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# The vacuolar proton-ATPase plays a major role in several membrane-bounded organelles in *Paramecium*

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## Summary

The vacuolar proton-ATPase (V-ATPase) is a multisubunit enzyme complex that is able to transfer protons over membranes against an electrochemical potential under ATP hydrolysis. The enzyme consists of two subcomplexes:  $V_0$ , which is membrane embedded; and  $V_1$ , which is cytosolic.  $V_0$  was also reported to be involved in fusion of vacuoles in yeast. We identified six genes encoding c-subunits (proteolipids) of  $V_0$  and two genes encoding F-subunits of  $V_1$  and studied the role of the V-ATPase in trafficking in *Paramecium*. Green fluorescent protein (GFP) fusion proteins allowed a clear subcellular localization of c- and F-subunits in the contractile vacuole complex of the osmoregulatory system and in food vacuoles. Several other organelles were also detected, in

particular dense core secretory granules (trichocysts). The functional significance of the V-ATPase in *Paramecium* was investigated by RNA interference (RNAi), using a recently developed feeding method. A novel strategy was used to block the expression of all six c- or both F-subunits simultaneously. The V-ATPase was found to be crucial for osmoregulation, the phagocytotic pathway and the biogenesis of dense core secretory granules. No evidence was found supporting participation of  $V_0$  in membrane fusion.

Key words: V-ATPase, RNAi, Contractile vacuole, Phagosome, Trichocyst

## Introduction

The vacuolar-proton-ATPase (V-ATPase) translocates protons across membranes against their electrochemical potential through ATP hydrolysis. The V-ATPase is responsible for the acidification of organelles like phagosomes, lysosomes, early endosomes, the trans-Golgi-network, dense core secretory granules and vacuoles of plants (Sun-Wada et al., 2003a; Nelson, 2003; Kluge et al., 2003). In some specialized cells of higher organisms, the enzyme is also localized in the plasma membrane and serves to secrete protons to the extracellular space. For example, the acidification of the extracellular matrix by proton secretion of osteoclasts is important for bone resorption (Toyomura et al., 2003). An important aspect of the proton translocation by the V-ATPase is the creation of an electrochemical potential that can be used for secondary active transport of ions. This mechanism is used in neuronal cells, where the neurotransmitters glutamate or  $\gamma$ -amino-butyric-acid are concentrated in the lumen of synaptic vesicles (Roseth et al., 1995; Fonnum et al., 1998) and in chromaffin granules (Apps, 1997).

The V-ATPase is composed of two subcomplexes: the cytosolic  $V_1$ -sector, where ATP binding and hydrolysis takes place; and a transmembranous  $V_0$ -sector, through which protons are tunneled. The holoenzyme in *Saccharomyces cerevisiae* consists of 14 different subunits (Kawasaki-Nishi et al., 2004; Sambade and Kane, 2004).  $V_1$  contains subunits A-

H in the stoichiometry  $A_3B_3CDEFG_2H_{1-2}$ .  $V_0$  is formed by the subunits a, c, c', c'', d and e in a stoichiometry of  $ade_xc_4c''$ . The V-ATPase was shown to act by a rotary mechanism similar to that of the structurally related F-ATPase (Imamura et al., 2003).

The enzyme activity can be regulated by reversible dissociation of  $V_1$  from  $V_0$ . It was found in yeast that the ratio of assembled/dissassembled enzyme is strongly influenced by the culture conditions. Most of the  $V_1$ -domains dissociate from their  $V_0$  counterparts at vacuoles in yeast when glucose is depleted in the medium (Kane and Smardon, 2003). Whether other physiological conditions exist that lead to a dissociation of the enzyme is unclear.

Apart from its enzymatic action as a proton pump, the  $V_0$ -subcomplex of the V-ATPase was found to be localized in the plasma membrane and to be involved in the release of neurotransmitter in mammalian cells (Falk-Vairant et al., 1996; Morel, 2003). A role of the  $V_0$ -sector was also shown in homotypic membrane fusion of vacuoles in yeast (Peters et al., 2001).  $V_0$ -subcomplexes were reported to build so-called 'trans-complexes' in opposing membranes and were postulated to facilitate membrane fusion. A knockout mutant of the vacuolar a-subunit of  $V_0$  (vph1) was shown to cause fragmentation of vacuoles, also indicating an involvement of the V-ATPase in membrane dynamics (Bayer et al., 2003).

*Paramecium* is a unicellular model organism, in which

several differentiated membrane traffic pathways coexist. Especially well characterized is the membrane traffic leading to regulated exocytosis of dense core granules called trichocysts (Vayssié et al., 2000; Plattner and Kissmehl, 2003) and the phagosomal/lysosomal system (Allen and Fok, 2000). Phagosomes are acidified to inactivate ingested bacteria (Fok and Allen, 1988). The V-ATPase is delivered by the fusion of small, acidic vesicles, the 'acidosomes' with newly formed food vacuoles. Acidification takes place only for a short period (3-5 minutes) before the luminal pH of the food vacuole raises to become nearly neutral (Fok et al., 1982). Additionally, there exist traffic routes in *Paramecium* that can be found only in free-living freshwater protozoans, such as the contractile vacuole complex (Allen and Naitoh, 2002) used to expel excess water that enters through the plasma membrane. The organelle consists of the contractile vacuole from which emerge 5-10 radial arms. The radial arms are complex membrane systems that direct a stream of water into the contractile vacuole, through which it is expelled to the extracellular medium by membrane fusion of the contractile vacuole with the plasma membrane. This membrane system consists of the collecting canals and the spongione, which is composed of the smooth and the decorated spongione as characterized by the different ultrastructure (Allen and Naitoh, 2002). The V-ATPase was shown to be concentrated in the decorated spongione by using an antibody against the B-subunit of V<sub>1</sub>, and in fact gives the 'decorated' appearance to these membrane tubules (Fok et al., 1995).

In this work, we identified essential genes encoding the V-ATPase subunits in *Paramecium tetraurelia* and studied their function by RNAi and their localization using GFP fusion genes. Surprisingly, we found the V-ATPase to be involved not only in acidic compartments, but also in all membrane compartments we were able to identify.

## Materials and Methods

### Culturing of *P. tetraurelia*

Wild-type *Paramecium* cells of stock d4-2 were used in all experiments. Cells were grown at 27°C in a wheat grass infusion (BHB, L'arbre de vie, Luçay Le Male, France; or WGP, Pines International, Lawrence, KA) bacterized with *Klebsiella pneumoniae* and supplemented with 0.4 µg/ml β-sitosterol according to standard procedures (Sonneborn, 1970).

### Cloning and sequencing of cDNA

Total *Paramecium* RNA was extracted as described earlier (Froissard et al., 2001). The reverse transcription reaction was carried out with the 5'-RACE System from Invitrogen according to the manufacturer's instructions. Primers used for the amplification of a 350 bp fragment of the F-subunit were: 5'-gcgctcgagcatccctagatcaattattggcg-3' and 5'gctgtagatcactaaatctgtaccatacaata-3'. The PCR reaction, cloning with *Xho*I and *Xba*I into pBluescript II SK<sup>-</sup> (Stratagene) and sequencing was carried out according to standard procedures in molecular biology.

### Gene silencing constructs

The double T7-promotor plasmid L4440 described in Timmons et al. (Timmons et al., 2001) was used; the complete macronuclear sequence of a gene from the start to the stop codon was amplified by PCR with PCR primers containing the restriction sites required for

cloning. Restriction sites used for cloning were: *Xho*I and *Acc*65I for *vha*c2; *Xho*I and *Eco*RI for *vha*c4; *Eco*RI and *Not*I for *vha*c6; and *Xba*I and *Xho*I for *vha*F1. The following primers were used [start codons atg and stop codons tca (reversed complement: tga) are underlined]: c2, 5'-gcgggtaccatgatgcttttcattcttgacacaatgg-3', 5'-gcgctcagatcatgagcgaagaatcaagg; c4, 5'-gcgctcgagatggctgagaacgatactattgagc-3', 5'-gcggaattctcatgatgtttatgataaaatcaagg-3'; c6, 5'-gcggaattctcatgctttttattctgatactatgg-3', 5'-gcggcgccgctcatgattatgataagatcaatga-3'; F, 5'-gcgctcgagatgtcaaaagaaatcttaagaatc-3', 5'-gcgtctagatcactaatatctgtaccatacaata-3'. PCR reactions and cloning was carried out according to standard procedures. For the extinction of more than one gene, the plasmids pL4440/c2/c6 and pL4440/c2/c4/c6 were constructed. As a negative control, the plasmid pL4440 was used. Positive control was the plasmid pL4440/NSF (Galvani and Sperling, 2002). Part of the dsRNA is evidently capable of leaving the food vacuole during feeding and arrives in the cytoplasm, where it is thought to be diced into siRNA. This makes it possible to silence more than one gene of interest by cloning the genes in tandem. Considering that only the delivery of siRNA is important for silencing, the organization of the genes on the dsRNA is not important because RNA processing disrupts this organization anyway. The approach to use one plasmid with several genes instead of feeding two or more *Escherichia coli* strains containing different plasmids has the advantage that the same amount of dsRNA of each gene of interest is delivered. *E. coli* strains with different pL4440 plasmids show remarkably different growth rates in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG), resulting in an uncontrolled change of the ratio of different feeding bacteria during the experiment.

