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Characterization of dominant lethal mutations in the yeast plasma membrane H^+-ATPase gene

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Abstract Site-directed mutants of yeast ATPase were studied after introduction of mutant alleles into a yeast strain where these alleles were constitutively expressed and the expression of the wild-type chromosomal *ATPase* gene was turned off. One objection to this constitutive expression system was made apparent recently, as dominant lethal mutations are lost by gene conversion with the wild-type allele during the process. Here, the phenotypes of the mutant alleles, which were studied in a constitutive expression system, are re-evaluated under conditions in which these site-directed mutants are conditionally expressed. We show that 12 of 25 site-directed mutations previously described are actually dominant lethal alleles. In addition, we show that dominant mutant proteins interfere with transport of wild-type ATPase to the plasma membrane.

Key words: H^+-ATPase; Plasma membrane; Dominant lethal mutation; *Saccharomyces cerevisiae*

1. Introduction

The yeast plasma membrane H^+-ATPase from *Saccharomyces cerevisiae* is an electrogenic proton pump essential for nutrient uptake and intracellular pH regulation [1,2]. The yeast ATPase belongs to the large family of cation ATPases, which forms an aspartyl-phosphate intermediate during catalysis and is vanadate sensitive [3].

Site-directed mutagenesis has been used to study structure–function relationships of the yeast enzyme. This approach has allowed the identification of several residues of the enzyme essential for catalysis [4–8]. In those studies, the mutant ATPase gene was constitutively expressed from its own promotor in a yeast strain in which the expression of the wild-type chromosomal ATPase gene (*PMA1*) is under control of the inducible promotor of the *GAL1* gene [9]. To study the mutant ATPases, the cells were switched to glucose-containing medium in which the chromosomal wild-type *PMA1* gene is turned off and only the mutant form is expressed. A drawback of this constitutive expression system was pointed out recently in the work of Harris et al. [10] and Supply et al. [11]. These authors show that since the mutant gene is co-resident in the cell with a wild-type gene, a dominant lethal mutation can be lost during sequence shuffling by gene conversion with either *PMA1* or its unexpressed homologous gene *PMA2*. In fact, the mutant allele *pma1-D378N*, reported to render a functional ATPase in a previous study [4], was later shown to be an example of gene conversion [12,13]. It is therefore important to determine whether any of the other site-directed mutants previously studied are in fact dominant lethals.

Here, we have investigated the dominant lethality of previously generated site-directed mutants [4–8]. The phenotype of the mutant alleles was re-evaluated using a galactose-inducible expression system. Twelve of 25 mutants exhibited a dominant negative phenotype. Immunofluorescence staining of HA-tagged wild-type Pma1 shows that dominant lethal proteins hamper the transport of the wild-type ATPase to the plasma membrane.

2. Materials and methods

2.1. Yeast strains and growth media

Saccharomyces cerevisiae strain XZ611 is Gal$^+$ *MAT*a *ura3 leu2 trp1* [13] and was used to determine the dominant lethality of *pma1* genes. Autodiploid W3O3, obtained from W3O3-1B (Gal$^+$ *MAT*α *ade2 ura3 his3 leu2 trp1 can1*) [14] by transformation with the *HO* gene [15], was used in immunofluorescence experiments. Synthetic media with 2% dextrose (SD), 2% lactic acid (SL) or 2% galactose (SG) and the appropriate requirements were used [16]. Yeast cells were transformed using the lithium acetate procedure [17], and transformants selected in SD medium. To test the dominant lethality of the *pma1* alleles, transformants were transferred to SL medium and, after growth, suspended in water to a cell density of 2×10^7 cell/ml and 5 μl were dropped on SG.

