Plasma-membrane ATPase action affects several stress tolerances of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* as well as the extent and duration of the heat shock response

BARRY PANARETOU and PETER W. PIPER*

Department of Biochemistry, University College London, London WC1E 6BT, UK

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The *pma1.1* mutations of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* decrease plasma-membrane ATPase activity. This study investigated how they affect different stress tolerances, and the extent and duration of the heat-shock response. *pma1.1* mutants exhibited higher resistance to ethanol and osmotic stress, but lower tolerance to ultraviolet damage, as compared to wild-type cells. *pma1.1* mutations also increased tolerance of the lethal temperature of 48 °C in cells in which no heat-shock response had been induced. However, after induction of a heat-shock response and elevated thermotolerance by a 25–38 °C upshift, then maintaining cells at 38 °C for 40 min, *pma1.1* lowered subsequent tolerances of much higher lethal temperatures. Analysis of pulse-labelled *S. cerevisiae* proteins revealed reduced heat-shock protein synthesis in the *pma1.1* mutant after a 25–38 °C heat shock. This may explain the greater increases in thermotolerance in wild-type as compared to *pma1.1* cells after both were given identical 25–38 °C shocks. With more severe treatment (25–42 °C), heat-shock protein synthesis in wild-type cells, although initially high, was switched off more rapidly than in the *pma1.1* mutant. These results indicate that plasma-membrane ATPase action exerts a major influence over several stress tolerances, as well as the extent and duration of heat-shock protein synthesis following induction of the heat-shock response.

Introduction

The ability of organisms to withstand cytotoxic agents or stressful situations is intimately affected both by their physiological state and by their capacity to induce protective responses. One of the best-characterized stress-induced responses is that elicited by heat shock. This 'heat shock response' (reviewed by Craig, 1986; Lindquist & Craig, 1988) entails the transient induction of a small number of heat-shock proteins (HSPs), and a simultaneous blocking of the synthesis of most of the proteins made prior to the shock. One of its most marked consequences is an increase in thermotolerance, measured as the ability to withstand normally lethal temperatures. A heat-shock response is displayed by most organisms and many of the induced genes show a remarkable degree of evolutionary conservation. Although experimentally the response is usually induced by temperature upshift, several potentially cytotoxic chemicals are also potent inducers. All these inducers may act through a common trigger, possibly the intracellular

accumulation of aberrant or misfolded protein (Ananthan *et al.*, 1986; Pelham, 1987).

Stress tolerances can be affected by many factors in addition to the actions of those proteins induced through the triggering of a stress response. Thus thermotolerance in *Saccharomyces cerevisiae* cannot be attributed solely to the actions of HSPs. It is also affected by growth state, being lowest in cells in rapid exponential growth and high in stationary-phase (G_0) cells (Iida & Yahara, 1984; Iida, 1988). The high thermotolerance of G_0 cells may be due in part to the induction of HSP genes with the decline in cyclic AMP levels as cells enter stationary phase (Boucherie, 1985; Iida & Yahara, 1984; Shin *et al.*, 1987). A few of the proteins made in heat-shocked *S. cerevisiae* correspond to glycolytic enzymes that are also made by unstressed cells (Piper *et al.*, 1986, 1988). Furthermore, we recently identified the gene for plasma membrane ATPase (*PMAl*) as one of the few in *S. cerevisiae* still efficiently transcribed after heat shock to 42 °C, approximately 3 °C above the maximum growth temperature. Polyadenylated RNA pulse-labelled *in vivo* at 42 °C hybridizes to relatively few yeast sequences, one of them being the *PMAl* transcribed region (Curran *et*

Abbreviation: HSPs, heat-shock proteins.

al., 1988). This led us to investigate mutants altered in plasma-membrane ATPase activity to obtain evidence of whether the activity of this important enzyme is a major determinant of the tolerances of budding (*S. cerevisiae*) and fission (*Schizosaccharomyces pombe*) yeast to heat shock and other stresses.

Plasma-membrane ATPase is an electrogenic proton pump crucial to all fungal and plant cells. It generates the electrochemical H^+ gradient essential for such important cellular functions as the secretion of acids, the uptake of nutrients, the maintenance of K^+ levels and the regulation of intracellular pH (Ulaszewski *et al.*, 1983, 1987; Serrano *et al.*, 1986; Eraso *et al.*, 1987; Eraso & Gancedo, 1987). One of the effects of heat-shock stress, at least in *S. cerevisiae* (Weitzel *et al.*, 1987), is a dissipation of this H^+ gradient. The resultant cytoplasmic acidification should be counteracted by plasma-membrane ATPase action, even though this enzyme has not previously been considered a possible influence on the tolerances or responses of fungi and plants to heat shock.

