Two members of the beige/CHS (BEACH) family are involved at different stages in the organization of the endocytic pathway in *Dictyostelium*

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Summary

Proteins of the Chediak-Higashi/Beige (BEACH) family have been implicated in the function of lysosomes, as well as in signal transduction, but their molecular role is still poorly understood. In *Dictyostelium*, at least six members of the family can be identified. Here cells with mutations in two of these genes, *LVSA* and *LVSB*, were analyzed. Interestingly both mutants exhibited defects in the organization of the endocytic pathway, albeit at distinct stages. In *lvsB* mutant cells, the regulated secretion of lysosomal enzymes was enhanced, a phenotype reminiscent of the Chediak-Higashi syndrome. *LvsA* mutant cells exhibited alterations in the organization and function of the early endocytic and phagocytic pathway. The LvsA protein may participate in the signaling pathway, which links adhesion of a particle to the subsequent formation of a phagocytic cup. Further genetic analysis will be necessary to determine whether other members of the BEACH family of proteins are also involved in controlling the organization of the endocytic pathway.

Key words: Beige, BEACH domain, Chediak-Higashi, phagocytosis, lysosome, Lvs, *Dictyostelium*

Introduction

The Chediak-Higashi syndrome (CHS) is a human autosomal disorder, which leads to albinism, coagulopathy and recurrent infections as well as other symptoms (Introne et al., 1999). At the cellular level, CHS is characterized by the presence of huge cytoplasmic granules containing lysosomal enzymes, which suggests that the organization of the late endocytic pathway is defective. In certain specialized cell types, these morphological alterations are also associated with defects in the secretion of lysosomal enzymes. Indeed release of lysosomal content into the extracellular medium is not observed in most cell types but is implicated in a number of specialized functions, such as the killing of virally infected cells by cytotoxic T cells (Page et al., 1998). In cytotoxic T cells originating from CHS patients the release of lysosomal constituents is defective, and this leads to a marked decrease in their ability to kill target cells (Baetz et al., 1995). Thus the protein responsible for CHS seems to be directly or indirectly involved in the intracellular transport steps from late endocytic compartments to the plasma membrane. Consequently it was named LYST for lysosomal trafficking regulator.

The gene affected in human CHS codes for a 3801 amino-acid protein, which has a predicted mass of 429 kDa (Nagle et al., 1996). The same gene is also affected in the related syndrome in mouse known as 'beige'. The LYST protein belongs to a large family of proteins characterized by the presence of a BEACH (beige and Chediak-Higashi) domain and several WD motifs at the C-terminus. At present, no function has been assigned to the BEACH domain. One other member of the BEACH family, the FAN protein, has been well characterized. It was found to associate with the tumor necrosis factor receptor and may therefore be involved in signal transduction and coupling to neutral sphingomyelinase (Adam-Klages et al., 1996). The organization of the C-terminal ends of LYST and FAN proteins is similar, with a BEACH domain followed by several WD repeats. At the N-terminus, however, both proteins diverge and FAN is significantly shorter (Fig. 1). Besides LYST and FAN, at least six other genes encoding proteins with a BEACH domain are found in the human genome. No information is available about their functions.

The amoeba *Dictyostelium discoideum* has been used extensively as a model organism to study the endocytic and phagocytic pathway (reviewed in Rupper and Cardelli, 2001). In these haploid cells, mutant cell lines defective for the function of a particular gene product can be obtained relatively easily. At least six proteins with BEACH domains have been identified so far in *Dictyostelium* sequence databases.

To obtain a more comprehensive view of the function of the BEACH family of proteins, two mutants were analyzed in *Dictyostelium*, each with a mutation in a gene encoding a member of the family. Both mutants exhibit defects in the organization and function of the endocytic pathway, albeit at distinct stages.

Materials and Methods

Cells and media

Wild-type DH1 and *lvsA* mutant cells (VIG9) used in this study were described previously (Cornillon et al., 2000; Kwak et al., 1999). Cells were grown at 21°C in HL5 medium (Cornillon et al., 1998) and subcultured twice a week. Cells were not allowed to reach a density of more than 10^6 cells/ml. Soerensen's buffer (SB; 2 mM Na_2HPO_4, 14.7 mM KH_2PO_4, pH 6.0) was used to induce starvation and the secretion of lysosomal enzymes.

The *lvsB* mutant cell was obtained essentially as described (Kuspa et al., 1995). The cDNA clone SLC545 (Morio et al., 1998) contains an insert of 1667 bp, from nucleotide 9240 of the *LVSB* gene to the stop codon (accession number AU034302). A blasticidin resistance cassette was inserted in a *ClaI* site situated 762bp 5′ (upstream) of the stop codon (i.e. within the coding sequence), and the resulting vector was used to transfect DH1 cells. In the case of *LVSA*, an insertion at an equivalent position was seen to result in a phenotype equivalent to that created by a deletion of the gene (Kwak et al., 1999). The insertion at the *LVSB* locus was confirmed by Southern blotting as described (Cornillon et al., 1998), using the insert contained in SLC545 as a probe. No defect in the growth or development of *lvsB* mutants was seen compared to wild-type cells. The entire coding sequence of *LVSB* is contained within contig c-JC2b375c04.r1. It can be obtained from the Genome Sequencing Centre Jena website at http://genome.imb-jena.de/dictyostelium/.

Cell transfection

Plasmid WF38 was a kind gift from P. Devreotes (Parent et al., 1998). This plasmid contains the sequence coding for the PH domain of CRAC fused to green fluorescent protein under the control of the actin promoter, for constitutive expression in *Dictyostelium* cells. To transfect cells, 8×10^6 cells were washed once in sterile ice-cold electroporation buffer (10 mM $NaPO_4$, pH 6.1, 50 mM sucrose), mixed with 20 μg of plasmid linearized with *NdeI* and electroporated using a Biorad Gene Pulser (0.4 cm cuvettes, 1 kV, 3 μF). Cells were then rapidly transferred to 30 ml of HL5 medium. G418 (Gibco, Basel, Switzerland) was added 24 hours later to a final concentration of 10 μg/ml. After 10 days of selection, cells were cloned by limiting dilution in 96 well plates.

