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Vacuole Membrane Protein 1 Is an Endoplasmic Reticulum Protein Required for Organelle Biogenesis, Protein Secretion, and Development

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Vacuole membrane protein 1 (Vmp1) is membrane protein of unknown molecular function that has been associated with pancreatitis and cancer. The social amoeba *Dictyostelium discoideum* has a *vmp1*-related gene that we identified previously in a functional genomic study. Loss-of-function of this gene leads to a severe phenotype that compromises *Dictyostelium* growth and development. The expression of mammalian Vmp1 in a *vmp1*$^-$ *Dictyostelium* mutant complemented the phenotype, suggesting a functional conservation of the protein among evolutionarily distant species and highlights *Dictyostelium* as a valid experimental system to address the function of this gene. *Dictyostelium* Vmp1 is an endoplasmic reticulum protein necessary for the integrity of this organelle. Cells deficient in Vmp1 display pleiotropic defects in the secretory pathway and organelle biogenesis. The contractile vacuole, which is necessary to survive under hypoosmotic conditions, is not functional in the mutant. The structure of the Golgi apparatus, the function of the endocytic pathway and conventional protein secretion are also affected in these cells. Transmission electron microscopy of *vmp1*$^-$ cells showed the accumulation of autophagic features that suggests a role of Vmp1 in macroautophagy. In addition to these defects observed at the vegetative stage, the onset of multicellular development and early developmental gene expression are also compromised.

INTRODUCTION

One of the hallmarks of eukaryotic cells is the presence of complex intracellular membrane-bound organelles dedicated to specific functions. Part of the structural and functional specificity of these organelles is based on their distinct complement of proteins and membrane lipids. The link between membrane traffic, protein traffic, and organelle biogenesis is now becoming evident in the context of the secretory pathway (Derby and Gleeson, 2007). Failure of any component within the pathway can lead to abnormal targeting of proteins and membrane components that are necessary for organelle function or biosynthesis (Howell *et al*., 2006). Furthermore, some of these defects in organelle biogenesis have been found to be associated with human diseases (Dhaunsi, 2005).

Vacuole membrane protein 1 (Vmp1) is a conserved putative membrane protein with no recognizable functional motifs. The function of Vmp1 is now beginning to be elucidated. Several lines of evidence suggest a possible role of this protein in membrane traffic and organelle organization. It has been described as a stress-induced endoplasmic reticulum (ER) protein in the rat exocrine pancreas that is highly expressed during acute pancreatitis (Dusetti *et al*., 2002; Vaccaro *et al*., 2003). Overexpression of this protein in cell culture leads to vacuole formation and cell death, a process that is observed in pancreatitis (Dusetti *et al*., 2002). A recent report also identified Vmp1 as a novel autophagy-related membrane protein involved in mammalian pancreatitis-induced autophagy (Ropolo *et al*., 2007). In *Drosophila*, Vmp1 (known as TANGO-5) was also identified in a functional genomic screen by using RNA interference. TANGO-5 was found to be required for protein secretion and Golgi organization (Bard *et al*., 2006). In another study, Vmp-1 was localized in the plasma membrane in the kidney cancer cell line Caki-2, and it was found to be essential for cell–cell contact (Sauermann *et al*., 2008). These results suggested a totally different function of Vmp1 in tumor cells. Therefore, the function of this protein remains controversial and seems to depend on the specific cell type studied.

Vmp1 is a conserved protein and the study of its complex function might benefit from the use of simple experimental systems such as *Dictyostelium discoideum*. Using the completed *Dictyostelium* genome sequence, we generated a collection of mutants by targeted disruption of genes with unknown function that are highly conserved between *Dictyostelium* and humans, but also absent from the genomes

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Abbreviations used: CV, contractile vacuole; ER, endoplasmic reticulum; GFP, green fluorescence protein; PDI, protein disulfide isomerase; VMP1, vacuole membrane protein 1; TEM, transmission electron microscopy.

of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Torija *et al*., 2006a,b). Among those genes, we identified Vmp1 (named DupF in that analysis). Its absence in any fungi makes *Dictyostelium* the simplest genetically tractable model system to address its function.