### Gene silencing by feeding

The gene silencing plasmids described above were transformed into the RNaseIII-deficient *E. coli* strain Ht115 (Timmons et al., 2001). An overnight culture was grown in LB medium with ampicillin (amp) and tetracyclin as selecting agents at 37°C in a shaker (Sambrook et al., 1989). The overnight culture was used to inoculate LB/amp 1/100 (v/v). The culture was grown to an optical density at 600 nm (OD<sub>600nm</sub>) between 0.2 and 0.4, and IPTG was added to a final concentration of 125 µg/ml. The culture was induced for 3 hours and induced bacteria were pelleted by centrifugation. The pellet was resuspended in *Paramecium* culture medium and the OD<sub>600nm</sub> was measured. The culture was diluted with medium to give a final OD<sub>600nm</sub>=0.25 and supplemented with 100 µg/ml amp, 125 µg/ml IPTG and 0.4 µg/ml β-sitosterol.

*Paramecium* cells were pelleted by centrifugation at 1200 rpm for 2 minutes at room temperature (RT) in a Sigma 6-15 centrifuge, washed twice in Dryl's buffer and starved for at least 1 hour in Dryl's at RT before used in a feeding experiment. In single cell experiments, one cell was added to 300 µl of the feeding solution in a depression well. For mass feeding experiments, 50 paramecia per ml were added to the feeding solution. Cells were cultured at 27°C during the experiment. The phenotype was analyzed 20-26 hours after the start of the feeding experiment. During the experiment, the cells were transferred every 24 hours to freshly prepared feeding solution.

### Picric acid test

Exocytotic capacity was analyzed by adding one cell in a minimal volume of medium into saturated picric acid.

### AED triggering

Cells were transferred in a buffer consisting of 10 mM TrisCl, 0.1 mM CaCl<sub>2</sub>, 0.2% (w/v) aminoethyl dextran, pH=7.0, and incubated for several minutes at RT (Plattner et al., 1984).

### Phagocytotic test

2 ml of an overnight culture of *E. coli* in LB medium was pelleted by centrifugation and resuspended in 1 ml Dryl's buffer. Bacterial cells were washed once more (as above), again resuspended in 1 ml Dryl's and 4  $\mu$ l of an Alexa594-coupled goat anti-mouse F(ab')<sub>2</sub> (Molecular Probes) was added and vortexed. Cells were incubated in this feeding solution for 30 minutes and processed for fluorescence microscopy (see below).

### Decoration of living paramecia with anti-GFP antibody

Living descendants of a cell transformed simultaneously with c1-, c4- and c5-GFP were incubated in Dryl's buffer containing 1/100 (v/v) of a mixture of two monoclonal mouse anti-GFP antibodies (Roche) for 30 minutes at RT, followed by three times washing in Dryl's. Then cells were incubated for 30 minutes at RT in Dryl's containing 1/300 (v/v) of the Alexa-coupled goat anti-mouse F(ab')<sub>2</sub>, followed by three times washing in Dryl's and preparation for light microscopy (see below). As a negative control, uninjected cells were prepared in the same way.

### GFP constructs

The eGFP-gene presented in Hauser et al. (Hauser et al., 2000a) was cloned into the *Acc65I* site of the *Paramecium* overexpression vector described in Haynes et al. (Haynes et al., 1995) referred to as pPXV-GFP. All genes were cloned between *SpeI* and *XhoI* sites in-frame with the gene encoding GFP. The stop codon of the genes was changed from tga to gga (coding for glycine). PCR primers used were [start codons atg and former stop codons changed to tcc (reversed complement of gga) are underlined]: c1, 5'-gcgactagtctgcagatgcttttattcttgacacaatgtag-3', 5'-gcgctcgagatcctgattgcgataagatcaagg; c4, 5'-gcgactagtctgcagatggctgagaacgatactattgag-3', 5'-gcgctcgagatcctgattgataaaatcaagg-3'; c5, 5'-gcgactagtctgcagatgctttttattcttgatgac-3', 5'-gcgctcgagatcctgattatgataagatcaatgac-3'; F, 5'-gcgactagtatgtcaaga-aactttaagaaatc-3', 5'-gcgctcgagttccctaaatctgtaccatacaata-3'; TMP1b, 5'-gcgactagtatgtataaattagcagctctgcacattg-3', 5'-gcgctcgagatccaaatgctcccttgagttggg-3'. So the gene of interest is separated from GFP by a 7 amino acid spacer: i.e. c-subunit1-GSRGTNNM-GFP (the underlined G for glycine corresponds to the former stop codon of the gene; the underlined M for methionine represents the start codon of GFP).

### Microinjection of GFP constructs

Plasmid DNA was prepared with a plasmid midi kit (Qiagen) according to the manufacturer's protocol. 50  $\mu$ g of plasmid DNA was linearized by digestion with 20 units of *SfiI* at 50°C overnight. The DNA was precipitated by adding 1/10 (v/v) 3M sodium acetate pH=5.2, 2.5 (v/v) ethanol and incubated for 60 minutes at -20°C. DNA was pelleted by centrifugation, washed with 70% (v/v) ethanol and dried. The DNA was resuspended in 10  $\mu$ l of Millipore-filtered water. The resuspended DNA was again centrifuged for 30 minutes at 4°C, and 8  $\mu$ l of the supernatant was taken off and transferred to a new tube to avoid the presence of any insoluble material that could block the microinjection needle. Microinjection of the DNA was carried out as described in Froissard et al. (Froissard et al., 2002).

### RNA extraction and northern hybridization

Clones injected with c1- or c4-GFP plasmids, or non-injected control cells, were grown in culture to a density of 600-800 cells/ml to a final volume of 400 ml. Cells were washed one time in 100 ml Dryl's buffer and concentrated by centrifugation. RNA was prepared using the guanidine method described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). c1- and c4-DNA probes were

labeled with [ $\alpha$ -<sup>32</sup>P]dATP (Hartmann Analytic) using the High prime DNA labeling kit (Roche). Approximately 15  $\mu$ g of total *Paramecium* RNA was loaded per lane on a formaldehyde/agarose gel. Hybridization was carried out at 42°C overnight in hybridization solution as described in Sambrook et al. (Sambrook et al., 1989). Autoradiography films (Fuji Film) were exposed between 1 and 120 hours.

### Fluorescence microscopy

For the GFP recording of living cells, *Paramecium* cells were washed two times in Dryl's buffer containing 0.2% bovine serum albumin (BSA). Cells were transferred into a small drop on a coverslip and overlaid with paraffin oil. Excess buffer was sucked off until the cells were immobile. GFP-labeled paramecia were also prepared for long-term storage by fixation in 2.5% formaldehyde in PHEM-buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl<sub>2</sub>, pH=6.9) at RT for 10 minutes, washed two times in TBS+BSA (10 mM TrisCl, 150 mM NaCl, 3% BSA, pH=7.4) and mounted in Citifluor AF2 (Citifluor). Cells were analyzed in a Zeiss Axioskop 2 plus fluorescence microscope equipped with a coolsnap cf camera (Zeiss). Images were processed using the Metamorph software (Universal Imaging).

### Electron microscopy

Cells were concentrated by centrifugation, quickly added into 2.5% glutaraldehyde in phosphate-buffered saline (PBS) containing 10 mM MgCl<sub>2</sub> and incubated for 1 hour at RT. Fixed cells were washed two times in PBS and transferred into 1% (w/v) osmiumtetroxide in 0.1 M cacodylate buffer. Cells were incubated for 1 hour at RT, washed twice with PBS and dehydrated in an ethanol series followed by embedding in Spurr's resin.

## Results

### Identification of c-subunits (proteolipids) of V<sub>0</sub> and F-subunits of V<sub>1</sub> in *P. tetraurelia*

Only one gene of the *Paramecium* V-ATPase has been previously described, encoding the B-subunit of V<sub>1</sub> of *P. multimicronucleatum* (Fok et al., 2002). In a pilot genome survey of *P. tetraurelia* (Dessen et al., 2001), a clone containing a sequence highly similar to a c-subunit (proteolipid) was identified, thus opening the possibility of gaining molecular knowledge on V<sub>0</sub>-subunits. By using this c-subunit gene as a probe to screen an indexed genomic library (described in Keller and Cohen, 2000), four c-subunits could be identified, namely Pt-VHAc1-c4.

