Rave Is Essential for the Efficient Assembly of the C Subunit with the Vacular H\(^{+}\)-ATPase*

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The RAVE complex is required for stable assembly of the yeast vacuolar proton-translocating ATPase (V-ATPase) during both biosynthesis of the enzyme and regulated reassembly of disassembled V\(_1\) and V\(_0\) sectors. It is not yet known how RAVE effects V-ATPase assembly. Previous work has shown that V\(_1\) peripheral or stator stalk subunits E and G are critical for binding of RAVE to cytosolic V\(_1\) complexes, suggesting that RAVE may play a role in docking of the V\(_1\) peripheral stalk to the V\(_0\) complex at the membrane. Here we provide evidence for an interaction between the RAVE complex and V\(_1\) subunit C, another subunit that has been assigned to the peripheral stalk. The C subunit is unique in that it is released from both V\(_1\) and V\(_0\) sectors during disassembly, suggesting that subunit C may control the regulated assembly of the V-ATPase. Mutants lacking subunit C have assembly phenotypes resembling that of RAVE mutants. Both are able to assemble V\(_1\)/V\(_0\) complexes in vitro, but these complexes are highly unstable in vivo, and V-ATPase activity is extremely low. We show that in the absence of the RAVE complex, subunit C is not able to stably assemble with the vacuolar ATPase. Our data support a model where RAVE, through its interaction with subunit C, is facilitating V\(_1\) peripheral stalk subunit interactions with V\(_0\) during V-ATPase assembly.

Vacuolar proton-translocating ATPases (V-ATPases)\(^2\) are conserved in all eukaryotic cells where they function to acidify internal organelles such as the lysosome/vacuole, Golgi apparatus, secretory vesicles, and endosomes. V-ATPases couple hydrolysis of cytoplasmic ATP to transport of protons from the cytosol into intracellular compartments. Organelle acidification is essential for a wide range of cellular processes including protein sorting in the biosynthetic and endocytic pathways; protein processing, activation, and degradation; cellular ion homeostasis; and coupled transport of small molecules (1–4). V-ATPases also have been identified in the plasma membrane of certain specialized cells where they pump protons from the cytosol out of the cell (1, 5). The structure and subunit composition of V-ATPases is very similar in all organisms from yeast to humans. They are multisubunit complexes composed of two domains. The V\(_1\) domain is a peripheral cytoplasmic complex composed of eight different subunits (subunits A–H), and it contains the sites of ATP hydrolysis. The V\(_0\) domain is an integral membrane complex that is composed of six different subunits (subunits a, d, e, c, c\(_1\), and c\(_2\)). It comprises the proton pore. The V\(_1\) and V\(_0\) domains must be structurally and functionally coupled for ATP-driven proton translocation to occur. V\(_1\) complexes that are not attached to V\(_0\) at the membrane cannot hydrolyze MgATP, and V\(_0\) complexes in the membrane that are not attached to V\(_1\) are not able to transport protons (6, 7).

The biosynthetic assembly pathway for V-ATPases is not completely understood. Free V\(_1\) and V\(_0\) complexes exist in vivo in both yeast and mammalian cells (8–11). Independent assembly of preassembled V\(_1\) and V\(_0\) subcomplexes, however, is probably not the predominant pathway for biosynthetic assembly. There is evidence that supports an integrated assembly of V\(_1\) and V\(_0\) subunits with initial association of individual V\(_1\) and V\(_0\) subunits followed by the addition of subunits from both domains (12, 13). In addition to the initial biosynthetic assembly, an important mechanism for regulation of V-ATPase activity is reversible disassembly of assembled complexes into free V\(_1\) and V\(_0\) domains in response to extracellular glucose concentrations (14). In yeast cells, glucose deprivation for as little as 5 min triggers dissociation of ~70% of V-ATPase complexes. The addition of glucose reverses this process, reassembling the V\(_1\) and V\(_0\) complexes to original levels (14). Dissoication of the V-ATPase is not unique to yeast. In Manduca sexta midgut epithelial cells, cessation of feeding during molting or starvation results in disassembly of fully assembled, active V-ATPase complexes into inactive cytosolic V\(_1\) and membrane-bound V\(_0\) complexes (6, 10). There is also recent evidence from renal epithelial cells and maturing dendritic cells that V-ATPase activity is regulated at the level of assembly in mammalian cells as well (15, 16). Reversible dissociation appears to be a widely used mechanism of V-ATPase regulation.