Dictyostelium is a eukaryotic microorganism used as a model to study basic cellular processes, including membrane traffic and the endocytic pathway (Maniak, 2003). These social amoebae live as solitary cells feeding on other microorganisms by phagocytosis. Laboratory strains are also capable of growth in axenic media that is taken up by macropinocitosis. The vacuoles of ingested material fuse with lysosomes and undigested residues are secreted by exocytosis. As in many soil microorganisms, water regulation is essential for survival. A specialized organelle, the contractile vacuole (CV) system, is composed of an independent network of membrane tubules and cisternae that fill up and expel water by transient fusion with the plasma membrane (Gabriel *et al*., 1999). Contractile vacuole biogenesis is dependent on clathrin-coated vesicles and the adaptor-protein complex 1 (AP-1) for transporting protein and membranes required for the CV formation (O'Halloran and Anderson, 1992; Lefkir *et al*., 2003). As a result, defects in AP-1 function lead to impaired osmoregulation.

Besides its interest as a cellular model, *Dictyostelium* has the exceptional ability to form a multicellular organism by aggregation of solitary cells. The differentiation program is triggered by starvation and leads to the formation of a fruiting body composed of spores supported by a stalk (Escalante and Vicente, 2000).

In this report, we describe the first loss-of-function mutation for a Vmp1 homologue in a model system. We have found that Vmp1 is an endoplasmic reticulum protein in *Dictyostelium* necessary for the integrity of this organelle. The lack of this ER protein has pleiotropic defects in several membrane traffic-dependent processes such as organelle biogenesis and structure, endocytosis, and protein trafficking. Our results also suggest that an aberrant pattern of protein secretion during starvation might in part account for the impairment in the transition from growth to development in *Dictyostelium*.

MATERIALS AND METHODS

Dictyostelium *Cell Culture, Transformation, and Development*

Cells were grown axenically in HL5 medium or in association with *Klebsiella aerogenes* in SM plates (Sussman, 1987). Transformations were carried out by electroporation as described previously (Pang *et al*., 1999). For synchronous development, axenically growing cells were washed from culture media by centrifugation, resuspended in PDF buffer (20 mM KCl, 9 mM K_2HPO_4, 13 mM KH_2PO_4, 1 mM $CaCl_2$, and 1 mM $MgSO_4$, pH 6.4) and deposited on nitrocellulose filters (Shaulsky and Loomis, 1993). Because Vmp1 mutant cells do not grow well in axenic HL5 media the strain was initially grown in association with bacteria. For most of the experiments (unless otherwise indicated), ~10,000 cells were mixed with *Klebsiella* (300 μl of an overnight culture) and plated in SM plates. After 3 d, the clear lawn of cells were taken from the plate and used directly or resuspended in HL5. The remaining bacteria were then washed out by centrifugation, and the cells were deposited again in an appropriate volume of HL5 during an overnight, unless otherwise indicated. For filter development mutant cells were taken from SM plates as indicated above, washed, and deposited on the filters. Mutant cells in HL5 or PDF remain viable, and no cell lysis occurs in these conditions.

Generation of Mutant Strains

Disruption of Vmp1 gene in *Dictyostelium* was performed as described previously (Torija *et al*., 2006a). Briefly, DNA fragments ranging from 2 to 2.5 kb containing the genes to be disrupted were cloned by polymerase chain reaction (PCR) from genomic DNA. Insertion of the blasticidin resistance cassette by in vitro transposition by using the vector was performed as described previously (Abe *et al*., 2003). The constructs containing the flanking regions and the transposon were amplified by PCR, and the products were transformed in *Dictyostelium* cells by electroporation. Transformants were plated in association with bacteria for clonal isolation and screened for homologous recombination by PCR by using oligonucleotides surrounding the site of insertion. The DNA of the transformant clones used for PCR was isolated from cells of the growing zone by using the Master amp DNA extraction solution from EPICENTRE.

