**VMA12** Is Essential for Assembly of the Vacuolar H\(^+\)-ATPase Subunits onto the Vacuolar Membrane in *Saccharomyces cerevisiae*

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**vmal2** mutants of the yeast *Saccharomyces cerevisiae*, which were originally identified as calcium-sensitive (cs) mutants that were also respiratory deficient (Rd-), have a defect in vacuolar membrane H\(^+\)-ATPase activity (Ohyay, Y., Umemoto, N., Tanida, I., Ohta, A., Iida, H., and Anraku, Y. (1991) *J. Biol. Chem.* 266, 13971-13977). The VMA12 gene was cloned by complementation of the growth defects of vmal2 mutants. The nucleotide sequence of the gene predicts a polypeptide of 215 amino acids (25.2 kDa) with two putative membrane-spanning domains. A null vmal2 mutant, constructed by chromosomal deletion of the gene, is viable but has completely lost the vacuolar membrane H\(^+\)-ATPase activity and exhibits the same growth defects as observed for the original vmal2 mutants. Synthesis and targeting of the subunits of the H\(^+\)-ATPase in the Δvma12 mutant cells were examined by Western blotting analyses of whole cell and vacuolar membrane protein extracts. None of the peripheral membrane subunits that we analyzed (the 69-, 60-, 42-, and 27-kDa subunits) was detected in the vacuolar membrane fractions, although the cellular levels of these polypeptides appeared to be normal. The 100- and 17-kDa integral membrane subunits of the enzyme were absent or present at a substantially reduced level in mutant vacuolar membrane fractions. Anti-Vma12p antibodies recognized a vacuolar protein with the expected molecular mass of 25 kDa. However, the Vma12 protein was not detected in the vacuolar membrane ATPase complex that had been solubilized with a zwitterionic detergent, ZW3-14, and purified by glycerol gradient centrifugation (Kane, P. M., Yamashiro, C., T., and Stevens, T. H. (1989) *J. Biol. Chem.* 264, 19236-19244). These results indicate that the VMA12 gene product is not a component of the active vacuolar ATPase complex and instead suggest that this protein is required during the process of assembly and/or targeting of the enzyme complex to the vacuolar membrane.

The yeast vacuole functions as a primary storage compartment for various ions and solutes and regulates cytosolic ion and pH homeostasis (1, 2). In addition, this acidic compartment contains a number of hydrolases and serves as a digestive organelle similar to the animal cell lysosome (3). The physiological importance of the vacuole, particularly under stressed conditions, has become clear from studies of yeast mutants that are defective in vacuolar functions and biogenesis (4-9).

The lumen of the vacuole is acidified by an electronegative H\(^+\)-translocating ATPase (H\(^+\)-ATPase) residing on the membrane of the organelle (10). The activity of the H\(^+\)-ATPase appears to be indispensable for many vacuolar functions. The enzyme generates a proton motive force across the membrane that drives various secondary transport systems on the membrane (11-13) and serves to acidify the vacuolar lumen, which may be important for vacuolar hydrolase function (2, 3).

The yeast vacuolar membrane H\(^+\)-ATPase is a well-characterized member of the "V-type" ATPases. V-type ATPases have also been identified in various endocytic and exocytic membrane compartments of eucaryotic cells (14). The V-type ATPases characterized thus far are multisubunit complexes with similar subunit compositions (14, 15). The yeast enzyme has a functional molecular mass of more than 500 kDa (16) and consists of at least eight polypeptides with apparent molecular masses of 100, 69, 60, 42, 36, 32, 27, and 17 kDa (17-20). Genes for all these subunits, except for the 32-kDa subunit, have been isolated and sequenced (18, 19, 21-27). The predicted primary structures of the subunits are very similar to those determined for the subunits of higher eucaryotic cells, illustrating that the yeast enzyme serves as a good model for studies on the structure and function of the V-type ATPase.

