

# Structure-Function Analysis of the BEACH Protein LvsA

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**Most eukaryotes have several members of the BEACH family of proteins but the molecular function of these large proteins remains unknown. The *Dictyostelium* BEACH protein LvsA is essential for cytokinesis and contractile vacuole activity. The functional contribution of different portions of LvsA was tested here by deletion analysis. The C-terminal WD domain was important for protein stability and C-terminal deletions resulted in loss of LvsA function. In contrast, N-terminal deletions yielded abundant protein expression that could be assayed for function. Despite very low sequence conservation of the N-terminal portion of LvsA, this region is important for its function *in vivo*. Deletion of 689 N-terminal amino acids produced a protein that was functional in cytokinesis but partially functional in osmoregulation. Further deletions resulted in the complete loss of LvsA function. Using *in vitro* fractionation assays we found that LvsA sedimented with membranes but that this association does not require the N-terminal portion of LvsA. Interestingly, the association of LvsA with the contractile vacuole was perturbed by the loss of drainin, a protein important for vacuole function. In drainin-null cells, LvsA bound irreversibly to engorged contractile vacuoles that fail to expel water. These experiments help delineate the biochemical and physiological requirements for function of one important BEACH protein, LvsA.**

**Key words:** BEACH domain, beige, CHS, cytokinesis, Lyst

**Received 14 November 2003, revised and accepted for publication 29 January 2004**

The novel family of BEACH proteins is a poorly understood group of proteins found in all eukaryotes (1). Sequence analysis suggests that these proteins can be classified into at least five distinct classes that may have different cellular functions (2). However, few BEACH proteins have been characterized and the details of their cellular role remain obscure. The human Chediak–Higashi Syndrome protein (also known as Lyst), was the first BEACH protein to be characterized (3,4). This protein and its orthologues in other mammals and the amoeba *Dictyostelium discoideum* are involved in controlling the fusion of endosomal

compartments (5,6). The mammalian BEACH protein FAN is a signaling protein involved in tumor necrosis factor (TNF) receptor signaling (7). Mammalian neurobeachin and *Drosophila* AKAP550 are BEACH proteins that bind to the regulatory subunit of protein kinase A (PKA) (8,9). The *Dictyostelium* LvsA protein is a BEACH protein required for cytokinesis and for the function of the contractile vacuole during osmoregulation (10,11).

The C-terminus of all members of this family contains the highly conserved BEACH domain as well as several WD motifs and a PH-like domain (2). The WD domain of these proteins is presumed to be a protein interaction domain. Indeed, the WD domain of FAN interacts with the cytosolic domain of the p55 TNF receptor (7). The structure of the BEACH domain has been solved and found to have a unique protein fold without any obvious catalytic or binding pockets (12). It is not clear whether this domain plays a structural role or whether it interacts with specific binding partners. With the exception of the mammalian protein FAN, most BEACH proteins are very large proteins that have a very large N-terminal domain. This domain, which accounts for about 75% of the protein size, shares little similarity among different BEACH proteins and does not have any clearly recognizable sequence motifs (2). The N-terminal domain of the *Drosophila* AKAP550 and mammalian neurobeachin proteins contains a binding site for the RII regulatory subunit of PKA but the significance of this binding has not been tested (8,9). Given the poor conservation of this domain among BEACH proteins, it is not evident whether this domain contributes any significant function to the cellular role of BEACH proteins.

Details about the subcellular localization of BEACH proteins are only known for neurobeachin and *Dictyostelium* LvsA. Neurobeachin is a brain-specific protein localized on membranes of unknown origin but which are near the *trans*-Golgi apparatus and also in puncta throughout the cell (8). The association of neurobeachin with these membranes might be regulated by G-proteins. *Dictyostelium* LvsA is localized on the contractile vacuole network and is also found in puncta throughout the cell (11). However, the association of LvsA with the contractile vacuole is transient, occurring only during the expulsion phase of the vacuole. An expanding vacuole is devoid of LvsA. After reaching its maximum diameter, the vacuole rapidly becomes labeled by LvsA and LvsA remains associated with the vacuole throughout the expulsion phase. Once the vacuole has collapsed, LvsA labeling is lost. Thus, like other BEACH proteins, LvsA appears to be a peripheral membrane protein important for the function of its associated membrane compartment, the contractile vacuole.

The structural and regulatory mechanisms of membrane association of LvsA or any other BEACH protein have not been defined. Here we use a combination of deletion analysis, biochemical fractionation and genetic epistasis to explore the function and regulation of the *Dictyostelium* LvsA protein.

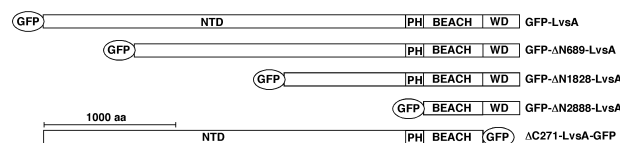
## Results

### Deletion analysis of *Dictyostelium* LvsA

*Dictyostelium* LvsA is the first BEACH protein shown to dynamically associate with a membrane compartment, the contractile vacuole (11). Like most other BEACH proteins, LvsA is a large protein (408 kDa) that contains multiple domains of unknown function. Since LvsA is essential for cytokinesis and contractile vacuole function, we wanted to determine whether these functions require some or all of the LvsA domains.

To determine the functional contribution of different portions of LvsA, we designed constructs to express GFP-tagged LvsA proteins with increasing deletions from the N-terminus or C-terminus (Figure 1). The three largest constructs were created by a 'knock-in' approach (10), inserting the Actin 6 promoter and GFP into the appropriate location on the *lvsA* gene. This approach has the advantage that the designed protein is expressed from the single copy of the *lvsA* gene in its original locus. As a result, only the GFP-tagged recombinant protein is expressed in each of these cell lines; the wild-type protein is absent. The smallest construct, which contained only the BEACH and WD domains, was cloned into a *Dictyostelium* expression vector and transformed into wild-type and LvsA-null cells.

Western blot analysis of these cell lines with anti-LvsA or anti-GFP antibodies demonstrated that the engineered cell lines expressed GFP-LvsA fusion proteins of the expected molecular mass. We found that the N-terminal deletions (GFP-ΔN689-LvsA and GFP-ΔN1828-LvsA) were expressed at higher levels than the full-length protein (Figure 2, lanes 2–4) even though these three proteins are expressed from a single copy of the *lvsA* gene driven by the same Actin 6



**Figure 1: Domain organization of LvsA and its truncation mutants.** The diagram indicates the domains found in LvsA and the relative position of the N-terminal and C-terminal truncation constructs used in this study. NTD is the large N-terminal domain of little homology to other BEACH proteins. PH is the region shown to fold like a PH-domain in neurobeachin. BEACH is the highly conserved BEACH domain. WD is the domain of LvsA that has six WD repeats.

promoter. In contrast, deletion of the C-terminal WD domain (ΔC271-LvsA-GFP) resulted in a drastic reduction of the amount of expressed protein (Figure 2, lanes 5 & 10). Cells with this construct had a phenotype indistinguishable from LvsA-null cells and will not be described further. Finally, the expression of GFP-ΔN2888-LvsA is much greater than the other constructs, presumably because it is expressed from a high copy-number expression plasmid (Figure 2, lanes 6–7).