Gene Expression Analysis, Northern Blots, and Western Blots

RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. The different RNAs were separated by electrophoresis, transferred to nylon membranes, and hybridized to the indicated radioactive-labeled PCR probes. All DNA/RNA manipulation and Western blot analysis were performed according to methods described previously (Ausubel *et al*., 1992).

Transmission Electron Microscopy

Wild-type (WT) and mutant cells were incubated in Petri dishes with HL5 overnight to allow the attachment of the cells. The media were discarded, and cells were rapidly fixed with 1.25% glutaraldehyde in 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer, pH 6.8, containing 1% sucrose and 2 mM Mg_2SO_4 for 60 min at 37°C. Cells were then gently scraped, pelleted at 200 × *g*, rinsed in PIPES buffer (3 times), postfixed with 1% (wt/vol) OsO_4, 1% (wt/vol) $K_3Fe(CN)_6$ in PIPES buffer for 1 h at room temperature in the dark, and rinsed in PIPES buffer. Cells were treated for 5 min with 0.1% tannic acid in PIPES buffer, dehydrated with graded ethanol solutions, and finally embedded in Epon plastic resin. Ultrathin sections were stained with 2% uranyl acetate for 30 min, and then with lead citrate for 10 min and observed with a JEOL 1010 transmission electron microscope operating at 80 kV with a Gatan BioScan model 792 module for acquisition of digital images with Digital. Micrograph 3.4.3 acquisition software (Gatan, Pleasanton., CA). ImageJ 1.37 software (National Institutes of Health, Bethesda, MD) was used for the morphometric analysis. Data are mean values with SD.

Endocytosis, Exocytosis, and Phagocytosis

Wild-type cells were grown axenically in HL5 and mutant cells were initially grown in SM-plates and then incubated overnight in HL5 before being used for the experiments. Endocytosis, exocytosis, and phagocytosis of fluorescent markers were performed according to Rivero and Maniak, 2006). Results are shown as mean values with SD from duplicates or triplicates of at least three independent experiments. Significance of differences between groups was determined by Student's *t* test.

Conditioned Media, In-Gel Digestion of Proteins, Matrix-Assisted Laser Desorption Ionization-Tandem Mass Spectrometry (MALDI-MS/MS), and Database Searching

Conditioned media were obtained by the incubation of cells in PDF at a concentration of 1 × 10E7 cells/ml during 7 h in shaking culture. The media were washed free of cells by centrifugation at 1000 rpm for 5 min. Conditioned media were subsequently used for the biological experiments and for analysis by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis the gel was silver stained with the silver staining kit from GE Healthcare (Chalfont St. Giles, United Kingdom), according to the instructions that allow the subsequent identification by MALDI-MS/MS. Differential bands between wild type and mutant were excised manually from the gel and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Bremen, Germany). The digestion protocol used was that described previously (Shevchenko *et al*., 2006), with minor variations: gel plugs were submitted to reduction with 10 mM dithiothreitol (GE Healthcare) in 50 mM ammonium bicarbonate (99.5% purity; Sigma-Aldrich, St. Louis, MO) and alkylation with 55 mM iodoacetamide (Sigma-Aldrich) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Merck, Darmstadt, Germany) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, Madison, WI) at a final concentration of 8 ng/μl in 50 mM ammonium bicarbonate was added to the dry gel pieces and the digestion proceeded at 37°C for 8 h. Finally, 0.5% trifluoroacetic acid (99.5% purity; Sigma-Aldrich) was added for peptide extraction.

