Mge1, a nucleotide exchange factor of Hsp70, acts as an oxidative sensor to regulate mitochondrial Hsp70 function

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ABSTRACT

Despite the growing evidence of the role of oxidative stress in disease, its molecular mechanism of action remains poorly understood. The yeast Saccharomyces cerevisiae provides a valuable model system in which to elucidate the effects of oxidative stress on mitochondria in higher eukaryotes. Dimeric yeast Mge1, the cochaperone of heat shock protein 70 (Hsp70), is essential for exchanging ATP for ADP on Hsp70 and thus for recycling of Hsp70 for mitochondrial protein import and folding. Here we show an oxidative stress–dependent decrease in Mge1 dimer formation accompanied by a concomitant decrease in Mge1–Hsp70 complex formation in vitro. The Mge1-M155L substitution mutant stabilizes both Mge1 dimer and Mge1–Hsp70 complex formation. Most important, the Mge1-M155L mutant rescues the slow-growth phenomenon associated with the wild-type Mge1 strain in the presence of H$_2$O$_2$ in vivo, stimulation of the ATPase activity of Hsp70, and the protein import defect during oxidative stress in vitro. Furthermore, cross-linking studies reveal that Mge1–Hsp70 complex formation in mitochondria isolated from wild-type Mge1 cells is more susceptible to reactive oxygen species compared with mitochondria from Mge1-M155L cells. This novel oxidative sensor capability of yeast Mge1 might represent an evolutionarily conserved function, given that human recombinant dimeric Mge1 is also sensitive to H$_2$O$_2$.

INTRODUCTION

Mitochondria are essential organelles involved in many cellular processes, such as energy metabolism and apoptosis. Although the mitochondrion has its own genome, it depends on the nucleus for optimal functioning (Chacinska et al., 2009). Based on their signal sequence, mitochondrial proteins encoded by nuclear DNA are targeted to different subcompartments of mitochondria through a translocase system present on outer and inner mitochondrial membranes known as the translocase of outer membrane (TOM) and translocase of inner membrane (TIM) complexes, respectively (Schulke et al., 1997, 1999; Endo et al., 2003; Kutik et al., 2007; Neupert and Herrmann, 2007). Targeting of precursor protein to the matrix involves an interplay among many proteins; however, the final step of this process is mediated by Tim44 and a translocation motor that contains mitochondrial heat shock protein 70 (mHsp70), Pam16, Pam18, and the nucleotide exchange factor Mge1 (Azem et al., 1997; Mokranjac et al., 2007; Stojanovski et al., 2007; Schiller et al., 2008). Hsp70, in combination with mHsp70, promotes a change from the high–substrate affinity conformation of mHsp70 to a lower–substrate affinity form with a concomitant release of precursor protein from mHsp70 to begin the next round of translocation...
Mge1 is a homologue of bacterial GrpE and interacts with mHsp70 to facilitate importation across the inner mitochondrial membrane. Yeast Mge1 shares 57% similarity with bacterial GrpE and can functionally substitute the *Escherichia coli* GrpE, demonstrating the conserved mechanism of action during evolution. Functional Mge1 exists as a dimer, and this dimerization seems to be critical for its interaction with mHsp70, as the monomeric form of Mge1 fails to interact with mHsp70, resulting in a delay in ATPase cycle and chaperonic function of mHsp70 (Moro and Muga, 2006). Besides acting as ATP–ADP exchanger, Mge1 acts as a thermosensor in bacteria and yeast (Grimshaw et al., 2001, 2003; Groemping and Reinstein, 2001). At high temperatures, Mge1 exists as a monomer and fails to interact with mHsp70 (Grimshaw et al., 2001; Moro and Muga, 2006). Further, temperature-sensitive mutants of Mge1 accumulate precursors in the cytosol, aggregate proteins in the matrix, and have reduced nucleotide-dependent dissociation of mHsp70 from Tim44 (Laloraya et al., 1995). It is also involved in maintenance of mitochondria during heat stress, sorting of proteins, degradation of misfolded proteins, and the formation of iron–sulfur clusters (Westermann et al., 1995; Dutkiewicz et al., 2006).

Reactive oxygen species (ROS) have been implicated in aging, as well as in a host of diseases (Mammucari and Rizzuto, 2010). The electron transport chain present in mitochondria is the major source for the generation of reactive oxygen species, and there has been an increased focus on identifying the protein(s) that alter activity during oxidative stress, in order to understand mitochondria-mediated ROS signaling and diseases (Drechsel and Patel, 2009; Murphy, 2009). Decreased import of mitochondrial DNA repair enzyme in aged samples (Szczesny et al., 2003) and reduced import of preproteins under pro-oxidant conditions directly imply a role for ROS in mitochondrial protein import (Wright et al., 2001). Further, oxidative stress impairs the growth of yeast (Demasi et al., 2006). However, no protein import factor or receptor of mitochondria has been implicated in the oxidative stress response in either lower or higher eukaryotes.

