PMA1, A P-TYPE PROTON ATPASE, IS A DETERMINANT OF CHRONOLOGICAL LIFESPAN IN FISSION YEAST

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Chronological lifespan is defined by how long a cell can survive in a non-dividing state. In yeast, it is measured by viability after entry into the stationary phase. To date some factors affecting chronological lifespan have been identified, however molecular details of how these factors regulate chronological lifespan has not yet been elucidated clearly. Because lifespan is a complicated phenomenon and is supposedly regulated by many factors, it is necessary to identify new factors affecting chronological lifespan in order to understand lifespan regulation. To this end, we have screened for long-lived mutants and identified that Pma1, an essential P-type proton ATPase is one of the determinants of chronological lifespan. We presented that partial loss of Pma1 activity not only by mutations but also by treatment with the Pma1-inhibitory chemical vanadate resulted in the long-lived phenotype in Schizosaccharomyces pombe. These findings suggest a novel way to manipulate chronological lifespan by modulating Pma1 as a molecular target.

In the natural environment, most microorganisms exhibit only brief periods of rapid growth. Nutrient starvation is the most common natural situation, so the ability to adapt to nutrient limitation is crucial for microorganisms. Cells respond to starvation by ceasing growth and entering the stationary phase differentiate in ways that allow them to maintain viability for extended periods in the absence of nutrients (1). In yeast, the period in which the cells keep their viability after entry into the stationary phase is recognized as the chronological lifespan (2).

In S. cerevisiae, two of the major pathways that control chronological lifespan have been identified: the Ras / PKA / Msn2/4 pathway and the Sch9 pathway (3,4). The down-regulation of either pathway promotes lifespan extension. Importantly, similar pathways (insulin/IGF-I-like) regulate longevity in higher eukaryotes, suggesting a common evolutionary origin for the life span-regulatory mechanisms (5,6).

In S. pombe, the Pka1 and Sck2 are regulators of chronological lifespan; each mutant shows a long-lived phenotype, and the pka1 and sck2 double mutant displays an additive effect on chronological lifespan extension, suggesting that these two factors regulate related but independent pathways (7). We determined previously that the mutant of lcf1+, which encodes a long-chain fatty acyl-CoA synthetase, showed rapid loss of viability after entry into the stationary phase, and suggested that fatty acid utilization and/or metabolism is important to determine viability in the stationary phase in fission yeast (8,9). Recently, we have identified a novel gene, ecl1+ which extends chronological lifespan of S. pombe when over-expressed (10). In addition, we also identified two paralogs and an ortholog of ecl1+ in S. pombe and S. cerevisiae, respectively (11,12). Based on these observations, we proposed that Ecl1 family proteins are novel regulators for chronological lifespan in yeast. Ecl1 family proteins seem to be specific in fungi groups and molecular
mechanism responsible for the regulation of lifespan has not yet been clarified.

Because lifespan is supposed to be regulated by many factors complicatedly and important factors for lifespan regulation were conserved in eukaryote, it is necessary to identify new conserved factors affecting chronological lifespan for understanding lifespan regulation (13). To accomplish this, we have screened and identified a mutant that extends chronological lifespan of S. pombe.

**Experimental Procedures**

*Strain and media*- S. pombe strain JY333 (h’ leu1-32 ade6-M216) was used for mutant screening. Strains were grown in SD medium (0.67 % yeast nitrogen base without amino acids [Difco], 2 % glucose) supplemented with necessary growth requirements in standard amounts at 30 °C. The analysis of viability was carried out as described previously (10).

*Mutation mapping*- To identify the mutation in L18 mutant, whole genome tiling array of S. pombe was constructed and used to map the L18 mutation. This analysis (Nandemo-Arei analysis) was carried out by GeneFrontier Inc.

