The SzA mutations of the B subunit of the *Drosophila* vacuolar H\(^+\) ATPase identify conserved residues essential for function in fly and yeast

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Summary

V-ATPases play multiple roles in eukaryotes: in *Drosophila*, null mutations are recessive lethal. Here, mutations underlying five extant lethal alleles of *vha55*, encoding the B subunit, were identified, including a premature termination codon and two mutations very close to residues thought to participate in the catalytic site of the enzyme. Lethality of these alleles could be reverted by transformation of flies with a wild type *vha55::GFP* fusion, confirming that the lethal phenotype described for these alleles was due to defects in V-ATPase function. The chimeric protein was correctly localised to the apical domain of the Malpighian (renal) tubule, and restored fluid transport function to wild-type levels. No dominant-negative phenotype was apparent in heterozygotes. When the *vha55::GFP* fusion was driven ubiquitously, fluorescent protein was only detectable in tissues known to contain high levels of V-ATPase, suggesting that *vha55* requires stoichiometric co-expression of other subunits to be stable. Yeast (*Saccharomyces cerevisiae*) deleted for the corresponding gene (Δvma2) demonstrated a pH-sensitive growth phenotype that was rescued by the *vha55::GFP* construct. Δvma2 yeast could not be rescued with fly cDNAs encoding any of the mutant *vha55* alleles, confirming the functional significance of the mutated residues. In yeast, bafilomycin-sensitive ATPase activity and growth rate correlated with the ability of different constructs to rescue the pH-sensitive conditional-lethal phenotype. These classical *Drosophila* mutants thus identify residues that are essential for function in organisms with wide phylogenetic separation.

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Introduction

Vacuolar H\(^+\) adenosine triphosphatase (V-ATPase) is a ubiquitous ATP-dependent proton pump, which transports H\(^+\) out of the cytoplasm to acidify eukaryotic endomembrane compartments (Futai et al., 2000; Nelson, 1992), and which also energizes ion transport across many plasma membranes (Harvey and Wieczorek, 1997; Wieczorek et al., 1999). It is structurally and evolutionarily related to the F\(_{1}\)/F\(_{0}\) ATPase of bacteria, chloroplasts and mitochondria, in that it is composed of two distinct catalytic (V\(_{1}\)) and transmembrane (V\(_{0}\)) sectors, comprising at least 14 subunits. Evolutionary studies have shown that subunits A and B of the V\(_{1}\) sector, and c of the V\(_{0}\) sector, are the most conserved (Nelson, 1991; Nelson, 1992). The discovery of V-ATPase subunits has been helped immeasurably by work using the simple model organism, *Saccharomyces cerevisiae*. Yeast display a pH-sensitive phenotype for mutants in V-ATPase subunits: mutant yeast grow at pH 5.5, but not at 7.5 (Anraku et al., 1992; Kane et al., 1992; Noumi et al., 1991). However, in animals, V-ATPases are found on apical plasma membranes of many epithelia, where they are capable of energising both proton translocation and secondary active transport processes (Harvey and Wieczorek, 1997). Recently, mutations in the human ATP6V1B1 gene, encoding the B1 renal and cochlear isofrom of V-ATPase, have been shown to be responsible for distal renal tubular acidosis associated with sensorineural deafness (Karet et al., 1999).

How distinct are the ubiquitous endomembrane 'housekeeping' and specialised plasma membrane roles for this ATPase? In its plasma membrane manifestation, V-ATPase accumulates to remarkable densities, forming semi-crystalline arrays of protein on the inner surface of the plasma membrane (Harvey et al., 1983). To study plasma membrane V-ATPase, a genetically tractable animal model system presents unique advantages beyond those offered either by yeast or human. In *Drosophila*, at least 31 genes encode the 14 subunits of the V-ATPase (Dow, 1999; Wang et al., 2004). Microarray analysis identified exactly one gene for each subunit that was both abundant and enriched in adult Malpighian (renal) tubules, strongly suggesting that these were the genes that contributed to the plasma membrane isoform of the ATPase (Wang et al., 2004). This was subsequently verified by in situ hybridisation and immunocytochemistry (Allan et al., 2005).

