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Antisense RNA inhibition of the putative vacuolar H^+-ATPase proteolipid of *Dictyostelium* reduces intracellular Ca^{2+} transport and cell viability

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SUMMARY

Transport of Ca^{2+} via a P-type pump into the contractile vacuole of *Dictyostelium discoideum* appears to be facilitated by vacuolar proton (V-H^+) ATPase activity. To investigate the involvement of the V-H^+-ATPase in this process using molecular techniques, we cloned a cDNA (*vatP*) encoding the putative proteolipid subunit of this enzyme. The deduced protein product of this cDNA is composed of 196 amino acids with a calculated M_r of 20,148 and the primary structure exhibits high amino acid sequence identity with V-H^+-ATPase proteolipids from other organisms. *vatP* is a single-copy gene and it produces one ~900 nt transcript at relatively constant levels during growth and development. Attempts to disrupt the endogenous gene using *vatP* cDNA were unsuccessful. But, expression of *vatP* antisense RNA reduced the levels of *vatP* message and V-H^+-ATPase activity by 50% or more. These antisense strains grew and developed slowly, especially under acidic conditions, and the cells seemed to have difficulty forming acidic vesicles. During prolonged cultivation, all of the antisense strains either reverted to a wild-type phenotype or died. Thus in *Dictyostelium*, unlike yeast, the V-H^+-ATPase seems to be indispensable for cell viability. When different antisense strains were analyzed for Ca^{2+} uptake by the contractile vacuole, they all accumulated less Ca^{2+} than control transformants. These results are consistent with earlier pharmacological studies which suggested that the V-H^+-ATPase functions in intracellular Ca^{2+} transport in this organism.

Key words: Antisense RNA, V-H^+-ATPase proteolipid, Ca^{2+} transport, *Dictyostelium*

INTRODUCTION

In eukaryotic cells, many intracellular compartments including fungal and plant vacuoles, lysosomes, clathrin-coated vesicles and chromaffin granules are acidified by a vacuolar-type proton-translocating (V-H^+) ATPase (Nelson, 1992). V-H^+-ATPases are large, complex enzymes composed of two distinct regions: a peripheral catalytic sector containing at least 6 subunits and a multimeric integral membrane sector (Kane and Stevens, 1992; Supekova et al., 1995). The best characterized integral protein is a small, extremely hydrophobic proteolipid which binds N,N′-dicyclohexylcarbodiimide (DCCD) and is thought to form a transmembrane proton pore (Uchida et al., 1985). Results of recent molecular genetic studies with *Saccharomyces cerevisiae* suggest that the V-H^+-ATPase and vacuolar acidification influence a wide range of cellular activities. For example, in this organism, disruption of genes encoding different subunits of the V-H^+-ATPase is non-lethal providing the cells are grown in a low calcium, acidic medium (Hirata et al., 1990; Nelson and Nelson, 1990). But at neutral pH, these V-H^+-ATPase-deficient strains are defective in many vacuolar activities as well as in a number of processes not obviously associated with the vacuole including copper detoxification, iron metabolism and normal mitochondrial function (Anraku et al., 1992; Eide et al., 1993).

In the cellular slime mould, *Dictyostelium discoideum*, V-H^+-ATPase activity and the distinctive peg-like enzyme structures are associated primarily with a buoyant membrane fraction (Nolta et al., 1991, 1993), probably a component of the contractile vacuole (CV) (Fok et al., 1993; Heuser et al., 1993). This organelle, which is found in protozoa and freshwater amoebae, is thought to function in osmoregulation, but it may be involved in other cellular processes as well (Patterson, 1980; Heuser et al., 1993). Recently, a high-affinity P-type Ca^{2+} ATPase has been localized to membranes of the CV (Milne and Coukell, 1989; Rooney and Gross, 1992; Rooney et al., 1994; Moniakis et al., 1995) and Ca^{2+} transport via this enzyme has been reported to be enhanced by V-H^+-ATPase activity and a low intraorganellar pH (Rooney and Gross, 1992; Rooney et al., 1994). These observations suggest that in *Dictyostelium* the CV might also function in Ca^{2+} regulation.

To learn more about the function of the V-H^+-ATPase in *Dictyostelium* during growth and development, and especially about its role in Ca^{2+} homeostasis, we have attempted to inactivate the V-H^+-ATPase by cloning a cDNA encoding a subunit of the enzyme and using it to disrupt the endogenous gene by homologous recombination and to reduce its expression by antisense RNA inhibition. To do this, we chose to target the gene for the V-H^+-ATPase proteolipid, since in *S.*

cerevisiae this subunit is essential for enzyme function and assembly (Nelson and Nelson, 1990; Noumi et al., 1991). This report describes the cloning of a cDNA encoding a *Dictyostelium* proteolipid and the effects of antisense RNA inhibition of this protein on Ca^{2+} transport into the CV and on cell viability.

MATERIALS AND METHODS

Materials

Chemicals were obtained from the following sources: $^{45}CaCl_2$ (9.5-12.2 mCi/mg Ca^{2+}), dinitrophenol (DNP), gramicidin D, folic acid (ICN); filipin, carbonylcyanide *m*-chlorophenylhydrazone (CCCP), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), sodium metavanadate, sodium azide (Sigma); concanamycin A (Fluka).

Strains and culture conditions

Wild-type *Dictyostelium discoideum* strain AX2 (Watts and Ashworth, 1970) and the thymidine-requiring mutant, JH10 (Hadwiger and Firtel, 1992), were used in this study. Both strains were grown in HL-5 medium (Cocucci and Sussman, 1970) supplemented with 100 μg of dihydrostreptomycin and 100 μg of thymidine (JH10 only)/ml, in Petri dishes or shake cultures at 22°C. The HL-5 medium normally had a pH of 6.7 but in some experiments the pH was adjusted by addition of H_3PO_4 or NaOH.

Isolation of *vatP* cDNA clones

We used the PCR and degenerate deoxyribonucleotide primers to highly conserved regions of the proteolipid subunits of V-H^+-ATPases of other organisms to amplify related sequences from *Dictyostelium* genomic DNA. The sense (5′-CGGGATCCG-GHGCHGCHTAYGGHACHGCHAA-3′) and antisense (5′-CGGAATTCDACDCCDGCRTCDCCDACDAT-3′) primers corresponded to amino acid residues in the 1st and 3rd putative transmembrane domains, respectively, and contained *Bam*HI or *Eco*RI restriction sites.