An aliquot of the above-mentioned digestion solution was mixed with an aliquot of cyano-4-hydroxycinnamic acid (Bruker Daltonics, Billerica, MA) in 33% aqueous acetonitrile and 0.25% trifluoroacetic acid. This mixture was deposited onto a 600 μm AnchorChip prestructured MALDI probe (Bruker Daltonics) and allowed to dry at room temperature. MALDI-MS(/MS) data were obtained in an automated analysis loop using an Ultraflex time-of-flight (TOF) mass spectrometer (Bruker Daltonics) equipped with a LIFT MS/MS device (Suckau *et al*., 2003). Spectra were acquired in the positive-ion mode at 50-Hz laser frequency, and 100-1000 individual spectra were averaged. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precur-

sors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector. Automated analysis of mass data was performed using the flexAnalysis software (Bruker Daltonics). Internal calibration of MALDI-TOF mass spectra was performed using two trypsin autolysis ions with m/z 842.510 and m/z 2211.105; for MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region. MALDI-MS and MS/MS data were combined through the BioTools program (Bruker Daltonics) to search nonredundant protein databases (National Center for Biotechnology Information, Bethesda, MD; and SwissProt, Swiss Institute for Bioinformatics, Switzerland) by using the Mascot software (Matrix Science, London, United Kingdom) (Perkins *et al*., 1999).

Green Fluorescent Protein (GFP) Expression Constructs, Immunocytochemistry, and Microscopy

The *Dictyostelium vmp1* gene was amplified from genomic DNA by using oligonucleotides containing targets for the restriction enzyme BamH1 and XbaI. The fragment was cloned in pGEMt-easy vector and sequenced to check for possible polymerase errors. The fragment was subsequently cloned into the BamH1 and XbaI sites of the GFP vector pDV-CGFP-CTAP, kindly provided by Pauline Shaap (University of Dundee, Dundee, United Kingdom). The construct, driven by actin15 promoter, contained the complete Vmp1 coding region fused to GFP-TAP. A similar approach was used for cloning the rat Vmp1 vector. In this case, the complete Vmp1 coding region was obtained by reverse transcription (RT)-PCR from RNA isolated from rat tissue.

For immunocytochemistry, WT and mutant cells (incubated previously in HL5 overnight), were allowed to adhere to coverslips and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. After two washes with PBS cells were permeabilized with chilled methanol during 2 min and incubated during 20 min in blocking buffer (0.2% bovine serum albumin in PBS). The samples were then incubated with the first antibody in blocking buffer for 1 h. After six washes with blocking buffer the appropriate secondary antibody (labeled with red Alexa 546) was added at a dilution of 1/1000 in blocking buffer for 30 min. After two washes with blocking buffer cells were mounted for microscopic observation. Confocal analysis was performed on a Leica TCS SP5 by using a PL APO 63×/1.4-0.6 objective and a LAS-AF (Leica Application Suite; Lecia, Wetzlar, Germany) software. For excitation of GFP a 488-nm argon laser was used. For fluorescence microscopy, an Olympus DP70 microscope with a Plan Neofluar 100×, 1.30 oil objective was used. The acquisition software was DP controller 2002, Olympus Optical CO.LTD. The antibodies and the dilution used for each were as follows: PDI (221-64-1 ascitis, mouse monoclonal) used at 1:1000; p80 (H161, mouse monoclonal) used at 1:10; Rh50 (rabbit polyclonal), used at 1:500. These antibodies were kindly provided by Pierre Cosson from the University of Geneva (Geneva, Switzerland). VatA (221-35-2 ascitis, mouse monoclonal) used a dilution of 1:3. Kindly provided by Marcus Maniak (Kassel University, Kassel, Germany). AprA (rabbit polyclonal) used at 1:1000. Kindly provided by Richard Gomer (Rice University, Houston, TX).