In this study, we find that recombinant yeast and human Mge1 fail to dimerize and interact with mHsp70 in the presence of H$_2$O$_2$ in vitro. In addition, by using Saccharomyces cerevisiae as a model system, we show that oxidative stress reduces interaction of mHsp70 with Mge1 in vivo. Of interest, a single amino acid substitution from methionine to leucine in Mge1 renders this protein resistant to oxidative stress in vitro and in vivo. Our studies suggest that Mge1 acts as a novel oxidative sensor of mitochondria to regulate mitochondrial protein import and folding.

**RESULTS**

**Oxidative stress reduces Mge1 and mHsp70 complex formation in vitro**

Some proteins undergo oxidation when they are exposed to H$_2$O$_2$ in vitro (Men and Wang, 2007). To investigate whether Mge1 responds to oxidative stress, we treated purified recombinant Mge1 (Figure 1A) with increasing concentrations of H$_2$O$_2$, followed by cross-linking with the amine reactive cross-linker bis(sulfosuccinimidyl) suberate (BS$_2^\text{S}$). The samples were resolved on SDS–PAGE, and Coomassie-stained gels were analyzed (Figure 1B). Mge1 migrates as a 27-kDa monomer in the absence of a cross-linker (Figure 1, A and B). In the presence of BS$_2^\text{S}$, Mge1 migrates as a ~60-kDa homodimer, reflecting the efficiency of the cross-linking agent. However, in the presence of H$_2$O$_2$, the appearance of monomer increased in a concentration-dependent manner despite the presence of cross-linker (Figure 1B, compare lane 3 with lanes 4–6). To determine whether the Mge1 response to H$_2$O$_2$ is conserved across species, we repeated the experiment, using purified human Mge1 (Figure 1A). Consistent with the observations made using yeast Mge1, the dimer form of human Mge1 is also susceptible to H$_2$O$_2$ (Figure 1C). These results suggest that Mge1 loses its dimer formation ability in the presence of oxidative stress triggered by H$_2$O$_2$.

Because the dimerization of Mge1 was shown to be crucial for its interaction with mHsp70, we evaluated the effect of H$_2$O$_2$ on this interaction, using recombinant purified yeast Mge1 and mHsp70 (Figure 1A). We incubated recombinant mHsp70 with Mge1 that was either left untreated or treated with H$_2$O$_2$ (Figure 1D). The reaction mixtures were cross-linked, resolved on SDS–PAGE, and stained with Coomassie blue (Figure 1D). A high–molecular weight cross-linked band was observed at ~130 kDa upon incubation of Mge1 with mHsp70 in the absence of H$_2$O$_2$ (Figure 1D, lane 4). Of interest, formation of this complex decreases with prior treatment of Mge1 with increasing concentration of H$_2$O$_2$ (Figure 1D, compare lane 4 with lanes 5 and 6). To confirm the constituents of the 130-kDa complex, we carried out Western blotting using antibodies specific for Mge1 (Figure 1E) and mHsp70 (Figure 1F). Both antibodies detected a similar strong, high–molecular weight (130 kDa) band in the absence of H$_2$O$_2$, suggesting that indeed Mge1 and mHsp70 exist in a high–molecular weight complex. To rule out the possible interference of H$_2$O$_2$ with BS$_2^\text{S}$ cross-linking efficiency, we used glutathione S-transferase (GST), a dimeric protein (Fancy and Kodadek, 1999), as a control and cross-linked with BS$_2^\text{S}$ in the presence and absence of H$_2$O$_2$. Most of the GST remains as a dimer in the presence of H$_2$O$_2$, indicating that BS$_2^\text{S}$ is not affected by H$_2$O$_2$ (Figure 1G). Taken together, our results clearly show that Mge1 is able to modulate its interaction with mHsp70 in a manner that is dependent on the oxidative stress in vitro. Mge1 is able to switch from an active dimer to an inactive monomer form by sensing the oxidative stress. Of interest, we find that recombinant Mge1 copurifies with a small amount of bacterial DnaK protein, the bacterial homologue of eukaryotic mHsp70 (Figure 1, asterisk). DnaK also forms a high–molecular weight complex with Mge1 (Figure 1B, double asterisks), and we find that this interaction is dependent on the Mge1 dimer, as pretreatment of Mge1 with H$_2$O$_2$ decreases the amount of the Mge1–DnaK complex (Figure 1B), thus indicating that the mechanism might have been conserved across evolution.