*Insertion of Km-resistant marker downstream of pma1+ gene*- To carry out linkage analysis, Km-resistant marker was inserted at 1824 to 1835 bp downstream of termination codon of pma1+ gene by the methods described (14). Both the upstream and downstream regions of the desired insertion region were PCR-amplified by using F1 and F2 primers and R1 and R2 primers, respectively, and both fragments were purified. After mixing both DNA fragments with pFA6a-kanMX6, a PCR reaction was performed with the F1 and R1 primers. JY336 (h’ leu1-32 ade6-M210) was transformed with the amplified DNA fragment, and stable G418-resistant transformants were selected. Then, the construct on the chromosome was confirmed by PCR using appropriate primers. The primers used were: F1, AGAAGTTATCGTGAGCTACG; F2, TTAATTACCCGGGATCCGGAATCAT TGAATTATCTATATAC; R1, GTCCTTGCTCGTGATCAACG; and R2, GTTTAAACGAGCTCGAATTCCATGGATA AGCTGCTAATCCATAAT.

*Preparation of Pma1 antibody*- Antibody for Pma1 was prepared by immunizing rabbit with a peptide, MMNGKPKESRNQRSIEDL which corresponds from 886 to 903 rd amino acid of Pma1 protein and purchased from Sigma-Aldrich.

*Pma1 ATPase assay*- ATPase assay was carried out essentially as described (15). The assay was carried out with or without 0.1 mM sodium vanadate, and the released inorganic phosphate was determined using the Phospha C-Test (Wako Co. Japan). The vanadate-sensitive ATPase activity was determined and expressed as Pma1 activity.

*Assay of glucose concentration*- Cells were grown in SD medium, and 20 ml each of culture was sampled along cell growth and the concentration of glucose in medium was assayed by using a kit, Glucose CII-test wako (Wako Co. Ltd).

**RESULTS AND DISCUSSION**

*Isolation of mutant that keeps viability after entry into the stationary phase.* To isolate long-lived mutants, the wild type-cells (strain JY333) were grown in SD liquid medium for 7 days and aliquot of them was transferred into the same fresh medium. It was known that the viability of wild type cells decrease to 1/10000 within 5 days in SD medium, so we repeated the 7 days incubation 5 times (8,10). From this old culture, we isolated one spontaneous mutant, named L18, which shows increase in viability after entry into the stationary phase. As shown in Fig.1A, wild type cells decreased its viability to 1/1000 within 3 days incubation. On the other hand, the L18 mutant kept its viability for a long period compared to wild type cell. It should be noted that the calculated doubling time at logarithmic growth phase of L18 and JY333 were identical, that is 209 min and 205 min respectively, under this growth condition. Next, to confirm that L18 mutant increases chronological survival under other growth condition, we analyzed the viability of the cells in water. Cells grown in SD medium until logarithmic growth phase of L18 and JY333 were identical, that is 209 min and 205 min respectively, under this growth condition. Next, to confirm that L18 mutant increases chronological survival under other growth condition, we analyzed the viability of the cells in water. Cells grown in SD medium until logarithmic growth phase (OD600 =1) were collected and then suspended in water. As shown in Fig. 1B, the viability of the L18 mutant was higher than that of wild type cells. Based on these results, we concluded that L18 is...
a chronologically long-lived mutant of *S. pombe*.

**Phenotypic characterization of L18 mutant.**  
To understand why L18 mutant showed long-lived phenotype, cell morphology was monitored along cell growth in SD medium. As shown in Fig. 1C, there was no difference in cell morphology between wild type and L18 mutant at the logarithmic growth phase. However after entry into the stationary phase, wild type cells showed abnormal morphology such as broken or shrunked figures. On the other hand, the L18 mutant showed normal morphology though the cells became a little bit longer. This phenotypic difference in morphology might be reflected in the different viability after entry into the stationary phase.

In *S. cerevisiae*, it has been reported that chronological long-lived mutants exhibit oxidative stress-resistant phenotype (3). Previously, we also have presented data that cells over-expressing Ecl1 family proteins, such as Ecl1, Ecl2 and Ecl3, were resistant to oxidative stress (10-12). Hence we analyzed oxidative stress sensitivity by spotting cells on a plate containing H2O2. As shown in Fig. 2A, L18 mutant cells were more resistant to oxidative stress (5mM H2O2) than wild type cells. Because the catalase encoded by *ctt1* gene was known to protect cells from the toxic effects of H2O2, we monitored the expression of *ctt1*-mRNA. As shown in Fig. 2B, *ctt1*-mRNA was highly expressed in L18 mutant compared to wild type cells, that is in good agreement with the phenotype. It is known that *ctt1* is controlled by transcription factors, Prr1 and Pap1 (16). Prr1 is a response regulator that is involved in the His-Asp phosphorelay system. Pap1 is controlled by the stress-activated MAP kinase, Sty1 that responds to a range of environmental stresses. So, L18 mutant may cause stress signals that activate the Sty1 MAP kinase and/or His-Asp phosphorelay system to induce the *ctt1* expression through Pap1 and/or Prr1, respectively. We analyzed other phenotypes such as sensitivity against high osmolarity (2M sorbitol) and heat stress (37℃), however no difference was found between wild type and L18 mutant (data not shown).

