The patB gene of *Dictyostelium discoideum* encodes a P-type H\(^+\)-ATPase isoform essential for growth and development under acidic conditions

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During growth and early development of *Dictyostelium discoideum*, the amoebae exhibit transient pH changes in their cytosol (pH\(_c\)) and external medium which correlate with the extrusion of H\(^+\) from the cell by a plasma membrane pump. Moreover, the changes in pH\(_c\) have been postulated to influence early prestalk/prespore differentiation during development. To learn more about the role of H\(^+\) fluxes in *Dictyostelium*, we cloned and analysed cDNAs of the gene patB, which appears to encode a P-type H\(^+\)-ATPase. The patB ORF encodes a protein (termed PAT2) of 1058 amino acids with a calculated molecular mass of 117460 Da. When aligned with other P-type ion-transport ATPases, PAT2 showed the greatest amino acid sequence identity with plasma membrane H\(^+\)-ATPases of plants and fungi and considerably lower identity with other monovalent cation pumps and with Ca\(^2+\) pumps. Northern and Western analyses revealed that patB is expressed at very low levels in cells growing at neutral pH, but it is up-regulated rapidly and dramatically when the cells are shifted to an acidic medium. Immunofluorescence analysis indicated that PAT2 resides on the plasma membrane. When patB was disrupted by homologous recombination, the cells grew and developed normally at neutral and slightly alkaline pHs but they were unable to grow or develop at pH 5-0, and they slowly died. In growth medium at pH 6-8, patB\(^+\) and patB\(^-\) cells exhibited similar levels of vanadate-sensitive ATPase activity. However, when the cells were shifted to pH 5-0, this activity rapidly increased about twofold in the control cells but not in the mutant cells. Despite the lower ATPase activity in patB\(^-\) cells, they showed relatively normal H\(^+\) fluxes and only a slight decrease in pH\(_c\) when incubated in acidic medium. Together, these results suggest that patB encodes an acid-inducible P-type H\(^+\)-ATPase which is indispensable for the survival of *Dictyostelium* cells in moderately acidic external environments.

Keywords: *Dictyostelium discoideum*, P-type H\(^+\)-ATPase, gene disruption, cell viability

INTRODUCTION

Fungal and plant cells possess plasma membrane P-type H\(^+\)-ATPases which function to pump protons out of the cell. This activity regulates cytosolic pH (pH\(_c\)) and generates an electrochemical gradient which drives secondary transporters involved in moving ions and metabolites across the cell membrane (reviewed by Goffeau & Slayman, 1981; Michelet & Boutry, 1995). Recently, it has been found that in many organisms these H\(^+\)-ATPases comprise a multienzyme family, with the various members exhibiting different catalytic properties and tissue distributions (Supply et al., 1993; Houlnew Boutry, 1994; Michelet et al., 1994). In most cases, however, it is unclear why the organism requires more than one form of this enzyme.

Abbreviations: pH\(_c\), cytosolic pH; GST, glutathione S-transferase; E-64, trans-epoxysuccinylleucyl amino (4-guanidino) butane.

The GenBank/EMBL accession number for the sequence reported in this paper is X98286.
**Dictyostelium discoideum** is a eukaryotic micro-organism which has been used effectively to identify and to analyse molecular mechanisms underlying basic cellular processes such as growth, differentiation and morphogenesis (see Loomis, 1982; Loomis et al., 1994). Amoebae of this organism normally feed on bacteria, but mutant strains have been isolated which also grow in axenic medium. When the amoebae are starved on a solid substratum, they aggregate in response to endogenously generated extracellular cAMP signals and form fruiting bodies composed of two major cell types, stalk cells and spores. During both growth and development, a variety of observations suggest that cell regulation might involve changes in pH. For example, when the amoebae are grown synchronously in axenic medium, the pH of the cells oscillates during the cell cycle in phase with protein synthesis and DNA replication. Moreover, during exponential growth, artificially increasing the pH of the cells stimulates their rates of protein and DNA synthesis while decreasing the pH has the opposite effect (Aerts et al., 1985). During early *Dictyostelium* development, amoebae in suspension exhibit spontaneous and cAMP-induced transient acidifications of their external medium (Malchow et al., 1978a b) which can be correlated with an increase of the pH. (Aerts et al., 1987). These pH changes appear to involve the extrusion of protons from the cells via a diethylstilbestrol-sensitive P-type plasma membrane H+ pump(s) (Aerts et al., 1987; Gross et al., 1988). Furthermore, it has been suggested that these changes in pH might influence early cell type differentiation with a decrease in pH favours prestalk differentiation and an increase in pH directing cells along the prespore pathway (Gross et al., 1983; Inouye, 1985; Aerts et al., 1987; Aerts, 1988). On the other hand, another study failed to detect significant differences in the pH of prestalk and prespore cells (Ratner, 1986).

In an attempt to use a molecular genetic approach to learn more about the mechanisms regulating pH changes in *Dictyostelium* cells and their possible roles in growth and early development, we have cloned and characterized the putative P-type H+-ATPase gene, *patB* (P-type ATPase gene B). Interestingly, although a functional *patB* product (PAT2) is essential for *Dictyostelium* growth and development under moderately acidic conditions, disruption of *patB* has little effect on H+ fluxes and only a small effect on pH.