Internalization assays

Internalization of fluid phase or particles was measured as described previously (Cornillon et al., 2000). Briefly, 10^5 cells were resuspended in 1 ml of HL5 medium containing 0.5 mg/ml of FITC-dextran (Molecular Probes, Eugene, Oregon) or 1 μl of 1 μm diameter fluoresbrite YG carboxylate microspheres (Polysciences Inc., Warrington, PA) or 5×10^7 TRITC-labeled bacteria (Cornillon et al., 2000). The cell suspension was incubated on a rotating shaker for the indicated time, then washed twice with ice-cold HL5. The internalized material was quantified using a Fluorescence activated cell sorter (FACSCalibur, Beckton-Dickinson, San Jose, CA).

To measure phagocytosis at very early time points, for each time point 2×10^5 cells were incubated in 450 μl of HL5 medium for 15 minutes before adding 0.5 μl of fluorescent microspheres in 50 μl of HL5. At each indicated time, 500 μl of a 4% solution of formaldehyde in HL5 was added to one tube to stop the uptake. Cells were then washed three times in PBS, and the internalized fluorescence quantified by FACS analysis as described above.

To measure the recycling of the internalized fluid phase, cells were first incubated with FITC-dextran in HL5 for 1 hour, then washed once with HL5 and resuspended in fresh HL5. An aliquot of cells was collected at each indicated time, washed twice with HL5, and the intracellular fluorescence was quantified by FACS analysis.

To observe the formation of macropinosomes, cells were grown on glass coverslips in HL5 medium and observed in phase contrast with a Zeiss Axiovert 100 microscope. Pictures were recorded every 10 seconds with a Hamamatsu Orca camera and analyzed with OpenLab 3 software.

Adhesion of cells to substrate

The cell detachment assay was adapted from Cozens-Roberts et al. (Cozens-Roberts et al., 1990). The technical details will be described in a separate publication (E. Décavé, D. Garrivier, Y. Bréchet et al.). Briefly, cells were spread evenly on a glass plate and allowed to settle for 15 minutes in the indicated buffer. A flat stainless steel disk pierced in its center was placed above. Medium was flowed at a constant rate for 7 minutes through the central orifice of the disk before removal of the disk and microscopic examination of the remaining cells. The radius at which 50% of the cells were detached was determined ($r_{50\%}$) and the stress at this distance to the center was $\sigma_{50\%}=3D\eta/\pi e^2 r_{50\%}$, where D is the flow rate, e the distance between the plate and the disk (0.21 mm for experiments in SB buffer, 0.56 in HL5 buffer) and η the fluid viscosity (10^{-3} Pa·s).

Secretion of lysosomal enzymes

Secretion of lysosomal enzymes was assessed essentially as described previously (Dimond et al., 1983). Briefly, to induce regulated secretion, cells were washed twice in ice-cold SB and resuspended in SB at 10^8 cells/ml. At time 0 the cells were diluted in 10 volumes of SB at 21°C and incubated with mild shaking. At each indicated time point, 1 ml of the cell suspension was recovered and centrifuged. Enzymatic activity was assessed in the supernatant and in the cell pellet. 0.1% TX100 was added to all samples to allow measurement of intracellular lysosomal enzyme activity. The cells were also counted at each indicated time, and their number did not vary during the experiment (data not shown), indicating that the release of lysosomal enzymes was not caused by cell lysis.

To examine the constitutive secretion of lysosomal enzymes, cells were grown in HL5 medium for three days at a final density of 2×10^6 cells/ml, and enzymatic activity was assessed in the supernatant and cell pellet.

To determine the enzymatic activity in each sample, 50 μl of sample was added to 50 μl of substrate mix (10 mM substrate in 5 mM NaOAc, pH 5.2) and incubated for approximately 1 hour at 37°C. The reaction was stopped by adding 100 μl of 1 M Na_2CO_3, and the optical density at 405 nm was determined in a microplate elisa reader.

Enzyme substrates (Sigma, St Louis, MO) were dissolved in DMF at a concentration of 250 mM and stored at –20°C. P-nitrophenyl phosphate, p-nitrophenyl N-acetyl β-D-glucosamide, and p-nitrophenyl α-D-mannopyranoside were used as substrates for acid phosphatase, N-acetylglucosaminidase and α-mannosidase, respectively.

Fluorescence microscopy

For immunofluorescence analysis, cells were grown on glass coverslips for three days, then fixed with picric acid and processed for immunofluorescence as described (Humbel and Biegelmann, 1992). This fixation protocol is optimal for the preservation of contractile vacuole structure. Briefly, cells were fixed for 30 minutes in picric acid solution (15% picric acid v/v, 2% paraformaldehyde w/v, 10 mM PIPES pH 6.5), rinsed twice with PBS, post-fixed for 10 minutes in 70% EtOH, rinsed twice with PBS, then incubated with the indicated antibodies. The antibodies used were a rabbit antiserum against the *Dictyostelium* Rhesus (Rh50) protein (Benghezal et al., 2001), a mouse monoclonal antibody against the vacuolar H^+-ATPase (221-35-2; (Neuhaus et al., 1998)) and H161, a mouse monoclonal against p80, a transmembrane protein present in the endocytic pathway (K. Ravanel, B. de Chassey, S.C. et al., unpublished). Cells were visualized with a Zeiss confocal microscope (LSM510).

To visualize phagocytic cups, 3×10^5 cells expressing the CRAC-GFP fusion protein were incubated with rhodamine-labeled yeast cells

for 1 hour in HL5 medium. They were then fixed in 4% paraformaldehyde, washed three times with PBS, and an aliquot of each sample was mounted for observation. Phagocytic cups were identified by the accumulation of CRAC-GFP fluorescence at the contact site between the *Dictyostelium* cell and a yeast particle and counted.