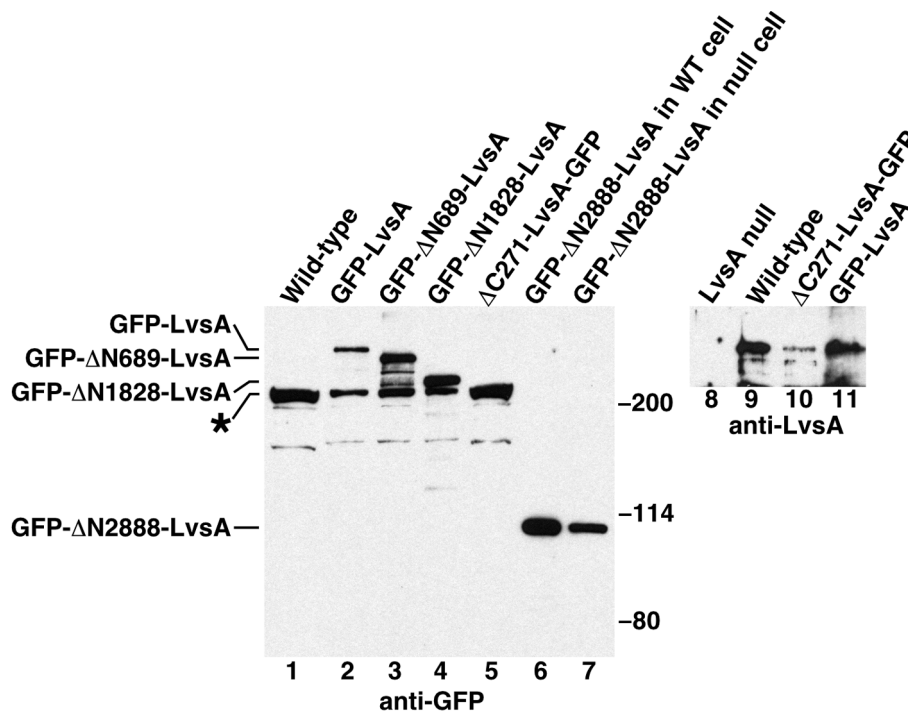
### Effect of LvsA truncations on cytokinesis

Due to a cytokinesis defect, LvsA-null cells are not able to divide in suspension cultures and become large multinucleated cells that eventually die (Figure 3) (10). We tested the ability of cells expressing each GFP-LvsA fragment to carry out cytokinesis in suspension culture. Cells expressing full length GFP-LvsA grew at the same rate as wild-type control cells demonstrating that the GFP tag does not affect the function of LvsA (Figure 3). Cells expressing GFP-ΔN689-LvsA were also able to grow in suspension culture. Cells expressing GFP-ΔN1828-LvsA and GFP-ΔN2888-LvsA behaved like LvsA-null cells, and did not grow in suspension and became multinucleate cells. Furthermore, overexpression of GFP-ΔN2888-LvsA (containing the BEACH and WD domains of LvsA) in wild-type cells (Figure 2, lane 7) did not disturb the function of the endogenously expressed LvsA protein and did not cause a dominant-negative cytokinesis defect in suspension (data not shown).

### Effect of LvsA truncations on osmoregulation

LvsA is localized on the contractile vacuole and is essential for the function of the contractile vacuole in hypoosmotic conditions (11). We tested the ability of our different cell lines to osmoregulate when exposed to distilled water or buffers of low osmolarity. Cells containing full length GFP-LvsA retained their shape and ability to move even after 30 min in water (Figure 4A–B). These cells displayed vigorous contractile vacuole activity throughout this period. In contrast, LvsA-null cells swelled rapidly and lost attachment to the slide (Figure 4I–J). Cells containing GFP-ΔN1828-LvsA and GFP-ΔN2888-LvsA also swelled rapidly and detached like LvsA-null cells (Figure 4E–H). Close inspection of these cells by differential interference contrast (DIC) microscopy did not reveal any contractile vacuole activity. Cells containing GFP-ΔN689-LvsA also swelled but not to the same extent as LvsA-null cells and many cells remained attached throughout the treatment period. These cells displayed contractile vacuole activity, although the vacuoles were less frequent and smaller than those found in wild-type cells (Figure 4C–D, arrowhead). Thus, deletion of the N-terminal 690 amino acids of LvsA renders a protein that is partially functional in osmoregulation.

Since LvsA localizes on the membrane of the contractile vacuole during the expulsion phase (11) we investigated the localization of the GFP-labeled LvsA truncation mutants. GFP-LvsA was readily detectable when it was bound to



