Altered expression of the 100 kDa subunit of the *Dictyostelium* vacuolar proton pump impairs enzyme assembly, endocytic function and cytosolic pH regulation

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Summary

The vacuolar proton pump (V-ATPase) appears to be essential for viability of *Dictyostelium* cells. To investigate the function of VatM, the 100 kDa transmembrane V-ATPase subunit, we altered its level. By means of homologous recombination, the promoter for the chromosomal vatM gene was replaced with the promoter for the act6 gene, yielding the mutant strain VatMpr. The act6 promoter is much more active in cells growing axenically than on bacteria. Thus, transformants were selected under axenic growth conditions, then shifted to bacteria to determine the consequences of reduced vatM expression. When VatMpr cells were grown on bacteria, the level of the 100 kDa V-ATPase subunit dropped, cell growth slowed, and the A subunit, a component of the peripheral catalytic domain of the V-ATPase, became mislocalized. These defects were complemented by transformation of the mutant cells with a plasmid expressing vatM under the control of its own promoter. Although the principal locus of vacuolar proton pumps in *Dictyostelium* is membranes of the contractile vacuole system, mutant cells did not manifest osmoregulatory defects. However, bacterially grown VatMpr cells did exhibit substantially reduced rates of phagocytosis and a prolonged endosomal transit time. In addition, mutant cells manifested alterations in the dynamic regulation of cytosolic pH that are characteristic of normal cells grown in acid media, which suggested that the V-ATPase also plays a role in cytosolic pH regulation.

Key words: *Dictyostelium*, V-ATPase, Endocytosis, pH regulation

Introduction

All eukaryotic cells contain an evolutionarily ancient, highly conserved enzyme, the vacuolar proton pump or V-ATPase, a rotary motor that transports protons across membranes at the expense of ATP (reviewed by Nelson and Harvey, 1999; Wieczorek et al., 1999; Forgac, 2000). The V-ATPase serves many functions, such as acidifying intracellular organelles (endosomes, lysosomes), energizing transport (as in uptake of neurotransmitter amines), and acidifying extracellular spaces (as in bone resorption by osteoclasts). The enzyme is composed of two functional domains: the V₁ domain, a peripheral complex of eight subunits that is responsible for ATP hydrolysis; and the V₀ domain, an integral complex of five subunits that is responsible for proton translocation across the membrane. The V-ATPases are structurally and evolutionarily related to the F-ATPases (ATP synthases), which are found in bacteria and in mitochondria.

Using the soil amoeba *Dictyostelium discoideum*, an organism in which genetic as well as biochemical tools can be used to explore protein function, we are analyzing the large (~100 kDa) transmembrane subunit of the V₀ domain of the V-ATPase (Liu and Clarke, 1996). Although this subunit has been the subject of study in both yeast and mammalian cells, its structure and function are not well understood. Three genes encoding isoforms with different tissue distributions have been demonstrated in vertebrates; one isoform may be specific to osteoclasts and other cells that target proton pumps to plasma membranes (Nishi and Forgac, 2000; Mattsson et al., 2000). *C. elegans* has four isoforms that are strongly expressed in distinct cells (Oka et al., 2001). In *S. cerevisiae*, there are two isoforms that are localized to different endomembranes (Manolson et al., 1992; Manolson et al., 1994). Initial results suggested that the yeast 100 kDa subunits may act to target the enzyme complex to particular endomembranes and/or regulate its function in different compartments (Manolson et al., 1994). Site-directed mutagenesis of one of the yeast isoforms (*VPH1*) confirmed that this subunit is important in enzyme organization (Leng et al., 1998) and showed that it is involved in proton translocation, which suggested that it is functionally related to the a subunit of the F-ATPase (Leng et al., 1996). Other data indicate that this subunit may provide a fixed structural link between the V₁ and V₀ domains, thereby acting as a stator for the rotary motor (Landolt-Marticorena et al., 1999; Landolt-Marticorena et al., 2000).

In *Dictyostelium*, vacuolar proton pumps are concentrated primarily in membranes of the contractile vacuole complex, an
osmoregulatory organelle (Heuser et al., 1993; Fok et al., 1993; Bush et al., 1994); they are also present in lesser abundance in membranes of the endo/lysosomal compartment (Rodriguez-Paris et al., 1993; Temesvari et al., 1994; Bush et al., 1994; Clarke and Heuser, 1997). Our studies of the 100 kDa V-ATPase subunit in Dictyostelium have detected only a single gene (vatM) encoding this subunit and have shown that the product of vatM is greatly enriched in contractile vacuole membranes (Liu and Clarke, 1996). Biochemical fractionation experiments have shown that the same protein is also present in endosomal membranes (Adessi et al., 1995). Our attempts to inactivate vatM by homologous recombination yielded no strains in which the gene had been disrupted. Instead, when transformants were screened by immunoblot to detect clones lacking the 100 kDa protein, only regulatory mutants with low vatM mRNA levels and a transient reduction in protein levels were obtained, and the protein soon returned to normal (Liu and Clarke, 1996). These results suggest that the level of the 100 kDa protein is tightly regulated and that a cell in which the gene has been fully inactivated is probably not viable.

To examine the function of this protein, a means of conditionally blocking expression of the vatM gene would be helpful. One possible means of accomplishing this would be through expression of an antisense transcript. Although this approach has been effective for several genes in Dictyostelium, for vatM an antisense-mediated reduction in mRNA levels had only a temporary effect on protein levels (Liu and Clarke, 1996). Accordingly, we developed an alternative strategy: we altered the expression of vatM by inactivating its endogenous promoter and substituting another promoter with a different pattern of regulation. We describe here the consequences of the altered expression of vatM in growing Dictyostelium cells.