The first animal knockout of a V-ATPase subunit was identified in *Drosophila* (Davies et al., 1996; Gausz et al., 1997). Recently, mutations in the human ATP6V1B1 gene, encoding the B1 renal and cochlear isofrom of V-ATPase, have been shown to be responsible for distal renal tubular acidosis associated with sensorineural deafness (Karet et al., 1999).
1979), conferring an early-larval lethal phenotype for disruptants of vha55, the single gene encoding the B subunit. *Drosophila*, as a simple animal with rich genetic resources, is thus ideal for a dissection of the endomembrane and plasma membrane functions of the V-ATPase on an organismal scale. To study V-ATPase structure and function further, we decided to combine the strengths of these two model systems, using the genetics, transgenics and organismal relevance of *Drosophila*, and the relative facility of generating and screening V-ATPase mutants of yeast. The starting points for analysis were five mutant fly lines (Gausz et al., 1979), with recessive-lethal phenotypes that are allelic to a P-element insertion in vha55, previously known as the SzA locus (Davies et al., 1996). A yeast chromosomal deletion of VMA2 (Δvma2), encoding the B subunit of V-ATPase is available (Anraku et al., 1992; Liu et al., 1996; Vasilyeva et al., 2000). VMA2 has 79% amino acid sequence identity to the *Drosophila* homologue. Here, we describe the molecular basis for the *Drosophila* mutations, demonstrate that viability and renal function can be rescued with a GFP-tagged vha55 transgene, and show that the mutations act similarly, though not identically, in yeast to affect a range of V-ATPase-related phenotypes.

**Results**

**Molecular basis of the SzA alleles of vha55**

The SzA alleles (Gausz et al., 1979) of vha55 represent the first ‘knockouts’ of a V-ATPase gene in an animal (Davies et al., 1996). They were shown to be allelic to a lethal P-element inserational mutant of vha55 (Davies et al., 1996), although this falls short of a rigorous proof that their lethality is due to a range of V-ATPase-related phenotypes.

Although 22 SzA alleles were originally identified (Gausz et al., 1979), only three fly lines (SzA1, SzA9 and SzA12) are still extant. All are embryonic or larval recessive lethal, although they were described as showing heterozygous phenotypes of varying severity. Two further alleles, vha55SzA14 and vha55SzA14, were available from stock centres. All were generated by chemical mutagenesis with EMS, and so the underlying mutations were likely to be single base changes or small deletions. Genomic DNA was extracted from each line, and the coding regions of vha55 scanned by single-strand conformation polymorphism (SSCP) analysis (Fig. 1A). In each case, a mis-sense mutation was identified in the coding region (Fig. 1B). For two of these lines, SzA9 and vha55SzA14, the change is a relatively benign replacement of glycine with valine; for one (vha55SzA14) it is a non-conservative substitution of acidic glutamate with basic lysine. One mutation, in SzA1, produces a premature stop codon early in the protein, and is thus likely to represent a functional null. A non-coding change in an intron within the 5’ UTR, and a silent mutation, were also identified. In all cases, the residues mutated are in areas of the protein that are absolutely conserved between fly, human, yeast and plant (Fig. 1B), consistent with essential roles in function.

The SzA alleles of vha55 are rescued by vha55::GFP

The identification of mutations in vha55 does not exclude the possibility that the SzA flies also carry further lethal mutations in other genes. Accordingly, flies were transformed with a vha55::GFP transgene, under control of the UAS promoter, and crossed into three vha55 mutant lines (vha55SzA1, vha55SzA9, vha55SzA14). When the transgene was driven by heat-shock GAL4, lethality of all lines was reverted (data not shown, but see phenotypic description below). Therefore, the lethal phenotypes of the SzA alleles indeed encode mutant VHA55, and that they can be rescued by expression of the wild-type gene.

![Fig. 1. Identification of point mutations in lethal alleles of *Drosophila vha55*. (A) Sample results from PCR-based SSCP gel, showing extra bands in mutants (arrows). C, control wild-type (Oregon R) genomic DNA; mutant strains are as described in the text. (B) Sequencing results. Point mutations and amino acid changes are indicated above the vha55 gene structure diagram. The four exons are labelled E1-E4; the introns are not to scale. The translated region is shown in black. Nucleotide numbers are relative to the Gadfly annotation for transcript A. Alignments for each of the mutated regions are shown: the mutated residue is underlined in the *Drosophila* sequence. The genes used are: D. melanogaster, vha55; H. sapiens, ATP6V1B2; S. cerevisiae, vma2; A. thaliana, At1g76030.](image-url)
viability to normally lethal homozygotes, it was also possible to study the secretion phenotype in such rescued flies. Accordingly, wild-type flies were compared with homozygous mutant flies expressing the vha55::GFP transgene under heat-shock control. Resting secretion rates were indistinguishable from wild-type, and maximally stimulated fluid secretion rates were about two-thirds that of the wild type (Fig. 2A-C); it is remarkable that rescue by this chimeric protein is so effective. Furthermore, it was possible to visualise the location of the transgenic protein in tubules by means of its GFP tag: the transgenic protein localised correctly to the apical domain (Fig. 2D, cf. Fig. 2E), as has been documented for the wild-type protein in several species (Weng et al., 2003). Rescue by the vha55::GFP transgene can thus be judged to be successful by three separate indicators: viability (i.e. survival to adulthood), correct targeting of protein, and restoration of epithelial transport phenotype.