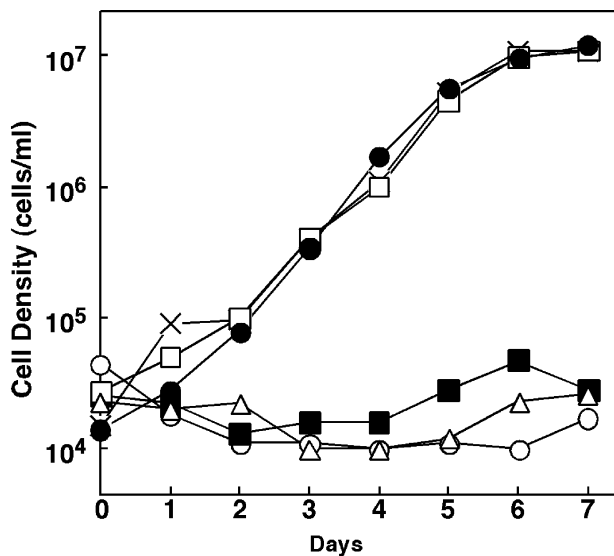
**Figure 2: Expression of GFP-tagged LvsA truncated proteins in *Dictyostelium*.** The expression of GFP-tagged LvsA proteins was demonstrated by Western blot analysis of the different cell lines with an anti-GFP antibody (lanes 1–7) or an anti-LvsA antibody (lanes 8–11). Lane 1, wild-type cells without GFP. Lane 2, cells expressing full length GFP-LvsA. Lane 3, GFP-ΔN689-LvsA. Lane 4, GFP-ΔN1828-LvsA. Lane 5, ΔC271-LvsA-GFP. Lane 6, GFP-ΔN2888-LvsA (in wild-type cells). Lane 7, GFP-ΔN2888-LvsA (in LvsA-null cells). Lane 8, LvsA-null cells. Lane 9, control wild-type cells. Lane 10, ΔC271-LvsA-GFP. Lane 11, full length GFP-LvsA. The relative levels of expression of full length and the N-terminal truncation mutants are seen in lanes 2–4 and 6–7. The expression of the smallest LvsA fragment (GFP-ΔN2888-LvsA) is so high that we loaded 20× fewer cells in lanes 6–7. The anti-GFP antibody reacts with a nonspecific band of about 200 kDa (asterisk) which can be used to see the relative amount of cell lysate loaded per lane. This band is not visible in lanes 6–7 because they contain fewer cells. The C-terminal truncation ΔC271-LvsA-GFP is expressed at very low levels and cannot be detected at this exposure even though 5 times more lysate was loaded on lane 5. A long exposure of a similar gel (lanes 8–11) demonstrates the expression of this protein and its relative level of expression compared to LvsA and to GFP-LvsA. The predicted molecular mass of ΔC271-LvsA-GFP is nearly identical to that of wild-type LvsA. GFP-LvsA is only 25 kDa larger than wild-type LvsA but this difference is not resolved in this 7.5% polyacrylamide gel. Lanes 1, 5 and 8–11 contain  $1 \times 10^6$  cells/lane; lanes 2–4 contain  $2 \times 10^5$  cells/lane and lanes 6–7 contain  $1 \times 10^4$  cells/lane.

the membrane of the contractile vacuole (Figure 5A and QuickTime movie 1, available at [http://www.traffic.dk/videos/5\\_5\\_3bis.asp](http://www.traffic.dk/videos/5_5_3bis.asp)). The fluorescence of GFP-LvsA in the cytosol was barely above background. Although the expression level of GFP-ΔN689-LvsA was higher than that of full length GFP-LvsA, we did not detect any association of this truncated protein with the small contractile vacuoles present in this cell line (Figures 4D and 5B). Similarly, we did not detect any membrane association of GFP-ΔN1828-LvsA (data not shown) or GFP-ΔN2888-LvsA (Figure 5C). These data suggest that deletions from the N-terminus severely impair the ability of LvsA protein to accumulate on the membrane of the contractile vacuole.

To examine in more detail the function of the different LvsA truncation mutants we examined the organization of contractile vacuole membranes in each mutant cell line using the marker GFP-dajumin (13). This integral membrane protein has been shown to label specifically the

contractile vacuoles and the network of tubules and sacs that are part of this organelle [(13) and Figures 5F and 6A, and QuickTime movie 2, available at [http://www.traffic.dk/videos/5\\_5\\_3bis.asp](http://www.traffic.dk/videos/5_5_3bis.asp)). The expression of GFP-dajumin is much higher than that of GFP-LvsA and the two truncation mutants GFP-ΔN689-LvsA and GFP-ΔN1828-LvsA. Thus, this protein can be readily detected above the very low fluorescence level of these truncated LvsA proteins (Figure 5D–F).

Using GFP-dajumin we confirmed our previous observations using an independent marker (the proton pump ATPase) that the contractile vacuole network is disrupted in LvsA-null cells (11). In these cells the network of tubules was broken down into many small vesicles that often clustered in a juxtanuclear position or associated with the plasma membrane (Figure 6B). No large contractile vacuoles were ever present in LvsA-null cells. We found that the distribution of GFP-dajumin in GFP-ΔN1828-LvsA

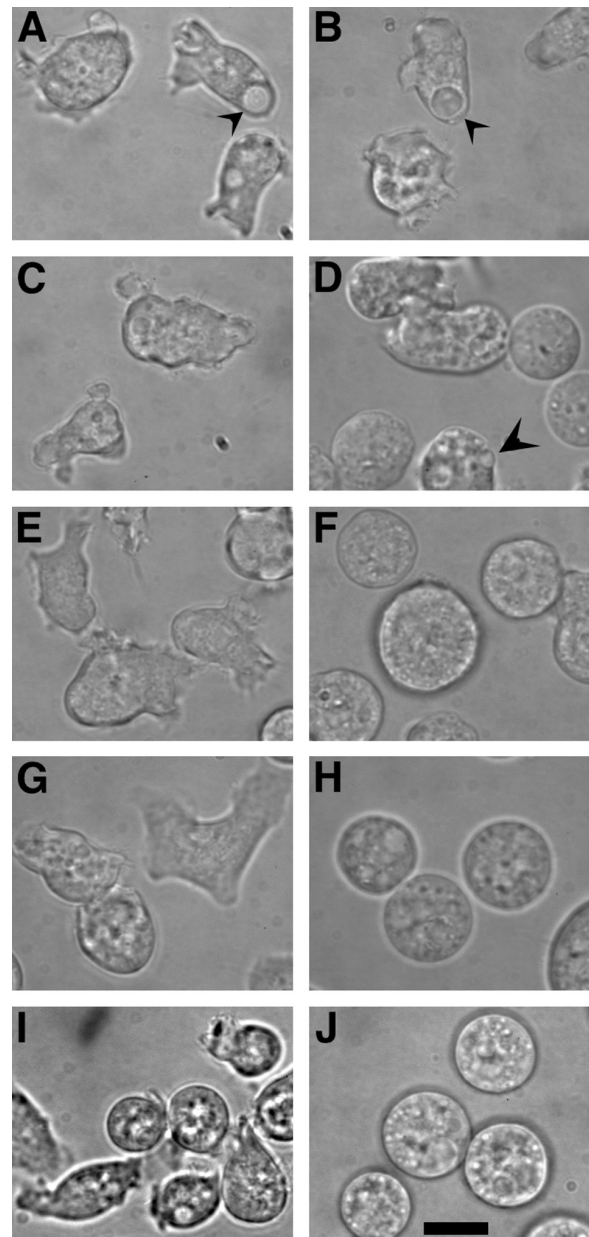


**Figure 3: The N-terminus of LvsA is not required for cytokinesis.** Different cell lines were incubated in suspension cultures and their growth was monitored daily. Wild-type cells (●), GFP-LvsA cells (X), and GFP-ΔN689-LvsA cells (□) grow normally under these conditions. In contrast, LvsA-null cells (■), GFP-ΔN1828-LvsA cells (△) and GFP-ΔN2888-LvsA cells (○) cannot carry out cytokinesis and fail to grow.

cells was similar to that seen in LvsA-null cells. We never observed contractile vacuole activity in these cells and their CV network was disorganized into small punctae near the nucleus or at the plasma membrane (Figure 6D). In contrast, we did observe GFP-labeled contractile vacuoles in the strain with GFP-ΔN689-LvsA, although they were organized as a group of small vacuoles clustered near the plasma membrane (Figure 6C). Occasionally, these small vacuoles expanded and contracted as in wild-type cells, indicating that they were able to expel water from the cells (QuickTime Movie 3, available at [http://www.traffic.dk/videos/5\\_5\\_3bis.asp](http://www.traffic.dk/videos/5_5_3bis.asp)).