Amplification conditions were as described previously (Coukell et al., 1995). The single 0.3 kb PCR product obtained was cloned into *Bam*HI/*Eco*RI-digested Bluescript II KS^+ vector (Stratagene), and sequenced. Double-stranded plasmid DNA was sequenced by the chain termination method using universal and internal strand-specific primers and a Sequenase Version 2.0 Kit (US Biochemical/Amersham). Analysis of this sequence revealed a single open reading-frame and the deduced protein product showed a high degree of amino acid sequence identity (~60%) with other proteolipids. This DNA fragment was used to probe a *Dictyostelium* 3 hour λgt11 cDNA library (provided by Dr P. Devreotes, The Johns Hopkins University School of Medicine). The largest insert (0.65 kb; cDNA1) was cloned into the *Eco*RI site of the KS^+ vector (designated pB0.65cV), and sequenced. cDNA1 contained a single long open reading-frame terminating with a TAA stop codon and a partial 3′-untranslated sequence (UTS). The deduced amino acid sequence of cDNA1 began with a methionine. However, since there was no additional 5′ DNA sequence and the amino acid sequence in this region showed no homology with other proteolipids, it wasn't clear if this methionine corresponded to the translation initiation site. To extend the 5′ sequence, a 0.20 kb fragment of cDNA1 spanning the 5′-end to a unique internal *Bgl*II site (see Fig. 1A) was used as a probe to rescreen the cDNA library. A 0.5 kb cDNA (cDNA2) was isolated which possessed a 24 bp 5′ extension but lacked the 3′ UTS. This additional 5′ sequence revealed that the ATG of the initial methionine in cDNA1 was in a consensus *Dictyostelium* translation initiation sequence and that it was preceded by an in-frame stop codon. To construct a full-length cDNA, a 5′ *Pst*I-*Bgl*II fragment of cDNA2 was cloned into *Pst*I/*Bgl*II-digested pB0.65cV to give plasmid, pBvatP. The sequence of this *vatP* (*v*acuolar *AT*Pase *p*roteolipid) cDNA was verified in the Molecular Biology Core Facility, York University, using Taq DyeDeoxy Terminator Cycle Sequencing and an automated DNA Sequencer Model 373A (Applied Biosystems).

Northern and Southern blot analysis

Total RNA was isolated from frozen cell pellets (2×10^7 cells), size-fractionated and transferred to nylon membranes as described previously (Coukell and Cameron, 1990). The filters were incubated with either full-length *vatP* cDNA labelled with ^{32}P by random priming or a strand-specific RNA probe prepared with a Riboprobe Gemini System II Kit (Promega) and linearized pBvatP. Hybridization and washing conditions were as described by Franke et al. (1987). The amount of *vatP* mRNA in each lane of the blot was determined on an InstantImager (Canberra Packard).

For Southern blots, 1-3 μg of *Dictyostelium* genomic DNA was digested with the appropriate restriction enzymes, fractionated on 0.7% agarose gels, transferred to nylon membranes, and probed with full-length *vatP* cDNA. High-stringency hybridization and washing conditions were as described previously (Coukell et al., 1995); low-stringency hybridization was at 37°C and the membranes were washed twice for 5 minutes in 2× SSC at room temperature, twice for 30 minutes in 2× SSC, 1% SDS at 37°C and then rinsed briefly in 2× SSC.

Construction of disruption vectors and transformation

To construct a single-crossover disruption vector, the full-length *vatP* cDNA was removed from pBvatP by digestion with *Eco*RI and cloned into the *Eco*RI site of plasmid pGEM#25 (a gift from Dr R. A. Firtel, UCSD), which carries the selectable Thy1 marker (Dynes and Firtel, 1989). A double-crossover disruption vector was constructed by introducing the Thy1P-42 insert from pGEM#25 as a filled-in *Hin*dIII fragment into the filled-in *Bgl*II site of *vatP* in plasmid pBvatP. The plasmid was linearized by digestion at the *Eco*RI site in the multiple cloning sequence before transformation. JH10 cells were transformed by electroporation (Dynes and Firtel, 1989) with 30-50 μg of plasmid DNA and transformants were selected for the ability to grow in HL-5 medium in the absence of thymidine. Transformants were purified by clonal growth on lawns of *Klebsiella aerogenes*.

Construction of antisense vectors, transformation, maintenance of antisense strains and development

Two antisense vectors were constructed. To make an integrative vector, full-length *vatP* cDNA derived from pBvatP by digestion with *Kpn*I and *Sac*I was inserted in an antisense orientation into *Kpn*I/*Sac*I-cut pVEII (Blusch et al., 1992; Liu et al., 1992). This construct was termed pVEII-AS. In pVEII, expression of the neomycin phosphotransferase gene (G418-resistance) is under the control of the constitutive actin 15 promoter while expression of sequences inserted at the multiple cloning site are driven by the inducible discoidin I promoter. This promoter is activated by prestarvation factor (PSF) which accumulates in proportion to cell density and it is repressed by folate (Clarke et al., 1987; Blusch et al., 1995). To construct an extrachromosomal antisense vector, a full-length *vatP* cDNA obtained from pVEII-AS by digestion with *Bam*HI was introduced into the *Bgl*II site of vector pRHI9 (provided by Dr R. Insall, University College, London). A plasmid with the *vatP* sequence in an antisense orientation was identified by restriction mapping, and designated pRHI9-AS. In this construct, both the neomycin phosphotransferase gene and *vatP* antisense RNA are expressed constitutively.

The antisense vectors (as well as pVEII and pRHI9) were introduced into AX2 cells by electroporation and transformants were selected in 96-well plates in HL-5 medium supplemented with 10 μg (pVEII and pVEII-AS) or 20 μg (pRHI9 and pRHI9-AS) of G418/ml. Individual transformants were cultured in 60 mm Petri dishes and the

G418 concentration was increased gradually to 30-40 μg/ml. To reduce expression of *vatP* antisense RNA in the pVEII-AS transformants, the HL-5 medium was supplemented with 1 mM folate and the cultures were diluted frequently to maintain a low cell density. To obtain sufficient cells for biochemical studies, the transformants were grown (−folate) in shake cultures (~250 rpm) to a density of 5-10×10^6 cell/ml. All of the antisense strains eventually reverted or died (see Results), and we were unable to recover antisense strains from frozen stocks, therefore new transformations were performed approximately every two months.

To observe development, cells were grown to mid-log phase, harvested by centrifugation (700 ***g***, 2 minutes), washed twice in ice-cold salt solution (SS) (Bonner, 1947) and resuspended in SS to 2×10^8 cells/ml. Duplicate 20 μl aliquots were spotted (~5×10^6 cells/cm^2) on PBS agar (40 mM Na_2HPO_4/KH_2PO_4, 20 mM KCl, 2.5 mM $MgSO_4$, 1.5% agar) adjusted to pH 5.5, 6.7 or 7.5 with H_3PO_4 or NaOH. Excess moisture was permitted to evaporate and the plates were incubated in a humid atmosphere at 22°C.

Neutral red staining and photography

Transformants were grown to mid-log phase, harvested, washed in SS and suspended in SS at 5×10^6 cells/ml. Neutral red was added to a final concentration of 0.01% and the cells were shaken at room temperature for 20 minutes. The cells were then washed twice in SS, observed and photographed on a Polyvar (Reichert-Jung) microscope using DIC optics with a 100× 1.32 NA objective. The images were visualized on a Hamamatsu (DVS-3000) image processor, recorded with a Hamamatsu (C2400) video camera and printed using a Mitsubishi thermal video printer. A green interference filter (BP 495-580) was used to intensify red-stained vesicles in the cells.

