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Biochemical support for the V-ATPase rotary mechanism: antibody against HA-tagged Vma7p or Vma16p but not Vma10p inhibits activity

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Summary

V-ATPase null mutants in yeast have a distinct, conditionally lethal phenotype that can be obtained through disruption of any one of its subunits. This enables supplementation of this mutant with the relevant subunit tagged with an epitope against which an antibody is available. In this system, the effect of antibody on the activity of the enzyme can be analyzed. Towards this end we used HA to tag subunits Vma7p, Vma10p and Vma16p, which are assumed to represent, respectively, the shaft, stator and turbine of the enzyme, and used them to supplement the corresponding yeast V-ATPase null mutants. The anti-HA epitope antibody inhibited both the ATP-dependent proton uptake and the ATPase activities of the Vma16p-HA and Vma7p-HA containing complexes, in intact vacuoles and in the detergent-solubilized enzyme. Neither of these activities was inhibited by the antibody in Vma10p-HA containing enzyme. These results support the function of Vma10p as part of the stator, while the other tagged subunits are part of the rotor apparatus. The HA-tag was attached to the N terminus of Vma16p; thus the antibody inhibition points to its accessibility outside the vacuolar membrane. This assumption is supported by the supplementation of the yeast mutant by the homologues of Vma16p isolated from *Arabidopsis thaliana* and lemon fruit c-DNA. Contrary to yeast, which has five predicted helices, the plant subunit Vma16p has only four. Our results confirm a recent report that only four of the yeast Vma16p complexes are actually transmembrane helices.

Key words: V-ATPase, subunit, antibody, proton uptake, yeast, lemon, ATPase.

Introduction

The eukaryotic V-ATPase is an ATP-driven proton pump responsible for the acidification of various intracellular compartments, which in turn is important for a variety of cellular functions. Among these are secondary transport of small molecules and ions, targeting of newly synthesized lysosomal enzymes and receptor-mediated endocytosis (Nelson and Harvey, 1999; Nishi and Forgac, 2002). V-ATPases also play a critical role in the maintenance of vacuolar homeostasis in plant cells and are involved in plants' defense against environmental stress (Taiz, 1992; Dietz et al., 2001). The V-ATPase is composed of two functional domains interconnected by peripheral and central stalks. The 640 kDa catalytic V_1 domain contains eight different subunits designated A–H, and the 260 kDa membranous V_0 domain contains five different subunits (*a*, *c*, *c′*, *c″* and *d*) (Arata et al., 2002). It is believed that the hydrolysis of ATP by V_1 rotates the central shaft (D subunit), which in turn causes the rotation of the membrane *c*-ring against its stator (E, G and a subunits), resulting in proton translocation into the vesicle (Nelson and Harvey, 1999). The V-ATPase is closely related to bacterial and mitochondrial F-ATPase, and they both share structural and functional similarities (Nelson et al., 2002). Unlike the F-ATPases, however, which contain a single type of proteolipid c subunit (8 kDa), the V-ATPase in yeast contains three different proteolipid subunits designated *c*, *c′* (16 kDa) and *c″* (21 kDa) (Vma3p, Vma11p and Vma16p, respectively) (Nelson and Nelson, 1989; Umemoto et al., 1990; Hirata et al., 1997). It is suggested that a single copy of both Vma11p and Vma16p and multiple copies of the Vma3p proteolipid are present per complex (Powell et al., 2000), although other studies have suggested that there may be two copies of Vma16p (Gibson et al., 2002).

The F-ATPase was directly shown to work by a rotary mechanism in which conformational changes in the catalytic sector cause a rotation of the γ subunit within it, leading to a counterclockwise turning of the membrane sector *c*-ring against the membranous large *a* subunit. The latter is held fixed relative to the headpiece by a peripheral stalk (Noji et al., 1997; Omote et al., 1999; Sambongi et al., 1999; Panke et al., 2000). A similar method was recently used to show the rotation mechanism of V-ATPase (Imamura et al., 2003; Yokoyama et al., 2003; Hirata et al., 2003). In order to support the rotary mechanism of the V-ATPase, and to elucidate the participating rotating subunits, we used a biochemical approach involving

specific antibodies. In contrast to single molecule studies that frequently rely on a small fraction of the total molecules, biochemical studies report on the total population and are able to quantify the proton uptake and ATPase activities simultaneously.

Antibodies have long been used as a powerful tool in the analysis of structure–function relationship in various enzymes and enzyme complexes. The influence of antibody binding to enzyme or enzyme complex on their activity can be positive, negative or neutral. The extent of inhibition or enhancement of activity is a reflection of the nature and distribution of the various antigenic determinants on the enzyme (Arnon, 1975). Antibodies are hydrophilic macromolecules and as such cannot penetrate biological membranes; this makes them applicable to the study of the topology of membrane proteins as was done for the F-ATPase *c* subunit (Girvin et al., 1989) and for the V-ATPase *c″* subunit (Vma16p), where it was shown that the C terminus of Vma16p is cytoplasmic (Nishi et al., 2001). In studies of the F-ATPase, monoclonal or polyclonal antibodies against the various subunits were used. Polyclonal antibodies against the *c* subunit of *E. coli*, recognizing epitopes on the cytoplasmic side (the loop region), prevented the binding of F_1 to F_0 and blocked proton translocation through the open F_0 channel (Deckers-Hebestreit and Altendorf, 1992). It was shown that ε subunit strongly binds the γ subunit, and is located between the F_1 and F_0 portions of the enzyme (Fillingame, 1999; Tsunoda et al., 2001). Polyclonal anti-ε sera caused near complete inhibition of the F-ATPase activity (Smith and Sternweis, 1982). The α subunit was subsequently shown to be non-rotary by the use of a monoclonal antibody against it. Binding of gradually increasing moieties to the α subunit by using the $(Fab)_2$, complete antibody or a complete antibody with increasing amounts of the secondary IgG did not change the extent of inhibition, which was 50% at most; hence leading to the conclusion that rotational catalysis of that subunit is most unlikely (Moradi-Ameli and Godinot, 1988).

When the effect of specific antibodies on V-ATPase was tested, only the antibody raised against subunit F (Vma7p) inhibited activity. In tobacco hornworm proton uptake as well as the ATPase activity could be inhibited to the same extent (Gräf et al., 1994). In the yeast *S. cerevisiae*, it was reported that the ATP-dependent proton uptake was inhibited by anti-HA epitope antibody added to vacuoles containing HA-tagged Vma7p V-ATPase (Nelson et al., 1994). Hence, the interaction of antibody with the V-ATPase subunits can be used to test the mobility of the various parts of the enzyme.

Up to now, homologues of Vma16p from plants have not been isolated (Sze et al., 1999). Here we report on the cloning of plant cDNAs encoding Vma16p and show that the subunit lacks the first putative transmembrane span but nevertheless supplements the yeast Vma16p-null mutant. Implications of these results on the yeast subunit led to construction of the N-terminal HA-tagged Vma16p, which was used along with tagged Vma10p and Vma7p V-ATPase subunits in our experiments. In order to learn more about the structure–function relationship of the specific subunits, with respect to the proposed rotary mechanism, the effect of an anti-HA antibody on V-ATPase containing a tagged subunit was tested. We report that, in contrast to the HA-tagged Vma10p, the binding of anti-HA antibody to epitope-tagged Vma7p or Vma16p-containing complexes inhibited both ATP-dependent proton uptake and ATPase activity.

