocin potentiates heat-mediated translation arrest

Current data show that formation of p-bodies is linked to decreasing translation initiation(6,8); however, previous data indicated a role of sphingoid bases in increasing translation initiation during heat stress, after an initial decrease(15). These data at first seemed inconsistent with data presented here supporting a role for sphingoid bases in p-body formation; however, it should be noted that the precise relationship of p-bodies to translation arrest is currently not clear. Some studies have suggested that p-bodies form downstream of translation arrest; however, a recent study indicated that p-body formation is required for the formation of stress granules, and that stress granules mediate translation initiation. Therefore one possibility is that p-body formation is required for sequestration of RNA in order to enhance resumption of translation. In this case, inhibition of p-body formation with myriocin should prolong the translation arrest following heat stress.

It should be also noted that the studies on sphingoid bases and translation initiation were conducted using the *lcb1-100* yeast strain temperature-sensitive for the serine palmitoyltransferase subunit LCB1(15). This yeast strain showed decreased protein synthesis compared to its parental background over a time course of heat stress; however, the use of this strain does not distinguish totally the roles of acutely generated sphingolipids from other basal changes, including effects on gene expression(14). Consequently, we sought to establish whether an acute inhibition of *de novo* sphingolipid synthesis in an otherwise healthy yeast strain would reveal a role for sphingoid bases in heat-mediated translation arrest. In this effort, the Dcp2-GFP expressing strain (yRP1727) was treated with myriocin or vehicle control at the onset of heat stress, and active translation was determined by analysis of polyribosome profiles. While log-phase yeast at 30°C demonstrated high polyribosome peaks relative to free 80S ribosomal subunit, heat stress for 15 minutes caused a decrease in polysome peak height and a concomitant increase in the free subunit peak, reflecting a decrease in active translation (Figure 5). Treatment with myriocin during heat stress potentiated this decrease, as demonstrated by the even greater height of the free ribosomal subunits peak relative to polysome peaks (Figure 5). Thus, these results agree with previous findings that sphingoid bases promote translation initiation (15). Moreover, the same study demonstrated that the *lcb4 /lcb5* double deletion strain, which has constitutively increased sphingoid bases, showed elevated rates of translation initiation compared to its parental strain. Together, these results demonstrate a direct correlation between p-body formation and rate of translation initiation, and suggest that sphingolipids mediate translation initiation by promoting p-body formation. Moreover, the results suggest that the key function of p-bodies during heat stress is to mediate translation initiation.

Discussion

The current study provides data demonstrating that heat stress induces p-body formation in *S. cerevisiae*, and also implicating the acute induction of sphingoid bases in this process. The results also provide evidence supporting a role for p-bodies in resumption of translation after heat stress, rather than in the initial arrest of translation which is observed after heat stress.

To our knowledge, these data represent the first observation of p-body formation during heat stress in yeast, though p-body formation in response to heat has been observed in mammalian cells (28), and stress granules, which are thought to form subsequent to p-bodies, have been observed in yeast (ref), though at severe heat shock conditions, rather than the mild heat stress conditions utilized in this study. We show here that p-bodies form during heat stress in yeast, though they are smaller and more faint than those formed under other stress conditions. Thus, p-body formation is emerging as a generalized response to various stress stimuli, though the precise directionality of mRNA flow through polysomes, p-bodies, and/or other mRNP particles such as stress granules remains unclear at present. Moreover, it is also unclear if mRNA dynamics differ based on the specific stress stimulus and/or the species under study.

Sphingolipid biosynthesis occurs in response to a broad array of cell stress conditions and sphingolipids including sphingoid bases in yeast, and ceramides, in mammalian cells, are emerging as key regulators of cell stress responses(10,13,29). This is best exemplified in the yeast *S. cerevisiae*, where genetics and biochemical techniques have enabled the identification of diverse roles for sphingoid bases in the response to heat stress(30). These roles include regulation of growth(21), transcription(14), protein degradation(16), and regulation of translation initiation(15). The current results expand these roles to include formation of p-bodies, as inhibition of *de novo* sphingolipid synthesis attenuated p-body formation during heat stress. The link between sphingolipid metabolism and p-body formation derives further support from the observations that addition of exogenous sphingoid bases or the use of the *lcb4 /lcb5* deletion strain, which shows high constitutive levels of sphingoid bases, p-bodies are readily observed at normal temperature. Thus, sphingoid base accumulation appears sufficient to drive p-body formation, and necessary for this process during heat stress.

