Targeted gene disruption reveals a role for vacuolin B in the late endocytic pathway and exocytosis

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SUMMARY

Cells of Dictyostelium discoideum take up fluid by macropinocytosis. The contents of macropinosomes are acidified and digested by lysosomal enzymes. Thereafter, an endocytic marker progresses in an F-actin dependent mechanism from the acidic lysosomal phase to a neutral post-lysosomal phase. From the post-lysosomal compartment indigestible remnants are released by exocytosis. This compartment is characterised by two isoforms of vacuolin, A and B, which are encoded by different genes. Fusions of the vacuolin isoforms to the green fluorescent protein associate with the cytoplasmic side of post-lysosomal vacuoles in vivo. Vacuolin isoforms also localise to patches at the plasma membrane. Since vacuolins have no homologies to known proteins and do not contain domains of obvious function, we investigated their role by knocking out the genes separately. Although the sequences of vacuolins A and B are about 80% identical, only deletion of the vacuolin B gene results in a defect in the endocytic pathway; the vacuolin A knock-out appeared to be phenotypically normal. In vacuolin B− mutants endocytosis is normal, but the progression of fluid-phase marker from acidic to neutral pH is impaired. Furthermore, in the mutants post-lysosomal vacuoles are dramatically increased in size and accumulate endocytic marker, suggesting a role for vacuolin B in targeting the vacuole for exocytosis.

Key words: Dictyostelium, Endocytosis, Exocytosis, Lysosome, Cytoskeleton

INTRODUCTION

In mammalian cells, endocytic cargo which is targeted for degradation first reaches the early endosomes from where carrier vesicles transport the material to the late endosomes and packaging into lysosomes occurs. In most cells the lysosome is considered to be a dead end for endocytic trafficking (Kornfeld and Mellman, 1989), though recent evidence suggests that its contents can be released when intracellular calcium is artificially increased (Rodríguez et al., 1997). Cells of the immune system are capable of secreting lysosomal contents in a regulated fashion. This process is involved in MHC class II antigen presentation (Harding, 1995) and cellular defence by eosinophiles or cytotoxic T cells (Griffiths, 1996).

In contrast to mammalian cells, the endocytic pathway in protozoa serves primarily nutritional purposes. Protozoa which phagocytose bacteria continuously release the remnants of lysosomal digestion by exocytosis. In ciliates like Paramecium, the endocytic pathway is linked to an ordered movement of vacuoles from uptake at a specific site of the surface, the ciliated cytopharynx, to release at the cytoproct (Fok and Allen, 1988). The spatial and temporal order of the pathway can be visualised with antibodies that recognise antigens specific for early or late endosomes (Allen et al., 1995).

Dictyostelium cells are more similar to mammalian phagocytes which are able to take up particles at any site of the cell surface. The uptake is sensitive to drugs that disrupt the actin cytoskeleton (Axline and Reaven, 1974; Maniak et al., 1995). In both types of cells phagocytosis involves talin (Greenberg et al., 1990; Niewöhner et al., 1997) and recruitment of the cytoskeletal protein coronin to the phagocytic cup (Maniak et al., 1995; Grogan et al., 1997; M. Thelen, personal communication). Dictyostelium cells take up fluid by macropinocytosis, which is also a process depending on the cytoskeleton (Hacker et al., 1997). For less than a minute after uptake, endosomes containing particles or fluid are surrounded by a coat of F-actin and the associated protein coronin (Maniak et al., 1995; Hacker et al., 1997). When this coat has dissociated, the vesicle content is acidified and digested. The acidic phase of endocytic vesicles is followed in Dictyostelium by a post-lysosomal phase, in which the pH reaches a value of 6.5 after 30 to 40 minutes.

Proteins that associate with vesicles during their transit through the digestive pathway of Dictyostelium have been isolated by feeding colloidal iron as a fluid-phase marker for various times, followed by magnetic fractionation to isolate endocytic vesicles of a given stage. Vesicles in the acidic phase are characterised by subunits of the vacuolar proton-ATPase (V-H+ATPase), lysosomal enzymes, and members of the rab family (Temesvari et al., 1994; Nolta et al., 1994; Bush et al., 1994; Adessi et al., 1995).