#### **The localization of LvsA on the CV is independent of drainin**

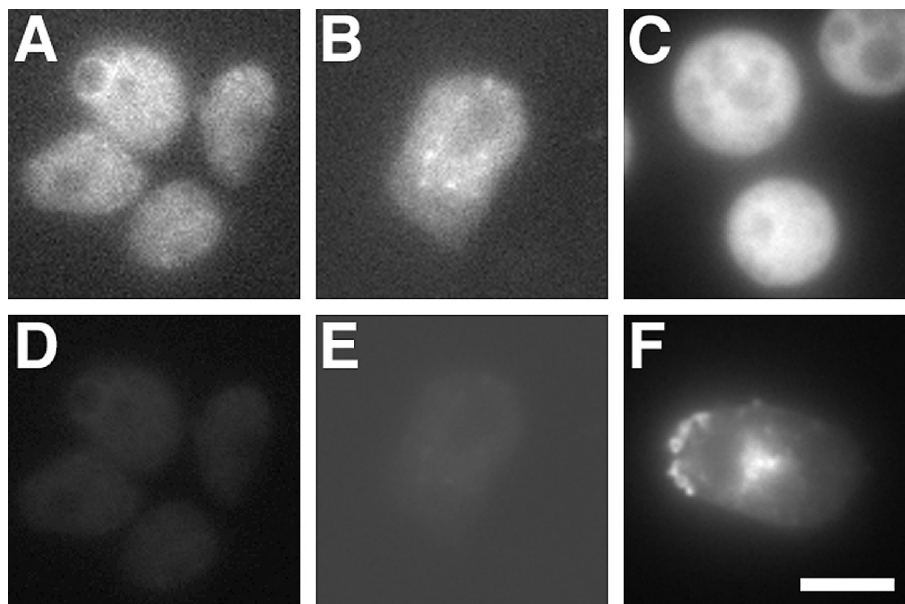
Drainin is a *Dictyostelium* protein that is also localized on the contractile vacuole and is required for the function of this organelle (14). Thus, it seemed possible that the localization and function of these two proteins might be interdependent. To test this hypothesis we disrupted the gene encoding drainin in our GFP-LvsA tagged cell line and also expressed GFP-tagged drainin in the LvsA-null cell line. We then determined the localization of the GFP-tagged proteins in each mutant cell line. As shown previously (14), we observed GFP-drainin associated with the contractile vacuole, although a large fraction of drainin is found diffused in the cytosol (Figure 7A). In contrast to LvsA, drainin was localized on the vacuole during both the expansion and contraction phases of the vacuole. Since the organization of the contractile vacuole is severely disrupted in



**Figure 4: Osmoregulation is defective in LvsA mutants.**

*Dictyostelium* cells with different LvsA constructs were exposed to water to trigger the activity of the contractile vacuole. Cells on the left column (A, C, E, G, and I) were exposed to water for 1 min and the cells on the right column (B, D, F, H, and J) for 30 min. Cells with full length GFP-LvsA (A, B), displayed vigorous contractile vacuole activity and retained the ability to move and change shape throughout the exposure period. The arrowheads indicate clearly visible contractile vacuoles. Cells with the smallest N-terminal deletion, GFP-ΔN689-LvsA (C, D), displayed few, very small contractile vacuoles and some cells became swollen. However, most cells remained attached and were able to change shape. The arrowhead indicates a contractile vacuole smaller than those seen in B. Cells expressing other LvsA truncation mutants, GFP-ΔN1828-LvsA (E, F), and GFP-ΔN2888-LvsA (G, H), and LvsA-null cells (I, J) did not have any visible contractile vacuoles, were unable to osmoregulate and swelled upon prolonged exposure to water. Bar = 10 μm.





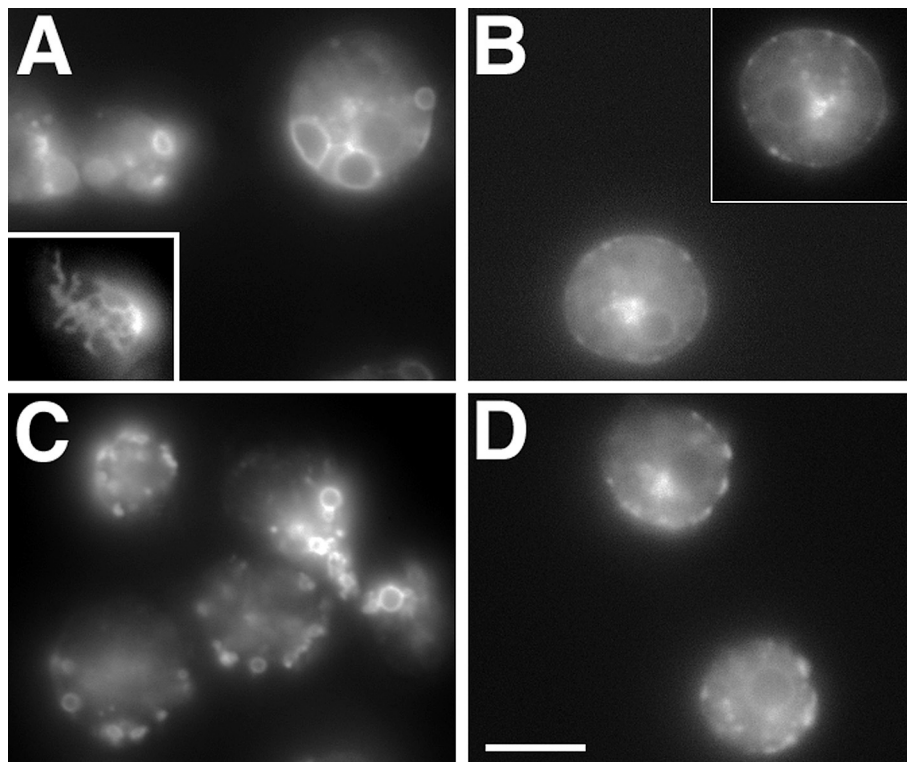
**Figure 5: The binding of LvsA to contractile vacuole membranes is lost in the N-terminal truncation mutants.** A) GFP-LvsA binds to the contractile vacuole (upper left corner) during the expulsion phase (see QuickTime Movie 1, available at [http://www.traffic.dk/videos/5\\_5\\_3bis.asp](http://www.traffic.dk/videos/5_5_3bis.asp)). In contrast, the truncation mutant GFP-ΔN689-LvsA (B) does not bind to the small contractile vacuoles present in this cell line (see Figure 4D). The small dotted structures visible in this image are due to autofluorescence of the nutrient medium inside endosomes. C) LvsA-null cells expressing GFP-ΔN2888-LvsA. These cells do not display any contractile vacuole activity and the bright GFP construct is found diffused in the cytosol. The expression of GFP-LvsA and GFP-ΔN689-LvsA is very low but that of GFP-ΔN2888-LvsA is very high (see Figure 2). This is illustrated by scaling the images in A and B to the same range as that used in C. D) Same image as in A but at the scale of C. E) Same image as in B but at the scale of C. F) Introducing an expression vector for GFP-dajumin into GFP-ΔN689-LvsA cells leads to high expression of GFP-dajumin, which can be easily detected above the background fluorescence of GFP-ΔN689-LvsA. This image is scaled at the same scale of C, D and E. Exposure time for these images is 200 ms. Bar = 10  $\mu$ m.

