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**Functional Biogenesis of V-ATPase in the Vacuolar
System of Plants and Fungi**

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Abstract

The vacuolar H⁺-ATPase (V-ATPase) is one of the most fundamental enzymes in nature. It pumps protons into the vacuolar system of eukaryotic cells and provides the energy for numerous transport systems. Through our BARD grant we discovered a novel family of membrane chaperones that modulate the amounts of membrane proteins. We also elucidated the mechanism by which assembly factors guide the membrane sector of V-ATPase from the endoplasmic reticulum to the Golgi apparatus. The major goal of the research was to understand the mechanism of action and biogenesis of V-ATPase in higher plants and fungi. The fundamental question of the extent of acidification in organelles of the vacuolar system was addressed by studying the V-ATPase of lemon fruit, constructing lemon cDNAs libraries and study their expression in mutant yeast cells. The biogenesis of the enzyme and its function in the Golgi apparatus was studied in yeast utilizing a gallery of secretory mutants available in our laboratories. One of the goals of this project is to determine biochemically and genetically how V-ATPase is assembled into the different membranes of a wide variety of organelles and what is the mechanism of its action. The results of this project advanced our knowledge along these lines.

New concepts of the structure and function of individual subunits in V-ATPase.

The general structure of F- and V-ATPases is quite similar. Both holoenzymes are composed of catalytic sectors, F₁ and V₁ respectively, and membrane sectors, F₀ and V₀ respectively (Nelson and Nelson, 1990; Nelson, 1992). Recent advancement in our knowledge about the mechanism of action of F-ATPase have led to a more precise definition of structure-function relations of individual subunits of the enzyme (Junge et al., 1997; Nelson and Harvey, 1999). Thus, knowledge gained in the study of F-ATPase was used to define the function of V-ATPase subunits in four parts of a mechanochemical machine: i) catalytic unit, ii) shaft, iii) hook and iv) turbine (Fig. 1). It is logical to assume that subunits A and B of the catalytic sector function in similar if not identical fashion to F-ATPase subunits β and α respectively, that subunit E and G (Vma4p and Vma10p) function in the "hook" and that the proteolipids (subunits c) function in the proton "turbine". Subunit D (Vma8p) is the best candidates for acting as the rotated "shaft" (analogous to subunit γ of F-ATPase). We initiated studies that include site directed mutagenesis and suppressor mutants to inactive mutations to analyze the above hypothesis.

Figure 1. Subunit structure of V-ATPase. The proposed organization of the various subunits is based on the assumption of common structure between F- and V-ATPases. The distribution of the various subunits in the four functional parts indicated.

The results reported in the previous year showed that Vma10p is insensitive to changes in its amino acid composition. Several replacement of amino acids did not yielded the pH sensitive phenotype of V-ATPase null mutations (Perzov et al., 1998). This finding indicate that Vma10p has very few contact sites that were not identified so far. This property is in line with its proposed assignment in the "hook" that functions in holding the A and B subunits and preventing them from rotating together with the "shaft". If the above mechanism of V-ATPase is correct the "shaft" must have a crucial function in the coupling of the ATPase and proton pumping activity of the enzyme. Therefore we analyzed subunit D (Vma8p) by site-directed mutagenesis (see Nelson et al., 1995). This subunit was assigned by us as the "shaft" that functions in the coupling of the scalar ATPase activity with the vectorial proton pumping. It is proposed that subunit D functions through a torque induced by the catalytic sector that is translated into a rotational moment at the membrane sector. This rotation drives the proton pumping across the membrane. We identified a semi-conserved amino acid sequence (NRRVN) in subunit D that may have similar function to an analog sequence in the γ subunit of F-ATPases (Fig. 2). The F₁ crystal structure suggest that this position in the γ subunit is responsible for inducing the torque (Abrahams et al., 1994; Junge et al., 1997). We designed random mutagenesis in this part of subunit D and analyzed several mutants. From 22 colonies that were analyzed, 14 were temperature sensitive at 37 °C in a medium buffered at pH 7.5. This finding prompted us to analyze the effect of several amino acid substitutions in subunit D on growth at pH 7.5. As shown in Table 1 most of the substitutions did not prevent growth at pH 7.5 but rendered the mutants temperature sensitive. These finding are in line with the proposed function of subunit D as mediator of torque between the "turbine" (proteolipids) and the catalytic sector (Nelson and Harvey, 1999).

urvey, 1999).