Rab proteins regulate vesicular traffic and serve as markers...
for endocytic compartments. A *Dictyostelium* rab4 homologue, rabD, localises to lysosomes and the osmoregulatory contractile vacuole system (Bush et al., 1994). Cells expressing a dominant negative mutation of rabD show a delayed delivery of marker to the neutral, post-lysosomal compartment. Exocytosis is consequently delayed by about 10 minutes (Bush et al., 1996). Rab7 is found on lysosomes, post-lysosomes (Buczynski et al., 1997) and early endosomes (i.e. 3 minutes after uptake) (Adessi et al., 1995). In cells transformed with a dominant negative mutant of rab7, internalisation of marker is reduced and neutralisation of the post-lysosomal compartment is affected (Buczynski et al., 1997).

The neutral compartment has been defined operationally with fluid-phase markers and pH probes (Nolta et al., 1994; Padh et al., 1993; Aubry et al., 1993). It consists of a few vacuoles of 2-3 μm in diameter which sequentially acquire coronin and vacuolin (Rauchenberger et al., 1997). Here we show that two isoforms of vacuolin exist, which both localise to the post-lysosomes and the plasma membrane. Analysis of *Dictyostelium* strains where the genes have been knocked out separately indicates that the genes are non-redundant. Loss of vacuolin A was without effect, while elimination of vacuolin B led to defects in the endocytic pathway which were restricted to the post-lysosome. The similarity of the vacuolin B phenotype to the defects observed in a rab7 mutant suggests that the two proteins may interact.

**MATERIALS AND METHODS**

**Molecular biology techniques**

A λgt11 cDNA library from cells developed for four hours (obtained from P. Devreotes) was screened with a radiolabelled vacuolin A (Rauchenberger et al., 1997) cDNA probe under low stringency conditions (30% formamide). From the clones obtained, those encoding vacuolin A were eliminated by a second round of high stringency hybridisation (50% formamide). The inserts of 12 positive phages were amplified using Taq polymerase and λgt11-specific primers and identified by digestion with restriction enzymes. The 4 largest PCR products were cloned into plasmid pT7Blue with T vector primers and the entire sequences of 2 clones encoding vacuolin B were determined starting from vector primers and completed using primers specific for internal sequences.

GFP-vacuolin B was expressed under control of the actin 6 promoter in a pDNeoII based vector (Witke et al., 1987). In this vector, a PCR-amplified coding sequence of GFP (Prasher et al., 1992) was inserted into the SphI and SstI sites. The GFP stop codon was eliminated so that translation proceeded into the polylinker at the 3’ end of GFP. A fragment of the vacuolin B cDNA, starting at amino acid position 25 (E) and comprising the entire 3’ untranslated region, was inserted in frame into the single BamHI site of the polylinker. The resulting protein has the sequence: MDGEDVqacmpMSK...(GFP)...LYKvEAS...(vacB)...LEK (uppercase lettering indicates amino acids derived from polylinker sequences).

The vector for the disruption of vacuolin A was constructed by cutting out a fragment of the coding region (corresponding to amino acid residues 128 to 461) with *Hpa*I and *Msc*I and replacing it with the blasticidin resistance cassette (Adachi et al., 1994). From the resulting plasmid a fragment from 60 bp upstream of the vacuolin A ATG to 19 bp before the stop codon was then used for *Dictyostelium* transformation. In the targeting vector for vacuolin B an internal HindII fragment (encoding amino acid positions 111 to 360) was eliminated before inserting the blasticidin resistance cassette. The fragment from this vector used for *Dictyostelium* transformation comprised 53 bp of noncoding region at the 5’ end of vacuolin B and 69 bp of 3’ untranslated region.

To obtain gene disruptions, *D. discoideum* wild-type AX2 cells were transformed by electroporation (Howard et al., 1988) and selected for resistance to 10 μg/ml blasticidin. For the expression of the GFP fusion, transformation was performed according to the method of Nellen and Saur (1988) and a stable clone (13C13) was selected and grown axenically in the presence of 20 μg/ml G418.

Genomic DNA was isolated as described by Nellen et al. (1987) and RNA was purified according to the method of Noegel et al. (1986). For analysis of the developmental regulation of the mRNA, cells were washed in 17 mM phosphate buffer, pH 6.0, and plated on black filters as described (Maniak and Nellen, 1988).