Recently, *Paramecium* whole-genome shotgun sequencing was undertaken at Genoscope (<http://www.genoscope.cns.fr>). A BLAST search was performed for V<sub>0</sub> c-subunits on the genome and two more genes, designated as Pt-VHAc5 and Pt-VHAc6, were identified. The six genes can be divided into three pairs, namely c1/c2, c3/c4 and c5/c6. The two genes within a pair share more than 85% sequence identity at the nucleotide level and introns are at the same position, though they may vary in length (Table 1). The proteins encoded by these genes are highly conserved in *Paramecium* (Fig. 1). They show considerable amino acid identity with c-subunits of other organisms, the same organization (with four membrane-spanning helices) (Flannery et al., 2004) and are highly hydrophobic molecules (Kawasaki-Nishi et al., 2003). The *Paramecium* c-subunits show the features described in other

**Table 1. Molecular characteristics of c-, F- and A-subunits of the V-ATPase in *Paramecium tetraurelia***

| Gene            | Length (bp) | Amino acids | Number of introns | Molecular mass (kDa) | Accession number |
|-----------------|-------------|-------------|-------------------|----------------------|------------------|
| <i>Pt-VHAc1</i> | 559         | 167         | 2                 | 17.3                 | AJ566616         |
| <i>Pt-VHAc2</i> | 561         | 167         | 2                 | 17.2                 | AJ566617         |
| <i>Pt-VHAc3</i> | 557         | 159         | 3                 | 16.1                 | AJ566618         |
| <i>Pt-VHAc4</i> | 557         | 159         | 3                 | 16.1                 | AJ566619         |
| <i>Pt-VHAc5</i> | 558         | 165         | 2                 | 17.1                 | CR764091         |
| <i>Pt-VHAc6</i> | 559         | 165         | 2                 | 17.1                 | CR764090         |
| <i>Pt-VHAF1</i> | 457         | 127         | 3                 | 14.4                 | CR764093         |
| <i>Pt-VHAF2</i> | 458         | 127         | 3                 | 14.4                 | CR764092         |
| <i>Pt-VHAA1</i> | 2008        | 618         | 6                 | 69.3                 | CR764097         |
| <i>Pt-VHAA2</i> | 2009        | 618         | 6                 | 69.3                 | CR764096         |
| <i>Pt-VHAA3</i> | 2011        | 618         | 6                 | 69.3                 | CR764095         |
| <i>Pt-VHAA4</i> | 2013        | 618         | 6                 | 69.3                 | CR764094         |

organisms; for example, this includes the conserved Glu137 in the yeast proteolipid vma3p (Hirata et al., 1997), and the three important Thr32, Phe136 and Tyr143 residues involved in

binding of the V-ATPase inhibitor bafilomycin identified in *Neurospora crassa* (Bowman and Bowman, 2002) (Fig. 1). It is important to note that the V-ATPase of *Paramecium* is only sensitive to concanamycin but not to bafilomycin (Fok et al., 1995), leaving the question open as to why bafilomycin does not act on *Paramecium* proteolipids when the residues required for binding are present. This fact might help to understand the binding and action of the two toxins. The classification of the c-subunits of *Paramecium* using the categories c, c', c'' of yeast is not possible because the proteins show higher amino acid identity with each other than with orthologs (Table 2).

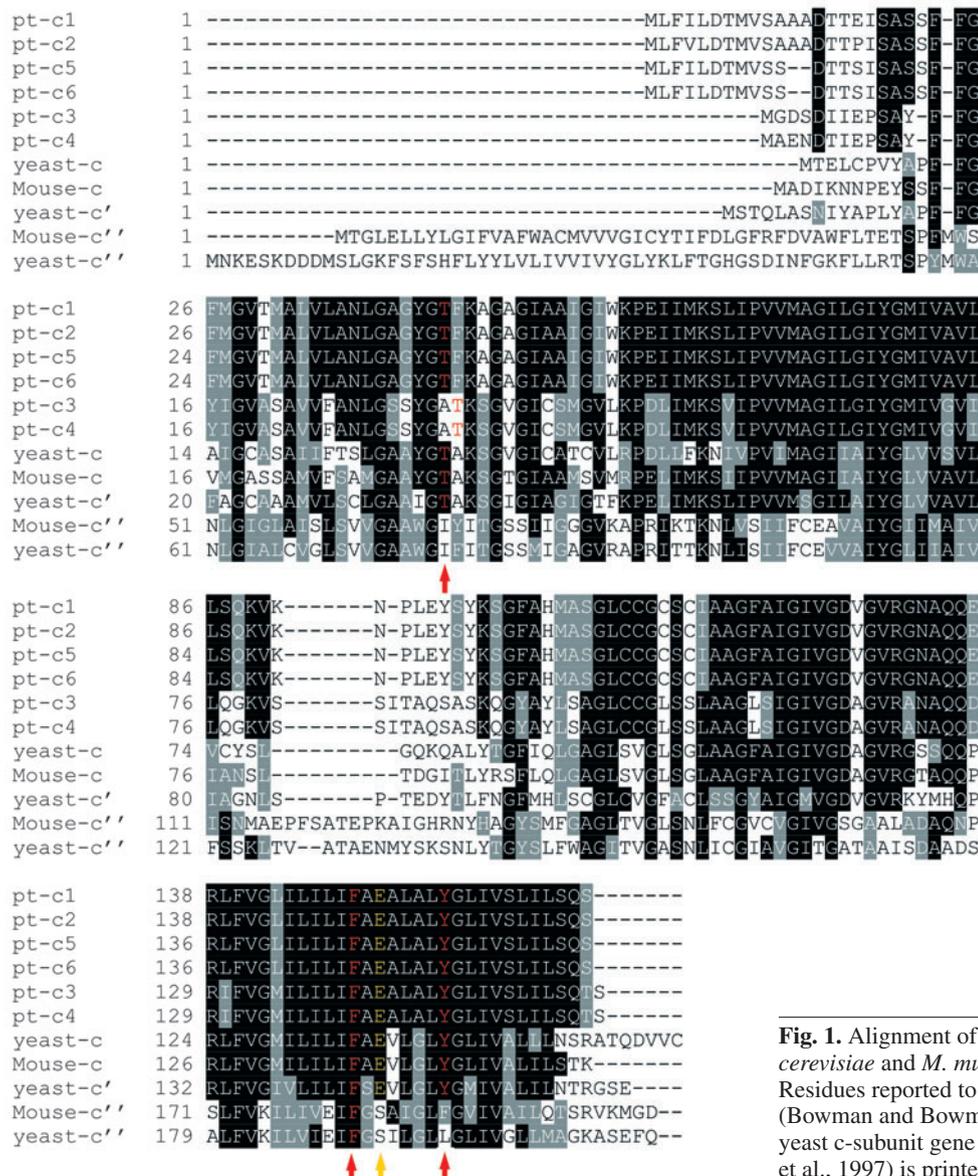
The *Paramecium* genome project database was also searched by BLAST for genes coding for the other subunits of the V-ATPase (Table 3). Homologs of all V<sub>1</sub>-subunits could be identified except subunit G, which is very small (approximately 13 kDa) and not sufficiently conserved. For investigating V<sub>1</sub>, we chose the F-subunits that are encoded by two genes, showing 89% identity at the nucleotide level and only one conserved amino acid change at the protein level. Both macronuclear F-subunit genes display a new type of intron not

previously observed in *Paramecium*. In both genes, the intron between nucleotides 170 and 195 lacks the canonical AG 3'-border but displays a TCG at the 3'-end. Amplification, cloning and sequencing of a 350 bp fragment of the F-subunit 1 from cDNA permitted confirmation of the existence and sequence of this new type of intron.

#### Localization of the V<sub>0</sub>- and V<sub>1</sub>-subcomplexes by GFP labeling

To investigate the subcellular localization of V<sub>0</sub>, three GFP fusion genes were constructed. Of each of the c-subunit pairs, one gene (c1, c4 and c5) was cloned into pPXV-GFP, the *Paramecium* overexpression vector in which a GFP tag is under the control of the calmodulin promoter (Haynes et al., 1995). The GFP gene was fused to the 3'-end of the c-subunit genes. These fusion constructs were introduced into *Paramecium* cells by microinjection into the macronucleus and GFP fluorescence was analyzed.

No difference in the localization between the gene fusions c1-GFP,



**Fig. 1.** Alignment of the c-subunits of *P. tetraurelia*, *S. cerevisiae* and *M. musculus* using the ClustalW algorithm. Residues reported to be involved in bafilomycin binding (Bowman and Bowman, 2002) are printed in red, Glu137 of the yeast c-subunit important for proton translocation (Hirata et al., 1997) is printed in yellow.

**Table 2.** Amino acid identity (%) of c-subunits from *P. tetraurelia*, *S. cerevisiae* and *M. musculus*, calculated by a ClustalW alignment

|           | Pt-c2 | Pt-c3 | Pt-c4 | Pt-c5 | Pt-c6 | Yeast-c | Mouse-c'' | Mouse-c | Yeast-c'' | Yeast-c' |
|-----------|-------|-------|-------|-------|-------|---------|-----------|---------|-----------|----------|
| Pt-c1     | 98.8  | 59.7  | 61.0  | 97.0  | 97.0  | 45.0    | 25.7      | 55.5    | 25.7      | 51.8     |
| Pt-c2     |       | 59.1  | 60.4  | 96.4  | 96.4  | 45.0    | 25.1      | 55.5    | 25.7      | 51.8     |
| Pt-c3     |       |       | 97.5  | 60.4  | 60.4  | 52.2    | 25.2      | 55.5    | 26.4      | 46.5     |
| Pt-c4     |       |       |       | 60.4  | 60.4  | 52.2    | 25.2      | 55.5    | 26.4      | 47.8     |
| Pt-c5     |       |       |       |       | 100   | 45.0    | 25.5      | 54.8    | 26.1      | 52.4     |
| Pt-c6     |       |       |       |       |       | 45.0    | 25.5      | 54.8    | 26.1      | 52.4     |
| Yeast-c   |       |       |       |       |       |         | 30.6      | 69.0    | 30.0      | 51.9     |
| Mouse-c'' |       |       |       |       |       |         |           | 28.4    | 47.8      | 26.2     |
| Mouse-c   |       |       |       |       |       |         |           |         | 29.0      | 60.0     |
| Yeast-c'' |       |       |       |       |       |         |           |         |           | 28.0     |

Amino acid identity within pairs of genes is shaded in black, identity between the pairs c1/2 and c5/6 is shaded in grey.

