The Pep4p vacuolar proteinase contributes to the turnover of oxidized proteins but *PEP4* overexpression is not sufficient to increase chronological lifespan in *Saccharomyces cerevisiae*

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Turnover of damaged molecules is considered to play a key role in housekeeping of cells exposed to oxidative stress, and during the progress of ageing. In this work, global changes in the transcriptome were analysed during recovery of yeast cells after H₂O₂ stress. Regarding induced genes, those associated with protein fate were the most significantly over-represented. In addition to genes encoding subunits of the 20S proteasome, genes related to vacuolar proteolysis (*PEP4* and *LAP4*), protein sorting into the vacuole, and vacuolar fusion were found to be induced. The upregulation of *PEP4* gene expression was associated with an increase in Pep4p activity. The induction of genes related to proteolysis was correlated with an increased protein turnover after H₂O₂-induced oxidation. Furthermore, protein degradation and the removal of oxidized proteins decreased in Pep4p-deficient cells. Pep4p activity also increased during chronological ageing, and cells lacking Pep4p displayed a shortened lifespan associated with higher levels of carbonylated proteins. *PEP4* overexpression prevented the accumulation of oxidized proteins, but did not increase lifespan. These results indicate that Pep4p is important for protein turnover after oxidative damage; however, increased removal of oxidized proteins is not sufficient to enhance lifespan.

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INTRODUCTION

Reactive oxygen species (ROS) produced as by-products of normal aerobic metabolism are scavenged by a system of both enzymic and non-enzymic antioxidant defences that maintain cellular redox homeostasis (Halliwell & Gutteridge, 1999). When the level of ROS exceeds the antioxidant capacity, redox homeostasis is disrupted, and molecules such as nucleic acids, lipids, proteins and

carbohydrates are oxidized. Studies using yeast cells have contributed to the characterization of cellular functions involved in protection against oxidative stress (Jamieson, 1998; Moradas-Ferreira & Costa, 2000; Thorpe *et al.*, 2004), and to the identification of the major protein targets oxidatively inactivated (Cabisco *et al.*, 2000; Costa *et al.*, 2002; Reverter-Branchat *et al.*, 2004). Protein oxidative modifications include the reversible oxidation of sulphur-containing amino acids, and the irreversible conversion of some amino acid residues to carbonyl derivatives. The oxidation of protein cysteine residues generates sulphenic acid derivatives that are S-thiolated with reduced glutathione (Grant *et al.*, 1999; Demasi *et al.*, 2003; Shenton & Grant, 2003), or further oxidized to sulphinic acid moieties. Protein activity is restored by the monothiol glutaredoxin Grx5p (Shenton *et al.*, 2002) or by sulphiredoxin (Biteau *et al.*, 2003), respectively. Protein methionine residues are oxidized to methionine sulfoxide, which can be reduced by methionine sulfoxide reductase (Moskovitz *et al.*,

Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; MIPS, Munich Information Center for Protein Sequences; ROS, reactive oxygen species.

The data from this study have been deposited in the microarray data public repository ArrayExpress under the accession number E-MEXP-326.

A summary of genes differentially expressed during the recovery of yeast cells from H₂O₂ stress is available as supplementary data with the online version of this paper.

1997). The accumulation of oxidized proteins has been implicated in the initiation and progression of numerous diseases and ageing (Petropoulos & Friguete, 2005). To prevent the accumulation of irreversibly oxidized proteins, cells have to respond by increasing the rate of proteolysis (Dunlop, 2002). Therefore, protein degradation, in addition to repair, plays a key housekeeping role for eliminating oxidized proteins.

Proteins can be degraded by the proteasome or by vacuolar/lysosomal proteases. The 26S proteasome is assembled by association of the 20S proteasome catalytic core with the 19S regulatory particle. A number of studies have indicated that the 20S proteasome, which degrades proteins in an ATP- and ubiquitin-independent manner, is responsible for the degradation of oxidized proteins. Indeed, ubiquitin-activating and -conjugating enzymes and the 26S proteasome are inhibited during oxidative stress, and cells deficient in ubiquitin-conjugating activity are able to degrade oxidized proteins at near normal rates (Shringarpure *et al.*, 2003). In yeast, exposure to H₂O₂ increases the activity of the 20S proteasome. Furthermore, cells deficient in the Rpn9p subunit of the 19S regulatory complex exhibit a higher activity of the 20S proteasome, and are able to degrade carbonylated proteins more efficiently than are wild-type cells (Inai & Nishikimi, 2002). The degradation of cytosolic components by lysosomes (autophagy) also plays an essential role in the maintenance of cellular homeostasis (Massey *et al.*, 2004). In mammalian cells, it has recently been shown that a chaperone-mediated autophagy pathway is activated by oxidative stress, and is important for the efficient removal of oxidized cytosolic proteins by lysosomes (Kiffin *et al.*, 2004).