**Methionine 155 in Mge1 is prone to oxidative modification in vitro**

Because pretreatment of Mge1 with H$_2$O$_2$ induces conformational change and affects its interaction with mHsp70, we further investigated the amino acid residues in Mge1 that are prone to oxidative modification. Generally, cysteine and methionine are most susceptible to oxidation, followed by tryptophan, histidine, and tyrosine (Kim et al., 2001). Surprisingly, yeast Mge1 protein is devoid of cysteine and contains only one methionine at the 155 position, and this methionine residue is highly conserved across species (Figure 2A). To test whether methionine 155 undergoes oxidative modification upon exposure to H$_2$O$_2$, we mutated methionine 155 to leucine (Mge1-M155L mutant) and investigated its response to oxidative stress in vitro. Recombinant purified Mge1-M155L mutant (Figure 2B) was treated with increasing concentrations of H$_2$O$_2$ and incubated without (Figure 2C) or with mHsp70 (Figure 2D). The cross-linked samples were resolved on SDS–PAGE and Coomassie stained for further analysis. Mge1-M155L mutant also forms a ~60-kDa dimer upon cross-linking (Figure 2C, lane 2), but we observed low amounts of dimer dissociation in
the presence of H$_2$O$_2$ (Figure 2C, lanes 3 and 4). In addition, this mutant forms a relatively stable complex with mHsp70 when compared with wild type even after pretreatment with H$_2$O$_2$ (Figures 2D, lanes 4–6, and 1D, lanes 4–6). We obtained similar results when we performed Western immunoprecipitation analysis of H$_2$O$_2$-treated, purified wild-type and Mge1-M155L mutant in the presence of mHsp70. We observed reduced coprecipitation of mHsp70 with Mge1 antibody when we treated wild-type Mge1 with H$_2$O$_2$ as compared with untreated or Mge1-M155L treated with H$_2$O$_2$ (Figure 2E). In addition, we modeled the structure of yeast Mge1 using the I-TASSER (Zhang, 2008; Roy et al., 2010) server and found that the methionine 155 position is present in the helical region of the protein (Supplemental Figure S1). The helical region is downstream of the dimerization domain. Of interest, this residue is present on the outer surface of the helical region and may be available for possible modifications. Like wild-type Mge1, Mge1-M155L also copurifies with a small amount of DnaK and forms a high–molecular weight, 130-kDa complex upon cross-linking (indicated by double asterisks). However, this complex is more susceptible to H$_2$O$_2$ treatment when compared with the Mge1-M155L–mHsp70 complex (Figures 1C, lanes 2–4, and 2D, lanes 4–6). These results suggest the likely involvement of methionine 155 in the oxidative response of Mge1.

**Altered structure of Mge1 with H$_2$O$_2$ treatment**

Because H$_2$O$_2$ induced the structural transition of Mge1 from dimer to monomer, we used circular dichroism (CD) spectroscopy to analyze structural changes in Mge1 in the presence of H$_2$O$_2$ (Figure 3) and quantified secondary structural alterations using CDNN 2.1 software. The CD spectra of wild-type Mge1 exhibited two negative bands in the ultraviolet region, at 208 and 218 nm. There is a gradual and significant alteration of the spectrum in wild-type Mge1 treated with increasing concentration of H$_2$O$_2$ (Figure 3A). Overall, $\alpha$-helical, $\beta$, and antiparallel turns in wild-type Mge1 are considerably altered with increasing concentration of H$_2$O$_2$ (Table 1). It is evident from the spectrum that the isodichroic point is at 203 nm and is not altered with varying concentration of H$_2$O$_2$, indicating the presence of a two-state conformation. This also suggests that loss of helical stability is possibly due to partial unfolding of Mge1 protein. In the case of Mge1-M155L mutant, the shape of the spectrum is similar to that of the wild type in the absence of H$_2$O$_2$ (Figure 3B). However, treatment of Mge1-M155L mutant with increasing concentrations of H$_2$O$_2$ does not appreciably alter the $\alpha$-helical and $\beta$-sheet structures (Table 2). These results show that M155L mutation stabilizes the Mge1 structure and protects it from H$_2$O$_2$-induced oxidative stress. This is consistent with our earlier observation of a stable Mge1-M155L–mHsp70 complex in the presence of H$_2$O$_2$ in vitro.
We grew the foregoing strains in liquid SC-Leu medium with or without 1 mM $H_2O_2$. All of the strains display similar growth profiles in SC-Leu medium (Figure 4B). However, consistent with our previous results on SC-Leu plates with $H_2O_2$, Mge1-M155L mutant is relatively more resistant to $H_2O_2$, whereas both wild-type and Mge1-H130L mutant are inhibited by $H_2O_2$. A single point mutation in Mge1 protein at M155L is able to rescue poor growth of wild-type yeast in the presence of $H_2O_2$. These results further show that Mge1 plays a significant role in the oxidative stress response and that methionine 155 is crucial for this activity.

To exclude the possibility that altered protein expression in Mge1-M155L mutant is responsible for the resistance to oxidative stress, we evaluated the steady-state levels of mitochondrial proteins in wild type and Mge1-M155L mutant by using antibodies specific for porin, Tim44, mHsp70, Tim22, CCPO, aconitase, Tim23, and Tom40 (Figure 4C). As shown in Figure 4C, there is no change in steady-state levels of mitochondrial proteins in Mge1-M155L mutant when compared with wild type.