**Determination of endocytosis, transit times, and vesicular pH**

The internalisation of fluid was measured as described previously (Maniak et al., 1995; Hacker et al., 1997), in cultures of equal cell number. The size of wild-type cells and vacuolin- mutants was indistinguishable. For measuring transit times and vesicular pH, 10 ml samples of 1×10^7 wild-type or vacuolin- mutant cells/ml were shaken in 25 ml Erlenmeyer flasks at 150 rpm. A mixture of 4 mg/ml TRITC-dextran and 0.4 mg/ml FITC-dextran (70 kDa, Sigma) in nutrient medium was added for 15 minutes. Thereafter, cells were washed once and resuspended in nutrient medium. Samples of 1 ml were withdrawn at 15 minute intervals, added to 100 μl of trypan blue solution to quench extracellular fluorescence and centrifuged for 3 minutes at 500 g. The cell pellet was resuspended in 1 ml of phosphate buffer and TRITC-fluorescence retained in the cells was measured at 544 nm for excitation and 574 nm for emission. FITC-fluorescence was determined at 480 nm and 520 nm, respectively. The ratio of FITC- to TRITC-dextran fluorescence was used to deduce vesicular pH from a standard curve.

**Monoclonal antibodies and immunofluorescence**

Endocytic vesicles from wild-type cells were prepared by magnetic fractionation (Maniak et al., 1995) and used to immunise Balb/c mice as described (Bomblies et al., 1990) using Alugel S as an adjuvant. Monoclonal antibody (mAb) 221-1-1 was directed against vacuolin (Rauchenberger et al., 1997). mAb 221-35-2 reacted with a fragment of 132 amino acid residues of the 70 kDa A-subunit of the V-H+ ATPase, encoded by a partial cDNA (GenBank/EMBL/DDBJ accession number L43966).

For immunofluorescence labelling, cells were diluted to 1×10^6 cells/ml of nutrient medium and allowed to adhere to glass coverslips for 30 minutes. The specimens were either fixed with picric acid/formaldehyde and post-fixed with 70% ethanol (Humbel and Biegelmair, 1992) as for Fig. 4, or fixed using –20°C methanol containing 1% formaldehyde (Clarke et al., 1987), as for Fig. 5. Specimens were incubated with rhodamine-phalloidin (Sigma), or hybridoma supernatant followed by TRITC-conjugated goat anti-mouse IgG (Jackson ImmunoLabs).

Except for Fig. 5, which was photographed using a Zeiss Axiosoph epifluorescence microscope, all images were taken as confocal sections by scanning one plane through the cells using a Zeiss LSM 410 inverted microscope. Optical equipment consisted of a 488 nm Argon-ion laser, a 543 nm HeNe laser and a Zeiss ×100 Plan-NEOFLUAR objective. The green and red contributions to the emission signal were separated using a bandpass filter of 510 to 525 nm and a longpass filter of 570 nm, respectively.

**Imaging of living cells**

GFP-vacuolin was imaged in living cells as described for immunolabelling. Then the cells were pulsed with 4 mg/ml TRITC-dextran for 10 minutes and chased with nutrient medium for 60
minutes. Alternatively, cells were exposed to 0.5 μM Neutral Red for 5 minutes.

For the observation of pH changes during endocytic traffic, cells were imaged in medium containing FITC- and TRITC-dextran as for the quantitative assay using the same confocal set-up as for immunofluorescence labelling. Marker concentration in the vacuoles was determined according to the grey-scale value of the TRITC-fluorescence image using the Photoshop software (Adobe).

RESULTS

Sequence and expression of vacuolin B

The monoclonal antibody 221-1-1 directed against vacuolin recognised a doublet of 65/67 kDa on western blots of phagosome proteins prepared by magnetic fractionation and has been used to isolate the cDNA encoding vacuolin A (Rauchenberger et al., 1997). Southern blots of genomic DNA probed with this cDNA under high and low stringency conditions (Fig. 1A,B) pointed to the presence of a related gene in the Dictyostelium genome. To identify this second gene, a λgt11 cDNA library was screened using the same hybridisation conditions as for Fig. 1B. A cDNA was isolated encoding vacuolin B, that was 79% identical to vacuolin A, and had the

![Fig. 1. Southern blot of vacuolin A and B genes. Genomic DNA from Dictyostelium AX2 cells was digested with restriction enzymes as indicated on top of each panel. The vacuolin A probe (A and B) used for hybridisation spans amino acid positions 422 to 578, while for C a probe encompassing amino acids 493 to 592 of vacuolin B was used. The bands detected by low stringency hybridisation (B) can all be assigned to originate either from vacuolin A (A) or vacuolin B (C). Size marker positions (kb) are shown on the left side.](image1)