2.2. Plasmids

The *pma1* genes containing different site-directed mutations were placed under control of the galactose-inducible promoter by subcloning either an *Eco*RV–*Bst*EII fragment (D143N→S234A mutations) into the plasmid pSN107 [13] or an *Bst*EII–*Hind*III fragment (R271T→T918A mutations) into the pRS427 [18]. All *ATPase* gene fragments containing the mutations were liberated from the corresponding *pma1* genes cloned into the plasmid pUC18. The construction of these *pma1* alleles has been described [4–7]. All the *pma1* alleles used in this study were re-sequenced to ensure that only the desired mutation was present. The plasmids pSN107 and pRS427 are derivatives of the *URA3* single-copy plasmid YCp50 and contain the entire *PMA1* gene joined to a galactose-inducible promoter. Both plasmids are identical except that the *URA3* gene of pSN107 was deleted and replaced by a new *URA3* gene lacking its *Eco*RV site and inserted into the 3′ end of *GAL1::PMA1* [13]. This modification facilitated subcloning of the *Eco*RV–*Bst*EII fragment of the mutant alleles. After the dominant lethality test, each plasmid was rescued from yeast and its corresponding *pma1* alelle sequenced to confirm the presence of the mutation. Plasmid pFP239 is a derivative of the *LEU2* single-copy plasmid pSB32 [19] and expresses an HA-tagged wild-type *PMA1* under the control of the galactose-inducible promoter. The nine-amino-acid hemagglutinin (HA) epitope was introduced after the second amino acid of the ATPase by site-directed mutagenesis (F. Portillo, unpublished data). This insertion has no deleterious effects on function [13] or regulation (F. Portillo, unpublished data) of the ATPase.

2.3. Fluorescence microscopy

Strain W3O3 was transformed with pFP239 and either pSN107 or pRS427 derivatives containing different dominant lethal mutations. The transformants were selected in SD medium. To induce expression of *GAL1::pma1* genes, cells were cultured in SL medium overnight at 30°C, collected, resuspended in SG medium and cultured for 5 h at 30°C. Cells were fixed and stained for immunofluorescence as described [20]. Localization of HA-tagged Pma1 was determined by

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immunofluorescence using a rhodamine-conjugated anti-HA antibody (Boehringer-Mannheim, Mannheim, Germany).

3. Results

3.1. Galactose-dependent lethality of pma1 mutations

A direct demonstration of the dominant lethal effect of several *pma1* mutations was obtained by transforming a wild-type strain with a centromeric plasmid carrying the mutant *ATPase* genes under the control of the *GAL1* promoter. Under these conditions, cells carrying a dominant negative allele arrest growth when they are transferred from glucose- to galactose-containing medium. An example of the growth pattern of yeast strains carrying representative dominant lethal alleles under the control of the galactose-inducible promotor is shown in Fig. 1. The results of this test for dominant lethality, together with the previously described phenotype for a number of different in vitro-generated mutations [4–8], are presented in Table 1. The *pma1-D378N* and *-K379Q* genes were described previously as dominant negative [12,13] and were included in this study as controls. All the 12 dominant negative allele genes identified in this work continued to exhibit dominant lethality when the wild-type gene was also expressed from the GAL promotor (data not shown), suggesting that lethality is not dose-dependent. It is interesting to note that, as expected, some dominant negative alleles (*pma1-D143N*, *-R271T*, *-C376L* and *-D378N* genes) exhibited a wild-type phenotype in the constitutive expression system, confirming that lethal mutations were lost by gene conversion. However, most lethal alleles exhibited either a recessive lethal (*pma1-D200N*, *-E233Q*, *-P335A*, *-D378E*, *-D378T* and *-D638N* genes) or recessive mutant (*pma1 -K379Q*, and *-K474R* genes) phenotype when constitutively expressed. This suggests that dominant lethality, as well as being lost by gene conversion, can also be suppressed by second-site mutations. In fact, second-site suppressor of the *K474R* dominant lethal allele have been recently obtained [21] which constitutes a direct evidence for this mechanism. It remains to be explained why some dominant lethal mutations are suppressed by gene conversion, while others are suppressed by secondary mutations.

Table 1
Dominant lethality of *pma1* genes

pma1 genes	Reported phenotype[a]	Dominant lethality
E129Q	wild type	–
D143N	wild type	+
D200N	recessive lethal	+
D226N	recessive mutant	–
T231G	recessive mutant	–
E233Q	recessive lethal	+
S234A	recessive mutant	–
R271T	wild type	+
P335A	recessive lethal	+
C376L	wild type	+
D378N	wild type	+
D378T	recessive lethal	+
D378E	recessive lethal	+
K379Q	recessive mutant	+
K474R	recessive mutant	+
K474H	recessive mutant	–
K474Q	recessive lethal	–
D638N	recessive lethal	+
N848D	wild type	–
Q908E	wild type	–
R909I	recessive mutant	–
S911A	wild type	–
S911A/T912A	recessive lethal	–
T912A	recessive mutant	–
T918A	wild type	–

[a]Data are from references [4,8].