The plasma-membrane ATPases of *Neurospora crassa*, *S. cerevisiae* and *Sch. pombe* all display extensive sequence homology, as well as similar hydrophobicity profiles, thought to indicate 8–10 transmembrane domains (Aaronson *et al.*, 1988). Plasma-membrane ATPases from flowering plants also have up to 36% amino acid sequence homology with these fungal enzymes (Harper *et al.*, 1989; Pardo & Serrano, 1989; Boutry *et al.*, 1989). This ATPase is so conserved in structure and function that should it be shown to influence stress tolerances in one organism, there would be grounds to suspect that its action might also determine tolerance levels in diverse fungi and plants.

Methods

Strains. The *S. cerevisiae* *pma1.1*, *pma1.2*, *pma1.3* and *pma1.4* mutants (strains MG2129, MG2130, MG2131 and MG2132 respectively), their PMA1⁺ prototrophic parent ϵ 1278b (Ulaszewski *et al.*, 1983), the *Sch. pombe* strain Jv66 (*h⁻ pma1.1, ade-413*) and its parent strain 972 (*h⁻ ade-413*) (Ulaszewski *et al.*, 1986) were all kindly provided by A. Goffeau (University of Louvain, Belgium).

Media. All tolerance experiments were done on cells that had been maintained in exponential growth at 25 °C on YEPD medium (2% bacto-peptone, 1% yeast extract, 2% glucose, all w/v) for more than 50 generations. Just prior to each experiment, *S. cerevisiae* cultures were briefly sonicated, just sufficiently for no cell aggregates to be seen by light microscopy. With the *Sch. pombe* cultures this sonication step was unnecessary.

Determination of killing kinetics. Thermotolerance experiments were done on exponential ($0.5\text{--}1 \times 10^7$ cells ml⁻¹) YEPD cultures. The non-acute heat-shock treatment used for induction of thermotolerance was a rapid shift of part of each culture (the 'induced' cells) from 25 °C to 38 °C, maintaining cultures at 38 °C for 40 min prior to an immediate

shift to 52 °C for a variable period. An identical portion of each culture (the cells 'uninduced' for thermotolerance) was immediately transferred from 25 °C to 48 °C for variable times, killing of these cells at temperatures higher than 48 °C being too rapid for accurate measurement. At intervals from 0 to 15 min, while cultures were at 48 °C or 52 °C, aliquots were rapidly diluted into 5 ml YEPD at room temperature (21–23 °C) and cells plated on YEPD plates within 20 min of the exposure to high temperature.

For measurements of ultraviolet killing, cells were diluted appropriately in YEPD and 0.15 ml aliquots spread on YEPD plates to give 300 cells per plate. Immediately after plating, the cells were exposed to an ultraviolet light source for various times.

To measure tolerance of high ethanol or salt concentrations, cells in exponential growth at 25 °C on YEPD ($0.5\text{--}1 \times 10^7$ cells ml⁻¹) were diluted appropriately in YEPD at 25 °C and additions made of either absolute ethanol or 4 M-NaCl (to final concentrations of 12.5%, v/v, and 2.5 M respectively). Aliquots of 0.1 ml were removed immediately (for the zero time point) and at subsequent intervals, these being immediately diluted 100- to 1000-fold in YEPD prior to plating on YEPD plates.

In all of these experiments, killing was measured from the colonies (including petites) on YEPD plates incubated at 28 °C. Each experiment was repeated at least five times, with similar results, each survival shown in Figs. 1, 2 and 4 being the results from a representative experiment. Since each time point involved the counting of at least 300 colonies the major error in these viability measurements was in the preparation of serial dilutions. This error was estimated as no more than 15%.

