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LvsA, a Protein Related to the Mouse Beige Protein, Is Required for Cytokinesis in *Dictyostelium*[V]

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We isolated a *Dictyostelium* cytokinesis mutant with a defect in a novel locus called *large volume sphere* A (*lvsA*). *lvsA* mutants exhibit an unusual phenotype when attempting to undergo cytokinesis in suspension culture. Early in cytokinesis, they initiate furrow formation with concomitant myosin II localization at the cleavage furrow. However, the furrow is later disrupted by a bulge that forms in the middle of the cell. This bulge is bounded by furrows on both sides, which are often enriched in myosin II. The bulge can increase and decrease in size multiple times as the cell attempts to divide. Interestingly, this phenotype is similar to the cytokinesis failure of *Dictyostelium* clathrin heavy-chain mutants. Furthermore, both cell lines cap ConA receptors but form only a C-shaped loose cap. Unlike clathrin mutants, *lvsA* mutants are not defective in endocytosis or development. The LvsA protein shares several domains in common with the molecules beige and Chediak–Higashi syndrome proteins that are important for lysosomal membrane traffic. Thus, on the basis of the sequence analysis of the LvsA protein and the phenotype of the *lvsA* mutants, we postulate that LvsA plays an important role in a membrane-processing pathway that is essential for cytokinesis.

INTRODUCTION

Cytokinesis is the complex process by which a mitotic cell separates into two daughter cells. This process appears to be morphologically quite different between plant and animal cells. Plants build a new cell wall at the equatorial plane to divide the two daughter cells (Wick, 1991). In contrast, animal cells divide by constriction of a cleavage furrow driven by the contractile ring (Rappaport, 1996). However, we believe that these apparent differences reflect specializations imposed by the presence of cell walls in plant cells rather than separate evolutionary origins of the mechanisms that regulate cell division. Indeed, a common origin for cytokinesis is reflected in organisms that diverged earlier than animal and plant cells (such as *Dictyostelium* and *Acanthamoeba*); these primitive cells divide by forming a contractile ring and a cleavage furrow (Fukui and Inoue, 1991; Yonemura and Pollard, 1992). Thus, it is likely that many of the molecular mechanisms that control cytokinesis are highly conserved throughout evolution.

The list of proteins that are known to be required for cytokinesis is continuously expanding and helping to delineate the molecular pathways involved in the regulation of cytokinesis. Among these, cytoskeletal components clearly play a central role in cell division. For example, mutations in myosin II (De Lozanne and Spudich, 1987; Chen *et al.*, 1994; Edamatsu and Toyoshima, 1996), tropomyosin (Chang *et al.*, 1996), profilin (Balasubramanian *et al.*, 1994; Haugwitz *et al.*, 1994), and cortexillin (Faix *et al.*, 1996) cause a strong defect in cytokinesis. Similarly, regulatory proteins, such as the rho

[V] Online version of this article contains video material for Figure 4. Online version available at www.molbiolcell.org.

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Abbreviations used: BEACH, beige and Chediak–Higashi; CHS, Chediak–Higashi syndrome protein; ConA, concanavalin A; DAPI, 4,6-diamidino-2-phenylindole; DIC, differential interference contrast; FAN, factor associated with neutral-sphingomyelinase activation; GFP, green fluorescent protein; *lvsA*, *large volume sphere* A; PBS, phosphate-buffered saline; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); REMI, restriction enzyme–mediated integration; TKMC, 20 mM *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 0.1 mM $CaCl_2$.

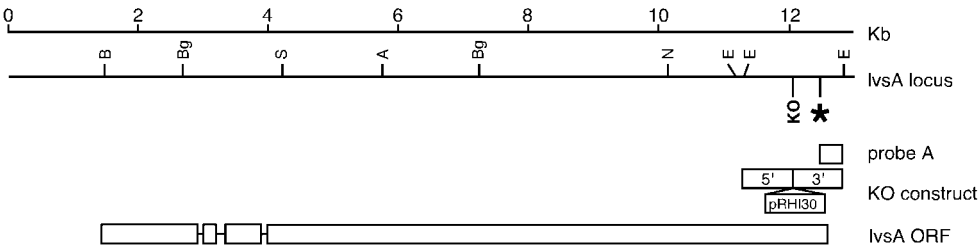

Figure 1. The *Dictyostelium lvsA* locus. The *lvsA* locus was mutated by REMI. The * denotes the site of insertion of plasmid pRHI30 used for REMI. Probe A is the portion of the *lvsA* gene that was cloned from the REMI mutant AD60. A larger fragment was used to design a knockout construct (KO construct) using plasmid pRHI30 as a transformation vector. The letters KO on the *lvsA* locus indicate the site of plasmid insertion in the knockout strains. The *lvsA* ORF is interrupted by three small introns at the positions indicated. Restriction sites indicated are the following: A, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; N, *Nde*I; and S, *Sph*I.

family of small GTPases (Mabuchi *et al.*, 1993; Dutartre *et al.*, 1996; Larochelle *et al.*, 1996), and the IQGAPs (Faix and Dittrich, 1996; Adachi *et al.*, 1997) are very important for the regulation of the cytoskeleton during cell division. In contrast, the roles of other proteins essential for cytokinesis are not well understood. For example, the septins are required for cytokinesis in yeast and fly cells, but their exact role has not been defined (Cooper and Kiehart, 1996). Similarly, an essential role for clathrin in cytokinesis has been demonstrated recently (Niswonger and O'Halloran, 1997b). These results suggest that multiple processes, including membrane transport pathways, may play important roles during cell division.

Recent studies have indicated that cells may use more than one mechanism to accomplish cytokinesis. *Dictyostelium* myosin II–null cells, which are completely unable to furrow and cleave in suspension culture, can still divide when attached to a substrate (Neujahr *et al.*, 1997b). This myosin II-independent process is not as efficient as that driven by myosin II but, nonetheless, is quite effective in dividing the cells (Zang *et al.*, 1997). Thus, cells may have myosin-dependent (cytokinesis A) and myosin-independent (cytokinesis B) processes to complete cell division (Zang *et al.*, 1997).

To define the cellular components that are required for cytokinesis, we devised a genetic screen to search for *Dictyostelium* cytokinesis mutants (Vithalani *et al.*, 1996). This screen (designed to isolate mutants that fail to divide in suspension culture) identified proteins required for cytokinesis independently of adhesion to the substrate. Using this screen, we identified previously a member of the rho family of proteins, racE, that is essential for cytokinesis (Larochelle *et al.*, 1996). Using the same screen, we have identified another novel *Dictyostelium* cytokinesis mutant. This article describes the cloning of the gene affected in this mutant (*large volume sphere* A [*lvsA*]) and phenotypic characterization of the mutant cells. This analysis suggests that the LvsA protein is a signaling molecule that may define a novel membrane-processing pathway required for cytokinesis.

MATERIALS AND METHODS

Nomenclature

We have followed the nomenclature guideline for *Dictyostelium* strains and genes as recommended in http://dicty.cmb.nwu.edu/dicty/dicty.html.