Materials and methods

Strains, media and reagents

The ‘wild type’ strain used was haploid *S. cerevisiae* W303 (*trp1 ade2 his3 leu2 ura3*). The other haploid strains used in this work were: *vma10Δ* (*trp1 ade2 his3 leu2 ura3 VMA10::URA3*); *vma16Δ* (*trp1 ade2 his3 leu2 ura3 VMA16::HIS3*) and *vma7Δ* (*trp1 ade2 his3 leu2 ura3 VMA7::URA3*); *vma11Δ* (*trp1 ade2 his3 leu2 ura3 VMA11::URA3*). *VMA3*, with the HA and six His residues inserted before the last cysteine, was integrated into the genome by homologous recombination to the *VMA3::URA3* mutant strain, and verified by Southern blot. The cells were grown in YPD medium containing 1% yeast extract, 2% bactopeptone and 2% dextrose (YPD) or 2% galactose with 0.2% fructose (YPGAL). The medium was buffered by 50 mmol l^{-1} MES or 50 mmol l^{-1} MOPS, and the pH was adjusted using NaOH (Noumi et al., 1991). Agar plates were prepared by the addition of 2% agar to the YPD-buffered medium at the given pH. Yeast transformation was performed as previously described (Ito et al., 1983; Elble, 1992) and the transformed cells were selected on minimal medium containing 0.67% yeast nitrogen base, 2% dextrose, 2% agar and the appropriate nutritional requirements. The growth phenotype of the transformed yeast null mutants was checked on YPD or YPGAL plates buffered at pH 7.5 and pH 5.5 (Nelson and Nelson, 1990).

Plasmid constructs

The coding region of *VMA7*, *VMA10* and *VMA16* genes was amplified from the yeast genomic DNA by polymerase chain reaction (PCR) with specific primers. The DNA fragments were cloned into BFG-1 yeast shuttle vector, which is a high copy number plasmid containing 2-micron (a yeast ori of replications), *LEU2* marker and a 3-phosphoglycerate kinase (PGK) promoter, followed by three copies of hemaglutinin (HG) epitope, a multiple cloning site and a PGK terminator. The yeast genes were cloned downstream to the HA epitope, into the *Xba*I and *Eco*RI sites (except *VMA11* that was cloned into *Eco*RI and *Xho*I sites), thus containing the tag in their N terminus. The cDNAs encoding Vma16p from *Arabidopsis thaliana* and lemon fruit were cloned into pYES2 shuttle vector (containing 2-micron and *URA3* marker) under the inducible GAL promoter.

Isolation of VMA16 cDNAs from Arabidopsis thaliana *and lemon fruit*

A search in the *Arabidopsis thaliana* database revealed two clones encoding Vma16p homologue. The longest one

(At4g32530) was amplified by PCR with specific primers from the *Arabidopsis thaliana* cDNA library (ATCC). The degenerative primers used for the isolation of the lemon fruit Vma16p homologue were prepared according to the deduced amino acid sequence of the *Arabidopsis thaliana* clone: forward-AWGIYITG and reverse-NAFGVII. These primers were used for PCR on the lemon fruit cDNA (Aviezer-Hagai et al., 2000). The DNA fragment that was obtained (240 bp) was used to construct specific primers: forward-GAT GGC ATA TCC AGC TCT TAG and reverse-CAC CTC CAA GAA TCT CAT CAG TGT. These primers were used by PCR with the lemon fruit cDNA N′ and C′ set of primers (see construction of specific primers; Aviezer-Hagai et al., 2000) to obtain overlapping fragments containing the 5′ and 3′ ends of the lemon cDNA. For cloning into the pYES2 plasmid, the coding region of the lemon fruit Vma16p homologue was obtained by PCR with primers containing the initiator methionine or the stop codon.

Western analysis

Western blots were performed as described by Nelson et al. (1994). The nitrocellulose filters were subjected to the ECL amplification procedure (Perkin Elmer Life Sciences, Boston, USA) and exposed to Kodak X-Omat LS film for 1–5 min. The antibodies used in this study were: monoclonal antibodies against the HA-tag (BabCO, 12CA5 mouse cell line) according to Nelson et al. (1994) and Vph1p (10D7-A7-B2; Molecular Probes, Inc.), both at a dilution of 1:1000 (v/v) and polyclonal antibodies against Vma5p and Vma1p from guinea pig at a dilution of 1:5000 and 1:1000 (v/v) (Supek et al., 1994), respectively. Secondary antibodies were horseradish peroxidase (HRP)-conjugated sheep anti-mouse Ig (Amersham International, Uppsala, Sweden), which also served as a primary antibody where indicated, and HRP-conjugated rabbit anti-guinea pig antibody (Sigma, St Louis, USA) at a dilution of 1:5000 (v/v).

Preparation of yeast vacuoles

Yeast vacuoles were prepared according to the method of Uchida et al. (1985) with the required modifications described by Perzov et al. (2002). For yeast strains transformed with plasmid, the cells were grown overnight in 1 liter of minimal medium lacking the appropriate amino acid to stationary phase; they were next diluted to 0.5× absorbance at 600 nm in 5 liters YPD, pH 5.5, and grown for 4–5 h. Then the cells were harvested and vacuolar membranes were isolated as previously described (Supek et al., 1994). Specific activity of each preparation was established, and to assess the effect of antibody on ATPase or proton uptake activity assays, a comparable amount was taken to give similar basal activities in all strains tested.

ATPase assay

Vacuolar membrane vesicles containing up to 1.5 mg protein were washed in 2 ml of 10 mmol l^{-1} Tris-HCl, pH 7.5, 1 mmol l^{-1} EDTA, 2 mmol l^{-1} dithiothreitol (DTT), 0.5 mmol l^{-1} phenylmethylsulfonylfluoride (PMSF), and recovered by centrifugation (Beckman Ti75, 200 000 ***g***, 30 min). The pellet was suspended in 500 µl of solubilization buffer containing 10 mmol l^{-1} Tris-HCl, pH 7.5, 1 mmol l^{-1} EDTA, 2 mmol l^{-1} DTT, 0.5 mmol l^{-1}, 0.5 mmol l^{-1} PMSF and 4.5% glycerol. To this suspension the detergent ZW3-14 was added at a final concentration of 0.5%, and after incubation at 4°C for 15 min (with gentle mixing every 5 min) it was centrifuged at 20 000 ***g*** for 30 min. 400 µl of the clear supernatant was recovered and layered on top of a 20%–50% (v/v) glycerol density gradient containing 10 mmol l^{-1} Tris-HCl, pH 7.5, 1 mmol l^{-1} EDTA, 2 mmol l^{-1} DTT, 0.5 mmol l^{-1} PMSF and 0.005% (w/v) ZW3-14. The gradient was separated by centrifugation in Beckman SW-60 435 500 ***g***, for 5 h. 12 fractions of 0.35 ml each were collected from the bottom of the tube. Bafilomycin A_1-sensitive ATP hydrolysis was tested by measuring the production of inorganic phosphate, using a modified McCusker et al. (1987) assay. ATP hydrolysis was assayed in 0.5 ml reaction mixture at a final concentration of 25 mmol l^{-1} MOPS-Tris, pH 7, 30 mmol l^{-1} KCl, 50 mmol l^{-1} NaCl, 5 mmol l^{-1} $MgCl_2$ and 5 mmol l^{-1} Na_2ATP. Briefly, 50 µl of gradient fractions were preincubated in 200 µl reaction mixture without Mg-ATP for 10 min at room temperature in the presence and absence of 4 µmol l^{-1} (final concentration) Bafilomycin A_1. They were then preincubated for another 1 h at room temperature in the presence and absence of 50 µl of 1:1000 anti-HA monoclonal antibody (BabCO, 12CA5 mouse cell line). Next, 10 µl of sonicated asolectin (5 mg ml^{-1}) was added. The reaction was initiated by addition of 250 µl 5 mmol l^{-1} $MgCl_2$ and 5 mmol l^{-1} Na_2ATP, allowed to proceed for 10 min at 30°C and stopped by addition of 0.5 ml combined stop–colorimetric development reagent consisting of 5% Fe_2SO_4, 1% ammonium molybdate and 1 mol l^{-1} H_2SO_4. Color development was allowed to proceed for 15 min at room temperature and was monitored at 660 nm. The same mixture but without the stop solution was used for the glycerol gradient fractionation in order to separate the unbound anti-HA antibody and demonstrate the anti-HA-V-ATPase complex.