Electron microscopy

To analyze the morphology and composition of phagosomes, cells were incubated with *Klebsiella* bacteria for 1 hour. They were then fixed for 1 hour at room temperature in a solution of 2% paraformaldehyde and 0.2% glutaraldehyde. The fixative was rinsed three times with PBS and the cells processed for cryosectioning essentially as described (Liou et al., 1996). Briefly, the cell pellet was infiltrated with sucrose and frozen in liquid nitrogen. Frozen sections were cut with a Leica FCS cryotome, transferred to grids, and incubated with an antibody to vacuolar H^+-ATPase, then with a gold-coupled antibody to mouse immunoglobulins. Grids were examined in a Philips CM10 transmission electron microscope. Gold particles associated with the membrane of early phagosomes (the vast majority in these experiments) were counted. Several late phagosomes (spacious and containing several bacteria (K. Ravanel, B. de Chassey, S. Cornillon et al., unpublished) were also seen and they were not considered in this study.

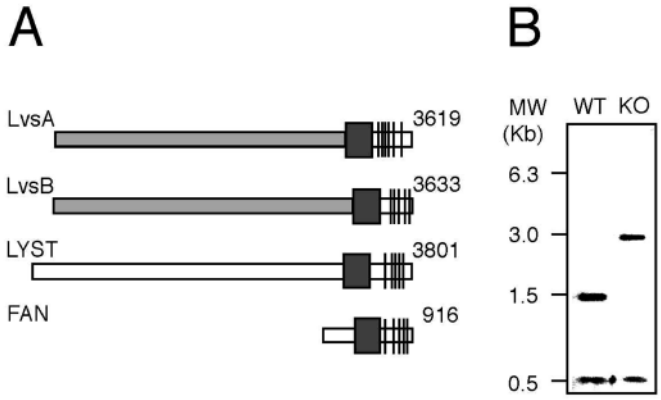

Fig. 1. The BEACH family of proteins. (A) A schematic drawing of four proteins of the BEACH family: *Dictyostelium* LvsA and LvsB and human LYST and FAN. The BEACH domains are indicated by dark gray boxes, the putative WD repeats by vertical black bars. The long N-terminal extension of LYST exhibits no homology with that of Lvs proteins. (B) Southern blot of the *lvsB* mutant. Genomic DNA was digested with *EcoRI*, migrated in an agarose gel, blotted and hybridized with an *LVSB* probe. The 1.5 Kb band in wild type was replaced by a 3 Kb band in the mutant clone owing to the integration of the bsr cassette (1.5 Kb).

Results

Regulated secretion of lysosomal enzymes is increased in *lvsB* mutant cells

In this study, two mutants were examined, each of which were disrupted in a gene encoding a member of the BEACH family of proteins. *LvsA* mutants were previously isolated in a screen for mutants defective in cytokinesis (Kwak et al., 1999). Mutant cells exhibit a strong cytokinesis defect in suspension, but divide and grow almost normally on the surface of a culture plate. The organization of the C-terminal segment of LvsA is similar to that of LYST, with a BEACH domain followed by several putative WD repeats. However the long N-terminal extension present in LvsA is extremely divergent from that of LYST (Fig. 1A).

The *LVSB* gene was identified by screening the *Dictyostelium* databases with the BEACH domain of LvsA. The *LVSB* gene encodes a protein of 3633 amino-acid residues and is weakly homologous to LvsA along its entire length (22% identity; 36% similarity over 3268 amino-acid residues). The structure of both proteins is very similar, with a BEACH domain at the C-terminus (58% identity; 75% homology) followed by several potential WD repeats (Fig. 1A). For the purpose of this study, *lvsB* mutant cells were obtained by targeted gene inactivation as previously described (Kuspa et al., 1995) (Fig. 1B).

At least four other proteins containing a C-terminal BEACH domain followed by several WD repeats can be identified in the *Dictyostelium* sequence database at present (N. Wang and A. De Lozanne, unpublished).

As the LYST protein has been implicated in the function of lysosomes in mammalian systems, mutant cells were first tested for alterations in the function of the lysosomal compartment. Three different lysosomal enzymes were examined: acid phosphatase, N-acetylglucosaminidase and α-mannosidase. When cells were grown in rich medium no significant secretion of lysosomal enzymes was observed for either wild-type, *lvsA* or *lvsB* mutant cells (data not shown). Secretion of lysosomal enzymes can be induced by starvation in wild-type *Dictyostelium* cells, and, as described previously, it proceeds with distinct efficiencies for different enzymes (Dimond et al., 1983) (Fig. 2). Although no significant difference was observed between wild-type and *lvsA* mutant cells in these conditions, secretion of all three lysosomal enzymes was markedly enhanced in *lvsB* mutant cells compared to wild-type cells (Fig. 2). Thus the physiology of the lysosomal compartment and the sorting of lysosomal enzymes are altered in *lvsB* mutant cells, a phenotype reminiscent of the Chediak-Higashi syndrome.

The organization of the early endocytic and phagocytic pathways is altered in *lvsA* but not *lvsB* mutant cells

To further characterize the phenotype of *lvsA* and *lvsB* mutant cells, intracellular sorting in the early endocytic compartments was examined. It must be emphasized that the morphology of the compartments considered here (endocytic compartments and contractile vacuole) is rather complex and somewhat similar, as they can both appear to be vacuolar. Thus, for immunofluorescence analysis we exclusively relied on colocalization experiments using three different membrane markers. In wild-type *Dictyostelium* cells, the contractile vacuole constitutes a network of ducts and cisternae, which are particularly apparent in a plane close to the substrate surface (Gabriel et al., 1999). It also extends around the entire cell body and towards the top of the cell, and it appears as a flat compartment apposed to the cell membrane, which is often more developed on one side of the cell (Gabriel et al., 1999). This was also observed when wild-type cells were labeled with an antibody to a marker of the contractile vacuole, the Rhesus (Rh50) protein (Benghezal et al., 2001) (Fig. 3A). The vacuolar H^+-ATPase is present in the early endocytic compartments and absent from later endocytic or phagocytic compartments. The bulk of this protein, however, is localized in the contractile vacuole (Neuhaus et al., 1998). This can be seen easily in wild-