The progressive accumulation of malfunctioning cell components during chronological ageing of post-mitotic cells is associated with oxidative damage (Reverter-Branchat *et al.*, 2004; Petropoulos & Friguete, 2005; Vijg & Suh, 2005; Harris *et al.*, 2005) and age-dependent decline of turnover rate and housekeeping (Chen *et al.*, 2004). Consistently, the deletion of *UMP1*, a gene necessary for 20S proteasome biogenesis, increases constitutive and ageing-induced protein oxidation in yeast (Chen *et al.*, 2004, 2005). The decrease in the activity of both the proteasomal system and the lysosomal proteases, accompanied by an increased accumulation of oxidized proteins, has been observed during senescence of non-dividing fibroblasts (Grune *et al.*, 2005).

In this study, we show that protein fate is a significantly over-represented function induced during recovery of yeast cells after H₂O₂ stress. In addition to genes encoding subunits of the 20S proteasome, the *PEP4* and *LAP4* genes, encoding vacuolar proteases, as well as genes associated with protein sorting into the vacuole and vacuolar fusion, were upregulated. Protein turnover increased after oxidative damage by a mechanism partially dependent on Pep4p, which is required for the post-translational precursor maturation of vacuolar proteinases (Woolford *et al.*,

1986). In agreement, the degradation of carbonylated proteins decreased in cells lacking Pep4p. The key role of vacuolar proteolysis in the removal of oxidized cytosolic proteins was further supported by data showing that Pep4p activity increased during chronological ageing, and *pep4Δ* mutants showed premature senescence associated with increased accumulation of carbonylated proteins. However, the increased removal of oxidized proteins by *PEP4* overexpression was not sufficient to enhance chronological lifespan.

METHODS

Yeast strains and growth conditions. The following *Saccharomyces cerevisiae* strains were used in this study: W303 (Matz, *ade2-1, can1-100, trp1-1, ura3-1, his3-11,15, leu2-3,112*) (Wallis *et al.*, 1989), W303 *pep4Δ::HIS3* (this work), W303 pDP34 (this work), W303 pDP34-*PEP4* (this work) and W303 p416ADH-*PEP4-GFP* (this work). For *PEP4* (= *PRA1*) disruption, a 3.0 kb deletion fragment containing *HIS3* and the flanking regions of *PEP4* was used. This fragment was obtained from pKS-*pra1ΔEN::HIS3* (Hirsch *et al.*, 1992), using the following specific primers: reverse (5'-ACACAGGAAACAGCTATGAC-3') and forward (5'-AGGG-TTTTCCCAGTCACGAC-3'). Yeast cells were transformed by electroporation. Gene disruption was confirmed by PCR analysis. Yeast cells were grown in YPD [1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) glucose] or in minimal medium [0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose], supplemented with appropriate amino acids (40 mg tryptophan l⁻¹, 40 mg histidine l⁻¹, 80 mg leucine l⁻¹, 40 mg methionine l⁻¹) and nucleotides (40 mg adenine l⁻¹, 40 mg uracil l⁻¹), to early exponential phase (OD₆₀₀ 0.6) or post-diauxic phase (OD₆₀₀ 7–8 in YPD; OD₆₀₀ 4 in minimal medium), in an orbital shaker, at 26 °C and 120 r.p.m.

H₂O₂ recovery and chronological lifespan assay. In H₂O₂ recovery experiments, yeast cells were grown in minimal medium to the exponential phase, treated with 1.5 mM H₂O₂ for 30 min, and centrifuged at 4500 r.p.m. for 5 min. Yeast cells were resuspended in minimal medium lacking H₂O₂, and allowed to recover for the indicated times. Chronological lifespan was assayed as previously described (Harris *et al.*, 2005). Yeast cells were grown to the post-diauxic phase, centrifuged at 4000 r.p.m. for 5 min, and washed twice with water. Cells were resuspended in water, and incubated at the indicated temperature. Cell viability was determined by standard dilution plate counts on YPD medium containing 1.5% agar. Colonies were counted after growth at 26 °C for 3 days.

Glucose assay and enzyme activities. Glucose in the growth medium was quantified by the glucose/peroxidase method. Samples (2 ml) of cultures were taken and centrifuged at 4500 r.p.m. for 5 min, and supernatants were stored at -20 °C until use. The TGO reagent [0.5 M Tris, pH 7.0, 20 U glucose oxidase ml⁻¹, 0.38 U peroxidase ml⁻¹, 0.05 mg *o*-dianisidine hydrochloride ml⁻¹, 1% (v/v) Triton X-100] (2.5 ml) was added to 0.5 ml of sample; the mixture was incubated for 20 min at 37 °C, and the absorbance at 420 nm was measured. Glucose was estimated by reference to a standard curve prepared with known amounts of glucose. For the proteinase A (Pep4p) activity assay, yeast extracts were prepared in 0.1 M Tris, pH 7.5, by vigorous shaking of the cell suspension, in the presence of glass beads, for 5 min. Short pulses of 1 min were used, at 1 min intervals, on ice. Pep4p activity was determined using 0.5 mg total protein, by measuring the release of tyrosine-containing acid-soluble peptides from acid-denatured haemoglobin [expressed as μg Tyr min⁻¹ (mg protein)⁻¹] (Jones, 1990). Glyceraldehyde-3-phosphate dehydrogenase activity (Tdh) was quantified as described by

Holland & Westhead (1973), using 25 µg total protein, and expressed as U (mg protein)⁻¹, or as percentage of the control. Protein content of cellular extracts was estimated by the Lowry method, using BSA as a standard.