Pulse labelling of *S. cerevisiae* proteins during heat shock. From 1×10^8 to 2×10^8 cells from exponential YEPD cultures of *S. cerevisiae* ϵ 1278b and MG2129 were collected by centrifugation (3000 g for 5 min), resuspended in 20 ml CMD medium at 25 °C (2% glucose, 0.67% yeast nitrogen base without amino acids), recentrifuged, resuspended in 20 ml CMD medium and incubated for 20 min at 25 °C prior to heat shock. During this 20 min period the cultures were subdivided into 1 ml aliquots in glass tubes. To heat shock the cells, aliquots were shifted to either 38 °C (Fig. 3a) or 42 °C (Fig. 3b) and, at variable times after this temperature upshift, were labelled with [³⁵S]methionine (10 μ Ci ml⁻¹; 370 kBq ml⁻¹). Each pulse-labelling was for 15 min. A control 1 ml aliquot was pulse-labelled for 1 h at 25 °C without any heat shock. The cells from each labelling were rapidly chilled, then used in the preparation of samples of total cell protein which were analysed on one dimensional 12.5% polyacrylamide gels as described by Piper *et al.* (1986).

Results

Influence of the pma1.1 mutations of S. cerevisiae and Sch. pombe on thermotolerance

When *S. cerevisiae* or *Sch. pombe* cells in exponential growth at 25 °C are placed at 48 °C they rapidly lose viability. Neither organism can synthesize HSPs above 42–43 °C (see Discussion). In both species, *pma1.1* caused higher thermotolerance levels at 48 °C, as manifested by the slightly greater survival of mutant as compared to wild-type cells in Figs 1(a) and 2(a). Similar results were obtained with *S. cerevisiae* *pma1.2*, *pma1.3* and *pma1.4* (data not shown). If, prior to exposure to lethal temperatures, the *S. cerevisiae* or *Sch. pombe*

Fig. 1. High temperature tolerance of the *S. cerevisiae* *pma1.1* mutant (strain MG2129) (○) and its wild-type parent (81278b) (●). Cultures were either (a) uninduced for thermotolerance, being shifted to 48 °C from 25 °C; or (b) had their thermotolerance elevated by prior heat shock from 25 °C to 38 °C for 40 min before being shifted to 52 °C.

Fig. 2. High temperature tolerance of the *Sch. pombe* *pma1.1* mutant (strain JV66) (△) and its wild-type parent (972) (▲). Cultures were either (a) uninduced for thermotolerance, being shifted to 48 °C from 25 °C; or (b) had their thermotolerance elevated by prior heat shock from 25 °C to 38 °C for 40 min before being shifted to 52 °C.

cultures were given a non-acute heat shock (25–38 °C temperature upshift then a 40 min incubation at 38 °C) this effect of *pma1.1* on thermotolerance was reversed (Figs 1b and 2b). This effect was also demonstrated with *S. cerevisiae* *pma1.2*, *pma1.3* and *pma1.4* (not shown). The non-acute heat shock to 38 °C enables the induction of HSPs, associated with a marked elevation of thermo-

tolerance (see Introduction). In both *S. cerevisiae* and *Sch. pombe* cells induced for this heat-shock response, *pma1.1* reduced viability at high temperature. Comparison of Fig. 1(a) with Fig. 1(b), and of Fig. 2(a) with Fig. 2(b), reveals that *pma1.1* reduces the increase in thermotolerance as the heat-shock response is induced by a 25–38 °C upshift.

Fig. 3. Proteins pulse-labelled in heat-shocked cells of *S. cerevisiae* ϵ 1278b and *pma1.1* mutant MG2129 after heat shock either to 38 °C (a); or to 42 °C (b). Cells were pulse-labelled with [35 S]methionine 10–25 min (2), 25–40 min (3), 40–55 min (4), or 55–70 min (5) after temperature upshift. Track 1 shows proteins labelled in unstressed cells at 25 °C. All gel samples contained protein from the same number of cells so the relative intensity of bands on the autoradiographs indicates relative protein labelling under these conditions. The indicated bands of 70, 82 and 96 kDa are the large heat-shock proteins HSP70, HSP82 and HSP96.

Fig. 4. Survival of yeast exposed to (a, d) 12.5% (v/v) ethanol; (b, e) 2.5 M-NaCl; and (c, f) ultraviolet irradiation. (a-c) Survival data for *S. cerevisiae* ϵ 1278b (●) and *pma1.1* mutant MG2129 (○); (d-f) data for *Sch. pombe* 972 (▲) and *pma1.1* mutant JV66 (△).

