Evidence that there are two copies of subunit c'' in V₀ complexes in the vacuolar H⁺-ATPase

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The proton-translocating core of eukaryotic vacuolar H⁺-ATPase (V-ATPase), V₀ consists of a hexameric arrangement of transmembrane α -helices formed from the related polypeptides, subunit c and subunit c''. The former is comprised of four transmembrane α -helices, whilst the latter has an extra transmembrane domain at its N-terminus. In addition, the fungal form of V₀ contains a minor subunit c-related polypeptide, subunit c'. All three are required for activity of the proton pump in *Saccharomyces cerevisiae*. We have introduced cysteine residues in the N-terminal extension of subunit c'' in a cysteine-free form. All mutant forms are active in the V-ATPase from *S. cerevisiae*. Oxidation of vacuolar membranes containing the cysteine-replaced forms gave a cross-linked product of 42 000 Da. Analysis of this species showed it to be a dimeric form of subunit c'', and further studies confirmed there are two copies of subunit c'' in the V-ATPases in which it is present. Co-expression of double cysteine-replaced forms of both subunit c and c'' gave rise to only homotypic cross-linked forms. Also, subunit c

oligomeric complexes are present in vacuolar membranes in the absence of subunit c'', consistent with previous observations showing hexameric arrangements of subunit c in gap-junction-like membranes. *In vitro* studies showed subunit c'' can bind to subunit c and itself. The extent of binding can be increased by removal of the N-terminal domain of subunit c''. This domain may therefore function to limit the copy number of subunit c'' in V₀. A deletion study shows that the domain is essential for the activity of subunit c''. The results can be combined into a model of V₀ which contains two subunit c'' protomers with the extra transmembrane domain located toward the central pore. Thus the predicted stoichiometry of V₀ in which subunit c'' is present is subunit c₃:subunit c'₁:subunit c''₂. On the basis of the mutational and binding studies, it seems likely that two copies of subunit c'' are next to each other.

Key words: proton-translocating proteins, *Saccharomyces cerevisiae*, subunit c.

INTRODUCTION

The vacuolar H⁺-ATPase (V-ATPase) is a universal proton pump of eukaryotes [1,2]. It establishes a proton gradient across membranes and provides the necessary protonmotive force to power vacuolar compartments such as Golgi, endosomes/lysosomes, plant and fungal vacuoles, and secretory organelles, including the synaptic vesicle. Many cell types also have V-ATPases present in large numbers at the cell surface [3], such as osteoclasts in which the proton pumping is a key element for bone resorption [4]. Moreover, in some cell types, such as macrophages [5], V-ATPases are involved in cytosolic pH regulation.

The V-ATPase is closely related to the familiar F-ATPase, but is more complex. Both comprise a large water-soluble sector (V₁ or F₁) which hydrolyses ATP, and an integral membrane sector (V₀/F₀) which is the site of proton translocation. The F-ATPase works by rotational catalysis [6,7]. Hydrolysis of ATP in F₁ causes rotation of a central spindle, which rotates the membrane complex, and this turbine movement is linked to proton translocation.

The turbine of F₀ is formed from a ring of 10–14 copies of subunit c [8–10], a small highly hydrophobic polypeptide soluble in organic solvents and so designated as a proteolipid. Each subunit c is around 70–80 residues in length and is structured as a simple hairpin of two transmembrane α -helices [9,11]. The C-terminal helices form the outer ring of the turbine, whilst the N-terminal helix forms an inner ring. Critical for proton translocation is an acidic residue in the second helix and switching

between protonation and deprotonation causes a large rotation of the C-terminal end of this helix, which is thought to be responsible for driving mechanical rotation [12]. This switching is presumed to occur as each subunit c rotates past the stator, or subunit a [6,12]. This subunit occurs as a single copy and is a polytopic transmembrane component which links F₀ to F₁.

V₀ is believed to work by a similar rotational mechanism [13], but it is thought that there is a fixed stoichiometry of six copies of subunit c-like proteolipids in each V₀. The reduced number is explained by each V₀ proteolipid being a tandem repeat of two F₀ subunit c-like domains, so having four transmembrane α -helices. Thus the overall mass contributed by the proteolipid complex is similar. However, there are multiple forms of subunit c in V₀. In yeast (*Saccharomyces cerevisiae*) there are three types, with subunit c being a major component and subunits c' and c'' being minor components [14]. All three are essential for full V-ATPase activity and have highest homology with each other in the transmembrane α -helices, although subunit c'' has an extra transmembrane domain located at its N-terminus. In addition, whereas the critical acidic residue for proton translocation is located on the fourth transmembrane (TM) α -helix of subunits c and c', in subunit c'' this residue is located on the equivalent second transmembrane domain (Figure 1). Genome sequencing has shown genes for subunit c'' in all other eukaryotes, but subunit c' appears to be restricted to fungi. A recent study has shown that whereas subunit c can complex with itself and with subunit c'', the latter cannot complex with itself [15].