A novel regulator of V-ATPase assembly, the heterotrimeric protein RAVE (regulator of the H\(^{+}\)-ATPase of vacuolar and endosomal membranes), mediates both the biosynthetic assembly and the glucose-induced reassembly of the V-ATPase in yeast (17, 18). RAVE was first identified as a Skp1-containing complex that binds to free cytosolic V\(_1\) complexes (18). RAVE is a stable complex composed of three subunits: Rav1p, Rav2p, and Skp1p. rav1Δ and rav2Δ mutants display a very similar...
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**EXPERIMENTAL PROCEDURES**

**Materials and Growth Media**—Oligonucleotides were synthesized by MWG Biotech. TaKaRa LA-Taq was purchased from Fisher, and Pfu I DNA polymerase was from Stratagene. Zymolyase 100T was from MP Biomedicals, and concanamycin A was from Wako Biochemicals. Restriction enzymes and other molecular biology reagents were from New England Biolabs. Alkaline phosphatase-conjugated secondary antibody and pGEM-T Easy TA cloning vector were from Promega. Horseradish peroxidase-conjugated c-Myc (9E10):sc-40 was from Santa Cruz Biotechnology. Yeast and _Escherichia coli_ media were from Fisher. All other reagents were purchased from Sigma. Yeast cells were grown in yeast extract – peptone – 2% dextrose (YPD) medium or fully supplemented minimal medium (SD) lacking individual nutrients as described previously (22). For growth and testing of _vma_ mutant strains, YEPD was buffered to pH 5.0 or 7.5 with 50 mM potassium phosphate, 50 mM potassium succinate.

**Strain Construction**—All of the strains were derived from SF838-5A (23) or from the two-hybrid strains PJ69-4A and PJ69-4a (24). Genotypes of strains used in this study are listed in Table 1, and oligonucleotides used to construct the strains are listed in Table 2. To delete the VMA5 gene we used oligonucleotides VMA5-600/VMA5+350 to amplify _vma5A::kanMX6_ from the BY4741 _vma5A::kanMX6_ deletion strain (25). The _vma5A::kanMX6_ PCR product was introduced into the SF838-5A strain using a modified lithium acetate transformation protocol (26), and transformants were selected on 200 μg/ml G418 and tested for integration by PCR and by testing for the Vma^- phenotype (lack of growth at pH 7.5 and 60 mM CaCl2 at elevated temperatures (33–37 °C) (18). Consistent with this phenotype, raviΔ mutants have a loss of quinacrine staining of the vacuole at elevated temperatures (37 °C), indicative of a loss of vacuolar acidification. Even at the permissive temperature of 30 °C, the raviΔ mutant shows a kinetic delay in reassembly of V-ATPase complexes after the readdition of glucose to glucose-deprived cells (18). Vacuoles isolated from cells lacking RAVE subunits have very low levels of V1 subunits and V-ATPase activity even when cells are grown in glucose at the permissive temperature (30 °C) (17). RAVE was shown to bind to V1 released from the vacuolar membrane by glucose deprivation and to release V1 upon glucose readdition (17). The RAVE complex appears to be aiding cytosolic V1 complexes to assemble with V0 at the membrane. The interaction between RAVE and V1 is lost in mutants lacking two V1 subunits, E and G (17). These two subunits, present in at least two copies/V1 complex, are believed to form one or more peripheral stalks connecting the catalytic head group and the V0 sector. Subunit C is another V1 peripheral stalk component that is associated with subunits E and G of the V1 complex and subunit a of the V0 complex in the assembled V-ATPase (19). Studies in yeast have produced genetic, biochemical, and structural data supporting a role for subunit C in both the stability of the V1/V0 holoenzyme and in regulation of reversible assembly/disassembly of the complex (20, 21).

In this work we provide evidence that RAVE interacts with subunit C in the cytosol and that this interaction is independent of RAVE/V1 binding. In the absence of RAVE function, subunit C is not able to stably assemble with the V1 and V0 subcomplexes of the V-ATPase at the vacuole. Our results support a model in which RAVE influences V-ATPase peripheral stalk subunit interactions via a chaperone type assembly function.