**Identification of mutation site in L18 mutant.**  
To identify the mutation in L18, the whole genome mutation mapping methods was adopted using tiling arrays of *S. pombe*. The result suggested that one mutation arose at nucleotide position 3873842 to 3873898 on chromosome 1 of L18 mutant. This region corresponded to the coding sequence of *pma1* gene which encodes P-type proton ATPase. Then we sequenced *pma1+* gene of L18 and identified C to A missense mutation that causes Ala-270 to Asp substitution (Fig. 3A and 3B). We named this mutation allele as *pma1-L18*. We then confirmed whether *pma1-L18* mutation is responsible for the chronologically long-lived phenotype of L18 as follows. The Km-resistant cassette was inserted in the chromosome at 1824 to 1835 bp downstream from termination codon of wild type *pma1* gene and then the Km-resistant strain was mated with L18 mutant to analyze the linkage between Km-resistant phenotype and chronologically long-lived phenotype. The phenotype of progeny after crossing revealed that the 100% of chronologically long-lived cell (n=20) was Km-sensitive, indicating that the *pma1-L18* mutation and the locus in which the Km-resistant cassette was inserted were closely located on the chromosome. Next we cloned wild type *pma1* gene on plasmid and introduced into L18 mutant and confirmed that the long-lived phenotype of L18 was partially complemented (data not shown). Moreover, after crossing of L18 mutant with wild type strain, we randomly isolated both long-lived cells (n=4) and non-long-lived cells (n=4), and sequenced their chromosomal regions corresponding to *pma1-L18* mutation. We confirmed that all of long-lived cells had *pma1-L18* mutation and all of non-long-lived cells had wild type *pma1* allele. Based on these results, we concluded that the *pma1-L18* mutation is the causative mutation that confers the long-lived phenotype.

**Analysis of ATPase activity of L18 mutant.**  
Pma1 is an essential P-type ATPase in plasma membrane and functions as hydrogen ion pump (17,18). To characterize the nature of Pma1-L18 protein, H⁺-ATPase activity of L18 mutant was analyzed. Whole cell extract was prepared, then H⁺-ATPase activity was assayed with or without vanadate. Because plasma membrane H⁺-ATPase is sensitive to vanadate, we estimated the vanadate-sensitive H⁺-ATPase
activity as plasma membrane H⁺-ATPase activity. Cells were grown to mid logarithmic phase (OD600 =1) and to stationary phase (OD600 =2) and the H⁺-ATPase activities were analyzed. As shown in Fig. 3C, the H⁺-ATPase activity of L18 mutant was about one half of that of wild type at both growth phases. In *S. cerevisiae*, it is known that the activity of Pma1 H⁺-ATPase is regulated by glucose, that is, when glucose was added to carbon-starved cells the ATPase activity increased (19,20). Then we analyzed the *S. pombe* Pma1 activity in response to glucose concentration. Wild type and L18 mutant cells were grown in SD medium until logarithmic growth phase, and then transferred into SD medium containing various concentration of glucose. Cells were grown in each medium for 90 min and H⁺-ATPase activity was analyzed. As shown in Fig. 3D, H⁺-ATPase activity of wild type cells seemed to be regulated by glucose. That is, the ATPase activity that is low in the medium containing no glucose increased in response to glucose concentration. In L18 mutant, the activity also increased in response to glucose. However, the activity was lower than that of wild type in medium containing 0.5 to 2% glucose. These results suggested that Pma1-L18 protein has a defect in the activity but not in the regulation by glucose. Taken together we concluded that the L18 mutant has low H⁺-ATPase activity.