Dictyostelium Mutagenesis and Screen for Cytokinesis Mutants

Dictyostelium cells were mutagenized by restriction enzyme–mediated integration (REMI) (Kuspa and Loomis, 1992) and screened for those mutants with defects in cytokinesis as described previously (Larochelle *et al.*, 1996; Vithalani *et al.*, 1996). Briefly, *Dictyostelium* DH1 cells were transfected with plasmid pRHI30 (linearized with *Bgl*II) in the presence of the restriction enzyme *Dpn*II. Individual colonies were selected in minimal medium in 96-well plates. The colonies were then replica-plated onto two 24-well plates. One 24-well plate was shaken at 240 rpm to maintain the cells in suspension, while the other plate remained stationary. After several days, plates were examined for clones that grew under stationary conditions but not under shaking conditions. After screening >10,000 mutagenized clones, we identified a total of six mutant cell lines: two in myosin II, two in the small GTPase racE (Larochelle *et al.*, 1996), one in an uncharacterized locus (Vithalani *et al.*, 1996), and one, called AD60, that is described here.

Cloning of the lvsA Gene

Initial attempts to rescue the plasmid inserted into the *lvsA* locus were not successful, possibly because of deletions or rearrangement of the sequences essential for plasmid maintenance in bacteria (Vithalani *et al.*, 1996). We then used the following PCR-based approach to clone the genomic sequences flanking the pRHI30 plasmid inserted within the *lvsA* gene. Genomic DNA from the cytokinesis-defective REMI cell line AD60 was digested with a panel of restriction enzymes, and the resulting fragments were analyzed on Southern blots using plasmid pRHI30 as a probe. This probe hybridized to an ~5-kb *Eco*RI genomic fragment that is slightly larger than plasmid pRHI30. Therefore, *Eco*RI-digested genomic DNA from this cell line was separated on a 0.7% agarose gel, and fragments in the region of 4.5–5.5 kb were isolated from the gel and purified by the Geneclean (Bio101, Vista, CA) protocol. This population of purified fragments was ligated to an *Eco*RI primer adapter consisting of the following annealed primers: 5′-AAT TCC ATG GCT GCA GTG GCC AGC G-3′ (AO-182) and 5′-CGC TGG CCA CTG CAG CCA TGG-3′ (AO-183). The primer AO-183 and a primer from the pRHI30 vector (AO-118) were used to amplify intervening sequences using PCR. This approach yielded a 350-bp fragment (Figure 1, probe A) from only one side of the inserted plasmid. The PCR-generated clone was subsequently used as a probe to screen multiple *Dictyostelium* genomic libraries. Additional *lvsA* gene sequences were obtained by plasmid rescue and inverted-PCR amplification. Finally, cDNA clones were obtained by reverse transcription–PCR.

The different clones were sequenced on both strands using manual or automated sequencing at the Duke Sequencing Facility (Durham, NC). The sequences were compiled into a contiguous sequence of almost 13 kb (GenBank accession number AF088979) using the Lasergene software (DNAstar, Madison, WI). Comparison

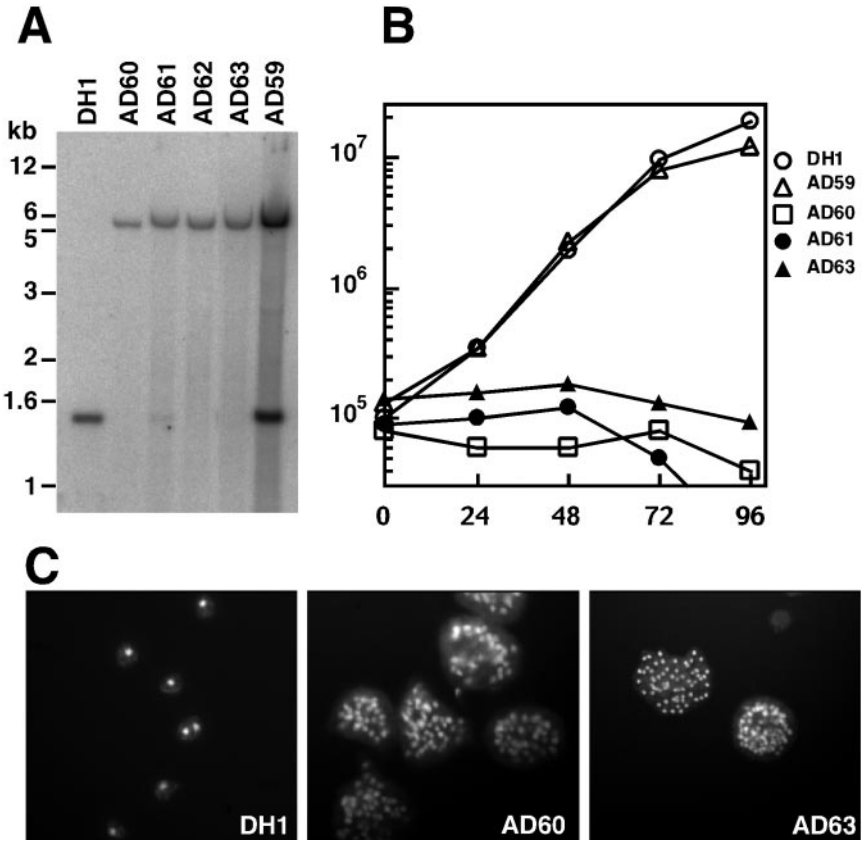

Figure 2. *Dictyostelium lvsA* mutants are defective in cytokinesis. (A) Southern blot analysis of genomic DNA from *Dictyostelium* cells is shown. The DNA was digested with *Eco*RI, and the blot was probed with probe A (Figure 1). This probe detects a 1.5-kb *Eco*RI band in DNA from wild-type (DH1) and control (AD59) cells. This band is replaced by a 6-kb band in the *lvsA* REMI mutant (AD60) and knockout strains (AD61–AD63). (B) *LvsA* mutants cannot grow in suspension culture. The different strains were placed in suspension culture in nutrient-rich medium. Wild-type and control cells grow at fast rates under these conditions. In contrast, the *lvsA* REMI mutant (AD60) and knockout strains (AD61 and AD63) fail to grow. (C) *LvsA* mutants fail in cytokinesis. Cells were fixed after being in suspension for 2 d and were stained with DAPI. Most wild-type (DH1) cells have one or two nuclei because they divide normally in suspension. The *lvsA*-mutant strains (AD60 and AD63) accumulate numerous nuclei because they fail to divide.

of the genomic and cDNA sequences revealed a large open reading frame encoding a protein (LvsA) of 3619 amino acids and predicted molecular mass of 408 kDa. The open reading frame is interrupted by three introns of 84, 127, and 82 bp