LvsA-null cells, we did not see any vacuolar localization of GFP-drainin in these cells. Nonetheless, LvsA-null cells contained membranous structures that were labeled with GFP-drainin (Figure 7B). These may represent remnants of the residual contractile vacuole network visualized by GFP-dajumin.

In drainin-null mutants we observed the reported formation of giant contractile vacuoles that are unable to expel water (14). However, we found that this phenotype was not completely penetrant since some cells contained functional contractile vacuoles while others did not. Interestingly, LvsA was able to associate with the aberrantly large contractile vacuoles in drainin-null cells (Figure 7C–D, and QuickTime Movie 4, available at [http://www.traffic.dk/videos/5\\_5\\_3bis.asp](http://www.traffic.dk/videos/5_5_3bis.asp)). This demonstrates that the binding of LvsA to contractile vacuole membranes is not dependent on drainin. Furthermore, we found that LvsA remained associated with the enlarged contractile vacuoles during the entire period of observation. In contrast, LvsA dissociated from normal contractile vacuoles as soon as they contracted. Thus, while the initial association of LvsA with the contractile vacuole is unaltered in the absence of drainin, the dissociation of LvsA from contractile vacuole membranes is dependent on vacuole contraction and, by extension, on drainin function.

#### **Association of LvsA with membranes *in vitro***

To understand the association of LvsA with membranes in better detail we analyzed the fractionation of LvsA with membranes *in vitro*. We tested the ability of GFP-tagged full-length LvsA (GFP-LvsA) to sediment with membranes at 100 000  $\times$ g in different buffer conditions. As expected for a peripheral membrane protein, LvsA was completely solubilized in buffers containing 1 M NaCl, 0.1 M Na bicarbonate or 1% Triton X-100 (data not shown). We found that pH strongly influenced the fractionation of LvsA. In a pH 7.5 buffer, conditions commonly used to fractionate *Dictyostelium* membranes, about 40–60% of GFP-LvsA sedimented with membranes (Figure 8). By contrast, 100% of GFP-LvsA sedimented at pH 6.5. Because this pH dependence of membrane association mirrors that of clathrin (Figure 8), we tested the possibility that the sedimentation of LvsA was due to an interaction with clathrin-coated membranes. We found that LvsA still sedimented with the same efficiency in clathrin heavy chain null mutants (Figure 8), indicating that this property is independent of clathrin. Similarly, the sedimentation of clathrin was not dependent on the presence of LvsA (data not shown). In addition, the loss of drainin did not disturb the ability of LvsA to sediment with the membrane fraction in drainin-null cells (data not shown).



**Figure 6: The contractile vacuole network is disrupted in LvsA mutants.** All cell lines were transformed with a GFP-dajumin expression vector to label their contractile vacuole network. A) GFP-dajumin is a membrane protein known to localize exclusively on the membranes of the contractile vacuole network, as shown in these wild-type cells. The inset shows the tubular network found near the bottom of the cells. B) In LvsA-null cells the organization of the contractile vacuole is severely disrupted. Most GFP-dajumin labeling is seen near the nucleus and at the plasma membrane. C) In GFP- $\Delta$ N689-LvsA cells, GFP-dajumin labels a collection of very small but functional contractile vacuoles near the plasma membrane of the cells. A tubular network was only rarely seen in these cells. D) The organization of the contractile vacuole in GFP- $\Delta$ N1828-LvsA cells was very similar to that of LvsA-null cells. Bar = 10  $\mu$ m.

Using this assay we examined the ability of the different LvsA truncation mutants to sediment with membranes *in vitro*. At pH 6.5, we found that GFP- $\Delta$ N689-LvsA, GFP- $\Delta$ N1828-LvsA and GFP- $\Delta$ N2888-LvsA completely sedimented, yet they only partially sedimented at pH 7.5 (Figure 8). At pH 7.5, GFP- $\Delta$ N689-LvsA sedimented almost to the same extent as full length LvsA; GFP- $\Delta$ N1828-LvsA sedimented to a lower extent. The highly overexpressed GFP- $\Delta$ N2888-LvsA sedimented about 50% at pH 7.5 in both wild-type and LvsA-null cells. Our data would suggest that the ability to interact with membranes in this sedimentation assay might involve interactions with the BEACH or the WD domains still present in all these constructs.

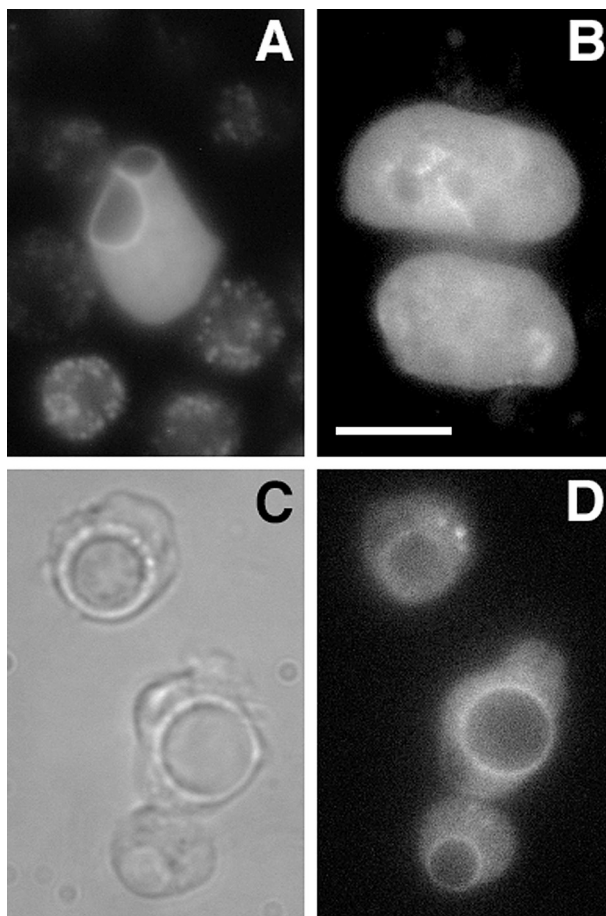
We also tested whether the membrane fraction of LvsA-null cells is competent to bind LvsA from wild-type cells. To do this we combined the supernatant fraction of GFP-LvsA cells at pH 7.5 containing soluble GFP-LvsA and mixed it with the membrane fraction from LvsA-null cells at pH 6.5 and 7.5. In the absence of added membranes, soluble GFP-LvsA did not sediment when centrifuged again at 100 000  $\times$  g (Figure 9A). In contrast, soluble GFP-LvsA sedimented readily when combined with the mem-