Materials and Methods

Cells and growth conditions

Dictyostelium discoideum strains were grown in HL5 medium (Clarke et al., 1980); for DH1, a uracil auxotroph derived from AX3 (Caterina et al., 1994), uracil (100 μg/ml) was added to the medium. Transformants of DH1 to uracil prototrophy were selected by growth in FM (Franke and Kessin, 1977). Prior to microscopic or spectrofluorimetric observations of axenically growing cells, the cells were shifted to low fluorescence (LF) medium for a period of 2-24 hours, as indicated. LF was identical to FM except that 5 g/l Bacto-Casitone (Difco) was substituted for the vitamins and amino acids present in FM, and the concentration of salts was reduced by one-half. Cells were also grown in association with K. aerogenes, either as a co-culture on SM nutrient agar plates (Loomis, 1975) or in a suspension of bacteria harvested from such plates in KP buffer (17 mM KH₂/K₂HPO₄ buffer, pH 6.4), as described (Rathi et al., 1991).

Construction and use of vector for promoter replacement

The vector for promoter replacement was prepared in three steps that are summarized here. (1) From plasmid pVM2 (Liu and Clarke, 1996) we isolated a fragment that contained 0.6 kb of vatM upstream DNA plus 10 vatM codons (i.e. up to the BglII site in the gene). This fragment was cloned into pBluescriptSK- (Stratagene) between the XbaI and BamHI sites. (2) The complete vatM cDNA was recovered as a polymerase chain reaction (PCR) product from the phagemid pVM1 (Liu and Clarke, 1996), using a forward primer 5'-GGATCCCATGAAGCTTTTTAAGACC-5' (which added a BamHI site immediately before the ATG translation initiation codon); the reverse primer was the M13 sequence that flanked vatM in the phagemid. The PCR product was cloned into the BamHI site of pDNeo67 (Da Silva and Klein, 1990), immediately downstream of the act6 promoter. A fragment containing the act6 promoter plus 1 kb of vatM coding sequence (to the XhoI site in vatM) was isolated and subcloned into the plasmid created in step 1, just downstream of the vatM promoter. (3) The pyr5-6 gene was recovered by PCR as a 1.8 kbp fragment from DIV2 (Kuspa and Loomis, 1994) and the gene was moved into the plasmid created in step 2, positioned between the vatM and act6 promoters. The final construct, pVATM-act6, is shown schematically in Fig. 1. DH1 cells were transformed with this plasmid using the electroporation protocol described previously (Kuspa and Loomis, 1994). The pVATM-act6 plasmid was cut with SacII and XhoI to free the vatM-containing segment from the pBluescript backbone and 40 μg of the linear vatM-containing segment was used to transform DH1 cells. Following transformation, the cells were plated in ten 96-well plates at a density expected to yield single clones (approximately 200 cells/well) and cultured axenically in FM medium.

Expression of vatM under its own promoter

From plasmid pVM2 an 0.6-kb Xbal/BglII fragment was isolated consisting of genomic DNA upstream of vatM plus the first few nucleotides of coding sequence (as described above). This fragment was used to replace the corresponding Xbal/BglII fragment of the vatM cDNA in pVM1. Finally, a 2.1 kbp Xbal fragment containing the G418 resistance gene TN903 flanked by the act15 promoter and terminator was recovered from pDNeo2 (Witte et al., 1987) and cloned into the XbaI site of this plasmid, yielding pVMop, a G418-selectable transformation vector containing vatM driven by its own promoter. This vector was used to transform VatMpr cells, and transformants were selected in HL5 containing 5 μg/ml G418 (Geneticin, Sigma).

Construction and use of the A15-pPHluorin vector

Ratiometric pHluorin (Miesenböck et al., 1998) (a gift of Dino De Angelis, Memorial Sloan-Kettering Institute, New York, NY) was received as coding sequence in the bacterial expression vector pGEX-2T. The downstream EcoRI site was converted to a XhoI site by insertion of the oligonucleotide AATTTTAACTCGAGTAAA, which also created a stop codon in the pHluorin reading frame. The pHluorin gene was then excised with BamHI and XhoI, and combined with the A15-promoter from A15-UGus (Zaccaria et al., 1998) and the A8-terminator from pDgal-17 (Harwood and Drury, 1990) in the V18Tn5-DRE Dictyostelium selection vector (Wetterauer et al., 1996; Deichsel et al., 1999). In the predicted protein, there are 13 N-terminal amino acids (MDGEDVQARSTG) in place of the wild-type-GFP initiation methionine, most of which arise from A15 coding sequence, while four amino acids (TGEF) at the C-terminus are derived from pGEX2T, and four further C-terminal amino acids present in the De Angelis construct are deleted. AX2, DH1 and VatMpr cells were transformed with this vector as described above, and transformants were selected as described [Wetterauer et al., 1996] agar method] using living K. aerogenes and a G418 concentration of 100 μg/ml. The selection plates were examined directly with fluorescent illumination, and transformants with uniform GFP expression were chosen. The cells were cultured in HL5 with 20 μg/ml G418 until required for experiments.

Detection of vatM DNA, mRNA and protein

Methods for preparing and analyzing DNA were as previously described (Liu and Clarke, 1996), except that the probe was the 1 kb EcoRI/XhoI fragment from pVM1 described in that report. For analysis of protein in cell lysates, the methods of sample preparation, electrophoresis and transfer were as previously described (Liu and Clarke, 1996), except
that nitrocellulose membrane (Schleicher and Schuell) was used instead of PVDF. After transfer, the membrane was cut in half using prestained molecular weight markers as guides. The upper portion was stained with N2 and sometimes also N4 hybridoma culture supernatants (1:100 and 1:30 dilutions, respectively) and the lower portion with anti-actin antibodies (C-4, 1:2000), as previously described (Liu and Clarke, 1996). The secondary antibody was alkaline phosphatase-conjugated anti-mouse IgG (Promega, 1:1000 dilution), used as directed by the manufacturer.

**Indirect immunofluorescence**

**Dictyostelium** cells of strains DH1, VatMpr, and VMop were grown in suspension in association with *K. aerogenes* for 2 or 3 days before being examined. Samples were taken from low density, exponentially growing cultures and washed free of bacteria by three cycles of centrifugation in 17 mM NaH/KH₂PO₄ buffer (pH 6.4). After agar-overlay, the cells were fixed in the same buffer containing 2% formaldehyde (5 minutes, room temperature), followed by 1% formaldehyde in methanol (5 minutes, −15°C). They were stained with N2 or N4 hybridoma culture supernatants (Fok et al., 1993) diluted 1:30 and 1:20, respectively, followed by FITC-conjugated donkey anti-mouse IgG (1:500, Jackson ImmunoResearch Laboratories) (for details, see Clarke et al., 1987).

