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MUTATIONS AT THE PUTATIVE JUNCTION SITES OF THE YEAST VMA1 PROTEIN, THE CATALYTIC SUBUNIT OF THE VACUOLAR MEMBRANE H+-ATPASE, INHIBIT ITS PROCESSING BY PROTEIN SPLICING

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Summary: A single gene, *VMA1*, encodes the 69-kDa subunit of the vacuolar membrane H+-ATPase in the yeast *Saccharomyces cerevisiae.* We have proposed that the subunit is synthesized as a precursor of 120 kDa (1,071 amino acids) and then converted to the 69-kDa form by an unusual processing reaction, which removes the internal domain of 454 amino acids (residues 284-737) and joins the N- and C-terminal domains. Cysteine to serine mutations at residues 284 and 738, the residues that bracket the internal domain, were introduced into the *VMA1* gene by site-directed mutagenesis, and the mutant genes were expressed in a null *vma1* mutant. Cells harboring either of the mutant *vma1* genes accumulate nonfunctional fragments of the subunit. The mutation of Cys-284 inhibited the cleavage of the N-terminal junction site. Cys-738→Ser mutation appeared to block the processing at both junction sites although the mutant gene yielded a small fraction of the functional 69-kDa subunit.

The vacuolar membrane H+-ATPase of the yeast *Saccharomyces cerevisiae* is composed of at least eight polypeptides with apparent molecular masses of 120-, 69-, 60-, 42-, 36-, 32-, 27-, and 17-kDa (1-4). The 69-kDa subunit, which has a catalytic site for ATP hydrolysis (3), is encoded by a single gene, *VMA1* (5). The gene contains an uninterrupted open reading frame, which is capable of encoding a polypeptide of 120 kDa (1,071 amino acids). The predicted amino acid sequence of the subunit shows high similarity to those of the "70-kDa" subunits of carrot and *Neurospora* vacuolar ATPase (5). However, the yeast sequence contains a nonhomologous region of 454 amino acids, which is inserted between the N- (residues 1-283) and C-terminal (residues 738-1,071) homologous regions. We have proposed that a novel processing mechanism [refered as "protein splicing" in (6)] operates on the *VMA1* gene product, which involves a posttranslational excision of the nonhomologous domain and a ligation of the N- and C-terminal homologous domains (5).

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Recently, Kane *et al.* (6) demonstrated that antibodies raised against a synthetic oligopeptide of the nonhomologous domain does not react with the ATPase subunit and instead recognizes a polypeptide of 50 kDa, which fits the calculated mass of the nonhomologous domain (*see* Fig. 1). They also found that frame-shift mutations in the nonhomologous region of the *VMA1* gene block the synthesis of both the 69-kDa subunit and the 50-kDa polypeptide (6). These results agree with the "protein splicing" hypothesis. However, the "120-kDa intermediate" has not been detected by pulse-chase experiments possibly because the reaction occurs very rapidly. The splicing reaction has been predicted to be an autocatalytic process (6), but it has yet to be determined how the processing reaction proceeds and where the processing activity resides in the molecule.

To understand the processing mechanism, we constructed mutant *vma1* genes with cysteine to serine substitutions at residues 284 and 738, which are located at the putative N- and C-terminal junction sites and analyzed the mutant gene products. Both mutations affected the production of the 69-kDa subunit; the Ser-284 mutation blocked the cleavage at the N-junction site, whereas the Ser-738 mutation affected the excision at both N- and C-junction sites. Possible roles of the residues in the processing reaction will be discussed later in this communication.

Materials and Methods

Strains and culture conditions. Yeast strains used were ANY21 (*MAT*a *leu2 trp1 ura3 his3 his4*) (5) and its *vma1*::*TRP1* derivative, RH103. The *VMA1* locus of RH103 was disrupted by replacing the 3.2-kb *Eco*RV-*Eco*RI region in the gene with the 0.95-kb *Eco*RI-*Hin*cII fragment from pRS314 (7) that contains *TRP1* gene. Yeast cells were grown in YPD [1% yeast extract (Difco), 2% polypeptone (Nihon-eiyou), 2% glucose], YPG (1% yeast extract, 2% polypeptone, 3% glycerol) or YNBD [synthetic minimal medium; 0.67% yeast nitrogen base (Difco), 2% glucose].