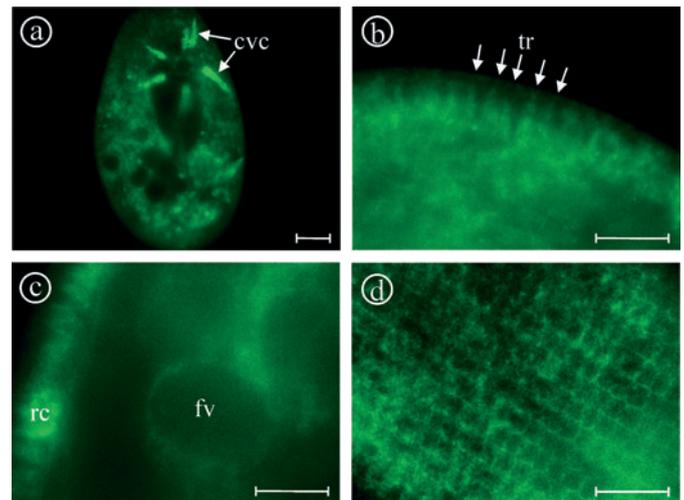
**Table 3.** V-ATPase subunits of *Paramecium*, mouse and yeast

| V <sub>0</sub> | <i>P. tetraurelia</i> | <i>M. musculus</i> | <i>S. cerevisiae</i> |
|----------------|-----------------------|--------------------|----------------------|
| a              | 17                    | 4                  | 2                    |
| c              | 6                     | 1                  | 1                    |
| c'             | 0                     | 0                  | 1                    |
| c''            | 0                     | 1                  | 1                    |
| d              | 3                     | 2                  | 1                    |
| e              | 2                     | Not identified     | 1                    |
| V <sub>1</sub> |                       |                    |                      |
| A              | 4                     | 1                  | 1                    |
| B              | 4                     | 2                  | 1                    |
| C              | 1                     | 3                  | 1                    |
| D              | 1                     | 1                  | 1                    |
| E              | 4                     | 2                  | 1                    |
| F              | 2                     | 1                  | 1                    |
| G              | Not identified        | 3                  | 1                    |
| H              | 4                     | 1                  | 1                    |

*Paramecium* genes were identified by tblastn searches in the assembly database (state Dec. 2003). Data are therefore preliminary. G subunits could not be identified owing to their small size and insufficient sequence conservation. Mouse data are from Sun-Wada et al. (Sun-Wada et al., 2003b), and yeast data from Graham et al. (Graham et al., 2003).

c4-GFP and c5-GFP was observed. The most striking labeling was at the contractile vacuole complex (Fig. 2a). It is well established that the V-ATPase is highly abundant in the contractile vacuole complex of freshwater protozoans (Fok et al., 1995). Membranes of phagosomes (or food vacuoles) were also revealed by the c-GFP gene fusions (Fig. 2c). Less stained but visible are the trichocysts (Fig. 2b). By focusing on the cortex of transformed cells, a stained network can be seen (Fig. 2d). A good candidate is the endoplasmic reticulum (ER), which in *Paramecium* expands into the cytoplasm in the cortical region of the cell (Ramoino et al., 2000; Hauser et al., 2000b). Finally, apart from this distinct staining of organelles, strong staining of the cytoplasm was visible, probably representing unidentified membranous structures.

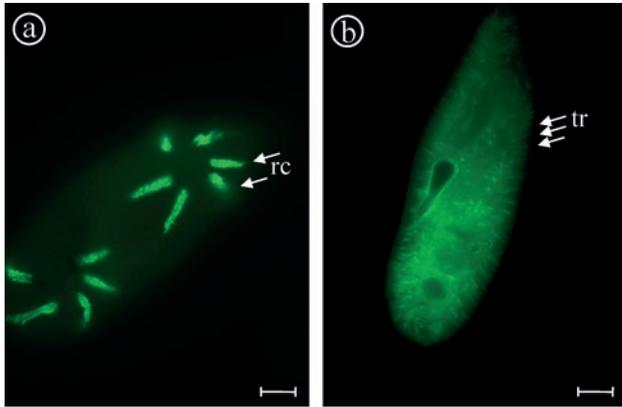
Localization of the soluble V<sub>1</sub>-subcomplex was achieved by tagging the F2-subunit gene with GFP. Injection of a moderate amount of this F-GFP plasmid resulted in bright staining of the



**Fig. 2.** Transformation of *Paramecium* cells with c-subunit-GFP (V<sub>0</sub>) constructs. Staining with the constructs c1-GFP, c4-GFP and c5-GFP showed no differences; thus, all the c-GFPs seem to be targeted to the same organelles. (a) c5-GFP, strong labeling of the radial canals (rc) of the contractile vacuole complex (cvc); (b) c4-GFP, trichocysts (tr) attached to the cortex are labeled but somewhat obscured by strong cytoplasmic labeling; (c) c4-GFP, the membrane of a food vacuole (fv) is labeled; (d) c4-GFP, when focused on the cell cortex, a regular network is visible, probably representing the ER. Bars, 10 μm.

radial arms of the contractile vacuole complex, reflecting the high binding capacity of this organelle of V<sub>1</sub> (Fig. 3a). Injection of a large amount of F-GFP plasmid clearly labeled the contractile vacuole complex, but also trichocysts, a punctate network at the cell surface and numerous vesicles at the cytotome, though cytoplasmic background was strong (Fig. 3b).

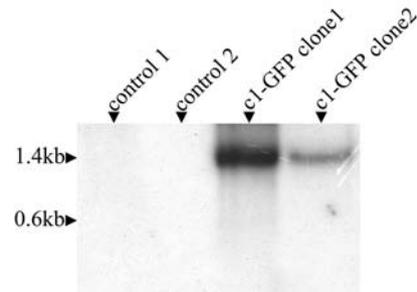
To evaluate the expression level of GFP fusion proteins in transformed cells, we first attempted to use an anti-c1-subunit antibody that turned out to be not reactive enough to reveal a signal on a western blot. We therefore decided to quantify the



**Fig. 3.** GFP localization of the F-subunits of  $V_1$ . (a) Injection of a moderate amount of F-GFP plasmid into the macronucleus resulted in bright staining of the radial canals (rc) of the contractile vacuole complex; (b) injection of a large amount additionally stained trichocysts (tr), a regular network at the cell cortex, and it caused considerable cytosolic background. Bar, 10  $\mu$ m.

messenger RNAs on northern blot. Surprisingly, autoradiography for 120 hours did not reveal the expression of the endogenous c-subunits, whereas the recombinant forms of c1 and c4 were easily detectable (Fig. 4). The endogenous mRNAs of both c1 and c4 were only detectable in RT-PCR (not shown). This means that c-GFP-subunits are highly overexpressed whereas the endogenous c-subunits are expressed but remain undetectable as transcripts in northern blots. Therefore, all the localizations observed in cells overexpressing the GFP fusion genes have to be taken with care, although they still yield significant information if interpreted in combination with the results of silencing experiments (see Discussion).

We were also interested in the question of whether the V-ATPase is localized in the plasma membrane of *Paramecium*, as shown for *Plasmodium falciparum* (Hayashi et al., 2000) and neuronal cells in *Torpedo marmorata* (Morel et al., 2003). This question cannot be answered by the GFP labeling of c-subunits alone, because any possible plasmalemmal staining would be obscured by the strong subcortical signal. Recently, c- and c'-subunits in yeast were shown to expose their N- and C-termini to the lumen of vacuoles (Flannery et al., 2004). In our study, we tagged the c-subunits C-terminally with GFP (see Materials and Methods), expecting that GFP should be exposed either to the lumen of organelles or to the extracellular space, if localized in the plasma membrane. We therefore applied an anti-GFP antibody to living cells transformed with all three c-GFP constructs, to decorate only c-subunits present in the plasma membrane and in other membranes exposed to the medium. Such cells showed a strong uptake of the antibody in food vacuoles and labeling of the membranes of these organelles, whereas no staining at the plasma membrane level could be detected under our conditions (Fig. 5a,b). Decoration of c-GFP in food vacuoles with the anti-GFP antibody shows that the orientation of the protein in membranes is as predicted and that the GFP tag is accessible to the antibody, thus providing a very good internal control for the labeling. It is noteworthy that the conditions in food vacuoles are far from favorable for antibody stability,



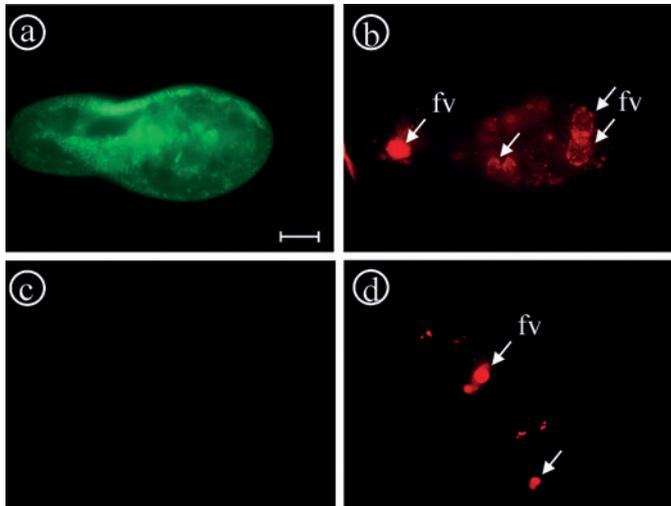
**Fig. 4.** Northern blot of two clones injected with c1-GFP in comparison with non-injected control clones. Overnight exposition of the film allowed the detection of the c1-GFP-mRNA at ~1.4 kb whereas the endogenous mRNA of c1 (~0.6 kb) is undetectable in transformed and control clones, even after 120 hours of exposure (not shown).

antigen binding and stability of the GFP tag because of the low pH and high protease activity in this organelle. The absence of labeling at the plasma membrane under far more favorable conditions thus means that  $V_0$  is not present in the plasma membrane or is present in only an extremely small amount.