**Assays for phagocytosis**

Phagocytosis of fluorescent yeast particles was measured essentially as described (Maniak et al., 1995). Fluorescent yeast particles were prepared by suspending 5 g of yeast (Sigma YSC-2) in 50 ml PBS (150 mM NaCl in 20 mM Na₂H/KH₂PO₄, pH 7.2); the suspension was stirred for 30 minutes in a boiling water bath, then washed five times in PBS and twice in KP. A pellet of 2×10^10 yeast particles was resuspended in 20 ml Na₂H/KH₂PO₄ (50 mM, pH 9.2) containing 2 mg tetramethylrhodamine isothiocyanate (TRITC, Sigma T3163, Isomer R) and incubated for 30 minutes at 37°C. The yeast particles were washed twice in the pH 9.2 phosphate buffer, then four times in KP buffer, and were stored at −20°C. The uptake of yeast particles was measured using cells that had been growing on a suspension of *K. aerogenes* for 2-3 days prior to the assay. The cells were collected from log phase cultures, washed three times in KP, and resuspended at 2×10⁶ cells/ml in KP containing 10% LF medium. The cells were then incubated on a rotary shaker for 30 minutes, and fluorescent yeast particles were added (1.2×10⁷ yeast particles/ml). Duplicate 1 ml samples were taken at 20 minute intervals; each was added to a tube containing 0.1 ml of trypan blue (2 mg/ml), which quenched the fluorescence of uningested particles (Maniak et al., 1995). The samples were mixed by inversion for 1 minute, then centrifuged (500 g, 4 minutes). The pellets were resuspended in 1 ml KP, and emission spectra were determined immediately in an SLM 8000C spectrofluorimeter (λ<sub>ex</sub>=544 nm, λ<sub>em</sub>=576 nm).

Phagocytosis of the fluorescent yeast particles and endocytosis of fluorescent dextrans were also monitored by confocal laser scanning microscopy using a Zeiss LSM510 equipped with a 100× 1.4 NA Plan-Neofluar objective. The 543 nm line of the HeNe laser was used for excitation of TRITC-labeled yeast or dextran, and the 488 nm line of the Argon laser for excitation of FITC-dextran. Cells that had been growing in LF were plated in one-third strength LF in dishes with coverslip glass bottoms (WillCo-Dish, WillCo Wells, The Netherlands). Bacterially grown cells were washed as described above, then plated in one-tenth strength LF (to maintain osmotic conditions similar to those in which the cells had been growing and to avoid triggering development) and incubated for 30 minutes at room temperature. For measuring endosomal transit time, a mixture of TRITC- and FITC-labeled dextrans (2 mg/ml and 0.2 mg/ml, respectively, average M<sub>r</sub> 70,000; Sigma) was added (Jenne et al., 1998; Maniak, 1999). For phagocytosis experiments, TRITC-yeast particles were added in one-tenth strength LF thirty minutes after the cells had been plated. The cells were then covered with a thin layer of agarose, excess fluid was removed, and the cells were observed using low laser power (10%).

**Confirmation of the cytosolic location of pHluorin**

Cells of AX2/A15-pHluorin, grown overnight in LF medium, were allowed to settle on coverslips and examined from below at 100× magnification using epifluorescent illumination (λ<sub>ex</sub>=405 or 480 nm, λ<sub>em</sub>=485 nm). At both excitation wavelengths the cell edge was sharply defined with conspicuous bright filopodia. Agar-overlayed cells presented a ‘Swiss cheese’ appearance with abundant dark vesicles. Ratio images showed an essentially uniform intracellular pH, with the only variations attributable to cellular motion between blue and UV exposures (data not shown).

**Spectral acquisition with axenically growing cells**

Cells were cultured overnight in LF medium that had been supplemented with 0.7 g/liter yeast extract. Cells grew with a normal
Cells were harvested after culture in bacteria for at least 48 hours, and spectral acquisition with bacterially grown cells. This was then subtracted from the spectrum of the cell suspension. Centrifugation, and a second spectrum recorded from the supernatant; this medium still contains 50 mM glucose, so the cells would not be expected to exit the growth phase into development for several hours (Marin et al., 1980). Fluorescence spectra were taken using a microscope, and the presence of fluorescent bacterial secretion products.) The fluorescence spectrum was recorded and the supernatant spectrum subtracted as above.

Spectral acquisition with bacterially grown cells

Cells were harvested after culture in bacteria for at least 48 hours, during which time the cell density was not allowed to exceed 1×10^6 cells/ml. The cells were collected by centrifugation and washed three times in PB6 (20 mM Na/K phosphate, pH 6.0, containing 10 mM CaCl₂ and 200 μM MgCl₂), then suspended in the buffer described above. (We were not able to work with cells directly in bacterial suspension because of light absorption and scattering by the bacteria and the presence of fluorescent bacterial secretion products.) The fluorescence spectrum was recorded and the supernatant spectrum subtracted as above.

Spectral analysis

The pHluorin excitation spectrum has two peaks: one at 400 nm and the other at 475 nm, whose ratio depends on pH (Miesenböck et al., 1998). To obtain pH values from experimental spectra, the ratio of peak heights was compared to a calibration table. To suppress noise in the experimental spectra these were first fitted as the sum of two Gaussian curves, using the ‘Solver’ feature of Microsoft Excel (97) to fit values of the mean, standard deviation and height with the sum of squared deviations between measurement and model as the goodness-of-fit criterion. The peak heights used for pH measurements were then determined from the fitted spectra using a calibration table. To create this table, we first prepared calibration spectra using cell extracts from AX2/A15-pHluorin cells that had been grown in LF medium and developed overnight in PB; comparison with similarly treated, untransformed AX2 showed that cellular autofluorescence is a negligible component of the total fluorescence in this material. The cell extracts were prepared in 20 mM Na/K phosphate buffer and titrated to round values of pH. The spectra differed slightly from the published curves, perhaps reflecting differences in the N- and C-termini of our construct; in particular, the 400 nM excitation peak at pH 7 is more nearly intermediate between the peaks at pH 6.5 and pH 7.5. We fitted our calibration spectra with paired Gaussian curves as above, and the ratios of the fitted curve heights were expressed as a function of pH. Finally, the last-mentioned curve was itself fitted with a sigmoid curve, whose value was calculated at 1000 points to construct the calibration table.