Disruption of the lvsA Gene by Homologous Recombination

To confirm that the REMI disruption of the *lvsA* gene caused a cytokinesis defect, we designed a construct to knock out the *lvsA* gene by homologous recombination. The 1.5-kb *Eco*RI fragment near the 3′ end of the *lvsA* gene (Figure 1) was divided into equally sized fragments using PCR amplification as follows. A Bluescript plasmid containing the 1.5-kb *Eco*RI fragment was used as a template in two PCR reactions: one with *lvsA* primer AO-197 (GAA GAT CTT CCA ATA CAA GAG ATT GGA CC) and a vector primer and the other with *lvsA* primer AO-198 (CCA TCG ATG GGG TAT TCA TGA TAC AAC TGG) and a vector primer. The *lvsA* primers incorporated a *Bgl*II and *Cla*I site, respectively. The PCR products were digested with *Eco*RI to eliminate vector sequences and were ligated together through their *Eco*RI ends. The ligation product was digested with *Bgl*II and *Cla*I and cloned into the *Bgl*II and *Cla*I sites of vector pRHI30. The resultant plasmid (pEKKO-1) has a single *Eco*RI site joining the 5′ and 3′ segments of the *lvsA* gene (Figure 1). Plasmid pEKKO-1 was linearized with *Eco*RI and introduced into DH1 cells by electroporation. Clones of transformed cells were selected in minimal medium in 96-well plates, and individual clones were tested for their ability to divide in suspension culture. Out of 94 clonal transformants, 11 clones failed to grow and became large and multinucleate. Southern blot analysis of three of these cell lines demonstrated that the knockout plasmid had been inserted by homologous recombination into the *lvsA* gene. Probe A hybridized to a 1.5-kb *Eco*RI fragment from the *lvsA* gene in both wild-type (DH1) and control (AD59) cells (Figure 2A). The same probe hybridized to a 6-kb *Eco*RI fragment in the REMI mutant (AD60) and knockout strains (AD61–AD63) because of the plasmid insertion in the *lvsA* gene.

Cell Culture

For most experiments, cells were grown on Petri dishes with HL5 medium (Sussman, 1987) supplemented with 60 U/ml penicillin and 60 μg/ml streptomycin. Cells that carried the green fluorescent protein (GFP)–myosin II expression vector (Moores *et al.*, 1996) were grown in medium supplemented with 10 μg/ml G418.

To enrich for mitotic cells, we used the protocol described previously (Gerald *et al.*, 1998). Briefly, cells cultured on Petri dishes were collected while still in log-phase growth. The cells were washed twice in fresh HL5 and allowed to attach to coverslips in a humid chamber or placed in a flask for shaking culture at 240 rpm. Samples were taken at various time points for fixation and microscopy.

Microscopy of Live Cells

Cells undergoing mitosis were viewed under phase-contrast microscopy in attached conditions as described previously (Gerald *et al.*, 1998). To observe cells in suspension conditions, we placed the cells in a solution of low–melting temperature agarose as described (Gerald *et al.*, 1998). Video images were digitized for montage and QuickTime movies as described (Gerald *et al.*, 1998). Ten video frames were averaged to form each digital image taken at 5-s intervals.

Microscopy of Fixed Cells

Cells were fixed for microscopy as described previously (Gerald *et al*., 1998). Briefly, adherent cells were fixed on coverslips with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min and permeabilized with 0.05% Triton X-100 in PBS for 2 min. Cells in suspension were fixed by adding an equal volume of cells to 2× fixative solution (7.4% formaldehyde in PBS) and inverting for 15 min. The cells were sedimented in a tabletop clinical centrifuge and allowed to settle onto poly-L-lysine–coated coverslips in the presence of fixative for 10–15 min. After attachment, the fixed cells were treated similarly to the adherent cells.

Cells were also fixed with picric acid as described (Humbel and Biegelmann, 1992; Neujahr *et al*., 1997a). Adherent cells were fixed with 2% paraformaldehyde, 15% of a saturated aqueous solution of picric acid, and 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 6.5, for 30 min. Cells were washed with 10 mM PIPES, pH 6.5, and then in PBS containing 100 mM glycine. Cells were post-fixed with 70% ethanol for 10 min with subsequent washes in PBS–glycine. For cells in suspension, an equal volume of cells was added to 2× fixative solution (4% paraformaldehyde, 30% picric acid, 10 mM PIPES, pH 6.5) and inverted for 15 min. Cells were spun down and allowed to settle onto poly-L-lysine coverslips in the presence of fixative for 10–15 min. Coverslips were then treated as adherent samples.

After fixation, nuclei were stained with 10 μM 4,6-diamidino-2-phenylindole (DAPI) and 50 mM ammonium chloride in PBS for 10 min. Cells were also stained for F-actin with 66 nM rhodamine–phalloidin (Molecular Probes, Eugene, OR) in PBS for 30 min in a covered humid chamber at room temperature. The coverslips were washed three times for 5 min in PBS, rinsed in distilled water, and mounted onto glass slides with 0.01 ml of mounting media (50% glycerol, 100 mg/ml 1,4-diazabicyclo-[2.2.2]octane in PBS).

To determine the distribution of myosin II, we transformed each cell line with a GFP–myosin II heavy-chain expression plasmid (Moores *et al*., 1996). The resulting cells were fixed as before and stained with DAPI. The fixation methods used here preserved the fluorescent GFP signal.

Samples were viewed on a Zeiss (Oberkochen, Germany) axioplan microscope equipped with a 1.4–numerical aperture 100× oil objective. Images were obtained with a Star I Photometrics (Tucson, AZ) cooled charge-coupled device camera using IPlab software (Signal Analytics, Vienna, VA). Some images were obtained from a Leica (Nussloch, Germany) upright microscope equipped a 1.4–numerical aperture 100× oil objective with a KHF1400 Photometrics cooled charge-coupled device camera using PMIS Image Processing Software (Photometrics). Adobe Photoshop 5.0 (San Jose, CA) was used to adjust the contrast of the digital images. The Photoshop unsharp mask filter was also applied to differential interference contrast (DIC) images. Images of the DAPI staining were merged with those of F-actin or GFP–myosin staining using the "add image" function of Photoshop.

Capping of Concanavalin A Receptors

The ability of the different strains to cap cell surface receptors was assayed using FITC-labeled concanavalin A (ConA; Sigma Chemical, St. Louis, MO) as described (Larochelle *et al*., 1996).

Dictyostelium Development

The development phenotype of the *lvsA*-mutant cells was assessed on a bacterial lawn on an SM/5 agar plate as described (Vithalani *et al*., 1996).

Pinocytosis Assay

Fluid-phase uptake of tetramethylrhodamine isothiocyanate-dextran (Sigma Chemical) was performed as described (Hacker *et al*., 1997). Cells were equilibrated 30 min at 21°C before beginning each assay. Two time points were averaged for each data point shown.