This led to the proposal that the stoichiometry of yeast V₀ is subunit c, c' and c'' in a 4:1:1 ratio respectively.

Abbreviations used: AEBF, 4-(2-aminoethyl)benzenesulphonyl fluoride; BCA, bicinchoninic acid; β -lac, β -lactamase; ORF, open reading frame; TM, transmembrane; V-ATPase, vacuolar H⁺-ATPase.

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Figure 1 Two proposed arrangements of the subunit c components in V_0

The TM α -helices are shown as circles. The diameter of helices 1 and 3 are smaller than helices 2 and 4, as the former helices are populated by glycine and alanine residues [8,11]. The position of the glutamic acid residue critical for proton translocation is shown as a black filled circle for subunits c, c' and c''.

Whereas V_1 appears to have a single function, that of hydrolysing ATP to create mechanical rotation, V_0 appears to be multifunctional. For example, it has recently been shown [16] that V_0 forms homotypic end-to-end associations between fusing membranes, and is thought to be the fusion pore first detected as a transient high-conductance channel. Indeed, re-constituted subunit c can allow bulk flow of molecules as large as neurotransmitters [17]. In addition, a form of subunit c – ductin – is present in a gap junction-like structure isolated from mammalian and crustacean tissues [18–20]. Ductin can substitute for subunit c in the V-ATPase [21], and these gap junction-like structures are reminiscent of the 'fusion pore' being an end-to-end association of V_0 -based complexes across two membranes [22].

It has been proposed that the helices of the V_0 subunit c are arranged the same as those of F_0 subunit c, as two concentric rings [16]. However, image analysis of the gap junction structure indicates six discrete densities [21,23], and cysteine and glutamate scanning studies [24–26] on subunit c have shown that the best arrangement of subunit c in V_0 is as a star-shaped hexamer that contains a water-accessible central pore (see Figure 1). Such a pore would account for the bulk flow of small molecules. The first transmembrane domain of subunit c is the innermost helix lining this central pore, whilst the fourth transmembrane is outermost, partly exposing the critical glutamic acid to the hydrophobic core of the membrane.

The arrangement of subunit c'' in V_0 is unknown, but if it has a similar arrangement to subunit c, then the extra N-terminal transmembrane domain may occupy the central pore. We have investigated this domain of subunit c'' and find it is essential for a functional V-ATPase in *S. cerevisiae* vacuoles. Cysteine-replacement studies suggest that it is centrally located, but unexpectedly show that there are two copies of subunit c'' in V_0 sectors in which it is present. Subunit c'' can also bind to itself, as it can to subunit c, but the extra TM domain reduces the extent of binding. However, subunit c'' is not essential for the assembly of subunit c.

EXPERIMENTAL

Yeast strains and culture conditions

The following yeast strains were used in the present study: YPH500 (*MAT α ade2, ura3, leu2, his3, trp1, lys2*), YPH500 C14 (as above $\Delta vma16::his2$), YPH500 C14-1 (as above, $\Delta vma16::HIS3$, $\Delta vma3::LEU2$). The YPH500 strain was a gift from Y. Anraku (Department of Biology, University of Tokyo, Tokyo, Japan). *VMA16* and *VMA3* genes were inactivated by replacement of the open reading frame (ORF) of each gene with the

Table 1 Mutations in *S. cerevisiae* subunit c''

Amino acids are given in one-letter code.

Type of mutation	Subunit C'' mutations
Cysteine	C-: C ^{67S/108S} C ⁵ : as in C-plus S ^{5C} C ²³ : as in C-plus Y ^{23C} C ²⁴ : as in C-plus L ^{24C} C ²⁵ : as in C-plus V ^{25C} C ²⁶ : as in C-plus L ^{26C} C ⁴³ : as in C-plus S ^{43C} C ^{6/C43} : as in C-plus S ^{5C/S43C}
Deletion	Δ TM-1: deleting from L ¹² to S ⁵⁵ inclusive
Hybrid	MoTM-1-Sc subunit c'': N-terminus of mouse to E ⁴⁴ followed by C-terminus of <i>S. cerevisiae</i> from T ³⁴ ScTM-1-Mo subunit c'': N-terminus of <i>S. cerevisiae</i> to R ⁵³ followed by C-terminus of mouse from T ⁴⁵
Extension	HA-1: HA1 epitope added at C-terminus from <i>S. cerevisiae</i> form from Q ²¹³ β -lac: β -lac fragment (60 residues) added at C-terminus (Q ²¹³) as described in Dunlop et al. [28]

ORFs of *S. cerevisiae* *HIS3* and *LEU2* genes, respectively. Cultures were maintained on YPD [1 % (w/v) yeast extract/2 % (w/v) peptone/2 % (w/v) glucose] or YNB [yeast nutrient broth, containing 0.67 % yeast nitrogen base without amino acids (Sigma) and 2 % (w/v) glucose] the latter supplemented with appropriate nutrients, as described by Sherman [27]. Growth at pH 7.5 was examined on YPD plates buffered with 50 mM Mes/Mops.