Studies on deletion mutants of the genes for the ATPase subunits (VMA genes) have revealed that the mutants exhibit a common, characteristic set of growth phenotypes. The mutant cells did not grow in YPD medium (1% yeast extract.
Role of Vma12p in Assembly of Yeast V-ATPase

(Difco), 2% Polypeptone (Nihon Seiyaku, Tokyo), and 2% glucose buffered to neutral pH (27–29, 32). YPD containing 100 mM Ca\(^2+\) (22, 25, 26, 29, 30, 32), or media containing nonfermentable carbon sources (22, 26, 30–32). Several additional members of VMA genes have been identified by screening mutants that show similar growth phenotypes as those of the \(vma\) mutant cells (18, 30, 32, 33). Ohya et al. (30) identified three new genes, \(VMA11\), \(VMA12\), and \(VMA13\), which are essential for expression of the vacuolar membrane \(H^+\)-ATPase, by characterizing the yeast calcium-sensitive (\(cls\)) mutants that also have a respiratory deficient phenotype (Pet:\(cls\) mutants; \(cls7\)-\(cls11\)) (34).

In this paper, we report the isolation and characterization of a gene, \(VMA12\), that was originally identified as \(CLS10\) (30, 34). Nucleotide sequence predicts, and biochemical studies confirm, that the \(VMA12\) gene product (\(Vma12p\)) is a polypeptide of 25 kDa. Deletion of the \(VMA12\) gene resulted in the loss of vacuolar membrane \(H^+\)-ATPase activity. Further analysis revealed that the ATPase subunits failed to assemble onto the vacuolar membrane in the \(vma12\) mutant cells. Finally, although \(Vma12p\) is detectable in the vacuolar membrane fraction in wild-type cells, this polypeptide did not copurify with the ATPase activity when the enzyme was isolated by glycerol gradient fractionation. These results suggest that \(Vma12p\) is essential for the assembly of the \(H^+\)-ATPase, although it is not itself a component of the purified vacuolar enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials—**Enzymes for recombinant DNA methods were purchased from Takara Shuzo (Kyoto). Modified T\(7\)-polymerase was from U. S. Biochemical Corp. \((\alpha-32P)dCTP\) (110 TBq/mmol) was from ICN K&K Laboratories Inc. Other chemicals were as described by Uchida et al. (20). Bafilomycin \(A_1\) was a generous gift from Dr. Karlheinz Altendorf (University of Osnabruck).

**Strains and Conditions—**Saccharomyces cerevisiae strains used are \(YPH499\) (\(MAT\(a\) leu2 ura3 trpl lys2 his3 ade2\)), \(YPH500\) (\(MAT\(a\) leu2 ura3 trpl lys2 his3 ade2\), \(YPH501\) (a diploid derivative of \(YPH499\)), \(YPH502\) (\(MAT\alpha leu2 ura3 trpl lys2 his3 ade2\)), \(YPH504\) (\(MAT\alpha leu2 ura3 trpl lys2 his3 ade2\)), \(YPH505\) (a diploid derived from a mating between \(YPH499\) and \(YPH500\) (35), \(NYU33\) (\(MAT\alpha\) \(vma12\)-1 \(leu2\) \(ura3\) \(lys2\) \(his3\)), \(NYU44\) (\(VMA12\)-LEU2 derivative of \(YPH499\)), and \(RH202\) (\(VMA12\)-TRP1 derivative of \(YPH500\)). \(NYU33\) was constructed by a cross between \(YOC18\) (\(vma1\)-1, \(leu1\)) (34) and \(YPH500\). Yeast cells were grown aerobically in the following media: YPD, YNB (synthetic minimal medium: 0.67% yeast nitrogen base (Difco) and 2% glucose), and YPG (1% yeast extract, 2% polypeptide, and 3% glycerol). When required, YNB was supplemented with amino acids and nucleic acids as described by Sherman (36). Cell growth was monitored by direct cell counts or by measuring the absorbance at 600 nm. For certain experiments, cell growth was measured using a Coulter counter (Beckman Instruments, Fullerton, CA).

**Antibodies—**Rabbits were immunized with the 100-kDa (7B1), 69-kDa (11E6), 60-kDa (13D11), and 42-kDa (7A2) vacuolar ATPase subunits as described (17). 3H- or \(^{125I}\)-labeled proteins were visualized by autoradiography. For some experiments, polyclonal antibodies were prepared in rabbits using synthesized peptides corresponding to the amino acids 601–605 of the \(Vma12p\) gene product.