RESULTS

Disruption of Vmp1 in Dictyostelium Leads to a Severe Defect in Osmoregulation

Dictyostelium vmp1 (DDB0234044) codes for a putative transmembrane protein of 403 amino acids. The level of identity between the human and the *Dictyostelium*-predicted proteins is 41% (Figure 1A). Homologues are also present in other organisms, including *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Arapidopsis thaliana* (Figure 1B). However, no homologues were found in any fungi. Interestingly, it is present in other protists such as pathogenic protozoa, suggesting a specific gene loss during fungi evolution. Furthermore, *Dictyostelium* Vmp1 is more similar to the vertebrate homologues than to other simpler organisms, including other protists. The hydropathicity profile between the *Dictyostelium* and human proteins also showed a high level of similarity, suggesting conservation in the predicted transmembrane domains (Figure 1C). The *Dictyostelium vmp1* gene was disrupted by homologous recombination using an optimized protocol based on in vitro transposition (Torija *et al*., 2006a,b). Figure 1D shows a scheme of the gene and the point of insertion of the blasticidin cassette. Two oligonucleotides surrounding the disruption were used to screen for homologous recombination by PCR as depicted in Figure 1D. Several independent mutants were obtained that showed the same phenotype, and one of them was chosen for further analysis. The lack of expression of *vmp1* mRNA in the disruptant strain suggests that the insertion generated a loss-of-function mutant (Figure 1E). *vmp1* disruption was also generated in other *Dictyostelium* strains (Table 1), and the phenotype was similar regardless of the strain background.

The major consequences of *vmp1* disruption are shown in Figure 2. Growth in association with bacteria was slightly affected as observed by the size of the clearing plaques (Figure 2A). These cells were also deficient in initiating development upon starvation (see also below). Cell growth in axenic media (HL5) was compromised in shaking culture. Cells grew slowly over the first 2 d in culture, and cell growth eventually stopped completely. However, when cells were set in Petri dishes, allowing them to attach to the plastic, their growth was very slow but sustained (data not shown). The possibility of a cytokinesis defect was studied by staining the cells with 4,6-diamidino-2-phenylindole (DAPI). No differences in the number of nuclei per cell were found in any growth condition (data not shown). Intriguingly, when mutant cells were directly taken from SM plates and incubated in water they rapidly rounded up and after few minutes a proportion of them began to burst. This can be recognized by the presence of remaining cell debris (Figure 2B). This aspect of the phenotype is more patently illustrated in Supplemental Movie 1. The same phenotype was observed in cells previously incubated overnight in HL5. *Dictyostelium* cells have a contractile vacuole (CV) system, which allows the cells to efficiently survive hypoosmotic conditions. The presence and the activity of these vacuoles that expel water outside the cell can be visualized under phase contrast microscopy. A high magnification of the cells (Figure 2B) showed that whereas wild type displayed abundance of clear vacuoles that eventually fused with plasma membrane, the mutant cells were round and had a flat appearance with no evident activity of the CV (Figure 2B).

These results suggested a defect in osmoregulation and more specifically in the activity of the CV. To confirm this hypothesis, we incubated wild-type and mutant cells in different concentrations of sorbitol to generate a wide range of osmotic pressure. Figure 2C shows how the morphology of mutant cells became less round as the sorbitol concentration increases. At 100 mM sorbitol the cells looked more normal in appearance but nevertheless they showed no clear vacuole activity as seen in wild type. Mutant cells were able to respond to hyperosmotic stress (400 mM sorbitol) by reducing their volume (also known as cringing) and they showed high refringence, as described for wild type cells (Kuwayama *et al*., 1996). These results suggested a specific defect of the mutant cells in coping with hypoosmotic conditions as a result of impaired CV activity.

Vmp1 Is Required for Contractile Vacuole Biogenesis

To characterize the function of the CV in vivo, we disrupted *vmp1* in a *Dictyostelium* strain expressing the protein Dajumin fused to GFP (see Table 1 for a complete list of strains used in this report). Dajumin is a protein specifically located in the CV (Gabriel *et al*., 1999). Figure 3A shows the expected pattern of fluorescence in wild type. However, GFP fluorescence was hardly detectable in the disruptant strain, confirming the absence of functional vacuoles as observed under phase contrast microscopy. For further confirmation, we used the CV-specific antibody Rh50 (Benghezal *et al*., 2001). Figure 3B shows the immunofluorescence staining in wild type with the expected pattern. However, in this case a weak