Plasmids

The p424 centromere-based plasmid was used for expression of the subunit c'' and cysteine-replaced mutants of subunit c''. The inserted gene is under the control of the ADH promoter and is constitutively expressed and the plasmid contains the *ADE2* gene. The pYES2 vector (Invitrogen) was used for the expression of mutant forms of the Norway-lobster (*Nephrops norvegicus*) subunit c and the N-terminal exchanges of subunit c''. The selectable marker is the *URA3* gene and the expression of the inserted gene is driven by the GAL1 promoter. For experiments using the pYES2 vector, glucose in the growth medium and spheroplasts buffer was replaced by 2 % (w/v) galactose/raffinose.

Construction of mutant forms of subunit c and subunit c''

Cloning and preparation of cysteine-free from (C^{54S}) and double cysteine-replaced form (S^{6C/S44C}) of the Norway-lobster subunit c has previously been described [24]. Cloning of subunit c'' from *S. cerevisiae* has been previously described [28]. The mouse form of subunit c'' was cloned from a 15-day-embryo library (Cambridge Bioscience, Cambridge, U.K.) and probed with a PCR product. The sequence of the mouse subunit c'' was identical with that described by Sun-Wada et al. [29]. Mutant forms of subunit c'' from *S. cerevisiae* and mouse were generated by conventional PCR strategies. The mutations are shown in Tables 1 and 2.

Antibodies

Monoclonal antibodies to the V-ATPase components subunit A (Vma1p) and Vph1p and to the epitopes HA1 and AU1 were

Table 2 Mutations in Norway-lobster subunit c

S, R and Y are serine, arginine and tyrosine in one-letter code.

Type of mutation	Norway-lobster subunit C mutations
Cysteine	C-: C ⁵⁴ S C ⁶ /C ⁴⁴ : as in C-plus S ⁶ C/S ⁴⁴ C
Extension	β -lac: β -lac fragment (60 residues) added at C-terminus as described by Dunlop et al. [28] ScTM-1-Ne subunit c: N-terminus of <i>S. cerevisiae</i> to R ⁵³ followed by C-terminus of subunit c from Y ⁸

purchased from Cambridge Bioscience. A rabbit polyclonal antiserum (Ab650) to the Norway lobster form of subunit c has previously been described [30].

Vacuole isolation

Vacuoles were isolated by the method of Uchida et al. [31] as described in Ashby et al. [32]. Yeast cultures (200 ml) were grown in minimal medium overnight at 30 °C to an attenuation (*D*) of between 0.5 and 1 at 600 nm. Glucose was added to restore the concentration to 2% (w/v) 2 h before the cells were harvested at 5000 *g* for 5 min at room temperature. The cells were then washed in 20 ml of phosphate buffer (50 mM K₂HPO₄/KH₂PO₄, pH 7.5) and spheroplasts were prepared by resuspending in 20 ml of digest buffer [1 M sorbitol/phosphate buffer (pH 7.5)/2% (w/v) glucose/0.4% (v/v) β -mercaptoethanol/0.5 mg/ml yeast lytic enzyme (ICN biochemicals; 70000 units/g)] and incubating at room temperature for 1 h. The spheroplasts were harvested at 2000 *g* for 5 min at room temperature under a cushion of 1.2 M sorbitol in the same buffer. All subsequent steps were carried out at 4 °C. The spheroplasts were resuspended in 6 ml of floatation buffer [8% (w/v) Ficoll 400/10 mM Mes/Tris (pH 6.9)/0.5 mM MgCl₂], which contained the protease inhibitors 0.1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF) and 1 mM benzamidine (Sigma–Aldrich). Spheroplasts were then lysed by four strokes in a loose-fitting homogenizer and the suspension was poured into a Beckman SW40 thin-walled polyallomer tube, overlaid with floatation buffer and centrifuged at 51900 *g* for 30 min. The layer of vacuoles was harvested from the top of the tube with a spatula and resuspended in 1.5 ml of buffer C [10 mM Mes-Tris (pH 6.9)/5 mM MgCl₂/25 mM KCl/0.1 mM AEBSF/1 mM benzamidine]. The vacuoles were harvested by centrifugation at 20000 *g* for 5 min, resuspended to approx. 2 mg/ml in buffer C and either used immediately or stored at –70 °C in buffer C containing 10% (w/v) glycerol.

Protein measurement

Protein was measured by using the bicinchoninic acid (BCA) method. BCA reagent, (Sigma–Aldrich) was prepared fresh according to the manufacturer's instructions. Samples were solubilized in 0.1% (w/v) SDS and the reaction mix incubated at 65 °C for 30 min. Absorbance at 562 nm was measured and the protein concentration calculated from a standard curve of BSA.