**METHODS**

**Strains and culture conditions.** The following axenic strains of *Dictyostelium discoideum* were used in this work: wild-type AX2 (Watts & Ashworth, 1970); the thymidine auxotroph, JH10 (Hadwiger & Firtel, 1992); HC390, a thyA, patB transformant of JH10; HC391 and HC392, thyA, patB transformants of JH10; HC392/patB, a patB-null transformant overexpressing PAT2 from a constitutive actin-15 promoter. All strains were grown axenically in Petri dishes or in shake cultures at 25° C in axenic medium. When the amoebae are grown synchronously in axenic medium, the pH of the cells oscillates during the cell cycle in phase with protein synthesis and DNA replication. Moreover, during exponential growth, artificially increasing the pH of the cells stimulates their rates of protein and DNA synthesis while decreasing the pH has the opposite effect (Aerts et al., 1985). During early *Dictyostelium* development, amoebae in suspension exhibit spontaneous and cAMP-induced transient acidifications of their external medium (Malchow et al., 1978a b) which can be correlated with an increase of the pH. (Aerts et al., 1987). These pH changes appear to involve the extrusion of protons from the cells via a diethylstilbestrol-sensitive P-type plasma membrane H+ pump(s) (Aerts et al., 1987; Gross et al., 1988). Furthermore, it has been suggested that these changes in pH might influence early cell type differentiation with a decrease in pH favours prestalk differentiation and an increase in pH directing cells along the prespore pathway (Gross et al., 1983; Inouye, 1985; Aerts et al., 1987; Aerts, 1988). On the other hand, another study failed to detect significant differences in the pH of prestalk and prespore cells (Ratner, 1986).

**Cloning and sequencing *patB*** DNA. The *patB* gene was initially detected as an ~0.8 kb PCR fragment isolated during the amplification of *Dictyostelium* genomic DNA sequences using degenerate primers based on conserved regions of P-type ion-transport ATPases (Moniakis et al., 1995). Using this DNA fragment as probe, partially overlapping cDNAs corresponding to ~98% of the *patB* ORF were isolated by screening a 3 h *Dictyostelium* JgtII cDNA library (provided by Dr P. Devreotes, The Johns Hopkins University School of Medicine). The extreme 5’ end of the ORF was obtained by constructing a partial genomic DNA library. AX2 genomic DNA was digested with Clal and EcoRI, size-fractionated on a 0.8% agarose gel, and fragments of 1–2 kb were isolated and cloned into Clal/EcoRI-digested Bluescript II KS(+) vector (Stratagene). The library was introduced into *Escherichia coli* strain DH5azE (Stratagene) by electroporation and ~6000 transformants were screened with the 0.42 kb 5’ *patB* probe (labelled with 32P by random priming) shown in Fig. 3(a). Two identical 1.5 kb genomic clones were obtained which contained ~0.9 kb of *patB* (including a single ~100 bp intron) and ~0.6 kb of 5’ untranslated sequence. The cDNA and genomic DNA clones were sequenced using a combination of exonuclease III/mung-bean-nuclease-generated deletions and sequence-specific primers. DNA sequencing was either performed manually using a Sequenase version 2.0 kit (USB/Amersham) or in the York University Molecular Biology Core Facility using DyeDeoxy Terminator Cycle Sequencing and an automated DNA Sequencer model 373A (Applied Biosystems).

**Northern and Southern blot analysis.** Poly(A)+ RNA was isolated from dry-ice/ethanol frozen cell pellets (9 x 107 total cells) and analysed as described previously (Moniakis et al., 1995). The membranes were probed with the 0.42 kb *patB* cDNA fragment. To assess gel loading, the membranes were stripped and reprobed with a 0.63 kb *vatP* cDNA (Moniakis et al., 1995; Xie et al., 1996). The amount of mRNA in each lane was determined on an InstantImager (Canberra Parkard).

For Southern blots, genomic DNA was isolated from individual transformants according to Reymond (1987). DNA (1–3 µg) from each transformant was digested with *HindIII* and analysed as described by Xie et al. (1996) using the 0.42 kb *patB* cDNA probe.

**Production of PAT2 antiserum, Western blot analysis and immunostaining.** A GST–*patB* gene fusion was constructed by PCR amplification of an ~3 kb *patB* cDNA possessing ~1 kb of 3’ untranslated sequence. The sense primer (5’-CGGGATCCCGTGGTTATCCACATGAT-3’) corresponded to nucleotides 2872–2889 of the ORF and possessed a *BamHI* restriction site on the 5’ end while the antisense primer (5’-CCGATCTCGAGATCTTTTTTTTTTTTTTT-3’) was a universal polyT primer with an EcoRI restriction site. Amplification conditions were as described by Coukell et al. (1995). The single PCR product of ~1.3 kb was cloned in-frame into *BamHI*/EcoRI-digested pGEX-2T vector (Pharmacia/LKB). The structure at the GST/*patB* junction was verified by DNA sequencing and the plasmid was transformed into *E. coli* DH5azF. Cultures of transformed cells were induced with 200 µM IPTG, sonicated in the presence of 1% Triton X-100, and a soluble 38 kDa fusion protein (GST–PAT2) possessing the C-terminal 114 amino acids of PAT2 was purified on glutathione-agarose beads as described by Smith & Johnson (1988). To produce PAT2 antiserum, a female New Zealand
White rabbit was inoculated intramuscularly with approximately 100 μg GST-PAT2 fusion protein at 4 week intervals for 3 months. One week after the final boost, the rabbit was bled and aliquoted serum was stored at −80 °C.

For Western blot analysis, ~10^7 mid-exponential-phase cells were collected by centrifugation (500 g, 2 min), washed once in 10 ml ice-cold salt solution (SS) (Bonner, 1947), pelleted in a microcentrifuge (5 s), aspirated dry and frozen at −80 °C. The cell pellets were dissolved in 250 μl cold 50 mM Tris/HCl, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet-40, 1 mM PMSF, 10 μg E-64 ml⁻¹ (pH 8.0), and the protein concentration was determined with a D\_ Protein Assay Kit (Bio-Rad). Proteins (20 μg per lane) were size-fractionated, transferred to nitrocellulose and probed with PAT2 antiserum (1:10000) as previously described (Moniak\_ is et al., 1995).