**Northern Blotting—**RNA was prepared from \(vma12\) null strains (expressing no \(Vma12p\)) and \(Vma12\) overexpressing strains (expressing higher than normal levels of \(Vma12p\)) using the guanidinium thiocyanate method (44) with the modification of Tabor and Richardson (45). Yeast RNA was isolated by the lithium acetate method of Ito et al. (47). Two synthetic oligonucleotide probes, \(5'\)-CAAGAAACACGATCCATCAGTG-3' (bases 897–916 with a linker sequence (underlined) to introduce an EcoRI site at the end of the PCR product), were used to amplify a gapped plasmid with a deletion in the \(VMA12\) gene. The PCR products were labeled with \({^{32P}}\)dATP and were used to probe genomic DNA of the \(VMA12\)-null strain and EcoRI digested genomic DNA of wild-type yeast. Hybridization was done according to standard procedures.

**SDS-PAGE and Western Blotting—**Protein extracts of whole cells and vacuolar membrane vesicles were prepared as described by Kane et al. (48). SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (49). Immunoblots were prepared and probed as described (17). Blots were probed with primary antibodies diluted 1:1,000. Alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibodies (Promega Biotech) were used as secondary antibodies, and bound alkaline phosphatase was visualized by the addition of 4-nitro blue tetrazolium (Sigma). Apparent molecular masses of proteins were determined relative to prestained molecular weight standards (Bethesda Research Laboratories).

**Antibodies—**The \(877\)-base pair BstUI-SpeI fragment in the \(VMA12\) gene was cloned into the \(E. coli\) expression vector pEXPl (pMH20) (50). The \(Vma12p\) antigen (amino acids 21–215) produced from this plasmid was purified from \(E. coli\) and injected into New Zealand White rabbits as previously described (51). Rabbit anti-\(Vma12p\) antibodies were affinity purified against antigen expressed from plasmid pMH20 as described (52). Monoclonal antibodies that recognize the 100-kDa (7B1), 89-kDa (11E6), 60-kDa (13D11), and 42-kDa (7A2) vacuolar ATPase subunits were prepared as described (46). Anti-Vma12p monoclonal antibody was prepared as described (17).

**Chloroform/Methanol Extraction of the Vacuolar Membrane Vesicles—**Vacuolar membrane vesicles were washed three times with a buffer containing 1 mM EDTA and 10 mM Tris/HCl, pH 7.5.

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1 The abbreviations used are: \(Vma12p\), \(VMA12\) gene product; kb, kilobase(s); PCR, polymerase chain reaction; PACE, polycarylamide gel electrophoresis; ZW-15, \(N\)-tetradecyl-\(N\)-dimethyl-3-amino-1-propanesulfonate.

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washed membranes were suspended in the same buffer (1 mg of protein/ml), and 5 volumes of chloroform/methanol (2:1) were added to the suspension. The suspension was incubated on ice for 1 h with occasional vortexing. The suspension was then centrifuged, and the upper aqueous phase and interphase were removed. Proteins in the organic phase were recovered by evaporating the solvent and were subjected to SDS-PAGE.

Other Methods—Preparation of vacuolar membrane vesicles and purification of the vacuolar membrane H+·ATPase were done as described previously (20, 53). Vacular ATPase, dipeptidyl amino-peptidase B (64), and α-mannosidase (53) activities were determined as previously described.

RESULTS

Isolation of the VMA12 Gene—The umal2-1 mutant was originally isolated as a Ca2+-sensitive mutant (cls10-1) that also exhibited a respiratory deficient phenotype (Pet-) (30). The VMA12 gene was isolated by complementation of the growth defect of the mutant cell. A umal2 mutant strain, NUY33 (uma12-1, leu2), was transformed with a yeast genomic DNA library carried on the yeast multicopy vector YEp13 (LEU2). Leu+ transformants were selected on synthetic complete plates lacking leucine and tested for growth on YPG and YPD supplemented with 100 mM CaCl2. A single clone, pNUVA401, restored the growth of NUY33 on both the glycerol and the Ca2+ plates in a plasmid-dependent manner.