Membrane isolation and ATPase assays

Membrane fractions enriched for V-H+-ATPase activity (CV membranes) were isolated from filter-broken cells and assayed for ATPase activities as described by Padh et al. (1989). Crude lysates and membranes were preincubated for 5 minutes with or without 25 μM NBD-Cl or 100 μM sodium vanadate and the reaction was initiated by addition of 2 mM ATP. Protein concentration was determined with a D_C Protein Assay Kit (Bio-Rad).

Ca^{2+}-transport assay

Intracellular Ca^{2+} transport was examined by assaying the uptake of $^{45}Ca^{2+}$ by filipin-permeabilized cells as described by Milne and Coukell (1989), except the concentration of filipin was reduced to 10 μg/ml. When >90% of the cells were permeable to Giemsa stain (20-30 minutes), they were pelleted by centrifugation at 1,100 ***g*** for 2 minutes, resuspended to 1×10^8 cell/ml in ice-cold uptake buffer, and assayed immediately. Inhibitors, dissolved in methanol, ethanol or DMSO, were added to the cells 5 minutes before initiating the reaction by addition of Ca^{2+} and ATP. The solvents never exceeded 1% of the total volume and alone they had no effect on Ca^{2+} uptake.

RESULTS

Cloning and characterization of a cDNA encoding a *Dictyostelium* proteolipid

Using the PCR and degenerate oligodeoxyribonucleotide primers synthesized to highly conserved regions of V-H+-ATPase proteolipids of other organisms, we amplified a single ~0.3 kb fragment from *Dictyostelium* genomic DNA. This DNA fragment was used as a probe to isolate overlapping cDNAs which were spliced together at a unique internal *Bgl*II site to produce a clone of 689 bp (Fig. 1A; EMBL accession number X90516). This cDNA contained a complete open reading-frame (ORF) of 588 bp encoding a putative protein of 196 amino acids with a calculated M_r of 20,148. The proposed ATG start codon is in the sequence AAA**ATG**TTT which agrees with the consensus *Dictyostelium* translation initiation

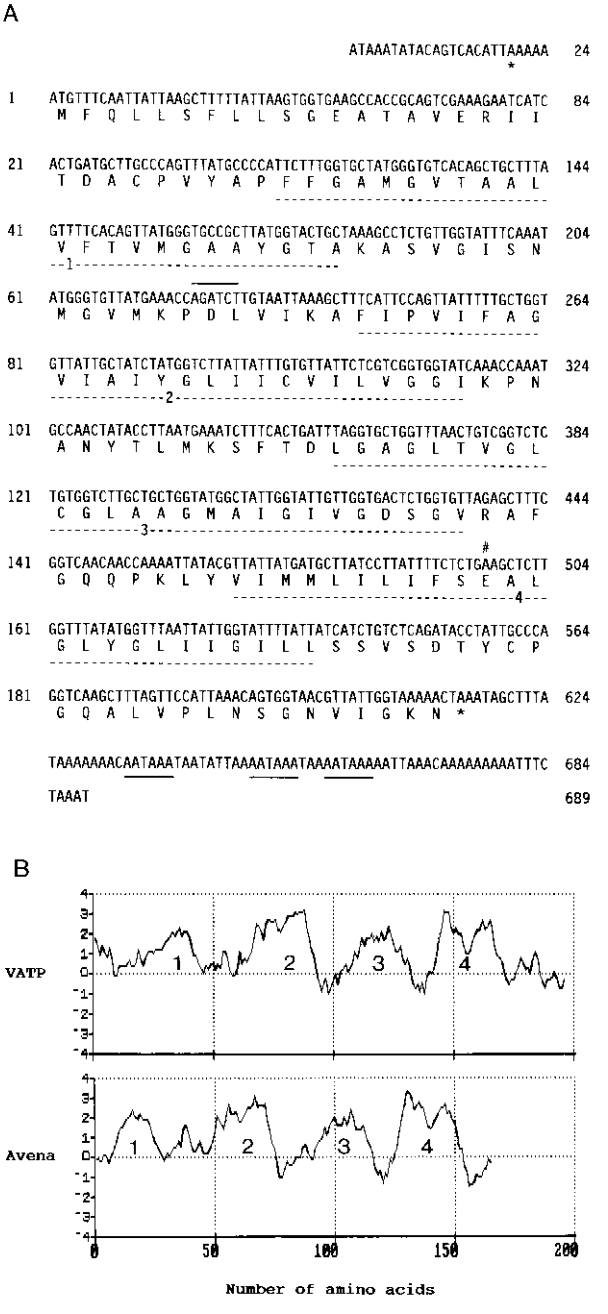

Fig. 1. Structure of the *Dictyostelium* proteolipid, VATP.
(A) Nucleotide and deduced amino acid sequence of the full-length *vatP* cDNA. Nucleotides are numbered on the right and amino acids on the left. In-frame TAA stop codons are indicated by asterisks. The four putative transmembrane domains are delineated by numbered dashed lines and the conserved glutamic acid residue in the 4th domain is denoted with a #. Three potential polyadenylation signals are underscored and the unique *Bgl*II restriction site is overscored. (B) Hydropathy plot comparison of VATP and the V-H+-ATPase proteolipid from *Avena*. Hydropathy analysis was performed using the Kyte-Doolittle algorithm of the PROSIS program with a window of 10. Positive values indicate hydrophobicity; negative values denote hydrophilicity. The four potential transmembrane domains are numbered.

sequence and it is preceded by an in-frame TAA stop codon 6 bp upstream. The ORF terminates at a TAA and the 3′ untranslated sequence contains several possible polyadenylation recognition sequences.

Like other proteolipids, the deduced protein product of this cDNA (VATP) possesses a high proportion of hydrophobic amino acids (~60%). Hydropathy analysis (Fig. 1B) revealed four potential transmembrane domains (designated 1-4) and an overall profile very similar to other V-H^+-ATPase proteolipid subunits (e.g. compare the profiles of VATP and the proteolipid from oats, *Avena sativa*). Furthermore, VATP possesses the highly conserved glutamate (E-158) in the 4th transmembrane domain which is thought to function in DCCD binding (Nelson, 1992). An alignment of the predicted amino acid sequence of VATP with sequences of proteolipids from other organisms disclosed a high degree of identity in the central ~160 amino acids of the protein (Fig. 2). However, the *Dictyostelium* proteolipid has extensions of ~15 amino acids on both the N- and C-termini when compared to most of the other proteins. Interestingly, the proteolipid from *Avena* contains a sequence at its N terminus which shows some identity to the N-terminal extension of VATP. Disregarding the terminal extensions, VATP is 55-63% identical to authentic V-H^+-ATPase proteolipid subunits from other organisms, but only 48% identical to the VMA11 gene product of *S. cerevisiae*, a proteolipid thought not to be a structural component of the enzyme (Umemoto et al., 1991).

Southern blot analysis of *Dictyostelium* genomic DNA at high and low stringency suggested that *vatP* is a single-copy gene and that there are no other sequences closely related to *vatP* in the genome of this organism (not shown).

Northern blot analysis of total RNA from cells during growth and development revealed a single transcript of ~900 nt. The abundance of this transcript was relatively constant at all stages, although it appeared to decrease somewhat at culmination (not shown).