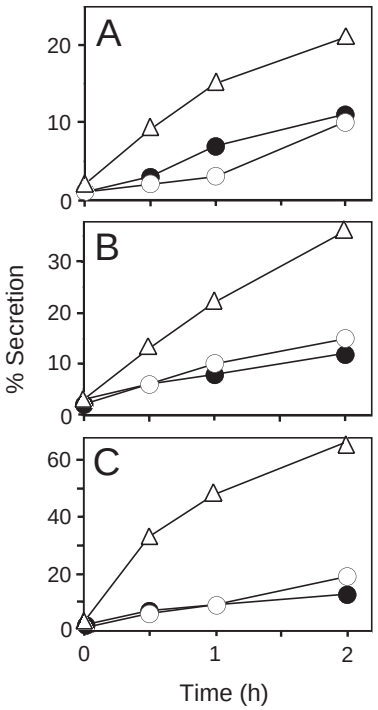

Fig. 2. Secretion of lysosomal enzymes in *lvsA* and *lvsB* mutant cells. Cells were incubated in SB medium to induce secretion of lysosomal enzymes. The activity of three lysosomal enzymes was determined in the cellular pellet and the supernatant at the indicated times. Enzymes tested were α-mannosidase (A), acid phosphatase (B) and N-acetylglucosaminidase (C). Over the course of the experiment, the total amount of the enzymatic activity did not vary significantly. The percentage of the total activity secreted in the medium is indicated. Filled circles, wild-type cells; open circles, *lvsA* cells; open triangles, *lvsB* cells.

type cells where virtually all the vacuolar H^+-ATPase was colocalized with the Rh50 protein in the contractile vacuole (Fig. 3A). However, in *lvsA* mutant cells a much larger fraction of the vacuolar H^+-ATPase was found in a compartment that contained no Rh50 protein and was thus presumably not the contractile vacuole (Fig. 3B). To further characterize the nature of this compartment, we performed colocalization experiments with the p80 protein, a membrane marker recently characterized in our laboratory. In wild-type cells the p80 transmembrane protein is present at the cell surface, in the early endocytic compartments, and concentrates in late endocytic compartments (K. Ravanel, B. de Chassey, S. Cornillon et al., unpublished). In wild-type cells, only a small amount of vacuolar H^+-ATPase is detected in a compartment containing low amounts of p80 but no Rh50 protein, that is, in early endocytic compartments (Fig. 3C, arrow). On the contrary, in *lvsA* mutant cells large amounts of vacuolar H^+-ATPase were found in this compartment (Fig. 3D, arrow). Remarkably, in these cells vacuolar H^+-ATPase was detected in the early endocytic compartments (p80-low, arrow) but not in more mature endocytic compartments (p80-high, arrowhead). This did not result from non-specific mixing of the endocytic compartments and the contractile vacuole, as the p80 protein and the Rh50 protein were never found colocalized (data not shown). In *lvsB* mutant cells, no significant difference in the localization of these three markers was seen compared to wild-type (data not shown). In summary, these results suggest that unusually high amounts of vacuolar H^+-ATPase are localized in the early endocytic compartments in *lvsA* mutant cells compared to wild-type cells. However, when assessed with LysoSensor Green DND-189 both the morphology and the lumenal pH of acidic endosomal compartments appeared unchanged in *lvsA* mutant cells (data not shown), indicating that the concentration of vacuolar H^+-ATPase is not the only determinant controlling the acidification of endosomes.

To verify if vacuolar H^+-ATPase was also mislocalized in the

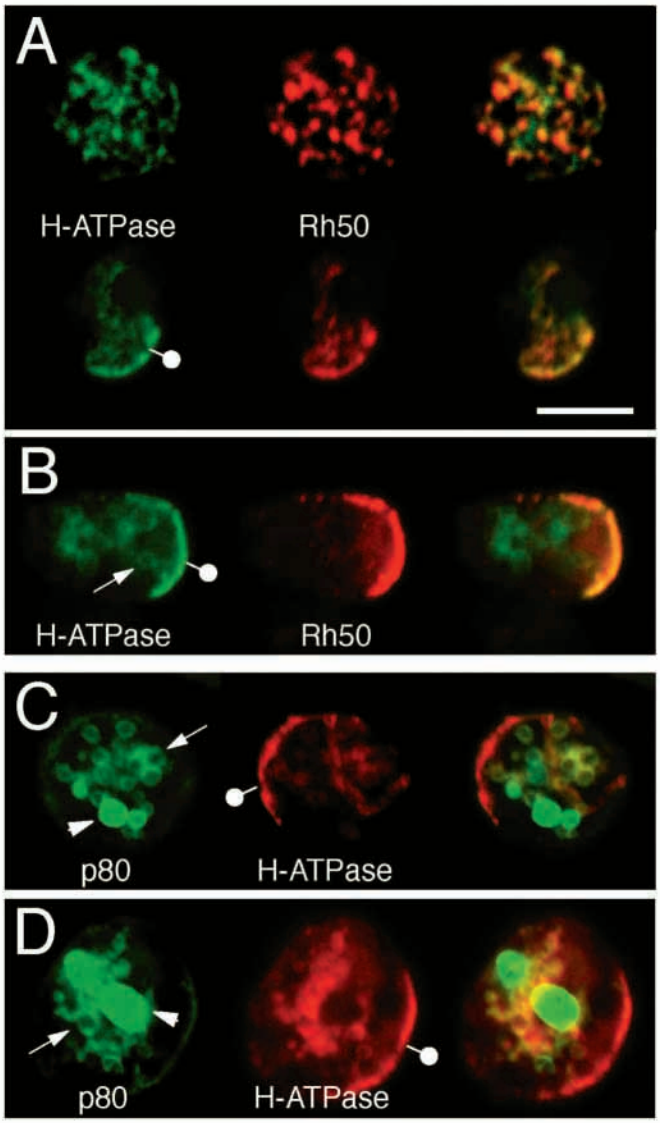

Fig. 3. Localization of the vacuolar H^+-ATPase in *lvsA* cells. Wild-type (A,C) or *lvsA* mutant cells (B,D) were fixed and labeled by immunofluorescence with the indicated antibodies. The top panel in (A) represents labeling in a plane close to the substrate, whereas in other panels pictures correspond to a plane towards the middle of the cell body. The circles indicate the contractile vacuole ($Rh50^+$, $HATPase^+$, $p80^-$), the arrows the early endocytic compartment ($Rh50^-$, $HATPase^+$, $p80^{low}$) and the arrowheads the late endocytic compartment ($Rh50^-$, $HATPase^-$, $p80^{high}$). Scale bar, 10 μm.