Expression profile of pma1+ mRNA and Pma1 protein. To know the expression profile of pma1+ gene, wild-type and L18 mutant cells were grown in SD medium, and pma1+ mRNA level was analyzed at several points during cell growth by Northern hybridization. As shown in Fig. 3E the expression of pma1+ mRNA was constant during analyzed periods and there is no obvious difference between L18 mutant and wild type. Next, we analyzed the amount of Pma1 protein by Western blotting under the same growth conditions. As shown in Fig. 3E, very similar amounts of the Pma1 protein were expressed in both wild type and L18 mutant. Based on these analyses, we concluded that there is no difference in the amount and stability of Pma1 protein expressed in L18 and wild type cells. This means that the specific activity for H⁺-ATPase of Pma1-L18 protein is lower than that of wild type Pma1 protein.

pma1-L18 mutant consumed less glucose. We next addressed the question why pma1-L18 mutation extends chronological lifespan. Previously, it was reported that in *S. pombe* pma1 mutant which has defect in ATPase activity, the rate and extent of extracellular acid secretion is lower compared to the wild type cells (13). So, we supposed that the difference of acidification of the growth medium might explain the phenotype of L18. But this was not the case. We analyzed the pH change of culture medium at several points during cell growth. As shown in Fig. 4A, pH decreased along cell growth but there was no obvious difference between wild and L18 mutant.

Another possibility was the defect in nutrient (glucose) uptake in L18 mutant. In *S. cerevisiae*, Pma1 H⁺-ATPase is responsible for H⁺-dependent nutrient uptake (20). Pma1 ATPase acts physiologically to pump protons out of the cell, creating the electrochemical gradient that drives solute uptake by an array of H⁺-coupled co-transporters. In *S. pombe*, glucose uptake was described to be energy dependent, driven by the plasma membrane ATPase-generated electrochemical gradient (21). Then we compared the consumption of glucose along cell growth between wild-type and L18 mutant (Fig. 4B). It was revealed that L18 mutant consumed less glucose compared with wild type cells. The difference in glucose consumption is likely to be caused by the difference in the Pma1 activity that provides energy for glucose uptake. Based on these observations, we propose that the L18 mutant suffers somewhat glucose-limiting condition, so the physiology of L18 mutant has been changed to adapt to this condition.

In *S. pombe*, chronological aging is accelerated by glucose signaling; cells bearing mutations in genes controlling this pathway, such as *pak1* and *sck2*, live longer (7). In *S. cerevisiae*, deletion of the genes *SCH9*, *CYR1*, and *RAS2*, which mediate glucose signaling, extends the chronological lifespan (4,22). To investigate the relationship between pma1-L18 mutation and glucose signaling in more detail, we analyzed cell viability in a glucose-limiting medium. The analysis of viability in a glucose-limiting medium is known as a calorie-restriction experiment, and calorie...
restriction is a condition that extends lifespan in a variety of species (23,24). As shown in Fig. 5A and 5B, calorie restriction extended the viability of both wild-type and L18 mutant cells. Under the calorie restriction condition, there was little difference in viability between wild-type and L18 mutant.

Based on these results, we propose the following scenario explaining the long-lived phenotype of L18 mutant. In L18 mutant, the reduced Pma1 activity causes some defect in glucose uptake. This might cause physiological changes that are equivalent to the changes caused by calorie restriction. In this study we presented evidence indicating that Pma1 is a factor affecting chronological lifespan. This highlights the new possibility for the regulation of chronological lifespan by modulating Pma1 activity. Then we tried to reduce Pma1 activity by adding vanadate, an inhibitor of P-type ATPase. If Pma1 activity was reduced by vanadate, it was supposed that the chronological lifespan was extended. This was the case as shown in Fig. 5C. When wild type cells were grown in medium containing various concentration of vanadate, the cell viability increased in the concentration dependent manner (Fig. 5C). On the other hand, the viability of L18 mutant was not affected by vanadate (Fig. 5D).

In summary, we presented evidence showing a link between Pma1 and lifespan for the first time. This suggested that the Pma1 ATPase activity is a crucial for the determination of chronological lifespan in S. pombe and implied that the chronological lifespan could be manipulated by modulating Pma1 activity in other organisms. Verification of the possibility for this novel way to regulate lifespan is waiting in the future.