Attempts to disrupt the endogenous *vatP* gene

Strain JH10 cells were transformed with single or double crossover disruption vectors (see Materials and Methods) in an attempt to disrupt the endogenous *vatP* gene. In addition to selecting transformants in normal HL-5 medium, they were also selected in acidic HL-5 medium (pH 6.2) and in HL-5 medium supplemented with 5 mM $FeCl_3$, conditions which permit *vma3* mutants (V-H^+-ATPase proteolipid subunit-deficient) of *S. cerevisiae* to grow (Nelson and Nelson, 1990; Eide et al., 1993). Transformants were purified on lawns of *K. aerogenes* and examined for growth rate and developmental phenotype. Genomic DNA was isolated from most transformants exhibiting aberrant growth and/or development as well as from many phenotypically normal transformants, and subjected to Southern analysis. None of the transformants appeared to carry a disrupted *vatP* gene.

Antisense RNA inhibition of *vatP* expression

Based on these results, we suspected that *vatP* might be an essential gene, therefore we decided to reduce the level of VATP in the cells by expressing antisense *vatP* RNA. Two antisense expression vectors were constructed (see Materials and Methods): an extrachromosomal vector (pRHI9-AS) possessing a constitutive actin 15 promoter and an integrative

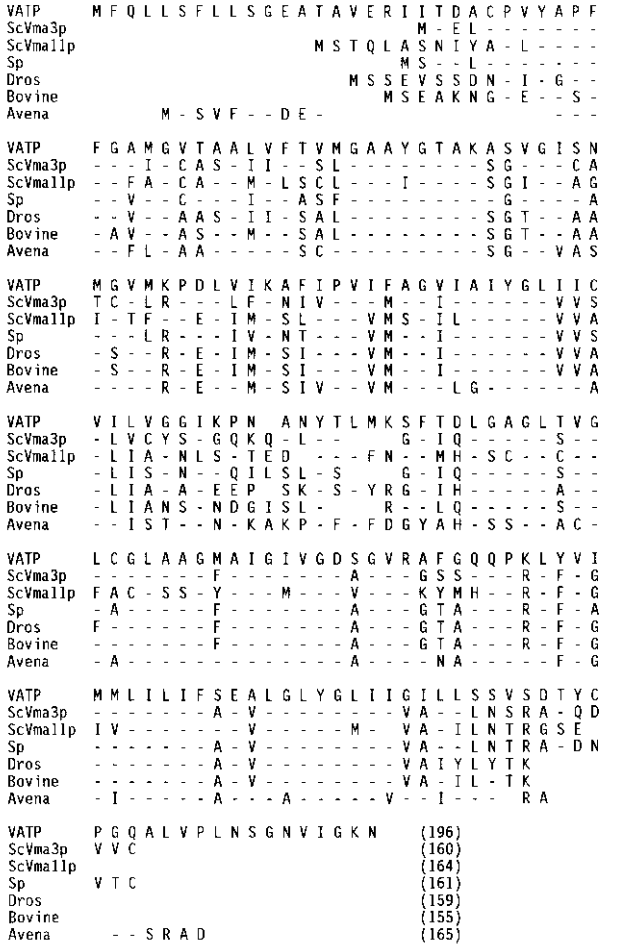

Fig. 2. Comparison of the derived amino acid sequence of VATP with proteolipids from *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Drosophila* (Dros), Bovine and *Avena*. The amino acid sequence of VATP is shown in single letter code. Sequences of the other proteolipids were aligned with VATP with the aid of the CLUSTAL program. Amino acids identical to those in VATP are indicated by a dash, those different are denoted by the appropriate code letter. Gaps have been introduced to maximize alignment. Amino acid sequences are from: ScVma3p (Nelson and Nelson, 1989); ScVma11p (Umemoto et al., 1991); Sp (Toyama et al., 1991); Dros (Meagher et al., 1990); Bovine (Mandel et al., 1988); Avena (Lai et al., 1991). Total numbers of residues are shown in parentheses.

vector (pVEII-AS) carrying an inducible discoidin promoter. AX2 cells were transformed with the antisense vectors (as well as with the vectors alone) and the different cell lines were cultured in HL-5 medium containing G418, which was increased gradually to a concentration of 30-40 μg/ml. Under these conditions, the level of *vatP* message in most antisense transformants examined was reduced by 40-60%; however, in a few cases, the level of transcript was <20% of the control (Figs 3, 4A). These latter strains normally died within a few days (see later).

Since antibodies specific to V-H^+-ATPase proteolipids were unavailable and we were unable to express the VATP as a fusion protein in bacteria (probably because of its extreme hydrophobicity), we decided to assess the effect of the antisense RNA on the V-H^+-ATPase by assaying the enzyme

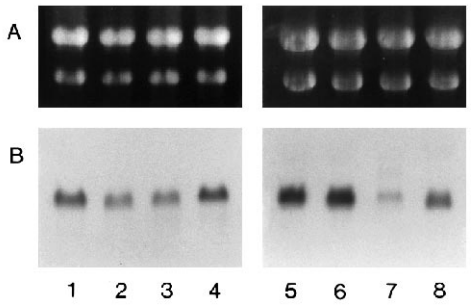

Fig. 3. Expression of *vatP* mRNA in control and antisense strains. Total RNA (20 μg/lane) from each cell line was fractionated on agarose gels, stained with ethidium bromide to evaluate loading (A), transferred to membranes and hybridized with a *vatP* mRNA-specific RNA probe (B). Cell lines: (1) AX2 (pVEII), (2) AX2(pVEII-AS1), (3) AX2(pVEII-AS2), (4) AX2(pVEII-AS) revertant, (5) AX2, (6) AX2(pRHI9), (7) AX2(pRHI9-AS1), (8) AX2(pRHI9-AS2). The levels of *vatP* mRNA in the antisense strains relative to controls were (2) 50%, (3) 50%, (7) <10%, and (8) 20%; the relative level of mRNA in the revertant (4) was 120%.

Table 1. NBD-Cl-sensitive and vanadate-sensitive ATPase activities in control and antisense strains

		Specific ATPase activity (nmol/min per mg protein)			
		NBD-Cl		Vanadate	
Strain	Cell fraction	sen.	% Change	sen.	% Change
AX2	Lysate	31.2		29.7	
AX2(pVEII-AS)	Lysate	19.6	–37	29.0	–2
AX2	Memb.	165.5		81.9	
AX2(pVEII-AS)	Memb.	85.8	–48	75.5	–8
AX2(pRHI9)	Lysate	30.6		26.5	
AX2(pRHI9-AS)	Lysate	11.6	–62	32.6	+23
AX2(pRHI9)	Memb.	195.8		90.6	
AX2(pRHI9-AS)	Memb.	88.7	–55	82.0	–9

All values are an average of duplicate or triplicate determinations. The levels of *vatP* mRNA in antisense strains AX2(pVEII-AS) and AX2(pRHI-AS) relative to controls were 50% and 20%, respectively.

