The Glycolytic Metabolite Methylglyoxal Activates Pap1 and Sty1 Stress Responses in Schizosaccharomyces pombe*

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Methylglyoxal, a toxic metabolite synthesized in vivo during glycolysis, inhibits cell growth. One of the mechanisms protecting eukaryotic cells against its toxicity is the glyoxalase system, composed of glyoxalase I and II (glo1 and glo2), which converts methylglyoxal into D-lactic acid in the presence of glutathione. Here we have shown that the two principal oxidative stress response pathways of Schizosaccharomyces pombe, Sty1 and Pap1, are involved in the response to methylglyoxal toxicity. The mitogen-activated protein kinase Sty1 is phosphorylated and accumulates in the nucleus following methylglyoxal treatment. Moreover, glo2 expression is induced by methylglyoxal and environmental stresses in a Sty1-dependent manner. The transcription factor Pap1 also accumulates in the nucleus, activating the expression of its target genes following methylglyoxal treatment. Our studies showed that the C-terminal cysteine-rich domain of Pap1 is sufficient for methylglyoxal sensing. Furthermore, the redox status of Pap1 is not changed by methylglyoxal. We propose that methylglyoxal treatment triggers Pap1 and Sty1 nuclear accumulation, and we describe the molecular basis of such activation mechanisms. In addition, we discuss the potential physiological significance of these responses to a natural toxic metabolite.

Cells adapt to stressful environments by inducing genetic responses. Oxidative stress is signaled by reactive oxygen species, a harmful byproduct of aerobic metabolism. Microbial oxidative stress responses sense an excess of either superoxide or hydrogen peroxide (H2O2) have been described in recent years (1, 2). The Schizosaccharomyces pombe Pap1 and Sty1/Spc1 pathways respond to increased intracellular concentrations of H2O2. Pap1 is a b-ZIP transcription factor, homologue to mammalian c-Jun, which upon activation by H2O2 activates the transcription of antioxidant genes such as trr1 or obr1 (3, 4). Unlike Pap1, the mitogen-activated protein (MAP) kinases Sty1 can be activated not only by H2O2 but also by osmotic stress, heat shock, or nutritional starvation (5, 6). The transcription factor Atf1, the main substrate of Sty1 (7, 8), up-regulates a wide variety of genes, such as gpx1, in response to stress.

Both Pap1 and Sty1 activities are regulated primarily by signal-dependent changes in subcellular localization. Sty1 phosphorylation occurs in response to different stimuli even though the upstream components involved in its activation seem to be signal specific. Pap1 is induced not only by H2O2 but also by the cysteine-alkylating agent diethylmaleate (DEM) (9). This transcription factor features two cysteine-rich domains, located at its center (N-CRD) and C-terminal (C-CRD) regions. Alkylation of any of the three cysteine residues at the C-CRD following treatment with DEM, or formation of a disulfide bond between Cys-278 at the N-CRD and Cys-501 or Cys-532 at the C-CRD in response to H2O2, can mask the accessibility of the nuclear exporter CRM1 to the C-terminal nuclear export signal (NES) of Pap1 and trigger its nuclear accumulation as well as Pap1-dependent gene expression (9, 10).

Methylglyoxal (MG) is also an endogenous toxic compound. It is primarily generated by the glycolytic enzyme, triosephosphate isomerase. Once believed to be an intermediate metabolite of glucose consumption, MG is now known to inhibit cell growth. It is not known whether MG synthesis is an undesirable side metabolite or whether its presence is physiologically beneficial for cell homeostasis and/or signaling as has been hypothesized for reactive oxygen species. The amount of MG capable of reacting and inactivating various biological molecules, however, is strictly controlled to prevent accumulation. This MG-metabolizing enzyme activity, or glyoxalase system, is composed of two enzymes: glyoxalase I catalyzes the conversion of MG and glutathione into D-lactoylglutathione, which is then transformed into lactic acid and glutathione by glyoxalase II. In mammalian cell studies, regulation of glyoxalase activity has been suggested to be of physiological interest because elevated MG levels have previously been detected in biological samples from diabetic individuals (11).

