Regulation of *Saccharomyces cerevisiae* Plasma membrane H⁺-ATPase (Pma1) by Dextrose and Hsp30 during Exposure to Thermal Stress

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Abstract Pma1p is an essential plasma membrane H⁺-pump in *Saccharomyces cerevisiae* that pumps out H⁺ at the expense of cellular ATP. Its activity is induced by glucose at 30°C and is inhibited by Hsp30 during exposure to heat shock conditions. To further investigate the regulation of Pma1 function by glucose and Hsp30 during exposure to thermal stress, we estimated Pma1 activity, its protein levels and ser-phosphorylation status in membrane fractions isolated from BY4741 and *hsp30Δ* cells grown in dextrose and sorbitol at 30°C, and following exposure at 40°C for 30 min. Our results demonstrate that Pma1 activity and protein levels were reduced in Hsp30⁺ cells following exposure to thermal stress in dextrose media. The above was not observed in *hsp30Δ* cells wherein Pma1 activity did not decrease following exposure to similar conditions. Although Pma1p levels decreased in heat-shocked *hsp30Δ* cells, it was lower compared to that observed in Hsp30⁺ cells. Total ser-phosphorylation of Pma1 also showed a decrease following exposure to heat shock condition in dextrose media in both BY4741 and *hsp30Δ* cells. Its levels were also reduced in BY4741 cells upon heat shock treatment in sorbitol unlike that observed in *hsp30Δ* cells wherein it was increased. Taken together the above indicate that heat shock induced reduction in Pma1 activity and protein levels in dextrose media required

Hsp30. To examine functional interactions between dextrose utilization, Hsp30 and the regulation of various aspects of Pma1, we determined if dextrose regulated other functions attributed to Hsp30. Results demonstrate that the deletion of *HSP30* rendered cells dependent on dextrose utilization for survival during exposure to lethal heat stress. Our study has hence been able to establish a functional relationship between glucose utilization, Hsp30 function and the regulation of Pma1 activity. Finally, since the deletion of *HSP30* renders Pma1p levels and its activity unresponsive to thermal stress in dextrose media, we concluded that Hsp30 is necessary to maintain Pma1 in a regulation competent conformation. Hsp30 may thus act as a chaperone in the *S. cerevisiae* plasma membrane.

Keywords *Saccharomyces cerevisiae* · Pma1 · Dextrose · Hsp30 · Chaperone

Plasma membrane H⁺-ATPase (Pma1) is an essential protein in *S. cerevisiae*, it pumps out H⁺ to maintain intracellular pH homeostasis as well as to generate an electrochemical gradient required to drive the import of metabolites into the cell [1]. The above activity of Pma1 utilizes a significant fraction of total cellular ATP. Its activity and expression are induced by glucose [2, 3]. Glucose induced phosphorylation of Pma1 in Ser-899, Thr-912 and Ser-911 [4, 5] correlated with increase in Pma1 catalytic activity. In glucose starved cells Pma1 is negatively regulated by Casein kinase I (Yck1p and Yck2p) phosphorylation [6], which is reduced in the presence of glucose. The mechanism of glucose-induced activation of Pma1 function is not clear at the present point in time; it is thought to involve phosphorylation of Ser-899 and Ser-911 and dephosphorylation of serine residues in Casein kinase I

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phosphorylation sites. Pma1 activation by glucose requires the participation of the glucose sensor Snf3p, the G-protein Gpa2p and transport and phosphorylation of glucose [7]. Pma1 expression induces oncogenic transformation of NIH 3T3 cells and causes increase in intracellular pH [8]. In recent years, Pma1 has attracted much attention as a probable target for the development of anti-fungal agents [9], its suitability as a biomarker for toxicant induced stress [10] and as a model for the study of membrane protein biogenesis [11].

Pma1 activity is negatively regulated by Hsp30 during exposure to heat shock conditions. Hsp30 thus contributes towards thermo-tolerance by lowering cellular ATP consumption during stress response [12]. *S. cerevisiae* Hsp30 is a plasma membrane heat shock protein. It has seven predicted trans-membrane domains and may belong to Family A of the G protein coupled receptor superfamily of proteins [13]. Hsp30 expression is induced during exposure to a variety of stress conditions including heat shock, exposure to weak organic acids, hyper-osmotic stress, oxidative stress, glucose limitation, exposure to alcohol and entry into stationary phase [14, 15]. Its role in providing resistance against multiple environmental stress conditions remains to be elucidated. *HSP30* expression is independent of Hsf1 and partially dependent on Msn2 and Msn4 [14]. Two other activators of *HSP30* expression namely Pci8 [16] and Sfl1p [17] have also been identified. It is part of the Pci8 induced stress regulon that is constituted of genes induced by various stress conditions.