ATP-dependent proton-uptake assay

Proton uptake activity was measured following the absorption changes at 490–540 nm of Acridine Orange as previously described by Perzov et al. (2002), with the appropriate modifications. Various amounts of purified vacuoles were added to the reaction mixtures (10 mmol l^{-1} MOPS-Tris, pH 7, 15 mmol l^{-1} KCl, 135 mmol l^{-1} NaCl) to achieve similar proton pumping activity. From the wild type (WT) strain, 6 µg of protein were used; from Vma10p-HA, 8 µg; from Vma7p-HA, 40 µg and from Vma16p-HA, 15 µg were used for this assay. 30 min preincubation at room temperature followed in the presence and absence of 1–5 µl of 1:1 diluted anti-HA monoclonal antibody (BabCO, 12CA5 mouse cell line). Next, Acridine Orange to 30 µmol l^{-1} final concentration were added followed by 10 µl of 0.1 mol l^{-1} MgATP. The reaction was terminated by the addition of 1 µl of 1 mmol l^{-1} carbonyl cyanide *p*-(trifluoromethoxy) phenyl-

hydrazone (FCCP). To analyze the extent of antibody binding to the holoenzyme, the same vacuolar preparation was used for glycerol gradient fractionation and the anti-HA antibody assay was performed (see below).

Anti-HA antibody binding assay

The anti-HA binding to the intact vacuoles was tested in the following way: 200 µl of vacuoles (~2 µg µl^{-1}) were diluted in 2 ml of the proton uptake assay buffer (see above) and incubated at room temperature with 50 µl anti-HA monoclonal antibody (BabCO, 12CA5 mouse cell line) for 30 min. The vacuoles were washed twice (Ti75, 165 000 ***g***, 30 min) and suspended in 200 µl of the same buffer. Next, the vacuolar vesicles were solubilized by incubation with 0.5% ZW3-14 (zwiterionic detergent), on ice for 15 min, and fractionated for 13 h (to provide the same conditions as in the ATPase assay; see below), 435 500 ***g*** on a glycerol density gradient as detailed above. Anti-HA binding to solubilized and fractionated vacuoles was tested following the ATPase assay procedure, but without the addition of the stop solution. The reaction mixture was then fractionated on a 20%–50% glycerol density gradient (as detailed above) for 13 h at 505 500 ***g***.

Results

Vma16p from plants complements the yeast null mutant

The yeast Vma3p and Vma11p subunits contain four transmembrane segments (TMs) while Vma16p was predicted to contain five TMs, according to the hydropathy plot analysis (Hirata et al., 1997). It is accepted that both the N terminus and C terminus of Vma3p and Vma11p face the vacuolar lumen, while Vma16p C terminus was shown to face the cytoplasmic side (Nishi et al., 2001); hence its N terminus was supposed to face the lumen. The plant V-ATPase is believed to have a similar subunit composition to the yeast enzyme. In order to examine this, we used a previously constructed cDNA library from the lemon fruit and isolated from it several subunits that complemented the yeast null V-ATPase mutants (Aviezer-Hagai et al., 2000). Here we report the cloning of the plant *VMA16* from lemon fruit and from *Arabidopsis thaliana*. To this end, we searched the *Arabidopsis thaliana* full genome database and found two clones possessing significant homology with yeast Vma16p (At2g25610 and At4g32530; Sze et al., 2002). Primers were designed according to the nucleotide sequence of the longest *VMA16* clone from *Arabidopsis thaliana*, and were used to obtain the cDNA

