Ribosome-associated peroxiredoxins suppress oxidative stress–induced de novo formation of the [PSI\(^+\)] prion in yeast

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Peroxiredoxins (Prxs) are ubiquitous antioxidants that protect cells against oxidative stress. We show that the yeast Tsa1/Tsa2 Prxs colocalize to ribosomes and function to protect the Sup35 translation termination factor against oxidative stress–induced formation of its heritable [PSI\(^+\)] prion conformation. In a \(tsa1\) \(tsa2\) \([psl]\) \([PIN]\) strain, the frequency of [PSI\(^+\)] de novo formation is significantly elevated. The Tsa1/Tsa2 Prxs, like other 2-Cys Prxs, have dual activities as peroxidases and chaperones, and we show that the peroxidase activity is required to suppress spontaneous de novo [PSI\(^+\)] prion formation. Molecular oxygen is required for [PSI\(^+\)] prion formation as growth under anaerobic conditions prevents prion formation in the \(tsa1\) \(tsa2\) mutant. Conversely, oxidative stress conditions induced by exposure to hydrogen peroxide elevates the rate of de novo [PSI\(^+\)] prion formation leading to increased suppression of all three termination codons in the \(tsa1\) \(tsa2\) mutant. Altered translational fidelity in [PSI\(^+\)] strains may provide a mechanism that promotes genetic variation and phenotypic diversity (True HL, Lindquist SL (2000) Nature 407:477–483). In agreement, we find that prion formation provides yeast cells with an adaptive advantage under oxidative stress conditions, as elimination of the [PSI\(^+\)] prion from \(tsa1\) \(tsa2\) mutants renders the resulting [psl] [pin] cells hypersensitive to hydrogen peroxide. These data support a model in which Prxs function to protect the ribosomal machinery against oxidative damage, but when these systems become overwhelmed, [PSI\(^+\)] prion formation provides a mechanism for uncovering genetic traits that aid survival during oxidative stress conditions.

Tsa1 functions as a peroxidase on ribosomes, as mutation of its peroxidatic cysteine residue, which inactivates its peroxidase but not its chaperone activity, results in sensitivity to translation inhibitors such as cycloheximide and hygromycin. Approximately 5% of cellular Tsa1 is associated with ribosomes during normal growth conditions (8). However, the function of this pool is unclear, as loss of \(TSA1\) does not affect the translation process during normal aerobic growth conditions.

The Tsa2 Prx is highly homologous to Tsa1 (86% amino acid identity) and possesses similar peroxidase and chaperone activities, although it is normally expressed at significantly lower levels compared with Tsa1 (4, 6). We show here that the majority of cellular Tsa2 localizes to ribosomes. This prompted us to examine whether the simultaneous deletion of \(TS\(A\)1 and \(T\(S\(A\)2 causes any defect in translation, and we find that cultures of \(tsa1\) \(tsa2\) mutants display increased suppression of all three translation termination codons. Elevated readthrough of termination codons is a well known phenotype of the yeast [PSI\(^+\)] prion, a conformationally altered form of the Sup35 translation termination factor (9). Sup35 is an essential protein, which, together with its interacting partner Sup45, facilitates the termination of translation and polypeptide chain release (10). Sup35 forms [PSI\(^+\)] prion aggregates that sequester it away from its normal function in translation termination, resulting in suppression of all three stop codons. [PSI\(^+\)] prion formation is known to increase in response to environmental stress conditions, including H\(_2\)O\(_2\) stress (11). We therefore examined [PSI\(^+\)] prion formation in the \(tsa1\) \(tsa2\) mutant and we show that this mutant shows a significantly elevated frequency of de novo formation of [PSI\(^+\)] that would account for the nonsens suppression observed in a culture of a \(tsa1\) \(tsa2\) strain. As [PSI\(^+\)] prion formation can provide a mechanism for uncovering hidden genetic variation that promotes phenotypic diversity (12, 13), the ability to form the [PSI\(^+\)] prion in the \(tsa1\) \(tsa2\) mutant may provide an adaptive advantage, as [tsa1] [tsa2] [pin\(^-\)] mutants, which are unable to form [PSI\(^+\)] prions, are hypersensitive to H\(_2\)O\(_2\) stress.