**TABLE 1**

Genotypes of strains used in this study

| Strain           | Genotype                                      | Source       |
|------------------|-----------------------------------------------|--------------|
| SF838-5A         | MATα ura3-52 leu2-3,112 his4-519 ade6         | Ref. 23      |
| SF838-5A         | HA-VMAS                                      |              |
| SF838-5A         | raviΔ HA-VMAS                                 | This study   |
| SF838-5A         | RAV1-Myc13 vma5Δ                              | This study   |
| SF838-5A         | RAV1-Myc13 HA-VMAS                            | This study   |
| SF838-5A         | RAV1-Myc13 vma5Δ                              | This study   |
| SF838-5A         | RAV1-Myc13 HA-VMAS                            | This study   |
| SF838-5A         | RAV1-Myc13 vma5Δ                              | This study   |
| SF838-5A         | MA2-GFP raviΔ                                 | This study   |
| SF838-5A         | MA2-GFP vma5Δ                                 | This study   |
| SF838-5A         | MA2-GFP vma10Δ                                | This study   |
| SF838-5A         | MA2-GFP raviΔ                                 | This study   |
| SF838-5A         | MA2-GFP vma5Δ                                 | This study   |
| SF838-5A         | MA2-GFP vma10Δ                                | This study   |
| SF838-5A         | MA2-GFP raviΔ                                 | This study   |
| SF838-5A         | MA2-GFP vma5Δ                                 | This study   |
| SF838-5A         | MA2-GFP vma10Δ                                | This study   |
| SF838-5A         | MA2-GFP raviΔ                                 | This study   |
| SF838-5A         | MA2-GFP vma5Δ                                 | This study   |
| SF838-5A         | MA2-GFP vma10Δ                                | This study   |
| PJ69-4A          | MATα trp1-901 leu2-3,112 his3-200 gal4Δ lys2Δ GAL1-HIS3 GAL2-ADE2 met2Δ2,7- lacZ | Ref. 24      |
| PJ69-4a vma4Δ    | MATα trp1-901 leu2-3,112 his3-200 gal4Δ lys2Δ GAL1-HIS3 GAL2-ADE2 met2Δ2,7- lacZ | This study   |
| PJ69-4A vma10Δ   | MATα trp1-901 leu2-3,112 his3-200 gal4Δ lys2Δ GAL1-HIS3 GAL2-ADE2 met2Δ2,7- lacZ | This study   |
| PJ69-4a vma10Δ   | MATα trp1-901 leu2-3,112 his3-200 gal4Δ lys2Δ GAL1-HIS3 GAL2-ADE2 met2Δ2,7- lacZ | This study   |
| PJ69-4a vma10Δ   | MATα trp1-901 leu2-3,112 his3-200 gal4Δ lys2Δ GAL1-HIS3 GAL2-ADE2 met2Δ2,7- lacZ | This study   |
CaCl₂). A single HA tag (YPYDVPDYA) was fused to the N terminus of VMA5 to generate the integrated HA-VMA5 strain using a fusion PCR protocol in which two individual PCR products were first produced using oligonucleotide pairs VMA5-600/VMA5-HT2 and VMA5-HT/VMA5+350 with genomic DNA as template. This was followed by a second PCR using oligonucleotides VMA5-600/VMA5+350 and the two PCR products previously produced and gel-purified as templates. The resulting fusion PCR product was transformed into the SF838-5A vma5Δ strain, and colonies able to grow on YEPD pH 7.5 plates were selected. The integrated HA-VMA5 was confirmed by DNA sequencing. To delete VMA4 in the RAV1-Myc13 and RAV1-Myc13 HA-VMA5 strains, the VMA4+200/ VMA4+200 oligonucleotide pair was used to PCR amplify vma4Δ::URA3 from the SF838-5A vma4Δ::URA3 (9) strain. The vma4Δ::URA3 fragment was used to transform the RAV1-Myc13 and the RAV1-Myc13 HA-VMA5 strains as described above and transformants were selected on supplemented minimal plates lacking uracil (SD −ura), followed by testing for integration by PCR and by testing for lack of growth on pH 7.5 and 60 mM CaCl₂. We used a previously constructed SF838-5A VMA2-GFP (27) strain to introduce the GFP tag at the C terminus of VMA2 in the SF838-5A raviΔ, SF838-5A vma5Δ and SF838-5A vma10Δ strains as described (28). The VMA4 open reading frame was deleted in the PJ69-4A and PJ69-4α strains by integrating a vma4Δ::URA3 PCR fragment as described above in the RAV1-Myc13 strains. The VMA10 open reading frame was deleted in a similar manner using the oligonucleotide pair VMA10-2/VMA10-200 to amplify the vma10Δ::URA3 PCR fragment from the SF838-5A vma10Δ::URA3 strain (12). Transformants were selected on supplemented minimal plates lacking uracil (SD −ura), and disruption of the VMA4 or VMA10 genes was confirmed by PCR and phenotype testing as described above.

