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SHORT COMMUNICATION

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Polyubiquitin gene expression contributes to oxidative stress resistance in respiratory yeast (*Saccharomyces cerevisiae*)

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Abstract *UBI4*, the polyubiquitin gene of *Saccharomyces cerevisiae*, is expressed at a low level in vegetative cells, yet induced strongly in response to starvation, cadmium, DNA-damaging agents and heat shock. *UBI4* is also expressed at a higher basal level in cells growing by respiration as compared to glucose-repressed cells growing by fermentation. This higher *UBI4* expression of respiratory cultures probably helps to counteract the greater oxidative stress of respiratory growth. The effects of inactivating *UBI4* on high temperature viability are more marked with respiratory cultures. Also loss of *UBI4* leads to a considerably increased rate of killing of respiring cells by hydrogen peroxide, whereas the same gene inactivation has relatively little effect on the peroxide sensitivity of cells in which mitochondrial functions are repressed. This is the first study to reveal that ubiquitin levels in cells can influence their ability to withstand oxidative stress.

Key words *Saccharomyces cerevisiae* · Oxidative stress · High temperature viability · Ubiquitin

Introduction

Ubiquitin is a very highly conserved, 76 amino acid peptide found in all eukaryotic cells. It acts by becoming covalently attached to the free amino groups of target proteins through its C-terminal glycine, an attachment catalysed by the ubiquitin ligases (Jentsch et al. 1990; Stadtman 1990; Finley and Chau 1991; Rechsteiner 1991). This "ubiquitination" is thought to be primarily a means of targetting proteins for cytoplasmic degradation, those proteins that are abnormal or short-lived becoming extensively polyubiquitinated immediately prior to degradation by the multicatalytic proteasome complex (Jentsch et al. 1990; Finley and Chau 1991; Rechsteiner 1991). In a few cases ubiquitination of a protein appears not to cause its degradation, since histones H2A and H2B, actin and integral membrane receptors are ubiquitinated apparently as part of their normal cellular functioning (Monia et al. 1990).

Stress-inducible polyubiquitin genes have been found in eukaryotes from yeast to man (Finley and Chau 1991). *UBI4*, the polyubiquitin gene of *Saccharomyces cerevisiae* is typical, its mRNA encoding a polyubiquitin comprising five head-to-tail ubiquitin repeats (Özkayzak et al. 1987). This polyprotein is rapidly cleaved to ubiquitin monomers after synthesis. Expressed at a low level in vegetative cells, *UBI4* is expressed at much higher levels under conditions of heat shock, starvation and **a**/α diploid sporulation, and during treatments with cadmium or DNA-damaging alkylating agents (Finley et al. 1987; Tanaka et al. 1988; Treger et al. 1988; Fraser et al. 1991; Jungmann et al. 1993). Inactivation of *UBI4* in yeast results in a considerably reduced resistance to starvation, higher cell mortality at the highest temperatures of growth, greater sensitivity to amino-acid analogues and alkylating agents, and a block to the sporulation of **a**/α *ubi4/ubi4* diploid cells (Finley et al. 1987; Tanaka et al. 1988; Treger et al. 1988; Fraser et al. 1991). These are all treatments or conditions which probably impose on the cell a need for greatly enhanced intracellular protein turnover. It is thought that *UBI4* induction under these situations provides the higher ubiquitin levels needed for this increased protein degradation.

Using an *S. cerevisiae* strain with an integrated *UBI4* promoter-*lacZ* gene fusion it has been observed that the basal activity of the *UBI4* promoter is approximately four-fold higher during respiratory growth on

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nonfermentable carbon sources (e.g. glycerol, acetate) compared to fementative growth on repressing sugars such as glucose (Kirk 1993). This promoter also has a sequence (TGATTGGT at positions − 542 to − 549 relative to the ATG start codon) that precisely matches the consensus for HAP2/HAP3 complex binding (5′-TNA/GTTGGT-3′; Bowman et al. 1992). The HAP2/HAP3 binding element is commonly found in the promoters of genes for mitochondrial components. It constitutes part of the system for activation of mitochondrial genes during catabolite derepression, when cells are adapting for respiratory growth and becoming competent in respiration (Hahn and Guarente 1988; Bolotin-Fukuhara and Grivell 1992). We reasoned that the higher levels of expression of *UBI4* during respiratory growth might help the cell to counteract the greater oxidative stress of respiratory as compared to fermentative growth. If this is true, a *UBI4* gene inactivation should have more severe effects during respiratory growth; also the same gene inactivation should result in increased sensitivity to oxidative damage in respiratory cultures with their intrinsically higher levels of *UBI4* expression.