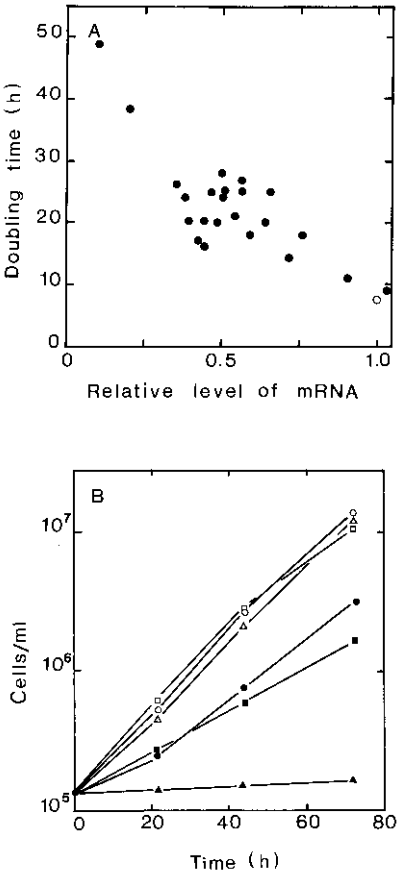

Fig. 4. Growth properties of *vatP* antisense strains. (A) A correlation of the doubling times and relative levels of *vatP* mRNA for 23 independent antisense cell lines (●) and a control transformant (○) growing in HL-5 medium (pH 6.7). (B) Growth of control and antisense strains at different pHs. AX2 cells transformed with pVEII (open symbols) or pVEII-AS (closed symbols) vectors were grown to mid-log phase in normal HL-5 medium containing 30 μg of G418/ml, diluted to 2×10^5 cells/ml in fresh medium at pH 7.5 (□,■), 6.7 (○,●) or 5.5 (△,▲), and shaken at 22°C.

activity in different cell lines. Table 1 shows NBD-Cl-sensitive (V-type) and vanadate-sensitive (P-type) ATPase activities in crude lysates and in CV membrane fractions of control cells and cells harbouring each type of antisense vector. Relative to control cells, both types of antisense cells exhibited an ~50% decrease in V-H+-ATPase activity; in contrast, the P-type activities showed little change. These results demonstrate that in addition to reducing the abundance of *vatP* transcript, the antisense RNA also decreases V-H+-ATPase activity.

Phenotype of antisense strains

Transformants carrying either type of antisense vector generally grew more slowly in normal HL-5 medium (pH 6.7) supplemented with G418 than control transformants. Doubling times of the antisense strains ranged from ~15 hours to >40 hours and they showed an inverse correlation with the level of *vatP* mRNA in the cells (Fig. 4A). At pH 7.5, doubling times for these strains were usually longer, and under acidic conditions (pH 5.5), they grew very slowly or not at all. Typical growth curves for AX2(pVEII) and an AX2(pVEII-AS) antisense strain at different pHs are shown in Fig. 4B; similar results were obtained with strains carrying the extrachromosomal vectors. After prolonged periods of growth in normal HL-5 medium, all of the slow-growing antisense strains either reverted to a wild-type growth rate or grew progressively more slowly, and then died. When examined by northern blot analysis, the revertants invariably exhibited wild-type levels of *vatP* mRNA (see Fig. 3). In general, transformants carrying the pRHI9-AS vector had slower growth rates, lower levels of *vatP* mRNA and were less stable than the pVEII-AS cell lines. The pVEII-AS strains could be maintained for several weeks when kept at a low cell density in Petri dishes in normal HL-5 supplemented with 1 mM folate. But, even under these conditions, the strains eventually reverted or died.

When antisense and control cells were stained with neutral red under hypotonic conditions and examined by light microscopy using Nomarski optics, two major differences were observed (Fig. 5). First, the controls cells possessed large numbers of intensely red-staining organelles while the antisense cells generally contained only small, weakly staining structures. Many of the antisense cell populations were heterogeneous in composition, however, possessing both stained and unstained cells (not shown). This heterogeneity in staining

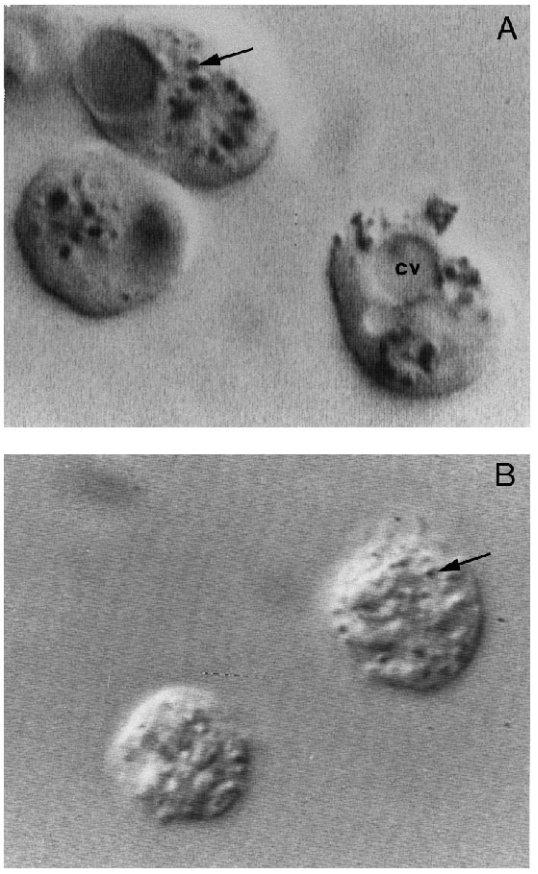

Fig. 5. Appearance of (A) control AX2(pVEII) and (B) antisense AX2(pVEII-AS) cells stained with neutral red. Transformants were grown, incubated with neutral red, and photographed as described in Materials and Methods. Contractile vacuoles (cv) and the intensely red-staining vesicles (arrows) are indicated. ×5,100.

might reflect differences in *vatP* antisense RNA expression in the various cells. Second, the antisense cells usually lacked the prominent, pale orange-staining vacuoles observed in the control cells. These structures are probably osmotically swollen CVs (Zhu and Clarke, 1992; Heuser et al., 1993).

To assess the ability of the antisense strains to develop, control and antisense cells suspended in SS were spotted on non-nutrient PBS agar buffered at pH 5.5, 6.7 or 7.5, and incubated in a humid environment. Under these conditions, the control cells formed fruiting bodies by 26 hours at all three pHs. Transformants carrying either type of antisense vector developed very poorly or not at all at pH 5.5, and slowly at the higher pHs. By 50 hours, antisense strains spotted at pH 6.7 or 7.5 usually formed a reduced number of small fruiting bodies which appeared to be proportioned normally (not shown).

When slow-growing antisense strains reverted to the control growth rate, the cells regained the control neutral red-staining pattern and the prominent vacuoles, and they developed at a rate comparable to control cells.