Immunostaining was performed essentially as described by Moniak\_ is et al. (1995). Cells were grown to a density of ~5 × 10^4 cells ml⁻¹ in HL-5 at the appropriate pH, washed and resuspended in 15 mM Na\textsubscript{2}HPO\textsubscript{4}/KHPO\textsubscript{4} (pH 6.1), and allowed to attach to glass slides at room temperature (30 min). After fixing and blocking, the cells were stained sequentially with PAT2 antiserum (1:1000) and FITC isomer-I-conjugated goat anti-rabbit IgG (1:500; Caltag). Immunostained cells were examined using a Bio-Rad MRC-600 confocal microscope.

**Overexpression of patB.** The entire coding region of the patB gene (plus at least one intron) was amplified from genomic DNA using the PCR. Primer PAT2-5′ (5′-GCACTAGT-AATGGATAAATCAG) corresponded to the N-terminus of the protein with three upstream As and an engineered Spe\_I restriction site on the 5′ end, while primer PAT2-3′ (5′-GACTCGTGGATTATATTCCGGT) encoded the five C-terminal amino acids, the TAA stop codon and a Xho\_I site. The reaction (25 μl) contained 100 pmol each primer, 20 nM each dNTP, ~250 ng Dictyostelium genomic DNA, 1 × Taq Extender buffer, 0.5 U Pfu DNA polymerase (Strata\_gene) and 1 U Tsg DNA polymerase (Sangon). Amplification was performed in a Perkin-Elmer Cetus thermal cycler using a protocol with a reduced extension temperature (Su et al., 1996). After denaturation at 94 °C for 5 min, the enzymes were added at 80 °C (3 min) followed by 30 cycles of 94 °C (20 s), 50 °C (30 s), 60 °C (3 min), and an additional 5 min at 60 °C. The single ~3.2 kb PCR product was cloned into pcRII vector using a TA Cloning kit (Invitrogen), excised by digestion with Spe\_I and Xho\_I and subcloned into the Spe\_I-Xho\_I site of the Dictyostelium expression vector EXP4 (+) (Dynes et al., 1994). Plasmid was introduced into the amoebae by electroporation (Dynes & Firtel, 1989) and transformants were selected by growth in HL-5 medium (pH 6.8) containing 10 μg G418 ml⁻¹. To select cells producing high levels of PAT2, HC392 transformants growing well at pH 6.8 in the presence of 30 μg G418 ml⁻¹ were subcultured sequentially to antibiotic-containing media buffered at pH 6.0, 5.5, and finally 5.0.

**Disruption of patB.** To construct a disruption vector, a 2.3 kb fragment of patB cDNA (nucleotides 843-3071 of the coding sequence) was cloned into the EcoRI site of pGEM-7Zf(+) (Promega). This plasmid was digested with Hind\_III, blunted-ended with Klenow DNA polymerase to destroy the Hind\_III site in the multiple cloning sequence, and religated. Next, the plasmid was linearized at the unique EcoRV site in the cDNA sequence, Hind\_III linkers were added, and the selectable tbyA gene from pGEM\_M25 (Dynes & Firtel, 1989), excised with Hind\_III, was cloned into the cDNA (see Fig. 3a). The plasmid was linearized by digestion in the multiple cloning sequence with Xho\_I, and transformed into strain JH10 by electroporation. Transformants were selected for the ability to grow in HL-5 medium (pH 6.8) in the absence of thymidine.

**Biochemical and physiological experiments.** Cell lysates, washed crude membranes and plasma membranes were prepared as described by Pogge-von Stram\_mann et al. (1984), except that the cells were broken by forced passage through polycarbonate filters (pore size, 3.0 μm). Total, azide- and vanadate-sensitive ATPase activities were determined as described by Pogge-von Stram\_mann et al. (1984).

To compare the growth properties of patB+ and patB- strains, cells growing exponentially in normal HL-5 medium (pH 6.8) were diluted to ~1–2 × 10^5 cells ml⁻¹ in freshly prepared HL-5 medium (with or without 10 mM potassium acetate) at various pHs. The cultures were shaken (~200 r.p.m.) at room temperature on a New Brunswick gyratory shaker and cell numbers were determined by counting with a haemocytometer at the times indicated. Cell viability was determined by diluting and plating duplicate samples of culture in SS and plating appropriate numbers of cells with K. pneumoniae on SM/5 agar plates. The plates were incubated at 22 °C and clones were counted after 3–6 d.

The ability of patB+ and patB- cells to develop at different pHs was determined as described by Xie et al. (1996).

Intracellular pH was measured by the null-point method (Rink et al., 1982; Aerts et al., 1985) using 0.1% digitonin (Sigma) to permeabilize the plasma membrane. Spontaneous and cAMP-induced extracellular pH changes in suspensions of patB+ and patB- cells were monitored as described previously (Malchow et al., 1978a, b), except the amoebae were starved by shaking for 3 h in 17 mM Na\textsubscript{2}HPO\textsubscript{4}/KHPO\textsubscript{4} at pH 6.8 rather than at pH 6.0. All pH measurements were made with an Accumet pH meter model 825MP equipped with an Accumet combination microprobe electrode and recorded using a Kipp & Zonen model BD41 chart recorder.