Results and discussion

The effects of UBI4 inactivation on high temperature viability are more marked with respiratory than with fermentative cultures

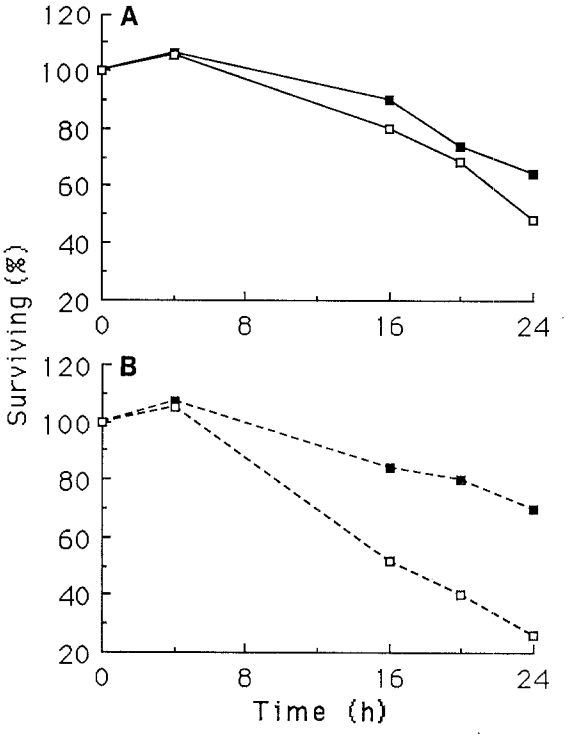
At the highest temperatures that permit *S. cerevisiae* cell division there is also significant cell death (Van Uden 1984). Inactivation of *UBI4* has been shown to increase the rate of this cell death in glucose-repressed, fermentative yeast cultures shifted from 28° C to 38.5° C (Finley et al. 1987). Heat shock to 38.5° C also causes a transient arrest of growing cells in the G1 phase of the cell cycle. This increase in the unbudded cell fraction probably arises from a temporary destabilisation of cyclins (reviewed in Piper 1993).

Cultures exposed to supra-optimal temperatures are subject not just to heat stress, but also an additional oxidative stress if they are growing by respiration (see Schnell et al. 1992 for references). To test whether the loss of *UBI4* causes heat-stressed cells to die more rapidly under respiratory than under fermentative conditions, we determined the high temperature viabilities of respiratory and fermentative cultures of the strains SUB61 (α *leu2 ura3 his3 UBI4*) and SUB63 (α *leu2 ura3 his3 ubi4::LEU2*). These two strains are completely isogenic but for a *LEU2* gene disruption of the *UBI4* gene in SUB63 (Finley et al. 1987). Vegetative cultures of SUB61 and SUB63 growing aerobically on either glycerol or glucose were shifted from 28° C to 38.5 °C and their viability determined at subsequent intervals (Fig. 1). At 38.5° C the *ubi4* mutant SUB63 died considerably more rapidly, relative to SUB61, when in respiratory growth on glycerol (Fig. 1A), whereas the effect of the *ubi4* mutation was considerably smaller during fermentative growth on glucose (Fig. 1B). This is consistent with the higher *UBI4* expression levels of respiratory cultures (Kirk 1993) contributing to survival of the additional oxidative stress of cells maintained by respiration.

UB14 inactivation causes increased sensitivity to hydrogen peroxide (H_2O_2) in respiratory cultures

If *UBI4* expression contributes to the removal of oxidatively damaged proteins from the cell, *ubi4* mutant cells should be more sensitive to inactivation by H_2O_2. To measure the influence of *UBI4* expression on cell killing by H_2O_2, the survival was measured of SUB61 and SUB63 cells incubated for different lengths of time

Fig. 1A, B UBI4 inactivation causes greater sensitivity to supra-optimal temperatures in cells in respiratory, as compared to fermentative, growth. Shake-flask cultures in mid-exponential aerobic growth at 28° C on either YPD (2% (w\v) bactopeptone, 1% yeast extract, 2% glucose) or YPG (2% (w\v) bactopeptone, 1% yeast extract, 3% glycerol) were shifted to 38.5° C at time zero. At the times indicated, cells were removed, diluted into YPD at 28° C and spread on the surface of YPD plates. These plates were then incubated at 28° C to allow colony formation by viable cells. **A** YPD cultures; **B** YPG cultures; *open symbols*, SUB63 (*ubi4* mutant); *closed symbols*, SUB61