In bacterially grown cells, cellular autofluorescence was evident as a peak or shoulder on the pHluorin excitation spectrum centered at λ_ex=360 nm, λ_em=510 nm. We corrected for this by subtracting the fluorescence of a matched suspension of identically treated untransformed AX2 cells. In some experiments an emission spectrum was recorded at λ_ex=390 nm, λ_em=410-550 nm; in this case cellular autofluorescence was evident as a peak that appeared to be centered below 410 nm. When emission spectra were available, they were used to estimate autofluorescence directly, and the result was used to correct the excitation spectra. The cytosolic pH was determined in all cases from corrected spectra.

Results

Replacement of the vatM promoter with the act6 promoter

A plasmid was constructed that contained in tandem the promoter region of vatM, a selectable marker (pyr5-6, which confers uracil prototrophy), the promoter region of the act6 gene, and 1 kb of vatM coding region, starting with the
Dictyostelium 100 kDa V-ATPase subunit

1911

translation initiation codon. Preparation of this plasmid, pVATM-act6, is summarized in Materials and Methods, and it is shown schematically in Fig. 1A. Homologous recombination involving a double crossover between this plasmid and the vatM region of Dictyostelium chromosomal DNA would separate the vatM promoter from the vatM gene and introduce a new promoter (act6) to control vatM expression (Fig. 1B). This plasmid was used to transform Dictyostelium strain DH1, a uracil auxotroph, and transformants were selected in defined liquid medium lacking uracil. A change in restriction fragment size was expected if homologous recombination between chromosomal vatM and the transforming DNA led to the insertion of the uracil marker plus the act6 promoter between vatM and its own promoter (Fig. 1B). Twenty-two transformants were obtained and analyzed by Southern blot. A single transformant manifested the larger BclI fragment size consistent with the desired double crossover (Fig. 1C). This clone was designated VatMpr (for promoter replacement).

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Effect of growth conditions on the level of VatM protein in VatMpr cells

The amount of the 100 kDa V-ATPase subunit, the protein product of vatM, was compared in VatMpr cells grown in liquid medium (HL5) and on bacteria. Axenically growing VatMpr cells were harvested during mid log phase growth. Cells growing in association with K. aerogenes on nutrient agar plates were also collected during exponential growth, prior to any visible clearing of the bacterial lawn, and the cells were washed free of bacteria. The lysate of an equal number of cells from each growth condition was electrophoresed on a polyacrylamide gel, and the level of the 100 kDa V-ATPase subunit was examined by immunoblot. The upper portion of the blot was stained with N2 monoclonal antibodies, which specifically recognize the 100 kDa subunit of the V-ATPase (Fok et al., 1993). The lower portion of the blot was stained with anti-actin antibodies, as a control for the amount of protein loaded in each lane. The results are shown in Fig. 2A for VatMpr and DH1. In the parental DH1 cells, growth conditions had no significant effect on the level of the 100 kDa protein. However, in VatMpr cells, the level of this V-ATPase subunit was much lower in cells grown on bacteria than those grown axenically. Phosphorimager analysis indicated that the amount of the 100 kDa subunit present in bacterially grown VatMpr cells (normalized for actin levels) was 29% of that present in axenically grown cells.

Time course experiments using suspension cultures of cells shifted from axenic to bacterial growth conditions showed that approximately 2 days of growth on bacteria were required to produce the maximal reduction in VatM protein levels. After 2 days, the level of VatM protein in VatMpr cells was typically one-third that of control levels (range 29-37%).

Growth properties of VatMpr cells correlate with the level of expression of vatM

We examined the consequences of the reduced level of the 100 kDa subunit on growth rate and other properties of VatMpr cells. When cultured axenically, DH1 and VatMpr cells grew at the same rate, with doubling times of approximately 12 hours. However, when cultured with K. aerogenes on nutrient agar plates, VatMpr cells cleared the bacterial lawn much more slowly than did DH1 (Fig. 3). A similar result was observed for cells grown on a suspension of bacteria. Under these
conditions, the doubling time was about 8 hours for VatMpr compared with 3 hours for DH1 cells. To determine whether the reduced level of the 100 kDa subunit was responsible for the poor growth of VatMpr cells on bacteria, we tested whether restoration of normal levels of this subunit would restore normal growth. We constructed a plasmid that contained the vatM coding region plus ~0.6-kb of genomic DNA upstream of vatM, presumed to include the endogenous promoter for this gene. After addition of a G418 drug resistance cassette, this plasmid was transformed into VatMpr cells. Several transformants were obtained that grew at normal rates on bacteria. In Fig. 3, the growth of one of these (VMop-11) on a bacterial lawn is compared with that of VatMpr and DH1. This figure shows the size of plaques formed by each strain on a lawn of *K. aerogenes* after 5 days and 6 days of growth. The very slow growth rate of VatMpr is evident from its tiny plaque size (Fig. 3A,B); addition of vatM driven by its own promoter was sufficient to restore normal growth (compare VMop-11 in Fig. 3E,F to DH1 in Fig. 3C,D). Immunoblot analysis confirmed that the level of the 100 kDa subunit had been restored to normal in VMop-11 cells (Fig. 2B). These data indicate that the reduced level of the 100 kDa subunit in VatMpr cells was sufficient to account for the slow growth of these cells on bacteria.