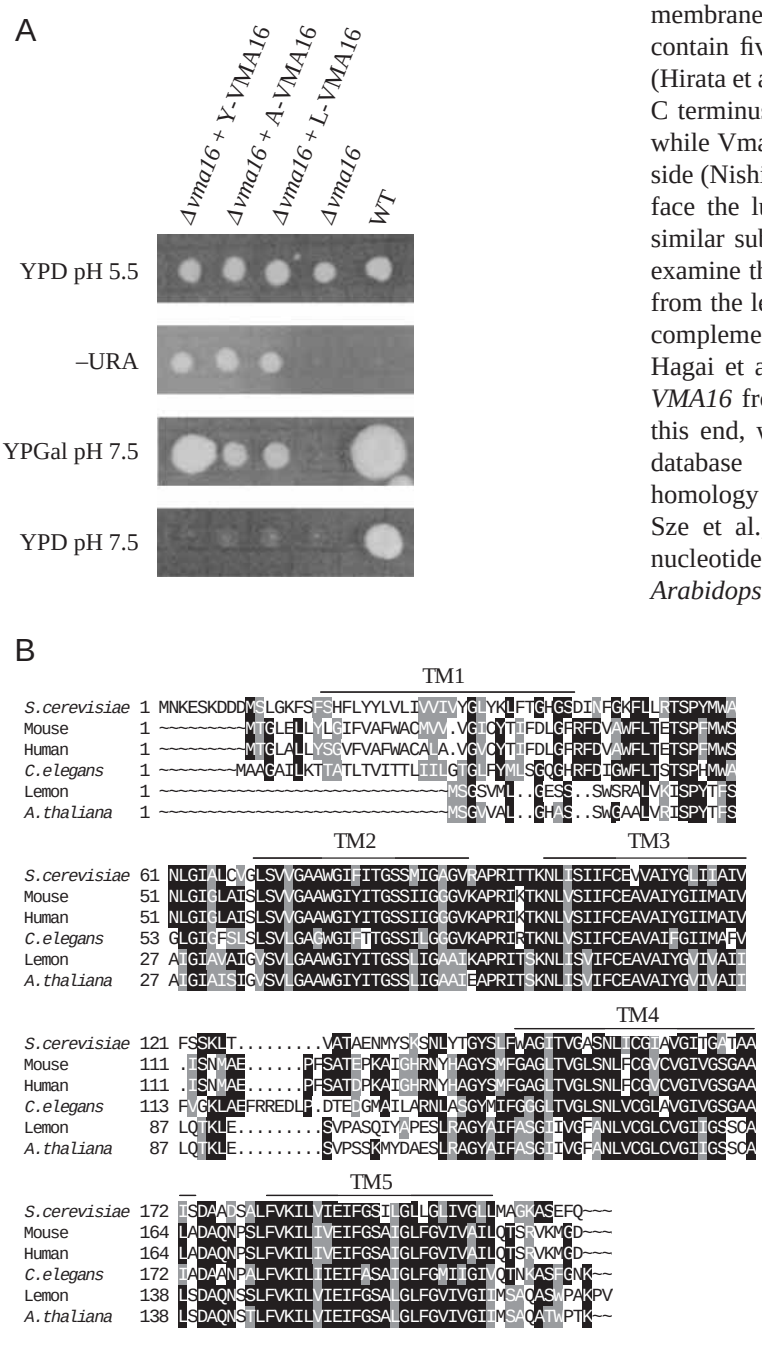

Fig. 1. Lemon fruit and *Arabidopsis thaliana* Vma16p homologues lack the first putative TM1, yet complement the *vma16*Δ yeast strain. (A) Complementation of the yeast null mutant by the plant *VMA16* cDNAs. Yeast cells lacking the *VMA16* gene were transformed with the pYES2 shuttle vector carrying the *Arabidopsis thaliana* (A-VMA16), lemon fruit (L-VMA16) or the endogenous (Y-VMA16) cDNA. The growth phenotype of the transformants was tested on YPD medium buffered at pH 5.5 or 7.5, on YPGal plates, pH 7.5, with 2% galactose and 0.2% fructose, and minimal medium without uracil (–URA). The yeast cells were grown for 4 days at 30°C, except for the YPGal plate that was left for 7 days at 30°C. WT, wild type. (B) Protein sequence alignment of Vma16p from various species: mouse (AF356006), human (D89052), *C. elegans* (Z68317), *Citrus lemon* (AY226999) (this paper), *Arabidopsis thaliana* (AY226998) (this paper) and *S. cerevisiae* (U_10399). Black boxes indicate identical residues; shaded boxes indicate similar residues. Putative transmembrane regions are indicated by dark lines labeled TM1–TM5.

encoding the predicted full-length protein from the *Arabidopsis thaliana (*ATCC). Degenerate primers according to the *Arabidopsis thaliana* gene were used for PCR of the lemon fruit cDNA library (Aviezer-Hagai et al., 2000) to isolate the gene from lemon (see Materials and methods). The genes were subcloned into the yeast shuttle vector pYES2 under the GAL promoter and complementation of the *vma16Δ* yeast null mutant was tested. Yeast lacking any of the V-ATPase genes (except Vph1p or Stv1p) displays a ΔVma conditional lethal phenotype, characterized by sensitivity to elevated pH, thereby unable to grow at pH 7.5 (Nelson and Nelson, 1990). Fig. 1A shows that the plant cDNAs, expressed under inducible GAL promoter, are able to complement the corresponding yeast null mutant, although their growth on YPGal buffered at pH 7.5 was slower than yeast cells carrying the endogenous gene under the same promoter. Fig. 1B shows sequence analysis of Vma16p from various sources. The overall identity of the lemon fruit Vma16p to the protein from human (Nishigori et al., 1998), mouse (Nishi et al., 2001), *C. elegans* (Oka et al., 1997), yeast (Apperson et al., 1990) and *A. thaliana* (present work) was 58%, 59%, 53%, 56% and 90%, respectively. Comparison of the amino acid sequence between species revealed that the predicted transmembrane regions TM2–TM5 are highly conserved. By contrast, the putative TM1 is less conserved amongst the species and is missing in plants. Taken together with the fact that the plant Vma16p was able to complement the *vma16* yeast null mutant, this may suggest that the N-terminal first hydrophobic helix of the yeast Vma16p is not a transmembrane domain, but rather a cytoplasmic segment. This was the rationale for binding of the anti-HA antibody to N-terminal HA-tagged Vma16p and a test of its influence on the activity of the holoenzyme.

Antibody interaction as a tool for structure-function implications

The use of monoclonal antibodies against tagged V-ATPase subunits in order to test their effect on the enzyme activity may advance our knowledge of the proposed rotary mechanism of the enzyme and the structure–function relationship of its specific subunits. Binding of a large moiety such as an antibody (approximately 150 kDa) to the rotating subunits should cause a steric interference, which might decrease the rate of rotation and hence inhibit activity. We tested the antibody effect on V-ATPase-containing tagged Vma16p or Vma7p representing the proteolipid-ring and shaft subunits (respectively), which are proposed to rotate against the stator subunits of the enzyme, represented by the tagged Vma10p. For that purpose, we cloned the yeast *VMA16*, *VMA7* and *VMA10* in-frame into BFG plasmid with the three HA epitopes at their N termini. The resulting plasmids were used to complement the corresponding yeast null mutants. Vacuoles containing the tagged V-ATPase complexes were isolated, and the binding of anti-HA antibody was tested. In general the activity of tagged strains was somewhat lower than the WT; however, for the various strains variable amounts of the vacuoles were used to give a similar basal activity in all the assays (see Materials and methods).

Only if the epitopes are exposed and accessible will the antibodies bind to them. Alternatively, when the HA-tag is on the lumenal side, detergents are used, and a prerequisite to anti-HA binding is that the HA-tag will be intact and not degraded by vacuolar proteases. This problem presented itself when the Vma3p-HA and Vma11p-HA were tested. Although the total vacuolar preparation displayed a positive signal with the anti-HA antibody, the glycerol gradient fractionation showed that the HA containing fractions were not active, and the active Bafilomycin A_1-sensitive ATPase fractions were devoid of HA-tag (results not shown). It was previously demonstrated (Hirata et al., 1997) that the tag on Vma11p-HA remained intact only in *pep4Δ* cells. However the question remains as to why the other inactive fractions maintain their HA-tag.

Binding of anti-HA antibody to tagged subunits in intact vacuoles

In order to test the accessibility of the epitope tags to the antibody, intact vacuoles containing the tagged subunits were preincubated for 30 min with anti-HA antibody. Next, the unbound antibody was washed twice, and the membranes were detergent-solubilized and immediately fractionated on a glycerol density gradient. The distribution pattern of bound antibody and the various subunit-containing fractions along the gradient were analyzed by western blot. Anti-Vma5p and Vma1p antibodies were used as markers for the catalytic sector of V-ATPase, anti-Vph1 antibody as a marker for the membrane sector, anti-HA antibody as a marker for HA-tagged subunits, and HRP-conjugated sheep anti-mouse Ig to trace the bound anti-HA antibody. The heavy fractions positive in all antibodies indicated the presence of the antibody-holoenzyme complex. As shown in Fig. 2B–Da, in the tagged Vma7pHA, Vma16pHA and Vma10pHA, bound anti-HA antibody is detected by anti-mouse Ig in heavier fractions (1–4). It coincides with the distribution of all V-ATPase subunit signals (Fig. 2B–D), including the HA. A small amount of non-specifically bound anti-HA-antibody is traced by the anti-mouse Ig antibody in the WT (Fig. 2Aa, fractions 6,7); this may be due to insufficient washing of the anti-HA prior to the application of the samples on the gradient.