Oxidation of vacuolar membranes

This was carried out as previously described [25]. Samples (approximately 50 μ g of protein) were suspended in TE buffer [10 mM Tris/HCl (pH 8.0)/1 mM EDTA] and Cu^{II}-phenanthro-

line added to a final concentration of 2.5 mM. Membranes were incubated at room temperature for 60 min and the reaction stopped by adding iodacetamide and *N*-ethylmaleimide to final concentrations of 0.5 and 2.5 mM, respectively. The membranes were pelleted in a bench microfuge (20000 *g* for 15 min) and washed several times in TE buffer.

Immunoblotting and dot blotting

Proteins were transferred from SDS/PAGE to Hybond nitrocellulose membrane (Amersham Biosciences, U.K.) using semi-dry blotting apparatus. The transfer buffer contained 100 mM Tris, 100 mM glycine, 0.1% SDS and 20% (v/v) methanol. The membranes were blocked with 5% (w/v) commercial skimmed milk powder in 10 mM Tris/HCl, pH 7.5, 50 mM NaCl and 0.01% (w/v) Tween for 30 min and incubated overnight with the appropriate antibody (1:500 dilution) and washed four times in the above buffer. Bound antibody was detected using the ECL[®] detection system (Amersham Biosciences).

In vitro synthesis and binding

RNA was prepared from PCR products using the Ambion T7 mMessage mMachine kit according to the manufacturer's protocol. A 1 μ g portion of capped RNA was used for each translation reaction, which contained rabbit reticulocyte lysate (Promega, Chilworth Research Centre, Southampton, U.K.), canine pancreatic microsomes (Promega), and [³⁵S]cysteine and [³⁵S]methionine (Redivue Pro-mix L-[³⁵S] *in vitro* Cell Labelling Mix, Amersham Biosciences) [28]. Translation was carried out according to the manufacturer's instructions. Microsomes were collected by centrifugation at 400000 *g* for 10 min and resuspended in 400 μ l of radio-immunoprecipitation (RIPA) buffer (20 mM sodium Mops, pH 7.5, 150 mM NaCl, 10 mM EDTA, 5 mg/ml Triton X-100, 5 mg/ml sodium deoxycholate and 1 mg/ml SDS). A 5 μ l portion of anti-HA1 (E5 proteins), anti-AU1 (Vma3p E137G) or antiserum to Norway-lobster subunit c was added to 200 μ l aliquots of the solubilized microsomes, to which a further 200 μ l of RIPA buffer was added. The mixture was then placed on a rotary mixer overnight at 4 °C. A 10 μ l portion of Protein D–Sepharose was added and mixed for 1 h at room temperature before harvesting the Sepharose by centrifugation at 20000 *g* for 5 min. The samples were washed three times in 500 μ l of RIPA, the final wash including incubation on a rotary mixer for 15 min. The final pellet was resuspended in 10 μ l of 2 $\times$ SDS loading buffer [100 mM Tris/HCl, pH 6.8, 10% (w/v) glycerol, 5% (w/v) SDS and 0.2% (w/v) Bromophenol Blue], which was electrophoresed on a Tris/Tricine 15% polyacrylamide gel to optimize the resolution of the small proteins. The gel was fixed in 50% (v/v) methanol/10% (v/v) acetic acid for 1 h, dried on to Whatman paper and used to expose film (Fuji super RX) for approx. 1 week.

RESULTS AND DISCUSSION

Cysteine scanning of the TM-1 domain of subunit c"

The two proposed arrangements [15,25,26] of the TM α -helices of the subunit c 'proteolipids' in V₀ are shown in Figure 1. In one model the TM α -helices are arranged in two concentric rings (Figure 1a), and in the other as a hexamer (Figure 1b). Cysteine and scanning studies [24,25] in subunit c are consistent with the second model, i.e. a hexameric arrangement of protomers. From this and a recent study suggesting there was one copy of subunit c" in V₀ [15], we predicted that the N-terminal TM domain (TM-1; Figure 1) of subunit c" would be located towards the centre of

Figure 2 Cysteine substitutions in subunit c and subunit c''

Cysteine substitutions were made in cysteine free forms of the Norway-lobster subunit c (positions C⁶ and C⁴⁴) (top panel) and *S. cerevisiae* subunit c'' (positions C⁵, C²³, C²⁴, C²⁵, C²⁶ and C⁴³) (Bottom panel). The positions of the essential glutamic acid residues in subunit c and subunit c'' are marked as black circles.

Figure 3 Growth in YPD medium at pH 5.5 and pH 7.5

C14 cells were transfected with either empty p242ADH vector, or with vector containing the gene encoding for wild-type subunit c'' or the various mutants. (1) Empty vector; (2) Wild-type subunit c''; (3) C-subunit c''; (4) C⁵ subunit c''; (5) C²³ subunit c''; (6) C²⁴ subunit c''; (7) C²⁵ subunit c''; (8) C²⁶ subunit c''; (9) C⁴³ subunit c''; (10) C⁵/C⁴³ subunit c''.

V₀. Such a location would place it close to the N-terminal region of subunit c. As this region in the yeast (*S. cerevisiae*) form of subunit c contained several cysteine (C) residues in the N-terminal region (C⁵, C¹⁷, C⁴⁰ and C⁴³), this provided the opportunity to introduce cysteine residues in the region of TM-1 to determine if this region of subunit c'' was capable of cross-linking to subunit c.