early phagocytic pathway of *lvsA* mutant cells, cells were allowed to phagocytose yeast particles for 15 minutes, before being fixed. The localization of vacuolar H^+-ATPase was then determined by immunofluorescence. After 15 minutes of phagocytosis, phagosomes exhibited almost undetectable levels of H^+-ATPase in wild-type cells (Fig. 4A), while in *lvsA* mutant cells clearly higher amounts of H^+-ATPase were seen (Fig. 4B). To measure this in a more quantitative manner the structure of phagosomes and the localization of vacuolar H^+-ATPase were examined by immunoelectron microscopy. For this, wild-type and *lvsA* mutant cells were incubated with *Klebsiella aerogenes* bacteria to allow phagocytosis, and cells were then fixed and prepared for

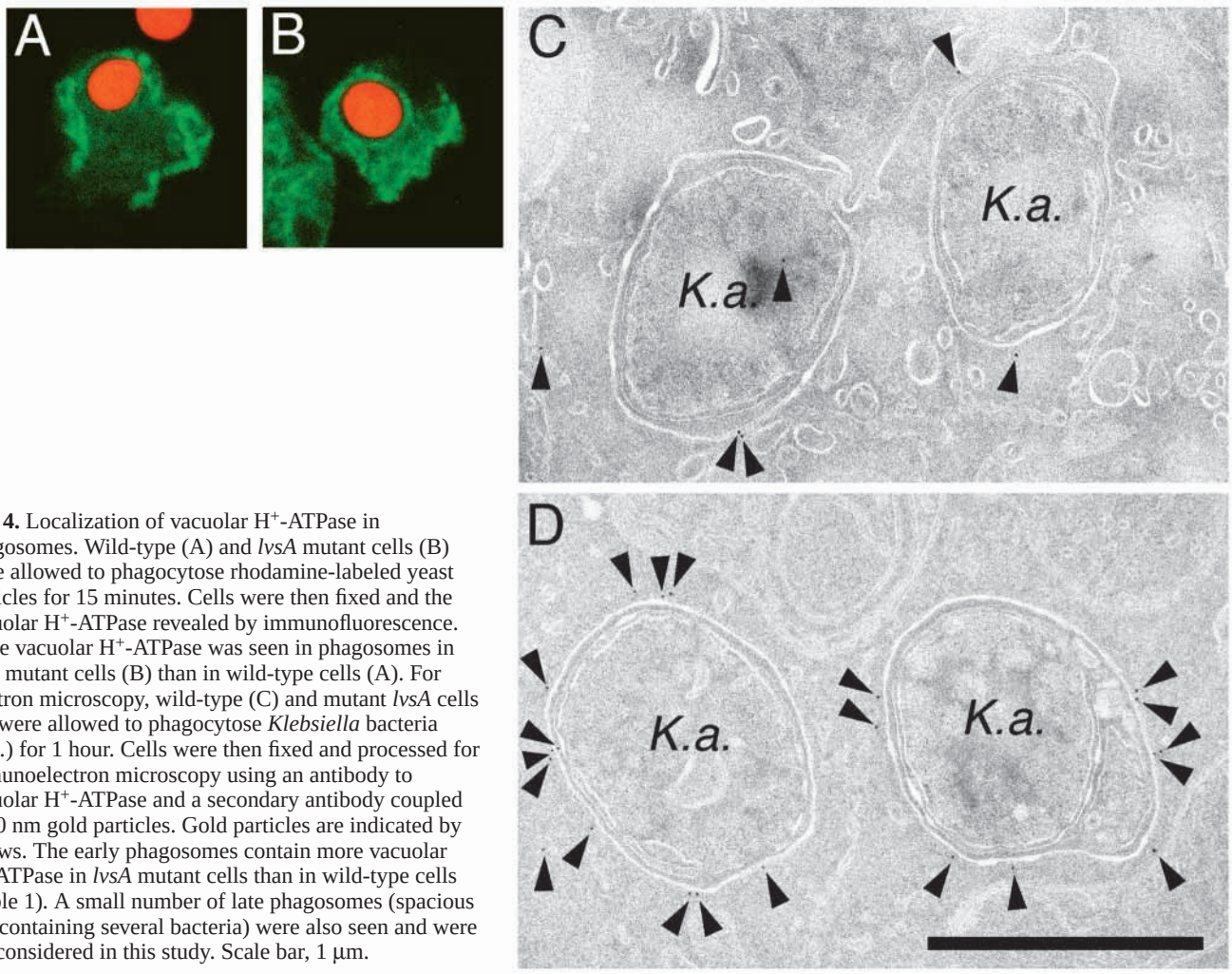

Fig. 4. Localization of vacuolar H^+-ATPase in phagosomes. Wild-type (A) and *lvsA* mutant cells (B) were allowed to phagocytose rhodamine-labeled yeast particles for 15 minutes. Cells were then fixed and the vacuolar H^+-ATPase revealed by immunofluorescence. More vacuolar H^+-ATPase was seen in phagosomes in *lvsA* mutant cells (B) than in wild-type cells (A). For electron microscopy, wild-type (C) and mutant *lvsA* cells (D) were allowed to phagocytose *Klebsiella* bacteria (*K.a.*) for 1 hour. Cells were then fixed and processed for immunoelectron microscopy using an antibody to vacuolar H^+-ATPase and a secondary antibody coupled to 10 nm gold particles. Gold particles are indicated by arrows. The early phagosomes contain more vacuolar H^+-ATPase in *lvsA* mutant cells than in wild-type cells (Table 1). A small number of late phagosomes (spacious and containing several bacteria) were also seen and were not considered in this study. Scale bar, 1 μm.