branes from LvsA-null cells (Figure 9A). Thus, even though the contractile vacuole network is disorganized in the absence of LvsA, the membrane fraction of LvsA-null cells is still able to recruit LvsA.

Several small GTPases have been shown to be important for the function of the contractile vacuole (15–17). Unknown GTPases have also been implicated in the association of neurobeachin with near-Golgi membranes (8). We explored the possibility that a small GTPase could regulate the association of LvsA with the membrane fraction. Figure 9B shows that the sedimentation of GFP-LvsA at pH 7.5 was not affected by concentrations of up to 30  $\mu$ M GTP $\gamma$ S. This assay suggests that the association of LvsA with membranes is not modulated by small GTPases.

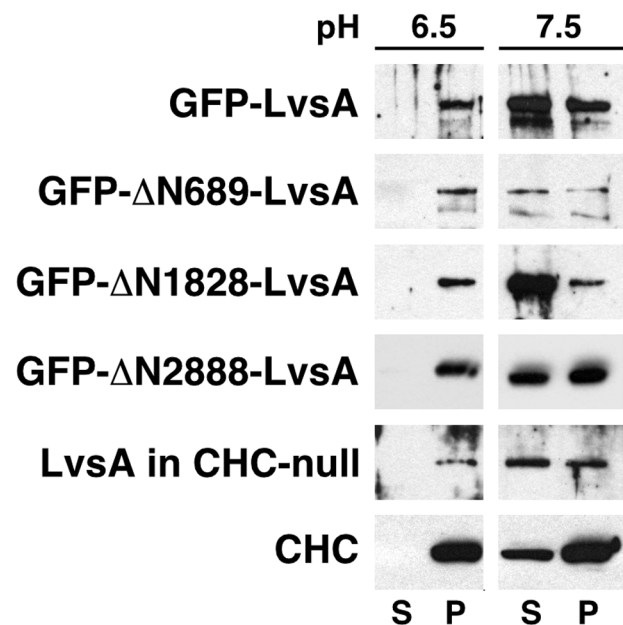
## Discussion

We have presented here a structural, biochemical and epigenetic analysis of LvsA function in *Dictyostelium* cells. We have shown that most of the LvsA protein structure is required for its function in both cytokinesis and

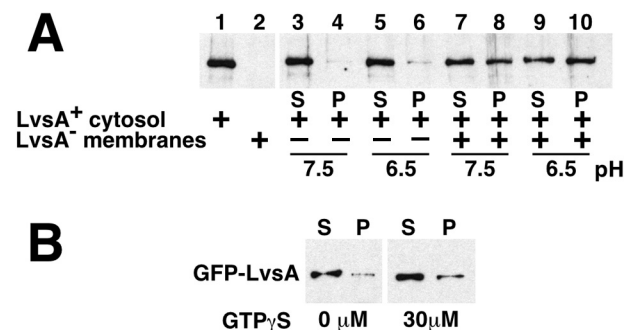


**Figure 7: Drainin is not required to recruit LvsA to the contractile vacuole but it is necessary for vacuole contraction and LvsA detachment.** Drainin is a peripheral membrane protein associated with the contractile vacuole of *Dictyostelium* cells (14). A GFP-drainin expression vector was introduced into wild-type (A) and LvsA-null cells (B). A) One wild-type cell expressing GFP-drainin is surrounded by three nonexpressing cells. GFP-drainin labels the contractile vacuole of the expressing cell. In LvsA-null cells (B), the contractile vacuole is disorganized and drainin is found mostly diffused in the cytoplasm and also bound to internal membranes. Loss of drainin function (drainin-null cells) leads to the formation of giant contractile vacuoles that are unable to expel water (14). We disrupted the *drainin* gene in our cell line expressing GFP-LvsA and observed the expected giant contractile vacuole phenotype (C). In these drainin-null cells (D), LvsA is still recruited to the enlarged contractile vacuoles but is trapped on the engorged vacuoles. Bar = 10  $\mu$ m.

osmoregulation. Despite the very low similarity between the N-terminal-domain of LvsA and that of other BEACH proteins, the majority of this region is needed for function. Even deletion of only 689 amino acids from the N-terminus of LvsA (19% of LvsA amino acid sequence) yielded a protein that was only partially functional. Cells expressing GFP- $\Delta$ N689-LvsA were able to grow in suspension at the same rate as wild-type cells but did not osmoregulate normally, and swelled when exposed to water. This defect can be attributed to the disorganization of the contractile



**Figure 8: Sedimentation of LvsA and fragments with membranes.** Cells expressing full length LvsA or truncated LvsA proteins were lysed in two different buffers (pH 6.5 and 7.5) and fractionated at 100 000  $\times g$  into pellets (P) and supernatants (S). Samples were analyzed by Western blots with antibodies against GFP, LvsA or clathrin heavy chain. At pH 6.5 all proteins partition completely with the pellet fraction, whereas at pH 7.5 they partition to various degrees between the supernatant and pellet fractions.



**Figure 9: LvsA binds to membranes *in vitro* in a GTP-independent manner.** A) To test whether the binding of LvsA onto membranes could be reconstituted *in vitro* we used a membrane fraction of LvsA-null cells and combined it with a cytosolic extract from GFP-LvsA cells. The high-speed supernatant of GFP-LvsA cells made in TKMC buffer pH 7.5 (lane 1) contains soluble LvsA that remains soluble even after re-centrifugation at 100 000  $\times g$  (at pH 7.5 or after changing the pH to 6.5, lanes 3–6). But when the same soluble LvsA is combined with the membrane fraction of LvsA-null cells (lane 2) it binds to those membranes and about 50% of it sediments at either pH (lanes 7–10). S=supernatant. P=pellet. B) To test whether the fractionation of LvsA is regulated by small GTPases we lysed GFP-LvsA cells in TKMC buffer pH 7.5 in the presence or absence of up to 30  $\mu$ M GTP $\gamma$ S. This and several more experiments demonstrated no effect of this nucleotide on the sedimentation of LvsA.

vacuole network of these cells into multiple small contractile vacuoles instead of one large vacuole per cell. Further deletions from the N-terminus of LvsA yielded proteins that were not functional in either cytokinesis or osmoregulation. These results suggest that the region between 690 and 1828 of LvsA is essential for its function in cytokinesis and osmoregulation.