To address whether the resistance of Mge1-M155L mutant to $H_2O_2$ is due to stable interaction of mHsp70-Mge1 complex under oxidative stress in vivo, we used antimycin A, an electron transport chain complex III inhibitor that is known to generate ROS in yeast (Smith et al., 2007). Generation of ROS by antimycin A was measured as described in Materials and Methods. Antimycin A induces Mge1 acts as an oxidative sensor in vivo

To understand the in vivo significance of Mge1 in oxidative stress, we generated chromosomal MGE1–deleted haploid yeast strains harboring and expressing MGE1 wild-type or MGE1 mutants under a high-copy plasmid. Methionine, cysteine, tryptophan, and histidine are amino acids that can be modified due to oxidative stress. Mge1 does not have any tryptophan or cysteine but has one methionine and four histidines. Because histidine is one of the other possible amino acid that can be modified by oxidative stress, we mutated histidine at the 130 position with leucine (Mge1-H130L) and used this as a control for any nonspecific activity of the Mge1-M155L mutant. We compared the phenotypes of wild-type MGE1 strain with those of MGE1 mutants on Synthetic Complete (SC)-Leu plates supplemented with 0.5 or 1 mM $H_2O_2$ (Figure 4A). Exponentially growing yeast cells expressing either wild-type Mge1 or Mge1 mutants were serially diluted and spotted on SC-Leu plates with increasing concentration of $H_2O_2$. We found that wild-type Mge1 and Mge1-M155L mutant have comparable growth on SC-Leu. However, Mge1-H130L mutant has poor growth. Wild-type Mge1 is sensitive to $H_2O_2$, as its growth is retarded even at 0.5 mM $H_2O_2$. Most important, Mge1-M155L mutant has considerable growth in the presence of both 0.5 and 1 mM $H_2O_2$ when compared with wild type. The slow growth of Mge1-H130L mutant is further exacerbated in the presence of $H_2O_2$. To confirm the results obtained on
To determine whether Mge1 containing high–molecular weight complexes indeed contain mHsp70, we treated wild-type and mutant mitochondria with antimycin A (10 and 20 μM) and cross-linked them with DSS. Later we solubilized mitochondrial samples, immunoprecipitated them with antibodies specific for Mge1 (Figure 5, D and E), and probed them with mHsp70 antibodies. We were able to detect mHsp70 (Figure 5D, lane 4) in Mge1-mediated immunoprecipitated samples of wild-type mitochondria in the absence of cross-linker. In the presence of cross-linker, we observed mHsp70 in a high–molecular weight complex (Figure 5D, double asterisks). The amount of Mge1–mHsp70 complex (indicated by double asterisks) immunoprecipitated with Mge1 antibodies is consistently reduced by 50–60% in the presence of 20 μM of antimycin A in wild-type mitochondria despite an equal amount of immunoglobulin G in all fractions (Figure 5D). However, we cannot rule out the possibility that one of the complexes (indicated by an asterisk) could be a nonspecific band or Mge1–Hsp70 complex, since background levels of the band are present when the mitochondria sample was used for immunoprecipitation either without cross-linker (Figure 5E, lane 1) or with preimmune serum (Figure 5E, lane 5). Nevertheless, there is no substantial decrease in Mge1–mHsp70 complexes in Mge1-M155L mutant mitochondria samples compared with wild type (Figure 5E). Our observations are consistent with our in vitro findings that Mge1 interaction with mHsp70 diminishes during oxidative stress and that M155L within Mge1 is important for imparting this sensitivity.

FIGURE 3: Circular dichroism analysis of Mge1 under oxidative stress. Purified and dialyzed Mge1 (40 μM) wild-type and Mge1-M155L mutant samples in phosphate buffer were used for CD spectra analysis at 20°C at a scan rate of 60°C/h in a 2-mm–path-length cuvette. Wild type (A) and mutant Mge1 (B) were pretreated with 0.5, 1, or 2 mM H2O2 for 15 min before spectral analysis.

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All at 200–260 nm.

TABLE 1: Secondary structural analysis of H2O2-treated recombinant wild-type Mge1 from Figure 3A.
import assay to determine the effect of antimycin A on protein import. We used \[^{35}\text{S}\]methionine-labeled matrix targeting cytochrome oxidase subunit IV (CoxIV) and Su9-DHFR for import assays. The import of CoxIV and Su9-DHFR is monitored by the processing of N-terminal mitochondrial presequence by mitochondrial processing peptidase and the protection of the mature form from an externally added protease (Sepuri et al., 1998a). We performed kinetics of import of CoxIV and Su9-DHFR in mitochondrial fractions isolated from wild type and Mge1-M155L mutant. As shown in Figure 6A, CoxIV and Su9-DHFR are effectively processed, and the mature form is protected from the externally added protease in wild-type and Mge1-M155L mutant mitochondria (Figure 6A, left). However, imported CoxIV and Su9-DHFR are poorly protected from protease treatment at early time points in wild-type mitochondria pretreated with 20 μM antimycin A (Figure 6A, top right). However, CoxIV and Su9-DHFR are protected at early time points in antimycin A–treated Mge1-M155L mutant sample and are comparable to what is observed in untreated samples at all time points (Figure 6A, bottom). Of interest, the processing of precursor protein requires membrane potential, as the addition of valinomycin inhibits the processing of preprotein and protection from protease treatment in wild-type and mutant mitochondria (Figure 6A). We conclude that ROS decreases the mHsp70 import cycle and Mge1-M155L mutation suppresses the defect caused by the alteration of the Hsp70–Mge1 interaction.