Ca^{2+} transport by cells expressing antisense *vatP* RNA

To determine if antisense RNA inhibition of V-H^+-ATPase activity affects intracellular Ca^{2+} transport, we measured $^{45}Ca^{2+}$ uptake by filipin-permeabililzed control and antisense cells. Since it was reported earlier (Rooney and Gross, 1992; Rooney et al., 1994) that ATP-dependent Ca^{2+} uptake by CV membranes is facilitated by a low intravesicular pH, we first examined the effects of protonophores and V-H^+-ATPase inhibitors on Ca^{2+} transport by permeabilized cells. Fig. 6A shows the kinetics of $^{45}Ca^{2+}$ uptake by filipin-permeabilized AX2 cells in the presence of mitochondrial inhibitors and 100 nM free Ca^{2+}. In most experiments, uptake continued for ~6 minutes and then leveled off or decreased slightly. Ca^{2+} uptake was ATP-dependent and strongly inhibited by 100 μM vanadate. To assess the effects of different protonophores and inhibitors on Ca^{2+} transport, the magnitude of Ca^{2+} accumulation by the permeabilized cells in the presence and absence of drugs was compared after 6 minutes of Ca^{2+} uptake (Fig. 6B). Addition of our standard mitochondrial inhibitor cocktail (100 μM sodium azide, 100 μM DNP, 50 uM CCCP) or the protonophore, gramicidin D (10 μM) alone, had no significant effect on Ca^{2+} uptake. This result with the mixture of mitochondrial inhibitors confirms our earlier findings (Milne and Coukell, 1989). In contrast, the V-H^+-ATPase inhibitors, concanamycin A (10 μM) and NBD-Cl (25 μM) reduced uptake by 50-60%, and the ion exchanger, nigericin (10 μM), inhibited the activity by ~85%. The kinetics of Ca^{2+} uptake in the presence of 25 μM NBD-Cl is shown in Fig. 6A. Increasing the concentration of each of these compounds to 100 μM had no greater effect. These results are consistent with those of Rooney and Gross (1992) and they suggest that the Ca^{2+}-uptake activity detected in filipin-permeabilized cells is the same activity measured in isolated CV membranes.

When slow-growing antisense strains were assayed for Ca^{2+} transport, the magnitude of uptake by these strains was reduced, compared to control transformants. Fig. 6A shows the kinetics of Ca^{2+} uptake by one antisense strain and the relative levels of uptake by five other antisense strains and one revertant are illustrated in Fig. 6C. Altogether ten independent antisense strains were analyzed and the average level of Ca^{2+} uptake relative to controls was 32% ± 8% (s.e.m.) with a range of values from <5% to 70%. Two revertant strains were also assayed and they exhibited relative Ca^{2+} uptakes of 107% and 117%.

DISCUSSION

To impair V-H^+-ATPase function in *Dictyostelium* by gene disruption or antisense RNA inhibition, we cloned a cDNA (termed *vatP*) which appears to encode the proteolipid subunit of this enzyme. The following properties of *vatP* suggest that we have identified the correct gene. First, the predicted translation product of this cDNA (VATP) has a primary structure and an overall topography very similar to authentic proteolipid subunits of V-H^+-ATPases from other organisms (Figs 1, 2). Moreover, VATP possesses all of the amino acids (or acceptable replacements) identified by site-directed mutagenesis to be essential for the function of the yeast proteolipid subunit (Vma3p) in the V-H^+-ATPase (Noumi et al., 1991). The amino acid sequence of VATP differs from those of other proteolipid subunits by the presence of extensions on both the N- and C-termini (Fig. 2). However, this structural difference might be less significant than it first appears because many *Dictyostelium* proteins possess additional terminal amino acids

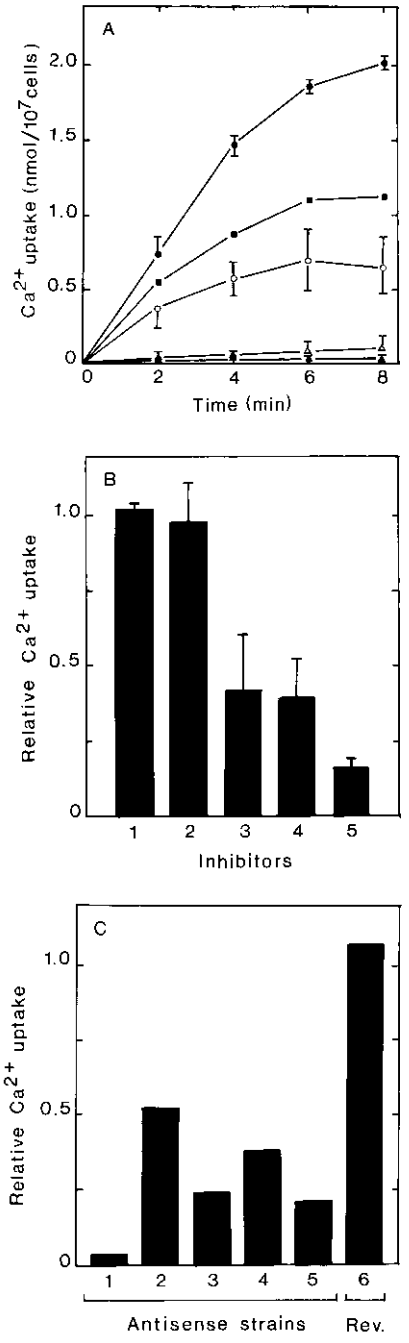

Fig. 6. Effects of inhibitors and antisense *vatP* RNA expression on $^{45}Ca^{2+}$ transport by filipin-permeabilized cells. (A) Kinetics of Ca^{2+} uptake by AX2 (●) and AX2(pRHI9-AS) antisense (■) cells in the absence of inhibitors and by AX2 cells in the presence of 25 μM NBD-Cl (○), 100 μM vanadate (△) or 1 mg of apyrase/ml and no ATP (▲). Results are means ± s.e.m. of values obtained in 3 experiments, except for (■) where the results are from a single experiment. (B) Ca^{2+} uptake by AX2 cells in the presence of (1) 100 μM sodium azide, 100 μM DNP, 50 μM CCCP, (2) 10 μM gramicidin D, (3) 10 μM concanamycin A, (4) 25 μM NBD-Cl, (5) 10 μM nigericin. Results are means ± s.e.m. of values obtained in 3 experiments and they are expressed relative to uptake in the absence of inhibitors. (C) Ca^{2+} uptake by five independent antisense cell lines (1-5) and by an antisense revertant (6). The result for each strain is from a single experiment and it is expressed relative to uptake by the appropriate control transformant. The levels of *vatP* mRNA in strains 1, 2, 4, and 6 relative to controls were 20%, 53%, 45% and 110%, respectively; strains 3 and 5 died before the levels of transcript could be determined.

when compared with homologs from other organisms (see e.g. Liu et al., 1992; Rutherford et al., 1992; Temesvari et al., 1994). Second, Southern blot analysis of genomic DNA using *vatP* cDNA as a probe revealed only a single gene even at very low stringency; thus there are unlikely to be other closely related proteolipids in this organism. Third, the level of *vatP* mRNA is maintained at a relatively constant level during growth and development, much like the expression of DVA41, another *Dictyostelium* gene encoding a V-H^+-ATPase subunit (Temesvari et al., 1994). This pattern of mRNA expression might be expected for components of an enzyme involved in a fundamental process such as organellar acidification. Finally, expression of antisense *vatP* RNA selectively reduces V-H^+-ATPase activity in crude lysates and on CV membranes (Table 1). Together, these results strongly support the idea that VATP is the proteolipid subunit of the V-H^+-ATPase. But, until the protein is actually purified from the enzyme complex, the possibility exists that VATP is like Vma11p of yeast (Umemoto et al., 1991), an accessory proteolipid essential for V-H^+-ATPase function but not a structural component.