#### RNA interference of $V_0$ and $V_1$

To investigate the function of the V-ATPase, we silenced genes using an RNAi approach by feeding (see Materials and Methods). In order to compare the effect of the depletion of functional  $V_0$ - and  $V_1$ -subcomplexes, we silenced the c-subunits of  $V_0$  and the F-subunits of  $V_1$ . Within a pair of c-subunits, the nucleotide identity is high enough to expect co-silencing (Ruiz et al., 1998). Because there are three pairs of very similar genes of c-subunits, we co-silenced all three pairs (c2/c4/c6) to avoid any functional compensation by unsilenced c-subunits. For silencing more than one pair, we used a novel strategy by cloning a representative of each c-subunit pair into the same feeding plasmid (see Materials and Methods). We were also interested in the degree to which c-subunits can replace each other. Therefore, single c-subunit silencing and the combination of the closest two pairs (c2, c6) was performed. Between the two F-subunit genes, the nucleotide identity is also high enough to expect co-silencing.

Whatever the silencing construct used to feed paramecia, the most obvious effect was a reduction of cell growth rate. Cells silenced in V-ATPase subunits (except for c-subunits 2 and 6 alone) made three fissions per day at 27°C and were blocked in growth thereafter. Control cells fed with bacteria containing only an empty plasmid made four fissions per day at 27°C without division arrest. The c2 and c6 constructs had less severe effects, and the growth rate was slightly reduced. If cells blocked in growth were transferred daily to freshly prepared feeding solution, they could be kept in the state of blocked growth. After 72 hours, the phenotype of a fraction of the cells became somewhat unstable. Some cells died whereas others overcame the feeding effect and started growing again. A careful examination of silenced cells allowed us to recognize several defects in organelle organization and function.



**Fig. 5.** Application of an anti-GFP antibody to living cells transformed simultaneously with, c1-, c4- and c5-GFP constructs. Food vacuole (fv) membranes are stained whereas the cell surface is devoid of any label; (a) GFP labeling by c-GFP fusion genes; (b) anti-GFP antibody, detected with an Alexa-coupled secondary antibody; (c) untransformed control cell; (d) anti-GFP antibody and secondary antibody applied on a control cell show neither cell-surface labeling nor staining of food vacuole membranes. Bar, 10  $\mu$ m.

#### Silencing of V-ATPase subunits induces abnormal functioning of the contractile vacuole complex

Silencing of any of the c-genes, alone or in combination, or silencing of the F-subunits led to severe perturbation of the contractile vacuole complex. The time of one pump cycle of the contractile vacuole was generally prolonged (from 5 to 10 seconds in control cells and from 30 to 60 seconds in c-subunit- or F-subunit-silenced cells) and extremely irregular. It was also possible to see morphological abnormalities in the pump cycle of this organelle. Some ampullae did not fuse with the contractile vacuole for two or three pump cycles, provoking swelling of the ampullae. The contrary was also observed, whereby the ampullae fused twice with the contractile vacuole

during one cycle. Sometimes collecting canals did not pinch off the contractile vacuole, pumping water continuously into it without any visible increase in the volume of the ampullae. Collecting canals were often inflated with fluid-filled lacunae within the canals (Fig. 6).

Electron microscopy of the contractile vacuole complex of cells, in which one pair of c-subunits or more were silenced, showed that the decorated spongiome was highly disrupted or absent (Fig. 7).

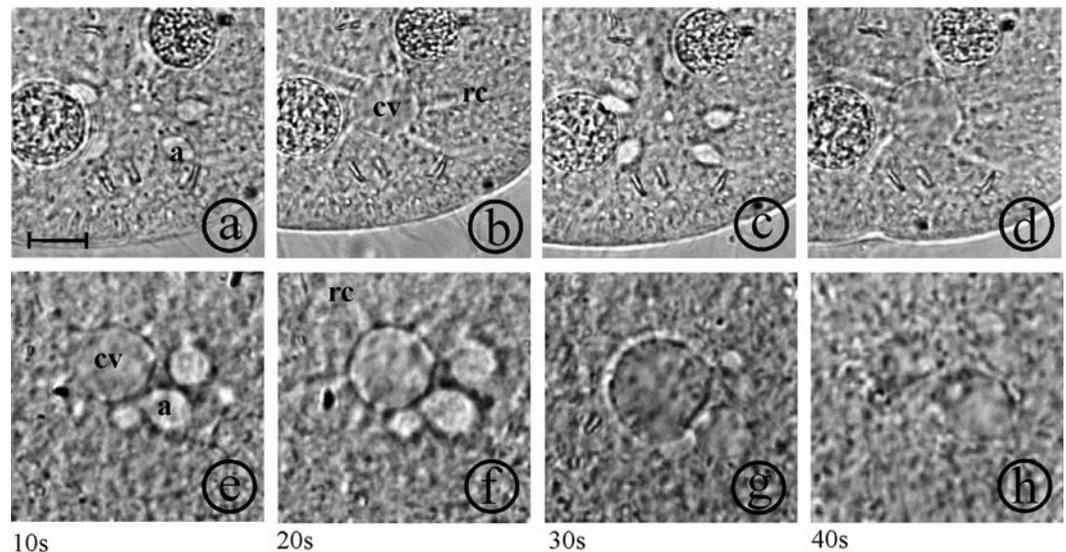
#### Silencing of V-ATPase subunit genes leads to impaired feeding

Since pH regulation is important for the inactivation of ingested microorganisms, membrane traffic (fusion of lysosomes with phagosomes) and activation of acidic hydrolases during the process of digestion (Fok and Allen, 1988), we analyzed the pH development of phagosomes in cells silenced in  $V_0$  or  $V_1$  using the pH-sensitive dye Congo Red. The number of acidified phagosomes in c- or F-subunit-silenced cells was drastically reduced after 20 hours of silencing (data not shown). However, this seemed to be a submaximal effect because, after 24-26 hours of silencing, the process of phagocytosis itself seemed to be heavily impaired. Cells fed for 30 minutes with a fluorescent *E. coli* suspension 24 hours after the beginning of the silencing experiment were found to have a significantly reduced number of phagosomes compared with control cells (Fig. 8). This reduced phagocytotic activity is likely to have an impact on the silencing process (see Discussion).

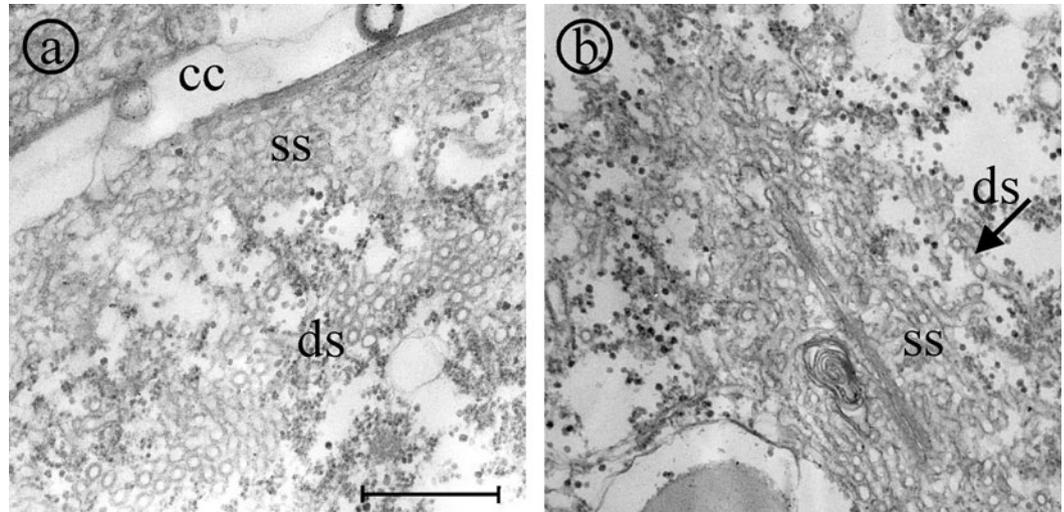
#### Silencing of V-ATPase subunits disturbs maturation of dense core granules

In *Paramecium*, a large number (approximately 1000 per cell) of trichocysts are synthesized through the maturation of post-Golgi vesicles. Trichocysts dock at the plasma membrane at preformed docking sites, ready for exocytosis upon trigger by an extracellular stimulus (Vayssié et al., 2000). The exocytotic capacity of trichocysts can be assayed using the picric acid test (see Materials and Methods). Cells silenced for all three c-

**Fig. 6.** Silencing of c-subunits of  $V_0$  or the F-subunit of  $V_1$  causes defects in the cycle of the contractile vacuole complex. (a-d) Control cell, showing normal cycles with filling of the ampullae, fusion with the contractile vacuole and fluid expulsion; (e-h) silencing of any V-ATPase subunit provoked serious perturbation in the series of events and a general prolongation of the cycle, illustrated in this figure by silencing of all c-subunits. Abbreviations: a, ampulla; cv, contractile vacuole; rc, radial canal. Bar, 10  $\mu$ m.