The effect of reduced VatM levels on the distribution and accumulation of VatA
Monoclonal antibodies specific for two different subunits of the *Dictyostelium* V-ATPase were used to examine the effects of the VatMpr mutation on the V-ATPase enzyme complex. N2 antibodies recognizing VatM and N4 antibodies recognizing VatA (subunit A of the peripheral V1 domain, responsible for ATP hydrolysis), were used to visualize V-ATPase distribution in bacterially grown VatMpr and DH1 cells (Fig. 4). In normal cells, the staining patterns of these two antibodies are indistinguishable; they both stain membranes of the contractile vacuole complex (Fok et al., 1993). The VatM-specific N2 antibodies gave only faint staining of VatMpr cells, in agreement with the immunoblot results shown in Fig. 2A. The cell population was somewhat heterogeneous with respect to staining intensity, but all VatMpr cells were very weakly labeled compared with DH1 (Fig. 4B versus 4A). Where staining was visible, N2 antibodies were associated with contractile vacuole membranes.

In contrast, N4 antibodies revealed a striking difference in the distribution of the A subunit in mutant versus control cells. In DH1 cells, these antibodies labeled membranes of the contractile vacuole complex in the usual manner (Fig. 4G). However, in bacterially grown VatMpr cells, N4 staining was mostly diffuse and cytoplasmic (Fig. 4H). Labeling of contractile vacuole membranes could still be detected in some cells (as expected owing to residual VatM in VatMpr cells), but this pattern was largely obscured by bright cytoplasmic fluorescence. Thus, in the absence of normal amounts of the 100 kDa subunit, much of the A subunit became mislocalized to the cytosol.

VatMpr cells were also examined under conditions in which normal levels of the 100 kDa subunit were present, that is, when growing axenically or after being transformed with the plasmid carrying vatM under the control of its own promoter. Cells of VatMpr and its transformant VMop-11, both harvested after growth on bacteria, were stained with N2 and N4 antibodies. In bacterially grown VMop-11 (Fig. 4I) and in axenically grown VatMpr (not shown), N2 and N4 antibodies stained contractile vacuole membranes, just as in wild-type cells. Thus, when approximately normal levels of the 100 kDa subunit were restored, mislocalization of the A subunit did not occur.

The degree of N4 staining in bacterially grown VatMpr cells (Fig. 4H) suggested that A subunit levels were not reduced, and, indeed, might be elevated, as a consequence of the 100 kDa subunit deficiency. To examine this possibility, we grew DH1 and VatMpr cells either axenically or for 2 days on a suspension of *K. aerogenes*, then subjected equal aliquots of the four cultures to electrophoresis and immunoblotting. The
The contractile vacuole system in VatMpr cells

Vacuolar proton pumps are heavily concentrated in membranes of the contractile vacuole complex of *Dictyostelium*, where they are responsible for the accumulation of water by this osmoregulatory organelle (Heuser et al., 1993; Bush et al., 1994; Clarke and Heuser, 1997). Electron microscopy of freeze-dried cells shows the V1 domains of the V-ATPase as a dense array of ‘studs’ on the cytoplasmic surface of contractile vacuole membranes (Heuser et al., 1993). Such images are shown in Fig. 6 for elements of the contractile vacuole system from DH1 and VatMpr cells examined after two days of growth on bacteria. In agreement with the immunostaining data reported above, the density of proton pumps in contractile vacuole membranes of VatMpr cells was found to be substantially reduced relative to that in control cells.

The possibility of impaired osmoregulatory function in VatMpr cells was tested by subjecting these cells to osmotic shock conditions that have been shown to kill *Dictyostelium* mutants defective in osmoregulation (Schuster et al., 1996). Mutant and parental cells were grown on a suspension of *K. aerogenes* for 2 days prior to osmotic challenge. Exponentially growing cultures at a density of 1x10^6 cells/ml were split in half, and sorbitol (final concentration 0.4 M) was added to one-half of each culture. After 2 hours, samples were taken from each culture and diluted 100-fold and 1000-fold with KP buffer; 10 minutes later, aliquots were plated to determine viable titer. No significant differences in viable titer were found between mutant and parental cells or between cells that had and had not been subjected to osmotic shock. Thus, the reduced levels of the 100 kDa subunit present in mutant cells did not significantly impair their ability to survive osmotic shock.

Contractile vacuole filling and emptying was also monitored in living cells by microscopy. Bacterially grown VatMpr cells, observed under hypotonic conditions using either interference reflection microscopy or styril dyes (Heuser et al., 1993), revealed an apparently normal, albeit somewhat sluggish, contractile vacuole cycle (J. Heuser, personal communication). Thus, VatMpr cells manifested no obvious impairment in osmoregulatory function.

Endo/lysosomal function in VatMpr cells

The slow growth of VatMpr cells on bacteria, evident in Fig. 3, raised the possibility that endo/lysosomal function might be affected. Accordingly, the endocytic and phagocytic capabilities of mutant and control cells were examined. For these experiments, cells were grown either axenically or for two days on bacteria prior to analysis. AX3 cells were used as controls in addition to (or instead of) DH1 cells in these experiments, so that wild-type and mutant cells could be analyzed in the same growth medium (i.e. without the 100 μM uracil required by DH1). During assays for both endocytosis and phagocytosis, care was taken to keep the cells in osmotic conditions similar to those in which they had been growing.

We found that if cells were shifted from growth on bacteria (suspended in 17 mM phosphate buffer) to the much greater osmotic strength of HL5 or LF medium, endocytosis and phagocytosis rates were significantly depressed for an hour or more, until the cells had time to adapt to the new conditions (data not shown).

Endosomal transit time was monitored using an assay developed by Maniak and co-workers (Jenne et al., 1998; Maniak, 1999). In this assay, cells are fed a mixture of TRITC and FITC-labeled dextrans (10:1 ratio) and monitored by fluorescence microscopy. FITC fluorescence is quenched at acidic pH, so endo/lysosomes appear red throughout most of their cycle. However, late in the pathway, prior to exocytosis of indigestible material, the pH of an endosome rises, restoring the FITC fluorescence and changing the color of the endosome from red to yellow. Thus, the time of appearance of yellow endosomes in a cell population is a convenient indicator of endosomal transit time.