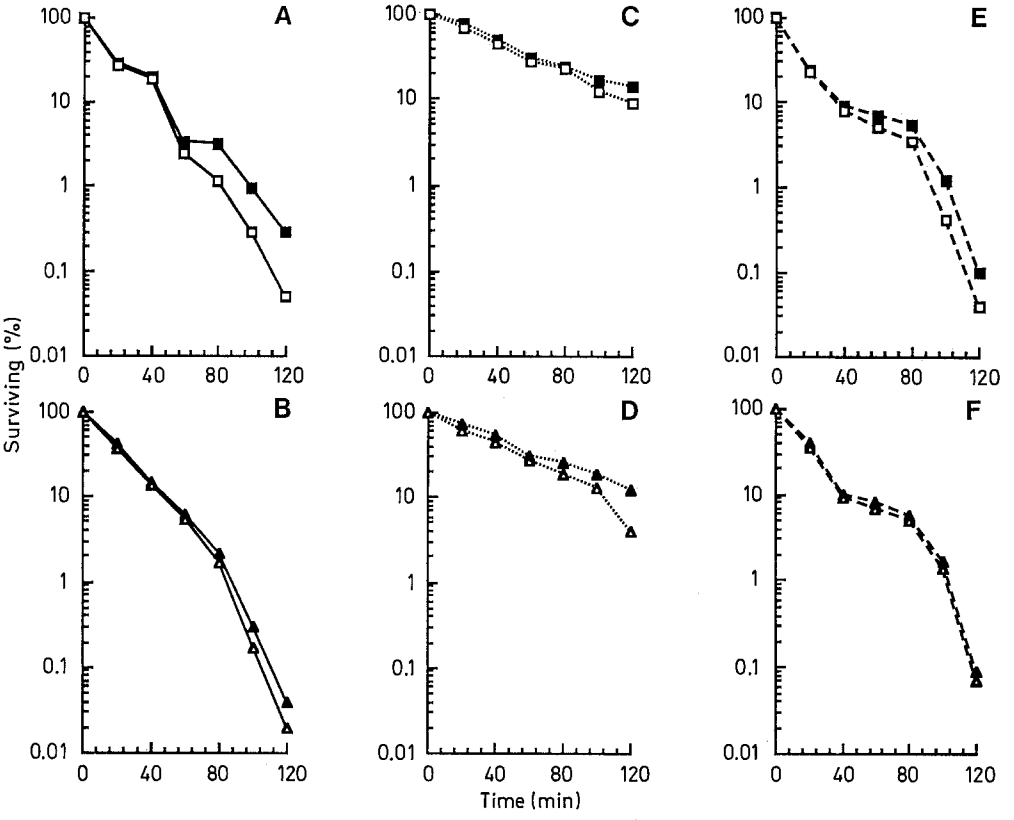


Fig. 2 UBI4 gene disruption has little effect on hydrogen peroxide (H_2O_2) inactivation of fermentative cultures. Flask cultures in exponential growth at 28° C on aerated YPD (A, C, E) or anaerobic YPD medium (the latter supplemented with 0.2% Tween 80 and 20 μg\ml cholesterol)(B, D, F) were treated with 4 mM H_2O_2 without any pretreatment for the times indicated (**A, B**) or pretreated 1 h with 0.4 mM H_2O_2 prior to treatment with 4 mM H_2O_2 at time zero (**C, D**) or heat-shocked 30 min at 38° C prior to 4 mM H_2O_2 treatment at time zero (**E, F**). After 4 mM H_2O_2 treatment for the times indicated, cells were removed from the cultures and the viable cell fraction determined as in Fig. 1. *Open symbols*, SUB63 (*ubi4* mutant); *closed symbols*, SUB61

in the presence of 4 mM H_2O_2. Aerobic (Fig. 2A) or anaerobic (Fig. 2B) SUB61 and SUB63 cultures growing by the fermentation of glucose showed relatively little difference in their rates of H_2O_2 inactivation. *UBI4* is only weakly expressed under these conditions (Finley et al. 1987; Tanaka et al. 1988; Treger et al. 1988; Fraser et al. 1991) and not induced by either H_2O_2 or the herbicide paraquat (methyl viologen; R. Watt, unpublished results). Paraquat is an agent known to cause increased intracellular production of superoxide radicals (Halliwell 1984). In contrast, when the same two strains were grown in aerobic respiratory conditions on glycerol, conditions under which *UBI4* is expressed more strongly (Kirk 1993), the *ubi4* mutation caused an appreciable increase in sensitivity to H_2O_2 (Fig. 3A). In addition the *UBI4* strain SUB61 is intrinsically more resistant to H_2O_2 when grown on a respiratory medium (compare Figs. 2A, B with Fig. 3A).