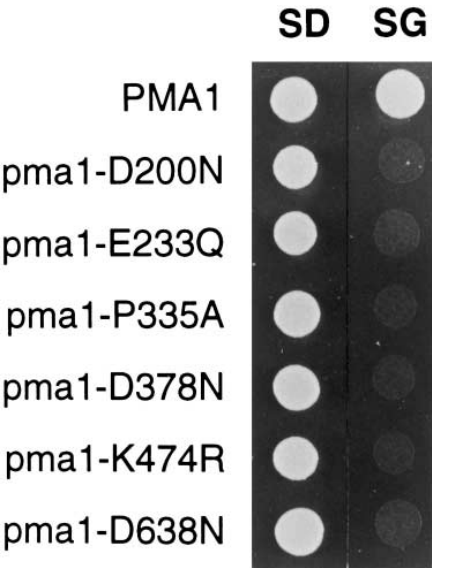

Fig. 1. Drop test for growth of yeast strains expressing dominant lethal alleles of PMA1. Strains are recombinant, carrying the *PMA1* wild-type allele or the indicated *pma1* gene under the control of the galactose-inducible promotor. The test was performed as indicated in Section 2. Plates were photographed after 36 h at 30°C.

3.2. Dominant lethal mutations interfere with the targeting of wild-type Pma1

With multimeric proteins, as may be the yeast ATPase [22], a mutant protein might be dominant negative by creating, in association with the wild-type protein, an inactive or unregulated oligomeric complex unable to perform its function. This would lead to lethality, since ATPase function is essential. Another possibility is that the mutant protein might interfere with wild-type protein transport to the membrane, decreasing the amount of correctly localized essential protein and arresting cell growth. This latter possibility has been recently shown to be true for several dominant negative *Pma1* mutants whose expression results in the trapping of the wild-type protein in subplasma membrane structures which also contain the endoplasmic reticulum marker protein Kar2 [23].

To test whether the dominant lethal alleles identified here also interfered with wild-type Pma1 targeting to the plasma membrane, we have investigated the transport of wild-type ATPase to the cell surface when dominant negative alleles are also expressed. To this end, we introduced the HA epitope in the N-terminus of the wild-type Pma1 protein and both the HA-tagged and dominant lethal genes were co-expressed under the control of the *GAL1* promotor in the same cell. The location of the wild-type HA-tagged protein was studied in galactose-cultured cells by immunofluorescent staining of wild-type Pma1 with the anti-HA monoclonal antibody (Fig. 2). When HA-tagged wild-type protein was induced simultaneously with a dominant lethal protein, the anti-HA antibody decorated a set of cytoplasmic structures identical to those observed for other dominant negative Pma1 proteins [13]. These cytoplasmic dots were not observed when the HA-