immunoelectron microscopy using an antibody against the vacuolar H^+-ATPase. In all the sections examined, the structure of early phagosomes appeared similar in wild-type and *lvsA* mutant cells, with each phagocytosed bacteria tightly surrounded with a cellular membrane. However, markedly higher amounts of vacuolar H^+-ATPase were seen in *lvsA* phagosomes (Fig. 4C,D). Indeed, although the total amount of vacuolar H^+-ATPase was the same in both cell types (data not shown), the *lvsA* phagosomes contained four times more vacuolar H^+-ATPase than phagosomes in wild-type cells (Table 1; mean±s.e.m.: 1.05±0.20 gold particles per phagosome in wild-type cells versus 4.19±0.38 in *lvsA* mutant cells; student's *t* test: $P<0.001$). Similarly, when cells were allowed to phagocytose bacteria for only 15 minutes, more H^+-ATPase was detected in phagosomes in *lvsA* mutant cells compared to wild-type (data not shown), although less phagosomes were observed and no statistical analysis was made.

Since the organization of the early endocytic and phagocytic pathway was altered in *lvsA* mutant cells, the ability of these cells to perform endocytosis and phagocytosis was examined next.

LvsA mutant cells exhibit reduced phagocytosis but normal macropinocytosis

To quantify the ability of cells to perform phagocytosis, cells were mixed with fluorescent latex beads or *K. aerogenes* bacteria, then washed and the internalized particles measured in a cell sorter. *LvsA* mutant cells exhibited a decrease of approximately 70% in their ability to phagocytose latex beads or bacteria compared to wild-type cells (Fig. 5A,B). This defect could be observed even after a few minutes of phagocytosis (Fig. 5, insert in A), suggesting that a very early stage of the phagocytic process is defective in *lvsA* mutant cells, that is adhesion to the particle or the formation of the phagocytic cup.

In *Dictyostelium*, internalization of the fluid phase occurs mostly by macropinocytosis, which involves actin rearrangement, and it takes place in a similar manner to internalization during phagocytosis (Hacker et al., 1997). Thus

Table 1. Vacuolar H^+-ATPase is enriched in the phagosomal membrane in *lvsA* mutant cells

Experiment	1		2		3	
Cell type	WT	*lvsA*	WT	*lvsA*	WT	*lvsA*
Number of investigated phagosomes	14	18	10	5	16	14
Gold particles/phagosome	0.71	3.44	1.00	3.20	1.38	5.50
Enrichment in *lvsA* phagosomes versus WT	4.85		3.20		3.99	

Wild-type and *lvsA* mutant cells with phagocytosed bacteria were prepared as described in Fig. 4 in three separate experiments. Gold particles associated with the limiting membrane of phagosomes were counted.

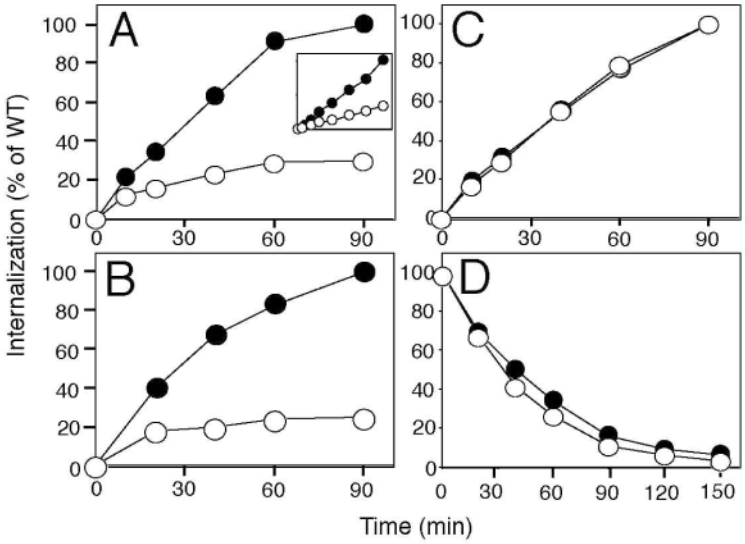

Fig. 5. Internalization of particles and fluid phase in *lvsA* mutant cells. Wild-type (filled circles) or *lvsA* mutant cells (open circles) were incubated for various times in the presence of fluorescent latex beads (A), rhodamine-labeled *Klebsiella aerogenes* (B) or FITC-dextran (C). The amount of internalized fluorescence was analyzed using a fluorescent-activated cell sorter (FACS). The results are expressed as a percentage of the internalization by wild-type cells after 90 minutes. Insert in (A), to check for fluorescent latex beads internalization at early time-points, cells were incubated with beads for various times up to 20 minutes, then fixed and analyzed by FACS. (D) To measure recycling of internalized fluid phase to the extracellular medium, cells were allowed to internalize FITC-dextran for 1 hour, then washed and incubated for the indicated times. The fluorescence remaining in the cells was then analyzed by FACS.

some mutants with alterations in the organization of the actin cytoskeleton also exhibit decreased uptake of the fluid phase as was observed for example for coronin mutants (Hacker et al., 1997; Maniak et al., 1995). The uptake of fluid phase (Fig. 5C), as well as its recycling from endosomes to the extracellular space via post-lysosomal compartments (Fig. 5D), was not affected in *lvsA* mutant cells. Furthermore the formation of macropinocytic cups was observed using live microscopy. Both the morphology and the kinetics of macropinocytosis appeared normal in *lvsA* mutant cells (Fig. 6). Together, these results demonstrate that in *lvsA* mutant cells, the reorganization of the actin cytoskeleton necessary for the formation of macropinocytic cups is not perturbed. Indeed no significant alteration in actin organization could be seen in *lvsA* or *lvsB* mutant cells by immunofluorescence (data not shown) (Kwak et al., 1999).