Pep4-GFP levels. Proteins were isolated from *S. cerevisiae* W303 p416-*PEP4-GFP* cells, as described for Pep4 activity, and separated by native PAGE (10% gel). After electrophoresis, Pep4-GFP was detected using a molecular imager (Typhoon; Amersham Biosciences Europe). For loading control, proteins were stained with 0.25% (w/v) Coomassie blue R250, 10% (v/v) acetic acid/45% (v/v) methanol, and destained with 10% acetic acid/45% methanol.

mRNA preparation, synthesis of cDNA, Genefilters hybridization and data analysis. Total RNA was isolated by the acid-phenol method (Ausubel *et al.*, 1998). Genefilters yeast microarrays (Research Genetics) were hybridized using [³²P]CTP-labelled cDNA, as described previously (Rep *et al.*, 2000). A Molecular Imager FX (Bio-Rad) was used to obtain a digital image of the filters. Images were converted to tagged image file format (TIFF), and the Pathways4 software (Research Genetics) was used for quantification of spot intensities, and for pairwise comparison of gene filter images. Prior to determination of changes in gene expression, all spot intensities were normalized by dividing sampled intensities by the mean sampled intensities of all clones. To determine the fold induction or repression, the relative mRNA level was expressed as the ratio H₂O₂/control (untreated) or recovery/H₂O₂. Genes that changed at least twofold were considered for further analysis. All values are means of the expression profiles of four experiments with similar results, using independent cultures grown or treated under the same conditions. Statistical analysis of the microarray data was performed by using BRB ArrayTools (version 3.3.0 beta1) developed by Richard Simon and Amy Peng Lam (<http://linus.nci.nih.gov/BRB-ArrayTools.html>). The data were deposited in the microarray data public repository ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/>) (Parkinson *et al.*, 2005) under the accession number E-MEXP-326. Statistical analysis of over-representation of functional groups was performed by using FUNSPEC (Robinson *et al.*, 2002). All available databases were addressed by using a probability cut-off of 0.01 and the Bonferroni correction for multiple testing.

Northern blotting. Total RNA (30 µg) was denatured with glyoxal and DMSO, blotted onto Hybond-N membranes, and probed, as described by Ausubel *et al.* (1998). The following probes were used: a 1 kb *HindIII-EcoRI* fragment of the *ACT1* gene, and a 787 bp fragment of the *PEP4* gene amplified by PCR, using PRA1F (5'-GGGAGCATCCTTTCTCACTG-3') and PRA1R (5'-TGCAG-AGATACAGGAGCCTGA-3') primers. Probes were labelled with [³²P]-dCTP using the Multiprime DNA Labelling System (RPN 1601Z; Amersham Biosciences Europe). Band intensities were evaluated by densitometry.

Pulse-chase. Yeast cells (5 ml per sample) were incubated with 3.7 × 10⁵ Bq [³⁵S]methionine ml⁻¹ for 30 min, centrifuged and resuspended in 5 ml minimal medium supplemented with methionine. Yeast extracts were prepared in 50 mM potassium phosphate buffer, pH 7.0, and 0.1 mM EDTA, containing a protease inhibitor cocktail (Complete, Mini, EDTA-free Protease Cocktail Inhibitor Tablets; Boehringer Mannheim), by vigorous shaking of the cell suspension, in the presence of glass beads, for 5 min. Short pulses of 1 min were used, at 1 min intervals, on ice. Proteins (500 000 c.p.m.) were separated by SDS-PAGE (12.5% gel). Gels were dried and exposed to an X-ray film. Band intensities were quantified by densitometry.

Protein carbonylation analysis. Yeast extracts were prepared in 50 mM potassium phosphate buffer, pH 7.0, and 0.1 mM EDTA, containing a protease inhibitor cocktail (Complete, Mini, EDTA-free

Protease Cocktail Inhibitor Tablets; Boehringer Mannheim), as described for Pep4 activity. For 1D analysis, proteins (15 µg) were derivatized with 2,4-dinitrophenylhydrazine (DNPH) (Levine *et al.*, 1994), and separated by SDS-PAGE (12.5% polyacrylamide gel). After electrophoresis, proteins were stained with Coomassie blue, or blotted onto Hybond-ECL membranes (Amersham Biosciences Europe). Slot blotting was performed using PVDF membranes (Hybond-PVDF; Amersham Biosciences Europe), as previously described (Costa *et al.*, 2002). The ECL or PVDF membranes were probed with rabbit IgG anti-DNP (Dako) at a 1:5000 dilution as the primary antibody, and goat anti-rabbit IgG-peroxidase (Sigma) at a 1:5000 dilution as the secondary antibody. Immunodetection was performed by chemiluminescence, using a kit from Amersham (RPN 2109). The membranes were exposed to a Hybond-ECL film (Amersham Biosciences Europe) for 15 s to 1 min, and the film was developed. Band intensities were quantified by densitometry.