Consistent with these results, our smallest construct containing only the BEACH and WD domains was not sufficient for function in an LvsA-null cell. However, unlike analogous proteins from other systems, this LvsA fragment did not produce a dominant negative effect when overexpressed in wild-type cells. Overexpression of C-terminal fragments of the protein FAN or the protein beige has been shown to generate a pronounced dominant negative effect (7,18). This difference in results may be due to the different fragments being expressed. The previously published experiments utilized a truncated BEACH domain together with the WD domain of those proteins. Our fragment GFP-ΔN2888-LvsA encompassed the entire BEACH and WD domains and about half of the PH-like domain that is found upstream of the BEACH domain. It is possible that the dominant-negative effect is exerted by a truncated BEACH domain and not by a complete BEACH domain.

One remarkable property of LvsA is its ability to bind to the membranes of the contractile vacuole at the point of its maximal expansion (11). This property clearly requires the first 689 amino acids since we did not observe any localization of GFP-ΔN689-LvsA on the small contractile vacuoles present in those cells. It is possible that this truncated protein may still bind to some extent to those contractile vacuoles, since they are functional in expelling water from cells. However, this interaction must be below the detection level of our assay. If the binding of GFP-ΔN689-LvsA were completely abrogated, then we would expect to see a complete failure in contractile vacuole function as it occurs in the LvsA-null cells or cells expressing the other truncated LvsA proteins.

In addition to the N-terminus of LvsA we found that the association of LvsA with the contractile vacuole is also controlled by the protein drainin. This protein is localized on the contractile vacuole during both the expansion and contraction phases and is important for water expulsion (14). Drainin-null mutant cells contain enlarged contractile vacuoles that fail to expel their contents. We found that drainin-null cells also contained functional contractile vacuoles in addition to the paralyzed and enlarged vacuoles. Interestingly, GFP-LvsA was localized on these enlarged vacuoles throughout the observation period, whereas it associated transiently with the functional vacuoles. These data suggest that the association of LvsA with the contractile vacuole occurs by a change in the contractile vacuole membrane that is independent of drainin. This change must represent a signal that the contractile vacuole has reached a minimum size required for

water expulsion. It has been proposed that drainin may be the sensor that signals when a vacuole has reached its filled diameter (14) but our data are not consistent with that hypothesis. It is possible that the function of drainin is to promote the formation of a docking site or pore on the plasma membrane to allow the expulsion of water. Our data suggest that the defective vacuoles of drainin mutant cells have already sensed that they have reached their maximum diameter and have recruited LvsA. Their expulsion defect seems to be downstream from LvsA binding onto the vacuole membrane, possibly in the formation of a pore or docking site. These observations also indicate that LvsA will remain on a contractile vacuole as long as it has not expelled its contents. The signal that recruited LvsA onto the expanded vacuole must be reversed when the vacuole has collapsed. Clearly, defining the molecular events that recruit and release LvsA from the contractile vacuole will be critical in understanding the pathways regulating this organelle.

To begin to define the biochemical requirements of LvsA binding to membranes we have shown here that LvsA is able to fractionate with membranes in an *in vitro* sedimentation assay. Using this assay we have demonstrated that LvsA behaves as a conventional peripheral membrane protein and that its fractionation with membranes is not modulated by GTPγS. The sedimentation properties of LvsA are also not affected by the loss of clathrin heavy chain or drainin, two important proteins required for the function of the contractile vacuole. The most important factor that modulates the sedimentation of LvsA is the pH of the assay buffer. At the slightly acidic pH 6.5, 100% of LvsA or its fragments sediment with membranes, whereas only about half sediment at pH 7.5. We find it interesting that the same behavior is observed in the sedimentation of clathrin. In the case of clathrin, it is well established that the acidification of the cytoplasm results in the formation of highly curved clathrin cages firmly attached to the plasma membrane (19). Perhaps LvsA, like clathrin, self associates into large structures that are stabilized in acidic conditions. Interestingly, we demonstrated that we could reconstitute the binding of LvsA to membranes using soluble LvsA from wild-type cells and membranes from LvsA-null cells. This assay should allow us to dissect in further detail the biochemical association of LvsA with membranes.

## Materials and Methods

### Cell culture

Cells were grown on Petri dishes with HL5 medium supplemented with 60 U/mL penicillin, 60 μg/mL streptomycin and 10 μg/mL G418 or 10 μg/mL blasticidin S as required.

### Constructs for expression of GFP-tagged LvsA fragments

We have described previously our knock-in approach to insert GFP at the amino terminus of LvsA (11). Briefly,



we engineered a construct that fused the GFP coding sequence in frame with the first 0.8 kb of the *lvsA* open reading frame. The GFP-fusion construct was placed under the control of the *Dictyostelium* actin-6 promoter. The construct also contained 1.5 kb of 5' *lvsA* untranslated sequences and a blasticidin-resistance cassette. Cell line MS2H10 is a well-characterized clone that contains our construct inserted at the *lvsA* locus and produces the full length LvsA protein tagged by GFP at its N-terminus. This cell line is referred in this paper simply as GFP-LvsA cells.

We used similar constructs to insert the actin-6 promoter/GFP portion at internal sites within the *lvsA* coding region, thereby producing LvsA N-terminus truncation mutants (Figure 1). For one construct we amplified by polymerase chain reaction (PCR) a 1.2 kb portion of the *lvsA* gene (from nucleotide 3729–4983 of the *lvsA* sequence AF088979) and cloned it in frame with the GFP coding region of the knock-in construct described above. Insertion of this construct into the *Dictyostelium* genome by a double cross-over resulted in the deletion of about 2.2 kb of the *lvsA* gene and in the expression of a GFP-tagged LvsA protein missing the first 659 amino acids. For another construct we amplified a 1.1 kb portion (from nucleotide 7227–8357) of the *lvsA* gene and cloned it in frame with GFP as above. This construct leads to the expression of a GFP-tagged LvsA protein missing 1828 amino acids from its N-terminus. These constructs were transformed into *Dictyostelium* NC4A2 wild-type cells, screened for the correct insertion by PCR and verified by Western blot analysis as described (11). Cell line MS17E3 is one clone in which the first knock-in construct inserted as predicted and is referred to in this paper as GFP-ΔN689-LvsA cells. Cell line MS6G5 is a clone generated with the second construct and is referred to here as GFP-ΔN1828-LvsA.