For these experiments, a cysteine-free (C-) form of subunit c'' was first prepared and single cysteine residues were introduced within the TM-1 (C²³, C²⁴, C²⁵ and C²⁶) and either side of the

Figure 4 Immunoblots of untreated and oxidized vacuolar membranes probed with HA1 antibodies

Vacuolar membranes from the C14 strain expressing the various mutant forms of subunit c'' were isolated and immunoblots probed with the HA1 antibodies after no treatment, or oxidation with Cu (II) phenanthroline. (1) C-subunit c''; (2) C⁵ subunit c''; (3) C²³ subunit c''; (4) C²⁴ subunit c''; (5) C²⁵ subunit c''; (6) C²⁶ subunit c''; (7) C⁴³ subunit c''; (8) C⁵/C⁴³ subunit c''.

TM-1 (C⁵, C⁴³), together with a double cysteine substitution (C⁵ C⁴³; see Table 1 and Figure 2). The wild-type and mutant forms of subunit c'' were tagged with a C-terminal HA1 epitope and expressed from the ADH promoter of the p242ADH vector in a genetically modified form (C14) of the haploid strain of *S. cerevisiae*, YPH500. In strain C14 the ORF of the *VMA16* gene (subunit c'') had been replaced by the *HIS2* gene. C14 cells exhibited the established VMA phenotype of failure to grow at pH 7.5, on medium containing 100 mM CaCl₂, or on a non-fermentable carbon source. The wild-type and all mutant forms of subunit c'' restored growth of C14 cells at pH 7.5 (Figure 3). Vacuolar membranes were prepared from C14 cells expressing the various forms of subunit c'' and oxidized with Cu^{II}-phenanthroline before probing immunoblots with HA1 antibodies to visualize subunit c'' and any cross-linked complexes.

In all cysteine replacements a higher-molecular-mass species with an apparent molecular mass of 42 kDa was observed but not, as expected, in the cysteine-free form (Figure 4). The 42 kDa species was removed by reduction to the expected size of subunit c'' of 22 kDa (Figure 4b). The degree of cross-linking varied with the position of the cysteine replacement. Those cysteine residues predicted to be extramembranous (C⁵ and C⁴³) showed most cross-linking and, indeed, the 42 kDa species could be detected before oxidation. Those in TM-1 showed varied ability to form cross-links: for example, the C²³ form showed little ability to cross-link compared with C²⁴, C²⁵ or C²⁶ mutant forms. The absence of the 42 kDa species without oxidation is consistent with this region of subunit c'' either being in the interior of the V₀ complex, or located in a hydrophobic environment (e.g. facing the alkane tails of the lipid). The double cysteine-replaced form has near complete conversion to the 42 kDa cross-linked species.

Figure 5 Immunoblots of vacuolar membranes from C14 cells expressing C⁵/C⁴³ subunit c'' after solubilization or glycerol-gradient centrifugation

(A) Vacuolar membranes were probed with HA1 antibodies before (lanes 1 and 2) or after (lanes 3 and 4) solubilization in *N*-dodecyl maltoside, with (lanes 2 and 4) or without oxidation (lanes 1 and 3). (B) Vacuolar membranes were solubilized in *N*-dodecyl maltoside and subjected to ultracentrifugation [32]. Fractions were collected and the V-ATPase activity measured [32] and immunoblots of each fraction probed with antibodies to Vph1p (V₀ marker; subunit a), Vma2p (V₁ marker; subunit B) and the HA1 antibody. Peak V-ATPase activity was in fractions 5 and 6.

The 42 kDa cross-linked species is predominantly formed within individual V₀ complexes

Although the study by Powell et al. [15] predicted that the cross-linked species would contain only one subunit c'' per complex, the size of the cross-linked species was larger than expected (maximum 38 kDa for one subunit c and one subunit c'' per complex) and was close to the expected size for a dimer of subunit c''. One possible explanation, which would be consistent with one copy of subunit c'' per V₀ and the 42 kDa being a dimer of subunit c'', was that the TM-1 of subunit c'' was situated to the outside of V₀, facing the membrane and not, as proposed, towards the centre. In this case, the apparent dimeric species would be the result of cross-linking between two V₀ sectors. Using C14 cells expressing the C⁵/C⁴³ form of subunit c'', we investigated this possibility in two ways: (1) examining the efficiency of cross-linking after solubilizing vacuolar membranes and (2) separation of V-ATPase complexes on glycerol gradients by centrifugation. Solubilizing membranes should markedly reduce the opportunity for contacts between V₀ sectors. Similarly, if there were complexes of two V₀ domains, then the 42 kDa species should sediment to a denser part of the glycerol gradient from the non-cross-linked subunit c''. The results of both experiments are shown in Figure 5.