To further analyze the role of oxidative stress on interaction of Mge1 with mHsp70 function, we used the steady-state ATPase activity of Hsp70 in the presence of Mge1. Mge1 assists in release of ADP for ATP on Hsp70 and thereby accelerates ATP hydrolysis. The ATPase activity of mHsp70 was measured in vitro as described in Materials and Methods. Recombinant mHsp70 alone shows a low level of ATPase activity (Figure 6B). Addition of wild type or Mge1-M155L mutant stimulates the ATPase activity of mHsp70. However, treatment of wild type with H\(_2\)O\(_2\) before incubation with mHsp70 reduces the ATPase activity of mHsp70 significantly when compared with Mge1-M155L mutant. Our results suggest that oxidative stress decreases the interaction of Mge1 with mHsp70 and thereby modulates its function.
pre-ornithine transcarbamylase in intact cells, as well as in vitro. Oxidative stress also leads to a decrease in the processing of pre-protein superoxide dismutase in Sf9 cells (Wright et al., 1997; Wright and Reichenbecher, 1999). The mechanism behind the reduced protein import into mitochondria in mammalian cells and in Sf9 cells is not known.

The present study stemmed from the observation that Mge1 acts as a thermosensor during heat stress and undergoes conformational changes that result in reduced ATPase cycle of mHsp70. Oxidant conditions also reduce the import of preproteins, we evaluated whether Mge1 has any relevance in protein import during oxidative stress. It is probable that alteration in Mge1 structure may be one of the reasons for alteration in the import of preproteins during oxidative conditions. We showed for the first time that Mge1, a cochaperone of mHsp70, can act as an oxidative sensor both in vitro and in vivo. Mge1 acts as a switch for sensing oxidative stress within the cell by changing its conformation from an active dimer to an inactive monomer. This conformational change in Mge1 is translated into a reduced interaction of Mge1 with mHsp70, thereby affecting the function of mHsp70 that is required for the vectorial

DISCUSSION

The mitochondrial electron transport chain is the major source of ROS generation within a cell (Brand, 2010). High levels of ROS lead to alteration in the structure of proteins, lipids, and DNA (Wright et al., 2001; Mammucari and Rizzuto, 2010). Further, oxidative stress in the form of H$_2$O$_2$ reduces the activity of several mitochondrial proteins, such as aconitase, α-ketoglutarate dehydrogenase, and succinate dehydrogenase, in mammalian cells (Bulteau et al., 2003). In addition, accumulation of oxidized proteins in the mitochondria is correlated with aging (Schoneich, 1999). Several lines of evidence suggest that the role of mitochondria-mediated oxidative stress in the progression of several neurological disorders (Lin and Beal, 2006; Zuin et al., 2008). However, very little is known about the role of mitochondria-generated ROS in regulating mitochondrial metabolism and biogenesis. One of the key regulators in mitochondrial biogenesis is translocation of nuclear-encoded proteins into various compartments of mitochondria. It has been shown that redox conditions influence the import of preproteins in higher eukaryotes. Pretreatment of mammalian mitochondria with paraquat, an oxidizing agent, causes the production of ROS at the level of complex I (Cocheme and Murphy, 2008) and causes reduction in the import of mitochondrial proteins (E, lane 6) to identify the mobility of mHsp70. Preimmune serum was used as a control (lane 5). We loaded 20 μg of mitochondrial (E, lane 6) to identify the mobility of mHsp70.