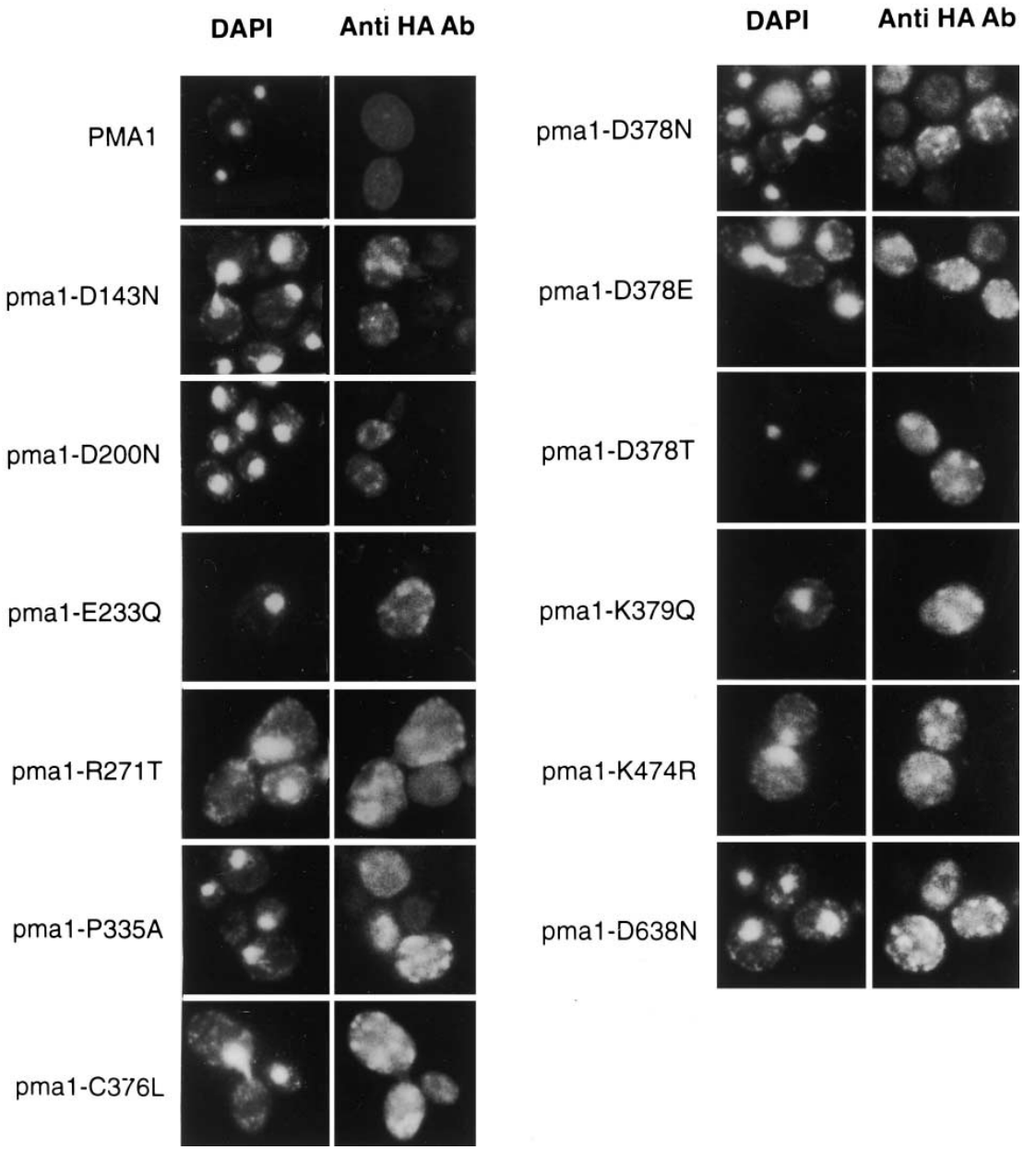

Fig. 2. Accumulation of wild-type HA-tagged Pma1 in cells expressing dominant lethal alleles. Transformants carried the HA-tagged *PMA1* gene and either the wild-type or the indicated dominant lethal allele under the control of the galactose-inducible promotor. Expression of the *GAL::pma1* genes was induced with galactose for 5 h. Panels show staining of the nucleus (DAPI) or immunofluorescence using a rhodamine-conjugated anti-HA antibody (Anti-HA Ab).

tagged gene was induced simultaneous with a wild-type protein. When only an HA-tagged dominant negative Pma1 protein was expressed in the cell, the same staining of cytoplasmic dots was also observed (data not shown). These results suggest that the dominant lethality of all 12 *pma1* alleles examined is due to the interference of the mutant proteins with wild-type Pma1 transport which results in the entrapment of the wild-type protein in some part of the secretory apparatus.

4. Discussion

Using site-directed mutagenesis in combination with a constitutive expression system of the mutants [4–8], a specific role for several amino acids in the catalysis and regulation of the H^+-ATPase had previously been assigned. In the light of the findings described here, the specific role of Asp-143, Arg-271, Glu-233, Cys-376 and Asp-638 residues in the the proposed model of structure-function of the ATPase [8] must be reconsidered.

Amino acids Asp-143 and Arg-271 were described as non-essential for ATPase as D143N and R271T mutations had no noticeable effect on ATPase activity [4,5]. Nevertheless, the fact that D143N and R271T mutations are here shown to be dominant lethal alleles suggests that Asp-143 and Arg-271 residues could be essential for the enzyme.