Do vha55 heterozygotes display a dominant-negative phenotype?
The V-ATPase alleles described all confer a homozygous late-embryonic or early-larval lethal phenotype (Allan et al., 2005; Gausz et al., 1979), and so are not amenable to physiological study. However, it is conceivable, given the high levels of V-ATPase expressed in insect epithelia (Harvey et al., 1981), that even the heterozygotes might display a phenotype; as there are three copies of the B-subunit in each holoenzyme, there is the potential for dominant-negative effects, whereby a single defective copy of VHA55 could disable the whole holoenzyme (Dow, 1999). Accordingly, adult heterozygote tubules were also compared with wild-type flies (Fig. 2). Secretion rates were measured both at rest and after maximal stimulation by the neuromodulatory neuropeptides Capa-1 (Kean et al., 2002) and drosokinin (Terhzaz et al., 1999), using standard protocols (Dow et al., 1994). The results show that, although vha55SzA9 heterozygotes appeared to perform better than the wild type, and vha557e1 and vha55SzA9 worse, the differences were not significant (Fig. 2). So any dominant-negative effect was too subtle to be detected by this assay. This might be because defective VHA55 protein is degraded quickly (before it can be incorporated into the holoenzyme) so in at least some alleles; this is discussed later.

Ubiquitous expression of the vha55::GFP transgene labels only tissues with high levels of V-ATPase
The UAS-vha55::GFP transgene is expected to label those parts of a cell with high levels of V-ATPase protein (e.g. Fig. 2F), though it would be surprising if ubiquitous expression of the transgene showed any cell-type specificity. However, when the transgene is driven ubiquitously in flies with a heat-shock GAL4 promoter, GFP fluorescence is observed only in restricted subsets of cells, specifically epithelia which have previously been implicated as sites of high-level plasma membrane expression (Fig. 3). Previous work (Allan et al., 2005) surveyed the expression of all V-ATPase genes in all tissues of the adult fly by in situ hybridization, and validated the predictions of a microarray study on Malpighian tubules (Wang et al., 2004), which had predicted the genes that contributed to the plasma membrane holoenzyme. For example, GFP is observed in salivary gland (Zimmermann et al., 2003), the cuprophilic cells of the midgut (Dubreuil et al., 2005) and the coprophilic cells of the hindgut (Vorburger et al., 1996). It is therefore possible that all tissues expressing V-ATPase have high levels of VHA55 protein, with the exception of the blood cells (Zimmermann et al., 2003).
Complementation between \textit{vha55} and VMA2

In 1998, the Malpighian tubules (Bertram et al., 1991) and hindgut (Phillips et al., 1996), all known sites of plasma membrane V-ATPase expression. Similarly, specific regions of the testes and ovaries are labelled. With the exception of the salivary gland, these are precisely the tissues (Malpighian tubules, ovaries, testes, midgut, hindgut and rectum) identified as expressing particularly high levels of endogenous \textit{vha55} (Allan et al., 2005). By contrast, when GFP alone is expressed under heat-shock control, nearly every tissue is labelled indiscriminately (not shown).

How are GFP fusions only stable in cells where the normal protein is naturally abundant? We suggest that, like the F-ATPase (Abrahams et al., 1994), the V-ATPase holoenzyme is held together by hydrophobic interactions between the different subunits. In cells with very high levels of V-ATPase expression, there is an abundance of the necessary subunits to bind the VHA55::GFP fusion protein. However, in cells that use V-ATPase only for vacuolar acidification, the relatively large excess of VHA55::GFP is not protected from surveillance by the ubiquitylation machinery of the cell (Bohley, 1996). Excess protein, beyond the stoichiometric ratio needed for the holoenzyme, is rapidly degraded. Thus, although V-ATPase is expressed ubiquitously as a housekeeping enzyme, it is only in regions of very high expression, such as V-ATPase-energised epithelia, where sufficient VHA55::GFP fluorescent protein accumulates to be visible.