RESULTS

Protein catabolism is a major function upregulated during recovery after H₂O₂ stress

Cells exposed to oxidative stress accumulate damaged molecules that have to be repaired or removed before growth is resumed. To understand the molecular mechanisms involved in cellular recovery after oxidative stress, yeast cells were exposed to 1.5 mM H₂O₂ for 30 min, and allowed to recover in fresh minimal medium without H₂O₂. Analysis of cellular viability, measured as percentage c.f.u., showed that 50% of the cells survived and were able to recover after exposure to H₂O₂. Cellular growth was almost completely absent during the first 4–6 h of recovery, subsequently becoming similar to the rate observed in control cells. However, cells remained metabolically active, as glucose consumption still occurred, although at a lower rate (Fig. 1).

To identify cellular functions that might be important for restoring cellular homeostasis, global changes in the transcriptome were analysed in yeast cells allowed to recover for 60 min after H₂O₂ treatment. Exposure to 1.5 mM H₂O₂ triggered a stress response (data not shown) that partially overlapped with that previously described in response to low concentrations (0.2–0.4 mM) of H₂O₂ (Godon *et al.*, 1998; Lee *et al.*, 1999; Gasch *et al.*, 2000). During cellular recovery after H₂O₂ stress (recovery versus H₂O₂) the mRNA level of 99 genes increased, whereas that of another 169 genes (including 88 encoding ribosomal proteins) was diminished (see Supplementary Table S1). Genes up- or down-regulated during recovery that were repressed or induced, respectively, during previous exposure to H₂O₂ were not considered to be specific, because these changes merely restored control mRNA levels. Genes differentially expressed were sorted into functional categories according to the Munich Information Center for Protein Sequences (MIPS). Our data showed that some categories were significantly more represented in cells after recovery, compared to those in H₂O₂-treated cells (Fig. 2): 57% of the genes down-regulated were associated with protein biosynthesis, whereas 32% of the upregulated genes were related to protein fate, and 37% to metabolism. To

Fig. 1. Cellular growth and glucose consumption during recovery of *S. cerevisiae* cells from H₂O₂ stress. *S. cerevisiae* W303 cells were grown in minimal medium to the exponential phase (OD₆₀₀ ~0.6). Control (●) and cells treated with 1.5 mM H₂O₂ for 30 min (▲) were centrifuged and resuspended in minimal medium without H₂O₂. Cell density (a) and percentage (w/v) glucose remaining in the growth medium (b) were determined during cellular recovery up to 24 h. Values are means ± SD of three independent experiments.

identify over-represented biological processes, gene lists were analysed by using the FUNSPEC software (Robinson *et al.*, 2002). Among induced genes, protein catabolism functions had the greatest significance (Table 1). The

induction (two- to threefold) of proteolytic activity was correlated with the upregulation of amino acid catabolism, and the down-regulation of amino acid biosynthesis. Genes encoding subunits of the 20S proteasome, as well as some associated with ubiquitin-dependent proteolysis, were found to be upregulated. These results are consistent with published data showing that the 20S proteasome is required for degradation of oxidized proteins (Shringarpure *et al.*, 2003; Inai & Nishikimi, 2002; Chen *et al.*, 2004, 2005).

In addition to proteasome subunits, genes related to vacuolar protein catabolism (*PEP4* and *LAP4*) were induced (two- and 2.5-fold, respectively) during recovery after oxidative damage. The *PEP4* gene encodes a vacuolar aspartyl protease that is required for the post-translational precursor maturation of vacuolar proteinases (Woolford *et al.*, 1986). *Lap4p* is a leucine aminopeptidase that is transported into the vacuole via an autophagosome (Suzuki *et al.*, 2002). Interestingly, genes associated with protein sorting into the vacuole, and with vacuolar fusion (*MVPI1*, 3.3-fold; *YHR138C*, 2.5-fold; see Supplementary Table S1), were also upregulated.

To confirm the induction of *PEP4* gene expression, mRNA-*PEP4*, *Pep4p* activity and *Pep4p* levels were analysed in cells allowed to recover for up to 4 h. The results obtained showed that mRNA-*PEP4* and *Pep4p* protein levels increased during the first 2 h of recovery (Fig. 3a, b). In agreement, *Pep4p* activity increased twofold (Fig. 3b). After 4 h of recovery, mRNA-*PEP4* returned to control levels; however, *Pep4p* levels and enzyme activity remained high. This transient upregulation of *PEP4* gene expression, leading to a more sustained increase in protein and enzyme activity, suggests that *Pep4p* is stable and has a long half-life.

The vacuolar protease *Pep4p* contributes to protein catabolism after oxidative damage

The upregulation of genes associated with protein catabolism suggests that cells respond to protein oxidative damage by increasing protein turnover. To test this hypothesis,

Fig. 2. Functional grouping of genes differentially expressed during recovery from H₂O₂ stress. *S. cerevisiae* W303 cells were exposed to 1.5 mM H₂O₂ for 30 min, and allowed to recover in H₂O₂-free medium for 60 min. Whole-transcriptome changes (recovery versus H₂O₂) were analysed using Genefilters (Research Genetics). Genes down-regulated (total 169) or upregulated (total 99) were sorted into functional categories according to the MIPS database. See Supplementary Table S1 for all data. Met, metabolism; Prot, protein biosynthesis; PF, protein fate; CT, cellular transport, transport facilitation and transport routes; CRD, cell rescue, defence and virulence; CTD, cell type differentiation; O/U, other/uncharacterized.