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Nucleotide Sequence of the VMA12 Gene—The nucleotide sequence of the 1.2-kb Xhol-Spel fragment in pNUVA420 was determined for both strands. A single open reading frame of 215 codons, capable of encoding a protein of 25.2 kDa, was found in this region (Fig. 2). Because no other open reading frame with significant length was found in this region, we concluded that the open reading frame specifies the VMA12 gene protein (Vma12p). Fig. 3 shows a hydropathy plot of the predicted Vma12p sequence drawn by the method of Kyte and Doolittle (55). At the C-terminal region of the sequence, two stretches of hydrophobic residues are present, suggesting that Vma12p is an integral membrane protein. No significant sequence similarities have been found between the Vma12p...
and any proteins in NBRF (release 31) or SWISS (release 20) protein data base. The \( VMA12 \) gene has proven to be identical defective in vacuolar acidification (33). It seems likely that \( vph \) mutants may include other \( uma \) genes.

Disruption of the \( VMA12 \) Gene—The chromosomal locus of the gene was disrupted by the method of Rothstein (56). The 1.2-kb \( XhoI-SpeI \) fragment containing the \( VMA12 \) gene was cloned into the vector pBluescript KS\(^+\). The disrupted allele of the gene was constructed on the plasmid by replacing the coding region of the gene with the \( TRP1 \) gene fragment (Fig. 4A). Almost the entire coding region was deleted in the construct. The disruption allele (\( \Delta vma12::TRP1 \)) was then liberated from the vector by \( XhoI-SpeI \) digestion and introduced into a wild-type diploid, YPH501 (\( trpl/trp1 \)). A \( Trp^+ \) transformant was picked, and substitution of one copy of the chromosomal \( VMA12 \) gene was confirmed by Southern blotting analysis (data not shown). The \( \Delta vma12::TRP1/VMA12 \) diploid cells were sporulated, and tetrads were dissected. All the tetrads analyzed (17 sets) yielded four viable spores, indicating that disruption of the gene was not lethal (data not shown). For the following analysis, we used a haploid \( \Delta vma12::TRP1 \) mutant, RH202 (Fig. 4), in combination with the wild-type parental strain YPH500. The \( \Delta vma12 \) mutant cells exhibited growth phenotypes identical to those of \( vma \) mutants defective for \( H^+\)-ATPase subunit genes (\( VMA1, VMA2, VMA3, \) and \( VMA4 \)).\(^5\) They did not grow in YPG medium, which contains glycerol as a sole carbon source, YPD medium supplemented with 100 \( \mu \)M CaCl\(_2\), or YPD buffered at neutral pH (Fig. 5). Vacuolar membranes isolated from the \( \Delta vma12 \) mutant cells lacked bafilomycin \( A_1 \)-sensitive ATPase activity (Table 1), indicating that \( Vma12p \) is essential for expression of this enzyme's activity. The morphology of the vacuoles in the mutant cells appeared normal. However, the mutant cells failed to accumulate the fluorescent dye quinacrine in their vacuoles (data not shown). Since quinacrine is known to concentrate within acidic membrane compartments (57), this result indicates that the \( \Delta vma12 \) mutant is defective in vacuolar acidification.

Assembly of the Vacuolar Membrane \( H^+\)-ATPase in the \( \Delta vma12 \) Mutant Cells—To investigate the role of \( Vma12p \) in expression of the ATPase activity, we examined the synthesis of any proteins in NBRF (release 31) or SWISS (release 20) protein data base. The \( VMA12 \) gene has proven to be identical defective in vacuolar acidification (33). It seems likely that \( vph \) mutants may include other \( uma \) genes.

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Assembly of the Vacuolar Membrane \( H^+\)-ATPase in the \( \Delta vma12 \) Mutant Cells—To investigate the role of \( Vma12p \) in expression of the ATPase activity, we examined the synthesis

\(^{1}\) A. Bachhawat and E. W. Jones, personal communication.