Membranes were solubilized in mild detergent (1% *N*-decyl maltoside) before oxidizing with Cu^{II}-phenanthroline. This detergent is known not to effect the integrity or the activity of the V-ATPase [32]. Prior solubilization had little effect on the extent of cross-linking (Figure 5a). Likewise the cross-linked 42 kDa species co-migrates with subunit c'' on glycerol gradients (Figure 5b). Both results show that there was unlikely to be cross-linking between V₀ sectors. As expected, the distribution of subunit c'' and the 42 kDa species overlaps with the distribution of other V-ATPase subunits (subunit B of V₁ and subunit a of V₀; Vma2 and Vph1p respectively in Figure 5b), which coincides with the peak ATPase activity. Therefore the 42 kDa cross-linked species is present within individual V₀ complexes.

Figure 6 Immunoblots of vacuolar membranes from C14-1 cells expressing C⁵/C⁴³ subunit c'' and C-Norway-lobster subunit c

Vacuolar membranes were isolated from C14-1 cells expressing C⁵/C⁴³ subunit c'' and C-Norway-lobster subunit c before oxidation (lanes 1 and 4), after oxidation (lanes 2 and 5), or after oxidation followed by reduction (lanes 3 and 6). Immunoblots were probed with either HA1 antibodies for C⁵/C⁴³ subunit c'' (A) or AU1 antibodies for Norway-lobster subunit c (B).

Figure 7 Immunoblots of vacuolar membranes from C14-1 cells expressing C⁵/C⁴³ or C-subunit c'' and C⁶/C⁴⁴ or C-Norway-lobster subunit c

Blots were probed simultaneously with HA1 and AU1 antibodies to detect subunit c'' and Norway-lobster subunit c'' respectively. (1) C-subunit c (no subunit c''); (2) C⁶/C⁴⁴ subunit c (no subunit c''); (3) C-subunit c and C-subunit c''; (4) C⁶/C⁴⁴ subunit c and C-subunit c''; (5) C⁶/C⁴⁴ subunit c and C⁵/C⁴³ subunit c''.

The 42 kDa species is a dimer of subunit c''

The nature of the 42 kDa species was investigated by co-expressing a cysteine-free form of subunit c derived from the Norway lobster and the C⁵/C⁴³ form of subunit c'' in C14-1 cells which are derived from C14 cells. C14-1 cells from C14 contain an inactivated *VMA3* gene (which encodes for subunit c) in C14 cells by substitution with the *LEU2* gene (*vma16::HIS2/vma3::LEU2*). Growth of C14-1 cells at pH 7.4 or in 100 mM CaCl₂ could be rescued by expression of subunit c and c'' together. The Norway-lobster cysteine-free form of subunit c (C-) was tagged at the C-terminus with the AU-1 epitope.

Oxidizing vacuolar membranes prepared from C14-1 cells expressing C⁵/C⁴³ subunit c'' and the cysteine-free form of the Norway-lobster subunit c still showed the presence of the higher-molecular-mass species of 42 kDa (Figure 6), showing that it was

not a complex that contained subunit c. It therefore was most likely a dimeric species of subunit c".

We had previously shown [24,25] that the C⁶/C⁴⁴ Norway-lobster subunit c readily forms inter-subunit homotypic dimers, and we therefore examined if we could induce cross-links between this form and the C⁵/C⁴³ form of subunit c". Although cysteine residues are located in the same sites in the endogenous form of subunit c, for reasons yet unclear, we could not induce cross-linking. One possible explanation is that cysteine residues close by on subunit c prevent intermolecular cross-links.

Oxidizing vacuolar membranes showed that although we could readily form homomeric complexes of subunits c and c", we could not produce heteromeric complexes between these two (Figure 7). Unexpectedly, there was equally efficient cross-linking between C⁶/C⁴⁴ subunit c in the absence of subunit c", suggesting the latter is not required for either the assembly of complexes of subunit c, or its movement to a vacuolar compartment (Figure 7, lanes 2, 4 and 5).

Subunit c" forms homotypic and heterotypic complexes *in vitro*

The simplest explanation of the above results was that there were two copies of subunit c" in each V₀ complex in which it was present. In addition, the disulphide bridges between the C⁴³ mutant of subunit c" indicated that they were adjacent to each other. We therefore investigated whether subunit c" can bind to itself and to subunit c.

We have previously shown [28], using an *in vitro* translation system, that newly synthesized subunit c can form oligomeric complexes with itself and with subunit c' in membranes derived from endoplasmic reticulum. We first investigated the binding between subunit c and subunit c", as our previous results showed only limited binding between these two, which appears to contradict the finding that subunit c" is an integral component of V₀.

cRNAs encoding for the *S. cerevisiae* subunit c" and the Norway-lobster subunit c were translated separately or together in the presence of pancreatic microsomes. An AU1 epitope tag was added to the C-terminus of subunit c". After translation, microsomes were pelleted and solubilized in RIPA buffer. Samples of the microsome fractions were separated on SDS/PAGE to examine the level of translation of each polypeptide in the separate and co-translations. Equal aliquots of the remaining separate translations were mixed and the solubilized membranes immunoprecipitated with monoclonal AU1 antibodies or a polyclonal antiserum (Ab650) to the Norway-lobster subunit c.