Results

The Tsa1 and Tsa2 Peroxiredoxins Localize with Ribosomes. Approximately 5% of Tsa1 is associated with ribosomes (8). We therefore examined Tsa2 to determine whether it is similarly ribosome-associated. Cell extracts were treated with cycloheximide to inhibit ribosomal translocation, and ribosomal fractions separated from soluble components by centrifugation through sucrose cushions. Known ribosomal markers including elongation factor

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Tef1 (EF-1A), termination factor Sup35 (eRF3), and ribosomal protein Rps3 were detected in ribosomal fractions as expected (Fig. 1A). Tsa1 was present in both ribosomal and soluble fractions, whereas another Prx, Ahp1, was only detected in the soluble fraction as previously described (8). Interestingly, Tsa2 was exclusively localized in ribosomal fractions and was not detected in the soluble fraction.

Tsa1 and Tsa2 are thought to have overlapping functions in protecting cells against oxidative stress (4), and their colocalization on the ribosome suggests that they play a common role in protecting the protein synthetic machinery against oxidative stress conditions. Although loss of TSA1 alone does not affect translational fidelity as measured by the readthrough of a UAA stop codon (8), stop codon readthrough was observed in a tsa1 tsa2 mutant of W303 as quantified using a reporter plasmid that carries the lacZ gene with a premature UAA, UAG, or UGA stop codon (14). Readthrough of all three stop codons was elevated in this tsa1 tsa2 mutant compared with the WT W303 strain ranging between threefold (UAA), fivefold (UAG), and 20-fold (UGA) (Fig. 1B). A similar increase in UAG codon readthrough was observed in the tsa1 single mutant, which may indicate that Tsa1 has an additional function on ribosomes distinct from that of Tsa2.

**Tsa1 and Tsa2 Peroxiredoxins Protect Against Sup35 Prion Formation.**

Given that elevated readthrough of termination codons is a well-known phenotype of the yeast [PSI+] prion, we examined whether the termination readthrough detected in the tsa1 tsa2 mutant culture correlated with an increase in [PSI+] prion formation in cultures of this mutant. The subcellular distribution of Sup35 was examined in exponential phase cultures of WT and Prx mutants, and this showed that Sup35 was present in the soluble fraction in WT and tsa1 and tsa2 single mutants, whereas in the tsa1 tsa2 double mutant, a significant proportion was present in an SDS-insoluble high molecular weight form (Fig. 2A). The de novo formation of Sup35 aggregates was also observed in tsa1 tsa2 cells using a fusion between the aminoterminal domain of Sup35 and GFP, under the control of the CUP1 promoter (15). In WT cells, strong cytoplasmic fluorescence comparable with a [psi+] control strain was seen (Fig. 2B), whereas many different-sized aggregates of Sup35 were detected in the majority (>95% of 200 cells examined) of tsa1 tsa2 mutants examined in two repeat experiments, similar to those seen in a [PSI+] strain (15).

One genetic criterion for a yeast prion is reversible curability (9). Guanidine hydrochloride (GdnHCl) blocks the propagation of yeast prions by inhibiting Hsp104, a molecular chaperone that is absolutely required for yeast prion propagation (16, 17). [PSI+] prions form SDS-resistant high molecular weight aggregates that can be detected using semidenaturing detergent agarose gel electrophoresis (SDD-AGE) (18). Such Sup35 aggregates were also observed in a tsa1 tsa2 mutant of W303 and in the control [PSI+] strain 74D-694 (Fig. 2C). Growth of the tsa1 tsa2 mutant in the presence of GdnHCl shifted Sup35 back to its monomeric state, confirming the requirement for Hsp104 to propagate [PSI+] prion formation in this strain.