Mutagenesis of the *VMA1* gene. The 0.6-kb *Pst*I-*Kpn*I (for a Cys-284→Ser mutant) or the 1.1-kb *Nsp*(7524)V (for a Cys-738→Ser mutant) fragment in the *VMA1* gene was cloned into bluescript KS+ and mutated by the method of Sayers *et al* (8). Two mutagenic probes, TATGTCGGGTCCTTTGCCAAG (for the Ser-284 mutation) and GTCCATAATTCCGGAGAAAGA (for the Ser-738 mutation), both of which contain a guanine to cytosine transversion (*underlined*), were used. The nucleotide sequences of the mutated fragments were confirmed by sequencing analysis. Mutant *vma1* genes *vma1*C284S (Cys-284→Ser), and *vma1*C738S (Cys-738→Ser) were constructed using the mutated fragments and expressed on the yeast single-copy plasmid pRS316 (7) or multi-copy plasmid pMK9 (5).

Other methods. Plasmid isolation, gel electrophoresis, ligation, restriction enzyme analysis and *E. coli* transformation were done as described by Ausubel *et al* (9). Yeast transformation was carried out by the lithium acetate method (10). DNA sequence was determined by the dideoxy-chain termination method (11). Vacuolar membrane vesicles and whole-cell extracts were prepared as described previously (5) from exponentially growing cells (1-2 x 10^7 cells/ml) in YNBD medium supplemented with 0.5% casamino acids. Protein determination, SDS-polyacrylamide gel electrophoresis and Western blotting analysis were as described previously (5). Quinacrine staining of vacuoles was done as in (12). ATPase was assayed as in (5) in the presence of 2 mM sodium azide, 1 mM sodium molybdate, and 0.1 mM sodium orthovanadate. Bafilomycin A_1 was added at the final concentration of 10 μM.

Materials. All the chemical reagents and enzymes for recombinant DNA methods were as described in (13).

Results

Expression of mutant *VMA1* genes *in vivo*. The nonhomologous domain of the *VMA1* gene product is bracketed by two cysteine residues, Cys-284 and Cys-738 (Fig. 1). The codons for Cys-284 and Cys-738 of the *VMA1* gene were replaced by serine codons, and the mutant genes *vma1*C284S and *vma1*C738S were expressed in a null *vma1* mutant, RH103. Fig. 2 shows the growth properties of the RH103 cells that carries the wild-type or the mutant *vma1* genes on a single-copy plasmid, pRS316. RH103 lacks almost the entire coding region of the chromosomal *VMA1* gene and does not grow in YPD medium supplemented with 100 mM $CaCl_2$ (Cls^- phenotype) and in YPG medium that contains glycerol as a sole carbon source (Pet^- phenotype) (14). This Pet^- Cls^- phenotype was fully complemented when the cells were transformed with the wild-type *VMA1* gene (Fig. 2). The *vma1*C738S gene partially restored the growth defect of RH103 both in the Ca^{2+}- and the glycerol-media, whereas the *vma1*C284S gene did not. The slow growth of *vma1*C738S cells was not suppressed by overexpression of the mutant gene on a multicopy plasmid (data not shown).