**RESULTS**

**Cloning and analysis of the patB gene.**

In an earlier study, we used the PCR and degenerate primers to amplify a family of fragments from D. discoideum genomic DNA with sequences related to P-type ion-transport ATPases of other organisms. One 0.95 kb PCR product was shown subsequently to be derived from the gene patA which encodes a Ca\textsuperscript{2+}-ATPase pump (PAT1) associated with the contractile vacuole of Dictyostelium (Moniak\_ is et al., 1995). Partial sequencing of another product (~0.8 kb) revealed a sequence more closely related to H\textsuperscript{+}-ATPases. This PCR product was used as a probe to isolate cDNAs and a genomic clone encoding an ORF of 3174bp termed patB. The proposed ATG start codon of patB is in the sequence CAAAATGG which agrees well with the consensus translation initiation sequence of D. discoideum. In addition, this sequence is preceded by 21 As and an in-frame TAA stop codon. The ORF also terminates with a TAA, the preferred stop codon for Dictyostelium genes. Analysis of the 5′ genomic clone revealed a single intron between nucleotides 675 and 676 of the ORF with consensus Dictyostelium 5′ (GTA-AAT) and 3′ (AG/) splice sites (Grant et al., 1990). Due to the extremely A+T-rich nature of the intron it was not sequenced completely, but it was estimated by partial sequencing and by PCR analysis to be ~100 bp
Amino acid sequences of various P-type pumps were aligned with the deduced sequence of PAT2 using the CLUSTAL program. Percentage amino acid sequence identity was calculated for each pair. SWISS-PROT accession numbers for the pumps are:

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in length. The patB gene has been mapped to the bottom of chromosome IV of *D. discoideum*, close to *gpaE* (W. F. Loomis, unpublished results).

The predicted protein product of *patB* (PAT2) is composed of 1058 amino acids with a calculated molecular mass of 117460 Da. As expected, the sequence contains the very highly conserved phosphorylation (CSDKTGT) and ATP-binding (GDGXNDXP) motifs characteristic of all P-type ATPases (Lutsenko & Kaplan, 1995). Further analysis revealed that PAT2 exhibits the greatest amino acid sequence identity with plasma membrane H⁺-ATPases of plants and fungi and considerably lower identity with other monovalent cation pumps and with Ca²⁺ pumps (Table 1). When aligned with PAT2, the various plant and fungal plasma membrane H⁺-ATPase isoforms showed an overall amino acid sequence identity of approximately 40 and 32%, respectively. In addition, PAT2 possessed 18/19 conserved sequences identified in other P-type H⁺-ATPases (Wach et al., 1992); the only region not highly conserved was sequence 'm' just C-terminal of the phosphorylation site. Although the PAT2 sequence is most homologous to plant plasma membrane H⁺-ATPases, it resembles more closely the fungal enzymes in having a greatly extended N-terminal domain. In PAT2, this region is even longer (by ~100 amino acids) than the N-terminal domains of the different fungal PMA1 enzymes, and, in this respect, PAT2 is more like the PMA2 isoforms of these organisms (Wach et al., 1992). Prosite analysis of the PAT2 sequence also identified three possible N-linked glycosylation sites in the N-terminal half of the protein.

### Expression of patB

Northern blot analysis of total RNA (20 μg per lane) isolated from *Dictyostelium* cells during vegetative growth and at 3 h intervals during development produced only a faint smear in the vicinity the 27S rRNA. When poly(A)+ RNA was examined, a single mRNA of ~4000 nt was detected and the level of this transcript was approximately the same in growing and developing cells (data not shown). The abundance of this message was comparable in cells grown at pH 6.8 or 7.5, but it was elevated ~fourfold when the cells were grown at pH 5.0 (Fig. 1a). Unlike patA expression which is up-regulated dramatically when the cells are grown in millimolar Ca²⁺ (Moniakis et al., 1995; J. Moniakis & M. B. Coukell, unpublished results), expression of *patB* was unaffected by the presence of this ion (data not shown). To monitor RNA loading in these experiments, the membranes were stripped and reprobed with *vatP* cDNA; expression of the ~900 nt *vatP* mRNA is not influenced appreciably by growth at different pHs or in the presence of Ca²⁺ (Xie et al., 1996; Moniakis et al., 1995).

Western blot analysis of total protein from AX2 cells grown in normal HL-5 medium using PAT2 antiserum revealed only a few very weak bands (Fig. 1b, lane 2). When the cells were shifted to pH 5.0 medium for 6 h, however, the ~120 and ~100 kDa bands were up-regulated substantially (Fig. 1b, lane 3). A similar pattern of up-regulation at pH 5.0 was observed with the *patB* strain Hc390 (Fig. 1b, lanes 4 and 5) but not with *patB*-disrupted cells (Fig. 1b, lanes 6 and 7) (see later). Thus, the major 120 kDa band is probably PAT2 since it agrees in size with the predicted size of the *patB* protein. The smaller 100 kDa band could be a degradation product of PAT2 or another acid-regulated protein. At present, we favour the latter possibility because (1) the intensity of this band was unchanged when the cells were lysed in the presence of a cocktail of 10 protease inhibitors, and (2) in several experiments (although not the one shown in Fig. 1b) a 100 kDa band was clearly visible on Western blots of *patB* cells incubated at pH 5.0. To examine the kinetics of PAT2 regulation by external pH, a double shift experiment was performed (Fig. 1c). When growing AX2 cells were shifted from pH 6.8 to 5.0, PAT2 was up-regulated within 1 h and it reached a maximum level by 4 h. Following a shift back to pH 6.8 medium, the level of PAT2 was reduced substantially by 5 h and it was virtually undetectable by 18 h. Therefore, *Dictyostelium* cells regulate *patB* expression rapidly in response to changes in ambient pH.
**Fig. 1.** Expression of the *patB* gene. (a) Northern blot analysis of poly(A)+ RNA isolated from AX2 cells grown in HL-5 medium buffered at pH 5.0, 6.8 or 7.5. (Top) RNA was size-fractionated on an agarose gel, transferred to nylon membrane, and probed with a 5' 0.42 kb *patB* cDNA fragment (see Fig. 2a). (Bottom) The membrane was then stripped and reprobed with a full-length *vatP* cDNA. (b) Western blot analysis. Strains AX2, HC390 (patB<sup>+</sup>) and HC392 (patB<sup>-</sup>) were grown to ~ 5 x 10<sup>6</sup> cells ml<sup>-1</sup> in HL-5 medium at pH 6.8, and samples were collected (lanes 2, 4 and 6). The remaining cells were pelleted by centrifugation, resuspended in HL-5 at pH 5.0, shaken for an additional 6 h, and sampled (lanes 3, 5 and 7). Strain HC392/patB<sup>+</sup> (lane 8) was grown at pH 5.0. Twenty micrograms of total protein from each sample was probed with PAT2 antiserum as described in Methods. (c) Kinetics of PAT2 regulation by extracellular pH. AX2 cells were grown to mid-exponential phase in normal HL-5 (pH 6.8), pelleted by centrifugation, resuspended in HL-5 at pH 5.0, shaken and sampled at the times indicated. After 6 h, the remaining cells were pelleted, resuspended in HL-5 at pH 6.8, shaken and sampled as indicated. The relative level of PAT2 in each sample was determined by Western blot analysis. In (b) and (c), the position of the 120 kDa band is denoted by an arrowhead. Results shown in each part (a–c) were confirmed in two or more additional experiments.