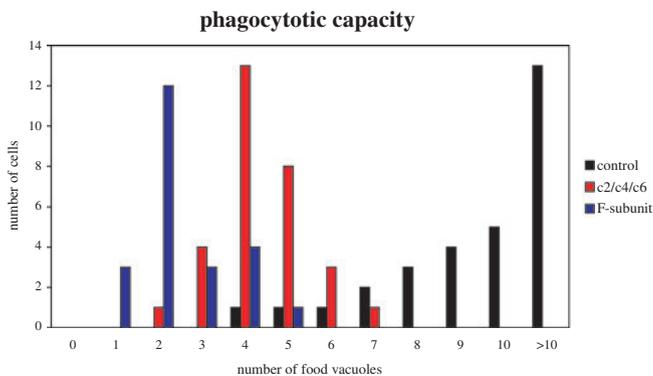
**Fig. 7.** EM analysis of the contractile vacuole complex. (a) Control cell compared with one silenced in all c-subunits (b). In cells silenced in any of the c-subunit pairs, the decorated spongiome (cross-sectioned in this figure) is highly disrupted (remaining decorated spongiome is indicated with an arrow) or absent, whereas the smooth spongiome seems to be unaffected. Abbreviations: cc, collecting canal; ds, decorated spongiome; ss, smooth spongiome. Bar, 0.5  $\mu$ m.



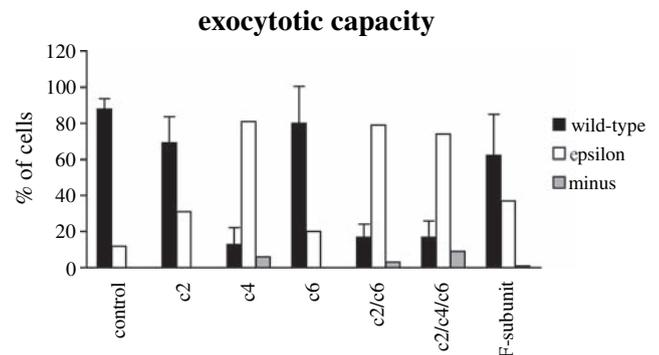
subunit pairs displayed a strongly diminished number of discharged trichocysts compared with control cells (Fig. 9). A similar effect was obtained in cells co-silenced for the c2 and the c6 genes, as well as in cells silenced for c4. By contrast, cells silenced for c2 or c6 alone were not significantly affected in their exocytotic capacity and displayed considerable variation between experiments. To visualize the reduced exocytotic capacity, a gene encoding a secretory protein of the trichocyst matrix, TMP1b (Gautier et al., 1996; Vayssié et al., 2001), was fused with the GFP gene. Descendants of *Paramecium* cells injected with a moderate amount of this construct showed strong fluorescence of functional trichocysts with slightly altered shape (Fig. 10b) but were fully able to respond to the vital trigger substance aminoethyl-dextran (AED) (Plattner et al., 1984) by normal exocytosis and decondensation of the contents to a 'needle'. Thus low-copy-number transformation with the TMP1b-GFP fusion protein does not interfere significantly with trichocyst maturation or exocytosis and was therefore considered to be a useful fluorescent marker for trichocysts. The TMP1b-GFP fusion construct was used to monitor the effects of c-gene

silencing on trichocyst formation and exocytosis. Silencing of all c-subunits resulted in a strongly decreased number of mature trichocysts attached to the cortex and a large amount of immature or misshaped trichocysts remaining in the cytoplasm (Fig. 10d-f). The defect in the formation of trichocysts was also examined by electron microscopy of cells in which c-subunits 1/2, 3/4, 5/6 were co-silenced. Various misshapings in late steps of granule maturation were observed (Fig. 11b).

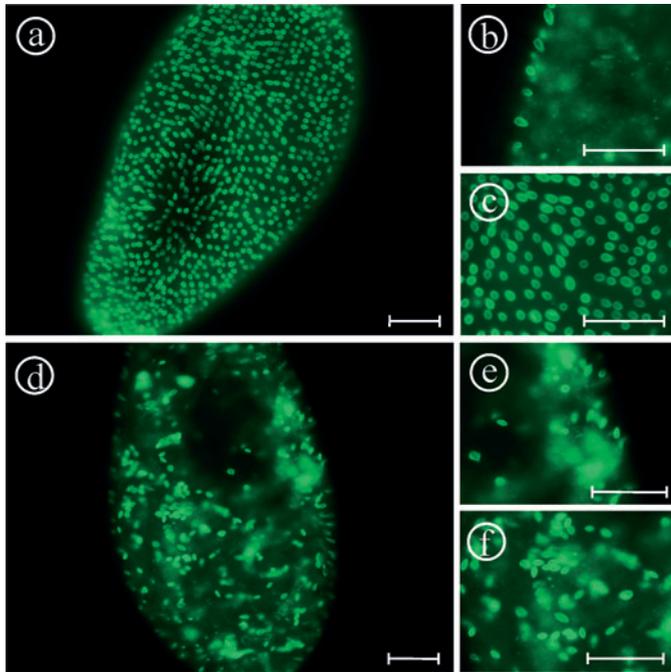
Silencing of the F-subunits of  $V_1$  gave similar perturbations of trichocyst biogenesis although with slower kinetics. The maximal effect of F-subunit silencing was obtained after 48 instead of 24 hours with the c-subunits of  $V_0$ . Exocytotic capacity after 48 hours was strongly impaired, especially when the cells were triggered after the first 24 hours of silencing with AED to remove trichocysts docked before the onset of the gene silencing experiment (see Materials and Methods). After 48 hours, as few as 5-50 trichocysts per cell were docked and exocytosis competent, thus reflecting an almost complete block of trichocyst formation. To confirm the silencing phenotype of



**Fig. 8.** Silencing of  $V_0$ - or  $V_1$ -subunits leads to strongly impaired feeding. Cells 24 hours after the beginning of the silencing experiment were fed for 30 minutes with an *E. coli* suspension containing a fluorochrome to visualize food vacuoles. After 30 minutes, cells were fixed and the number of food vacuoles of at least 25 cells was counted.



**Fig. 9.** Comparison of the exocytotic capacity of wild-type paramecia compared with cells silenced in V-ATPase subunits. The exocytotic capacity of c4, c2/c6 and c2/c4/c6 silenced cells is greatly reduced after 24 hours. Exocytotic capacity was analyzed using the picric acid test and three classes of cells were distinguished: 'wild-type' with more than 500 expelled trichocysts, 'epsilon' with less than 100, and 'minus' with no expelled trichocysts. Data were collected in three different experiments from at least 20 cells per group and the variation was calculated between the experiments.



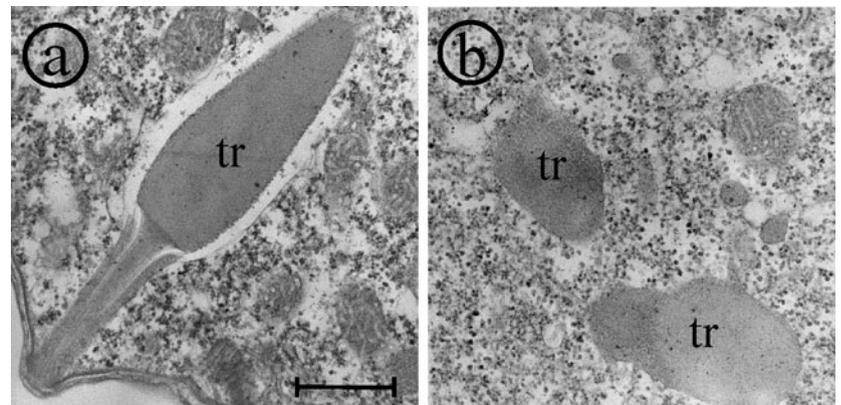
**Fig. 10.** Cells transformed with trichocyst matrix-protein1b-GFP were silenced in all c-subunits. (a,b,c) control cells show trichocysts docked at the cell cortex at high density; (d,e,f) cells co-silenced in all c-subunits have very few trichocysts attached to the cortex whereas the cytoplasm contains clusters of misshapen trichocyst precursor structures. Bars, 10  $\mu$ m.