Influence of pma1.1 on HSP synthesis in S. cerevisiae

S. cerevisiae ϵ 1278b and MG2129 were labelled with [35 S]methionine both before, and at intervals after, a 25–38 °C heat shock. Their labelled proteins were then separated on a one-dimensional gel (Fig. 3a). After this

relatively mild heat shock (38 °C is below the maximum growth temperature on glucose media), cells resumed an almost normal pattern of protein synthesis within about 1 h (Fig. 3a). However, synthesis of the major HSPs was dramatically reduced in the *pma1.1* mutant strain. Therefore a partial suppression of the heat-shock

response in mutant, compared to wild-type, cells is the probable reason for the lowered induction of thermotolerance by 25–38 °C heat shock in *pma1.1 S. cerevisiae* (Fig. 1).

When this labelling was repeated under conditions of considerably more severe (25–42 °C) heat shock a rather different result was obtained (Fig. 3*b*). The temperature of 42 °C is about 3 °C above the maximum for growth, and close to the maximum temperature at which *S. cerevisiae* will still display synthesis of HSPs (Piper *et al.*, 1986, 1988). Normally, after cells are shifted to 42 °C, an initial 'burst' of HSP synthesis is followed by a progressive cessation of all protein synthesis over about 1 h. This is apparent from the labelling of *S. cerevisiae* ϵ 1278b in Fig. 3(*b*). HSP synthesis in the *pma1.1* mutant at 42 °C, although initially similar to that of wild-type cells, was not subject to such rapid inhibition and was sustained for at least 70 min at 42 °C (Fig. 3*b*).

These experiments indicate that, depending on the severity of the heat shock, *pma1.1* can affect either the extent or the duration of HSP synthesis in *S. cerevisiae*. They were not repeated on *Sch. pombe* strains JV66 and 972 because growth of these strains in minimal medium is severely restricted by their adenine auxotrophy even in the presence of exogenous adenine (Ulaszewski *et al.*, 1987).

Influence of pma1.1 on ethanol tolerance, osmotolerance and tolerance of ultraviolet irradiation

The *pma1.1* mutations of *S. cerevisiae* and *Sch. pombe* increased tolerance of brief exposures to high ethanol and high salt concentrations, and decreased tolerance of ultraviolet irradiation (Fig. 4). Similar results were obtained with *S. cerevisiae pma1.2*, *pma1.3* and *pma1.4* (data not shown).

Discussion

S. cerevisiae pma1.1, *pma1.2*, *pma1.3* and *pma1.4*, and *Sch. pombe pma1.1*, were isolated as spontaneous mutants showing resistance to Dio-9, a non-specific inhibitor of plasma-membrane ATPase (Ulaszewski *et al.*, 1983, 1986). They exhibit several similar changes in the *in vitro* properties of plasma-membrane ATPase, which is why they were investigated in parallel in this study. These changes include: (i) a lower specific activity; (ii) a modified K_m for MgATP; and (iii) strong resistance to vanadate (Ulaszewski *et al.*, 1983, 1987). The *Sch. pombe pma1.1* mutation corresponds to a single amino acid substitution in the ATPase, namely Gly to Asp at residue 268 (Ghislain *et al.*, 1987).

These mutants were used to obtain evidence as to whether plasma-membrane ATPase action exerts a major influence on tolerances to elevated temperatures and other forms of stress. The fact that mutation to Dio-9 resistance causes identical changes to several tolerances in two very distantly related species shows that the profound metabolic changes that ensue from alteration to plasma-membrane ATPase activity are an important determinant of these tolerance levels. Also, protein pulse-labelling in *S. cerevisiae* indicated that *pma1.1* can affect the extent and duration of HSP synthesis induced by the heat-shock response.