Table 1. Functional categories over-represented in microarray data on cell recovery after H₂O₂ stress, according to the Gene Ontology (GO) database (see Supplementary Table S1 for all data)

Gene lists were analysed by FUNSPEC (Robinson *et al.*, 2002). *P* values represent the probability that the intersection of a given list of genes with any given functional category occurs by chance. 'e' is the base of natural logarithms.

Annotation	<i>P</i> value	Genes in category from cluster
Induced		
Protein catabolism	1·3e ⁻⁷	<i>PRE7 UMP1 CDC48 UBC13 UBC1 RPT3 UBP6 RAD6 PRE9 PRE3 LAP4 UBA1 PRE6 PEP4</i>
Ubiquitin-dependent protein catabolism	1·2e ⁻⁶	<i>PRE7 CDC48 UBC13 UBC1 RPT3 UBP6 RAD6 PRE9 PRE3 UBA1 PRE6</i>
Vacuolar protein catabolism	0·00483	<i>LAP4 PEP4</i>
Protein folding	0·00045	<i>SSA1 CNS1 PDI1 CCT4 SSA4</i>
Metabolism	0·00163	57 genes
Amino acid catabolism	0·00300	<i>ARO10 PUT1 CAR2</i>
Iron transport	0·00029	<i>FRE1 FET3 ENB1</i>
Fructose metabolism	0·00235	<i>HXK1 SOR1</i>
Glycerophospholipid biosynthesis	0·00743	<i>CDS1 OPI3 GPI12</i>
Repressed		
Protein biosynthesis	1·0e ⁻¹⁴	95 genes
Ribosomal subunit assembly	7·2e ⁻⁹	15 genes
Amino acid biosynthesis	0·00251	<i>CIT2 LYS21 LYS20 LEU1 ARG4 LYS12 LYS9 ARG1</i>
Lysine biosynthesis, aminoadipic pathway	0·00095	<i>LYS21 LYS20 LYS12 LYS9</i>
Amino acid metabolism	0·00418	<i>CIT2 LYS21 LYS20 LEU1 ARO8 ARG4 LYS12 AAT2 LYS9 ARG1</i>
Arginine biosynthesis	0·00998	<i>ARG4 ARG1</i>
Metabolism	1·0e ⁻¹⁴	124 genes
Cell growth and/or maintenance	0·00007	135 genes
Regulation of translational fidelity	0·00023	<i>RPS11B RPS9B RPS11A RPS2 RPS23A RPS9A</i>
Iron-sulphur cluster assembly	0·00210	<i>JAC1 QCR9</i>

S. cerevisiae W303 cells were pulse-chased with [³⁵S]methionine and the fate of [³⁵S]-labelled proteins was assessed during recovery from H₂O₂ stress. The results showed that protein turnover increased after H₂O₂-induced damage (Fig. 4a). In control cells (untreated), protein degradation was not observed at up to 4 h of recovery (data not shown).

The requirement of *de novo* protein synthesis to restore damaged protein function is consistent with the metabolic activity of the cells. As Tdh is a major target inactivated during exposure to H₂O₂, by both S-glutathionylation and carbonylation, this protein was used as a biomarker to assess the contribution of *de novo* protein synthesis to the recovery of enzyme activity. Protein carbonylation, in contrast to S-glutathionylation, is irreversible. Thus, Tdh dethiolation contributes to, but may be insufficient to fully restore, Tdh activity when cells return to non-stress conditions. To test this hypothesis, Tdh activity was initially measured in protein extracts prepared from controls and cells exposed to H₂O₂. The effect of Tdh dethiolation on enzyme activity was investigated by treating extracts from cells exposed to H₂O₂ with the reducing agent DTT (25 mM). In H₂O₂-treated cells, Tdh activity decreased to 41·1 ± 0·7 % of that in untreated cells. *In vitro* Tdh dethiolation led to a partial increase in Tdh activity up to 56·6 ± 6·0 % of that in

untreated cells (*P* < 0·01). The importance of *de novo* protein synthesis was studied *in vivo* by assessing the effect of cycloheximide in the recovery of Tdh activity. In the absence of this protein synthesis inhibitor, Tdh activity increased to 80 % of control levels after 6 h of recovery (Fig. 5). In cycloheximide-treated cells, Tdh activity increased to < 60 % of control levels (Fig. 5). Interestingly, Tdh activity after cell recovery was similar to that achieved by *in vitro* dethiolation. These results indicate that *de novo* protein synthesis contributes to restoration of the active enzyme.