With respect to Cys-376, the fact that the C376L mutation results in a dominant negative protein suggests that this residue is essential for the enzyme. Nevertheless, the recent re-

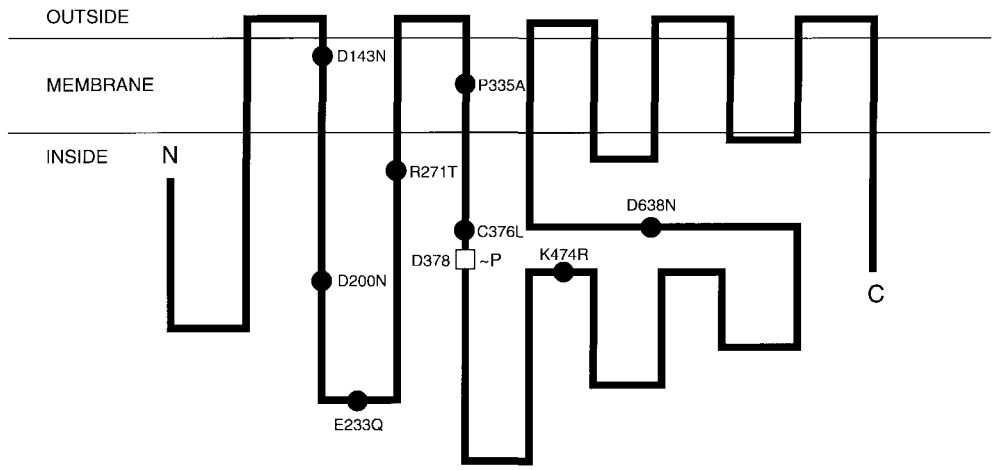

Fig. 3. Location of H^+-ATPase mutants displaying dominant lethality. Phosphorylation site (Asp-378) and positions of the dominant lethal mutations found in this work are indicated within the proposed structural topography of the H^+-ATPase as determined from hydropathy analysis [6,26].

port by Petrov and Slayman [24] that Cys-376 can be replaced by Ala with no significant loss of activity indicates that Cys-376 is not required for enzyme activity. An explanation for these results may be that the introduction of the more bulky Leu residue adjacent to Asp-378 could interfere, due to a steric effect, with the formation of the phosphorylated intermediate and result, as for the D378N mutation, in a dominant negative protein.

The Glu-233 residue was proposed to form part of a phosphatase domain, as the E233Q mutation blocked hydrolysis of the phosphorylated intermediate [4]. Amino acid Asp-638 was suggested to be involved in ATP binding to the adenine ring since the D638N mutation altered the enzyme's nucleotide hydrolysis specificity [4]. The fact that E233Q and D638N mutations result in dominant negative proteins suggests that these two amino acids must be important for enzyme function although a specific role cannot be assigned.

Concerning to the non-dominant lethal mutations described in this study (E129Q, D226N, T231G, S234A, K474H, K474Q, N848D, Q908E, R909I, S911A, S911A/T912A, T912A and T918A) it cannot be completely discarded that the previously described phenotype obtained using the constitutive expression system results from gene conversion or reversion. Because a functional H^+-pump is essential, there is strong selective pressure for the accumulation of revertants during growth of cells harboring variants with low activity. Work in progress using an alternative expression system will help to elucidate the phenotype of the non-dominant lethal mutations described here.

To date, dominant lethal mutations have been restricted to the phosphorylation site, to the first two putative transmembrane helices of the ATPase [12,13] and to the fifth putative membrane span [25]. Six of the dominant mutations described here were found to be located in three new regions of the enzyme (Fig. 3): three (D200N, E233Q and R271T mutations) in the small hydrophilic loop between hypothetical membrane segments 2 and 3, an additional two (K474R and D638N mutations) within the predicted nucleotide binding loop and the last (P335A mutation) in the putative fourth transmembrane helix.

Futher work involving isolation of second-site suppressor mutations will help to understand the mechanism of dominant lethality exhibited by the *pma1* allele described here and will allow assignation of a specific role for the essential residues.

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