![Fig. 2. Sequence of vacuolin B (lower) and alignment with the A isoform (upper). The amino acid residues as derived from the cDNAs are numbered on the left. Short gaps (-) were introduced to obtain maximal homology. Identical residues are shaded, and conservative exchanges are boxed. These sequence data are available from GenBank/EMBL/DDJB under accession numbers AF014049 for vacuolin A and AF014050 for vacuolin B.](image2)
same predicted molecular mass of 66.2 kDa (Fig. 2). A Southern blot hybridised with a vacuolin B cDNA probe (Fig. 1C) confirmed that this cDNA originated from the second gene detected in Fig. 1B and the pattern complementary to vacuolin A was obtained (Fig. 1B). No other cross-hybridisation was observed. Both vacuolin genes were expressed in growing cells. The amount of vacuolin A mRNA transiently increased in early starvation and remained detectable throughout development (Fig. 3A). In contrast, the mRNA of vacuolin B was most strongly expressed in vegetative cells with a sharp decline during early development (Fig. 3B), a pattern of expression that paralleled endocytic activity in Dictyostelium cells.

Vacuolin B localises to the post-lysosomal compartment

To localise vacuolin B, Dictyostelium wild-type cells were transfected with a fusion of the cDNA to the C-terminal end of GFP. By western blotting with a polyclonal antibody against GFP, a 92 kDa protein was detected in these transformants, which corresponded to the expected size of the fusion protein (data not shown). As seen in Fig. 4A, GFP-vacuolin B localised to discrete vacuoles. To identify the compartment labelled, cells were pulsed with the fluid-phase marker TRITC-dextran. This marker reached vacuoles decorated with the GFP-vacuolin B fusion protein after 60 minutes (Fig. 4B). The pH of their lumen was close to neutral, as evidenced by the exclusion of Neutral Red, which accumulates only in acidic compartments (Fig. 4D). Since vesicles are acidified by the V-H+ATPase, we stained fixed cells with mAb 221-35-2 (Fig. 4F) and obtained a pattern of vacuoles and tubules, which did not overlap with the compartment decorated with GFP-vacuolin B (Fig. 4E). To confirm that the 70 kDa A-subunit of the V-H+ATPase was present on endocytic vacuoles in addition to the osmoregulatory organelle of Dictyostelium, cells were fed with latex beads and stained with mAb 221-35-2 (Fig. 4G,H). The post-lysosomal neutral compartment was also characterised by a coat of filamentous actin as shown by phalloidin staining (Fig. 4J) around vacuoles identified by the GFP-vacuolin B fusion-protein (Fig. 4I). In summary, the vacuoles identified by GFP-vacuolin B shared all features with the post-lysosomal neutral compartment previously characterised by a fusion of vacuolin A to GFP (Rauchenberger et al., 1997). One remarkable difference was that GFP-vacuolin B also localised to the plasma membrane (Fig. 4A,C,I).
indicating that the post-lysosome was the compartment from which exocytosis occurred.

**Targeted disruption of vacuolin genes**

To analyse the role of vacuolin gene products in *Dictyostelium* we replaced a portion of the vacuolin A coding region with a cassette conferring blasticidin resistance to transformed cells. By the analysis of genomic DNA of 36 single clones four were found to lack the vacuolin A gene. One example where the band of the endogenous gene was shifted upwards in the mutant and represented the only band detected with a probe specific for the resistance cassette is shown in Fig. 5A. The vacuolin B gene was eliminated using a similar approach (Fig. 5B). Control hybridisations confirmed that the vacuolin A gene was not affected in the vacuolin B− mutant and vice versa (data not shown).

Immunofluorescence labelling with mAb 221-1-1 revealed staining of numerous vacuoles and patches within the plasma membrane in wild-type cells (Fig. 5D). In vacuolin A− mutants these structures were not labelled (Fig. 5F). In vacuolin B− mutants, the obvious phenotype was the presence of mostly one single large vacuole as seen by phase contrast microscopy (Fig. 5G). Staining with mAb 221-1-1 for the presence of vacuolin A indicated that this vacuole was the enlarged homologue of the post-lysosomal compartment of wild-type cells (Fig. 5H).