**Fig. 2.** Disruption of the *patB* gene. (a) Targeting strategy showing relevant features of the disruption vector, the wild-type *patB* gene and the disrupted *patB* gene. Coding regions of *patB* are depicted as open boxes while the 3.8 kb genomic DNA fragment carrying the *thyA* gene is shown as a black box. The solid bar represents the 420 bp cDNA fragment (base pair 392 of the coding sequence to the internal EcoRI site) used to probe the Southern and Northern blots. Restriction enzyme abbreviations: E, EcoRI; H, HindIII; K, KpnI; R, EcoRV. Southern blot of genomic DNA (b) and Northern blot (c, top) of poly(A)+ RNA; (c, bottom) the membranes were then stripped and reprobed with the *vatP* probe. Lanes: 1, parental strain JH10; 2, transformant HC390; 3, HC391; 4, HC392. Results in (b) and (c) were confirmed in two or more additional experiments.
Disruption of \( \text{patB} \)

To understand the role of \( \text{PAT2} \) in \( \text{Dictyostelium} \) growth and development, the endogenous \( \text{patB} \) gene was disrupted by insertion of the selectable \( \text{thyA} \) gene using the strategy illustrated in Fig. 2(a). To identify \( \text{patB} \)-disrupted strains, genomic DNA isolated from the parental \( \text{thyA} \) strain, JH10, and from purified \( \text{thyA}^+ \) transformants was digested with HindIII and analysed on Southern blots using a 5' \( \text{patB} \) cDNA probe. As shown in Fig. 2(b), DNA from strain JH10 and from a majority of the transformants (e.g. strain HC390) gave a single band of \( \sim 12 \) kb while about one-third of the transformants (e.g. strains HC391 and HC392) produced a smaller band of \( \sim 6.5 \) kb. Detection of only one band in each strain suggests that \( \text{patB} \) normally exists as a single-copy gene. Northern blot analysis of these transformants (Fig. 2c) revealed that \( \text{patB} \) mRNA is present in strain HC390 but absent in strains HC391 and HC392; this result is consistent with the presence of a disrupted \( \text{patB} \) gene in the latter two transformants. Also, acid pH shift experiments showed that the 120 kDa \( \text{PAT2} \) protein is up-regulated in strain HC390 but not in strain HC392 (Fig. 1b, lanes 4–7).

Localization of \( \text{PAT2} \)

To determine the cellular distribution of \( \text{PAT2} \), \( \text{patB}^+ \) and \( \text{patB} \) cells grown at different pHs were examined by indirect immunofluorescence microscopy using \( \text{PAT2} \) antiserum. HC390 (\( \text{patB}^+ \)) cells grown at pH 6.8 showed a reticular pattern of cytoplasmic staining and weak staining of the plasma membrane. The unstained internal region likely corresponds to the contractile vacuole since the cells were permitted to swell in hypotonic buffer (Fig. 3a). The same cells grown at pH 5.0 exhibited a comparable level of cytoplasmic staining but greatly enhanced staining of the plasma membrane (Fig. 3b). In contrast, the plasma membranes of HC392 (\( \text{patB} \)) cells at pH 6.8 were unstained (Fig. 3c); however, these membranes became intensely stained when \( \text{PAT2} \) was overexpressed from a plasmid (Fig. 3d). These results suggest that \( \text{PAT2} \) is associated with the plasma membrane while the internal staining, observed in all of the cells, probably represents cross-reaction of the \( \text{PAT2} \) antiserum with other proteins (see Fig. 1b, c).