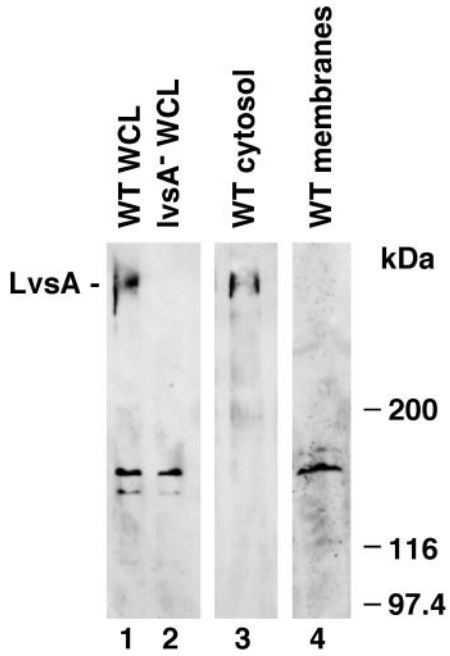

Figure 3. The LvsA protein is absent in *lvsA*-mutant cells and partitions in the cytosol. Whole-cell extracts (WCL) from wild-type (WT) and *lvsA*-mutant (*lvsA*$^-$) cells were analyzed by Western blot analysis with a polyclonal anti-LvsA antibody. A band of the predicted molecular weight for LvsA (408 kDa) is present in wild-type cells but not in the *lvsA*-mutant cells. The same band is present in the cytosolic fraction and absent from the membrane fraction of extracts from wild-type cells. Bands below 200 kDa are nonspecific and present in the preimmune serum.

Fractionation of Cell Extracts

Dictyostelium membranes were prepared as described previously (Cardelli *et al*., 1987). Briefly, 2 × 10^8 DH1 cells were pelleted at 1000 × *g* for 3 min and washed in cold 2-(*N*-morpholino)ethanesulfonic acid buffer (20 mM 2-[*N*-morpholino]ethanesulfonic acid, pH 6.8, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$). Cells were pelleted and resuspended to 4 × 10^7 cells/ml in 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 0.1 mM $CaCl_2$ (TKMC) with 0.25 M sucrose. Cells were lysed through a 25-mm syringe filter holder (Gelman, Ann Arbor, MI) containing two prewet 5-μm polycarbonate filters (Poretics, Livermore, CA). Protease inhibitors were added immediately (0.1 mg/ml PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 0.1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone, 0.1 mM *N*∀-*p*-tosyl-L-lysine chloromethyl ketone, 20 mM sodium bisulfite). Unlysed cells and nuclei were pelleted at 1000 × *g* for 3 min. The postnuclear supernatant was loaded over 4.5 ml of TKMC in 15% sucrose with a 0.3-ml TKMC–70% sucrose cushion and spun at 100,000 × *g* for 30 min at 4°C. Cell membranes settled in a band at the 70% sucrose interface. The top 0.75 ml of the 0.25 M sucrose fraction was collected as the cytosolic sample. Intervening fractions were carefully removed and discarded, and membranes were collected along with the 70% sucrose cushion.

Western Analysis

We raised a polyclonal antibody against the C-terminal 82.5-kDa portion of the LvsA protein. This portion of the LvsA protein was expressed in bacteria as a fusion protein with GST. The fusion protein was purified as described previously for racE–GST fusion proteins (Vithalani *et al.*, 1998). The fusion protein was used to raise polyclonal anti-LvsA antibodies in rabbits (Cocalico Biologicals, Reamstown, PA). The polyclonal anti-LvsA antibodies were affinity purified to the GST–LvsA fusion protein blotted onto nitrocellulose as described (Pollard, 1984). Purified antibody was used at a 1:100 dilution on Western blots.

Dictyostelium whole-cell lysates (1×10^6 cells/lane) or the fractionated membranes (5×10^6 cells/lane) and cytosol (3×10^5 cells/lane) were separated on a 7.5% low-bis-acrylamide SDS-PAGE as described (Koonce and McIntosh, 1990). The gel was then processed for Western blot analysis with the anti-LvsA antibody described above.

RESULTS

lvsA, a Novel Locus Required for Cytokinesis in Dictyostelium

Using a genetic screen designed to identify *Dictyostelium* proteins required for cytokinesis (Larochelle *et al.*, 1996; Vithalani *et al.*, 1996), we identified a novel mutant cell line (AD60) that failed to grow in suspension cultures. Under these conditions, these cells were unable to divide (Figure 2B) although they continued to progress through the cell cycle. Consequently, the AD60 cells became very large and multinucleate (Figure 2C) and died after several days in culture (our unpublished observations). We named the locus affected in the mutant cells *lvsA* for the bloated and round appearance of these cells when grown in suspension. Because the AD60 cell line was generated by restriction enzyme–mediated integration (Kuspa and Loomis, 1992), we used the inserted plasmid (pRHI30) to clone the *lvsA* locus (Figure 1).

Sequencing of the entire *lvsA* locus revealed a large open reading frame encoding a predicted protein (LvsA) of 3619 amino acids. This analysis revealed that the plasmid insertion in the REMI mutant AD60 occurred near the 3′ end of the *lvsA* locus (Figure 1, asterisk).

To confirm that the deficiency in cytokinesis exhibited by strain AD60 was caused by a plasmid insertion into the *lvsA* gene, we disrupted the copy of this gene in a wild-type strain (DH1). We designed a knockout construct using a portion of the *lvsA* gene and plasmid pRHI30 (Figure 1). Homologous recombination of this construct into the *lvsA* gene resulted in the insertion of plasmid pRHI30 ~400 bp upstream of the original REMI insertion, well within the coding region of the *lvsA* gene (Figure 1). Every cell line that harbored a disruption of the *lvsA* gene failed to grow in suspension and became large and multinucleate (Figure 2, cell lines AD61–AD63). Cells in which the *lvsA* gene was not disrupted did not have any defect and behaved like wild-type cells (Figure 2, cell line AD59). These results corroborated the requirement of the *Dictyostelium lvsA* gene for cytokinesis in suspension culture.

Although both insertional mutations described above occur near the 3′ end of the long *lvsA* gene, we found two lines of evidence that these mutations produce a null phenotype with the total loss of LvsA protein. First, although we could readily detect *lvsA* mRNA by reverse transcription–PCR in wild-type cells, we could not do so in any of our mutant cell lines (our unpublished observations). Second, we performed Western analysis of wild-type and mutant cell lysates using a polyclonal anti-LvsA antibody. This antibody detected an ~400 kDa band in wild-type cells that was absent in the mutant cells (Figure 3, lanes 1 and 2).

The Cytokinesis Defect of lvsA Mutants

To understand the nature of the cytokinesis defect in *lvsA* mutants, we compared the mitotic behavior of both wild-type and mutant cells. When attached to a substrate, both cell lines formed cleavage furrows that divided the cells swiftly (our unpublished observations). In these conditions, *lvsA* mutants did not accumulate a large number of nuclei (our unpublished observations). Thus, *lvsA*-mutant cells did not exhibit a defect in cytokinesis when attached to a substrate.

In contrast to the attached conditions, cytokinesis in *lvsA*-mutant cells was completely abrogated in suspension culture (Figure 2, B and C). Interestingly, *lvsA*-mutant cells initiated cytokinesis in suspension quite normally. The cells elongated and formed an initial cleavage furrow (Figure 4C, time 0). However, as the cells progressed through cytokinesis, the ingression of the cleavage furrow was disrupted by the appearance of a large "bulge" in the middle of the cell (Figure 4, B and C, arrowheads, and QuickTime movies). This abnormal structure could be interpreted as a bleb (a random extrusion of membrane as seen in *racE*-null cells), but we believe it is a different structure for several reasons. In contrast to such blebs, the bulge formed very slowly and contained cytoplasmic organelles (see Figure 6, DIC). Furthermore, the bulge often changed in size, sometimes reaching the same diameter as the rest of the cell (Figure 4 and QuickTime movies). Eventually, the cells rounded up and failed in their cleavage attempt.