To produce a C-terminus truncated mutant we engineered a construct in which the GFP coding region was fused in frame with a 0.9 kb portion of the *lvsA* coding region (from nucleotides 10875–11788). This fusion construct was not preceded by the actin-6 promoter. This portion was then followed by the blasticidin-resistance cassette and a 1 kb fragment (nucleotides 11802–12864) from the 3' end of the *lvsA* gene. Insertion of this construct into the *lvsA* gene leads to the expression (driven by the endogenous *lvsA* promoter) of an LvsA protein in which 271 amino acids from its C-terminus (the WD domain) have been replaced by GFP. Cell line WW1C4 is one clone that contained the appropriate insertion and is referred to here as ΔC271-LvsA-GFP.

The smallest truncation mutant (GFP-ΔN2888-LvsA) was small enough to clone in a *Dictyostelium* expression vector. We cloned a 2.4 kb fragment (nucleotides 10409–12818) encoding amino acids 2889–3619 of LvsA at the 3' end of the GFP coding region in the expression vector pTX-GFP (20). This is an extrachromosomal high copy number vector that carries a G418-resistance selectable

marker. This vector was introduced into wild-type NC4A2 cells and into LvsA-null cells (strain VIG9).

### Disruption of drainin in GFP-LvsA cells

To observe the distribution of GFP-LvsA in the absence of the protein drainin we disrupted the *drainin* gene in our cell line MS2H10 that expresses GFP-LvsA.

We engineered a drainin knockout construct designed to insert a G418-selectable marker within the second exon of the *drainin* gene. Two segments of the *drainin* gene were amplified from *Dictyostelium* genomic DNA using primer pairs GGATTTCAAAACCATCAAAACAAAGC + CATCTAGTTTCTCTTTTCTTTTGCTTCTC and AGATGCAAGAAAGGTATGGGAAGAGG + GATCTATTTAATG-GATTTTACCACCCCTG. The amplified fragments of 716 bp and 1192 bp were cloned flanking a G418-selectable cassette. The final construct was linearized and transformed into MS2H10 cells. Clonal transformants were screened by PCR for those that contained the plasmid inserted into the *drainin* gene.

### Expression of GFP-dajumin and GFP-drainin

Expression vectors for the production of GFP-dajumin (13) and GFP-drainin (14) were the kind gift of Dr. Guenther Gerisch (Max-Planck-Institut für Biochemie). These vectors carry the G418 selectable marker and were introduced into wild-type and LvsA mutant cells.

### Live observation of GFP-labeled proteins

Cells expressing the different GFP-labeled proteins were allowed to adhere to #1 coverglass-chambers (Nalge-Nunc Int., Naperville, IL) and were incubated in low fluorescence (LoFlo) medium (21) for 1–2 h to reduce autofluorescence from the HL5 nutrient medium. To observe the osmoregulatory response of the cells, the LoFlo medium was replaced with distilled water. Cells were imaged on an inverted Nikon Microscope TE200 (Nikon Instruments, Dallas, TX) equipped with a 100 × 1.4 NA PlanFluor Objective, shuttered illumination, and a Quantix 57 camera (Roper Scientific, Tucson, AZ) controlled by Metamorph (Universal Imaging Corp., West Chester, PA). Epifluorescence illumination was shuttered at 100 ms and the interval between acquisitions was 5 s.

### Protein fractionation assays

To determine the fractionation properties of LvsA and its truncation mutants, cells were collected by centrifugation at 1000 × g for 3 min and washed in either cold Isolation Buffer (10 mM morpholinoethanesulfonic acid, pH 6.5, 50 mM KOAc, pH 6.5, 0.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM dithiothreitol, and 0.02% NaN<sub>3</sub>) or MES wash buffer (20 mM morpholinoethanesulfonic acid, pH 6.8, 2 mM MgSO<sub>4</sub>, 0.2 mM CaCl<sub>2</sub>). Cells were pelleted and resuspended to 4 × 10<sup>7</sup> cells/mL in cold Isolation Buffer or TKMC Buffer (20 mM N-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.5, 25 mM KCl, 5 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>) supplemented

with 1:100 Fungal Protease Inhibitor Cocktail (Sigma, St. Louis, MO). Immediately, cells were lysed by shearing through a 25-mm syringe filter holder (Gelman, Ann Arbor, MI) containing two prewet 5- $\mu$ m polycarbonate membrane filters (Osmonics, Minnetonka, MN). The lysate was centrifuged at  $3000 \times g$  for 5 min. The supernatant was collected and centrifuged at  $100\,000 \times g$  for 1 h at 4°C. Membrane pellet and cytosolic supernatant samples were resolved on 7.5% SDS-PAGE gels, transferred to membranes and probed with anti-GFP or anti-LvsA polyclonal antibodies following standard methods.

To determine whether the association of LvsA with the  $100\,000 \times g$  pellet was typical of a peripheral membrane, protein membrane fractions were prepared in either Isolation Buffer or TKMC Buffer as described above. After centrifugation at  $3000 \times g$ , the supernatant was supplemented with 1 M NaCl, 1% Triton X-100, or 100 mM  $\text{Na}_2\text{CO}_3$ . Membrane and cytosolic samples were fractionated at  $100\,000 \times g$  for 1 h at 4°C and analyzed as before.

To determine whether soluble LvsA could bind to membrane pellets from LvsA-null cells, we prepared supernatant and pellet fractions from wild-type and LvsA-null cells in TKMC Buffer as described above. After sedimentation at  $100\,000 \times g$ , the wild-type cytosolic sample was combined with the LvsA-null membrane fraction. The pH of the wild-type cytosolic fraction (with or without LvsA-null membrane fraction) was maintained at its original pH, 7.5, or lowered to pH 6.5. These samples were fractionated once more at  $100\,000 \times g$  for 1 h at 4°C and the pellet and supernatant fractions analyzed as before.

To determine whether GTP $\gamma$ S influenced the fractionation of LvsA, we prepared lysates from wild-type cells in the presence of increasing concentrations of GTP $\gamma$ S. Samples were fractionated at  $100\,000 \times g$  and analyzed as before.

## Acknowledgments

We wish to thank all members of the De Lozanne and O'Halloran Laboratories for their comments and input throughout this work. We also thank Dr. Guenther Gerisch (Max-Planck-Institut für Biochemie) for providing us with expression vectors for GFP-dajumin and GFP-drainin. This work was supported by NIH grant GM48745.

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