**Endocytic performance of vacuolin A− and B− mutants**

To study the progression of marker through endocytic compartments, cells were pulse-labelled with a mixture of FITC- and TRITC-dextran. From the differential pH-sensitivity of these dyes, information on the pH of the endocytic compartments was derived. In vacuolin A− mutants and wild-type cells, the average pH of endocytic vesicles returned from 5.7 to a value of 6.6 in about 45 minutes, while an increase to the value of 6.0 took two hours in the vacuolin B− mutant (Fig. 6A). To assess the duration of endocytic transit, the amount of TRITC-dextran retained in the cells was analysed. Again, vacuolin A− mutants were similar to wild-type cells, while marker release was delayed by more than one hour in the vacuolin B− mutant strain and exocytosis occurred at 60% of wild-type rate (Fig. 6B).

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**Fig. 5.** Targeted disruption of vacuolin genes and phenotype of mutant cells. (A,B) Southern blots of genomic DNA isolated from wild-type AX2 or vacuolin− mutants. The hybridisation probes used to identify the vacuolin genes (vacA, vacB) or the resistance cassette (bsr) are indicated underneath. Positions of a molecular size marker (in kb) are given on the right. (A) A 0.9 kb NdeI and HindIII restriction fragment from the vacuolin A gene of wild type (AX2) is shifted to 2.8 kb in the vacuolin A− mutant (A−). (B) The size of a 1.1 kb vacuolin B fragment originating from a HindIII and Scal digest is increased to 1.6 kb in the vacuolin B− mutant (B−). (C,E,G) Phase contrast photographs and (D,F,H) immunofluorescence images using mAb 221-1-1 on fixed cells of (C,D) AX2 wild type, (E,F) vacuolin A− mutant, and (G,H) vacuolin B− mutant. Confocal analysis of the staining in the vacuolin A− mutant (F) reveals that weakly labelled vacuoles were hidden in the background and suggested that vacuolin B was less abundant or inefficiently recognised by mAb 221-1-1. In less than 5% of the vacuolin A− mutants clear label of a vacuole could be seen, which must have originated from a massive accumulation of vacuolin B protein (data not shown). Bar, 10 μm.
Despite these differences in endocytic traffic, the growth of vacuolin B\(^{-}\) mutants in axenic medium, as determined by the generation time, was equivalent to the growth of wild-type cells and vacuolin A\(^{-}\) mutants (data not shown). We therefore measured uptake of fluid at 15 minute intervals. Wild-type cells and vacuolin A\(^{-}\) mutants reached steady state level after 75 minutes. Initial rates of uptake in vacuolin B\(^{-}\) mutants were similar to wild-type cells and vacuolin A\(^{-}\) mutants, but diverged at later timepoints (Fig. 6C). When fluid-phase uptake was measured over prolonged periods of time, the vacuolin B\(^{-}\) mutants approached steady state levels more than one hour later than vacuolin A\(^{-}\) mutants or wild-type cells (Fig. 6D).

Since the steady state level reflects the equilibrium of endocytosis and exocytosis, this observation was in good agreement with the delay of marker release observed in the vacuolin B\(^{-}\) mutant (Fig. 6B).

**Post-lysosomal function is impaired in vacuolin B\(^{-}\) mutants**

To visualise the pH changes that occur during endocytic traffic in individual cells, we used a confocal laser scanning microscope. Fig. 7A shows one confocal section through cells of wild type and vacuolin B\(^{-}\) mutants exposed to a mixture of FITC- and TRITC-dextran in nutrient medium. Loss of fluorescein signal indicates a pH decrease in the vesicles, while rhodamine served as an internal pH-independent standard. After 14 minutes, both strains contained acidic (red) vacuoles; 30 minutes later, a fraction of vacuoles in the wild type had become neutral (yellow). The low number of vacuoles of intermediate (orange) pH value indicated that this process is completed within a few minutes in the wild type. The post-lysosomal compartment in vacuolin B\(^{-}\) mutants was defined by the labelling with vacuolin antibody (Fig. 5H). It could be easily identified because of its increased size (Fig. 5G). In the vacuolin B\(^{-}\) mutant the pH increase started at 77 minutes (large orange vacuoles) and only after 154 minutes clearly reached wild-type values (yellow vacuoles). From this experiment it became clear that within the post-lysosomal vacuole the pH gradually increases from acidic to neutral values over more than one hour.