A possible mechanism for the cytokinesis failure in *lvsA*-mutant cells is a defect in the organization of their cytoskeleton during this complex process. To test this possibility, we determined the distribution of actin and myosin II in cells undergoing mitosis. When attached, wild-type cells distributed their actin filaments at the cell cortex, with a particularly prominent accumulation of actin at their polar regions in filopodia and membrane ruffles (Figure 5, top left). The *lvsA*-mutant cells also accumulated actin at polar ruffles and filopodia, but in contrast to wild-type cells, actin was also accumulated on broad lamellipodia that were firmly attached to the substrate (Figure 5, bottom left). In these attached conditions, both wild-type and mutant cells concentrated myosin II in the cleavage furrow region (Figure 5, right). This relatively normal cytoskeletal distribution mirrored our observations of apparently normal cytokinesis in attached *lvsA*-mutant cells.

In suspension conditions, wild-type cells accumulated actin at their cortex and polar regions and localized myosin II at their cleavage furrows (Figure 6, top). In the *lvsA*-mutant cells, the distribution of the cytoskeleton appeared normal during the initial stages of cytokinesis. Actin concentrated in polar ruffles, and myosin II began to concentrate at the incipient cleavage furrow (Figure 6, middle). However, as described above, the cleavage furrow was then disrupted by the appearance of a bulge (Figure 6, bottom). Remarkably, myosin II remained at the equatorial region of the cell and

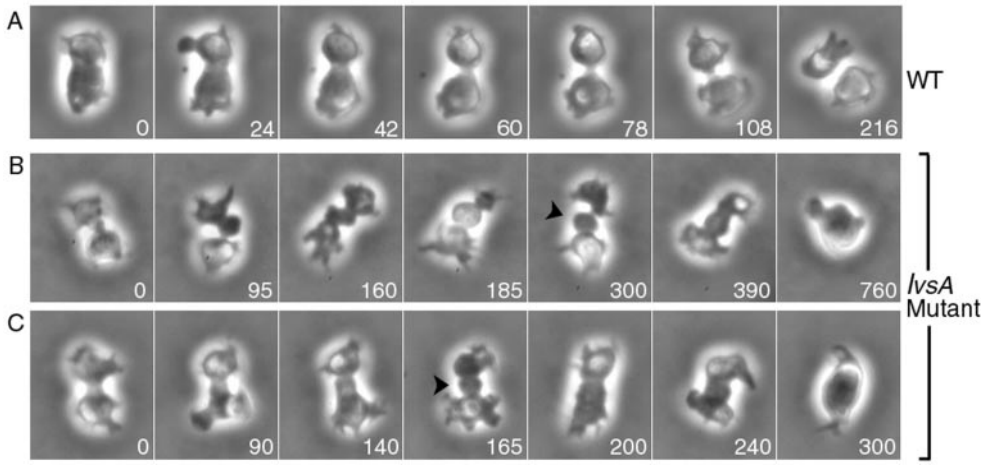

Figure 4. *LvsA*-mutant cells fail in cytokinesis with an abnormal morphology. *LvsA*-mutant and control cells were videotaped while undergoing mitosis in suspension conditions. (A) Control cells can form a normal cleavage furrow that rapidly cleaves the cell in two (QuickTime movie available online). (B and C) Early in cytokinesis, the *lvsA*-mutant cells form what appears to be a normal cleavage furrow (QuickTime movies available online). However, the furrow is then disrupted by the formation of a large bulge in the middle of the cell (arrowheads). This bulge grows and shrinks as the cells attempt to divide. Ultimately, cytokinesis fails, and the cells revert to a round shape.

often bounded the bulge (Figure 6, bottom), sometimes forming what appeared to be two distinct contractile rings (our unpublished observations).

lvsA Mutants Share Some Phenotypic Characteristics of Dictyostelium Clathrin Mutants

Our observations of the *lvsA*-mutant cells indicate that their cytokinesis phenotype is quite distinct from that of most previously characterized *Dictyostelium* cytokinesis mutants. Interestingly, the abnormal morphology of dividing *lvsA*-mutant cells is most reminiscent of that of clathrin-null mutants. These cells, which were shown to be defective in cytokinesis (Niswonger and O'Halloran, 1997b), also form a bulge in the middle of their furrows as they try to divide (Gerald, Damer, and O'Halloran, unpublished observations). Because in addition to the cytokinesis deficiency the *Dictyostelium* clathrin-null cells display several characteristic phenotypes (Ruscetti *et al*., 1994; Niswonger and O'Halloran, 1997a), we compared the phenotype of clathrin and *lvsA* mutants to determine whether they shared other cellular functions.

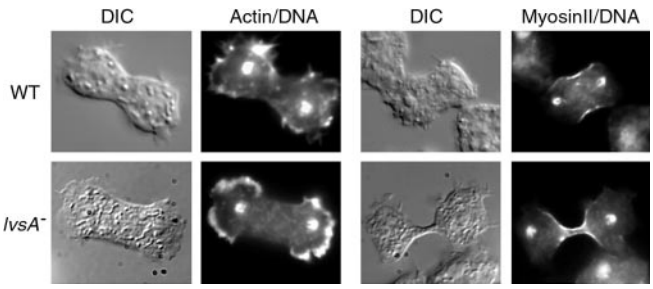

Figure 5. Actin (left) and myosin (right) distribution in wild-type (top) and *lvsA* mutant (bottom) cells in attached conditions. Cells attached to coverslips were fixed and stained with DAPI to visualize their nuclei (DNA). Some cells were stained with rhodamine-labeled phalloidin to visualize actin filaments (Actin) or were transfected with an expression vector for the production of GFP–myosin II (MyosinII). The cells were then photographed by DIC microscopy (DIC) and fluorescence microscopy. The signal from the DAPI staining was merged with that from actin or myosin imaging. In attached conditions *lvsA*-mutant cells can organize their cytoskeleton like wild-type cells. Actin is found at the cortex of the cells predominantly at the poles, and myosin II is concentrated at the cleavage furrow. *LvsA*-mutant cells also display broad lamellipodia at the poles of the cells, but this does not seem to affect their ability to divide on a substrate.

Clathrin-null cells have a strong defect in pinocytosis (O'Halloran and Anderson, 1992; Ruscetti *et al*., 1994). Thus, we examined the ability of wild-type, clathrin, and *lvsA* mutants to internalize a fluid phase marker, rhodamine-labeled dextran. As has been shown previously, clathrin-mutant cells were severely impaired in their ability to pinocytose compared with wild-type cells (Figure 7). Under the same conditions, the *lvsA*-mutant cells demonstrated only a slight reduction in their internalization rates but not to the same extent as the clathrin mutants.