These results indicate that the HA-epitope on the tagged subunits in the V-ATPase complex is exposed and accessible to the anti-HA antibody, that did indeed bind to it. Specifically, these results support our previous assumption regarding the number of TMs in Vma16p (four) and the topology of its N terminus (cytoplasmic). While this manuscript was in preparation, Nishi et al. (2003) reported that the deletion of the first 41 amino acids from the Vma16p N terminus resulted in an active V-ATPase. They also used cysteine labeling to conclude that the first presumed helix is located on the cytoplasmic side of the vacuolar membrane (Nishi et al., 2003).

Effect of bound antibody on ATP-dependent proton uptake into vacuoles

Once the binding of the anti-HA antibody was established, we investigated its influence on ATP-dependent proton uptake

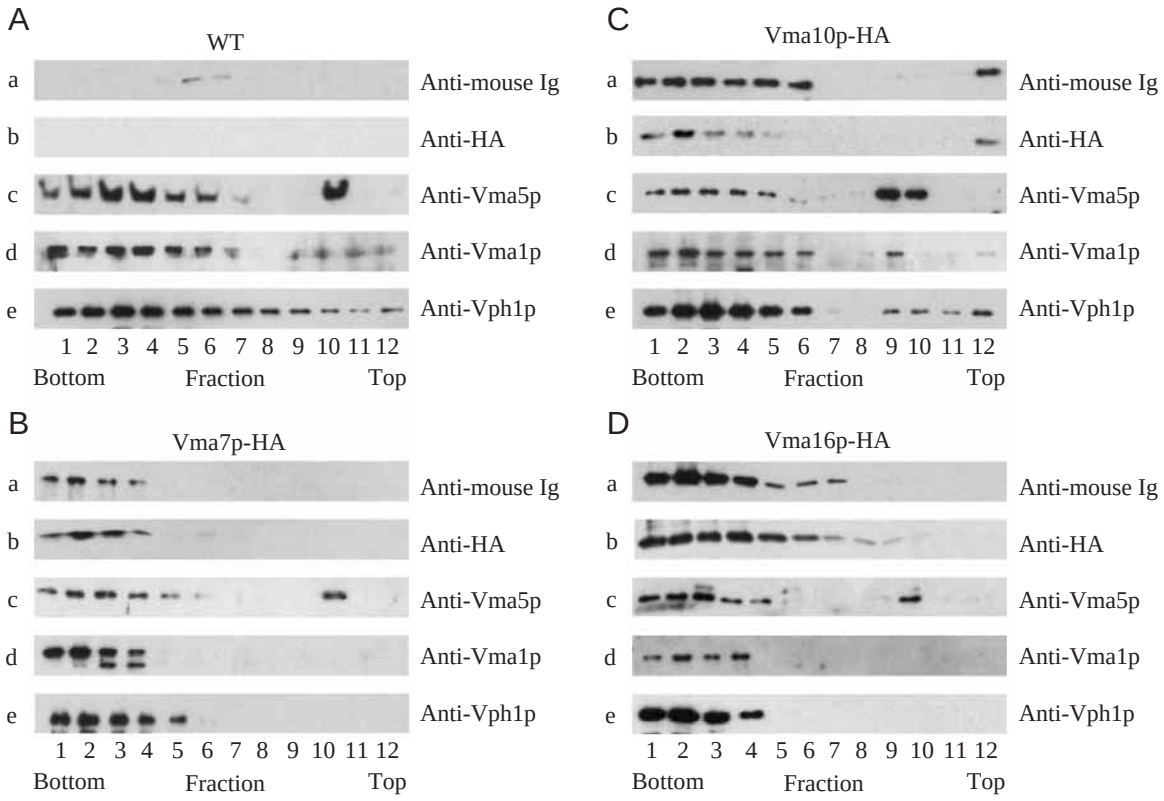

Fig. 2. Detection of bound anti-HA antibody to tagged subunits in intact vacuoles. (A) Wild type (WT), (B) Vma7p-HA, (C) Vma10p-HA, (D) Vma16p-HA. Approx. 400 μg of vacuoles were diluted and incubated for 30 min at room temperature with 50 μl of monoclonal anti-HA antibody. After two washes with the dilution buffer to remove the unbound anti-HA antibody, the vacuoles were solubilized by detergent, loaded on top of a 20%–50% glycerol density gradient and centrifuged at 435 500 ***g*** for 13 h. 12 fractions were collected from the bottom of the gradient. Protein samples from all fractions were analyzed by western blot. Lanes a were decorated with HRP-conjugated sheep anti-mouse Ig antibody; lanes b, anti-HA antibody, lanes c, anti-Vma5p antibody, lanes d, anti-Vma1p antibody and lanes e, anti-Vph1p antibody (see Materials and methods).

activity into intact vacuoles expressing the tagged subunits. To that end, vacuoles were preincubated with anti-HA antibody, and proton uptake activity was then tested by following the change in absorbency of Acridine Orange. As mentioned above, it was previously shown that anti-HA antibody could inhibit the proton uptake activity of V-ATPase containing Vma7p with a single HA-tag at its C terminus (Nelson et al., 1994). As shown in Fig. 3, the monoclonal antibody strongly inhibited the proton uptake into vacuoles expressing V-ATPase containing Vma7p-HA with three HA tags at its N terminus. Similarly, the antibody inhibited V-ATPase containing Vma16p-HA. The inhibition observed for both tagged complexes was dose-dependent, although the antibody had a more detrimental effect on the Vma7p-tagged complex, where 2 μl of antibody almost completely abolished the proton uptake by Vma7p-tagged complex, whereas 5 μl were needed for the Vma16p-tagged complex. In contrast, the anti-HA antibody did not inhibit the V-ATPase containing Vma10p-HA, even though the HA-epitope was retained on this subunit in the active enzyme and the anti-HA binds to it, as shown in Fig. 2Cb. Actually, the proton uptake activity of WT cells (diluted twice before the reaction) was slightly accelerated by the anti-HA antibody, similar to the results obtained by others when non-specific antibody was used (Gräf et al., 1994). These results show that the anti-HA antibody effect on the V-ATPase activity is genuine and specific to the tagged subunits.