**Two-hybrid Analysis**—To introduce RAV1 and RAV2 into the pAS2 two-hybrid plasmid (29), RAV1 and RAV2 were amplified from wild type genomic DNA with primer pairs RAV1NBamHI/YJR9 and RAV2NBamHI/YDR9, respectively. BamHI sites were introduced just upstream of the ATG start codon. The resulting PCR fragments were cloned into the pGEM-T Easy TA cloning vector and sequenced for accuracy. RAV1 and RAV2 inserts were excised using BamHI and Sall and cloned into the BamHI/Sall cleaved pAS2 vector. A similar method was used to clone RAV1, RAV1, RAV2, and VMA10 into the pACT2 (29) two-hybrid vector with the BamHI sites in frame with the activation domain of the pACT2 plasmid and using primer pairs RAV1NBamHI/YJR9, RAV2NBamHI/ YDR9, VMA4BamHI/VMA4+200, and VMA10NBamHI/ VMA10-2, respectively. PCR inserts were cloned into the pGEM-T Easy vector and sequenced, and inserts were excised with BamHI and Sall and cloned into the BamHI/XhoI cleaved pACT2 vector. To clone VMA5 into the pACT2 vector, the oligonucleotide pair VMA5-5′+3′ was used to amplify VMA5 from wild type genomic DNA and processed as above except that BamHI and XhoI were used to excise the VMA5 insert from the pGEM-T Easy vector and cloned into the BamHI/XhoI cleaved pACT2 vector. pAS2 plasmids were introduced into the two-hybrid reporter strains PJ69-4A (MA7a), PJ69-4A vma4Δ, and PJ69-4A vma10Δ, and pACT2 plasmids were introduced into PJ69-4α (MATa), PJ69-4α vma4Δ, and PJ69-4α vma10Δ reporter strains as described above, and transformants were selected on SD plates lacking tryptophan or leucine for selection of the pAS2 and pACT2 plasmids, respectively. To test for two-hybrid interactions, a MATa strain containing a pAS2 plasmid construct was crossed to a MATa strain containing a pACT2 plasmid and diploids were selected on SD −trp, −leu. The resulting diploid strains were tested for two-hybrid interaction by plating onto SD plates lacking tryptophan, leucine, adenine and histidine (SD −trp −leu −ade −his) (24).

**Immunoprecipitations and Immunoblots**—For immunoprecipitations of Rav1pMyc with HA-Vma5p and Vma2p, cytosolic fractions were obtained from the Rav1p-Myc HA-Vma5p strains as described (30). Briefly, the cells (100 A₆₀₀ of each strain) were lysed by agitation with glass beads, and cytosol was obtained by centrifugation for 30 min at 100,000 × g in a Beckman TLA-100 ultracentrifuge. Protein concentrations of the cytosolic fractions from the various strains were measured by Lowry assay (31); 0.4 mg of protein was directly precipitated with trichloroacetic acid, and 4.0 mg was combined with 100 μl
of anti-Vma2p monoclonal antibody (13D11) or 6 μl (~30 μg) anti-HA (16B12) monoclonal antibody followed by the addition of 60 μl of a 50% suspension of protein A-Sepharose CL4B. The trichloroacetic acid-precipitated and immunoprecipitated proteins were solubilized at 75 °C in cracking buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% SDS, 5% β-mercaptoethanol) for analysis on SDS-PAGE and immunoblotted as described below.

Other Biochemical Techniques—Vacuolar vesicles were isolated as described (32). ATP hydrolysis activity was determined at 37 °C using a coupled enzyme assay (33) in the presence and absence of 100 nm concanamycin A. For immunoblotting, vacuolar vesicles were solubilized in cracking buffer. For all immunoblots, the samples were separated by SDS-PAGE and then transferred to either nitrocellulose or polyvinylidene difluoride membranes. The blots were probed with monoclonal antibodies 13D11, 7A2, and 10D7 against V1 subunits B and C and V0 subunit α, respectively (34). Primary antibodies were bound by alkaline phosphatase-conjugated goat anti-mouse secondary antibody and detected by colorimetric assay as described (35). Western blot signals were quantitated using Image J (National Institutes of Health). Rav1p-Myc was detected with mouse monoclonal antibody 9E10 or horseradish peroxidase-conjugated 9E10 antibody. The antibodies were detected on blots either as described above or by using ECL detection reagents from Amersham Biosciences to detect the primary horseradish peroxidase-conjugated 9E10 antibody.

Microscopy and Immunofluorescence—Strains carrying the VMA2-GFP construct were grown in YEPD at 30 °C to 1.0 A600/ml, and GFP fluorescence was observed on a Zeiss Axioskop II microscope under fluorescein fluorescence optics. The images were captured using a Hamamatsu CCD camera. Indirect immunofluorescence microscopy was performed as described (32). Anti-Vma2p (13D11) and anti-HA monoclonal antibodies were used at dilutions of 1:10 and 1:100, respectively. Secondary Alexa Fluor 488 goat anti-mouse IgG (2 μg/μl) was used at a 1:200 dilution. The cells were visualized by fluorescence microscopy as described above.