Clathrin-null cells are known to have a defect in the *Dictyostelium* developmental program (Niswonger and O'Halloran, 1997a). In contrast, when we plated *lvsA*-mutant cells on a lawn of bacteria on an agar plate, we found that they were able to phagocytose the bacteria and formed plaques of normal size. After clearing the bacteria, the mutant cells initiated the developmental program and culminated with the formation of mature fruiting bodies (our unpublished observations). These fruiting bodies contained fully differentiated spores that germinated when placed in nutrient medium. Thus, unlike the clathrin-mutant cells, *lvsA* mutants were competent to complete the entire developmental program.

Finally, we challenged clathrin- and *lvsA*-mutant cells with FITC-labeled ConA to test the ability of their actomyosin cytoskeletons to effect the capping response. As expected, wild-type cells formed well-defined caps within 5 min of ConA treatment (Figure 8A). The *lvsA* mutants were able to congregate ConA to one pole of the cell in 5 min but never formed the tight cap that wild-type cells formed. Even after extended incubation periods, the *lvsA* mutants formed only "C"-shaped caps (Figure 8, C and D). Interestingly, the

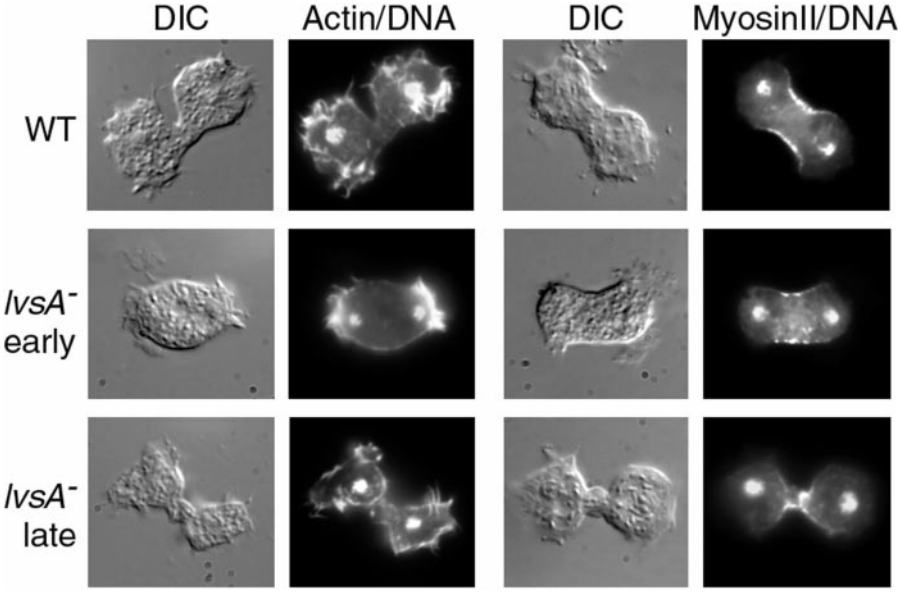

Figure 6. Actin (left) and myosin (right) distribution in wild-type (top) and *lvsA* mutant (middle and bottom) cells in suspension conditions. Cells were fixed in suspension and processed for actin and myosin II localization as described in Figure 5. In these conditions both wild-type and *lvsA*-mutant cells localize actin to their cortex and poles. Wild-type cells concentrate myosin into their cleavage furrows. However, myosin II distribution is clearly affected in the mutant cells. Early in cytokinesis (middle), myosin II begins to concentrate in the presumptive furrow, but this distribution is patchy and not as strong as that in wild-type cells. Later in cytokinesis (bottom), the distribution of myosin II is disrupted by the formation of a bulge in the middle of the cleavage furrow.

clathrin-mutant cells exhibited a very similar capping response (Figure 8B) (Niswonger and O'Halloran, 1997b).

We have attempted to determine the localization of LvsA protein to see whether it is associated with a similar compartment as clathrin. Unfortunately, our initial attempts have not been successful. However, when we fractionated *Dictyostelium* lysates on a sucrose gradient, we found that LvsA was associated with the soluble cytoplasmic fraction (Figure 3, lanes 3 and 4). Thus, it appears that clathrin and LvsA reside in different compartments in the cell.

The Dictyostelium lvsA Gene Is Related to Mammalian Trafficking and Signaling Molecules

Because the *lvsA*-mutant cells shared some (but not all) characteristics with the clathrin mutants, we postulated that

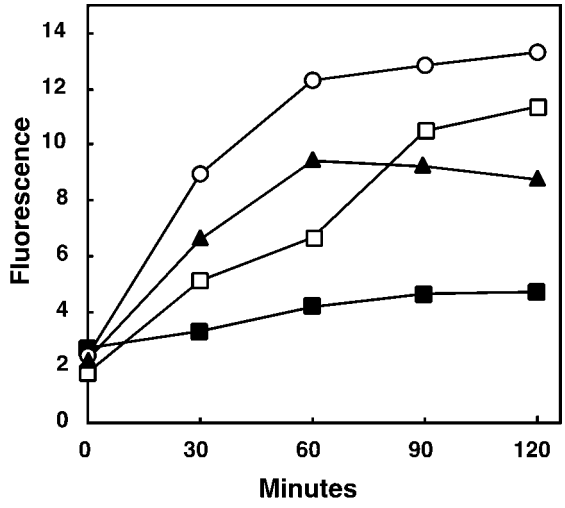

Figure 7. Pinocytosis in *lvsA* and clathrin-null mutants. Endocytosis of rhodamine-labeled dextran was determined as described (Hacker *et al.*, 1997). Clathrin-null cells have a strong defect in their ability to internalize this fluid phase marker. *LvsA*-mutant cells can endocytose fluid but at a slightly reduced rate compared with wild-type cells. Each data point is the average of duplicate measurements. Symbols indicate the following: ○, wild-type DH1 cells; ■, clathrin-null cells; □, *lvsA* REMI mutant AD60; and ▲, *lvsA* knockout mutant AD63.

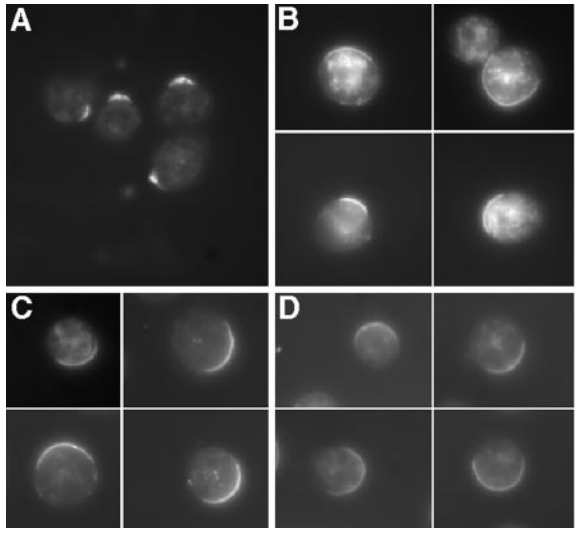

Figure 8. Capping of cell surface receptors in *lvsA* and clathrin-null mutants. Different *Dictyostelium* strains were challenged with FITC-labeled ConA to determine their capping response. (A) Wild-type (DH1) cells were able to form very tight caps within 5 min. (B) Clathrin-null cells formed caps, but only to a limited extent. (C and D) *LvsA*-mutant strains AD60 (C) and AD63 (D) also formed caps with a C shape similar to that formed by the clathrin mutants. Incubation time for all three mutant strains was 30 min.