Effect of bound antibody on ATPase activity of the solubilized enzyme

In order to test the influence of the anti-HA antibody on the ATPase activity of the V-ATPase containing tagged subunits, we first solubilized the isolated vacuoles in order to allow maximal ATPase activity and to avoid unnecessary background activity. The detergent-solubilized vacuoles were fractionated on a glycerol density gradient for 5 h (see Materials and methods). 12 fractions were collected from the bottom and assayed for their Bafilomycin A_1-sensitive ATPase activity. The most active fraction was further tested for the influence of anti-HA antibody on the ATPase activity. The samples were preincubated for 1 h at room temperature with an excess of anti-HA monoclonal antibody in the ATPase reaction mixture without ATP. Next, MgATP was added and the enzyme was allowed to work for 10 min at 30°C. The same treatment was performed for the control but without the antibody. Finally, the

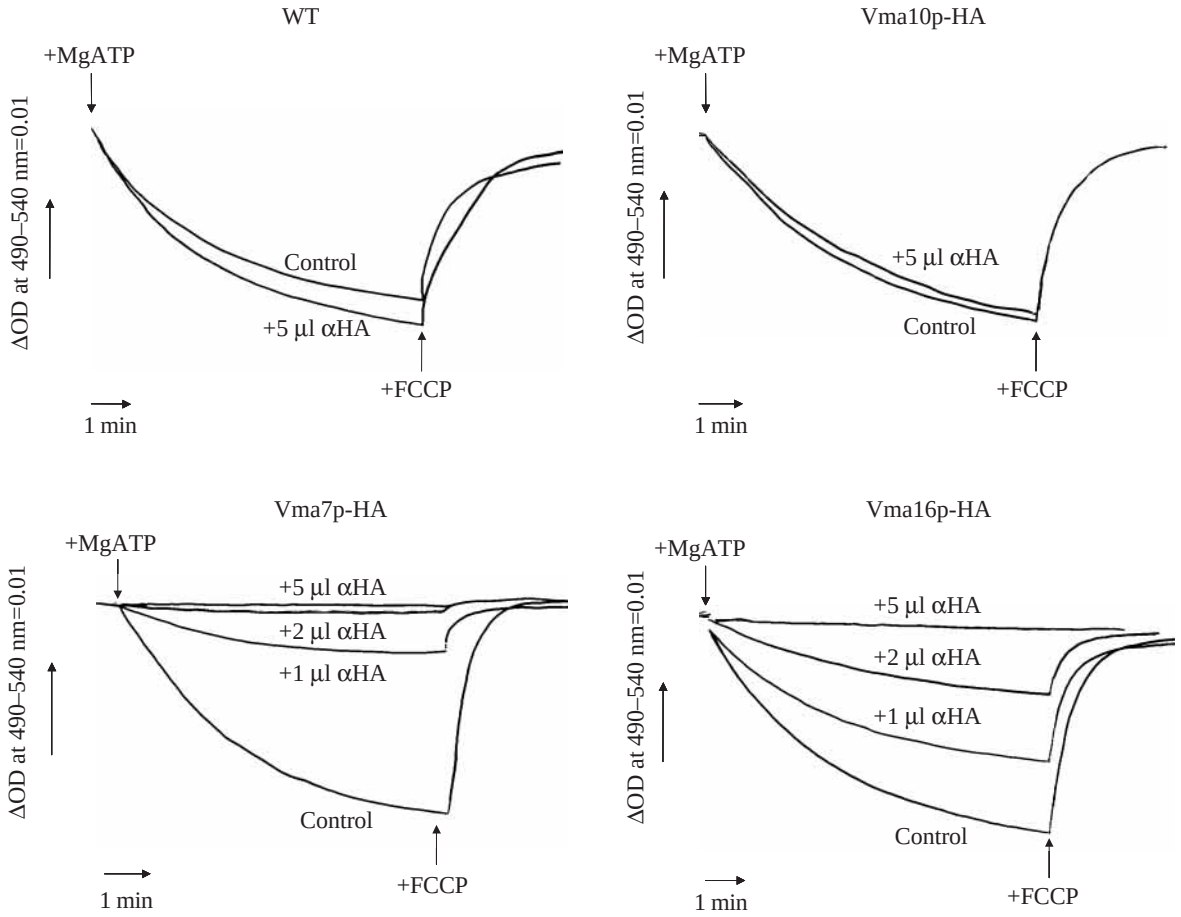

Fig. 3. The effect of the anti-HA antibody on ATP-dependent proton uptake activity in yeast expressing the HA-tagged subunits. Vacuoles were isolated from the various yeast strains and preincubated for 30 min at room temperature in 1 ml of the proton-uptake reaction mixture (see Materials and methods) in the presence or absence of 1, 2 or 5 µl of 1:1 diluted monoclonal anti-HA antibody (αHA). The ATP-dependent proton uptake activity was measured by following the decrease in absorption difference at 490–540 nm of Acridine Orange. Where indicated, 1 µmol of MgATP or 1 nmol of carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) was added to the reaction mixture. WT, wild type.

reaction was stopped and monitored. The Bafilomycin A_1-sensitive ATPase activity of the solubilized vacuolar preparation of each strain (without the antibody) served as a control (100% activity) to the same strain's preparation treated with antibody. As shown in Fig. 4, the anti-HA monoclonal antibody had no effect on the Vma10p-HA containing V-ATPase complex and even slightly increased the activity, similar to the WT yeast cells. However, there was nearly 80% inhibition of the activity of Vma16p-HA containing V-ATPase, and about 70% of the enzyme expressing the HA-tagged Vma7p, each in comparison with their respective controls.

Duplicate samples of the activity assays, without stop solution, were utilized to test the anti-HA binding to its specific tag in the solubilized V-ATPase. These samples were loaded directly on top of a glycerol density gradient and fractionated for 13 h, to allow a better separation of the unbound from the bound antibody as the excess of the unbound antibody was not washed out. 12 fractions were collected from the bottom and analyzed by western blot. The distribution of the HA-tagged subunits was detected by the use of the native anti-HA antibody as a primary antibody, while the pre-bound antibody was detected using an HRP-conjugated sheep anti-mouse Ig in the western analysis. Colocalization of the anti-HA and the anti-mouse Ig antibodies indicated successful antibody binding to the tag in the V-ATPase complex. In all tagged subunits, the anti-HA antibody and the anti-mouse Ig showed a common peak in heavier fractions, in addition to the peak of the non-specific or unbound anti-mouse Ig that was also present in the WT (results not shown).

In summary, the monoclonal antibody was able to bind to the HA-tagged subunits in intact vacuoles as well as in the solubilized enzyme. In the case of the HA-tagged Vma16p and Vma7p, binding of the antibody inhibited both the ATP-dependent proton uptake into vacuoles and the ATPase activity of the V-ATPase enzyme, yet it did not interfere with either activity in the Vma10p-HA containing V-ATPase.

The suggested model for the antibody inhibition

The fact that binding of the antibody to Vma10pHA did not affect the activity of the enzyme, yet it inhibited the complexes

with the other HA-tagged subunits, supports the suggested rotation mechanism of action in V-ATPase. By this assumption, Vma16p and Vma7p are part of the rotor and shaft, respectively, whereas Vma10p is implicated in the peripheral complex, serving as a stator that fixes the V_1 sector to the *a* subunit against which the *c*-ring is rotating.

Fig. 5 is a schematic representation of the subunits involved in the rotation within the ATPase enzyme and the alpha helix packing of the *c*-ring. In it, the putative TM1 and the C terminus of Vma16p are located on the cytoplasmic face of the membrane. The interference by the antibody bound to the HA epitopes is likely to be due to its collision with one of the other subunits that are attached to the membrane: for example, subunit *a*, which results in inhibition of rotation, hence the activity inhibition. We suggest that the binding of the antibody to Vma7p-HA inhibited activity by a similar mechanism. On the other hand, although accessible as are the above subunits, Vma10p is static during the catalysis; therefore the bound anti-HA has no effect on its V-ATPase activity.