Cells from exponentially growing axenic or bacterial *Dictyostelium* cultures were plated on glass coverslips in low fluorescence (LF) nutrient medium (see Materials and Methods for details). At T0, they were fed a FITC/TRITC-dextran mixture and observed by confocal laser scanning microscopy. For all axenically grown cells (AX3, DH1 and VatMpr), yellow endosomes first appeared after about 1 hour (range 52-68 minutes in four experiments). For bacterially grown AX3 and DH1 cells, similar timing was observed (range 65-75 minutes). However, for bacterially grown VatMpr cells, orange or yellow endosomes first appeared after about 2 hours (range 110-140 minutes in five experiments). Thus, the endosomal transit time for bacterially grown VatMpr cells was approximately twice as long as that of control cells or axenically grown VatMpr cells.
To monitor phagocytosis, we labeled heat-killed *S. cerevisiae* with TRITC and fed the TRITC-yeast to Dictyostelium cells, as described (Maniak et al., 1995). For cells growing in suspension, uptake was monitored by harvesting aliquots at 20 minute intervals after yeast were added. Quenching the fluorescence of undigested particles and determining the cell-associated fluorescence spectrofluorimetrically (see Materials and Methods for details). For bacterially grown cells, the rate of uptake by VatMpr cells was one-half that of AX3 cells (Fig. 7).

Uptake of TRITC-yeast was also monitored by fluorescence microscopy of cells plated on coverslips. The behavior of AX3 cells and axenically grown VatMpr cells was similar; after 30-40 minutes, most cells in the population had ingested one or two yeast particles (Fig. 8A,C). For bacterially grown VatMpr cells, only rare cells had ingested more than a single yeast particle. Even after 70 minutes, many VatMpr cells contained no yeast, although yeast particles were enriched at the surface of the cells (Fig. 8B), suggesting that binding was not impaired.

Cytosolic pH in VatMpr cells

Ratiometric pHluorin, a pH-sensitive variant of GFP (Miesenböck et al., 1998), was used to examine VatMpr for possible alterations of cytosolic pH or its regulation. For axenically growing cells, cytosolic pH was 7.55±0.07 (n=10) for AX2/A15-pHluorin and 7.55±0.08 (n=10) for VatMpr/A15-pHluorin. For cells grown on bacteria, we obtained values suggesting that the cytosolic pH is unaltered in AX2 (7.6±0.24/0.11) but slightly reduced in VatMpr (7.3±0.18/0.06) (mean±s.d./s.d.m.). Although this difference is formally significant at the 1% level, we view it with caution, since the expression of pHluorin was much reduced in cells growing at low density on bacteria, presumably owing to the lowered activity of the A15 promoter (Cohen et al., 1986). Autofluorescence and bacterial fluorescence, which were negligible in comparison with the signal from axenic cells, made significant contributions to the spectra of cells cultured on bacteria. Corrections for these were necessarily imperfect, resulting in significantly larger standard deviations for measurements made on bacterially grown cells. Thus, our results suggest but do not conclusively establish a decrease in cytosolic pH on the order of a few tenths of a pH unit for bacterially grown VatMpr cells.

We next explored the possibility that VatMpr cells might be maintaining their cytosolic pH with the help of mechanisms normally used only under acid growth conditions. We probed for this situation in the two strains by measuring the dynamic responses of the cytosolic pH to increased acid loading. Bacterially grown cells were washed and suspended in PB6, and the fluorescence at the ‘acid’ pHluorin peak (λex=475 nm, λem=510 nm) was monitored continuously while acetate was added to the medium without a change in extracellular pH. In bacterially grown AX2, the fluorescence increased smoothly with time, indicating a decrease in pH with roughly inverse-exponential kinetics and an estimated half-time of 70 seconds. Bacterially grown VatMpr initially acidified in a manner similar to AX2, but the curve abruptly flattened, reaching a plateau at 30-45 seconds (Fig. 9A).

This type of very rapid pH stabilization is characteristic of cells that have been growing under conditions of acid stress, as illustrated in Fig. 9B. In this experiment we analyzed AX2 cells that had been grown overnight in LF medium at pH 7.0, then either left in this medium or transferred to LF medium buffered to pH 5.0. Four hours later, the two cultures were subjected to acetate challenge as described above. AX2 cells that had been grown at pH 5.0 exhibited very rapid pH regulation, closely resembling the behavior of bacterially grown VatMpr.

Discussion

The use of the act6 promoter to alter vatM gene expression

The haploid nature of Dictyostelium cells is a great advantage when using molecular genetic approaches to examine the function of nonessential genes. However, the analysis of essential gene products is less straightforward. Studies of the V-ATPase in *S. cerevisiae* have shown that this enzyme is not required for growth of yeast cells under standard culture conditions, although cells lacking V-ATPase function are sensitive to elevated pH or high calcium levels (reviewed by Nelson and Harvey, 1999; Forgac, 2000). In contrast, in Dictyostelium, attempts to disrupt genes encoding vatM and other V-ATPase subunits have been unsuccessful (Liu and Clarke, 1996; Xie et al., 1996; see also Clarke and Heuser, 1997) (V. Burdine and M.C., unpublished), which implies that these genes are essential in Dictyostelium. Disruption of genes encoding V-ATPase subunits is lethal in *Drosophila melanogaster* (Davies, S. A. et al., 1996) and probably also in mice (Inoue et al., 1999), consistent with this interpretation. It has therefore proved necessary to seek means other than gene disruption to analyze V-ATPase function in Dictyostelium.

Several laboratories have attempted antisense mutagenesis of Dictyostelium V-ATPase subunits using an inducible promoter, discoidin-Iy, to drive expression of the antisense transcripts (Liu and Clarke, 1996; Xie et al., 1996; Davies, L. et al., 1996). This approach was ineffective in altering VatM
protein levels (Liu and Clarke, 1996) but reductions in Ca\textsuperscript{2+} transport and acidic vesicle content were detected in vatP antisense cells (Xie et al., 1996). Further, poor development was reported for antisense strains of vatP (Xie et al., 1996) and vatB (Xie et al., 1996; Davies, L. et al., 1996). However, each group found that the ability to induce the antisense effects was lost over time. Moreover, even initially, a mixture of affected and unaffected cells is to be expected, since transcripts regulated by the discoidin-I promoter are not uniformly expressed in a cell population (Clarke and Gomer, 1995).