Similar experiments were performed with *lvsB* mutant cells. The internalization of fluid phase was not affected, and only a minor phagocytosis defect was observed (data not shown) (Fig. 7B). The phagocytosis defect in *lvsB* mutant cells was hardly significant and not studied further. It could be a small indirect effect resulting from alterations in the function of late endocytic compartments.

LvsA mutant cells are not defective for adhesion to the substrate

Several phagocytosis mutants previously isolated in this laboratory were found to have a specific defect in adhesion to certain phagocytic particles. In these mutants, the phagocytosis defect depends on the type of phagocytic particle that is used. For example *phg1* mutant cells hardly phagocytosed latex beads or *E. coli* bacteria but efficiently internalized *K. aerogenes* (Cornillon et al., 2000). We therefore tested the ability of *lvsA* mutant cells to phagocytose latex beads and various types of bacteria. These cells exhibited a strong phagocytosis defect (60 to 70% inhibition), and similar results were obtained for all types of particles tested (Fig. 7A), which suggested that the mutation did not specifically affect the function of a subset of phagocytic receptors at the surface of the amoebae. The phagocytosis of *lvsA* mutant cells might however result from a general adhesion defect. To test this, the ability of cells to adhere to a glass surface was tested. In previous experiments this test revealed a strong adhesion defect in *phg1* mutant cells (Cornillon et al., 2000). This was not the case for *lvsA* mutant cells, however, and in fact *lvsA* mutant cells adhered more strongly to glass than wild-type cells (Table 2).

The induction of phagocytic cups is decreased in *lvsA* mutant cells

Is the phagocytic defect observed for *lvsA* mutant cells caused by a slow extension of the phagocytic cup or are phagocytic cups induced less often? In the first case one would expect phagocytosing *lvsA* cells to exhibit a number of phagocytic cups comparable to or greater than the number observed in wild-type cells. On the contrary, in the latter case a decrease in the number of phagocytic cups should be observed. In order to distinguish between the two possibilities, cells expressing a fusion protein of CRAC with the green fluorescent protein (CRAC-GFP) were used. CRAC translocates from the cytosol to the plasma membrane and is associated transiently with the leading edge of cells, as well as with forming macropinosomes and phagosomes (Parent et al., 1998; Tuxworth et al., 2001). Thus

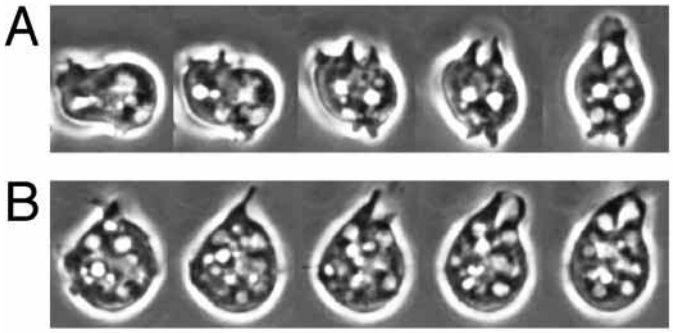

Fig. 6. Formation of macropinosomes is normal in *lvsA* mutant cells. Wild-type (A) and *lvsA* mutant cells (B) grown in HL5 medium were observed in phase contrast with a Zeiss Axiovert 100 microscope. Pictures were recorded every 10 seconds. In both series of pictures the cells can be seen to extend pseudopods and form macropinosomes with similar morphologies and kinetics.

Table 2. Adhesion of *lvsA* and *lvsB* mutant cells to glass

	WT	*lvsA*	*lvsB*
HL5	0.18±0.08 Pa	0.51±0.05 Pa	0.10±0.01 Pa
SB	1.6±0.2 Pa	2.1±0.2 Pa	1.6 ±0.2 Pa

Cells adhering to glass in HL5 or SB were submitted to a flow of medium at a constant rate for 7 minutes. The strength needed to detach 50% of cells was calculated as described in the Materials and Methods section.

in cells expressing the CRAC-GFP fusion protein, fluorescence accumulates around the forming phagocytic cup and is lost later from mature phagosomes (Tuxworth et al., 2001). *LvsA* and wild-type cells were transfected with the CRAC-GFP construct. After phagocytosis of rhodamine-labeled yeast particles, cells were fixed and examined. It was seen that in *lvsA* mutant cells CRAC-GFP also accumulates in the forming phagocytic cups (Fig. 8B) as in wild-type cells (Fig. 8A). Fluorescent CRAC-GFP did not decorate mature phagosomes (Fig. 8). Fluorescent phagocytic cups were counted and their number was found to be reduced in *lvsA* cells compared to wild-type cells (Table 3). This result suggests that *lvsA* mutant cells exhibit a defect in the very early steps of phagocytic cup formation, presumably in the initiation of a phagocytic cup upon adhesion to a particle.

Discussion

In this study, two mutants, which harbored disruptions in members of the BEACH family of proteins, were studied in *Dictyostelium discoideum*. The *lvsB* mutant secreted more lysosomal enzymes when starved than wild-type *Dictyostelium* cells. In *lvsA* mutant cells, secretion of lysosomal enzymes was normal. Instead defects in the organization of the early endocytic and phagocytic pathway were observed, with an