Discussion

A major difference between V-ATPase and F-ATPase is the presence of three different proteolipid subunits *c*, *c*′ and *c*″ (Vma3p, Vma11p and Vma16p, respectively) within the V_0 sector of V-ATPase compared with a single subunit *c* in F_0. All three proteolipids are required for functional yeast V-ATPase (Hirata et al., 1997). Vma3p and Vma11p are predicted to contain four putative transmembrane segments (Mandel et al., 1988; Hirata et al., 1997). By contrast, Vma16p was predicted to have five transmembrane segments (Hirata et al., 1997). Expression of epitope-tagged forms of the V-ATPase subunit *c* and *c*″ suggested that the C terminus of subunit *c* is lumenal, whereas the C terminus of subunit *c*″ is cytoplasmic; hence the N terminus was assumed to face the lumenal side of the vacuole (Nishi et al., 2001). Interestingly, up to now, homologues of subunit *c*″ have not been isolated from plants (Sze et al., 1999). In the *Arabidopsis thaliana* database, five isoforms of genes encoding subunit Vma3p and two isoforms of Vma16p were identified (Sze et al., 2002). When we analyzed the cDNAs encoding the Vma16p subunit from *Arabidopsis thaliana* and from the lemon fruit it was apparent that the first helix is missing in those subunits, yet nevertheless they complemented the yeast null mutant *vma16Δ* (Fig. 1). All plant subunit *c* isoforms cloned so far are homologues of the yeast *VMA3* gene. However, although a functional similarity exists between the yeast and plants vacuoles (Taiz et al., 1992), there are few examples of complementation of yeast null mutants with proteolipids from heterologous sources (Ikeda et al., 2001; Aviezer-Hagai et al., 2000). Recently, it was reported that despite the relatively high similarity between the mouse and yeast Vma16p subunit, the mouse homologue, which possesses the extra first helix, albeit not conserved, did not complement the growth defect at pH 7.5 of the *VMA16* deletion strain (Nishi et al., 2001). These results imply that the strong interactions between the different *c*-ring subunits in the membrane sector are important. Therefore the mouse subunit cannot supplement the yeast corresponding mutant while the plant Vma16p can, due to less interference by ‘cross-talk’ between the various proteolipids of the yeast V_0.

Vma16p has two negatively charged glutamyl residues in the transmembrane parts, but only the one in TM2 (previously referred to as TM3), the analog of that in TM4 of Vma3p and Vma11p, is necessary for V-ATPase activity (Hirata et al., 1997). These features rendered the *c*-ring an asymmetric structure, in contrast to the potentially symmetric structure of the corresponding part in F-ATPase (Fillingame et al., 2002). From our results of antibody binding and those of others (Nishi et al., 2001, 2003), the asymmetry of the *c*-ring in V-ATPase is twofold: one is the different subunit composition and the other is the opposite membrane orientation of subunit Vma16p as compared to Vma3p and Vma11p. This puts the important catalytic glutamyl residue of Vma16p in an nonparallel helix. It was proposed that the special arrangement of the V-ATPase *c*-ring subunits is also the key to understanding the inability of the enzyme to reach thermodynamic equilibrium (Nelson et al., 2002; Moriyama and Nelson, 1988). The lemon fruit is a rare example having very low pH (pH ~2) in the vacuole, where the

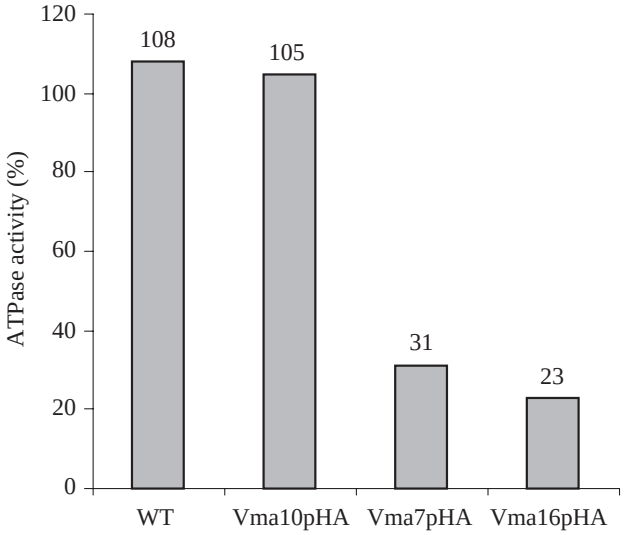

Fig. 4. The effect of anti-HA antibody on the ATPase activity in yeast expressing the HA-tagged subunits. Bafilomycin A_1-sensitive ATPase activity was measured for solubilized and fractionated vacuolar membranes with or without anti-HA antibody. Relative activity is given for each strain as compared to its specific ATPase activity without the antibody (100% activity). For each assay 50 µl (~2 µg protein) of the fraction with most active ATPase (fraction no. 5 out of the 4–6 active fractions for Vma16p-HA and Vma10p-HA; fraction no. 4 out of the 4–7 fractions for Vma7p-HA; and fraction no. 7 out of the 5–8 active fractions for wild type, WT), was preincubated in the presence and absence of 50 µl of 1:1000 (w/v) diluted anti-HA monoclonal antibody. 5 mmol l^{-1} MgATP was added, and following 10 min incubation at 30°C, the reaction was stopped and absorbance at 660 nm monitored. Each value represents a one single experiment out of three.

V-ATPase might be operating close to its thermodynamic equilibrium (Muller et al., 1999, 2002). In summary, even though there are differences in subunit composition, in catalysis and in coupling, the general structure of the two ATPases, the F- and V-ATPase, is so similar that a rotary mechanism of action is the suggested mechanism.