The giant size of the post-lysosomal compartment could indicate a reduced frequency of exocytosis. If this assumption was true, marker should become concentrated in the neutral compartment of the vacuolin B\(^{-}\) mutant to higher levels than in wild type. We therefore loaded cells overnight with a mixture of FITC- and TRITC-dextran, and took images as for Fig. 7A.
FITC-fluorescence was used to identify compartments of acidic or neutral pH and the intensity of the TRITC-fluorescence was measured as a pH-independent indicator of marker concentration in endocytic vacuoles (Fig. 7B). In wild-type cells, the average concentration of marker in acidic vacuoles corresponded to that of the extracellular medium, while the concentration in neutral vacuoles was 2.3-fold higher. In the vacuolin B− mutant, the giant post-lysosomes contained marker that was concentrated by at least a factor of 3.7 as compared to extracellular medium. Acidic vesicles did not differ between mutant and wild type indicating that the vacuolin B defect was strictly confined to the post-lysosomal vacuoles.

**F-actin integrity is required for lysosomal to post-lysosomal transport**

Because the post-lysosomal vacuole was a compartment coated with F-actin (see Fig. 4J), we analysed whether endocytic transport was also sensitive to treatment with cytochalasin A. When a pulse of marker was followed through the endocytic pathway, the pH-increase which accompanies progression from the lysosomal to the post-lysosomal phase was dependent on the dose of cytochalasin A (Fig. 8A). The increase of pH over time was the result of a transient cytochalasin effect as seen earlier (Maniak et al., 1995; Hacker et al., 1997). A concentration of 3 μM blocked marker release completely until it resumed after a delay of 45 minutes (Fig. 8B) when the average vacuolar pH reached a value above 6. Treatment with 10 μM of cytochalasin A resulted in a complete inhibition of pH increase and marker release over the time of the assay (Fig. 8A,B). The disruption of the F-actin cytoskeleton produced a defect similar to the vacuolin B mutation. However, cells treated repeatedly with 10 μM of cytochalasin A did not reveal an increased size of the GFP-vacuolin decorated compartment over 4 hours (data not shown), which is conceivable, because the drug entirely inhibits the uptake of fluid (Hacker et al., 1997).
In the endocytic pathway of Dictyostelium cells four phases can be distinguished: the uptake of particles and fluid, an acidic phase, initiated by the association of the V- H+ ATPase with the membrane. In the post-lysosome of vacuolin B-compartment (Buczynski et al., 1997). This compartment probably corresponds to the large vesicles in the vacuolin B-mutant which slowly progress from acidic to neutral pH (Fig. 7A). Since both proteins localise to the post-lysosome of wild-type cells (Buczynski et al., 1997, and Fig. 4), vacuolin B may be a target for rab7 in regulating late endocytic transport in Dictyostelium.

From the post-lysosome two trafficking steps exist. Exocytosis of marker is the final step in anterograde transport, while retrieval of membrane proteins occurs via retrograde vesicles. In order to retrieve a relatively large area of membrane together with only little volume, recycling vesicles are small and numerous. In the compartment from which they originate, the decrease in membrane area leads to an increased concentration of a non degradable, membrane impermeable marker substance. The concentration of endocytic marker in wild-type post-lysosomes is more than twofold increased as compared to lysosomes and the surrounding medium (Fig. 7B), indicating that retrograde traffic indeed exists. For rab7, Buczynski et al. (1997) favour a role in this retrograde transport, which is thought to retrieve lysosomal enzymes and the V- H+ ATPase.

One problem, however, is that neither rab7, nor vacuolin co-localise with vesicles containing the proton pump (Buczynski et al., 1997, and Fig. 4E,F). The strict localisation of vacuolin antibody and GFP-vacuolin to post-lysosomes and to patches of the plasma membrane (Figs 4, 5) rather supports the idea that the vacuolin-decorated vacuoles fuse with the membrane. In the post-lysosome of vacuolin B- mutants marker accumulates to even higher concentrations than in wild-type cells (Fig. 7B), indicating that retrieval of membrane from this compartment is operating while the discharge of its contents is impaired. Therefore, vacuolin is obviously altered in the progression through the developmental cycle (data not shown). The expression of vacuolin B is restricted to the vegetative phase (Fig. 3B), which is the phase of highest endocytic activity in the Dictyostelium life cycle (Takeuchi et al., 1983). A vacuolin B- mutant shows severe defects in the late endocytic pathway and exocytosis, indicating that the vacuolin isoforms are non-redundant.