The reversible curability of the [PSI+] prion in the W303 tsa1 tsa2 mutant was confirmed using a genetic assay in a different strain background. TSA1 and TSA2 were deleted in a [psi+] derivative of the 74D-694. This strain contains an ade1-14, a nonsense (UGA) mutant allele that confers adenine autotrophy, and 74D-694 [psi+] cells accumulate a red pigment. Suppression of the ade1-14 nonsense mutation by [PSI+] gives rise to white/pink Ade+ colonies. The tsa1 tsa2 mutant of 74D-694 gave rise to white/pink Ade+ colonies at a very high frequency (Fig. 2D) and the Ade+ phenotype was eliminated by growth in the presence of 3 mM GdnHCl, suggesting that this was a result of the de novo formation of [PSI+] in these cells (Fig. 2E). GdnHCl also caused loss of the diagnostic SDS-resistant Sup35 polymers in this.
putative [PSI+] tsa1 tsa2 strain (Fig. S1), and expression of the ATPase-deficient allele of Hsp104 (hsp104::K218T, K620T) also generated red Ade+ colonies (Fig. 2E), as has been previously described for [PSI+] cells (19).

Using the 74D-694 tsa1 tsa2 mutant we differentiated between [PSI+] prion formation and any spontaneous nonsense suppressor mutations that might have arisen as a result of the previously described mutator phenotype displayed by tsa1 tsa2 mutants (5). Colony-purified red Ade+ 74D-694 tsa1 tsa2 mutants were streaked for single colonies on Yeast Extract Peptone Dextrose (YEPD) media (Fig. 2D) and 120 of the independently arising white/pink colonies were checked for curability of the Ade+ phenotype by growth in the presence of 3 mM GdnHCl. Eighty-five percent of the mutants examined were curable, indicating that these were [PSI+] strains rather than nuclear-encoded nonsense suppressors. GdnHCl treatment also cures the [PIN+] prion, which is absolutely required for de novo formation of [PSI+] (20), and so we also tested 108 of the cured 74D-694 tsa1 tsa2 mutants for their ability to form [PSI+] prions. In all cases, few white/pink colonies were detected and all 13 such independent Ade+ revertants tested were noncurable on 3 mM GdnHCl, i.e., were not [PSI+]. These data also show that the absence of Tsa1 and Tsa2 does not override the need for the [PIN+] prion for de novo formation of [PSI+].

As tsa1 tsa2 mutants were sterile, we were unable to carry out genetic crosses or cytoduction experiments to confirm the presence of a transmissible infectious entity in the spontaneous [PSI+] tsa1 tsa2 mutants. We therefore resorted to the “protein transformation” method recently described by Tanaka et al. (21) to demonstrate that one of the proteins that [PSI+] strains derive from W303 contained such infectious entities that could be transmitted to a [psi−] strain. Of 49 transformants derived using this extract, 13 were [PSI+], and 11 of 54 transformants obtained with an extract of the 74D-694 [PSI+] strain were [PSI+]. No [PSI+] cells were obtained among 74 transformants using an extract from a [psi−] 74D-694 derivative.

Prion Formation in the tsa7 tsa2 Mutant Requires Oxygen. As Tsa1 can function as both a peroxidase in the detoxification of hydroperoxides, and as a molecular chaperone that protects against protein misfolding, we determined which of these activities are important for preventing Sup35 aggregation and de novo formation of the [PSI+] prion. Introduction of a WT plasmid copy of TSA1 into the Ade− W303 tsa1 tsa2 mutant resulted in Sup35 being largely redistributed into the soluble fraction (Fig. 3A). Mutants lacking the redoxreactive (Cys47) or the resolving (Cys170) cysteine residue cannot function as peroxidases, but maintain chaperone activity (6). Sup35 aggregation was still detected in cells expressing various Tsa1 cysteine mutants (Tsa1C47S, Tsa1C170S, Tsa1C47S/C170S), indicating that the peroxidase activity, rather than the chaperone activity, protects against de novo [PSI+] prion formation (Fig. 3A).