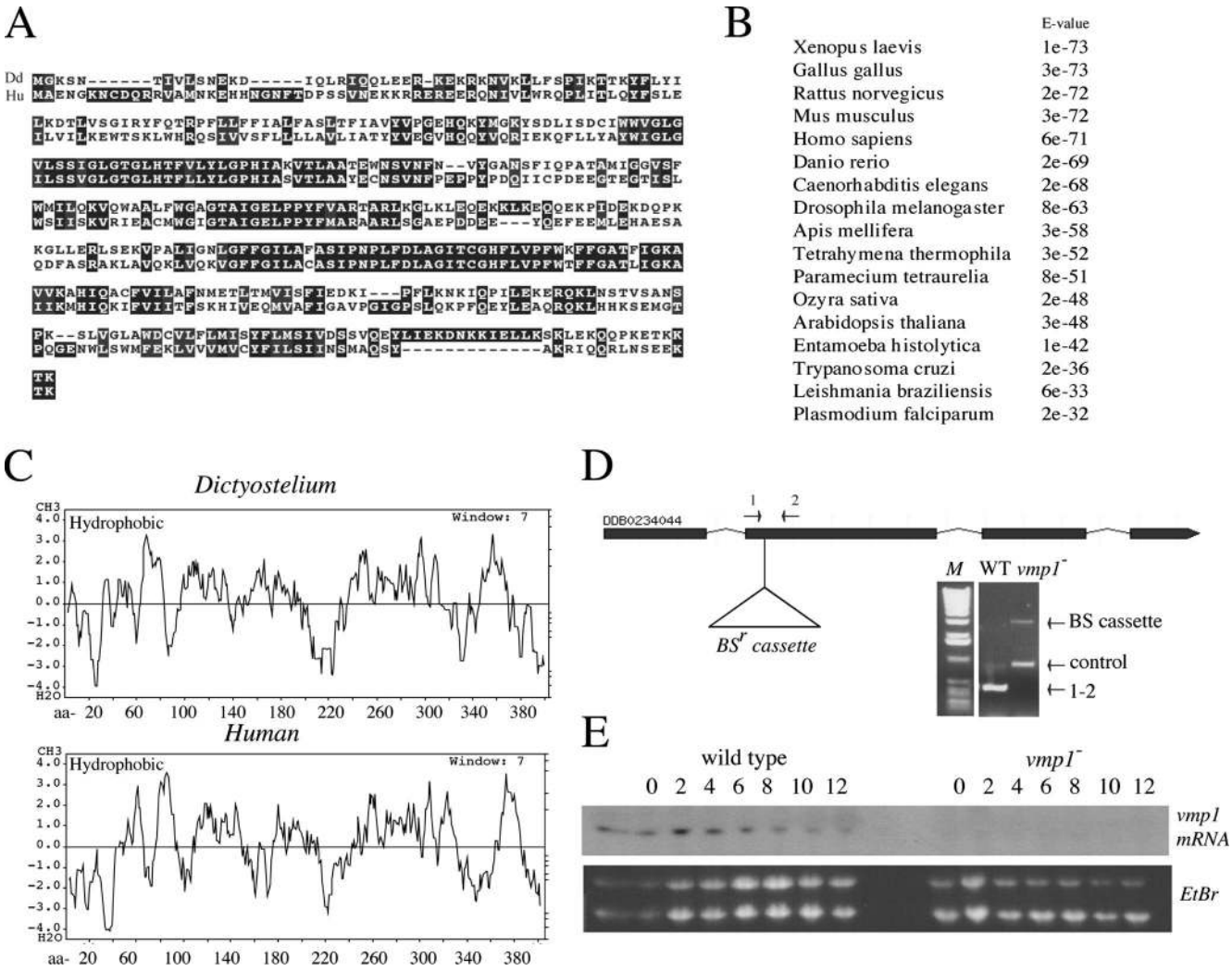

Figure 1. Disruption of the conserved gene *vmp1* in *Dictyostelium*. (A) The sequence of *Dictyostelium* Vmp1 predicted protein (DDB0234044) was aligned with the human homologue (CAG38552) by using the ClustalW program. The letters with black background correspond to identical residues and the gray background to similar residues. (B) The most similar proteins to the *Dictyostelium* Vmp1 are listed in order, from the highest identity to the lowest. (C) The hydropathicity plot of *Dictyostelium* and human Vmp1 predicted proteins are compared. The analysis was performed by the method of Kyte and Doolittle by using the online program at http://workbench.sdsc.edu/. (D) The structure of the *vmp1* gene is shown. Open boxes correspond to coding regions and the thin lines represent the introns. The insertion of the blasticidin cassette took place at the beginning of the second exon (amino acid 89) as indicated below. Disruption of the gene was assessed by PCR using oligonucleotides located at both sides of the insertion as indicated by the arrows. A pair of oligonucleotides from an unrelated locus was used as internal control of the PCR reaction. The panel below shows a typical analysis using DNA isolated from wild type (labeled as WT in the figure) and a disruptant strain (*vmp1*$^-$). The expected band (1–2) shifts to a higher band (BS-cassette) as a consequence of the insertion. M correspond to DNA size markers. (E) RNA isolated at different times of development was hybridized with a radioactive probe derived from the coding sequence of Vmp1. The band detected in wild type is absent in the disruptant strain.

signal was detected in the mutant, although the intensity of the staining and the size and number of vacuoles was greatly reduced (Figure 3B).