When *S. cerevisiae* or *Sch. pombe* cells actively growing at 25 °C are shifted to 48 °C there is rapid protein synthesis arrest; no HSPs are synthesized and these cells, uninduced for thermotolerance, quickly die. Pulse-labelling studies have shown that neither yeast can synthesize HSPs above 42–43 °C (Piper *et al.*, 1986; also unpublished observations). At 48 °C *pma1.1* conferred higher thermotolerance (Figs 1*a* and 2*a*). This result was not entirely unexpected for *S. cerevisiae*, since *pma1.1* causes slightly slower growth of prototrophic strains on YEPD, and thermotolerance levels are generally thought to be inversely related to the growth rate of this organism (Iida & Yahara, 1984; Iida, 1988). Figs 1(*b*) and 2(*b*) show survival of the same *S. cerevisiae* and *Sch. pombe* cultures after they had been shifted from 25 °C to 38 °C for 40 min and then exposed to a considerably higher temperature (52 °C). This non-acute 38 °C heat shock induces the synthesis of heat shock proteins, and causes a marked elevation of thermotolerance (for evidence of this in *S. cerevisiae* see Piper *et al.*, 1986, 1987, 1988). However, the increase in this thermotolerance was less for *pma1.1* than for wild-type cells given the same heat shock, in both *S. cerevisiae* and *Sch. pombe* (Figs 1 and 2). As a result, *pma1.1* cells were killed more rapidly in such cultures induced for thermotolerance, the converse of the situation found in the uninduced cultures. Both this observation and the reduction in HSP synthesis (Fig. 3*a*) are consistent with *pma1.1* causing a partial suppression of the heat-shock response normally caused by a 25–38 °C temperature shift. Important considerations here may be the extent of cytoplasmic acidification that accompanies heat shock (Weitzel *et al.*, 1987) and the rate at which it is counteracted by plasma-membrane ATPase action. Since the *pma1.1* mutant of *Sch. pombe* maintains a smaller transmembrane H^+ gradient than wild-type *Sch. pombe*, with a cytoplasmic pH 0.4 units lower (Ulaszewski *et al.*, 1987), there are grounds for expecting smaller decreases in cytoplasmic pH with heat-shock stress in this mutant. This may in turn lead to a reduced heat-shock response. The thermal stability of the ATPase must also be an important factor. Although the wild-type enzyme is the more active under most assay

conditions, *in vitro* studies have shown the *S. cerevisiae* *pma1.1* enzyme to be less prone to inactivation at 50 °C (Ulaszewski *et al.*, 1983).

The *pma1.1* mutants of *S. cerevisiae* and *Sch. pombe* exhibit greater tolerances of ethanol and high salt than the wild-type (Fig. 4). Low cyclic AMP levels in *S. cerevisiae* also cause higher ethanol tolerance (Iida, 1988). Ethanol diffuses rapidly into *S. cerevisiae* cells (Guijarro & Lagunas, 1984) and affects membrane order, as shown by Walker-Caprioglio & Parks (1987), using the lipophilic probe 1,6-diphenyl-1,3,5 hexatriene. This change in membrane order causes enhanced proton influx (Leao & VanUden, 1985), and ethanol therefore has marked effects on the maintenance of cytoplasmic pH and H⁺-coupled transport of solutes across the plasma membrane. In contrast to ethanol tolerance, osmotolerance may primarily reflect intracellular levels of osmoregulatory solutes. Fungi that grow in media of high osmotic strength synthesize polyols (e.g. glycerol, arabitol) at high levels; these increase osmotolerance by functioning as compatible solutes. Their levels change with growth state and are major determinants of osmoregulation. Glycerol, probably the most important osmotic effector in exponentially-growing salt-stressed yeast (Reed *et al.*, 1987), is made at higher levels in yeast exposed to hypertonic media (Maiorella *et al.*, 1984).

The reduction in plasma-membrane ATPase activity due to *pma1.1* causes a slight reduction in growth rate on rich medium and a considerable reduction in growth on minimal medium containing ammonia as nitrogen source. Amino-acid and purine auxotrophies reduce the growth rate still further (Ulaszewski *et al.*, 1987). Physiological changes due to adaptation of *pma1.1* cells to slower growth presumably cause some of the tolerance effects observed in this study, but may not be the only cause of the observed differences. Thus *pma1.1* might even allow higher plasma-membrane ATPase activity under high-ethanol or dehydrating conditions, just as it renders this enzyme less susceptible to vanadate inhibition (Ulaszewski *et al.*, 1983). The available evidence highlights the need to relate tolerance measurements to other physiological studies (e.g. those measuring intracellular pH shifts under stress conditions) and *in vitro* studies of plasma-membrane ATPase action. However, the results with *pma1.1* mutant strains described in this paper show that plasma-membrane ATPase action cannot be ignored as a factor affecting tolerances of a variety of potentially cytotoxic agents (Figs 1, 2 and 4) and as a determinant of the extent and duration of at least one inducible stress response (Fig. 3).

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