FIGURE 5: Effect of oxidative stress on the oligomeric state and interaction of Mge1 with mHsp70 in mitochondria. (A) ROS measurement was carried out as described in Materials and Methods; the fluorescence signal derived from wild type and Mge1-M155L mutant in the absence or presence of increasing concentrations of antimycin A is shown. Mitochondria were isolated from wild-type (B, D) or Mge1-M155L mutant (C, E) strain. (B, C) Mitochondria were treated without (lanes 1 and 2) or with antimycin A (lanes 3 and 4) and subjected to cross-linking with membrane-permeable cross-linker DSS (lanes 2–4). Samples were incubated at room temperature for 30 min, and the reaction was quenched by addition of Tris-HCl (pH 7.5) buffer. Samples were separated on SDS-PAGE, Western transferred, and probed with antibodies specific for Mge1. (D, E) Wild-type Mge1 (D) and Mge1-M155L mutant (E) mitochondria were treated with or without antimycin A and subjected to cross-linking (D, lanes 1–3 and 5; E, lanes 2–5) with the membrane-permeable cross-linker DSS. Samples were incubated at room temperature for 30 min, and the reaction was quenched by addition of Tris-HCl (pH 7.5) buffer. The samples were subjected to immunoprecipitation with Mge1 antibodies and immunodecorated with mHsp70 antibodies. Preimmune serum was used as a control (lane 5). We loaded 20 μg of mitochondria (E, lane 6) to identify the mobility of mHsp70.
Our ATPase assays (Figure 6B) indicate that Mge1-M155L is efficient in stimulating ATPase activity of mHsp70 in the presence of $H_2O_2$. In addition, import of mitochondrial proteins and growth of yeast cells are not significantly reduced in the presence of Mge1-M155L mutant and $H_2O_2$ (Figure 6A). This persistent active Mge1-M155L during oxidative stress may be harmful to cells, as continuous import of unfolded proteins would cause protein aggregation in mitochondria. However, Mge1 plays a role in folding and degradation of misfolded proteins by interacting with chaperones (Westermann et al., 1995). It is possible that the mutant enhances transport of proteins across the mitochondrial cell membranes (Figure 7).

On the basis of our results, we speculate that during oxidative stress, mHsp70-ADP remains associated with precursor protein that is being translocated across the inner membrane of mitochondria. Our studies show that there is reduced interaction of Mge1 with mHsp70 during oxidative stress, and this might be reflected in decreased ADP-to-ATP exchange on mHsp70. As a result, mitochondria might be stalling the transport of proteins into the mitochondrial matrix to prevent accumulation of unfolded proteins in the mitochondria. Our ATPase assays (Figure 6B) indicate that Mge1-M155L is efficient in stimulating ATPase activity of mHsp70 in the presence of $H_2O_2$. In addition, import of mitochondrial proteins and growth of yeast cells are not significantly reduced in the presence of Mge1-M155L mutant and $H_2O_2$ (Figure 6A). This persistent active Mge1-M155L during oxidative stress may be harmful to cells, as continuous import of unfolded proteins would cause protein aggregation in mitochondria. However, Mge1 plays a role in folding and degradation of misfolded proteins by interacting with chaperones (Westermann et al., 1995). It is possible that the mutant enhances...
Methionine, cysteine, tryptophan, histidine, and tyrosine are prone to modifications during oxidative stress (Kim et al., 1998). In the presence of oxidative stress these amino acids are modified by large polar sulf oxide groups that increase the affinity for hydrophobic bonding, leading to destabilization of the proteins. It is assumed that methionine serves as a ROS scavenger in the cell to protect cellular structure and integrity (Levine et al., 1999). Of interest, yeast Mge1 protein lacks cysteines and contains only one methionine. Mutating the lone methionine to leucine shows the effect on Mge1 regulation during oxidative stress both in vitro and in vivo (Figures 1, 2, and 4–6).

In summary, we provided clear evidence that Mge1 responds to oxidative stress and its activity depends on the metabolic and redox environment of the matrix. Our results also suggest that Mge1 plays a key role in regulating the mitochondrial Hsp70 chaperone system and thereby mitochondrial metabolism during oxidative stress. This mechanism of Mge1 action during oxidative stress appears to be conserved across evolution from yeast to humans. It is likely that there are other stresses besides heat and oxidative stress that might elicit the same response from Mge1.

MATERIALS AND METHODS

Plasmid construction

The complete coding sequence of yeast MGE1 was amplified without the mitochondrial targeting sequence (A43) by using forward primer MGE1_Fwd1 (5′-CCCCAGAATTCACATTGAGTAAGCCCATCC-3′) and reverse primer MGE1_Rev1 (5′-ATCTCGAGGATATGCTCTCCGCCCCCTAACAATCC-3′) and cloned into an E. coli expression vector pET28a* to generate a hexahistidine tag at the N-terminal of MGE1 to generate pNB128. Human MGE1 without mitochondrial targeting sequence was amplified using HeLa cells cDNA as template, forward primer MGE1_Fwd2 (5′-CAAAAGATCCACATTGAGTAAGCCCATCC-3′), and reverse primer MGE1_Rev2 (5′-ACCCCTCGAGCTAGCTTCCGGTACGCC-3′) and cloned into pET28a* vector to generate pNB182. Plasmids pNB184 harboring MGE1-M155L and MGE1-H130L, respectively, were created by site-directed mutagenesis of MGE1 in pNB128. Wild-type MGE1 with presequence was amplified by using yeast genomic DNA using forward primers MGE1_Fwd3 (5′-CCCAAGATTCACATTGAGTAAGCCCATCC-3′) and reverse primer MGE1_Rev3 (5′-ACCCCTCGAGCTAGCTTCCGGTACGCC-3′) and cloned into pET28a* vector to generate pNB182.