An immediately apparent defect in the vacuolin B- mutant is a delay in the release of fluid-phase marker. Whereas in the wild-type strain exocytosis of marker starts after 30 minutes after its application, it starts in the vacuolin B- mutant at 90 minutes (Fig. 6B). During most of this period fluid-phase remains at low pH (Fig. 6A). When the vesicular pH begins to rise, exocytosis begins in the vacuolin B- mutants (Figs 6A, 7A). Since a similar effect is provoked in cells treated with cytochalasin (Fig. 8), the vesicular pH is assumed to control exocytosis in Dictyostelium. On the contrary, precocious release of endocytic marker is observed in mutants where endosomal pH remains close to neutral, due to an acidification defect (Aubry et al., 1993).

One protein, which regulates the balance of acidic and neutral phase in the endocytic pathway, is rab7 (Buczynski et al., 1997). A constitutively active form of rab7 shortens the acidic phase and causes premature exocytosis. A dominant negative mutant of rab7 prolongs the acidic phase and delays exocytosis (Buczynski et al., 1997). This phenotype resembles that of the vacuolin B- mutant (Fig. 6A, B). Dominant negative mutants of rab7 contain an enlarged acidic endocytic compartment (Buczynski et al., 1997). This compartment probably corresponds to the large vesicles in the vacuolin B- mutant which slowly progress from acidic to neutral pH (Fig. 7A). Since both proteins localise to the post-lysosome of wild-type cells (Buczynski et al., 1997, and Fig. 4), vacuolin B may be a target for rab7 in regulating late endocytic transport in Dictyostelium.
likely to play a role in anterograde traffic from the post-
lamellar, namely exocytosis.

Two discrete endocytic compartments, acidic and neutral,
cannot account for the kinetics of marker endocytosis, transit
and exocytosis experimentally observed in Dictyostelium.
From theoretical considerations, a model involving at least
nine endocytic compartments has been postulated (Aubry et
al., 1995). In principle, this requirement is fulfilled by
repeated cycling of marker through two compartments.
According to this idea, the pH of an endocytic vesicle would
fluctuate as a consequence of fusion with vesicles that deliver
the V-H\(^+\)ATPase and lysosomal enzymes or vesicles that bud
off to retrieve these proteins. In addition, pH changes may
occur upon homotypic fusion between endosomes of different
pH. We assume that any time, when the vesicle is neutralised,
vacuolin can associate with the vesicle membrane to prevent
further cycling and homotypic fusion. The duration of this
stable neutral phase could be determined by rab7 or one of
the other rab proteins, which are known to act as molecular
timers (Rybin et al., 1996). If during the neutral phase the
vesicle contacts the plasma membrane, exocytosis follows.
Otherwise vacuolin dissociates and the vesicle re-enters the
cycling phase and fuses with other endocytic vesicles. Cycles
of this kind would explain the common observation that
endocytic marker, which is taken up during a pulse of a few
minutes, is released from the cell over a period of several
hours (e.g. Fig. 6B).

The concept of endosomal cycling has also been used to
describe the relationship between late endosome and lysosome
in mammalian cells (Mellman, 1996). Fluctuations of
lysosomal pH, which may be associated with cycling, have
been observed (Butor et al., 1995). The progression of
endocytic marker from a late endosomal to a lysosomal stage
in mammalian cells or from acidic to neutral phase in
Dictyostelium depends on the F-actin cytoskeleton and can be
disrupted with cytochalasin (van Deurs et al., 1995; Durrbach
et al., 1996 and Fig. 8A), further emphasising the similarities
between the endocytic pathways.

However, in contrast to Dictyostelium, most mammalian
cells retain the contents of their lysosome. Cells of the
haematopoietic lineage including osteoclasts (Salo et al., 1997)
secrete their lysosomal contents (reviewed by Griffiths, 1996).
A mutation affecting the lysosomal system of the
haematopoietic lineage is the Chediak-Higashi syndrome (CHS)
in human patients which corresponds to the beige
mutation in mouse: mutant cytototoxic T lymphocytes contain
giant lysosomes which are unable to fuse with the plasma
membrane (Baetz et al., 1995). Though the defect of these cells
is strikingly similar to the phenotype of vacuolin B
mutants, the gene affected in CHS (Barbosa et al., 1996; Perou et al.,
1996) does not share obvious sequence characteristics with
vacuolin. It would be worthwhile to search for a vacuolin
homologue in haematopoietic cells and conversely, a CHS gene
in Dictyostelium cells to analyse on the molecular level how
closely the endocytic pathways are related.

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