Pretreatment of yeast with sublethal concentrations of H_2O_2 induces resistance to a subsequent, more severe H_2O_2 challenge (Collinson and Dawes 1992). When aerobic or anaerobic, glucose-repressed (YPD medium) cultures were pretreated with 0.4 mM H_2O_2 for 1 h, resistance to 4 mM H_2O_2 increased, yet there was again little difference in the H_2O_2 inactivation rates of SUB61 and SUB63 (compare Fig. 2A with 2C; also Fig. 2B with 2D). *UBI4*$^+$ and *ubi*4 mutant cells in respiratory (YPG medium) growth also showed this increased H_2O_2 resistance following a 0.4 mM H_2O_2 pretreatment, yet the increased susceptibility of the *ubi4* mutant (SUB63) was maintained (compare Figs. 3A and 3B).

Figs. 2 and 3 show the effect of preconditioning the SUB61 and SUB63 cultures by heat shock at 38° C for 30 min. This pretreatment will increase *UBI4* expression in SUB61 but not SUB63 (Finley et al. 1987). Also, at least in strains wild-type for ubiquitin genes, heat shock is reported to induce resistance to the lethal effects of H_2O_2 (Watson 1990; Collinson and Dawes 1992). The increases in H_2O_2 resistance due to this heat preconditioning were relatively small (compare Fig. 2A to 2E; Fig. 2B to 2F; and Fig. 3A to 3C). Even after this heat shock pretreatment, marked effects of the *ubi4* mutation were again obtained only with respiratory and not with fermentative cultures (compare Figs. 2E, F with Fig. 3C).

A number of other components of the antioxidant defences of yeast are heat inducible and probably mitigate the effects of oxidative stress associated with respiratory growth at high temperature

Superoxide radicals and H_2O_2 are produced as side-products of the metabolism of all living organisms. The major agent causing oxidative damage is thought to be the highly reactive hydroxyl radical that these species generate in metal-catalysed Haber-Weiss reactions

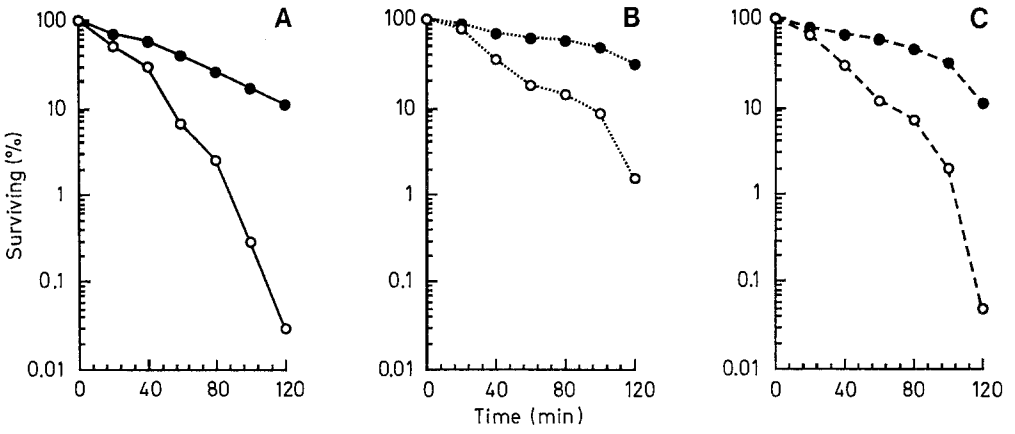


Fig. 3 The ubi4 mutation causes a considerable increase in lethality due to H_2O_2 in respiratory cultures. Flask cultures in exponential 28° C growth on aerated YPG were treated with 4 mM H_2O_2 at 28° C for the times indicated without pretreatment (**A**), after 1 h pretreatment with 0.4 mM H_2O_2 (**B**) and after a 30 min 38° C heat shock (**C**) Viability was measured by plating on YPD plates as in Fig. 1. *Open symbols*, SUB63 (*ubi4*); *closed symbols*, SUB61