The PCR product was digested with EcoRI/Xhol restriction enzymes and cloned into high-copy URA3 and high-copy LEU2 plasmids under TEF promoters at similar restriction sites to generate pNB185 and pNB186, respectively. Site-directed mutagenesis was used to create pNB187 and pNB188 harboring MGE1-M155L and MGE1-M155L, respectively, from pNB186.

Mitochondrial HSP70 plasmid in pet21b vector (pNB200) was a kind gift from Debkumar Pain (UMDNJ–New Jersey Medical School, Newark, NJ).

Yeast strains

A heterozygous diploid strain of MGE1, yNB39 (European Saccharomyces cerevisiae Archive for Functional Analysis [EUROSCARF], Institute for Molecular Biosciences, Johann Wolfgang Goethe-University Frankfurt, Frankfurt, Germany) (Mat/alpha:his3A1/3his3A1;Leu2A0;Leu2α0;lys2A0/LYS2;MET15/met15α0;ura3A0/ura3A0;OR232w::kanMX4/YOR232w), a derivative of BY4743, was used for making haploid strains expressing either wild type or MGE1 mutants for in vivo studies. Briefly, yeast strain yNB39 was transformed with pNB185 to generate strain yNB59. yNB59 strain was grown in 2% KOAc with minimal amino acids to induce sporulation. Random sporulated colonies were selected for haploid yNB65 by markers present on the genetic background. Haploid strain yNB65 was confirmed by PCR (primer sequence from the EUROSCARF website).

The high-copy URA3 plasmid in the yNB65 strain was replaced with either pNB186 or pNB188 or pNB187 by plasmid shuffling to generate yNB71, yNB72, or yNB73 strains carrying wild-type MGE1 or MGE1-H130L or MGE1-M155L mutants, respectively. Briefly,
yNB65 strain transformed with Leu plasmids was treated with 5-fluoroorotic acid for two generations to remove URA3-borne wild-type copy, and the loss of the URA3 plasmid was confirmed by lack of growth on SC-Ura plate. The resulting strains yNB71 (MGE1 wild type), yNB72 (MGE1-H130L), and yNB73 (MGE1-M155L) were used for further analysis.

**Yeast media**

Standard yeast protocols were used for culturing yeast strains. Wild-type yeast strains were grown in 1% yeast extract, 2% peptone, and 2% dextrose (YPD). Synthetic dextrose (SD) minimal medium contained 0.73% yeast nitrogen base with amino acids, 0.4% ammonium sulfate, and 2% glucose. For mitochondria isolation, we used semisynthetic lactose medium containing 2% lactose with yeast extract, ammonium chloride, calcium chloride, magnesium chloride, potassium phosphate, and glucose as described (Sepuri et al., 1998b). Yeast cells were grown at 30°C. Yeast transformations were performed by following the standard lithium acetate method (Gietz and Woods, 2006).

**Purification of recombinant proteins**

_E. coli_ BL21 (DE3) Codon Plus (RIL) cells were transformed with pNB1128, pNB182, pNB183, and pNB200 plasmids carrying wild-type MGE1, MGE1-M155L, human MGE1, and mHsp70, respectively. Expression of proteins was induced in the presence of 1 mM isopropyl-1-thio-β-D-galactopyranoside. The soluble histidine-tagged, wild-type, mutant yeast Mge1, human Mge1, and mHsp70 proteins were purified using Talon metal affinity resin (Invitrogen, Carlsbad, CA) and concentrated. Based on silver staining, wild type and mutant Mge1 constituted ∼95% of the total purified protein, with a minor DnaK contaminant. Yeast mHsp70 purified as a single protein. Antibodies against yeast Mge1 were raised in rabbit, and monospecific antibodies were purified by using CNBr antigen–coupled Sepharose beads. Purified GST protein was a kind gift from Bramanandam Manavathi (University of Hyderabad, Hyderabad, India).

**Preparation of H_{2}O_{2} solution**

Initially a stock of 1 M H_{2}O_{2} was prepared from commercially available 30% (vol/vol) H_{2}O_{2} (Merck, Darmstadt, Germany) by taking 102.14 μl of H_{2}O_{2} and diluting it to 1 ml with deionized water. The concentration of H_{2}O_{2} was measured spectrophotometrically at OD_{240} by placing 1 ml of a 10 mM solution in a quartz cuvette. The concentration of H_{2}O_{2} was calculated by using its extinction coefficient value (E_{240} = 43.6 M^{-1} cm^{-1}).