In the present communication, we report on the regulation of Pma1 activity by dextrose and Hsp30 during exposure to thermal stress.

Materials and Methods

Growth Media, Growth Conditions and Strains

Unless otherwise stated yeast cells were grown at 30°C in YPD. *S. cerevisiae* strains used in the present study were BY4741 (*Mata his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), BY4741 *hsp30Δ::LEU2* (constructed in this laboratory) and BY4741 *hsp30Δ::kanMX* (Open Biosystems).

Exposure of Yeast Cells to Lethal Heat Stress and Cell Survival Assay

Log phase cultures of BY4741 or BY4741*hsp30Δ* in minimal media containing appropriate supplements were subjected to lethal heat stress at either 50°C for different intervals of time or left untreated at 30°C (control). Cells were then spotted in 10 fold serial dilutions followed by incubation at 30°C for 3 days before photography.

Isolation of Total Membrane Fraction

Total membrane fractions were isolated from 25 ml log phase cultures of BY4741 and BY4741*hsp30Δ* grown either at 30°C or following exposure to either 40°C for 30 min as described earlier [18, 19]. Briefly, cell lysates prepared by the glass bead method in buffer L [30 mM Tris–HCl (8.5), 5 mM EDTA, 1 mM PMSF, 1 μM Pepstatin and 6 μM Leupeptin] were clarified by centrifugation at 700 g for 10 min and the supernatant was centrifuged at 20,000 g for 30 min to precipitate the membrane fraction. The crude membrane fraction was suspended in buffer S [10 mM Tris–HCl (7.5), 0.2 mM EDTA, 0.2 mM DTT and 20% (v/v) glycerol]. For isolation of membrane fraction used to estimate Pma1 phosphorylation, BY4741 and *hsp30Δ* cells were grown to log phase in YPD containing 4% dextrose, cells were precipitated, washed in sterile water and incubated at 30°C in sterile water for 1 h [5] followed by growth in either 250 mM sorbitol (glucose starved) or 4% (w/v) dextrose for 1 h. Cells were then subjected to heat shock at 40°C for 30 min or left untreated at 30°C and membrane fractions were isolated from all the cultures as above except that all buffers contained 2 mM NaF and 1 mM ammonium molybdate in addition to all the other components.

Immunoblotting

Immunoblotting was performed as described earlier [20]. Anti-Pma1 (yN-20) Ab (Santa Cruz Biotechnology, Inc.), alkaline phosphatase conjugated anti Rabbit-IgG and anti Goat-IgG (Santa Cruz Biotechnology, Inc.), Monoclonal anti-phospho-serine Ab (abcam) and HRP conjugated anti-mouse IgG (Thermoscientific) were used in dilutions recommended by the manufacturer. Pma1p blots were developed with alkaline phosphatase substrate BCIP and NBT and phospho-serine blots were developed with HRP substrate.

Assay for Pma1-ATPase Activity

Pma1 ATPase activity in total membrane fraction was assayed as described in [21]. The assay was performed in the presence and absence of diethylstilbestrol—a specific inhibitor of Pma1-ATPase to determine the contribution of Pma1 towards total membrane ATPase activity. One unit of Pma1 ATPase activity generates 1 μmol Pi per min by hydrolysis of ATP.

Measurement of Extracellular Acidification

Log phase BY4741 and *hsp30Δ* cells were precipitated, washed with water and re-suspended in sterile water at a

concentration of 150 mg cells (wet weight) per 10 ml water. Dextrose or sorbitol was then added to final concentrations of 2% (w/v) and 250 mM respectively to the cell suspension and incubated at either 30 or 40°C; pH was measured at 1 min intervals by a pH meter [22].

Results and Discussion

To investigate the regulation of Pma1 function by dextrose and Hsp30 during exposure to heat shock conditions, we estimated Pma1 activity its protein levels and ser-phosphorylation status in membrane fractions isolated from BY4741 and *hsp30Δ* cells grown either in dextrose or in sorbitol at 30°C and following exposure to 40°C for 30 min.