Is it possible that overexpressed VHA55::GFP protein is stabilised in certain cells by the abundance of particular proteins? Obviously, the other proteins that have been shown to be constituents of the V1 headgroup – A, D, E and F (Graf et al., 1996) – are strong candidates. Of the genes that encode the possible isoforms of these proteins, exactly one per subunit shares the same expression pattern as \textit{vha55} (Allan et al., 2005), suggesting that VHA55 might be stabilised in the cytoplasm by one or more of VHA68-2, VHA36-1, VHA26, VHA14-1 and VHA13. In principle, co-overexpression experiments may help to test the model.

**Complementation of the yeast \textit{\Delta vma2} mutation by \textit{Drosophila vha55}**

The \textit{ScA} alleles identify residues are essential for V-ATPase function in \textit{Drosophila}. However, it is important to establish whether these results are specific to the \textit{Drosophila} B-subunit, or whether they identify residues that are crucial in other species. All the affected residues are identical in yeast. Accordingly, yeast deleted for the corresponding gene, \textit{\Delta vma2}, was tested for functional complementation with both wild-type and mutant \textit{Drosophila vha55}.

To determine whether the \textit{Drosophila vha55} gene can functionally complement the yeast VMA2 gene, a yeast \textit{\Delta vma2} strain (Yamashiro et al., 1990) was transformed with Met expression vectors containing the \textit{Drosophila vha55} gene, both wild-type and \textit{vha55::GFP}. Transformants were plated initially onto selective medium containing Met to repress expression, at pH 5.0. Subsequent transfer of transformants to similar medium lacking Met resulted in induction of the \textit{vha55} gene, before final transfer to selective medium at pH 7.5.

\textit{Drosophila vha55} was able to complement yeast \textit{\Delta vma2} (Fig. 4). Furthermore, GFP-tagged \textit{vha55} rescued \textit{\Delta vma2} better than the wild-type \textit{vha55} gene, presumably because the much larger chimeric transcript is translated significantly
slower than wild-type. We ascribe these results to dosage sensitivity in assembling the V-ATPase holoenzyme, consistent with the recent observation that high levels of expression of Vma5p and Vma13p can be detrimental to V-ATPase function in yeast (Keenan Curtis and Kane, 2002). Consistent with this, rescue with a (less-physiological) high-copy vector was poorer than with the centromeric (low-copy) vector (Fig. 4).

Residues identified by Drosophila vha55 point mutations are essential for yeast function

Having established that Drosophila vha55 is capable of functional complementation of yeast Δvma2, the effects of mutations at the residues defined by the SzA alleles were investigated. The same mutant residues were introduced into low-copy plasmids carrying vha55::GFP. The Δvma2 strain cannot survive at pH 7.5, but with vha55::GFP, the mutant strain grew like the wild type (Figs 4 and 5). Overexpression of any of the mutations, identified in Fig. 1, in vha55::GFP did not rescue Δvma2 at pH 7.5 (Fig. 5). Therefore, these conserved residues are all essential for V-ATPase function in yeast.

Effect of V-ATPase mutations on yeast growth

Growth rates were determined, both by serial dilutions onto nutrient plates (Fig. 6), and by densitometric measurement of doubling times in liquid culture, in order to establish whether there was a quantitative effect of B-subunit deficiency. Δvma2 carrying vha55 or vha55::GFP in centromeric (low-copy) plasmids grew as rapidly as the wild type VMA2 strain at pH7.5, with an average doubling time of 3-3.5 hours (Fig. 6A). However, Δvma2 mutant strains carrying point mutations of vha55 in the vha55::GFP construct, all failed to grow at pH 7.5 (Fig. 6A,B).

Complementation of vacuole acidification function and V-ATPase activity

The B subunit of V-ATPase has been suggested to have multiple roles. For example, it has been shown to bind directly to F-actin (Holliday et al., 2000). It is thus of interest to see whether the rescue of the pH-sensitive conditional-lethal phenotype by wild-type and mutant constructs correlated with any other functional properties of V-ATPases, for example, the ability to acidify the vacuole, or biochemical ATPase activity. To determine whether VHA55 protein fully rescued the V-ATPase activity of Δvma2, we first examined the ability of wild-type and mutant constructs to acidify yeast vacuoles in vivo. The vacuole was strongly stained by the lysosomotropic dye Quinacrine in Δvma2 strains carrying VMA2 or wild-type Drosophila vha55 (Fig. 7A), confirming that the vacuole was acidified. By contrast, the vacuole of Δvma2 strains carrying mutations in vha5 (Fig. 7B), did not stain (because of elevated vacuole pH). These mutations thus all disrupt the ability of V-ATPase to acidify the yeast vacuole.