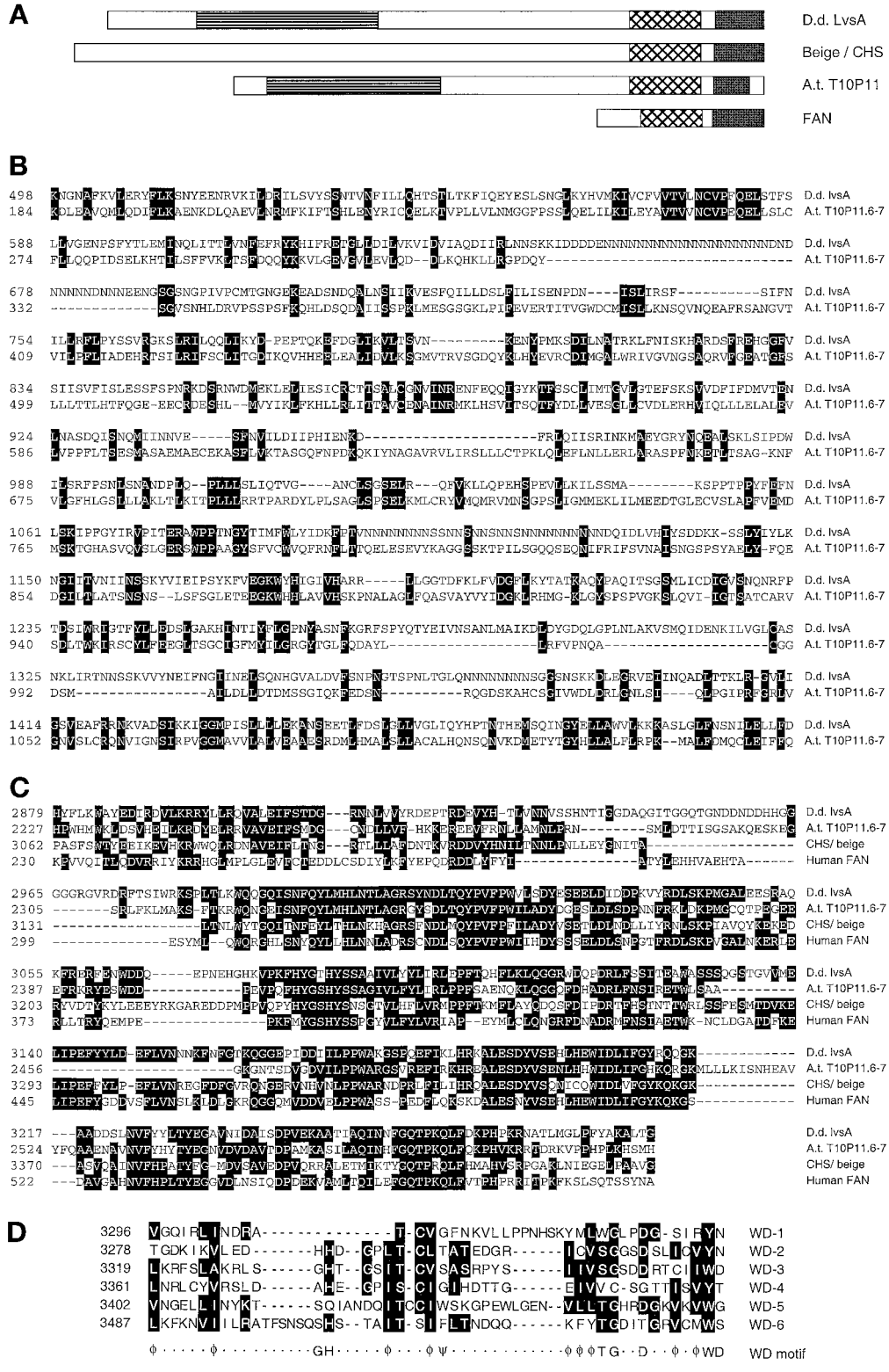

Figure 9. The *Dictyostelium lvsA* protein contains domains similar to those found in beige and CHS, FAN, and a plant protein. (A) Diagram indicating the relative size of the mouse beige and human Chediak–Higashi syndrome proteins (Beige/CHS), the human FAN protein (FAN), and a hypothetical protein found in the *Arabidopsis thaliana* genome (T10P11.6–7, accession numbers 2262139 and 2262140 [A.t. T10P11]). The

the LvsA protein might be similar to one of the other structural components of the clathrin-mediated membrane-trafficking pathway. However, we found that the *lvsA* mRNA is of extremely low abundance (our unpublished observations), suggesting a regulatory or signaling role rather than a structural role.

Comparison of the predicted LvsA protein sequence with the GenBank database revealed a striking similarity to the mouse beige protein and its ortholog, the human Chediak–Higashi syndrome protein (CHS). These proteins are known to be important for the traffic of lysosomal vesicles in mammalian cells (Burkhardt *et al.*, 1993). The portion that is most similar between LvsA and these proteins is a region of 400 amino acids near the C terminus (Figure 9, A and C). This region was recognized recently as a domain present in several proteins in the GenBank database and is now called the beige and Chediak–Higashi (BEACH) domain (Nagle *et al.*, 1996). Among the proteins that share this domain is a mammalian protein called the factor associated with neutral-sphingomyelinase activation (FAN) (Adam-Klages *et al.*, 1996). FAN is required for the activation of *N*-sphingomyelinase in a specific signaling pathway elicited by tumor necrosis factor receptor stimulation.

All proteins with BEACH domains also possess several WD motifs (Neer *et al.*, 1994) at their C terminus (Figure 9A). These motifs are known to fold into antiparallel beta-sheets that form a propeller-like structure (Sondek *et al.*, 1996). This structure provides an appropriate surface for the specific interaction of binding partners. LvsA contains at its C terminus six motifs that conform to the WD motif consensus (Figure 9D). Interestingly, although the WD motifs of LvsA share many similar residues with WD motifs from other proteins, the best BLAST scores obtained with this region of LvsA are with the WD regions of beige and related proteins (our unpublished observations). This indicates that the WD region in beige-related proteins may interact with a similar class of binding partners.

Although the N-terminal portion of LvsA shares limited similarity to the beige protein and CHS, this region shows significant similarity with a hypothetical protein (accession number 2262139) of unknown function from *Arabidopsis thaliana* (Figure 9, A and B). Interestingly, the open reading frame for this protein is adjacent to that of another ORF (accession number 2262140) that is clearly related to the beige protein and CHS (Figure 9C). Analysis of the genomic sequence of this locus (accession number AC002330) revealed that these two ORFs are in fact contiguous, without a stop codon between them. Thus, we believe that they represent the gene for a single protein that is very similar to LvsA. At present the function of this plant protein is unknown; it will be interesting to determine whether this protein has a similar function in the control of cytokinesis in plant cells.