Detection of the mutant *vma1* gene products. Growth phenotypes of the cells carrying the mutant *vma1* genes suggested that both mutations affect the synthesis of the functional subunit. We analyzed plasmid-born Vma1 polypeptides by Western blotting analysis using anti-69-kDa subunit monoclonal antibodies, R70 and 5M39, which recognize the N- (31 kDa) and C- (37 kDa) terminal homologous domains, respectively (*see* Fig. 1)[3]. To facilitate the detection of the gene products, we used the cells carrying the wild-type or mutant *vma1* gene on a multicopy plasmid, pMK9. The wild-type *VMA1* gene yielded a single immunoreactive polypeptide with an apparent molecular mass of 69 kDa (Fig. 3). The 69-kDa polypeptide was not detected in the mutant extracts under the same experimental conditions. Instead, a R70-reactive polypeptide of 80 kDa and a 5M39-reactive one of 40 kDa were detected in the two mutant extracts (Fig. 3). The cells harboring *vma1*C738S had an additional major polypeptide of 120 kDa that was recognized by the two antibodies. As we used monoclonal antibodies, Vma1-polypeptides that lack epitope sites for the antibodies should not be detected in these experiments. However, judging from the intensity on the blots, we think that these polypeptides were the major products of the mutant genes.

Properties of the vacuolar membranes from the cells carrying the mutant *vma1* genes. To characterize further the mutant gene products, vacuolar membrane vesicles were prepared from the cells bearing the mutant plasmids. The

[3] N. Umemoto, R. Hirata, and Y. Anraku, unpublished results

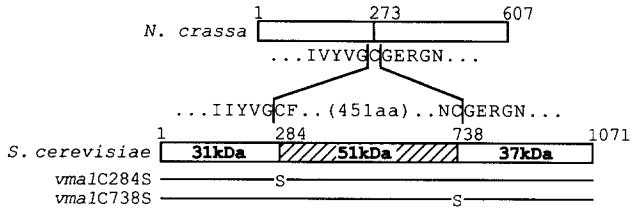

Fig. 1. Construction of the mutant *vma1* genes. *Open* and *hatched boxes* represent the homologous and nonhomologous domains, respectively. The amino acid sequences around the putative cleavage sites are shown by one-letter codes. Monoclonal antibodies, R70 and 5M39, recognize 31-kDa N-terminal and 37-kDa C-terminal domains, respectively.

vacuolar membrane prepared from *vma1*C738S-expressing cells contained a low but detectable ATPase activity that was sensitive to Bafilomycin A_1, a specific inhibitor for vacuolar ATPases (Table I). Acidification of vacuoles was also observed in the *vma1*C738S cells *in vivo* by staining the cells with a fluorescent dye, quinacrine (data not shown), which is known to concentrate into acidic membrane compartments (12).