Properties of \( \text{patB-null} \) cells

\( \text{patB} \) cells grew as well as \( \text{patB}^+ \) cells in HL-5 medium at pH 6.8 (Fig. 4a) or at higher pHs (not shown), but they grew more slowly than \( \text{patB}^+ \) strains at pH 6.0 (Fig. 4b) and they were unable to grow at pH 5.0 (Fig. 4c). Moreover, when the \( \text{patB} \) cells were shaken in HL-5 medium at pH 5.0, they decreased in size, became granular in appearance, and slowly died (Fig. 4d). When the HL-5 medium was supplemented with 10 mM potassium acetate, the growth rates of \( \text{patB}^+ \) and \( \text{patB} \) cells at pH 6.8 were unaffected (Fig. 4a), but at pH 6.0 the \( \text{patB}^+ \) cells grew more slowly and the \( \text{patB} \) cells failed to grow (Fig. 4b). At pH 5.0, the presence of 10 mM potassium acetate also completely inhibited the growth of the \( \text{patB}^+ \) cells (Fig. 4c). In both cases, cells inhibited by the presence of acetate ions showed the morphological changes and loss of viability characteristic of \( \text{patB} \) cells in normal HL-5 at pH 5.0. Growth of \( \text{patB} \) cells at pH 5.0 could be completely restored by
Fig. 4. Growth and viability of strains HC390 (patB+) and HC392 (patB) in axenic medium at different pHs. Late-
exponential-phase cultures of strains HC390 (●, ■) and HC392 (○, □) were diluted to 1-2 × 10^5 cells ml^-1 in HL-5
medium buffered at (a) pH 6.8, (b) pH 6.0 or (c) pH 5.0. Certain cultures (■, □) were supplemented with potassium
acetate to give a final concentration of 10 mM. In (c), HC392/patB+ cells overexpressing PAT2 are indicated by (△).
The cultures were shaken at 22 °C and counted at the times indicated. (d) HC390 and HC392 cultures were diluted in HL-5
medium at pH 5.0 to ~1 × 10^5 cells ml^-1, and shaken at 22 °C. At the times indicated, duplicate samples were removed,
diluted in 5S, and plated in duplicate with K. pneumoniae on SM/5 agar to determine numbers of viable cells. Values
given are means of the four plates. Results are representative of three to seven experiments at each pH (a-c) or three
experiments (d).

overexpressing PAT2 from a plasmid (Fig. 1b, lane 8; 4c).

During exponential growth in normal HL-5 medium at
pH 6.8, patB+ and patB cells had approximately the
same mean diameters. But, at stationary phase, the patB
cells increased in size and exhibited a mean diameter
~25% greater than the patB+ cells [e.g. HC390 (patB+)
mean diameter = 14.8 μm, n = 40; HC392 (patB) mean
diameter = 18.5 μm, n = 80].

patB cells failed to grow when plated with K. pneumoniae
on normal SM agar. This inability to grow on
cultured bacteria was probably caused by the slightly
acidic conditions (pH ~ 6) and/or secreted metabolites
in the rapidly growing bacterial lawns rather than to a
defect in phagocytosis because the same cells grew well on *K. pneumoniae* when the bacteria were washed and resuspended in 17 mM phosphate buffer, pH 6.8. Also, the growth rate and plating efficiency of *patB* cells was comparable to *patB*+ cells when they were plated with bacteria on SM/5 agar or on modified SM/5 agar in which only the glucose concentration was reduced fivefold. In contrast to the *patB*-null cells, *patB* cells overexpressing PAT2 grew and developed normally when plated with *K. pneumoniae* on SM agar (not shown).

When *patB*+ and *patB* cells were plated on non-nutrient agar buffered at pH 6.8 or higher, both types of cells proceeded through development at the same rate and formed relatively large fruiting bodies which were virtually identical in appearance (Fig. 5). In contrast, when the same cell populations were plated at pH 6.0, the *patB* cells developed more slowly than the *patB*+ cells and they formed small, spindly fruiting bodies. At pH 5.0, the *patB* cells fail to aggregate even when incubated up to 48 h.

Since analysis of the *patB* gene and the growth and developmental properties of *patB*-null strains at various pHs suggested that this gene encodes a P-type H+-ATPase, we assayed total cell lysates, crude membranes and purified plasma membranes of *patB*+ and *patB* cells grown under different conditions for vanadate-sensitive ATPase activity. Like Pogge-von Strandmann *et al.* (1984), we found that *patB*+ cells grown at pH 5.0 had two- to threefold higher plasma membrane vanadate-sensitive ATPase activity than cells grown at pH 6.8. But we could not detect reproducible differences in the ATPase activities of *patB*+ and *patB* cells grown at pH 6.8. If, however, cells of the two strains were grown at pH 6.8, and then shaken for 4 h in HL-5 medium at pH 5.0, the vanadate-sensitive ATPase activity associated with crude membranes of the *patB*+ cells increased almost twofold while the corresponding activity of the *patB* cells showed little change (Fig. 6). These results suggest that the higher vanadate-sensitive ATPase activity of pH 5.0-grown *patB*+ cells is due, at least in part, to the up-regulation of PAT2.

### Involvement of PAT2 in H+ fluxes

To determine if PAT2 plays a major role in regulating cytoplasmic pH in *D. discoideum*, we measured the pH of HC390 (*patB*+) and HC392 (*patB*) cells exposed to various extracellular pHs. In preliminary experiments,
Vanadate-sensitive ATPase activity in HC390 (patB+) and HC392 (patB) cells incubated in neutral and acidic medium. Cells of the two strains were grown to mid-exponential phase in normal HL-5 medium (pH 6.8), pelleted by centrifugation, resuspended to ~5 x 10^6 cells ml\(^{-1}\) in HL-5 at pH 6.8 and 5.0, and shaken at room temperature for 4 h. Washed crude membranes of each cell suspension were prepared according to the method of Pogge-von Strandmann et al. (1984) and assayed in duplicate for vanadate-sensitive ATPase activity and protein. Results are means ± SD of values obtained in three experiments.