As an alternative means of exploring the function of the 100 kDa transmembrane subunit of the 
Dictyostelium\hspace{1pt}V-ATPase, we altered the regulation of the chromosomal copy of vatM by replacing its promoter. Our choice of the act6 promoter for this purpose was based on the observations of Knecht and Loomis, who generated myosin-deficient 
Dictyostelium cells by expressing an antisense transcript of the myosin heavy chain gene under the control of the act6 promoter (Knecht and Loomis, 1987). They observed high levels of the antisense transcript (and a mutant phenotype) when the cells were grown axenically, but not when they were grown on bacteria. Thus, expression of a transcript driven by the act6 promoter appeared to be dependent on growth conditions. Furthermore, although an increase in act6-regulated transcription was detected at the onset of development (Romans et al., 1985; Knecht and Loomis, 1987), there was a steep decline in act6 mRNA in the later stages of development (McKeown and Firtel, 1981; Romans et al., 1985), which suggested that a transcript controlled by the act6 promoter would be absent in late development. Both of these features seemed useful for our analysis of VatM.

Substitution of the act6 promoter for that of vatM in VatMpr cells was confirmed by the expected changes in the restriction map of the gene as assessed by Southern blot, and by the diminished abundance of the VatM protein in bacterially grown cells. Although not shown here, it was also confirmed by the pattern of vatM mRNA accumulation during development, which matched that of act6 rather than wild-type vatM (T.L. and M.C., unpublished). However, the correct double crossover leading to promoter replacement was not a frequent event; only one such mutant was found among 40 clones from two transformations that were analyzed by Southern blot.

**Effect of reduced levels of VatM on proton pump assembly**

In bacterially grown VatMpr cells, immunostaining revealed that the A subunit of the V-ATPase was mostly cytoplasmic rather than membrane associated, and electron microscopy showed that the density of proton pumps in contractile vacuole membranes was reduced. These results indicate that assembly of the V-ATPase enzyme complex is limited by availability of the 100 kDa transmembrane subunit. A related finding was reported by Manolson and co-workers, who disrupted the VPH1 gene in 
*S. cerevisiae* and demonstrated by cell fractionation that the peripheral nucleotide-binding subunits of the V-ATPase became mislocalized (Manolson et al., 1992). Similarly, certain mutations introduced into VPH1 by site-directed mutagenesis impaired assembly of the enzyme complex (Leng et al., 1996). We conclude that in the 
Dictyostelium as a yeast, the 100 kDa subunit plays an essential role in V-ATPase localization and assembly.

Our data also showed that an elevated amount of A subunit protein was present in cells containing reduced levels of the 100 kDa subunit. Thus, the synthesis and/or stability of V\textsubscript{1} components does not depend upon the availability of sufficient V\textsubscript{0} domains to allow assembly of the holoenzyme. Instead, our results suggest that 
Dictyostelium cells have a means of monitoring the amount of functional V-ATPase present in the cell and adjusting expression of the subunits accordingly. In VatMpr cells, this homeostatic mechanism is deranged because vatM expression cannot be properly coordinated with that of the other subunits. However, it is noteworthy that VatM subunit levels remain as high as they do, since other studies have shown that the act6 promoter has very little activity in bacterially grown cells (Wetterauer et al., 1996; Souza et al., 1998). Strong
post-transcriptional regulation of VatM was also evident on our earlier attempts to alter protein levels using anti-sense RNA (Liu and Clarke, 1996).

Use of a single isoform of VatM in contractile vacuoles and endosomes
Multiple isoforms of the 100 kDa V-ATPase subunit have been detected in organisms ranging from yeast to mammals and their localization in distinct cell populations suggests that they possess specialized functions (see Introduction). PCR-based efforts to detect additional isoforms in Dictyostelium were not successful (Liu and Clarke, 1996), although the possibility that another isoform(s) exists cannot be ruled out until the sequencing of the Dictyostelium genome has been completed. However, the results of the present study are consistent with biochemical evidence that the same VatM subunit is present in both contractile vacuole and endosomal membranes (Adessi et al., 1995; Clarke and Liu, 1996). Furthermore, although the two organelles are physically separated in the cell (Gabriel et al., 1999), mutations in the clathrin heavy chain (O’Halloran and Anderson, 1992; Ruscetti et al., 1994) and in a Rab4-like GTPase (Bush et al., 1996) affect both compartments in a manner suggestive of a membrane trafficking relationship between them. Additional confirmation that the same VatM subunit is present in both contractile vacuole and endosomal membranes has come from imaging of the dynamics of VatM in living cells, visualized by expression of green fluorescent protein fused to VatM (M.C., J. Köhler, Q.A., T.L. and G. Gerisch, unpublished). The presence of this subunit in two compartments whose membranes differ so greatly in proton membranes has come from imaging of the dynamics of VatM in living cells, visualized by expression of green fluorescent protein fused to VatM (M.C., J. Köhler, Q.A., T.L. and G. Gerisch, unpublished). The presence of this subunit in two compartments whose membranes differ so greatly in proton pump density demonstrates that the abundance of proton pumps in a particular endomembrane is not determined by the isoform of the 100 kDa subunit that resides there.

Effect of reduced levels of VatM on cell growth and endocytic function
VatMpr cells grew slowly on bacteria and manifested clear endocytic defects under restrictive conditions. Both the rate of particle uptake by phagocytosis and the transit time of fluorescent dextrans through the endo/lysosomal pathway were affected by a factor of about two.