To gain further insight into the cellular function of MG as well as the physiological inducers of stress responses, recent studies have focused on the relationships among MG, the glyoxalase system, and stress pathways in the yeast Saccharomyces cerevisiae. For example, the YAP1 transcription factor, a homologue to Pap1 in S. pombe, is activated by MG (either by extracellular medium containing the natural metabolite or by increased intracellular MG in a Δglo1 strain) (12). Furthermore, MG can also trigger the Sty1 homologue HOG1 pathway (13). Despite similarities in the molecular components and sequence homologies of the various stress pathways between budding and fission yeasts, fundamental differences have been identified in recent years, prompting us to verify the universality of MG reactivity with stress pathways by characterizing the response of S. pombe to this metabolite. Indeed, we have demonstrated in the present study that both the Pap1 and Sty1 pathways are triggered by extracellular MG. We have determined that MG interacts...
with Pap1 through its C-terminal domain around the NES, resembling a pattern common to DEM, consequently differing from that of H$_2$O$_2$. Although Sty1 phosphorylation and nuclear localization are similarly triggered by MG, higher concentrations are required. MAP kinase can also be activated on initiation of stationary phase, and the possible involvement of intracellular MG in such activation is discussed.

**EXPERIMENTAL PROCEDURES**

**YEAST STRAINS, PLASMIDS, AND GROWTH CONDITIONS**—We used the wild-type strains 972 (h$^+$), HM123 (h$^+$ leu1), EHH14 (h$^+$ his2 ura4 pap1::ura4 leu1 nmt:GFP-pap1-leu1), and mutant derivatives EHH14.C501A, EHH14.C523A, EHH14.C352A, EHH14.C501,523A, EHH14.C278A, and EHH14.C501,523,532A (9). To construct *S. pombe* strains with specific loci deleted, we transformed strain 972 with linear plasmid pFR6a-kanMX6 (15). To construct *S. pombe* strains with specific loci deleted, we transformed strain 972 with linear fragments containing ORF:kanMX6, obtained by PCR amplification using open reading frame-specific primers and plasmid pFA6a-kanMX6 as a template (15). We obtained AV18 (h$^+$ sty1::kanMX6), AV25 (h$^+$ pap1::kanMX6), AV15 (h$^+$ atf1::kanMX6), and MSS (h$^+$ pcr1::kanMX6). To construct an *S. pombe* strain expressing green fluorescent protein (GFP)-tagged Sty1, we transformed strain HM123 with a linear fragment containing sty1::GFP:kanMX6, obtained by PCR amplification using sty1-specific primers and plasmid pFA6a-GFP(S65T)-kanMX6 as a template (15), yielding strain EHH15 (h$^+$ leu1 sty1::GFP:kanMX6). We used plasmid pRep41-GFP-pap1 (4) to trigger the expression of a GFP-Pap1 fusion protein in *S. pombe* cells. pEH104, designed to trigger the expression of a GFP-tagged truncated Pap1 in which the central cysteine-rich domain had been deleted, was constructed by religating plasmid pRep41-GFP-pap1 previously digested with NsiI and SpeI (the NsiI and the SpeI sites were chewed and filled in, respectively, before ligation so that the truncated gene would be in-frame), pEH66, used to trigger the expression in *S. pombe* of GFP-Pap1ΔCRDNESPK1, was constructed by digesting pRep41-GFP-pap1 with SpeI and SmaI, purifying the large fragment and ligating it to two annealed primers designed to contain SpeI and SmaI at the ends, and encoding the NES of protein kinase inhibitor (16). Cells were grown in rich medium (YE5S, with 3% glucose) or in synthetic minimal medium (MM, with 2% glucose) as described (17, 18).

**MG SENSIVITY ASSAY**—*S. pombe* strains were grown in liquid MM or rich (YE5S) medium to an A$_{600}$ of 0.5. Cells were then diluted in the same medium, and the indicated number of cells in 5 µl was spotted onto MM or YE5S agar medium containing, or not, 5 or 7.5 mM MG (Sigma). The spots were allowed to dry and the plates were incubated at 30 °C for 2–4 days.