Expression of antisense *vatP* RNA severely inhibited *Dictyostelium* growth and development, especially under acidic conditions (Fig. 4). In contrast, V-H^+-ATPase-deficient strains of *S. cerevisiae* are unable to grow at neutral pH but they grow reasonably well in medium at pH 5.5, the approximate internal pH of the vacuole. It has been suggested that these mutants use fluid-phase endocytosis to equilibrate their vacuolar system with the external medium (Nelson and Nelson, 1990). In *Dictyostelium*, unlike yeast, the V-H^+-ATPase is associated with a variety of organelles including lysosomes, phagosomes and the CV (Nolta et al., 1993). Perhaps in *Dictyostelium* one or more of these organelles cannot be acidified from the medium. Alternatively, the V-H^+-ATPase might function in important cellular processes in addition to organellar acidification. For example, this enzyme might assist the plasma membrane H^+-ATPase (Pogge-von Strandmann et al., 1984) in maintaining a neutral cytosolic pH by extruding excess protons through the CV system (Heuser et al., 1993). If this were the case, then, under acidic conditions, impaired V-H^+-ATPase activity in antisense strains could result in a suboptimal internal pH and reduced rates of growth and development.

During this study, all of the dozens of *vatP* antisense strains constructed either died or reverted to a form indistinguishable from wild type. Similar problems were encountered during the expression of *Dictyostelium* calmodulin antisense RNA (Liu et al., 1992). This difficulty in maintaining *vatP* antisense strains together with the inability to isolate a cell line with a disrupted *vatP* gene suggests that in *Dictyostelium*, unlike *S. cerevisiae*, the V-H^+-ATPase is probably essential for cell viability.

The primary aim of this project was to use a molecular genetic approach to assess the role of the V-H^+-ATPase in Ca^{2+} transport into the CV of *Dictyostelium*. In an earlier biochemical study, we observed that Ca^{2+} uptake by a P-type Ca^{2+} ATPase into vesicles, subsequently identified as the CV, was completely resistant to the protonophores, CCCP and DNP (Milne and Coukell, 1989). In contrast, Rooney and Gross

(1992) found that the activity of this pump in isolated CV membranes was reduced ~50% by the V-H^+-ATPase inhibitor, bafilomycin A_1 or the K^+/H^+ exchanger, nigericin. They proposed that Ca^{2+} is transported into the CV via a H^+-countertransporting Ca^{2+}-ATPase and that the low intravesicular pH is generated by a V-H^+-ATPase. Subsequently, they suggested that the inhibitors only reduce uptake by 50% because only one-half of the Ca^{2+}-sequestering vesicles possess the V-H^+-ATPase (Rooney et al., 1994). In the present study using filipin-permeabilized cells, we confirmed both seemingly contradictory observations: the protonophores CCCP, DNP and gramicidin had no effect on Ca^{2+} transport while the V-H^+-ATPase inhibitors, NBD-Cl and concanamycin A reduced uptake by 40-60%. In addition, under our conditions, nigericin strongly inhibited (~85%) Ca^{2+} accumulation (Fig. 6B). In general, these results support the model of Rooney and his colleagues, although it is unclear why nigericin should be more effective in permeabilized cells than in isolated CV membranes. It is also puzzling why the classical protonophores failed to collapse the pH gradient in these vesicles. Their inability to function, however, might be related to the nature of the CV membrane since this structure is known to possess a distinctive lipid and protein composition (Nolta et al., 1991; Zhu and Clarke, 1992). When *vatP* antisense cells were assayed for Ca^{2+} uptake under the same conditions, they always accumulated less Ca^{2+} than control transformants or antisense revertants (Fig. 6C). Furthermore, cell lines exhibiting the most severe effects of antisense *vatP* RNA expression generally showed the smallest uptake. These molecular genetic results are consistent with the pharmacological studies implicating the V-H^+-ATPase in Ca^{2+} transport into the CV. However, since antisense *vatP* RNA inhibited cells are unhealthy, it is difficult to say if the reduced Ca^{2+} uptake by these cells is due solely to an effect on the V-H^+-ATPase.

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REFERENCES

Anraku, Y., Umemoto, N., Hirata, R. and Ohya, Y. (1992). Genetic and cell biological aspects of the yeast vacuolar H^+-ATPase. *J. Bioenerg. Biomembr.* **24**, 395-405.

Blusch, J., Morandini, P. and Nellen, W. (1992). Transcriptional regulation by folate: inducible gene expression in *Dictyostelium* transformants during growth and early development. *Nucl. Acids Res.* **20**, 6235-6238.

Blusch, J., Alexander, S. and Nellen, W. (1995). Multple signal transduction pathways regulate discoidin I gene expression in *Dictyostelium discoideum*. *Differentiation* **58**, 253-260.

Bonner, J. T. (1947). Evidence for the formation of cell aggregates by chemotaxis in development of the slime mold *Dictyostelium discoideum*. *J. Exp. Zool.* **106**, 1-26.

Clarke, M., Kayman, S. C. and Riley, K. (1987). Density-dependent induction of discoidin I synthesis in exponentially growing cells of *Dictyostelium discoideum*. *Differentiation* **34**, 79-87.

Cocucci, S. and Sussman, M. (1970). RNA in cytoplasmic and nuclear fractions of cellular slime mold amebas. *J. Cell Biol.* **45**, 399-407.

Coukell, M. B. and Cameron, A. M. (1990). Calcium depletion of *Dictyostelium* cells selectively inhibits cyclic nucleotide phosphodiesterase synthesis at a post-transcriptional step. *J. Cell Sci.* **97**, 649-657.

Coukell, B., Moniakis, J. and Grinberg, A. (1995). Cloning and expression in *Escherichia coli* of a cDNA encoding a developmentally regulated Ca^{2+}-binding protein from *Dictyostelium discoideum*. *FEBS Lett.* **362**, 342-346.

Dynes, J. L. and Firtel, R. A. (1989). Molecular complementation of a genetic marker in *Dictyostelium* using a genomic DNA library. *Proc. Nat. Acad. Sci. USA* **86**, 7966-7970.

Eide, D. J., Bridgham, J. T., Zhao, Z. and Mattoon, J. R. (1993). The vacuolar H^+-ATPase of *Saccharomyces cerevisiae* is required for efficient copper detoxification, mitochondrial function, and iron metabolism. *Mol. Gen. Genet.* **241**, 447-456.