To determine whether oxygen is required for the spontaneous de novo formation and propagation of the [PSI+] prion in a tsa1 tsa2 mutant, we exploited a can1-100 nonsense (UAA) allele of the arginine permease gene CAN1, which is present in W303 and confers resistance to the toxic arginine analogue canavanine (Fig. 3B). Suppression of the UAA codon leads to a canavanine-sensitive phenotype, and this was observed with cultured cells of W303 tsa1 tsa2 mutant. That this canavanine sensitivity was dependent on [PSI+] prion formation was confirmed by comparing with a parallel culture grown in the presence of 3 mM GdnHCl (Fig. 3B). Interestingly, the same tsa1 tsa2 strain grown in low oxygen conditions was far less resistant to canavanine, suggesting that [PSI+] prion formation in this strain might be dependent on the presence of oxygen. We confirmed this hypothesis by examining the subcellular distribution of Sup35 in the W303 tsa1 tsa2 mutant grown under anaerobic conditions, in the absence of atmospheric oxygen. Subculturing the cells five times under anaerobic growth conditions resulted in Sup35 shifting from an SDS-insoluble high molecular weight form into a soluble form (Fig. 3C). This behavior was specific to the tsa1 tsa2 mutant, as the propagation of the [PSI+] prion in a control [PSI+] TSA1 TSA2 strain was unaffected by anaerobic growth. These results suggest that the accumulation of ROS in the tsa1 tsa2 mutant specifically triggers [PSI+] prion formation.

Curable Prion Formation Is Induced by Oxidative Stress. Tsa1 and Tsa2 have best been characterized as antioxidants that protect cells against oxidative stress induced by H2O2. Additionally, several unrelated environmental stress conditions including H2O2 have been reported to increase the frequency of [PSI+] prion formation de novo (11). We therefore examined whether [PIN+] prion formation is elevated in a tsa1 tsa2 mutant exposed to H2O2 (Fig. 3D). This behavior was specific to the tsa1 tsa2 mutant, as the propagation of the [PSI+] prion in a control [PSI+] TSA1 TSA2 strain was unaffected by anaerobic growth. These results suggest that the accumulation of ROS in the tsa1 tsa2 mutant specifically triggers [PSI+] prion formation.
the GdnHCl cured the UAA and UAG stop codons was restored to WT levels in the presence of Sup35 de novo, was hypersensitive to H$_2$O$_2$ and lost greater than 99% viability following peroxide treatment.

**Discussion**

The [PSI$^+$] prion is an infectious, self-perpetuating form of Sup35, an essential translation termination factor. When Sup35 shifts to its transmissible prion conformation, it becomes unavailable for translation termination, resulting in readthrough of nonsense codons (9). The molecular basis of how yeast prions such as [PSI$^+$] nucleate de novo into the infectious amyloid-like structures that underlie the [psi$^-$]-to-[PSI$^+$] transition is poorly understood at present.

Here, we show that the Ts1/Tsa2 Prxs suppress oxidative stress–induced de novo formation of the [PSI$^+$] prion. This raises the question how oxidation influences the formation of the Sup35 prion conformation. Hsp104 and a number of molecular chaperones are required for the continued propagation of [PSI$^+$] and other yeast prions (22). Hsp104 expression is induced in response to oxidative stress conditions, but this cannot account for the high frequency of de novo [PSI$^+$] prion formation in the tsa1 tsa2 mutant as elevated levels of Hsp104 promote [PSI$^+$] loss rather than facilitating its de novo formation (19). However, it is possible that oxidant-induced changes in expression of the protein homeostasis network may account for [PSI$^+$] prion formation. Alternatively, direct oxidation of Sup35 may generate structural conformations that favor a shift to the transmissible amyloid-like form. This would be analogous to various environmental factors which have been proposed to trigger misfolding of the mammalian prion protein (PrP$^\text{Sc}$) into the aggregated scrapie form (PrP$^\text{Sc}$) (23).

All amino acids in proteins are potential targets for oxidation, but cysteine and methionine residues are particularly susceptible to oxidation by ROS. Sup35 can be divided into three functional domains demarked by methionine residues: an N-terminal region (residues 1–123) that is essential for prion formation, a highly charged middle (M) region (residues 124–253), and a C-terminal region (residues 254–685) that is required for translation termination activity (24). The N and M regions contain no cysteine residues, but the M region is flanked by methionine residues. Interestingly, oxidation of methionine residues in purified PrP$^\text{Sc}$ may cause the α-β-β structural conversion that underlies the sporadic formation of PrP$^\text{Sc}$ (25). Direct oxidation of Sup35 methionine residue (or other residues) may therefore facilitate the conformational conversion to the prion form.