(Halliwell 1984; Zhu and Scandalios 1992; Schnell et al. 1992). Cells have evolved diverse ways of protecting themselves from the toxic effects of these reactive oxygen species. In eukaryotic cells the antioxidant defences are both nonenzymatic (primarily the glutathione pool) and enzymatic (notably peroxidase enzymes, superoxide dismutase and catalase). Superoxide dismutase catalyses the dismutation of two superoxide radicals into molecular oxygen and H_2O_2, whereas catalase degrades two H_2O_2 molecules to water and molecular oxygen. Studies with the appropriate mutants have shown two enzymes to be important for the survival of oxidative stress in yeast: the mitochondrial, manganese form of superoxide dismutase (MnSOD) (Van Loon et al. 1986) and cytoplasmic catalase T (the *CTT1* gene product; Bissinger et al. 1989; Belazzi et al. 1991; Weiser et al. 1991; Marchler et al. 1993). We show here that ubiquitin gene expression, part of the system for degradation of aberrant proteins (Finley et al. 1987; Jentsch et al. 1990; Stadtman 1990; Finley and Chau 1991; Rechsteiner 1991) also contributes to resistance to oxidative stress in respiratory yeast. This indicates that the ubiquitination system for protein turnover is important in the degradation of oxidatively damaged proteins.

MnSOD is a nuclear-encoded enzyme that is translocated to the mitochondrial matrix during maturation. Like most mitochondrial enzymes, it is repressed by high glucose concentrations. Elevation of its activity by heat or ethanol stress (Costa et al. 1993) may allow more efficient trapping of superoxide radicals within the mitochondria, limiting their escape to the cytosol and thereby minimising oxidative damage to proteins, nucleic acids and membrane lipids. The primary role of cytoplasmic catalase T is to protect against the damage caused by H_2O_2. It is induced by heat shock but, as with MnSOD, this induction does not occur in glucose-grown cells with high cyclic AMP-dependent protein kinase activity, since the catalase T gene is under negative regulation by this kinase (Marchler et al. 1993).

Peroxides induce the synthesis of heat shock proteins in *Salmonella typhimurium* (Morgan et al. 1986). In yeast also there may be a partial overlap between the heat- and peroxide-induced responses, since sublethal amounts of H_2O_2 and mild heat both independently induce resistances to the lethal effects of high H_2O_2 concentrations (Watson 1990; Collinson and Dawes 1992). In *S. cerevisiae* heat shock does not alter glutathione levels (Costa et al. 1993). However, certain proteins induced by heat shock are also induced by H_2O_2 treatment (Collinson and Dawes 1992; Marchler et al. 1993; Gropper and Rensing 1993). H_2O_2 inducibility is not however a property of all yeast heat shock genes since the *UBI4* polyubiquitin gene is not H_2O_2 inducible (our unpublished observations). This study shows that the lack of *UBI4* causes increased H_2O_2 sensitivity in respiratory cultures (Fig. 3), yet has relatively little influence on the H_2O_2 sensitivity of glucose-repressed cells (Fig. 2). It also provides evidence that the higher *UBI4* expression in cells growing by respiration (Kirk 1993) helps counteract the higher oxidative stress of respiratory growth relative to glucose-repressed fermentative growth at 38.5° C (Fig. 1). At 38.5° C *S. cerevisiae* cultures suffer appreciable cell death (Van Uden 1984; Finley et al. 1987) and numerous physiological changes due to heat stress (Piper 1933). Ubiquitin (Finley et al. 1987; Tanaka et al. 1988), MnSOD (Costa et al. 1993) and catalase T (Bissinger et al. 1989; Belazzi et al. 1991; Weiser et al. 1991; Marchler et al. 1993) are all heat inducible in yeast, although expression of high levels of catalase T requires a combination of stresses (either heat shock or nitrogen starvation and the presence of oxygen (oxidative stress signalled by haem) (Belazzi et al. 1991; Weiser et al. 1991; Marchler et al. 1993)). The heat inducibility of these diverse components of stress protection appears to help counteract the tendency to incur more severe oxidative damage at higher temperatures, damage that will be more acute in respiratory cultures.

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