**RNA PREPARATION FOR NORTHERN BLOT ANALYSIS**—Cells, grown in minimal medium to a final A$_{600}$ of 0.5, were left either untreated or treated for 60 min with H$_2$O$_2$, MG at the indicated concentrations or heat shocked at 39 °C. RNA was then isolated and Northern blot performed as described before (19). The blots were hybridized with [³²P]dCTP-labeled glo2, the Pap1-dependent trr1 or obr1, or the Sty1-dependent gpix1 probes.

**Fluorescence Microscopy**—Fluorescence microscopy and capture imaging was performed as described before (19).

**Preparation of Boiled *S. pombe* Extracts for Detection of Phosphorylated Sty1-GFP**—2 × 10$^6$ *S. pombe* cells (A$_{600}$ of 0.5) were harvested by centrifugation, washed once with distilled water, and resuspended in HB buffer (25 mM MOPS (pH 7.5), 60 mM β-glycerolphosphate, 15 mM p-nitrophenylphosphate, 1% Triton X-100, 15 mM MgCl$_2$, 15 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride). The cell suspension was boiled at 100 °C during 6 min and disrupted by vortexing with glass beads. For immunoblotting, samples of *S. pombe* extracts were electrophoresed in SDS-polyacrylamide gels and transferred to membranes. Phosphorylated Sty1 was detected in filters with a commercial anti-p38 MAP kinase antibody (New England Biolabs).

**Preparation of Trichloroacetic Acid *S. pombe* Cell Extracts and Immunoblot Analysis of Oxidized Pap1**—We obtained trichloroacetic acid, iodoacetamide-alkylated extracts as described before (19). Samples were separated by non-reducing SDS-PAGE. Gels were transferred to membranes, and filters were probed with a polyclonal anti-Pap1 antiserum (19).
Stationary Phase Conditions—*S. pombe* strains were grown in liquid MM or rich (YE5S) medium till they reached stationary phase, at an approximate $A_{600}$ of 5–8, depending on the strains and growth conditions.

RESULTS

Resistance to MG Is Impaired in Mutants of the Pap1 and Sty1 Stress Pathways—The glyoxalase system is encoded in *S. pombe* by *glo1* (20) and most likely by SPAC824.07/glo2 (the closest homologue of the two *S. cerevisiae* glyoxalase II-encoding genes, *glo2* and *glo4*) (21). Microarray analysis showed that *glo1* message levels were induced 2- or 3-fold by various stresses (22). *glo2* mRNA levels increased 2- or 3-fold under similar stress conditions according to both microarray (22) and Northern blot analyses (Fig. 1A). *glo2* expression is also triggered upon stationary phase (data not shown). At least for *glo2*, gene induction in response to environmental stress (Fig. 1A), as well as stationary phase entry (data not shown), is Sty1-dependent and Pap1-independent. Indeed, *glo2* induction did not occur at low (0.2 mM) $H_2O_2$ concentrations, which are known to fully activate Pap1 but not Sty1 (data not shown).

We then examined whether the Sty1 and Pap1 pathways contributed to *S. pombe* cell susceptibility to MG. As shown in Fig. 1, $B$ and $C$, fission yeast is very sensitive to the glycolytic metabolite; concentrations over 10 mM MG completely inhibited cell growth on either rich or minimal medium plates (data not shown). We also observed toxicity at 5 mM MG in wild-type cells, which was exacerbated when strains were lacking the MAP kinase Sty1. An intermediate sensitivity was observed in a strain lacking Pap1 (Fig. 1, $B$ and $C$). Interestingly, the lack of Atf1 did not render cells MG sensitive, a finding consistent with the fact that *glo2* expression is not *atf1*-dependent in response to multiple stresses, as determined by Northern blot analysis (data not shown). A Δper1 knockout strain showed intermediate MG sensitivity, similar to cells lacking Pap1 (Fig. 1, $B$ and $C$).