Figure 2. Identification of conserved amino acids sequences among various γ and D subunits.

Cloning of plant cDNAs encoding V-ATPase subunits and metal-ion transporters by complementation of yeast null mutants

It is likely that the plant V-ATPase will have similar subunit structure to the yeast and mammalian enzymes (Nelson, 1992; Nelson and Klionsky, 1996). Some of the plants genes encoding V-ATPase subunits were as yet not identified. In this part of the project we intended to clone the missing cDNAs by complementing the yeast null mutants in individual V-ATPase subunits. The yeast mutant *Δvma10* was transformed by a cDNA Arabidopsis library cloned into NotI site of the plasmid PFL61. The transformed yeast cells were grown overnight on a minimal medium without uracil. Glycerol was added to the cell suspension to give 15% V/V and aliquots were rapidly frozen and kept at -80 °C. Samples were thawed and spread on YPD plates buffered at pH 7.5 by 50 mM MOPS. Colonies that grow under this condition were grown on minimal medium lacking uracil and their plasmids were isolated by published procedures. The functionality of the cDNA was verified by transformation of yeast strains and growth analysis at pH 7.5. Sequencing of one of the positive cDNA clones revealed an amino acid sequence that is 34% identical to the yeast Vma10p. Although the conservation of this V-ATPase subunit is relatively poor plant protein effectively substitutes the yeast subunit.

One of the phenotypes of V-ATPase null mutations is their sensitivity to high and low metal-ion concentrations in the growth medium (Nelson and Harvey, 1999). Thus, V-ATPase is intimately involved in metal-ion transport. This observation led us to discover a novel gene family encoding metal-ion transporters, that is present not only in yeast but also in bacteria, plants, insects and mammalian cells (Supek et al., 1996, 1997). We undertook a similar approach to the cloning the plant homolog of VMA10 to clone a novel cDNA encoding a plant metal-ion transporter. Another member of this family (*OsNramp*) was recently cloned from *Oryza sativa* (Belouchi et al., 1997). Our cDNA encodes a related protein that complements a triple knockout yeast mutant in which the genes encoding Smf1p, Smf2p and Smf3p were interrupted. These successful complementation indicate that our approach of studying the slip mechanism in lemon fruit by complementing yeast V-ATPase null mutants is quite feasible.

Construction of cDNA library from lemon fruit

A key point in this project is the preparation of a cDNA library from lemon fruit. This part of the project faced unexpected obstacles that resulted from the low pH of the fruit and the large amounts of irrelevant material. We now overcame these obstacle and constructed a representative cDNA library. The mRNA was isolated from green lemons with a diameter of 1 to 2 cm. At this stage the fruit is not sour and its pH is higher than 4.5.