That Ts1 and Tsa2 are required to maintain genome stability during growth on glucose-based media suggests that sufficient endogenous ROS are generated to damage cellular macromolecules during normal aerobic growth (5). Loss of TSA1 has been correlated with protein damage and aggregation during similar growth conditions (6, 7), and we show here that [PSI$^+$] prion formation increases significantly in the tsa1 tsa2 mutant during normal aerobic growth conditions. We confirmed that this is an oxygen-dependent mechanism, as growth in the absence of molecular oxygen significantly reduces the de novo appearance of the [PSI$^+$] prion in a tsa1 tsa2 mutant. Oxidative stress conditions induced by exposure to H$_2$O$_2$ were found to further elevate the frequency of de novo formation of the [PSI$^+$] prion, suggesting that the extent of Sup35 aggregation correlates with the degree of oxidative stress suffered by the cell.

The loss of the nonsense suppression phenotype from the tsa1 tsa2 [PSI$^+$] strains when Hsp104 activity is inhibited by GdnHCl is consistent with a self-replicating prion state. For example, cells expressing an aggregating but noninheritable form of Sup35p (e.g., those formed by an Ap–Sup35p fusion) do not show loss of the nonsense suppression phenotype in the presence of GdnHCl (26). The demonstration that a transmissible prion entity is present in a tsa1 tsa2 [PSI$^+$] coupled with the presence of SDS-resistant
aggregates of Sup35 in the same strain confirms that transmissible forms of the [PSI+] prion are formed in the tsa1 tsa2 mutant.

Tsa1 and Tsa2 are the only antioxidants that have so far been shown to localize on ribosomes. Despite the finding that Tsa1/ Tsa2 apparently function to protect Sup35 on ribosomes, it is not clear why the protein synthetic machinery should be a particular target of oxidative damage. It may simply reflect the relative abundance of ribosomes and ribosome-associated proteins. There is also increasing evidence that the translocated apparatus is a target of oxidative damage and such damage has been implicated in the pathogenesis of a number of major neurodegenerative disorders, including Alzheimer disease and other protein misfolding disorders (27, 28).

Tsa1 is the major yeast Prx (4), and we show here that loss of both TSA1 and TSA2 results in a 50% decrease in cell viability following exposure to a normally nonlethal treatment with H2O2. H2O2 treatment also leads to a significant elevation in the frequency of de novo [PSI+] prion formation (11), which in turn increases the readthrough of all translation termination codons. The elimination of [PSI+] by GdnHCl from the tsa1 tsa2 mutant caused the mutant to become hypersensitive to H2O2, reducing cell viability to less than 0.1% of the WT strain following the same oxidant treatment. This suggests that the ability to form the [PSI+] prion provides a selective advantage to the tsa1 tsa2 mutant during exposure to oxidative stress conditions. However, it remains possible that [PIN+] or even other GdnHCl-curable prions could be responsible for relieving the peroxide sensitivity of the tsa1 tsa2 mutant without the appearance of [PSI+]. Resistance to several environmental stress conditions does correlate with the [PSI+] versus [psi−] status of cells (12, 13). Additionally, [PSI+]–induced effects on frame shifting increase the levels of antizyme, a negative regulator of polyamine biosynthesis. The resulting alterations in polyamine synthesis can account for many of the reported changes in sensitivity to environmental insults which have been correlated with the [PSI+] prion status of cells (29). The shift to the [PSI+] prion state may therefore allow cells to reprogram gene expression such that new genetic traits become uncovered that aid survival during the altered conditions (11). Our findings support the hypothesis that prion-induced changes in gene expression provide a mechanism to recover normally hidden genetic traits, which in this case aid survival under oxidative stress conditions. Tsa1 and Tsa2 normally act to prevent oxidant-induced [PSI+] prion formation, but overwhelming this defense system provides an evolvable mechanism to survive the ensuing oxidative stress conditions.

Experimental Procedures

Yeast Strains, Plasmids, and Growth Conditions. The WT strain W303 (MATa ura3-52 leu2-3,112 trp1-1 ade2-1 his3-11 can1-100, [PIN−]) and its iso-