RESULTS

RAVE Binds to Subunit C—The binding of RAVE to V1 is almost completely lost in the vma4Δ and vma10Δ mutants, which lack subunits E and G, respectively (17). Based on these results we hypothesized that RAVE binds to the stalk region of the cytosolic V1 complex, specifically subunits E and/or G. To examine further the nature of RAVE/V1 binding, we looked at pairwise interactions between RAVE subunits and V1 subunits in two-hybrid assays (see “Experimental Procedures”). We found that Rav1p interacts with Rav2p and Skp1p of the RAVE complex and Vma4p (subunit E), Vma10p (subunit G), and Vma5p (subunit C) of the V1 complex. Rav2p interacts with Rav1p and Vma5p, but not with Skp1p, Vma4p, or Vma10p (Fig. 1A). The remaining V1 subunits (Vma1p, Vma2p, Vma7p, Vma8p, and Vma13p) did not interact in two-hybrid tests with Rav1p or Rav2p (data not shown).

The disruption of the RAVE/V1 interaction in subunit E and G deletion strains (17) predicted positive interactions in the two-hybrid assay between RAVE and subunits E and/or G, but the RAVE interaction with subunit C was surprising. As noted above, subunit C is released from both V1 and V0 domains during disassembly. Subunit C is isolated only at very low levels with cytosolic V1 subcomplexes from glucose-deprived cells or yeast cells containing only V1 subcomplexes because of a deletion of a V0 subunit (36). An interaction between RAVE and subunit C raises the question as to whether this interaction is independent of the RAVE/V1 interaction. It is possible that the two-hybrid interactions between the RAVE subunits and Vma5p are not due to direct binary interactions but instead result from other V1 subunits bridging the interaction. To address this question we tested these interactions in two-hybrid
between Rav1p and subunit C in vivo. Immunoprecipitation with anti-Vma2p (V1) in a vma5Δ strain is able to pull down Rav1p-Myc, demonstrating that RAVE can interact with V1 in the absence of its interaction with subunit C (Fig. 2A, right panel, first lane).

The two-hybrid data above suggested that the Rav1p/subunit C interaction occurs independent of the RAVE/V1 interaction. To verify this in vivo, we deleted the VMA4 gene in the Rav1p-Myc HA-Vma5p strain so that we could repeat the immunoprecipitation in a background where RAVE is not able to interact with V1. Fig. 2B (right panel, center lane) shows that HA-Vma5p coimmunoprecipitates Rav1p-Myc in a vma4Δ strain, confirming that RAVE can interact with subunit C in the cytosol independent of its interaction with V1. Loss of coimmunoprecipitation of this same strain with anti-Vma2p (subunit B) verifies that Rav1p does not interact with V1 when VMA4 is deleted (Fig. 2B, right panel, last lane).

As noted above, stalk subunit C is required for activity and proper assembly of the yeast vacuolar ATPase, but after in vivo dissociation of the V-ATPase induced by glucose deprivation, very little subunit C is bound to either V1 or V0 (14, 40). Yeast strains lacking subunit C (vma5Δ) have a strong Vma+ phenotype characterized by failure to grow at pH 7.5 and high Ca2+ concentrations. vma5Δ mutants are distinct from most other V1 subunit deletion strains, however, in that both V1 and V0 subcomplexes assemble (9). Additionally, isolated vacuoles from vma5Δ strains, although they have no V-ATPase activity, do have low levels of V1 subunits at the vacuole (40). An interaction between RAVE and subunit C suggests that RAVE may be involved in establishing a functional interaction between subunit C and the V-ATPase.

**RAVE and Subunit C Stabilize V-ATPase Assembly**

To further characterize and compare assembly defects of V-ATPase complexes in rav1Δ and vma5Δ mutants, we isolated vacuolar vesicles from these strains and also from a vma3Δ strain that is not able to assemble V0 or V1/V0 complexes at the vacuolar membrane (34). Immunoblots of the

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**FIGURE 2. Interaction of cytosolic RAVE with V1 and subunit C in wild type, vma4Δ, and vma5Δ Cells.** A, cytosolic fractions were prepared from a strain carrying a C-terminal Myc-tagged RAV1 and a vma5Δ mutation (Rav1p-Myc vma5Δ) or an N-terminal HA-tagged VMA5 (Rav1p-Myc HA-Vma5p). Cytosolic proteins (4 mg) from both strains were immunoprecipitated separately with an antibody against the HA epitope (16B12) or a monoclonal antibody against subunit B (13D11). Immunoprecipitates were solubilized in cracking buffer, separated by SDS-PAGE, and immunoblotted with an antibody against the Myc epitope (9E10). Cytosolic fractions were prepared from a Rav1p-Myc vma4Δ strain and from a Rav1p-Myc HA-Vma5p vma4Δ strain. Protein was directly trichloroacetic acid-precipitated from 0.4 mg of cytosol (input) from each strain, and 4 mg of cytosol from each strain was immunoprecipitated separately with an antibody against the HA epitope (16B12) or a monoclonal antibody against subunit B (13D11). Myc-tagged Rav1p was detected as in A.

strains that lacked either subunit E or subunit G; loss of these subunits disrupts the RAVE/V1 interaction. Rav2p is able to interact with subunit C in these deletion strains (Fig. 1B). Similar tests with Rav1p were not possible because we were unable to make the appropriate diploid in a vma5Δ background. Overexpression of either Rav1p or Vma5p is somewhat toxic (37, 38), and this toxicity may be exacerbated in the vmaΔ strains. Nevertheless, the results suggest that the interaction between Rav2p and subunit C does not occur via a bridging interaction with V1 subunits and that the RAVE/V1 and RAVE/subunit C interactions can occur independently.