The young lemons were freshly picked up from the tree, peeled up from their skin by a sterile scalpel and frozen immediately in liquid nitrogen. The frozen lemon fruits were stored at -80 °C until used for mRNA preparation. About 5 g fruits were crushed to dust in liquid nitrogen by a sterile mortar and pestle. For the isolation of total RNA an SV Total RNA Isolation System (Promega) was used, and PolyAtract mRNA Isolation System III (Promega) was used for the isolation of poly A containing mRNA. Reverse transcriptase (Gibco BRL) was used for the synthesis of the first strand cDNA. And the cDNA was amplified by PCR with a cDNA library construction kit - Smart PCR (Clontech). Up to 7 kb DNA fragments were obtained and the DNA was size fractionated on agarose gel to 0.6-1.1 kb, 1.1-2.0 kb and 2-10 kb. The DNA fractions were incubated at 72 °C for 20 min in PCR reaction mixture containing the recommended nucleotide concentrations. The free nucleotides were removed by a spin-column containing Sephadex G-50 coarse equilibrated with 10 mM Tris-Cl (pH 7.5) and 1 mM EDTA. About 200 ng DNA of each size range were ligated into 350 ng of pGEM-easy plasmid (pGEM-easy cloning system, Promega). After ligation at 16 °C overnight the free nucleotides were removed by a spin-column containing Sephadex G-50 coarse equilibrated with 10 mM Tris-Cl (pH 7.5) and 1 mM EDTA, and the plasmids were used for the transformation of *E. coli* competent cells (Stratagene XL-2 blue). About 10⁵ independent colonies were harvested and were kept at -80 °C in the presence of 15% glycerol.

Negative-dominant mutations that induce growth at pH 7.5 in null mutants of V-ATPase

A sporadic growth of V-ATPase null mutants attracted our attention a long time ago. Following EMS treatment, several colonies in each plate were grown in a medium buffered at pH 7.5. Since the V-ATPase null mutants are constructed by the deletion of most of the reading frames of their genes, it is unlikely that the EMS treatment revived the V-ATPase and proton pumping activity of the enzyme. Moreover, experiments with quinacrine uptake by these mutant and ATP-dependent proton uptake into isolated vacuoles, demonstrated that their V-ATPase is not active (Perzov et al., 1998; Cohen et al., 1999). The mutants were denoted as *svf* mutants (Suppressor of V-ATPase Function). The large number of colonies, that grew at pH 7.5 after EMS treatment, suggests a big complex with multiple subunits as the target for mutagenesis. Several complementation groups were detected in these mutants. It is also likely but not necessary that the mutagenesis results in the inactivation of this protein complex. To analyze this possibility, a *VMA10::URA3 svf* mutant that can grow at pH 7.5 was crossed with the original *VMA10::URA3* null mutant that could not grow at pH 7.5. The resulting diploid strain was

unable to grow on a medium buffered at pH 7.5 (Fig. 3). Therefore, the mutation obtained by the EMS treatment inactivated a protein complex and the inactivation of this factor resulted in growth at pH 7.5 even when the V-ATPase is totally inactive. The presence of one intact copy of the same gene in diploid cells reversed the phenomenon and resulted in growth arrest on a medium buffered at pH 7.5. The observed phenomenon is very intriguing and if the responsible protein complex is identified, it may shed light on an important and novel cellular biology aspect of V-ATPase and the crucial function of acidification of parts of the vacuolar system.

Figure 3. The negative dominant phenotype of the EMS-generated suppressors of V-ATPase null mutants. *svf1-1* mutant was generated by EMS treatment of the V-ATPase null mutant *VMA3Δ* (MAT α) by a selection for growth at pH 7.5. It was crossed with *VMA3Δ* (MAT α) to give the diploid strain *svf1/VMA3Δ*. 1) Wild-type W303. 2) *svf1-1* 3,4) *svf1/VMA3Δ* 5) *VMA3Δ* (MAT α).

Mutation	Growth at pH 7.5	
	30° C	37° C
V8K	+	+
R12E	+	+
M13K	+	-
K19E	+	-
L22K	+	-
L31K	+	-
L32K	+	-
K35E	+	-
E37K	+	-

Table 1. Temperature sensitivity caused by amino acid substitutions in subunit D.