Wild-type and disrupted cells were shaken at a concentration of 2 x 10^7 cells ml\(^{-1}\) for ~1 h in phosphate buffer at pH 5.0, 6.0, 6.8 or 7.8, and then the pH\(_{i}\) of the cells was determined by the null-point technique (see Methods). In all cases, the pH\(_{i}\) of the cells was 7.2-7.4, which is comparable to values reported previously for vegetative AX2 cells (Aerts et al., 1985; Klein et al., 1989). To apply greater acid stress to the cells, they were shaken for ~16 h in HL-5 medium at pH 6.8 (control) and at pH 5.5 prior to pH\(_{i}\) measurements (Aerts et al., 1987). At pH 5.5, patB cells grow poorly but remain reasonably viable (data not shown). After this treatment, the following pH\(_{i}\) values were measured: patB+ (6.8) = 7.31 ± 0.05 (sd) (four experiments); patB (6.8) = 7.23 ± 0.04 (3); patB+ (5.5) = 7.33 ± 0.03 (3); patB (5.5) = 7.1 ± 0.09 (3). Thus, even after prolonged exposure to a pH of 5.5, the pH\(_{i}\) of the patB cells was only ~0.2 pH units lower than that of the patB+ cells.

To determine if patB-null cells are able to regulate the pH of their external environment, patB+ and patB cells were analysed as described by Malchow et al. (1978a, b). When HC390 (patB+) cells were starved for 3-4 h and then aerated in an unbuffered salt solution, the extracellular pH began to oscillate with a period and an amplitude comparable to those reported earlier for AX2 cells (Fig. 7a). Also, as described previously, prior to the onset of spontaneous pH oscillations and after they had ceased, addition of cAMP (1 nM or higher) caused a slow, transient decrease in the external pH which peaked after ~3 min (Fig. 7b). In contrast, in four experiments with patB cells (three with strain HC392 and one with strain HC391), spontaneous pH oscillations were never observed (Fig. 7c). However, when these cell populations were treated with cAMP, they responded by transiently decreasing the pH of the external medium with kinetics very similar to those of patB+ cells (Fig. 7d). In all four experiments, repeated stimulation of the patB cells with cAMP over a period of 1-2 h failed to initiate spontaneous pH oscillations.

Cultured plant cells respond to osmotic stress by activating a plasma membrane H+-ATPase-mediated H+-efflux which results in the uptake of more K+ (Curti et al., 1993). To assess the ability of patB-null cells to osmoregulate, the growth rates of strains HC390 (patB+) and HC392 (patB) were measured in HL-5 medium (pH 6.8) in the presence and the absence of 125 mM sorbitol.

### Fig. 6
Vanadate-sensitive ATPase activity in HC390 (patB+) and HC392 (patB) cells incubated in neutral and acidic medium. Cells of the two strains were grown to mid-exponential phase in normal HL-5 medium (pH 6.8), pelleted by centrifugation, resuspended to ~5 x 10^6 cells ml\(^{-1}\) in HL-5 at pH 6.8 and 5.0, and shaken at room temperature for 4 h. Washed crude membranes of each cell suspension were prepared according to the method of Pogge-von Strandmann et al. (1984) and assayed in duplicate for vanadate-sensitive ATPase activity and protein. Results are means ± SD of values obtained in three experiments.

### Fig. 7
Extracellular pH changes in suspensions of HC390 (patB+) and HC392 (patB) cells. Three-hour-starved cells of each strain were washed in S5, resuspended to 2 x 10^7 cells ml\(^{-1}\) in the same solution and bubbled with water-saturated air at 23 °C. (a) Spontaneous pH oscillations in a HC390 cell suspension; (b) cAMP-induced pH decrease in a HC390 cell suspension after the spontaneous oscillations had ceased (~ 3 h aeration); (c) absence of spontaneous pH oscillations in a HC392 cell suspension, and (d) cAMP-induced pH decrease in a HC392 cell suspension after ~3.5 h aeration. Results are representative of two experiments with HC390 and three experiments with HC392. In another experiment, HC391 (patB) cells gave results similar to the HC392 cells.
Also, the cells were shaken in 17 mM phosphate buffer (pH 6·1) containing 400 mM sorbitol for 60 min, diluted and plated clonally with \textit{K. pneumoniae} on SM/5 agar as described by Schuster \textit{et al.} (1996). No appreciable differences were observed in the growth rates or in the cell viabilities of the two strains in these experiments (data not shown).

**DISCUSSION**

In this paper, we describe the cloning and the characterization of the \textit{patB} gene of \textit{D. discoideum}. A variety of results suggest that \textit{patB} encodes a P-type plasma membrane H\textsuperscript{+}-ATPase in this organism. First, a search of DNA and protein databases revealed that \textit{patB} (and the \textit{patB} protein, \textit{PAT2}) show the highest overall sequence homology to P-type ion-transport ATPases, and more specifically, to H\textsuperscript{+}-ATPases (Table 1). In addition, the \textit{PAT2} primary structure contains perfectly conserved E1–E2 phosphorylation and ATP-binding motifs and most of the other conserved sequences characteristic of this class of H\textsuperscript{+} pumps (Wach \textit{et al.}, 1992). Interestingly, \textit{PAT2} appears to be more closely related to the H\textsuperscript{+}-ATPases of plants than to the fungal enzymes and it lacks the conserved 'RRKK' and 'DIDALI' sequences present in the N-terminal domains of most fungal H\textsuperscript{+}-ATPases (Schlesser \textit{et al.}, 1988; Wach \textit{et al.}, 1992). While this finding might seem surprising, in fact, many \textit{Dictyostelium} proteins exhibit greater amino acid sequence identity to proteins from higher organisms (animals and plants) than to their fungal homologues (Pitt \textit{et al.}, 1992; Moniakis \textit{et al.}, 1995; Xie \textit{et al.}, 1996). \textit{patB} expression is regulated rapidly and dramatically by changes in extracellular pH (Fig. 1b, c) and the \textit{patB} protein is associated primarily with the plasma membrane (Fig. 3). Third, disruption of \textit{patB} by homologous recombination results in cells which are unable to grow or develop at pH 5·0 (Figs 4c and 5), and the cells eventually die (Fig. 4d). The genetic defect in these \textit{patB}-null cells appears to be limited to the \textit{patB} gene because acid-tolerant growth can be restored to the cells by specifically overexpressing \textit{PAT2} from a plasmid (Fig. 1c, 4c). When \textit{patB} cells are cultured in less acidic medium (pH 6·0), the acid-sensitive phenotype of these cells is enhanced greatly by a decrease in pH of \textit{Dictyostelium} cells (Inouye, 1983). Like the \textit{patB}-disrupted cells, many mutants of \textit{Saccharomyces cerevisiae}, defective in plasma membrane H\textsuperscript{+}-ATPase activity, also fail to grow at low pH and in the presence of weak acids (McCusker \textit{et al.}, 1987). Finally, incubation at pH 5·0 rapidly elevates the vanadate-sensitive ATPase activity of \textit{patB}\textsuperscript{+} cells but not of \textit{patB} cells (Fig. 6).