**RAVE Binds Subunit C Independent of V1 in Vivo**—Based on the two-hybrid data presented in Fig. 1A above and on previous immunoprecipitation data in strains deleted for individual RAVE and V1 subunits, Rav1p appears to connect Skp1p, Rav2p, and V1 (17, 18, 39). To confirm a RAVE/subunit C interaction in vivo, we tested the ability of Rav1p to coprecipitate subunit C. A strain that expressed Vma5p containing a single HA epitope tag at the N terminus was constructed (see “Experimental Procedures”). HA-VMA5 was integrated into the yeast genome of a previously constructed Rav1p-Myc strain (17) creating a strain in which the sole copies of both VMA5 and RAV1 are epitope-tagged. This strain has a wild type growth phenotype and a V-ATPase complex with wild type assembly and activity properties in isolated vacuolar vesicles (data not shown). A cytosolic protein extract from the Rav1p-Myc HA-Vma5p strain was immunoprecipitated with anti-HA under native conditions, blotted, and probed with anti-Myc. Fig. 2A (left panel, second lane) shows that HA-Vma5p coimmunoprecipitates Rav1p-Myc, confirming an interaction...
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isolated vesicles shown in Fig. 4A indicate that although both rav1Δ and vma5Δ vesicles have levels of V0 subunit a approximately equal to wild type vesicles, they have much lower levels of V1 subunits A and B. The rav1Δ mutant vesicles, however, have significantly higher levels of subunits A and B than the vma5Δ vesicles, and also a small amount of subunit C can be detected in rav1Δ vesicles. By quantitating bands on immunoblots containing different dilutions of wild type and mutant vesicles (Fig. 4B), we were able to estimate that rav1Δ vesicles have ~9.3% of the V1 B subunit when compared with wild type and less than 2% of the C subunit. vma5Δ vesicles have less than 2% of subunit B compared with wild type. These results correlate well with previous observations in rav1Δ and vma5Δ mutant vesicles (17, 40). Although the Vma2p-GFP data described above indicate that V1/V0 is able to assemble in rav1Δ and vma5Δ mutants in vivo, these complexes are highly unstable in vitro because very small percentages of V1 subunits are found in isolated vacuolar vesicles from these strains, and V-ATPase activity is extremely low in isolated vesicles from these mutants. rav1Δ V-ATPase activity is 5.3%, and vma5Δ is 2.0% of wild type activity. Although the assembly defects in the rav1Δ and vma5Δ strains have striking similarities, rav1Δ mutants have more V1 subunits in isolated vesicles, including a small amount of subunit C, when compared with vma5Δ vesicles, and rav1Δ mutants are able to produce a population of V-ATPase complexes in vivo that provide some function at the permissive temperature of 30 °C. A similarity in V-ATPase assembly phenotypes in RAVE mutants and subunit C mutants could be explained by a loss of functional association of subunit C with the V-ATPase in the absence of RAVE.
RAVE Is Required for Subunit C Binding at the Vacuole—The localization of Vma2p-GFP in a rav1Δ strain shown in Fig. 3 indicates that a fairly significant amount of V₁ subunits assemble at the vacuole in vivo. However, isolated vacuoles from the rav1Δ mutant strain shown in Fig. 4B have only 9.3% of subunit B compared with wild type vacuoles. Presumably the unstable V-ATPase complexes in the rav1Δ mutant do not remain intact through the vacuole vesicle isolation process, and significantly, the fraction of subunit C in rav1Δ vesicles is less than 20% of the fraction of subunit B found in rav1Δ compared with wild type vesicles (Fig. 4B). This result suggests that subunit C is depleted from V-ATPase complexes in rav1Δ mutants.