Subunit a - dilemma that is being solved

The cDNA encoding this subunit of approximately 100 kDa was first cloned from rat brain (Perin et al., 1991). Subunit a is composed of an N-terminal hydrophilic domain of approximately 45 kDa and a C-terminal hydrophobic domain of approximately 55 kDa, with six or seven putative transmembrane segments. The hydrophilic globular domain is facing the cytoplasmic side of the membrane (Jackson and Stevens, 1997). Only a single gene encoding this subunit was identified in mammalian cells although splicing variations have been reported (Peng et al., 1994). In yeast, subunit a is encoded by two genes that were named *VPH1* and *STV1* (Manolson et al., 1994, 1996). In contrast with all the other V-ATPase subunits, null mutation in each of the two genes could grow at high pH and with high or low calcium concentrations in the medium. However, disruption of both genes resulted in a phenotype similar to all the other V-ATPase null mutations (Manolson et al., 1996). This observation suggests that one copy of subunit a is necessary for the activity and/or assembly of the enzyme. It was proposed that Vph1p is the analog of subunit a of F-ATPases (Leng et al., 1996). The main obstacle for accepting this proposal was the clear biochemical demonstration that this subunit is not present in V-ATPases isolated from plants and insects (Sze et al., 1992; Wiczorek, 1992). Recently this dilemma was put to rest by cloning cDNA encoding this subunit in insects (Wiczorek et al., 2000), and by cloning in our laboratory cDNAs from Arabidopsis and lemon encoding the plant subunit (K. Hagai, H. Nelson and N. Nelson manuscript in preparation).

Study of yeast proteins that function in the biogenesis and assembly of V-ATPase

Selective export of newly-synthesized proteins from the ER is achieved by the capture and concentration of cargo into COPII-coated vesicles (Kuehn and Schekman, 1997; Kuehn et al., 1998). How proteins are recognized as suitable cargo is not well understood. We are interested in how assembly of a multi-subunit complex, the V_o sector of the yeast H^+ -ATPase (V-ATPase), is coupled to its export from the ER.

Three genes, *VMA12*, *VMA21* and *VMA22*, encode ER-resident proteins that are not themselves components of the V-ATPase, but instead are required for the maturation of the V_o sector into functional form. In strains lacking any one of these ER-accessory proteins (Hirata et al., 1993; Hill and Stevens, 1994, 1995), Vph1p (the 100 kD subunit of V_o) is largely retained in the ER and is rapidly degraded. Vma22p is a soluble protein that achieves its ER localization via a stable interaction with an integral membrane protein, Vma12p. The complex of Vma12p and Vma22p has been shown to interact specifically

with newly-synthesized Vph1p. The other subunits of Vo and the other accessory protein, Vma21p, appear not to be included in the Vma12p/Vma22p/Vph1p complex. It has been suggested that Vma21p may interact with the other subunits of Vo in an assembly pathway that parallels the Vma12p/Vma22p/Vph1p pathway (Graham et al., 1998). Figure 3 depicts the steps involved in the assembly of the yeast V-ATPase.

Fig. 4. Steps and subcomplexes involved in the assembly of V-ATPase

Vma21p is an integral membrane protein that possesses an amino acid sequence at its C-terminus (-KKXX) that is a well characterized Golgi-to-ER retrieval signal, and that has been shown to be required for Vma21p's steady-state localization in the ER (Hill and Stevens, 1994). The presence of this retrieval signal suggests that Vma21p cycles between the ER and the Golgi, and that it may direct the assembled Vo sector into COPII-coated vesicles that transport proteins from the ER to the Golgi.

We have employed biochemical assays for protein-protein interactions and *in vitro* assays that recapitulate the formation and delivery of COPII-coated transport vesicles to learn more about the role of Vma12p and Vma21p in V-ATPase assembly and transport. We have found that Vma12p function is restricted to the ER and that in its absence an unproductive Vo complex is assembled. We suspect that Vma12p acts as a folding chaperone for Vph1p. Unlike Vma12p, Vma21p is required for the assembly of a complete Vo complex; it interacts physically with the assembled Vo, and accompanies Vo into COPII-coated transport vesicles. These observations suggest that Vma21p serves as a

cargo receptor, coordinating Vo assembly with transport out of the ER. We speculate that Vma21p may also regulate the assembly of enzymatically active VoV₁.