REFERENCES


**FOOTNOTES**

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**FIGURE LEGENDS**

**Fig. 1.** Phenotypes of mutant that increases cell viability after entry into the stationary phase. A, B. Cell growth (upper panels) and cell viability (lower panels) of wild type cells (open circles) and L18 mutant (closed circles) in SD medium (panel A) and in H2O (panel B) were monitored. Data shown represent the average of three independent experiments ±s.d.. C. Cell morphology at various growth phases. Wild type cells (open circles) and L18 mutant (closed circles) were sampled at each time points (a to c) indicated in growth curve and subjected to microscopic observation.

**Fig. 2.** L18 mutant is resistant to oxidative stress. A. Wild type cells and L18 mutant were spotted on SD plate or SD plate containing 5 mM H2O2 with serial dilution. The plates were incubated for 4 days at 30 °C and photographed. B. Total RNAs were isolated from wild type and L18 mutant cells grown in SD medium until mid-log phase and subjected to Northern blotting analysis with radiolabeled *ctt1* probe.

**Fig. 3.** L18 has a mutational lesion in *pma1*+ gene that causes decrease in Pma1 ATPase activity. A. *pma1* gene of L18 mutant was sequenced and an identified mutation (C to A substitution) was shown with arrowhead. B. Schematic representation of topology of Pma1 protein in plasma membrane. The mutation site (Ala-270 to Asp) was indicated with a star. C. Pma1 activities in wild (open bar) and L18 mutant (filled bar) were assayed using cell lysate prepared from cells grown in SD medium until OD600 =1 or 2. Data shown represent the average of three independent experiments ±s.d.. D. Pma1 activities in wild (open bar) and L18 mutant (filled bar) were assayed using cell lysate prepared from cells grown in SD medium containing indicated concentration of glucose. E. Expression profile of Pma1 along with growth. Wild type and L18 mutant were grown in SD medium at 30 °C. Total RNAs were isolated from cells at each OD indicated and subjected to Northern blotting analysis with radiolabeled *pma1*+ probe (upper panel). The ethidium bromide stained gel showing total RNA is presented for loading control (middle panel). Wild type and L18 mutant were grown as described above, and cell lysate was prepared. Amount of Pma1 protein was analyzed by Western blotting using anti Pma1 serum (lower panel).

**Fig. 4.** pH change and consumption of glucose in the growth medium. A. Cells were grown in SD medium at 30 °C and change of pH in the growth medium of wild type (open circles) and L18 mutant (closed circles) were analyzed. The growth of wild type (open triangles) and L18 mutant (closed triangles) were monitored at OD600. B. Cells were grown in SD medium at 30 °C and concentration of glucose in the medium of wild type (open triangles) and L18 mutant (closed triangles) were analyzed. The growth of wild type (open circles) and L18 mutant (closed circles) were monitored at...
Fig. 5. Effect of calorie restriction and vanadate, a Pma1 inhibitor, on cell viability after entry into the stationary phase. A, B The effect of calorie restriction. Wild-type cells and L18 mutant were grown in SD medium (2% glucose) or SD low-glucose medium (0.5% glucose). Cell growth (panel A) and viability (panel B) were monitored. The data were shown with symbols as follows; wild-type cells in SD medium (open circles), wild-type cells in SD low-glucose medium (open triangles), L18 mutant in SD medium (closed circles), and L18 mutant in SD low-glucose medium (closed triangles). Data shown represent the average of three independent experiments ±s.d.. C, D. The effect of vanadate. Wild-type cells (panel C) and L18 mutant (panel D) were grown in SD medium (open circles) or SD medium containing 100 mM (closed circles) and 300 mM (open triangles) vanadate and cell viability was monitored. Data shown represent the average of three independent experiments ±s.d..
Figure 2
Figure 3
Figure 4

A

B

Growth (OD600)

Time (hr)

Od600

Time (hr)

pH (---)

Time (hr)

Glucose conc. (mg/ml)

Time (hr)
Figure S

A

B

C

D

Growth (OD600)

Viability

Viability

Viability

Time (hr)

Time (hr)

Time (hr)

Time (hr)
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