Direct assay of V-ATPase activity

To confirm that the results observed were due to variation in V-ATPase activity, vacuole membranes were isolated from the strains, and bafilomycinA1- sensitive V-ATPase activity quantified. Surprisingly, all the strains carrying wild-type vha55 only showed 56-76% of the ATP hydrolysis activity of the VMA2 plasmid in Δvma2 (Fig. 8), although there was no difference in their growth phenotype compared with the wild type (Fig. 8). This implies that such V-ATPase levels are adequate for growth. In the strains carrying the point mutations in vha55 constructs, the mutants all had less than 25% of the wild-type V-ATPase activity (Fig. 8).
Functional expression of VHA55 protein in yeast
To confirm successful protein expression, V-ATPase B-subunit levels in protein extracts were assessed by western blotting with polyclonal antibodies raised against an epitope that is well conserved between the Drosophila and yeast B-subunits. Overexpression of VHA55 protein in yeast was carried out in selective medium, pH 5, lacking Met. The level of protein varied according to mutation (Fig. 9): protein was undetectable in vha55SzA12, found at low level in vha55SzA9, and was abundant in vha55SzA1 mutations, in the vha55SzA9 silent mutation and in vha55::GFP. Taken together, these results imply that, for at least some mutations, the aberrant protein is rapidly detected and degraded, and so does not persist after translation. The lack of protein in vha55SzA1 is unsurprising, as this encodes a severely truncated peptide (Fig. 1). However, we speculate that the relatively modest change (G-V) in vha55SzA1 is sufficient to prevent assembly, and so expose the defective subunit to degradation. This might also explain why dominant-negative effects were not observed in the fluid secretion assay (Fig. 2).

Discussion
SzA corresponds to vha55
These results provide a rigorous genetic proof of the earlier prediction (Davies et al., 1996) that the SzA alleles (Gausz et al., 1979) are defective in the single gene encoding the B-subunit of the V-ATPase in Drosophila. They also provide information about the molecular nature of the deficits, and demonstrate that vha55 is capable of complementing the corresponding yeast gene (VMA2). This observation is explained by the remarkable conservation of sequence in these ancient transport proteins (vha55 and VMA2 have 79% sequence identity at the amino acid level) across a very wide phylogenetic distance (Nelson et al., 1989). This proof that the SzA lethal complementation group corresponds to alleles of the V-ATPase B subunit allows the original painstaking description of the mutant phenotype (Gausz et al., 1979) to be reinterpreted. The SzA alleles were generated in a saturating mutagenesis of region 87C of Drosophila chromosome III, in which dozens of recessive-lethal mutations were identified (Gausz et al., 1979). These were exhaustively intercrossed, and four lethal complementation groups (SzA-SzD) identified. Although some alleles of SzA were sub-lethal (a few escapers survived to adulthood) most were embryonic and larval recessive lethal. Within this group, some trans-heterozygotes of individually lethal alleles performed unusually; they could survive to adulthood, with either mild (slight wing droop) or severe (crumpled wings, darkened abdomen) phenotypes. The SzA alleles all showed a transparent tubule phenotype, which was cell-autonomous in transplants of homoyzogous tubules into wild-type abdomens. We hypothesised, and recently showed, that this phenotype was caused by failure to precipitate uric acid crystals in the tubule lumen, and that this phenotype is shared by lethal mutants of nearly every plasma membrane V-ATPase subunit (Allan et al., 2005). Of course, the identity of SzA as a V-ATPase gene is consistent with this phenotype, and the plasma membrane location of the V-ATPase would explain the cell-autonomy of transplants. Two trans-heterozygote SzA combinations showed temperature-sensitive lethality (Gausz et al., 1979). Tantalisingly, most of these potentially valuable alleles are no longer extant. However, the three lethal lines that still exist, SzA1, SzA9 and SzA12 (Gausz et al., 1979), together with two more recently generated alleles, have been validated here as V-ATPase functional null mutations, in a range of assays in both fly and yeast.

The molecular nature of the mutations
Do these mutations cast any light on V-ATPase function in general? V-ATPase and F-ATPases originated from a common ancestor, and the A and B subunits of the V-ATPase show particularly close similarity with the β and α subunits, respectively, of the F-ATPase (Nelson and Nelson, 1989). A crystal structure for the F1 head-group of the F-ATPase was previously used to inform a site-directed mutagenesis of yeast VMA2 (Liu et al., 1996). Remarkably, two of the mutations described here are within three residues of those selected for mutagenesis of the catalytic site. The Gly-Val substitution in vha55SzA1 is only three residues from Tyr352, and the Ser-Leu substitution in vha55SzA12 is only two residues away from Arg381 of vma2p. These relatively modest substitutions could thus alter the shape of the catalytic region, explaining their lethality. Additionally, both Y352S and R381S mutations in vma2p abolished V-ATPase activity (Liu et al., 1996), as the equivalent V-ATPase alleles do (Fig. 8). The R381S substitution also affected V-ATPase assembly (Liu et al., 1996), which might explain the intermediate levels of VHA55 protein observed when VHA55 carrying the vha55SzA12 mutation is expressed in yeast (Fig. 9).
GFP-tagging the V-ATPase B-subunit does not affect function