Vma12p function

To determine whether Vma12p/Vma22p is required for Vo assembly, we assessed whether Vph1p could be co-immunoprecipitated in association with Vma3p, another subunit of Vo, in *vma12* and *vma22* mutants. We first confirmed the validity of our approach by analyzing the Vma3p-Vph1p interaction in wild-type and *vma11* mutant cells. Vma11p is a subunit of the Vo sector that has been shown to be required for Vo assembly. We found that, in detergent extracts from metabolically labeled wild-type cells, approximately 90% of the total Vph1p was co-immunoprecipitated with antibodies against Vma3p. As expected, less than 5% of Vph1p was co-immunoprecipitated with antibody against Vma3p in extracts prepared from *vma11* mutant cells. Surprisingly, approximately 80% of Vph1p associated with Vma3p in both *vma12* and *vma22* mutants.

If the Vo sector assembles efficiently in *vma12* and *vma22* mutants, why does it fail to be transported out of the ER and, instead, become targeted for degradation? How improperly folded/assembled proteins are distinguished from properly folded/assembled proteins is a critical unanswered question at the heart of the ER quality control system. We propose that the Vo complex assembled in the *vma12* mutant remains in the ER, in part, because it fails to present an ER-export signal that promotes efficient incorporation into COPII-coated vesicles.

We employed an *in vitro* assay for COPII vesicle formation to determine whether newly-synthesized Vph1p molecules present in the *vma12* mutant can be incorporated into COPII vesicles. Vesicle budding reactions were performed using membranes prepared from cells that had been radiolabeled for a short period of time, such that only newly-synthesized proteins would contain radiolabel. To these membranes we added various amounts of purified COPII proteins. The amount of newly-synthesized Vph1p released into vesicles was quantified. We found that low concentrations of COPII proteins supported Vph1p budding from wild-type membranes, but not from *vma12* membranes. However, high concentrations of COPII drove Vph1p budding from *vma12* membranes at levels comparable to wild-type membranes. These data suggested to us that the COPII proteins are directly involved in recognizing the properly assembled Vo complex. It is possible that the Vo complex assembled in the *vma12* mutant failed to exit the ER efficiently, because an ER signal contained in Vo is poorly presented. In the *in vitro* assay, high concentrations of COPII proteins allow the improperly assembled Vo complex possessing a weakened ER-export signal to be captured into vesicles.

Another possibility is that the Vo complex assembled on the *vma12* mutant is actively retained in the ER. *In vitro* this retention may be overcome at high concentrations of COPII. To distinguish between these two models, we plan to do experiments that assess the ability of Vph1p to enter 'pre-budding' complexes formed by COPII proteins prior to vesicle formation. If Vma12p is required for proper presentation of an ER-export signal, then pre-budding complexes should form with reduced efficiency on *vma12* membranes. However, if an ER-export signal is properly presented, but overridden by a retention mechanism, then pre-budding complexes should form as efficiently in *vma12* mutant membranes as they do in wild-type membranes.

Vma21p function

Assembly: We used the co-immunoprecipitation procedure described above to test whether Vma21p is required for Vo assembly. We found that Vph1p did not interact substantially (>5%) with Vma3p in extracts prepared from *vma21* mutant cells. However, Vma3p maintains interactions with two other Vo subunits, Vma11p and Vma16p, in the *vma21* mutant. It appears that Vma21p is required for a late stage in the assembly of Vo; bringing the proteolipid components (Vma3p, Vma11p, and Vma16p) together with Vph1p and Vma6p.

Transport: We speculated that Vma21p cycles between the ER and the Golgi, perhaps guiding the assembled Vo out of the ER. If so, we expect that: i) Vma21p enters into COPII vesicles generated *in vitro*; ii) Vma21p physically associates with the fully assembled Vo sector; iii) the Vma21p-Vo complex is enriched in COPII-coated vesicles; iv) Vma21p possesses an ER-export signal that directs it into COPII-coated vesicles.