$V_1$  on trichocyst biogenesis, we used the A-subunit of  $V_1$  as an additional control. The four genes identified in *Paramecium* (Table 1) show nucleotide identities greater than 88% with each other, which is considered to be sufficient for co-silencing (Ruiz et al., 1998). The open reading frame of one gene (A3) was cloned into the feeding plasmid and the effect of this construct on trichocyst biogenesis was analyzed. We found that silencing of all the A-subunits also leads to a severe reduction of released trichocysts in the picric acid test after 48 hours, especially when preceded by AED triggering after 24 hours. Therefore, the slower kinetics of the effect of  $V_1$  silencing on trichocyst biogenesis seems not to be an intrinsic characteristic of the F-subunit, but rather of  $V_1$  itself, though trichocyst biogenesis is dependent on both  $V_0$  and  $V_1$ .

#### Epistatic effect of c-subunit silencing on F-subunit stability and localization

Since the cytosolic  $V_1$ -sector of the V-ATPase depends on anchoring to the membranous  $V_0$ -

**Fig. 11.** EM analysis of trichocysts in  $V_0$ -silenced cells. (a) Control cell showing a trichocyst of normal shape docked to the cell cortex; (b) cells silenced in all c-subunits show various trichocyst misformations, e.g. absence of a proper tip and failure to form the typical spindle-shaped body, thus resembling misshapen trichocysts of the mutant *tam38* (Gautier et al., 1994); tr, trichocyst. Bar, 1  $\mu$ m.



sector, we investigated the effect of c-subunit silencing on  $V_1$ , using a *Paramecium* strain expressing F-GFP. At three different times of silencing (16, 25 and 40 hours), cells were fixed and examined in the fluorescence microscope. After 16 hours,  $V_0$  silencing provoked a strong decrease of the fluorescence intensity of the radial arms of the contractile vacuole complex, whereas cytoplasmic background increased slightly (Fig. 12d). After 25 hours, the contractile vacuole complex was still faintly stained, whereas the cytoplasmic background level decreased to that of the non-injected control cells. After 40 hours, only remnants of the contractile vacuole were barely visible or not visible at all. This indicates that excess  $V_1$ -subcomplexes or free F-subunits occurring transiently in the *Paramecium* cytosol are eliminated. This is different from observations in yeast, where it was shown that the amount of  $V_1$ -proteins is unchanged in knockout mutants of  $V_0$ -subunits (Parra et al., 2000). The level of free F-subunits in *Paramecium* must be regulated post-transcriptionally because our expression of  $V_1$ , F-GFP construct was driven by the constitutive calmodulin promoter, which excludes regulation at the transcriptional level.

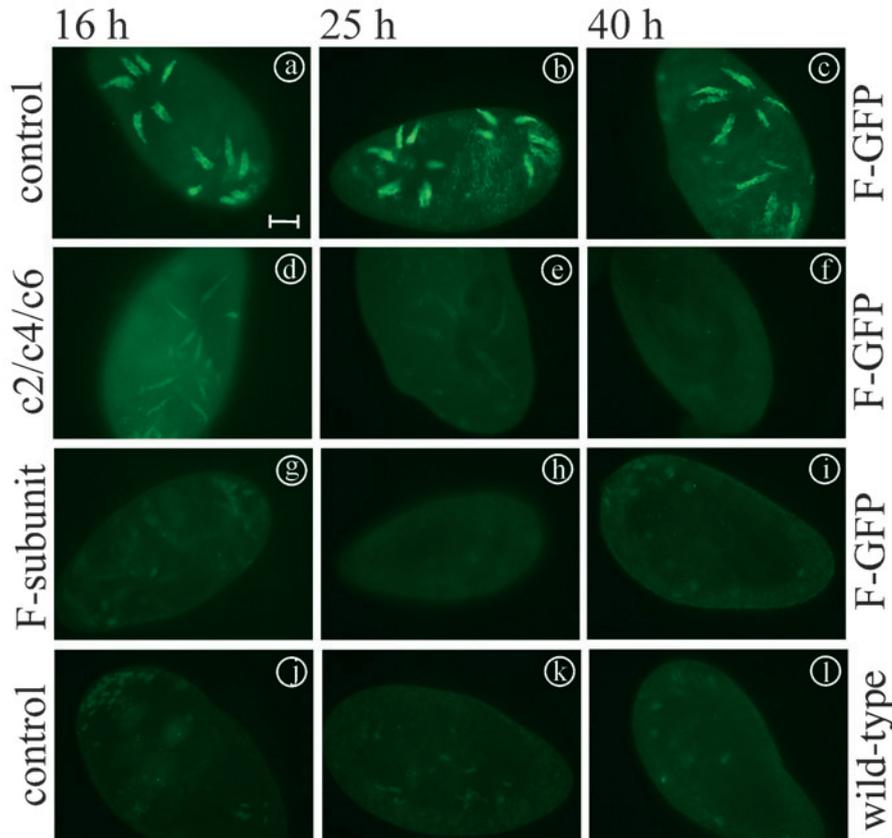
Silencing of the F-subunit in the same F-GFP-expressing cells resulted in complete elimination of fluorescence within 16 hours, demonstrating the rapid and powerful block of gene expression produced by the feeding approach. The remaining fluorescence intensity is indistinguishable from the autofluorescence of uninjected control cells (Fig. 12j-l). However, it should be noted that a relatively high concentration of GFP is needed to reach the detection limit in *Paramecium* (Hauser et al., 2000a). This means that a low concentration of the endogenous F- and F-GFP-subunits remains after 16 hours, because the cell biological defects caused by  $V_1$  silencing just start to become visible after 16 hours.

## Discussion

In this study we identified essential components of  $V_1$  and  $V_0$  of the V-ATPase in *Paramecium* and used them as markers to study the localization and functional significance of the two subcomplexes. For  $V_0$ , we used the c-subunits, encoded by six genes that can be grouped in three pairs. For  $V_1$ , we used the F-subunits as a marker, encoded by two almost identical genes.

### Localization of the V-ATPase

By labeling c-subunits with a GFP tag, it was possible to



**Fig. 12.** Cells derived from a clone transformed with F-GFP plasmid were subjected to gene silencing of all the c-subunits or the F-subunit itself (a-i); 16, 25 and 40 hours after the beginning of feeding, samples were taken and fixed. Control cells (a-c) show no difference in fluorescence within 40 hours, in contrast to cells after silencing of the c-subunits, which causes displacement of the F-subunit from the contractile vacuole complex after 16 hours (d). After 25 hours, only remnants of the contractile vacuole complex are visible and these have disappeared after 40 hours of feeding. Silencing of the F-subunit shows very rapid elimination of the fluorescence of F-GFP already after 16 hours of feeding (g). (j-l) Non-injected control cells showing autofluorescence typical for *Paramecium*. Silencing of the F-subunit as well as the c-subunits reduces fluorescence of F-GFP to the level of autofluorescence within 40 hours. Bar, 10  $\mu$ m.

localize  $V_0$  in organelles known to contain the V-ATPase, such as the contractile vacuole complex and phagosomes, but also in organelles not known to contain a proton pump, such as the ER and trichocysts. Care has to be taken in the interpretation of GFP images, because we cannot precisely control the amount of DNA injected. Northern blot analysis of cells transformed with c1-GFP or c4-GFP confirmed that, at least at the transcriptional level, c1- and c4-GFP are highly overexpressed in comparison with untransformed control cells (Fig. 4). As it is known from yeast, transmembranous  $V_0$ -subunits are synthesized at the ER and assembled in a tightly regulated series of events to form the  $V_0$ -subcomplex that binds the cytosolic  $V_1$  sector and is exported to various organelles (Graham et al., 2003). Only  $V_0$ -subcomplexes that are correctly assembled leave the ER. For this reason, it is unlikely that the overexpression of a single subunit causes a mislocalization of the  $V_0$ -subcomplex to wrong compartments, although changes in the stoichiometry may lead to mistargeting of unassembled c-subunits. GFP localization of proteolipids in the ER is not an indication of a functional V-ATPase in this compartment, but may as well represent excess proteolipids that are not incorporated in  $V_0$ -subcomplexes. However, despite these cautions in the interpretation, significant conclusions can be drawn from correlated results obtained by different approaches. As described in the rest of the discussion, most of the locations visualized by GFP fusion proteins correspond to organelles affected by RNAi experiments, strongly arguing for the implication of V-ATPases in their functioning.

$V_1$ -subunits are thought to be synthesized and assembled to the complex in the cytosol. This subcomplex is membrane

associated by binding to  $V_0$ . Localization of  $V_1$  was achieved by tagging the F-subunit with GFP. Interestingly, the GFP tag does not interfere in a perceptible way with the function of the V-ATPase, although the F-subunit was shown to be a part of the rotor connecting  $V_1$  with  $V_0$  (Imamura et al., 2004). Low-copy-number transformation led only to labeling of the contractile vacuole complex, which is known to contain a huge number of V-ATPase molecules: estimations range from 40 to 80 millions in a *Paramecium* cell (Fok et al., 1995). To achieve significant labeling of other cellular structures by F-GFP, the binding capacity of  $V_1$  of the contractile vacuole complex has to be saturated, which was achieved by injection of a large DNA amount of the F-GFP plasmid. Overexpression of this protein may cause mislocalization, therefore the localization results of  $V_1$  should be considered with caution. But we can be confident in the localization of the V-ATPase in organelles that were shown to contain a V-ATPase by GFP tagging of both  $V_0$  and  $V_1$ .