Figure 9 (Cont). horizontal-hatched bars indicate the portions of LvsA (*D. discoideum* LvsA [D.d. LvsA]) that are similar to A.t. T10P11 (shown in B). The cross-hatched bars indicate the BEACH domain (shown in C). The black bars indicate the regions containing WD motifs (shown in D). (B) Region of homology between LvsA (accession number AF088979; D.d. lvsA) and the hypothetical *A. thaliana* protein T10P11.6–7 (A.t. T10P11.6–7). (C) Alignment of the BEACH domains from LvsA, human Chediak–Higashi syndrome protein (accession number U67615; CHS/beige), human FAN protein (accession number Q92636), and the hypothetical *A. thaliana* protein T10P11.6–7. (D) The WD motif region of LvsA. The six WD motifs from LvsA are aligned with each other. White letters on a black background indicate those positions at which at least three repeats have amino acids with the same chemical properties. Dashes are gaps inserted to align the sequences. The consensus WD motif based on the structure of G_β (Sondek *et al.*, 1996) is shown below the six LvsA repeats. Φ indicates hydrophobic residues; ψ indicates aromatic residues.

DISCUSSION

We have identified a novel protein, LvsA, that is essential for cytokinesis in *Dictyostelium* cells grown in suspension. Although we do not yet know the exact role of LvsA in cytokinesis, it is clearly different from the role of other proteins required for cytokinesis in *Dictyostelium*. Myosin II mutants fail in cytokinesis because of an inability to form a cleavage furrow in suspension conditions (Zang *et al.*, 1997). Mutants in the actin-binding proteins profilin and cortexillin also fail early in cytokinesis because of a severe disorganization of the actin cytoskeleton (Haugwitz *et al.*, 1994; Faix *et al.*, 1996). These proteins clearly play major structural and regulatory roles of the actin cytoskeleton. In addition to these proteins, the regulatory proteins racE, rasG, and IQGAP-like proteins play important but more subtle roles in cytokinesis. *racE*-null cells fail in cytokinesis by regression of the cleavage furrow (Gerald *et al.*, 1998). This regression is produced by a weakly organized actin cytoskeleton as evidenced by the profuse blebbing of *racE*-null cells undergoing cell division. RasG and the IQGAP-like proteins are required late in cytokinesis during cleavage of the cytoplasmic bridge that connects the daughter cells (Adachi *et al.*, 1997; Tuxworth *et al.*, 1997). In contrast to mutants in these proteins, *lvsA*-null cells fail by formation of an unusual bulge in the middle of the cleavage furrow. This bulge is different from the blebs formed in *racE*-null cells because it is formed slowly and persists for the entire period that the cells attempt to divide. The blebs in *racE*-null cell are formed and resorbed rapidly at multiple locations on a dividing cell (Gerald *et al.*, 1998). Thus, the difference in phenotype of *lvsA* and other mutants suggests that there are multiple regulatory pathways that are essential for cytokinesis in *Dictyostelium*.

LvsA Is Related to a Novel Class of Signaling Proteins

The most salient feature of the sequence of the LvsA protein is its similarity with an emerging class of molecules involved in signaling and membrane traffic. The prototypes that define this class are the mouse beige and human CHS (Barbosa *et al.*, 1996; Nagle *et al.*, 1996; Perou *et al.*, 1996). These proteins are required for the proper traffic of lysosomal membranes, and mutations in these proteins can lead to the formation of giant lysosomes (Burkhardt *et al.*, 1993).

The exact function of beige and CHS is not known, but an interesting possibility is suggested by the function of another member of the beige-related proteins. The human protein FAN is known to mediate the activation of the neutral sphingomyelinase that resides at the plasma mem-

brane (Adam-Klages *et al*., 1996). On the basis of their sequence similarity, it is tempting to postulate that beige is involved in the regulation of the acidic sphingomyelinase that resides in the lysosomal compartment. If this is the case, then the function of the BEACH domain, which is the signature of this novel class of proteins, may be to regulate sphingomyelinases or similar enzymes in cells. This scenario suggests several possible roles for LvsA during cytokinesis in *Dictyostelium*.

Activation of a sphingomyelinase by LvsA may serve a signaling role by the production of ceramide. This molecule is known to act as a second messenger in multiple cellular processes, from cell cycle arrest to apoptosis (Hannun, 1996). Thus, it is conceivable that ceramide, or another sphingolipid derivative, may play an important role as a second messenger during cytokinesis.

LvsA and Membrane Traffic

Another potential role of LvsA may be to regulate membrane traffic in dividing cells. The specific transport of membrane vesicles may be vital during furrow formation in cytokinesis. For example, it is known that new membrane is inserted at the cleavage furrow of dividing eggs (Byers and Armstrong, 1986; Drechsel *et al*., 1997). Furthermore, mutations in the membrane-trafficking protein clathrin severely affect cytokinesis (Niswonger and O'Halloran, 1997b). A role for LvsA in membrane traffic would not be exclusive of a possible role in signaling mediated by sphingomyelinase activation. For example, by activating a specific sphingolipid hydrolase, LvsA could control a membrane-trafficking pathway that is required for cytokinesis.

The role of LvsA in membrane traffic is also suggested by the similarity in phenotype of dividing *lvsA* and clathrin-null mutants. Both strains attempt to form a normal cleavage furrow but then fail with the formation of a bulge in the middle of the furrow region. This phenotype is not caused by a general failure of clathrin-mediated vesicle transport in the *lvsA* mutants; the *lvsA* mutants can still perform many cellular processes that are defective in clathrin mutants. Thus, it is tempting to speculate that a specific membrane-trafficking pathway, which requires both clathrin and LvsA, is crucial for the correct formation of a cleavage furrow.

Finally, another possible role for LvsA may be to regulate the remodeling of the membrane lipids at the furrow region for successful cytokinesis. There is evidence that the plasma membrane at the cleavage furrow may require a special lipid composition to accomplish furrowing and membrane fusion at the end of cytokinesis. Recent studies demonstrated that the furrow region is specifically labeled by a peptide that binds to phosphatidylethanolamine (Emoto *et al*., 1996). It is not clear how phosphatidylethanolamine is concentrated in this region of the cell at this point of the cell cycle, but it may be a direct result of the remodeling of lipids at the cleavage furrow. LvsA could participate in this remodeling by activating a lipid hydrolase during cytokinesis. Such activation may have profound effects on the fluidity and even the curvature of the plasma membrane. A dramatic example of the effects caused by the hydrolysis of sphingomyelin in the outer surface of cells was published recently (Zha *et al*., 1998). Upon addition of sphingomyelinase, the surface of the cells invaginated and formed small vesicles in the absence of any coat proteins. It is possible that the lipid composition of the cleavage furrow may need to be changed in such a way that it facilitates the ingression of the furrow. This scenario may explain the bulge found in the furrow of the *lvsA* mutants. The wrong lipid composition at the furrow may cause the membrane to bulge out instead of invaginating in the normal manner, as is observed in the *lvsA*-mutant cells.

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