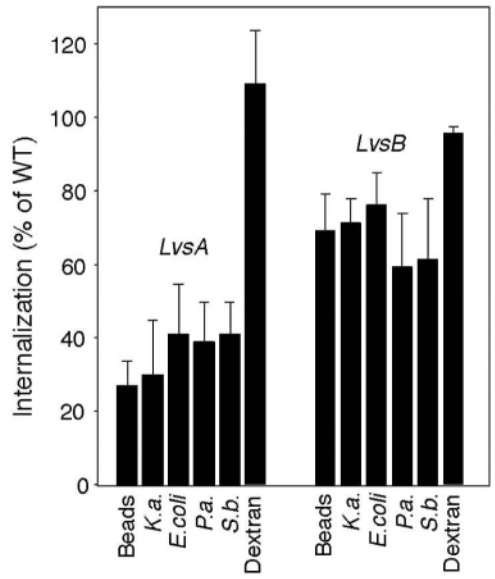

Fig. 7. Phagocytosis of different particles by *lvsA* and *lvsB* mutant cells. Cells were incubated for 1 hour with the indicated particles. The amount of internalized fluorescence was determined by FACS. The results are expressed as a percentage of the internalization by wild-type cells in the same experiment. The indicated values are the mean and standard deviation of seven independent experiments. Beads, latex beads, *K.a., Klebsiella aerogenes, E. coli, Escherichia coli, P.a., Pneumophila aeruginosa, S.b., Streptococcus bovis.*

Table 3. The number of phagocytic cups is decreased in lvsA mutant cells

Experiment	1		2	
Cell type	WT	*lvsA*	WT	*lvsA*
Number of CRAC-GFP positive structures	48	21	42	13
Relative abundance of CRAC-GFP positive structures (*lvsA*/WT)	44%		31%	

Wild-type and lvsA mutant cells were prepared as described in Fig. 8 in two independent experiments. For each sample 7.5×10^4 cells were examined for the presence of structures positive for CRAC-GFP (phagocytic cups or newly formed phagosomes).

increased concentration of vacuolar H^+-ATPase in early endo/phagocytic compartments and a defect in phagocytosis.

These results suggest that LvsA and LvsB proteins may be involved in the function of the early and late endocytic pathway, respectively. These results are reminiscent of observations made in mammalian cells. Indeed, in mammalian cells the LYST protein seems to play a role in the organization and function of lysosomes, as its absence induces morphological alterations in the late endocytic pathway and defects in the secretion of lysosomal enzymes in certain cell types. The FAN protein is the only other mammalian member of the BEACH family analyzed so far. It is associated with the receptor to tumor necrosis factor, which is present at the cell surface and in the endocytic pathway. Thus in mammalian cells as well as in *Dictyostelium* cells, members of the BEACH family seem to play a role predominantly in the endocytic pathway. Recently the LvsA protein was found to localize to elements of the contractile vacuole in addition to small vesicles in the cytosol (N. Wang and A. De Lozanne, unpublished). The identity and function of these cytoplasmic vesicles remains to be established. This result is compatible with the notion that the LvsA protein might directly participate in membrane trafficking in the early endo/phagocytic compartments and controls the transport of some membrane components between the contractile vacuole and endocytic compartments.

A second parallel between *Dictyostelium* and mammalian cells is the putative role of BEACH proteins in intracellular signaling. In mammalian cells, FAN plays a role in intracellular signaling and coupling to the neutral sphingomyelinase (Adam-Klages et al., 1996). The LYST protein was also proposed to be involved in a signal transduction pathway, as it is necessary for the secretion of lysosomes in response to the activation of the T-cell receptor at the cell surface.

Similarly, it seems likely that LvsA is involved in the intracellular signaling that allows the formation of a phagocytic cup in response to a local stimulus. Indeed *lvsA* mutant cells are not defective for adhesion to phagocytic particles but have problems inducing phagocytic cups. It is also striking that phagocytosis, but not macropinocytosis, is affected in *lvsA* mutant cells. Macropinocytosis is morphologically similar to phagocytosis, and the two processes share molecular components such as actin and coronin. However, there is some evidence to suggest that distinct signaling pathways are involved in induction of these. In particular several mutations have been identified that affect only one of the two processes (Rupper and Cardelli, 2001). Together, these observations support the notion that LvsA plays a role in some of the local transduction events specifically involved in the formation of the phagocytic cup.

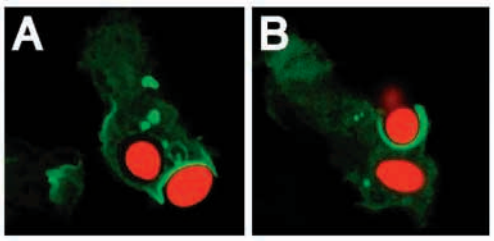

Fig. 8. Formation of the phagocytic cup in *lvsA* mutant cells. Wild-type (A) or *lvsA* mutant cells (B) expressing CRAC-GFP fusion protein were incubated with rhodamine-labeled yeast particles for 1 hour. Cells were then fixed and observed with a confocal fluorescence microscope. CRAC-GFP accumulates on the cytosolic face of the phagocytic cup but is not observed on mature phagosomes.

In *Dictyostelium discoideum*, secretion of lysosomal enzymes is also a response to changes in environmental conditions and occurs essentially upon amino-acid starvation (Marin, 1976). The signaling pathway involved has not yet been determined. The fact that LvsB is implicated in the secretion of lysosomes in response to cell starvation suggests that it may play a role in the underlying signaling events, presumably as a negative regulator.

In summary, all members of the BEACH family studied to date are implicated in situations where intracellular signaling is occurring. This suggests that they might be involved at some stage in the signaling cascade. This does not exclude the possibility that they might also play a structural role in the endocytic compartments.

Sequence analysis suggests that the LvsA and LvsB proteins are not the *Dictyostelium* equivalents of FAN and LYST proteins. All members of the BEACH family exhibit similar degrees of conservation within their BEACH domains (45 to 55% identities for LvsA and LvsB compared with LYST and FAN proteins). However, the N-terminal domains vary considerably. The long N-terminal extension in the LYST protein is very different from that found in LvsA and LvsB. The FAN protein exhibits only a very small N-terminal extension. It is striking that distinct members of the BEACH family, although in very different cellular contexts, all appear to be involved in the organization of the endocytic pathway and its modulation in response to extracellular stimuli. This may represent a conserved feature of many or even all the members of the BEACH family of proteins. *Dictyostelium* may prove to be a very good cellular model to analyze the functions of other members of the BEACH family of proteins.

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