It proved possible to rescue V-ATPase function in both fly and yeast with a GFP-tagged fly vha55 transgene. In flies, the rescued homozygous mutant survives to adulthood (this is the first reported rescue of a V-ATPase mutation in an animal), and so it is possible to assay V-ATPase function physiologically in the Malpighian tubule (Fig. 2), a tissue in which V-ATPase plays a plasma membrane role (Allan et al., 2005). Rescued mutant tubules perform statistically indistinguishably from either wild-type and heterozygous tubules, confirming the adequacy of rescue. As this cDNA was a translational fusion with enhanced GFP, we can conclude that neither V-ATPase

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**Fig. 7.** Functional assay of vacuole acidification by V-ATPase. (A) Quinacrine staining of acidified vacuoles for Δvma2 strains carrying wild-type Drosophila vha55 constructs in low-copy plasmids. Upper panels show Quinacrine-stained cells under epifluorescence; lower panels are phase-contrast images. A bright vacuole indicates functional acidification; a dark vacuole indicates inactivation of the V-ATPase. The vacuolar acidification phenotype is thus rescued both by VMA2, and by all constructs encoding vha55. (B) As A, except that yeast were carrying vha55::GFP plasmids with mutations corresponding to the alleles shown. Although the yeast can survive under these permissive conditions, none of the constructs rescue the acidification phenotype. (Although the vacuole in e.g. vha557e1 can appear less dark, this is due to an out-of-focus contribution from the cytoplasm; the contrast with functional vacuoles in the top panel is clear.)

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**Fig. 8.** Rescue of V-ATPase activity by vha55 and VMA2 constructs. V-ATPase activities were measured as V_{max} in yeast vacuole preparations. Black bars, wild-type, Δvma2 and rescued yeast; empty bars, Δvma2 yeast rescued with vha55 cDNA mutated with SzA defects. Data are mean ± s.e.m. of assays on three independent cultures, and are expressed as a percentage relative to wild-type yeast; the average V_{max} of control preparations was 2.3±0.5 μmol Pi/minute/mg protein, comparable with values reported by others (Curtis et al., 2002; MacLeod et al., 1998).
assembly nor function are compromised by this large C-terminal addition to the B subunit, in either fly or yeast holoenzymes. Indeed, a yeast stock with GFP-tagged VMA2 is now commercially available (www.invitrogen.com).

These results have provided several insights into V-ATPase function, and in particular the role and disposition of the B subunit. They have shown the first rescue of a lethal V-ATPase mutation in an animal, and that despite the large phylogenetic distance, an insect B-subunit can rescue the corresponding yeast null. The vha55::eGFP fusion offers a valuable tool to assess the dynamics of this important enzyme in an organotypic context, or even in vivo. It is also satisfying to reconcile molecular function with classical mutant phenotypes; the $S_A$ alleles (Gausz et al., 1979) thus find new life as models of a large, complex and multifunctional transport protein (Allan et al., 2005; Futai et al., 2000; Harvey et al., 1998).

Materials and Methods

Fly and yeast strains

Oregon R (wild-type) and mutant flies $l(3)S_A^{AT3}$, $l(3)S_A^{VTM3}$, $l(3)S_A^{VTM3}$ (kindly provided by J. Gausz, University of Szeged, Hungary), vha55$^{VTM3}$, y$^{kn1} w^{1118}$ sep$^{ab}$ Ubx$^{10-10}$ e se$^{8}$ and vha55$^{MKR5}$, kar$^{u} r^{y}$ Sb$^{4}$ (from Umea Stock Centre) used for the study were reared at 25°C, 55% humidity on a 12:12 hour light:dark cycle, on standard yeast cornmeal, sucrose and agar medium.

Yeast strains of wild-type SF38-5A and mutant SF38-5AV2 (vma2) which have been described previously (Liu et al., 1996) were kindly provided by P. Kane (Upstate Medical University, New York, NY). SF38-5A is leu2-3, ura3-52, ade6; SF38-5AV2 is leu2-3, ura3-52, ade6, vma2-2; URA3).