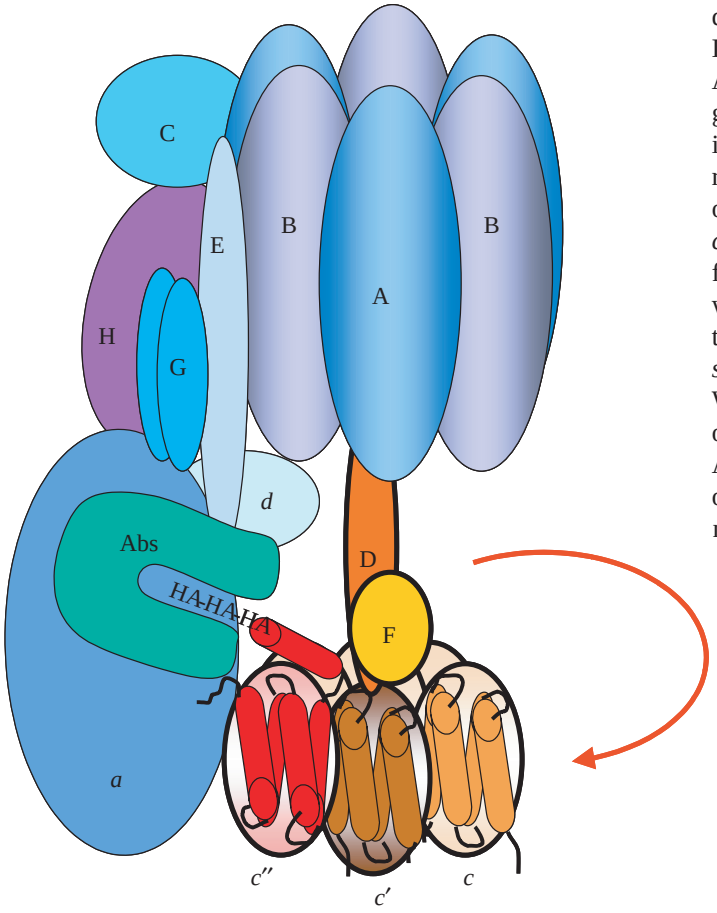

Fig. 5. Schematic representation of the effect of bound antibody to Vma16p-HA on V-ATPase activity. The V-ATPase is composed of two functional domains: the catalytic V_1 (A and B subunits) and the membranous V_0 (*a*, *c*, *c′*, *c″* and *d* subunits), which are interconnected by peripheral (C, E, G, H subunits) and central (D and F subunits) stalks, respectively. It is assumed that hydrolysis of ATP by V_1 rotates the central shaft (D and F subunits), which in turn causes the rotation of the membrane *c*-ring against its stator (E, G and N′-*a* subunits), resulting in proton translocation into the vesicle (Nelson et al., 2002; Nishi and Forgac, 2002). It is now clear that, similar to *c* and *c′* (Vma3p and Vma11p, respectively), the *c″* subunit (Vma16p) contains four TMs, although in an opposite arrangement in the membrane with both N′ and C′ pointing to the cytoplasm as shown above. The Vma16p (red) was tagged with three HA-epitopes at its N terminus. When the resulting tagged V-ATPase was incubated with anti-HA antibody, an inhibition of activity was observed. This suggests that the binding of antibody (green) to the HA-tags interfered with the rotation of the *c*-ring and shaft subunits (all presumed rotating subunits are framed with a thick line). This interference is probably due to a collision with the static subunits, and primarily with subunit *a*, against which the rotor is turning. A similar effect was observed when antibodies were allowed to bind to the Vma7p-HA (F subunit)-containing complex. This is in contrast to the effect of antibody on the Vma10p-HA (G subunit)-containing V-ATPase. Abs, bound anti-HA antibodies.

Very recently, several reports have directly demonstrated the rotational catalysis of single molecules of V-ATPase. In the thermophilic eubacterium *Thermus thermophilus* an ATP-dependent, counterclockwise rotation of beads attached to the D (Vma8p) or F (Vma7p) subunits was demonstrated when the A subunit (Vma1p) of the V_1 sector was immobilized onto a glass surface (Imamura et al., 2003). In the same manner of immobilization of the whole complex, an ATP-dependent rotation of a bead attached to a proteolipid subunit of V_o was obtained (Yokoyama et al., 2003). In the *Saccharomyces cerevisiae* V-ATPase, counterclockwise rotation of an actin filament attached to the G (Vma10p) subunit was observed when the enzyme was immobilized on a glass surface through the *c* subunit (Hirata et al., 2003). In this report we use an *in situ* biochemical approach to support the rotary mechanism. We report results of the effect of monoclonal anti-HA antibody on the ATP-dependent proton uptake activity of the tagged V-ATPase population embedded in the vacuolar membrane, and on the Bafilomycin A_1-sensitive ATPase activity of those membranes.

According to the suggested rotary mechanism, the most significant effect of antibody inhibition on a V-ATPase subunit carrying an HA-tag will be exhibited in the proteolipid subunits in the V_o sector and in the shaft subunits of V_1. The inhibition of the Vma7p-HA carrying enzyme has previously been demonstrated (Gräf et al., 1994; Nelson et al., 1994) and we used it as a control in our experiments. When we tried to tag the membrane subunits Vma3p and Vma11p with the HA-tag, we discovered that although the HA signal in total vacuolar preparations was present, the ATPase active fractions did not contain this epitope on them. As the N- and C-terminal segments of Vma3p and Vma11p are assumed to face the lumen, the HA-tag was probably degraded by proteolytic activity in the vacuole (Hirata et al., 1997). Therefore the only subunit remaining from the membrane sector for use in our assay was Vma16p. The fact that the plant Vma16p lacking the first α-helix supplemented the phenotype of the yeast mutant, led us to assume that the first α-helix of the yeast Vma16p might be outside the vacuole (as was later demonstrated by Nishi et al., 2003) and would be suitable for HA-tagging. Indeed, the tag on this subunit was retained in the active complex fractions and, as expected, the enzyme was inhibited by the anti-HA antibody. These results show for the first time that, by binding an antibody to tagged Vma16p-HA, we can inhibit the activity of the holoenzyme, in the presence of ATP.

Subunit G (Vma10p) exhibits structural homology to subunit *b* of F-ATPase (Supekova et al., 1995), which forms a stator (peripheral stalk) together with subunit *a*, and prevents the $\alpha_3\beta_3$ catalytic head from rotating when the *c*-ring and the shaft (subunits γ and ε) are rotating (Tsunoda et al., 2001). The main homology is in the N-

terminal half of these subunits, a major difference being the lack of a transmembrane domain in subunit G (Supekova et al., 1996). Further analysis showed that the N terminus of the G subunit might fold into an α helix in which one face has highly conserved residues in both the V_1 G and F_0 b subunits (Hunt and Bowman, 1997). Because of this structural resemblance, it was suggested that subunit G, together with subunit E, act like a 'hook' or a 'stator' similar to *b* in F-ATPase (Nelson and Harvey, 1999; Arata et al., 2002). Mutational analysis of the conserved face of the G subunit α helix revealed that several mutations were tolerated and even stabilized the complex, while others rendered the complex unstable (Charsky et al., 2000). Therefore it was interesting to test the effect of antibody binding to HA-tagged Vma10p. This binding does take place (Fig. 2), which means that subunit G is exposed in the V-ATPase complex, which supports previous results obtained from studies on accessibility to trypsin cleavage (Gruber et al., 2000). The bound antibody did not inhibit the activities of the Vma10p-HA-containing V-ATPase (Figs 3, 4) which is in agreement with Vma10p being a part of the stator, and not participating in the rotor apparatus, as previously suggested (Nelson and Harvey, 1999; Charsky et al., 2000; Arata et al., 2002). It appears that the G subunit is present at 2–3 copies per complex (Supekova et al., 1995; Hunt and Bowman, 1997). The large mass of the anti-HA antibody attached to the G subunits does not interfere with the enzyme's activity because it binds to the static part of the complex. On the other hand, the fact that the anti-HA antibody, while bound to Vma7p-HA and Vma16pHA, inhibited both ATP-dependent proton uptake and ATPase activities (Figs 3, 4), suggests that the two subunits are located in the rotating segment of the V-ATPase. With this in mind we suggest the model shown in Fig. 5 that depicts this steric interference of the antibody with the rotary movement mechanism of the V-ATPase complex.

It would be interesting to test the other V-ATPase subunits by the same method. Not all the HA-tagged or overexpressed V-ATPase subunits complement their corresponding null mutants, either because of problems of overexpression (Curtis and Kane, 2002), or proteolytic degradation of the tag. Nevertheless we intend to exploit the same method for analysis of other V-ATPase subunits.

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