Regulation of Pma1 H⁺-ATPase Activity

Pma1 ATPase specific activity was reduced in membrane fractions isolated from heat shocked BY4741 cells grown in dextrose media. The same was not reduced in *hsp30Δ* cells exposed to similar conditions (Fig. 1a). Thermal stress induced alterations in ATPase activity correlated with changes in extracellular pH. In agreement with decreased ATPase activity, BY4741 cells showed increased extracellular pH when incubated at 40°C (Fig. 1b). In contrast, the *hsp30Δ* strain showed a slight decrease in extracellular pH upon incubation at 40°C (Fig. 1c). Under conditions of dextrose starvation, Pma1 ATPase activity was low in BY4741 cells but increased in *hsp30Δ* cells (Fig. 1d). Extracellular pH of BY4741 and *hsp30Δ* cells in sorbitol did not show any alteration at either 30 or 40°C, which suggested that cellular H⁺

pumping activity, was reduced in dextrose-starved cells (Fig. 1e, f). Pma1 activity was hence regulated primarily by dextrose, which was further influenced by Hsp30 during exposure to thermal stress in BY4741 cells.

Regulation of Pma1 Protein Levels

Pma1 protein levels were down-regulated in BY4741 cells during exposure to heat shock conditions in media containing dextrose. In *hsp30Δ* cells, Pma1 protein levels were also reduced under similar conditions (Fig. 2a, b) but to a lesser extent. A comparison of the effect of dextrose on Pma1p levels in BY4741 and *hsp30Δ* cells indicated that its levels were reduced in the presence of dextrose in Hsp30⁺ cells but induced in *hsp30Δ* cells (Fig. 2; Table 1). Its levels were however higher in dextrose starved Hsp30⁺ cells compared to that in *hsp30Δ* cells under similar conditions.

Cellular Pma1p levels were hence controlled by a number of factors. It was regulated by thermal stress in both Hsp30⁺ and *hsp30Δ* cells, by dextrose availability in *hsp30Δ* cells and by Hsp30 during dextrose starvation.

Regulation of Pma1 Ser-phosphorylation

Total ser-phosphorylation of Pma1 as estimated by the ratio of phosphorylated Pma1 to total Pma1 protein was reduced in the presence of dextrose in both BY4741 and *hsp30Δ* cells (Fig. 3a, b). It was however higher in *hsp30Δ* cells at both 30°C and following exposure to heat shock conditions compared to Hsp30⁺ cells (Fig. 3) in dextrose media. Overall ser-phosphorylation of Pma1 was hence regulated primarily by dextrose that was independent of Hsp30.

Fig. 1 The effect of dextrose, heat shock and Hsp30 on Pma1 activity. **a** and **d** Pma1-ATPase specific activity in strains and conditions as indicated in the figure was determined by the method of Serrano (21). Average data $\pm$ SD are shown. * Indicates $P < 0.05$. Alteration in extra-cellular pH of BY4741 and *hsp30Δ* cells grown in dextrose [(b) and (c)] and in sorbitol [(e) and (f)] at 30°C (open squares) and 40°C (open triangles), experiments were performed as described in “Materials and Methods” section

Fig. 2 Regulation of Pma1p levels by dextrose, heat stress and Hsp30. **a** Pma1 protein levels were determined in 40 μ g membrane fraction isolated from strains and conditions as indicated in the figure by western blotting using anti-Pma1 Ab. The blot was initially used to detect ser-phosphorylation levels of Pma1 by anti-phospho-serine Ab, stripped and re-probed with anti-Pma1 Ab. *Lower panel* non-specific bands recognized by anti-Pma1 Ab was used as a loading control. **b** Densitometric scans of **a** using ImageJ software

Saccharomyces cerevisiae hsp30Δ cells were More Sensitive to Heat Stress in the Presence of 2-Deoxyglucose (2-dg)

Results presented above demonstrate that Hsp30 influenced Pma1 activity, protein levels and phosphorylation in the presence of dextrose. To examine possible relationships between glucose, Hsp30 and regulation of various aspects of Pma1 we determined if dextrose regulated other functions attributed to Hsp30. We determined cell survival of BY4741 and *hsp30Δ* cells in the presence and absence of 2-dg following an exposure to lethal heat stress at 50°C for different intervals of time. The *hsp30Δ* mutant was slightly

Fig. 3 Regulation of ser-phosphorylation of Pma1 by dextrose. **a** Ser-phosphorylation levels of Pma1 were determined in 40 μ g membrane fractions isolated from strains and conditions as indicated in the figure by immunoblotting using monoclonal anti-phospho-serine Ab. *Lower panel* a non-specific band recognized by the anti-phospho-serine Ab was used as a loading control. **b** Densitometric scans of **a** using ImageJ software

more sensitive to an exposure at 50°C as compared to BY4741 cells (Fig. 4a). The deletion mutant was however considerably more sensitive to 50°C in the presence of 2-dg even in YPD (Fig. 4b), thus indicating a requirement for dextrose utilization in *hsp30Δ* cells for survival during exposure to thermal stress. Taken together the results (Fig. 4a, b) indicated a relationship between Hsp30 and cellular glucose utilization, the later in turn regulates Pma1 activation.