Pap1 Nuclear Localization and Gene Expression Is Activated by Extracellular MG—As shown above, MG is toxic for *S. pombe* cells, and the lack of either Sty1 or Pap1 increases the sensitivity of fission yeast to the glycolytic metabolite. We therefore tested whether MG would directly regulate the activity of the Pap1 and Sty1 stress pathways.

As shown in Fig. 2, a GFP-Pap1 fusion protein became nuclear upon MG treatment. 1 mM extracellular MG was sufficient to trigger Pap1 nuclear accumulation at 30 min; higher concentrations induced a faster nuclear localization of the fusion protein (Fig. 2A). Pap1 nuclear accumulation occurred concomitantly with Pap1-dependent gene expression, as shown in Fig. 2B. The GFP-Pap1 fusion protein did not return to its cytosolic localization for as long as 240 min (Fig. 2C). Nevertheless, washing MG from the medium did accelerate Pap1 inactivation,
because 60 min after resuspending cells previously treated with 4 mM MG in MG-free medium Pap1 returned to the cytoplasm (Fig. 2C).

We analyzed the putative role of Pap1 as a sensor of intracellular MG. We constructed a \( \text{glo1}^{-} \) mutant and measured the intracellular concentration of the glycolytic metabolite, which was indistinguishable from that of its isogenic wild-type strain (data not shown). The GFP-Pap1 fusion protein did not constitutively accumulate at the nucleus in the \( \text{glo1}^{-} \) background (Fig. 2D). Significantly, the nuclear accumulation of Pap1 was achieved by lower concentrations of MG in \( \text{glo1}^{-} \) compared with wild-type cells, suggesting that intracellular MG levels of \( \text{glo1}^{-} \) cells increase faster than that of wild-type cells (data not shown). Taken together, these data have led us to postulate that intracellular MG may be one of the factors affecting the localization of Pap1, despite the fact that \( \text{glo1}^{-} \) deletion neither significantly increases the intracellular levels of MG nor activates Pap1.

**Only the C-terminal Domain of Pap1 Is required for MG Sensing**

We examined which cysteine residues in Pap1 were required for MG sensing. As previously described for DEM (9), single cysteine substitutions in a GFP-Pap1 fusion did not constitutively accumulate at the nucleus in the \( \text{glo1}^{-} \) background (Fig. 2D). Significantly, the nuclear accumulation of Pap1 was achieved by lower concentrations of MG in \( \text{glo1}^{-} \) compared with wild-type cells, suggesting that intracellular MG levels of \( \text{glo1}^{-} \) cells increase faster than that of wild-type cells (data not shown). Taken together, these data have led us to postulate that intracellular MG may be one of the factors affecting the localization of Pap1, despite the fact that \( \text{glo1}^{-} \) deletion neither significantly increases the intracellular levels of MG nor activates Pap1.

The \( \text{C-terminal Domain of Pap1} \) is required for MG Sensing—

We examined which cysteine residues in Pap1 were required for MG sensing. As previously described for DEM (9), single cysteine substitutions in a GFP-Pap1 fusion did not prevent MG activation of the transcription factor (Fig. 3A). We were unable to test whether the absence of all three cysteine residues at the C-CRD would be sufficient to abrogate Pap1 activation by MG, because the triple substitution mutant exhibited a constitutive nuclear localization (9) (Fig. 3A). Nevertheless, a GFP-Pap1 protein lacking the N-CRD, but not the C-CRD, was still sensitive to MG, whereas substitution of NES-containing C-CRD of Pap1 by a canonical NES from protein kinase inhibitor (23) prevented activation of the transcription factor (Fig. 3B). Thus, we concluded that the C-CRD of Pap1 is both necessary and sufficient for MG sensing.

We confirmed that MG treatment of \( S. \text{pombe} \) did not induce the formation of an intramolecular disulfide bond in the protein. We had previously demonstrated that \( \text{H}_{2}\text{O}_{2} \) oxidation of Pap1 leads to a protein conformational change, detectable by electrophoresis under non-reducing conditions (19) (Fig. 3C, GFP-Pap1 ox). As suspected, following MG treatment we did not observe the disulfide-dependent migration shift of Pap1 (Fig. 3C, MG).