The complete medium for yeast growth was YEPE, containing 1% yeast extract (YE), 2% peptone (P) and 2% glucose. Yeast was grown on Leu-selective medium, which is 0.06% SD (BIO 101), 0.67% yeast nitrogen base (DIFCO); and supplied with 2 mg/ml Met and 2% glucose. Both complete and selective media were buffered with 1× succinic acid buffer (50 mM succinic acid and 50 mM K$_2$HPO$_4$) to pH 5 or pH 7.5 for different experimental purposes.

Drosophila transgenesis

A vha55::GFP fusion construct was generated by fusion PCR, cloned into the P-element vector pUAST (Thummel et al., 1988), and co-injected with $\Delta 2.3$ helper plasmid into w$^{1118}$ mutant Drosophila embryos at the syncytial blastoderm stage, according to standard fly protocols (Ashburner, 1989). Transformants were selected on the basis of red or pink eye colours, and the chromosome of insertion established by crossing to marked flies. In pUAST, the transgene is under the control of the UAS promoter, and can be driven by any of the many GAL4 enhancer traps lines available for Drosophila (Brand and Perrimon, 1993; Kaiser, 1993; Stoeck et al., 1997). In this case, ubiquitous expression was driven by crossing to flies transgenic for GAL4 under heat-shock control. Progeny were heat-shocked daily to 37°C for 15 minutes to maintain a steady level of transgenic VHA55::GFP to allow rescue of $S_A$ mutant phenotypes.

Localization of VHA55 in Drosophila tubule epithelium

Tubules were dissected from 1-week-old adult flies in Schneider's Drosophila medium (Gibco). For visualisation of the VHA55::GFP fusion protein, they were lightly fixed (10 minutes) in 2% paraformaldehyde and counterstained with DAPI, then viewed by epifluorescence microscopy under Fluorescein optics, as described previously (Brodierick et al., 2004; Radford et al., 2002).

PCR-based SSCP detection of mutations

19 pairs of overlapping primers spanning the vha55 gene were designed, and Drosophila EMS point mutations were characterized by PCR single-strand conformation polymorphism (PCR-SSCP) (Orita et al., 1989a; Orita et al., 1989b). Briefly, genomic DNA was extracted from wild-type and vha55 mutant heterozygous flies by standard methods, and each sample amplified with each of the 19 primer pairs. 5 µl of each PCR product was mixed with 2 µl high-density loading buffer and 10 µl formamide, heat-denatured at 90°C for 5 minutes, placed on ice for 10 minutes and loaded onto a 0.5-1.0% MDE gel (Flowgen) with 0.5% APS and 10 µl TEMED. The gel was run in 0.5× TBE buffer at 200 V for 1-2 hours. Any single-stranded DNA mobility changes due to mutations within the amplifiers were detected by comparison with control samples.

Site-directed mutagenesis of VMA2

PCR-based site-directed mutagenesis was performed according to the Stratagene Quickchange site-directed mutagenesis manual. Mutations were induced into a ready-made plasmid, DV-GFP, by PCR, with 1× Pfu buffer, 10 mM dNTPs, 20 ng methylated plasmid, 125 ng of each primer pair and 2.5 U Pfu Turbo DNA polymerase in a final volume of 50 µl with DW. The reaction was carried out off-cycle at 95°C for 30 seconds, 12 cycles of 95°C for 30 seconds, 55-67°C for 1 minute, 68°C for 20 minutes and 1 cycle of 98°C for 5 minutes for inactivation of the reaction. The methylated non-mutated parental plasmid was digested by adding 10 U Dpn I enzyme (Promega), which recognizes the restriction site 5'-Gm6-3' in the PCR product, and incubated at 37°C for 1 hour. The circular nicked dsDNA was then transformed into XL1 blue supercompetent cells (Stratagene), and mutated plasmids were recovered by screening the colonies on LB plates with ampicillin (100 µg/ml). Mutated sites were confirmed by sequencing.