Most fungi possess two plasma membrane H\textsuperscript{+}-ATPases: a major enzyme encoded by the \textit{PMA1} gene and a minor isoform encoded by \textit{PMA2} (reviewed by Wach \textit{et al.}, 1992). In \textit{S. cerevisiae}, for example, the \textit{PMA1} enzyme is expressed at relatively high levels making up a major fraction of the ATPase activity in the plasma membrane, and it is essential for haploid growth (Serrano \textit{et al.}, 1986). In contrast, \textit{PMA2} expression is virtually undetectable at either the RNA or the protein level and disruption of the gene has no effect on cell proliferation at any pH (Schlessner \textit{et al.}, 1988). In \textit{Dictyostelium}, the level of \textit{patB} expression under normal growth conditions is extremely low (Fig. 1) and disruption of \textit{patB} has no effect on growth or on development at neutral pH, although below \textit{pH} 5·5 the cells become inviable (Figs 4 and 5). Also, like fungal \textit{PMA2} isoforms, \textit{PAT2} has a very long N-terminal domain when compared to the \textit{PMA1} enzymes. The significance of this structural feature (if any) is unknown. Together, these observations suggest that \textit{PAT2} is probably a homologue of the fungal \textit{PMA2} enzymes. Moreover, they predict that \textit{Dictyostelium} should possess at least one additional plasma membrane H\textsuperscript{+}-ATPase. One possible candidate is the acid-regulated 100 kDa protein which appears to cross-react with the \textit{PAT2} antiserum (Fig. 1b, c); most \textit{PMA1}-encoded enzymes are approximately this size (Wach \textit{et al.}, 1992).

What is the function of \textit{patB} in \textit{Dictyostelium}? Under laboratory conditions, where the environmental pH is usually maintained near neutrality, \textit{patB} expression is extremely low and the \textit{patB}-null cells appear capable of growing, developing and osmoregulating normally. It was observed that \textit{patB} cells are unable to acidify their external medium spontaneously when aerated in unbuffered suspension near pH 7. But when these cells were stimulated with cAMP, they responded much like \textit{patB}\textsuperscript{+} cells (Fig. 7). Since the spontaneous extracellular pH oscillations are thought to be cAMP-mediated (Malchow \textit{et al.}, 1978a, b), this aberrant behaviour of the \textit{patB} cells suggests that these strains might be defective in cAMP signalling in suspension. This deficiency is probably minor, however, because the cells developed well on agar (Fig. 5). Thus, at neutral pH, \textit{PAT2} probably does not play a major role in cell regulation. In contrast, when the amoebae were exposed to moderately acidic conditions, wild-type \textit{patB} expression was up-regulated rapidly and the \textit{patB}-null cells failed to grow or develop. In addition, following prolonged incubation in acidic media, there was a small (< 0·2 units) but reproducible decrease in the pH\textsubscript{1} of the \textit{patB} cells. Although this decrease is small, pH changes of this magnitude (<0·3 units) have been associated with a number of metabolic and developmental transitions, including cell cycle changes, in a wide variety of eukaryotic micro-organisms and in specific cells of higher organisms. In general, mitosis and meiosis are inhibited by a decrease in pH\textsubscript{1} while the rate of DNA synthesis is enhanced and the cell cycle is shortened at higher pHs (reviewed by Busa & Nuccitelli, 1994). In \textit{Dictyostelium}, it has been reported that there is a cycle of pH\textsubscript{1} changes which correlates closely with the cell cycle; between interphase and S phase, the pH\textsubscript{1} increases 0·2–0·3 units. Interestingly, artificially increasing the pH\textsubscript{1} by ~ 0·1 unit stimulates the rates of DNA and protein synthesis severalfold while a comparable decrease in pH\textsubscript{1} inhibits both processes (Aerts \textit{et al.}, 1985). Although
the mechanism(s) underlying this phenomenon is unknown, these observations suggest that the small pH change detected in patB cells in acidic media might be sufficient to inhibit the growth and reduce the viability of these cells. Taken together, these results suggest that patB probably functions as an auxiliary H+-ATPase gene which can be up-regulated in response to a decrease in extracellular pH. In the controlled conditions of the laboratory, patB appears to be a nonessential gene, but in a natural environment, rich in acidic bacterial and fungal metabolites, this gene might play a crucial role in ensuring the survival of Dictyostelium cells.

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