Proteolysis can be carried out by proteasomes or by vacuolar proteases. The role of the 20S proteasome in degradation of oxidized proteins has been reported elsewhere (Inai & Nishikimi, 2002). As *PEP4* and *LAP4* genes were induced, and Pep4p activity increased during recovery from H₂O₂ stress, we raised the hypothesis that vacuolar proteolysis contributes to protein turnover after oxidative damage. To test this hypothesis, the *PEP4* gene was disrupted by homologous recombination, and the rate of protein degradation in *pep4Δ* mutants was compared with that of parental cells. Notably, loss of Pep4p significantly decreased protein degradation during cellular recovery (Fig. 4a). The correlation between protein turnover and removal of oxidized proteins was further analysed by measuring protein carbonyl content. Consistent with earlier studies (Inai &

Fig. 3. Analysis of Pep4 levels (mRNA, activity and protein) during recovery from H₂O₂ stress. *S. cerevisiae* W303 cells were treated with 1.5 mM H₂O₂ for 30 min, and allowed to recover in H₂O₂-free minimal medium for up to 4 h. (a) Northern blot analysis. The mRNA-*PEP4* hybridization signal was quantified by densitometry, and corrected for *ACT1* (RNA loading control). Values are fold-changes relative to those in untreated cells. Results of a representative experiment are shown (out of three independent experiments). (b) Pep4p activity and protein levels. Pep4p activity was determined as described in Methods, and expressed as the ratio recovery/control (untreated cells). Pep4p activity in control cells was 1.1 ± 0.1 mg Tyr min⁻¹ (mg protein)⁻¹. For the analysis of Pep4p levels, proteins were isolated from yeast cells expressing Pep4-GFP and separated by native PAGE. Fluorescence was detected using a molecular imager. The gel was stained with Coomassie blue (loading control; only a region of the gel is shown). Results of a representative experiment are shown (out of three independent experiments). Values are means $\pm$ SD of three independent experiments. **P* < 0.05, ***P* < 0.01 [recovery versus H₂O₂ (*t*₀)].

Nishikimi 2002), protein carbonylation increased during exposure of parental cells to 1.5 mM H₂O₂, and decreased as cells were allowed to recover (Fig. 4b). The deficiency in

Fig. 4. Role of Pep4p in protein degradation and removal of carbonylated proteins during recovery from H₂O₂ stress. (a) *S. cerevisiae* W303 and *pep4Δ* cells were pulse-chased with [³⁵S]methionine, exposed to 1.5 mM H₂O₂ for 30 min, and allowed to recover in H₂O₂-free minimal medium supplemented with methionine for up to 4 h (Rec). C, untreated cells. Proteins were separated by SDS-PAGE and exposed to an X-ray film. Results of a representative experiment are shown (out of three independent experiments). (b) Yeast cells were treated with H₂O₂, and allowed to recover, as described in (a). Cellular extracts were derivatized with DNPH, and proteins were separated by SDS-PAGE, and stained with Coomassie blue (loading control), or blotted onto Hybond-ECL membranes. Immunodetection of protein carbonyl groups was performed using an anti-DNP antibody, as described in Methods. Results of a representative experiment are shown (out of three independent experiments). Molecular mass is shown on the left.

Pep4p led to an increase in the basal levels of protein carbonyl groups. The analysis of carbonyl content by slot blot analysis confirmed this increase ($146 \pm 14\%$, *pep4Δ* versus W303, *P* < 0.001; data not shown). Furthermore, although H₂O₂-induced protein carbonylation in the *pep4Δ* mutants was of the same order of magnitude as that of the parental strain, the decrease in carbonyl content during cellular recovery occurred at a much lower rate (Fig. 4b).

Fig. 5. Effect of cycloheximide on recovery of Tdh activity after H_2O_2 stress. *S. cerevisiae* W303 cells treated with 1.5 mM H_2O_2 for 30 min were allowed to recover in H_2O_2 -free minimal medium for up to 6 h, in the presence (closed bars) or absence (open bars) of cycloheximide ($100 \mu\text{g ml}^{-1}$). Tdh activity was determined as described in Methods, and expressed as a percentage of the activity in untreated cells [$0.246 \pm 0.051 \text{ U (mg protein)}^{-1}$]. Tdh activity in untreated cells remained unchanged for up to 6 h. Values are means $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

The vacuolar protease Pep4p is required for the removal of oxidized proteins during chronological ageing, but *PEP4* overexpression is not sufficient to increase lifespan

The progressive accumulation of malfunctioning cell components due to oxidative damage is associated with ageing. In yeast cells, chronological lifespan has been studied by measuring the capacity of post-mitotic cells (growth-arrested in water) to maintain viability over time. Under these conditions (nutrient starvation), intracellular vacuolar catabolism is activated, and protein-based nitrogen recycling is important for cell survival (Teichert *et al.*, 1989; Levine & Klionsky, 2004). To investigate if the homeostatic function of the vacuole is important for the turnover of oxidized proteins during chronological ageing, yeast cells were grown in YPD medium to the post-diauxic (respiratory) phase, growth-arrested in the G_0 phase, and incubated at 37°C . The results showed that parental cells started to lose viability after 15 days of ageing (Fig. 6a). As previously shown (Harris *et al.*, 2005), cell ageing was correlated with protein oxidation: the protein carbonyl content increased two- and sixfold in yeast cells aged for 7 and 15 days, respectively (Fig. 6b). This dramatic increase in total protein carbonyl content preceded a rapid decline in cell viability between the 15- and 21-day time points. Yeast cells lacking the Pep4p proteinase displayed premature ageing associated with increased accumulation of protein carbonyl groups (Fig. 6a, b): at day 2, 100 % of parental cells remained viable,

and protein carbonyl content was similar to that in the controls; and in *pep4Δ* mutants, only 3 % of the cells remained viable, and carbonyl content increased 3.4-fold. The accumulation of oxidized proteins has also been linked to an age-dependent decline in turnover rate and house-keeping (Chen *et al.*, 2004). Therefore, Pep4p activity was determined during chronological ageing. Our data showed an age-dependent increase in Pep4p activity, and did not reveal a decrease when cells started to lose viability (Fig. 6c), suggesting that Pep4p activation was not sufficient to counteract the protein oxidation rate.