Construction of plasmids and lithium acetate transformations

High or low-copy-number shuttle vectors for expression of the Drosophila vha55 gene in yeast are derived from pRS424/p415, which contains the LEU2 gene and the 2µ/CEN ori (Mumberg et al., 1994) (Fig. 3). The centromeric vector is held at only 1-2 copies per cell, and thus obviates problems associated with high copy-number vectors (Mumberg et al., 1994). pRS425/p415 has a Mat-repressible promoter inserted between SacI-XhoI sites of the ampicillin-resistance gene of pSp425/p415. For constructs DV425, VMA2425 and CVG415, Drosophila vha55 or yeast VMA2 coding sequence were subcloned from EST clone LP114411 (Accession no. AAI0192), and plasmid pCY3 37 (kind gift of P. Kane), respectively. A vha55::GFP fusion was made by fusion PCR, with primers CCGCGCGG-CTCAGACGTCGTTTGAGCTCA and CTGGCCGCTGCTCCATCACCGCGA- GTCCCTAGG, with 5-base-pair complementarity to eGFP (primers CTACC-TAGGACGTGCGGATGAGCAAGGGC and GTCTAGACTTGTACAGC- CTGCTCATGGCGAG) amplified from plasmid pGFP-N1 (Clontech). Fusion reactions were carried out at 95°C for 10 minutes to denature the two products, followed by a 30-minute extension at 45°C with 1× HiFi PCR buffer, 50 µM dNTPs and 10 U HiFi DNA polymerase (Roche). 1 µl fusion product was added to 49 µl PCR mix of 1× HiFi PCR buffer, 200 µM dNTPs, 300 nM primers (with HindIII and PstI sites) and Hi Fi DNA polymerase. The inserted fragments span the region of nucleotides 87(ATA)-1559(TAG) of the vha55 cDNA; the region of nucleotides 218-2386 of yeast VMA2 and a vha55::GFP, respectively. A high efficiency lithium acetate transformation protocol for yeast was used (Gietz and Woods, 2002). The cell suspension was plated onto Leu- Met+ selective medium with 2% glucose at pH 5.0, and incubated at 30°C for 2-4 days.

Yeast growth rate measurement

Growth rates were monitored by densitometric growth curves and by colony-forming dilution experiments. Transformant yeast cells were cultured in selective medium, pH 5.0 with 2 mg/ml Met, overnight to reach a density of 2×10$^{6}$ (mid-log phase), then diluted to 2% in selective medium without Met at pH 7.5, and grown at 30°C for 24 hours. Cell densities were monitored versus time by spectrophotometry at 600 nm. For the colony-forming dilutions, 1×10$^{6}$ cells were harvested at mid-log phase and serially diluted 1.5 times eight times. Equal amounts of each dilution were spotted onto pH 5 and pH 7.5 selective medium lacking Met, and grown at 30°C for 2-3 days.
Vacuole functional studies: purification, bafilomycin-sensitive V-ATPase assay and vacuole staining Wild-type. Δvma2 and vha55 transformed yeast were grown overnight in 500 ml selective medium, pH 6.0 adjusted to OD560 of 1.0 and 4 x 10^7 cells harvested. A modified version of the yeast vacuole vesicle preparation (Kakimura et al., 1981) was used. Spheroplasts were prepared by resuspending cells in 100 ml of 1 M sorbitol and adding 1 ml of lysozyme solution (50 mM Tris-HCl, pH 7.7, 1 mM EDTA, 50% glycerol, 400 U/ml lysozyme) for 10 min at 25°C. After centrifugation, cells were washed twice and diluted to 1 l litre in Met- medium, grown to OD600 of 1.4 and 4 x 10^8 cells collected. 

Ficoll was collected and homogenized in 6 ml buffer A. The suspension was centrifuged at 2200 g for 5 minutes, adding 1.5 ml buffer A and centrufuging at 30°C for 15 minutes to the V1 and V0 sectors separated. Cells were collected and washed twice in 1 M sorbitol. Spheroplasts were lysed by resuspending the final pellet in 25 ml buffer A (10 mM MES/Tris-HCl, pH 6.9, 0.1 mM MgCl2, 12% Ficoll 400) and homogenizing at 0°C. Unlysed spheroplasts were removed by centrifuging the lysate at 2200 g for 10 minutes at 4°C. Vacuoles were purified by flotation: the supernatant was transferred to a polycarbonate tube of 38 ml, carefully overlaid with 13 ml buffer A and centrifugation at 60,000 g in a SW 28 rotor for 30 minutes. The white 'wafer' at the top of the Ficoll was collected and homogenized in 6 ml buffer A. The suspension was transferred into a 5 ml polycarbonate tube, overlaid with 5 ml of buffer B (10 mM MES/Tris-HCl, pH 6.9, 0.5 mM MgCl2, 8% Ficoll 400), centrifuged at 60,000 g in a SW 28 Ti rotor for 30 minutes. The final white wafer on the top of the tube was collected and resuspended in a small volume (0.2-1 ml) of buffer C (10 mM MES/Tris-HCl, pH 6.9, 5 mM MgCl2, 5% Glycerol, 50 mM Na2HPO4 pH 7.6, containing 2% glucose). Vacuoles were stored at –70°C. 

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