The current results provide important insight into the regulation of translation in response to heat, and the role of sphingoid bases and p-bodies in this process. Specifically, previous studies demonstrated a role for sphingolipids in translational regulation during heat stress(15), which we hypothesized could potentially also involve p-body formation. However, whereas p-bodies are associated with inhibition of translation(3), sphingoid bases do not appear to play a role in the translation arrest but promoted resumption of translation during heat stress(15). Thus, it was initially difficult to reconcile the role of sphingoid bases in promoting p-body formation and the proposed role of p-bodies in translational arrest. However, recent findings and the results from this study demonstrating a role for sphingoid bases in resumption of translation support the recently proposed notion of an 'mRNA cycle' (ref) such that p-bodies form in response to translation arrest, from proteins and mRNAs stalled in translation, and then transfer these mRNAs to stress granules, mRNP particles distinct from p-bodies, which contain translation initiation machinery and which mediate resumption of translation (31). Thus, our data are consistent with a model whereby heat stress precipitates a decrease in translation initiation and an increase in sphingoid bases by separate mechanisms; sphingoid bases then mediate p-body formation, which, possibly through facilitating stress granule formation, is required for the subsequent resumption of translation initiation (Figure 6).

Data suggest that different stress conditions may regulate p-bodies differently (3,8,31). Indeed, the dynamics of mRNA flow between polysomes and cytosolic mRNP particles in response to different stressors, and possibly between different organisms, remain to be resolved. The current data suggest that during heat stress in *S. cerevisiae*, p-bodies form upstream of translation initiation, and sphingoid bases mediate both p-body formation and initiation of translation. Whether this order is specific for heat stress and/or for *S. cerevisiae* remains to be determined. Moreover, the data pose the intriguing possibility that

sphingolipids may also mediate translation and/or mRNA movement in higher organisms. In conclusion, data presented here underscore the fundamental role of sphingolipids in the complex regulation of eukaryotic stress responses.

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A**Early log
phase** **Saturated
culture** **B****heat stress,
min**

0

10

20

30

45

60

C**30°C** **39°C**

Figure 1. Heat stress induces p-bodies in *S. cerevisiae*

A. Yeast expressing Dcp2-GFP grown in YPD at 30°C at log-phase or saturated (2 days continuous culture) growth. B. Log-phase yeast in YPD were grown at 30°C and shifted to 39°C for the indicated times. Live yeast were quickly visualized by confocal microscopy. C, Log-phase cultures expressing mCherry-tagged Edc3p were grown in YPD and shifted to 39°C for 25 minutes prior to visualization as above.

A

Figure 2. Inhibition of heat-induced sphingolipid synthesis attenuates p-body formation

A, Yeast were grown in YPD to mid-log phase at 30°C and treated with 1 μ M myriocin or vehicle for 5 minutes prior to shifting culture temperature to 39°C. Sphingoid bases were extracted and measured as described in methods. Values are mean $\pm$ S.E.M. of experiments performed in triplicate. B Log-phase yeast grown in YPD at 30°C were treated with 1 μ M myriocin or vehicle prior to shifting culture to 39°C for the times indicated. Live yeast were quickly imaged by confocal microscopy.

Figure 3. Treatment of yeast with sphingoid bases, but not other lipids, causes p-body formation
Log-phase yeast at 30C were treated with 10 μ M of each lipid as indicated, or vehicle (DMSO for phytosphingosine, dihydrosphingosine, sphingosine, and stearylamine; ethanol for ceramide) for 20 minutes. Live yeast were quickly visualized by confocal microscopy.

A

B

Figure 4. Elevation of endogenous sphingoid bases promotes p-body formation at non-stress temperature

A, Yeast deleted for sphingoid base kinases (*lcb4* /*lcb5*) and their parental background strain (JK93d) were grown on YPD media at 30°C to mid log phase and then shifted to 39°C for 5 minutes. Sphingoid bases were measured by HPLC as described in Methods. **B**, yeast were transformed with the plasmid pRP1574 expressing Edc3p-mCherry and grown at 30°C on uracil drop-out selection media. Live yeast were quickly visualized by confocal microscopy as described in Methods.

Figure 5. Myriocin potentiates heat-induced decrease in translation initiation

Log-phase yeast were grown in rich media at 30°C, treated with vehicle (DMSO) or myriocin, and shifted to 39°C for 30 minutes. Polysomes were isolated and resolved by gradient centrifugation, and profiles were determined by spectrophotometric analysis as described in methods.

Figure 6.

A model of sphingoid base-mediated regulation of translation during heat stress.

Table 1

Yeast Strains Used in this study

Strain name	genotype	reference
yRP1727	<i>MATa leu2-3,112 trp1 ura3-52 his4-539 cup1::LEU2/PGK1pG/MFA2pG DCP2- GFP (NEO)</i>	(4)
JK93d	<i>MAT leu2-3,112 ura3-52 trp1 his4 rme1 HMLa</i>	(32)
RCD165	<i>MAT leu2-3,112 ura3-52 trp1 his4 rme1 HMLa lcb4 :KanMX lcb5 : KanMX</i>	(26)