To confirm the functional role of Pep4p in degradation of oxidized proteins, total protein carbonyl content was determined during chronological ageing of cells over-expressing *PEP4*. *S. cerevisiae* W303 cells were transformed with pDP34 (empty vector, multicopy) or pDP34-*PEP4* (Rupp & Wolf, 1995), and grown in minimal medium to the post-diauxic phase. Enzyme activity increased 5.7-fold in *PEP4* cells (data not shown). The lifespan of cells previously grown in minimal medium was significantly reduced relative to that in cells grown in rich medium (data not shown). Therefore, chronological lifespan was measured at the less stressful temperature of 30°C . As shown in Fig. 7, *PEP4* overexpression did not increase lifespan, although it completely prevented the increase in protein oxidation associated with cell ageing. The overall results suggest that the vacuolar protease Pep4p is important for maintaining cellular homeostasis by degrading oxidized proteins, in addition to its well-characterized function of supplying nitrogen under nutritional stress conditions (Teichert *et al.*, 1989). However, increased recycling of damaged proteins was not sufficient to extend lifespan. This may be explained by the fact that *PEP4* overexpression does not prevent the accumulation of other oxidatively modified molecules (lipids and DNA) that is associated with cell death.

DISCUSSION

The accumulation of oxidized molecules is a characteristic feature of cells undergoing oxidative stress. Cells recovering from acute oxidative stress have to repair or degrade the damaged molecules. To identify genes that are important for restoring cellular homeostasis, whole-transcriptome changes were analysed during recovery of yeast cells after H_2O_2 stress. Most of the genes specifically repressed were related to amino acid and protein biosynthesis. It is likely that these changes help to preserve mass and energy for use in housekeeping activities that are critical to restore internal homeostasis. Indeed, cellular growth was impaired, and among the genes induced during recovery, protein catabolism was the most significantly over-represented function. In agreement, the rate of proteolysis increased during recovery after oxidative damage, and concomitantly, protein carbonyl content decreased. Tdh is a major target that is carbonylated in cells treated with H_2O_2 (Cabiscol *et al.*, 2000; Costa *et al.*, 2002). Supporting the hypothesis that protein turnover is important for cellular recovery after

Fig. 6. Role of Pep4p in chronological lifespan. Yeast cells were grown in YPD medium to post-diauxic phase, and growth-arrested in water at 37 °C. (a) Chronological lifespan was analysed in *S. cerevisiae* W303 (▲) and *pep4Δ* (■) cells by measuring cell viability on the days indicated. (b) Carbonyl content in W303 (open bars) and *pep4Δ* (closed bars) cells. Proteins were isolated from control and aged yeast cells, derivatized with DNPH, and slot-blotted onto Hybond-PVDF membranes. Immunodetection was performed using an anti-DNP antibody, as described in Methods. Band intensities were quantified by densitometry. (c) Pep4p activity was determined in *S. cerevisiae* W303 cells, as described in Methods, and expressed as the ratio aged/control. Pep4p activity in control cells was 3.4 ± 0.1 mg Tyr min⁻¹ (mg protein)⁻¹. Values are means $\pm$ SD of three independent experiments; * $P < 0.01$ (*pep4Δ* versus W303 cells), ** $P < 0.01$ (aged versus control).

agreement, the expression of several genes encoding 20S proteasome subunits (*PRE3*, *PRE6*, *PRE7*, *PRE9* and *UMP1*) increased during recovery. Although we also observed the induction of genes encoding ubiquitin-activation (*UBA1*)

Fig. 7. Effect of Pep4p overexpression on chronological lifespan. *S. cerevisiae* W303 pDP34 (▲) and pDP34-PEP4 (■) cells were grown in minimal medium to post-diauxic phase, and growth-arrested in water at 30 °C. Chronological lifespan (a) and carbonyl content (b) were measured as described in the legend to Fig. 6. Values are means $\pm$ SD of three independent experiments; * $P < 0.05$, ** $P < 0.01$ (W303 pDP34 versus pDP34-PEP4 cells).

oxidative damage, we showed that *de novo* protein synthesis contributes to restoring Tdh activity.

Several studies have indicated that the 20S proteasome plays a key role in the removal of irreversibly oxidized proteins (Shringarpure *et al.*, 2003; Inai & Nishikimi, 2002). In

and -conjugation (*UBC1*, *RAD6*) enzymes, a ubiquitin-specific protease (*UBP6*) and one 26S proteasome subunit (*UBC13*), the degradation of carbonylated proteins does not require ubiquitination. Indeed, cells deficient in Doa4p, which contain reduced levels of free ubiquitin (Swaminathan *et al.*, 1999), degrade carbonylated proteins as efficiently as do parental cells (data not shown), and protein multi-ubiquitination is not observed in H_2O_2 -treated cells (Inai & Nishikimi, 2002).