To compare the localization of subunit B and subunit C in the rav1Δ mutant in vivo, we examined these subunits using indirect immunofluorescence microscopy. Subunit B can be detected by the monoclonal antibody 13D11 in immunofluorescence, and Vma5p was visualized via an HA epitope (14). As shown in the top row of Fig. 5A, subunit B localizes predominantly at the vacuolar membrane in wild type cells. The bottom row in Fig. 5A shows significant staining of subunit B at the vacuolar membrane in rav1Δ cells with some cytosolic staining as well. This agrees with VMA2p-GFP results shown in Fig. 3. In wild type cells subunit C (HA-Vma5p) displays essentially the same staining pattern at the vacuolar membrane as subunit B. Conversely, subunit C in rav1Δ cells shows a diffuse cytosolic staining pattern and little or no vacuolar localization (Fig. 5B, bottom row). This result suggests that RAVE is required for subunit C to assemble efficiently with the vacuolar ATPase. This experiment also suggests that the depletion of subunit C from the vacuole in rav1Δ mutants (Fig. 4B) is not caused by loss of subunit C from the cell. To confirm that subunit C was not destabilized in the rav1Δ mutant, we compared the total cell levels of HA-tagged subunit C in wild type and rav1Δ mutant cells by immunoprecipitation with the anti-HA antibody and immunoblotting. Very similar levels of HA-tagged subunit C are present in the two cell types (data not shown).

DISCUSSION

RAVE and Subunit C Stabilize V-ATPase Stator Stalk Assembly—The prior identification of subunits E and G as mediators of the RAVE-V₁ interaction suggested an involvement of the RAVE complex in assembling the V₁ peripheral stalk to the membrane-bound V₀ complex. The results reported here provide additional support for the notion that RAVE, through its interaction with subunit C, may be facilitating V₁/V₀ peripheral stalk subunit interactions during V-ATPase assembly. There is significant homology between the V-ATPase and the bacterial and mitochondrial F₁F₀-ATP synthase (F-ATPase) in both structure and mechanism of action (1). Both the V-ATPase and the F-ATPase utilize a rotational catalytic mechanism (41). In the V-ATPase, ATP hydrolysis on the V₁ A subunits drives rotation of the central stalk that is composed of the V₁ subunits D and F and the V₀ subunits d and the proteolipid ring (41, 42). Proton translocation occurs at the interface between the rotating proteolipid ring and the fixed V₀ a subunit. For proper coupling of ATP hydrolysis at the catalytic sites and proton transport across the membrane, a stator structure is required that prevents rotation of the static domain (43). Numerous studies have shown that the stator of the E. coli F-ATPase is composed of a dimer of the b subunit of F₀ (44). V-ATPase subunit G shows some homology to subunit b of the F-ATPase, and subunit G has been shown to interact strongly with subunit E (45–47). The V-ATPase E-G complex could be functioning in a manner similar to the b dimer of the F-ATPase, but it is notable that unlike the b subunit, subunit G has no membrane domain. Unlike the F-ATPase, the activity of the V-ATPase is down-regulated by reversible dissociation of the V₁ and V₀ domains of the enzyme in response to low extracellular glucose, and also unlike the F-ATPase, the dissociated V₁ and V₀ complexes are enzymatically inactive (6, 7). The role of the stator stalk (or stalks) of the V-ATPase, therefore, is 2-fold. It not only must act to stabilize the interaction between the V₁, and V₀ sectors but also must allow for the regulated disassembly/reassembly of the enzyme. Most of the V-ATPase subunits
have functional homologues (although not necessarily sequence homologues) in the F-ATPase. The exceptions are V-ATPase peripheral stalk V1 subunits C and H and V0 subunit d and the cytoplasmic domain of subunit a.

Recent studies have implicated V1 subunit C as part of the stator holding the V1 and V0 domains together and as the primary regulator of reversible dissociation of the complex. Based on cysteine-mediated cross-linking, subunit C binds to key subunits of the peripheral stalk and to subunit a of the V0 complex (19). This localization of subunit C has been confirmed by immunolectron microscopy that shows subunit C bound to the stalk region at the V1/V0 interface (21). Interestingly, subunit C binds on the opposite side of V1V0 from subunit H, the other V1 subunit implicated in bridging subunits E and G of V1 and V0 subunit a (21, 48). This arrangement suggests that the two copies of subunits E and G (45) might be present as two distinct EG stalks, one bridged to V0 by subunit H and the other by subunit C. In yeast, subunit C deletion (vma5Δ) mutants display a typical V-ATPase null phenotype, but a closer examination of the vma5Δ mutant reveals characteristics that distinguish it from other V1 subunits. In most V1 deletion mutants the V1 subcomplex is not able to assemble in the cytosol, whereas vma5Δ mutants can assemble both V1 and V0 subcomplexes (12). In this study we show that in a vma5Δ mutant a significant portion of a GFP-tagged V1 subunit is able to bind V0 at the vacuolar membrane in vivo. These V1V0 complexes, however, are not stable in in vitro isolations and do not support V-ATPase activity. We conclude from these data that a non-functional V1V0 complex is able to form in the absence of subunit C. RAVE mutants, rav1Δ and rav2Δ, show many similarities to vma5Δ mutants. A GFP-tagged V1 subunit in a rav1Δ mutant shows a localization that is very much like that seen in cells lacking subunit C. RAVE mutants have a partial Vma phenotype that supports some assembly and some activity of the V-ATPase in vivo in contrast to the strong Vma phenotype of vma5Δ mutants. Interestingly, a number of subunit C mutations exhibit growth phenotypes that are identical to rav mutants; that is they do not have a strong Vma phenotype in vivo, but the V-ATPase complexes show assembly defects in vitro (49). The similarity in defects in subunit C function and RAVE function suggested to us that RAVE might be involved in the attachment of subunit C with the peripheral stalk of the V-ATPase.