In conclusion, results summarized in Table 1 demonstrate that, Pma1 activity and protein levels were reduced in *Hsp30*⁺ cells upon exposure to thermal stress in dextrose unlike that observed in *hsp30Δ* cells wherein Pma1 activity and protein levels were rendered unresponsive to heat

Table 1 Summary of results

Parameters measured	Environmental conditions							
	Dextrose				Sorbitol			
	BY4741		<i>hsp30Δ</i>		BY4741		<i>hsp30Δ</i>	
	30°C	40°C	30°C	40°C	30°C	40°C	30°C	40°C
% Pma1 specific activity	100	56.4	72.3	78.5	0.1	0.01	28.4	34
Pma1p levels ^a	100	68	110	92	106	114	80	70
Pma1-P: Pma1p ^a	100	77	126	98	183	177	168	187
Dextrose requirement during lethal heat stress	Necessary but not essential				Essential			

^a Based on densitometric scanning of Figs. 2a and 3a

Fig. 4 Cell survival of BY4741 and *hsp30Δ* cells following exposure at 50°C. **a** Log phase BY4741 and *hsp30Δ* cells were exposed to 50°C in minimal media for intervals of time as indicated in the figure and spotted in tenfold serial dilutions, plates were further incubated at 30°C for 3 days before being photographed. **b** Cell survival in the

presence of 2-dg: log phase BY4741 and *hsp30Δ* cells were spotted in tenfold serial dilutions on YPD plates containing 200 µg/ml 2-dg and either exposed to 50°C for 2 h or left untreated; cells were then allowed to grow at 30°C for 3 days before being photographed

shock treatment. The above suggested that Hsp30 was necessary for the regulation of different aspects of Pma1 during exposure to heat-shock conditions. We observed increased Pma1 activity in membrane fractions isolated from *hsp30Δ* cells grown in sorbitol compared to its wild type counterpart grown under similar conditions. The data suggest that Hsp30 inhibited Pma1 activity in dextrose-starved cells at 30°C and following heat shock treatment. A comparison of the effect of dextrose on various aspects of Pma1 (Table 1) indicate that Pma1 activity, protein levels and phosphorylation were regulated differently in *hsp30Δ* cells compared to Hsp30⁺ cells in response to dextrose. While dextrose regulation of Pma1 activity was quantitatively different in *hsp30Δ* cells compared to its wild type counterpart, the regulation of Pma1p levels and phosphorylation status by dextrose in *hsp30Δ* cells was qualitatively different compared to Hsp30⁺ cells. We hence concluded that the deletion of *HSP30* renders the above parameters refractory to regulation by dextrose.

Pma1 phosphorylation in ser residues was reduced in BY4741 cells following heat-shock treatment in both dextrose as well as in sorbitol media and in *hsp30Δ* cells in dextrose media. It did not show a similar decrease in *hsp30Δ* cells in sorbitol wherein Pma1 phosphorylation was increased upon heat shock treatment (Table 1). Pma1 activity is regulated by multiple phosphorylation events and its activation by glucose is thought to involve phosphorylation of Ser-899 and Ser-911 and dephosphorylation of serine residues in Casein kinase I phosphorylation sites. Our data on Pma1 phosphorylation is limited to net ser-phosphorylation of Pma1 under different conditions and does not reflect the effect of ser-phosphorylation of Pma1 on its activity. However, it demonstrates increased phosphorylation of Pma1 in *hsp30Δ* cells in the presence and

absence of dextrose as compared to Hsp30⁺ cells. The above suggests that ser-phosphorylation of Pma1 is regulated by Hsp30 in addition to its regulation by glucose.

Given the above along with the requirement of dextrose as a carbon source by *hsp30Δ* cells for survival during exposure to lethal heat stress, our study has been able to establish a functional relationship between glucose utilization, Hsp30 function and the regulation of Pma1 activity. Since the deletion of *HSP30* renders Pma1 activity, its protein levels and phosphorylation comparatively refractory to heat shock and dextrose availability, it is possible that Pma1p undergoes structural alterations in the absence of Hsp30 that makes it non-responsive to regulatory events. Hsp30 may thus act as a plasma membrane chaperone in *S. cerevisiae*.

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