Our data show that genes associated with vacuolar proteolysis are also upregulated during recovery after oxidative damage. These include *PEP4*, which encodes a vacuolar aspartyl protease required for the post-translational precursor maturation of vacuolar proteinases (Woolford *et al.*, 1986), and *LAP4*, which encodes a vacuolar aminopeptidase, as well as genes related to protein sorting into the vacuole, and to vacuolar fusion. Consistent with a role for vacuolar proteolysis in cellular recovery from oxidative damage, Pep4p levels and enzyme activity increased during recovery, and this induction was correlated with a higher rate of proteolysis, and a decrease in protein carbonyl content. In addition, the protein degradation rate and the capacity to remove oxidized proteins decreased in *pep4Δ* mutants. Importantly, the oxidized protein level was constitutively higher in *pep4Δ* mutants, indicating that vacuolar proteolysis has a major role in the turnover of proteins oxidized by endogenously generated or exogenously added ROS. These results are in agreement with data suggesting that protein sorting, vacuole function and vacuolar acidification are core functions required for broad resistance to oxidative stress in yeast (Thorpe *et al.*, 2004). In yeast, a mechanism of transport into the vacuole independent of the secretory pathway has been described for a few cytosolic proteins, namely the gluconeogenic enzyme fructose-1,6-bisphosphatase (Brown *et al.*, 2000) and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Horst *et al.*, 1999). Members of the Hsp70 family play a role in the import of these cytosolic proteins into the vacuole. In mammalian cells, it has recently been shown that a chaperone-mediated autophagy pathway is activated by oxidative stress, and contributes to degradation of oxidized cytoplasmic proteins inside the lysosomes (Kiffin *et al.*, 2004). The present study identified several genes associated with protein folding and stabilization that are upregulated during recovery after H_2O_2 stress, and therefore, might be important for the selective import of oxidized proteins into the vacuole. However, it has recently been shown that cells treated with H_2O_2 release Pep4p into the cytoplasm (Mason *et al.*, 2005). It is therefore conceivable that oxidized proteins may be degraded by Pep4p in the cytoplasm.

Studies in yeast and other model organisms have shown that the increased scavenging of ROS by overexpression of antioxidant defences delays ageing (Sun *et al.*, 2002; Harris *et al.*, 2003, 2005), and defective removal of damaged proteins by the 20S proteasome promotes senescence (Chen

et al., 2004, 2005). The key role of Pep4p in the turnover of damaged proteins after oxidative stress led us to investigate if this homeostatic function is important for chronological lifespan. It has been shown that Pep4p is important for survival of stationary cells, as it recycles nitrogen in starved cells (Teichert *et al.*, 1989; Levine & Klionsky, 2004). The present study showed that the premature senescence of *pep4Δ* mutants was associated with increased accumulation of carbonylated proteins. It should be noted that protein carbonyl content in the parent strain at day 15, when cells began to lose viability, was 1.8-fold higher than the amount in aged *pep4Δ* mutants (day 2) (Fig. 6b). The dramatic shortening of the chronological lifespan in cells lacking Pep4p was probably due to the sum of two detrimental effects: nitrogen starvation and accumulation of damaged proteins.

In parental cells, Pep4p activity increased during cell ageing, indicating that lifespan was not limited by the loss of Pep4p function. Thus, protein oxidation rate may exceed the capacity of aged cells to degrade oxidized proteins in the vacuole. A progressive decline in the capacity to deliver cargo into the vacuole would also explain these results. Interestingly, orthologues of yeast autophagy genes are essential for lifespan extension by calorie restriction, and for the increased longevity of *Caenorhabditis elegans* mutants with defective Daf-2 protein, an insulin receptor analogue (Melendez *et al.*, 2003).

The steady accumulation of carbonylated proteins over time was prevented in cells overexpressing *PEP4*, supporting the role of Pep4p in the turnover of oxidized proteins. Despite this, the increased Pep4p activity did not extend yeast chronological lifespan. These results indicate that removal of oxidized proteins is not the (only) limiting factor in lifespan. This may be explained by the fact that increased levels of Pep4p do not prevent the increase of other hallmarks of cell ageing, such as lipid peroxidation and oxidative DNA damage (Halliwell & Gutteridge, 1999). In contrast, overexpression of free-radical-scavenging enzymes (primary defences) decreases intracellular oxidation, preventing oxidative damage to cellular molecules, and therefore extending lifespan (Sun *et al.*, 2002; Harris *et al.*, 2003, 2005). Nevertheless, this does not exclude the hypothesis that the accumulation of oxidized proteins promotes cell death associated with ageing.

In summary, the overall results indicate that vacuolar proteolysis contributes to the efficient removal of proteins oxidized during exposure to H_2O_2 , or by ROS generated as by-products of normal aerobic metabolism. This work also provides new evidence that failure to complete house-keeping due to defective vacuolar proteolysis contributes to the progressive accumulation of molecular damage associated with chronological ageing. However, removal of oxidized proteins is not the only limiting factor, as *PEP4* overexpression prevents the accumulation of carbonylated proteins, but is not sufficient to extend lifespan.

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