**High Extracellular Concentrations of MG Trigger the Sty1 MAP Kinase**

We tested whether the MAP kinase pathway was also induced by MG. When Sty1 is activated by environmental stresses, either osmotic or oxidative, the MAP kinase is transiently accumulated at the nucleus (24). Using a strain encoding Sty1-GFP fusion protein, we determined that MG could also trigger Sty1 nuclear accumulation, with varied kinetics depending upon metabolite concentrations (Fig. 4A). The extracellular levels required to trigger activation of the MAP kinase were at least 4-fold greater than those activating Pap1 (compare Figs. 2A and 4A).

**Sty1, but Not Pap1, Is Activated at the Stationary Phase Entry**—Having established that extracellular MG levels regulate both Pap1 and Sty1 stress pathways, we then tried to discover a physiological correlation...
between this metabolism-derived signal and Pap1 and/or Sty1. Previous studies in budding yeast have reported that cellular MG levels increased with cell growth, reaching a maximum at the onset of stationary phase (12). We therefore tested whether these stress pathways are regulated with respect to cell growth.

Phosphorylation of Sty1, as well as nuclear accumulation of MAP kinase and Sty1-dependent gene expression, occurred at the transition between exponential and stationary growth (Fig. 5A). Nonetheless, we did not observe Pap1 accumulation at the nucleus or Pap1-dependent gene expression when the culture reached maximum absorbance at 600 nm (Fig. 5B). These results have led us to conclude that Sty1 activation at the onset of the stationary phase does not result from intracellular increases of MG. Indeed, Pap1 is more sensitive to the glycolytic metabolite than Sty1 (compare Figs. 2A and 4A) and does not become nuclear at the initiation of stationary phase. It is worth pointing out that the transcription factor Pap1 is, at this stage of the growth curve, still susceptible to extracellular MG activation (Fig. 5B) and that this protein would therefore detect intracellular increases.

DISCUSSION

In the present study we have demonstrated that the glycolytic metabolite MG is toxic to S. pombe cells. In fact, fission yeast seems to be more sensitive to MG than the budding yeast (15 mM does not inhibit the growth of S. cerevisiae in plates (12), whereas concentrations as low as
Role of MG in Pap1 and Sty1 Activation

7.5 mM are sufficient to inhibit the growth of S. pombe by 1000-fold; Fig. 1C). Here we have shown that cells lacking Sty1 or Pap1 are more sensitive to MG-mediated killing than wild-type cells and that extracellular MG can strongly induce both pathways.

With regards to Sty1 pathway induction, we do not yet know how this signal is sensed by the MAP kinase. In fact, little is known about the interaction of the multiple components of the Sty1 pathway and its various inducers. Nevertheless, it is easy to hypothesize that Sty1 may be required for MG resistance because of its crucial role in glo2 gene induction, a requirement for MG detoxification. Interestingly, glo2 is one of the few genes triggered by many of the same inducers known to activate the Sty1 pathway that is not dependent on Atf1, the main substrate of the kinase (data not shown). Moreover, cells lacking Atf1 are consistently less sensitive to MG than are cells lacking Sty1.

Pap1 and Sty1 seem to have complementary roles against oxidative stress, each providing a specific response to different concentrations of H₂O₂ (19, 25). Thus, the Sty1 MAP kinase pathway preferentially responds to high doses of H₂O₂ (25), whereas Pap1 is oxidized and activated by low H₂O₂ concentrations (0.2 mM). Similarly, although both pathways are also activated by the secondary metabolite MG, Pap1 is more sensitive than Sty1. Nevertheless, the presence of Sty1 seems to be more important for MG resistance than Pap1, at least in solid plates. In fact, induction of the glo2 gene is not dependent on Pap1. Therefore, we do not know the exact role of Pap1 in countering an extracellular burst of MG.

The reactivity of MG with lysine, arginine, and cysteine residues has been proposed to constitute the basis of MG toxicity (26). We plan to investigate this hypothesis upon initiation of the stationary phase, such as decreases in culture glucose concentrations, are currently being studied.

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