Fok, A. K., Clarke, M., Ma, L. and Allen, R. D. (1993). Vacuolar H^+-ATPase of *Dictyostelium discoideum*: a monoclonal antibody study. *J. Cell Sci.* **106**, 1103-1113.

Franke, J., Podgorski, G. J. and Kessin, R. H. (1987). The expression of two transcripts of the phosphodiesterase gene during the development of *Dictyostelium discoideum*. *Dev. Biol.* **124**, 504-511.

Hadwiger, J. A. and Firtel, R. A. (1992). Analysis of $G_\alpha 4$, a G-protein subunit required for multicellular development in *Dictyostelium*. *Genes Dev.* **6**, 38-49.

Heuser, J., Zhu, Q. and Clarke, M. (1993). Proton pumps populate the contractile vacuoles of *Dictyostelium* amoebae. *J. Cell Biol.* **121**, 1311-1327.

Hirata, R., Ohsumi, Y., Nakano, A., Kawasaki, H., Suzuki, K. and Anraku, Y. (1990). Molecular structure of a gene, VMA1, encoding the catalytic subunit of H^+-translocating adenosine triphosphatase from vacuolar membranes of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265**, 6726-6733.

Kane, P. M. and Stevens, T. H. (1992). Subunit composition, biosynthesis, and assembly of the yeast vacuolar proton-translocating ATPase. *J. Bioenerg. Biomembr.* **24**, 383-393.

Lai, S., Watson, J. C., Hansen, J. N. and Sze, H. (1991). Molecular cloning and sequencing of cDNAs encoding the proteolipid subunit of the vacuolar H^+-ATPase from a higher plant. *J. Biol. Chem.* **266**, 16078-16084.

Liu, T., Williams, J. G. and Clarke, M. (1992). Inducible expression of calmodulin antisense RNA in *Dictyostelium* cells inhibits the completion of cytokinesis. *Mol. Biol. Cell* **3**, 1403-1413.

Mandel, M., Moriyama, Y., Hulmes, J. D., Pan, Y.-C. E., Nelson, H. and Nelson, N. (1988). cDNA sequence encoding the 16-kDa proteolipid of chromaffin granules implies gene duplication in the evolution of H^+-ATPases. *Proc. Nat. Acad. Sci. USA* **85**, 5521-5524.

Meagher, L., McLean, P and Finbow, M. E. (1990). Sequence of a cDNA from *Drosophila* coding for the 16 kD proteolipid component of the vacuolar H^+-ATPase. *Nucl. Acids Res.* **18**, 6712.

Milne, J. L. and Coukell, M. B. (1989). Identification of a high-affinity Ca^{2+} pump associated with endocytotic vesicles in *Dictyostelium discoideum*. *Exp. Cell Res.* **185**, 21-32.

Moniakis, J., Coukell, M. B. and Forer, A. (1995). Molecular cloning of an intracellular P-type ATPase from *Dictyostelium* that is up-regulated in calcium-adapted cells. *J. Biol. Chem.* **270**, 28276-28281.

Nelson, H. and Nelson, N. (1989). The progenitor of ATP syntheses was closely related to the current vacuolar H^+-ATPase. *FEBS Lett.* **247**, 147-153.

Nelson, H. and Nelson, N. (1990). Disruption of genes encoding subunits of yeast vacuolar H^+-ATPase causes conditional lethality. *Proc. Nat. Acad. Sci. USA* **87**, 3503-3507.

Nelson, N. (1992). Organellar proton-ATPases. *Curr. Opin. Cell Biol.* **4**, 654-660.

Nolta, K. V., Padh, H. and Steck, T. L. (1991). Acidosomes from *Dictyostelium*: initial biochemical characterization. *J. Biol. Chem.* **266**, 18318-18323.

Nolta, K. V., Padh, H. and Steck, T. L. (1993). An immunocytochemical analysis of the vacuolar proton pump in *Dictyostelium discoideum*. *J. Cell Sci.* **105**, 849-859.

Noumi, T., Beltran, C., Nelson, H. and Nelson, N. (1991). Mutational analysis of yeast vacuolar H^+-ATPase. *Proc. Nat. Acad. Sci. USA* **88**, 1938-1942.

Padh, H., Lavasa, M. and Steck, T. L. (1989). Characterization of a vacuolar proton ATPase in *Dictyostelium discoideum*. *Biochim. Biophys. Acta* **982**, 271-278.

Patterson, D. J. (1980). Contractile vacuoles and associated structures: their organization and function. *Biol. Rev.* **55**, 1-46.

Pogge-von Strandmann, R., Kay, R. R. and Dufour, J.-P. (1984). An electrogenic proton pump in plasma membranes from the cellular slime mould *Dictyostelium discoideum*. *FEBS Lett.* **175**, 422-428.

Rooney, E. K. and Gross, J. D. (1992). ATP-driven Ca^{2+}/H^+ antiport in acid vesicles from *Dictyostelium*. *Proc. Nat. Acad. Sci. USA* **89**, 8025-8029.

Rooney, E. K., Gross, J. D. and Satre, M. (1994). Characterization of an intracellular Ca^{2+} pump in *Dictyostelium*. *Cell Calcium* **16**, 509-522.

Rutherford, C. L., Peery, R. B., Sucic, J. F., Yin, Y., Rogers, P. V., Luo, S. and Selman, O. (1992). Cloning, structural analysis, and expression of the glycogen phosphorylase-2 gene in *Dictyostelium*. *J. Biol. Chem.* **267**, 2294-2302.

Supekova, L., Supek, F. and Nelson, N. (1995). The *Saccharomyces cerevisiae* VMA10 is an intron-containing gene encoding a novel 13-kDa subunit of vacuolar H$^+$-ATPase. *J. Biol. Chem.* **270**, 13726-13732.

Temesvari, L., Rodriguez-Paris, J., Bush, J., Steck, T. L. and Cardelli, J. (1994). Characterization of lysosomal membrane proteins of *Dictyostelium discoideum*: a complex population of acidic integral membrane glycoproteins, Rab GTP-binding proteins and vacuolar ATPase subunits. *J. Biol. Chem.* **269**, 25719-25727.

Toyama, R., Goldstein, D. J., Schlegel, R. and Dhar, R. (1991). A genomic sequence of the *Schizosaccharomyces pombe* 16 kDa vacuolar H$^+$-ATPase. *Yeast* **7**, 989-991.

Uchida, E., Ohsumi, Y. and Anraku, Y. (1985). Purification and properties of H$^+$-translocating, Mg^{2+}-adenosine triphosphatase from vacuolar membranes of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **260**, 1090-1095.

Umemoto, N., Ohya, Y. and Anraku, Y. (1991). VMA11, a novel gene that encodes a putative proteolipid, is indispensable for expression of yeast vacuolar membrane H$^+$-ATPase activity. *J.Biol. Chem.* **266**, 24526-24532.

Watts, D. M. and Ashworth, J. M. (1970). Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem. J.* **119**, 171-174.

Zhu, Q. and Clarke, M. (1992). Association of calmodulin and an unconventional myosin with the contractile vacuole complex of *Dictyostelium discoideum*. *J. Cell Biol.* **118**, 347-358.

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