One notable difference between the rav1Δ mutant and the vma5Δ mutant is the temperature conditional nature of the Vma phenotype in the rav1Δ mutant. We cannot explain this difference at present. It may be that there is a more stringent requirement for V-ATPase function at high temperature, so that the rav1Δ mutant, which retains a low level of V-ATPase activity (Fig. 4A), can support sufficient V-ATPase function at 30 °C, but not at 37 °C. It could also be that there is an alternate,
inefficient route of C subunit assembly in the \textit{rav1}\Delta mutant that is functional at 30°C but not at 37°C or that the C subunit assumes a conformation at 37°C that makes it less readily assembled in the absence of RAVE. Further experiments will be necessary to distinguish these possibilities.

RAVE \textit{Is Required for Efficient Assembly of Subunit C with the V-ATPase}—Vacuolar vesicles isolated from a \textit{rav1}\Delta mutant strain show lower levels of all \(V_1\) subunits, but Vma5p is present at significantly lower levels than the other \(V_1\) subunits. This suggests that in the absence of RAVE function, the association of subunit C with \(V_1\) is defective. Further evidence for this assumption is provided in the immunofluorescent localization of HA-Vma5p in a \textit{rav1}\Delta strain. We show in Fig. 5 that although a significant fraction of \(V_1\) subunit B is found at the vacuolar membrane in a strain lacking Rav1p, subunit C localizes to the cytosol and shows no staining at the vacuole. Our data support a model in which RAVE is directly involved in the attachment of subunit C to the peripheral stalk either just prior to or during the assembly of the \(V_1\) complex with the membrane-bound \(V_o\).

RAVE \textit{Delivers Subunit C to \(V_1/V_o\) in Biosynthesis and Reassembly}—Both biosynthetic assembly of the enzyme and reversible disassembly of the assembled complexes establish the overall level of assembly of the V-ATPase. The RAVE complex is required for stable V-ATPase assembly in both processes, but where and when in the assembly process do \(V_1\), subunit C, and \(V_o\) come together? Does RAVE play a similar role in the assembly of the C subunit in both biosynthetic assembly and regulated reassembly? A clue to the assembly function of RAVE was provided by the independent isolation of a mutation in Rav1p (\textit{soi3-1}) that suppresses the mislocalization of a Kex2p mutant with a defective \textit{trans}-Golgi network localization signal (50). These studies identified a membrane-bound fraction of Rav1p in addition to the cytosolic fraction. In sucrose gradient fractionation, the membrane-associated Rav1p cofractionated in a dense fraction characteristic of early endosomes and localization of Rav1p-GFP was also consistent with localization to early endosomes (50). The conclusion from this work was that acidification of early endosomes was required for proper localization of \textit{trans}-Golgi network proteins and that this acidification is regulated at the level of assembly of the V-ATPase. Therefore, a mutation in Rav1p (\textit{soi3-1}) resulted in defective V-ATPase assembly at the early endosome. One possibility is that the fraction of Rav1p that is in the early endosome is functioning in the initial biosynthetic assembly of the V-ATPase, and the Rav1p fraction in the cytosol is involved in the regulated reassembly of disassembled V-ATPase complexes. The biochemical function of the RAVE complex could be the same in both places, and based on our current findings, RAVE is likely “chaperoning” or directing subunit C to properly orient the peripheral stalk subunits of the \(V_1\) and \(V_o\) complexes and assemble a stable active V-ATPase (Fig. 6).

Why \textit{Would Subunit C Need a Specialized Chaperone}?—If there are two peripheral stalks, as suggested by electron microscopy (21), then forming distinct interfaces with the \(V_o\) sector may require RAVE intervention during biosynthesis. In addition, RAVE may serve to keep subunit C away from cytosolic \(V_1\) sectors. If subunit C is, as has been proposed, the regulator of reversible dissociation of the V-ATPase, a chaperone may be needed to ensure that \(V_1\) and \(V_o\) functionally interact only when conditions in the cell require an active V-ATPase.

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