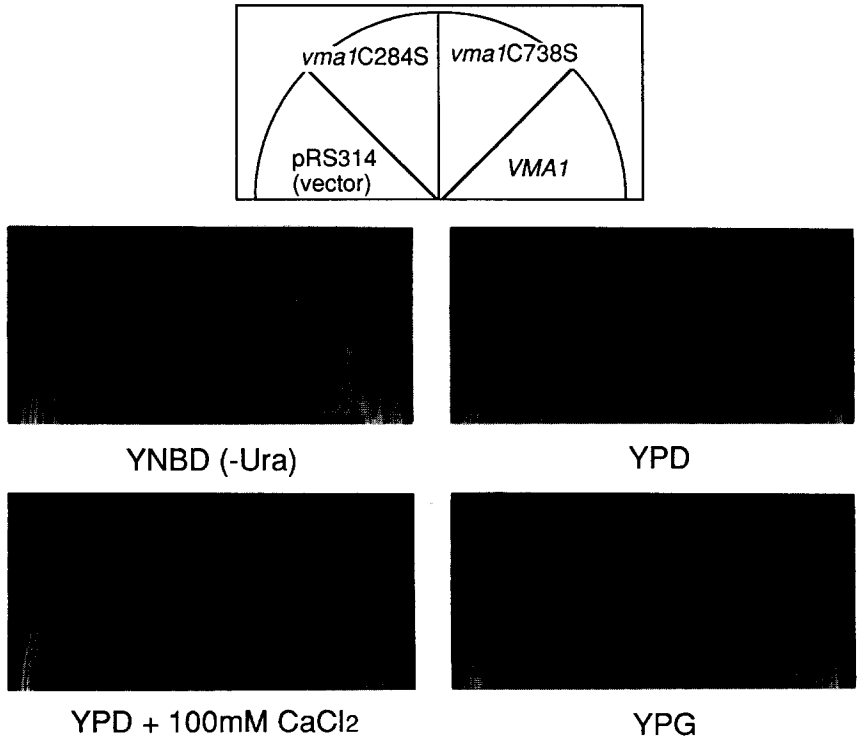

Fig. 2. Growth phenotypes of the cells carrying the wild-type and the mutant *vma1* genes. Wild-type and mutant *vma1* genes were cloned into a single-copy plasmid pRS316 (*URA3*) and introduced into a null *vma1* mutant, RH103. The transformants were streaked on the media indicated and incubated at 30°C for 3 days (for YPD, YPD supplemented 100 mM $CaCl_2$) or 5 days (YNBD and YPG)

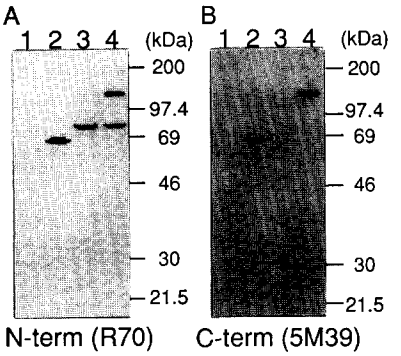

Fig. 3. Detection of the mutant *vma1* gene products in whole-cell extracts. Whole-cell extracts were prepared as described under "Materials and methods" from RH103 carrying the vector pMK9 (lane 1), the wild-type *VMA1* gene (lane 2), *vma1*C284S (lane 3) or *vma1*C738S (lane 4). Fifty micrograms of proteins were resolved in SDS-polyacrylamide gels (10% acrylamide) and transferred onto nitrocellulose membrane filters. Vma1-related polypeptides were probed with anti 69-kDa subunit monoclonal antibodies R70 (A) or 5M39 (B).

The *vma1*C284S mutant cells were completely devoid of the vacuolar membrane H^+-ATPase activity (Table I).

We next analyzed the mutant vacuolar membranes by Western blotting analysis (Fig. 4). All the Vma1-polypeptides that were observed on the blots of the whole-cell extracts were detected in the vacuolar membrane fractions. In addition to these polypeptides, a polypeptide of 69 kDa was detected in the membrane of the cells expressing *vma1*C738S. This polypeptide co-migrated with the wild-type subunit and recognized by both R70 and 5M39. The *vma1*C738 mutant membrane also contained the 60-kDa subunit of the vacuolar membrane H^+-ATPase. In addition, the 60-kDa subunit appeared to exist on the membrane in the proportionately small amount as the 69-kDa polypeptide although the cellular level of the 60-kDa subunit was unchanged by the *vma1* mutations (data not shown). We therefore concluded that the 69-kDa polypeptide that was yielded from *vma1*C738S is structurally and catalytically functional.

Table I
Vacuolar enzyme activities in the cell expressing the wild-type or mutant *vma1* genes

		α-mannosidase		ATPase	
		spheroplast lysate	vacuolar membrane vesicles	vacuolar membrane vesicles	
		(nmol/min/mg protein)		(μmol/min/mg protein)	
ANY21		0.08	2.73	0.342	(100)
RH103	+pMK9 (vector)	0.24	3.75	0.003	(<1)
	+pMVMA1 (wild)	0.10	3.66	0.252	(74)
	+pMVMA1C284S	0.29	5.91	0.006	(2)
	+pMVMA1C738S	0.17	2.63	0.075	(22)

Enzyme assay was done as described under "Experimental Procedures."