When translated separately, the AU1 immunoprecipitate contained, as expected, subunit c" with little or no subunit c (Figure 8A). Likewise, the Ab650 immunoprecipitate contained Norway-lobster subunit c with little subunit c". However, the co-translations showed detectable amounts of both proteins in the respective immunoprecipitates, indicating some binding between the two proteins. The amounts were considerably less than we had previously found for homologous subunit c interactions [28].

We next examined if subunit c" was also capable of homologous binding. A β -lactamase (β -lac) tail was added to subunit c" and the cRNAs translated separately or together, as before. The results showed that subunit c" can bind to subunit c" β -lac, indicating homologous interactions (Figure 8B), but again, the level of binding appeared lower than homologous interactions between subunit c. We therefore explored the possibility that the TM-1 domain of subunit c" would reduce the extent of heterologous and homologous binding.

Various forms of subunit c" and subunit c were constructed by PCR (Figure 8F), including a truncated form of subunit c"

(Δ TM-1 subunit c") and a N-terminally extended form of Norway-lobster subunit c (TM-1 subunit c) which contained the subunit c" TM-1 domain. To distinguish the various forms of subunit c and c" in the immunoprecipitation assays, the β -lac tail was added to the C-termini of some of the constructs. Removing the N-terminal domain of subunit c" increased its binding to subunit c and to itself (Figures 8C and 8D), whilst adding this domain to subunit c decreased homologous binding to a level similar to the full-length subunit c" (Figure 8E). The conserved 'core' of subunit c" thus appears to be capable of extensive binding.

Requirement of TM-1 for full activity of subunit c"

The above finding suggested that TM-1 was not an essential domain of subunit c" in interacting with other components of V₀ and, indeed, may function to limit the extent of interaction and thus restrict the number of copies of subunit c" in V₀. We therefore examined whether TM-1 was an essential domain for an active V-ATPase.

C14 cells were transfected with the pYES2 vector containing either subunit c" or Δ TM-1 subunit c" and grown at either pH 5.5 or pH 7.4, with either glucose or galactose as the carbon source. The inserted cDNA is under control of the GAL4 promoter and therefore only expressed in the presence of galactose. The results showed that, whilst the subunit c" restored growth at pH 7.5, Δ TM-1 subunit c" did not (Figure 9; results only shown for subunit c").

Genome sequencing programmes have identified genes from *Caenorhabditis elegans*, mouse and humans encoding for subunit c". As with the *S. cerevisiae* form, they all contain TM-1, although this region is less conserved than the other four TM domains. As the Norway-lobster form of subunit c can substitute for the endogenous form in *S. cerevisiae*, we cloned the mouse and *C. elegans* genes encoding subunit c" and examined their ability to rescue the V-ATPase activity of C14 cells. Neither could rescue growth at pH 7.5 (results not shown). This observation has recently been made by others [33]. However, exchanging the N-terminal extension of either the mouse or *C. elegans* form for that of *S. cerevisiae* rescued C14 cells, although growth was poorer than the endogenous form. Thus, whilst TM-1 is necessary, this region of subunit c" can tolerate substantial changes in its primary structure.

Comparison with other membrane complexes containing subunit c

Our data shows that cysteine-substituted subunit c" is capable of cross-linking to form a 42 kDa species and that this species is a dimer of subunit c" for the following reasons: (1) The migration of the species is close to the expected position of a dimeric subunit c" species; (2) the same 42 kDa species was generated in different positions of the cysteine substitution; (3) formation of the 42 kDa species occurred in the presence of a cysteine-free form of subunit c, the only other likely partner of subunit c", (4) *in vitro* analysis shows that subunit c" can bind to itself.

The double cysteine replacement (C⁵/C⁴³) shows a near-complete conversion into the dimeric species after oxidation. The best explanation of our data is that there are two copies of subunit c" present in each V₀ sector in which it is a constituent. However, this contradicts the conclusion of a recent study [15] using immunoprecipitation which failed to co-precipitate different tagged forms of subunit c", although complex formation could be detected between subunit c and c". A possible explanation is that, for some unclear reason, V₀ complexes containing subunit c" are partially unstable in the detergent conditions used for immunoprecipitation.