**In vitro cross-linking**

Cross-linking of purified yeast wild-type Mge1, Mge1-M155L mutant, human Mge1, and GST protein was performed with BS3 (Pierce, Indianapolis, IN) at room temperature in 20 mM sodium phosphate (pH 7.4), 150 mM NaCl, and 50 mM borate. We preincubated 2.5 μg of purified proteins with increasing concentrations of H_{2}O_{2} (0.5, 1, and 2 mM) at room temperature for 20 min and added BS3 to a final concentration of 0.1 mM. The reaction was quenched by addition of 40 mM Tris-HCl (pH 7.5) and analyzed by SDS–PAGE.

**In organelle cross-linking and immunoprecipitation**

Mitochondria were isolated from yeast as described (Sepuri et al., 2007, 2012). Mitochondria isolated from yNB71 and yNB73 were preincubated with increasing concentrations of antimycin A on ice for 20 min in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4), followed by addition of the membrane-permeable cross-linker disuccinimidyl suberate (DSS; Pierce) at 100 μM final concentration. The reaction was incubated for 30 min at room temperature and quenched by addition of 40 mM Tris-HCl (pH 7.5). Samples were either analyzed directly by SDS–PAGE or subjected to immunoprecipitation.

Mitochondria were solubilized in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors) and incubated on ice for 30 min. Lysates were centrifuged for 15 min at 13,000 rpm at 4°C. The clear supernatant fractions were incubated with protein A beads for 1 h at 4°C to remove nonspecific interactions. Precleared lysates were incubated with Mge1 antibody for 5 h at 4°C on a rotator. Protein A beads were added to the lysates and further incubated for another 4 h at 4°C. The beads were washed four times with mitochondrial lysis buffer. Samples were resolved on SDS–PAGE, Western transferred, and probed with antibodies specific to mHsp70.

**Circular dichroism spectroscopy**

Circular dichroism spectra and oxidative denaturation curves were obtained in a Jasco J-810 spectropolarimeter using a 2-mm-pathlength cuvette. Samples were dialyzed against 20 mM sodium phosphate (pH 7.0) overnight. Four spectra were recorded between 260 and 190 nm and averaged. The secondary structure was determined using CDNN 2.1 software for protein secondary structure analysis.

**Measurement of ROS**

Yeast strains yNB71 and yNB73 carrying wild-type Mge1 and Mge1-M155L were grown overnight in YPD medium. Cells (10^7 cells/ml) were preincubated in YPD medium with 40 μM dichlorofluorescin diacetate at 30°C for 1 h, followed by incubation with increasing concentrations of antimycin A at 30°C for 1 h. Then the cells were isolated by centrifugation and were washed with phosphate-buffered saline. The resulting cell pellet was resuspended in 100 μl of phosphate-buffered saline. Fluorescence intensity of the cell suspension containing 10^7 cells was read with a fluorescence spectrophotometer (PerkinElmer, Waltham, MA) at an excitation wavelength of 480 nm and an emission wavelength of 525 nm (Ryu et al., 2003).

**In vitro protein import**

Radiolabeled precursor proteins were generated by in vitro transcription/translation in the presence of [35S]methionine by using rabbit reticulocyte lysate (Promega, Madison, WI; Tammineni et al., 2012). Mitochondria from Mge1 wild type and M155L mutant were treated with either 20 μM antimycin A or 5 μM valinomycin on ice for 15 min. The mitochondrial samples were incubated with import buffer (fatty acid–free bovine serum albumin, sucrose, 80 mM KCl, 5 mM MgCl2, 5 mM methionine, 10 mM 3-(N-morpholino)propanesulfonic acid/KOH, pH 7.0, 5 mM ATP, pH 7.0, and 1 mM dithiothreitol) with [35S]methionine-labeled precursor proteins at 30°C for different time points. After the indicated times, mitochondria were isolated by centrifugation and washed in SEM buffer. The samples were either directly subjected to SDS–PAGE or protease protection analysis in the presence of trypsin for 15 on ice, followed by inhibiting the trypsin by soybean trypsin inhibitor and washing the mitochondria samples in SEM buffer. The samples were separated on SDS–PAGE and analyzed by autoradiogram.

**ATPase assay of mHsp70**

The ATPase activity of mHsp70 was measured by the release of P_i from [γ-32P]ATP using the method as described (Vitanen et al., 1990). Recombinant mHsp70

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(1 μg) was incubated with or without 5 μg of wild type or Mge1-M155L mutant in an ATPase assay buffer (50 mM HEPE/S KOH, pH 7.2, 5 mM MgCl₂, and 100 mM KCl) in the presence of 0.05 mM [γ-32P]ATP (3000 Ci/mmoll). At different time intervals 25 μl of reaction mixture was taken and mixed with seven volumes of 1 M perchloric acid and 1 mM sodium phosphate and kept on ice. After addition of 16 volumes of 20 mM ammonium molybdate and isopropyl acetate, the samples were mixed and centrifuged at 15,000 × g for 30 s in a microfuge to separate organic and inorganic phases. The amount of radioactive P was released as phosphomolybdate complex in organic phase was measured by scintillation counting. Control reactions were performed to eliminate the background nonspecific hydrolysis of ATP.

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