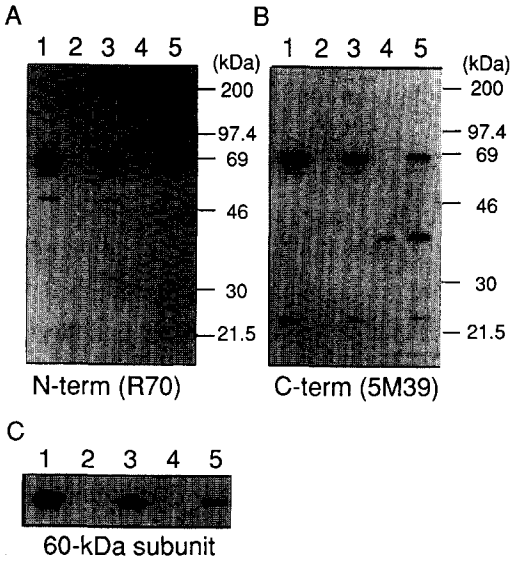

Fig. 4. Detection of the mutant *vma1* gene products and the 60-kDa subunit in vacuolar membranes. Vacuolar membrane vesicles were prepared as described under "Materials and methods" from ANY21 (wild-type strain; lane 1), RH103 carrying the vector pMK9 (lane 2), the wild-type *VMA1* gene (lane 3), *vma1*C284S (lane 4) or *vma1*C738S (lane 5). Forty micrograms of proteins were resolved in SDS-polyacrylamide gels (10% acrylamide) and transferred onto nitrocellulose membrane filters. Antibodies used are: anti 69-kDa subunit monoclonal antibodies, R70 (A) and 5M39 (B), and rabbit anti 60-kDa subunit antiserum (C).

Discussion

We constructed mutant *vma1* genes and analyzed the gene products. The residues chosen for mutagenesis (Cys-284 and Cys-738) are located at the putative cleavage sites of the "protein splicing" reaction. The Ser-284 mutation inhibited the synthesis of the functional subunit. *vma1*C284S failed to complement the null *vma1* mutation of the host strain and yielded no 69-kDa subunits as assessed by a series of the Western blotting analysis. On the other hand, a functional subunit was synthesized in the *vma1*C738S cells. We think that the 69-kDa species detected in *vma1*C738S membranes should be the functional form for the following reasons. First, this polypeptide was selectively concentrated in the vacuolar membrane fraction. Second, the 60-kDa subunit resided proportionately to the 69-kDa polypeptide in the mutant membrane. Immunoblot analysis of the whole-cell extracts failed to detect the 69-kDa species. We think that this is due to the low abundance of the polypeptide in the mutant cells. Umemoto *et al.* (12) have shown that there is a substantial cytoplasmic pool for the 69-kDa subunit. The small amount of the processed 69-kDa subunit may be sufficient to support the growth of the null *vma1* mutant cells in the selective media.

The cells expressing the mutant *vma1* genes accumulated non-functional Vma1-polypeptides with apparent molecular masses of 120-, 80- and 40-kDa. We propose that these polypeptides were formed in consequence of the incomplete processing of the 120-kDa precursor. Calculated masses of the N-, C- and internal

domains are 31, 37 and 51 kDa, respectively (Fig. 1). Therefore, a single proteolytic event at the putative C-terminal junction site should yield polypeptides of 82 kDa and 37 kDa. The sizes and the antigenicities of the 80- and the 40-kDa species agree with our hypothesis. The reason why the mutations block the synthesis of the correctly processed subunit is yet unknown. However, it is noteworthy that the Ser-738 mutation apparently affects the cleavage of peptide bond at both the N- and C-junction sites. This finding suggests that Cys-738 is operationally involved in a protein processing mechanism. Posttranslational processing of the jack bean concanavalin A, which involves cleavage and formation of a peptide bond has been reported (15). In this case, the cleavage site of the precursor molecule is the C-terminus of an asparagine residue. Cys-738 of the *VMA1* gene product is also preceded by an asparagine residue, Asn-737 (Fig. 1). We have observed that the Asn-737→Val mutation of the *VMA1* gene inhibits the synthesis of the functional subunit[4]. The Cys-738 and Asn-737 residues may cooperate during the processing reaction that directs the peptide excision and ligation of the junction sites by a tightly coupled mechanism. Further characterization of the mutant gene products is now in progress.

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