#### RNAi with the V-ATPase

It is not known whether all c-subunits of *Paramecium* coexist in the same V-ATPase complex or whether there exist V-ATPases with different c-subunit composition. This fact made it necessary to inactivate all proteolipid genes in gene silencing studies to be sure that no rescue by other c-subunits could occur. For this reason, we worked out a novel strategy to provoke RNAi by feeding with more than one gene by cloning the genes of interest in tandem in the feeding plasmid. The obstacle of possible rescue by related genes does not exist in

the case of the A- or F-subunits, because these subunits are encoded by families of genes that are nearly identical and are therefore co-silenced by RNAi. Depletion of V-ATPase subunits clearly disturbs the three fundamental processes of osmoregulation, phagocytosis and secretory vesicle formation.

#### Contractile vacuole and osmoregulation

The contractile vacuole complex proved to be extremely sensitive to RNAi of the corresponding V-ATPase gene. Silencing of single c-subunit pairs had a dramatic impact on function and structural integrity of the organelle. This is in good agreement with previous studies that showed the V-ATPase to be densely packed in the decorated spongiome (Fok et al., 1995), that the V-ATPase is necessary for fluid segregation (Grønlien et al., 2002) and that water is eliminated through the contractile vacuole complex by the creation of an ion gradient that is at least partially established by the electrochemical potential built by the V-ATPase (Stock et al., 2002).

#### Food vacuoles and phagocytosis

Phagocytosis in *Paramecium* is a complex process in which the organellar pH plays a central role in coordinating the temporal series of events. It is known that acidosomes, vesicles that contain a high number of V-ATPase complexes, are docked to the nascent food vacuole and fuse with the food vacuole as soon as it leaves the cytopharynx, leading to rapid acidification of the food vacuole (Fok and Paeste, 1982). Fusion with lysosomes (and therefore digestion) only takes place if the phagosome is acidified (Fok and Allen, 1988). RNAi with  $V_0$  or  $V_1$  probably disturbs this process. Food vacuoles that are not sufficiently acidified supposedly fail to fuse with lysosomes and are thus blocked at this step. As a consequence, the cellular pool of acidosome membranes is trapped and its titration inhibits further feeding. This result is consistent with the observation that, in *Dictyostelium*, a reduced expression level of the a-subunit of  $V_0$  also leads to decreased phagocytotic activity (Liu et al., 2002).

The block of feeding influences further delivery of dsRNA by *E. coli* bacteria to *Paramecium* cells. As soon as inhibition of phagosome formation takes place, the levels of dsRNA and siRNA in the cytoplasm drop, again allowing gene expression of V-ATPase subunits that were formerly silenced as feeding RNAi effects are reversible (Galvani and Sperling, 2002). When the formation of food vacuoles restarts, the levels of dsRNA and siRNA in the cytoplasm rise again and the phenotype becomes more severe. For this reason, a complete knock-down of the V-ATPase using the feeding approach is probably not possible, so no lethal phenotype has been observed. Consistently, gene silencing of all c-subunits by injection of a very high copy number of a plasmid containing only the open reading frames of the c-subunits, as established by Ruiz et al. (Ruiz et al., 1998), killed *Paramecium* cells within 48 hours (not shown).

#### Trichocysts and regulated secretion

Silencing of the c-, A- or F-subunits led to a block in trichocyst biogenesis although with remarkable differences between

silencing constructs. Among the c-subunits of  $V_0$ , silencing of c1/2 or c5/6 alone did not have a significant effect, but either co-silencing or simple silencing of the c3/c4 pair did have a significant effect. By contrast, the silencing of even single c-subunit pairs had a dramatic impact on the contractile vacuole. Silencing of the single pair c3/c4 had a maximal effect on both organelles. There are two types of explanations possible, a qualitative or a quantitative one. On the one hand, the differences in amino acid sequence between c1, c2 and c5/c6 (c1 differs from c2 by two, and from c5/6 by three, amino acid residues) might have a functional significance in the contractile vacuole complex, whereas they play the same role in trichocyst biogenesis. On the other hand, the decrease of the global level of c-subunits by silencing only one pair c1/c2 or c5/c6 may be enough to cause dysfunctioning of the contractile vacuole complex, but not of the trichocyst biogenesis. Whatever the explanation, this question is tightly linked to that of the organization and stoichiometry of proteolipids of  $V_0$  in different organelles of *Paramecium*.

When all  $V_0$  c-subunits were silenced after 24 hours, the pool of dischargeable trichocysts was strongly reduced, whereas it took 48 hours of silencing with  $V_1$ -subunits A and F. Why is the kinetics of the appearance of the trichocyst phenotype different between silencing of  $V_0$  and  $V_1$ ? The most likely answer lies in the different nature of these proteins, i.e. c-subunits are four-helix transmembrane proteins whereas A- and F-subunits are part of a soluble complex (Kawasaki-Nishi et al., 2003; Wilkens, 2001). Silencing of c-subunits acts rapidly on trichocyst maturation, because the formation of the organelle is probably paralleled by the synthesis of  $V_0$ -subunits. Interference with  $V_0$ -subunit synthesis leads to the absence of these molecules in trichocyst precursor membranes, as well as to an absence of V-ATPase activity and a block of the maturation process. With  $V_1$ , the situation is different: in the cell, a pool of  $V_1$  is bound but not strictly anchored to other organelles. This is apparent in the F-GFP experiment where silencing of  $V_0$  resulted in a delocalization of  $V_1$  to the cytosol after 16 hours (Fig. 12d). So it is possible for trichocysts to attract free  $V_1$  to form a functional V-ATPase for a longer time than when silencing of  $V_0$  takes place, where the effects are location specific and therefore immediate.

Interestingly, the phenotype of trichocysts strikingly evokes the shape of mutant trichocysts such as the football A or tam 38 mutants (Ruiz et al., 1976). This kind of mutant phenotype was shown to be a consequence of defective traffic early in the secretory pathway (Gautier et al., 1994). It is possible that in our silencing experiments, too, a deleterious effect of the silencing of the V-ATPase, for example on the ER, produces a similar trichocyst phenotype. If so, this would be in good agreement with the possible labeling of the ER by the V-ATPase-subunit/GFP-fusion genes. It should be noted that trichocysts are not remarkably acidic (Lumpert et al., 1992; Garreau de Loubresse et al., 1994), so that the V-ATPase seems to participate rather in membrane trafficking leading to the assembly and maturation of trichocysts. However, gene silencing experiments do not inform us on the possible later roles of the V-ATPase, which was detected in mature and anchored trichocysts by the GFP constructs. The silencing approach cannot provide such information, since any effects downstream of the earliest blocked step are impossible to observe. Among the late roles, we considered a possible

involvement of the  $V_0$  sector alone, as shown for the homotypic fusion of vacuoles by Peters et al. (Peters et al., 2001).  $V_0$  was proposed to play a catalytic role in membrane fusion in addition to its role of anchoring  $V_1$  and translocating protons. Could this proposed role of  $V_0$  in membrane fusion be a general feature in all traffic routes? The exocytotic site of trichocysts is assembled during docking of the organelle to the cell cortex and remains established until the trichocyst undergoes exocytosis and is discharged. The docking site is formed by six to eight intramembranous particles, forming the so-called 'rosette', as was shown by freeze fracture electron microscopy (Beisson et al., 1976). Under the assumption that  $V_0$  may be a general fusion factor, it was asked by Froissard et al. (Froissard et al., 2002) and Plattner and Kissmehl (Plattner and Kissmehl, 2003) whether  $V_0$  may be present at the trichocyst-docking site and therefore could represent the rosette particles. But the fact that, by the combination of the anti-GFP antibody with our overexpressed c-subunit/GFP fusion genes, we were unable to detect any c-subunits in the plasma membrane, makes it improbable that  $V_0$  is localized at the trichocyst-docking site and thus can represent the rosette.

#### Does the V-ATPase have a general role in membrane fusion?

Although we cannot exclude that only a few V-ATPase molecules per membrane are needed for membrane fusion and that we may not have reached this level in our silencing experiments, two arguments plead against a general role of the V-ATPase in membrane fusion.

First, impaired membrane fusion in *Paramecium* is most obvious by swelling of the ER, fragmentation of the Golgi and clumping of vesicles that are unable to undergo membrane fusion as it was shown by silencing of N-ethylmaleimide (NEM)-sensitive factor (NSF), a general fusion factor (Kissmehl et al., 2002; Jahn and Südhof, 1999). Such signs of impaired membrane fusion could not be observed by silencing all c-subunits of  $V_0$ , even at the level of electron microscopy.

Second, if  $V_0$  generally takes part in membrane fusion it would be expected that silencing of  $V_0$  would lead to defects in membrane trafficking in addition to all other defects produced by impaired proton pumping. By contrast, silencing of  $V_1$  should only affect processes that require proton translocation coupled to ATP hydrolysis. In our experiments, all major defects induced by gene silencing of  $V_0$  were the same as those resulting from  $V_1$  silencing.

Another important discovery of this study is the high number of genes by which some V-ATPase subunits are encoded in *Paramecium* compared with other organisms, especially the numbers of c- and a-subunit genes (Table 3). From studies in yeast, it is known that the two a-subunits are targeted to different organelles (Kawasaki-Nishi et al., 2001). To understand the significance of 17 a-subunits with regard to functional differentiation of the V-ATPase in *Paramecium* will be the goal of our future work.

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