\(^{2}\) \( VMA1 \) is the same gene as \( TFP1 \), \( VMA2 \) equals \( VAT2 \), and \( VMA11 \) equals \( TFP3 \) (18, 19).

\(^{3}\) \( VMA1 \) equals \( TFP1 \), \( VMA2 \) equals \( VAT2 \), and \( VMA11 \) equals \( TFP3 \) (18, 19).

\(^{4}\) Table 1

| Vamous mutant cells | Vamous enzyme activities were assayed as described under “Experimental Procedures.” |
|---------------------|-------------------------------------------------------------------------|
| YPH500 (Vma\(^{+}\)) | 15.9 6 900 |
| RH201 (\( \Delta vma12 \)) | 15.9 6 6 |

\(^{5}\) Activity that is sensitive to 5 \( \mu \)M bafilomycin A\(_1\).
disruption of the VMA12 gene, which is essential for the expression of the vacuolar membrane H\textsuperscript{+}-ATPase. The vma12 mutant was originally isolated as a type IV cls (or Pet\textasciitilde cls, cls7-cls11) mutant, cls10 (30, 34). The VMA12 gene was cloned by complementation of the \textit{vma12}-1 mutation and sequenced. The nucleotide sequence of the gene predicts an integral membrane polypeptide of 25.2 kDa.

Disruption of the \textit{VMA12} gene was not lethal but conferred upon yeast cells a Ca\textsuperscript{2+} and pH sensitivity, as well as a respiratory deficient phenotype. Vacular membrane vesicles isolated from the \textit{vma12} mutant cells were devoid of ATPase activity and the vacuoles in these mutant cells did not accumulate the fluorescent dye quinacrine, indicating that \textit{vma12} mutant vacuoles were not acidified. The phenotypes exhibited by the \textit{vma12} mutant were indistinguishable from those of other \textit{vma} mutants, suggesting that screening new Pet\textasciitilde cls mutants may identify additional VMA gene products that are required for the expression and molecular organization of the vacuolar membrane H\textsuperscript{+}-ATPase (30, 32).

We were interested in determining what effect the loss of Vma12p had on the synthesis and assembly of the subunits of the vacuolar membrane H\textsuperscript{+}-ATPase. Our results show that the synthesis and/or stability of the 100-kDa integral membrane subunit was reduced 5–10-fold in \textit{vma12} cells, whereas the steady-state levels of the peripheral membrane subunits (69, 60, 42, and 27 kDa) were unaffected. We then analyzed vacuolar membrane vesicles prepared from \textit{vma12} cells and determined that none of these ATPase subunits was associated with the vacuolar membrane in this mutant. The level of the 17-kDa subunit in vacuolar membranes was also decreased in the mutant cells. We concluded, therefore, that Vma12p is essential for the normal synthesis and/or stability of the integral membrane portion of the V-ATPase (100- and 17-kDa polypeptides) and for the targeting of both the peripheral and integral membrane ATPase subunits to the vacuolar membrane. These results are similar to the findings for \textit{umal3} and \textit{umal11} mutant cells (33, 48). Deletion of \textit{VMA} genes encoding the integral membrane polypeptides, Vma3p or Vma11p, from yeast cells affects the targeting of the

DISCUSSION

In this paper, we report the isolation of the yeast \textit{VMA12} gene, which is essential for the expression of the vacuolar membrane H\textsuperscript{+}-ATPase. The \textit{vma12} mutant was originally isolated as a type IV cls (or Pet\textasciitilde cls, cls7-cls11) mutant, cls10 (30, 34). The VMA12 gene was cloned by complementation of the \textit{vma12}-1 mutation and sequenced. The nucleotide sequence of the gene predicts an integral membrane polypeptide of 25.2 kDa.