We first performed *in vitro* COPII vesicle budding assays to determine if Vma12p or Vma21p are taken up into these transport vesicles. Consistent with a role in the folding of Vph1p, Vma12p was not incorporated into COPII-coated vesicles. Its function appears to be restricted to the ER. In contrast, Vma21p was taken into COPII-coated vesicles generated *in vitro*, consistent with a role in transport of the Vo out of the ER.

Second, we looked for proteins that interact with Vma21p using a co-immunoprecipitation protocol similar to that used to assess Vo assembly. We found that anti-Vma21p antibodies precipitated all five Vo subunits in addition to Vma21p itself. The identity of the co-immunoprecipitated proteins was confirmed by performing this experiment using strains in which the genes for the individual Vo subunits were deleted. The analysis of deletion mutants also suggested that Vma11p bridged Vma21p to the other subunits of the Vo complex.

With both the *in vitro* COPII budding reaction and the Vma21p-Vo interaction in hand, we tested whether the Vma21p-Vo complex was enriched in COPII vesicles. Enrichment of the Vma21p-Vo complex in COPII vesicles would suggest that Vma21p is not solely required for Vo assembly, but also plays a role in guiding Vo into transport vesicles. To test this possibility, we prepared membranes in which newly-synthesized proteins were radiolabeled. We then added purified COPII proteins to generate transport vesicles and separated the vesicles from the donor ER membranes by centrifugation. The percent of total Vo (Vph1p actually) associated with Vma21p in the vesicle and in the ER fractions was determined by co-immunoprecipitation. We found that the complex consisting of Vma21p and the fully assembled Vo sector was enriched two-fold in the vesicle fraction. The enrichment of the Vo sector itself into vesicles is no greater, arguing that the Vma21p-Vo complex is what is recognized as appropriate cargo for export out of the ER. Figure 4 shows the various intermediates that are involved in the biogenesis and assembly of the membrane sector Vo.

Fig. 5. The function of Vma12p, Vma21p and Vma22p in the assembly of Vo.

Cooperation

Since 1979 we established a fruitful and challenging exchange of ideas as well as the sharing of antibodies and yeast strains between the two laboratories. Due to the BARD grant, we established a common research program utilizing the unique systems available in the two

laboratories. This approach yielded new insights into the functional assembly of V-ATPase as well as the discovery of a novel family of membrane chaperones.

Dr. Schekman is highly recognized for his groundbreaking study of the yeast secretory pathway. This study later proved to be vital for understanding the secretory pathways of every eukaryotic cell including ER and Golgi transport systems as well as neurosecretion. Dr. Nelson was one of the pioneers in the study of V-ATPases and contributed to the current understanding of the structure, function and biogenesis of the enzyme, as well as its involvement in several physiological processes. The Israeli P.I. visited the US laboratory and the US P.I. visited our laboratory on January 1999. During this visit Dr. Schekman was a keynote speaker in an Israeli Cell Biology meeting and thus contributed to the advancement of science in Israel.

Conclusion

During the course of these experiments we encountered the phenomenon of *svf* mutations that circumvent the need for intact V-ATPase for growth at pH 7.5. This phenomenon presents an interesting challenge and a hidden clue for the function of V-ATPase in eukaryotic cells and the necessary metabolic factors that cause the growth arrest in the null mutants. Therefore, we set up to clone the genes whose inactivation results in growth at pH 7.5 even in the absence of active V-ATPase. We were able to show that VTC1 gene is one out of several genes that their inactivation caused V-ATPase null mutants to become *svf*.

The availability of the lemon fruit cDNA library set up experiments to unravel one of the most important aspect of this project - the mechanism of proton slip in V-ATPase. All the experimental tools are now available to address this important aspect. The cloning of the lemon subunit a of V-ATPase may shed light on the mechanism of the enzyme.

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