Figure 8 *In vitro* binding activities of various forms of subunit c and c''

S. cerevisiae subunit c'' and Norway-lobster subunit c polypeptides were mutated as described below and in Tables 1 and 2. Translation (Trans) and immunoprecipitation (IP) results are shown for each experiment. **(A)** Lane 1, translation of cRNA for subunit c; lane 2, translation of cRNA for subunit c''; lane 3, co-translation of cRNAs for subunit c and subunit c''; lane 4, lane 1 and 2 mixed and immunoprecipitated with Ab650; lane 5, lane 1 and 2 mixed and immunoprecipitated with AU1; lane 6, lane 3 immunoprecipitated with Ab650; lane 7, lane 3 immunoprecipitated with AU1. **(B)** Lane 1, translation of cRNA for subunit c'' AU1; lane 2, translation of cRNA for subunit c'' β-lac; lane 3, co-translation of cRNAs for subunit c'' AU1 and subunit c'' β-lac; lane 4, lanes 1 and 2 mixed and immunoprecipitated with AU1; lane 5, lane 3 immunoprecipitated with AU1. **(C)** Lane 1, translation of cRNA for ΔTM-1 subunit c'' AU1; lane 2, translation of cRNA for ΔTM-1 subunit c'' β-lac; lane 3, co-translation of cRNAs for ΔTM-1 subunit c'' AU1 and ΔTM-1 subunit c'' β-lac; lane 4, lanes 1 and 2 mixed and immunoprecipitated with AU1; lane 5, lane 3 immunoprecipitated with AU1. **(D)** Lane 1, translation of cRNA for ΔTM-1 subunit c AU1; lane 2, translation of cRNA for ΔTM-1 subunit c'' β-lac; lane 3, co-translation of cRNAs for ΔTM-1 subunit c AU1 and ΔTM-1 subunit c'' β-lac; lane 4, lanes 1 and 2 mixed and immunoprecipitated with AU1; lane 5, lane 3 immunoprecipitated with AU1. **(E)** Lane 1, translation of cRNA for TM-1 subunit c AU1; lane 2, translation of cRNA for subunit c; lane 3, co-translation of cRNAs for TM-1 subunit c AU1 and subunit c; lane 4, lanes 1 and 2 mixed and immunoprecipitated with AU1; lane 5, lane 3 immunoprecipitated with AU1. **(F)** Constructs used for *in vitro* binding analysis.

Figure 9 Rescue of growth at pH 7.5 by hybrid constructs of subunit c''

C14 cells were transfected with pYes2 vector containing inserts that encoded for the *S. cerevisiae* subunit c'', mouse subunit c'', or hybrid inserts containing the TM-1 domain of mouse at the N-terminus of the *S. cerevisiae* Δ TM-1 subunit c'' (MoTM-1-Sc subunit c''), or the TM-1 domain of *S. cerevisiae* at the N-terminus of the mouse Δ TM-1 subunit c'' (ScTM-1-Mo subunit c''). The expression of the insert is under control of the GAL4 promoter. See Tables 1 and 2 for details of the constructs.

Figure 10 Revised model of the *S. cerevisiae* V_0 core of subunit c complex

The basic arrangement is that shown in the second model of Figure 1, with the transmembrane (TM) α -helices being shown as circles. Two copies of subunit c'' are depicted, consistent with cysteine-replacement experiments in subunit c''. The TM-1 domain of each subunit c'' is placed innermost in the hexameric complex to account for the production of dimers of subunit c'' when cysteine replacements are made in this domain. This results in the critical glutamic acid residue of subunit c'' in TM2 (shown as the small dark circle) occupying a location within the interior of the complex and not partially exposed to the hydrophobic core of the membrane bilayer, as they are for subunits c and c'.

The cysteine-replacement study shows that the N-terminal TM-1 domains of two subunit c'' molecules are close to each other. Only one mutant, C²³, does not form efficient homotypic cross-links, but this would be expected if TM-1 is an α -helix. The close location of two TM-1 domains is best achieved in a model of V_0 in which these domains are innermost, essentially occluding the central pore (Figure 10). As mentioned above, this might

serve as a mechanism to limit the copy number of subunit c''. The extensive cross-linking between neighbouring molecules of C⁶/C⁴⁴ subunit c can be best accounted for by a hexagonal-based arrangement, rather than by two concentric rings of α -helices (Figures 1a, 1b and 10). Thus, as well as having a mixed composition of subunit c-like components, the V_0 appears to have a different fundamental arrangement of core helices. Additionally, the model predicts that the transmembrane glutamic acid residue critical for proton translocation of subunit c'' may occupy an internal environment unlike the external environment of subunit c and the F_0 subunit c [8,9,11,12]. Studies are in progress to confirm this location.

The presence of two copies of subunit c'' in V_0 has implications for the gap-junction-like form of subunit c (ductin). Only subunit c has been detected in this form [20], which suggests that such gap-junction membranes do not have a V_0 origin and therefore must arise from another cellular pool of complexes containing subunit c. The ability to generate oligomeric complexes of subunit c in vacuolar membranes in the absence of subunit c'' suggests the latter is not essential for assembly. Indeed, we have recently observed a labile V-ATPase in the C14 strain (G. Whyteside, L. Gibson, M. Scott and M. E. Finbow, unpublished work). This indicates there may be different forms of V_0 varying in the number of subunit c and subunit c''. In the case of the *S. cerevisiae* form of V_0 , which contains subunit c'', the stoichiometry is likely to be 3:1:2 of subunits c, c' and c'' respectively (Figure 10).

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