Light microscopy revealed that some cells in both wild-type and mutant cell populations did not take up particles (or fluid, in the case of fluorescent dextrans) during a given time interval, although a cell inactive during one time interval could be active during another. Such cell-to-cell variation within a population may reflect a dependence of endocytic activity on the cell cycle, although this possibility has not been explored. Whatever the basis of this variation, the bacterially grown VatMpr population contained a larger fraction of inactive cells (as illustrated in Fig. 8), contributing to the reduced rate of particle uptake. However, uptake was not the only activity affected. Endosomal transit time was also prolonged, a property measured not as a population average but as the earliest appearance of yellow endosomes in individual cells. Thus, the processing of endocytosed material was slower, even in the endocytically active fraction of the VatMpr population.

There are several ways in which the VatMpr mutation could slow endocytic activity. The number of proton pumps delivered to endosomes might be reduced as it is in the contractile vacuole system, or their rate of delivery might be slower, with possible consequences to acidification kinetics. However, the luminal pH of organelles is not determined uniquely by pump number but rather by a complex interplay of proton pumps with ion channels and transporters (Futai et al., 1998; Grabe and Oster, 2001). Consequently, there is no direct relationship between pump number and pH (as the contractile vacuole system also makes evident). Another step that might be affected is the delivery of lysosomal enzymes from the Golgi to endo/lysosomes; this and other types of membrane trafficking also depend on the V-ATPase (Gueze et al., 1983; Mellman, 1996). Finally, there may be general effects on the efficiency of energy utilization in mutant cells, as discussed below.

Effect of reduced levels of VatM on cytosolic pH regulation
Possible effects of altered VatM expression on cytosolic pH were examined with ‘ratiometric pHluorin’ (Miesenböck et al., 1998), a pH-sensitive GFP variant. We found that this probe gives intracellular pH values similar to those estimated using null-point lysis (Aerts et al., 1985; Coukell et al., 1997) and NMR (Satre et al., 1989), and allows excellent compartmental and temporal resolution. Under axenic growth conditions, the cytosolic pH of VatMpr was indistinguishable from that of the wild-type control. During growth on bacteria, VatMpr appeared to have a slightly lowered cytosolic pH, while that of AX2 seemed to be unchanged. These results, while statistically significant, should nevertheless be viewed with caution owing to technical difficulties in collecting spectra from bacterially grown cells. It is important to note that even a small drop in pH could have important effects on growth, as a decrease of 0.2 pH units in the Dictyostelium cell cycle is associated with a threefold decrease in the rate of protein synthesis (Aerts et al., 1985), and lowering cytosolic pH by comparable amounts quite generally inhibits cell cycle entry in cultured metazoan cells (Grinstein et al., 1989).

When we examined the dynamic regulation of cytosolic pH in bacterially grown VatMpr and AX2 cells, we found clear evidence of changes in VatMpr. In AX2 subjected to a sudden increase in acid loading, the cytosolic pH decreased asymptotically with a half-time of about 70 seconds. The acidification curve for bacterially grown VatMpr, although initially very similar, showed an abrupt plateau at about 45 seconds. This suggests that the mutant cells employ a mechanism for pH homeostasis that is not used by cells containing normal levels of vacular proton pumps. In particular, it appears that bacterially grown VatMpr cells make use of a system that is inactive in wild-type cells under standard conditions, but can be induced in the latter cells when these are grown in acid media. The system appears to require 30 seconds to 1 minute after acid challenge to become fully active, so that the acidification curve initially appears identical to that of uninduced cells, but then suddenly flattens. In S. cerevisiae, the V-ATPase plays a major role in cytosolic pH homeostasis (Nelson and Harvey, 1999; Forgac, 2000), but a P-type H+-ATPase in the yeast plasma membrane assists in pH regulation (reviewed by Portillo, 2000). This enzyme, Pma1p, is the major plasma membrane proton pump and is essential for viability. It
is activated, probably by phosphorylation (Goosens et al., 2000), when cells are exposed to any of a number of environmental factors, the most prominent of which are glucose and acid pH. At least in the response to glucose stimulation, Pma1p seems to require about 1 minute to reach maximal activity (Serrano, 1983). Yeast cells also contain a second plasma membrane H+-ATPase, Pma2p, which is not essential for growth and is expressed at a much lower level; its function has not been determined.

The Dictyostelium genome contains an open reading frame (aa-numgf1149 in the genome sequencing database) that encodes a sequence 32% identical to Pma1p. This predicted Pma1p homologue has not yet been characterized. However, a Dictyostelium P-type H+-ATPase homologous to Pma2p is induced under conditions of mild cytosolic acidification (Coukell et al., 1997), and our preliminary data suggest that the latter enzyme (Pat2) helps to defend cytosolic pH in the presence of proton ionophores (C.M. and H.M., unpublished). Our working hypothesis is that the V-ATPase is normally responsible for pH regulation in Dictyostelium, but that reduced expression of VatM leads to the induction of P-type proton ATPase(s) to assist in pH regulation. This would account for the ability of VatMpr to respond more rapidly than AX2 to acid challenge. It would also be consistent with the report that, in Neurospora, inhibition of a V-type ATPase led to the emergence of mutations in a P-type ATPase gene, which increased the in vivo activity of that enzyme (Bowman et al., 1997).

This strategy raises certain questions of energy economy: V-type ATPases pump up to two protons per ATP hydrolyzed (Grabe et al., 2000), or twice the yield of P-type enzymes (Davies et al., 1994; Briskin et al., 1995). Even under the most extreme conditions of our experiments – a gradient of about 2.5 pH units and a moderate membrane potential (van Duijn et al., 1988) – the energy of ATP hydrolysis should suffice for V-ATPase function (Wiecekorek et al., 1999), and the substitution of P-ATPases for V-ATPases would thus seem to squander ATP. However, V-type ATPases are huge molecules with relatively low turnover number, while P-type enzymes have about an eighth the mass and a much higher throughput. It thus makes at least intuitive sense for a cell to employ V-type enzymes for predictable base load applications but use P-type transporters to handle load peaks. Since use of P-type ATPases entails additional ATP consumption, activation of this mechanism could impose a brake on other cell functions, thereby contributing to the growth phenotype of VatMpr. Studies to test this hypothesis are in progress.

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