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FIG. 8. Detection of Vma12p in glycerol gradient fractions. Solubilized vacuolar membrane vesicles were applied to a 20–50% glycerol gradient and fractionated as previously described (20). Twenty-two fractions of ~500 µl each were collected from the bottom of the centrifuge tube. Each fraction was assayed for ATPase and dipeptidyl aminopeptidase B activities. The fractions exhibiting maximal enzyme activities are shown. The proteins present in each fraction were prepared for gel electrophoresis as described (20). A constant percentage of each fraction was separated by SDS-PAGE on 10% acrylamide gels. Total proteins present in each fraction were detected by staining with Coomassie Brilliant Blue (A). Western blots of the same glycerol gradient fractions were probed with anti-Vma2p and anti-Vma12p antibodies (B). The proteins above the 69-kDa marker are being recognized by the anti-Vma12p antibody. However, they are not VMAL2-encoded protein, because they are still present in Δvma12 cells.

remaining peripheral and integral membrane subunits of the ATPase onto the vacuolar membrane. In contrast, results obtained for Δvma1 or Δvma2 mutant cells demonstrate that deletions of the genes encoding these peripheral membrane subunits affect only the assembly of the remaining peripheral subunits onto the vacuolar membrane (26, 48). The 100- and 17-kDa integral membrane VMA gene products are present at wild-type levels in vacuoles from either Δvma1 or Δvma2 cells. Therefore, the phenotypes associated with the Δvma12 mutant are similar to mutants lacking VMA integral membrane polypeptides in that the assembly of all of the H+-ATPase subunits onto the vacuolar membrane is disrupted.

Finally, we were interested in determining whether Vma12p was a subunit of the vacuolar membrane H+-ATPase. Our results indicate that although this protein associated with vacuolar membranes, Vma12p did not copurify with glycerol gradient-purified vacuolar membrane H+-ATPase. Instead, Vma12p sediments in a manner similar to other small integral membrane proteins (<150 kDa). We therefore conclude that Vma12p is not a subunit required by the vacuolar membrane H+-ATPase for ATP hydrolysis and may not be a component of the fully assembled enzyme complex.

There are several possibilities as to the function of Vma12p. One possibility is that Vma12p is actually a component of the vacuolar membrane H+-ATPase that is required for either proton pumping or regulation of the enzyme activity but is detached from the enzyme complex in the course of purification. Another possibility is that Vma12p facilitates the assembly and/or targeting of the H+-ATPase subunits onto the vacuolar membrane. This possibility necessitates that proteins other than the prominent subunits of the vacuolar membrane H+-ATPase are required for the expression of this enzyme’s activity. Examples of proteins that affect the assembly of enzyme complexes but are not subunits of the final complex have been reported. Recently, Ackerman and coworkers (58–62) reported the isolation of four genes, ATP10–ATP13, that are required for the expression of the mitochondrial ATPase activity but are not structural genes of the enzyme. Of these, ATP13 is required for transcription of at least one of the ATPase subunits, subunit 9 (58). The ATP10–
The binding of this multisubunit complex onto the vacuolar membrane is predicted to regulate the function of Vma12p in the synthesis and assembly of the vacuolar membrane H+-ATPase, as well as an assembly/targeting factor. If, as we predict, the vacuolar membrane H+-ATPase is assembled onto membranes in an early secretory pathway organelle, one could envisage that Vma12p could associate with the H+-ATPase to control H+ translocation and thus regulate organelle pH in the various secretory pathway compartments that the H+-ATPase traverses en route to the vacuole. In this scenario, Vma12p could reside predominantly in membrane compartments outside the vacuole (e.g., endoplasmic reticulum, Golgi bodies, and/or endosomal membranes), where the H+ translocation activity of the vacuolar H+-ATPase may need to be very tightly controlled. Therefore, further studies on the function and localization of Vma12p might be important in establishing where and when the subunits of the vacuolar H+-ATPase are assembled onto the membrane to form the active enzyme complex.

The characterization of the VMA12 gene product and the subsequent effects of vma12 deletions in yeast have generated fundamental questions regarding the biogenesis, assembly, and molecular organization of the vacuolar membrane H+-ATPase complex. Future efforts will be directed toward elucidating the function of Vma12p in the synthesis and assembly of this multisubunit complex onto the vacuolar membrane. These studies should further our general understanding of the coordinated assembly of multisubunit enzyme complexes in membranes.

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