Dictyostelium cells contain many large vacuoles, including CVs and also vacuoles of the endocytic pathway, which are mainly devoted to nutrition in *Dictyostelium* (Maniak, 2003). Some of these vacuoles can be distinguished by electron microscopy. As seen in Figure 4, WT cells showed a high number of large electroluscent vesicles. Some of them contained spongy material that is believed to correspond to different degrees of digestion of the axenic media that has been internalized by macropinocytosis (Ryter and de Chastellier, 1977). Some of these vacuoles were completely electron transparent and probably correspond to contractile vacuoles (Ryter and de Chastellier, 1977). Mutant cells showed some striking differences with WT cells. First, there was a remarkable reduction in the number of electroluscent vacuoles, suggesting a defect not only in the biogenesis of the CV system but also in the endocytic pathway (aspect addressed below). Second, mutant cells showed an abnormal accumulation of electrodense vacuoles enclosing large granular and membranous material (Figure 4). The ratio of the number of electroluscent/electrodense vacuolar profiles ($\geq$200 nm) per μm^2 of cytoplasm was determined by morphometric analysis. This ratio in WT was 3.45 $\pm$ 0.98 (number of cells analyzed, n = 14), and it was significantly higher ($p \leq 0.0001$) than that obtained in the mutant, 0.43 $\pm$ 0.11 (number of cells analyzed, n = 14). These electrodense vesicular profiles are very similar to those described in macroautophagy mutants in *Dictyostelium* that are believed to be

Table 1. Strains used in this study

Strain name	Characteristics	Genotype	Parental strain	Reference
AX4	Wild-type strain			
AX2	Wild-type strain			
vmp1$^-$	Gene disrupted at aa-89	BS^r	AX4	This report
vmp1$^-$*/act15*::DdVmp1	Complemented strain by the expression of *Dictyostelium* Vmp1-GFP fusion protein	G418^r; BS^r	Vmp1$^-$	This report
vmp1$^-$*/act15*::rVmp1	Complemented strain by the expression of rat Vmp1-GFP fusion protein	G418^r; BS^r	Vmp1$^-$	This report
Vmp1-GFP	Complemented strain by single homologous recombination	G418^r; BS^r	Vmp1$^-$	This report
act15::ratVmp1	Expression of rat Vmp1 in wild type	G418^r	AX2	This report
Golvesin(C)-GFP	Marker for the Golgi apparatus	G418^r	AX2	Schneider *et al.* (2000)
Golvesin(C)-GFP/Vmp1$^-$	Marker for the Golgi apparatus in *vmp1*$^-$	G418^r; BS^r	Golvesin(C)-GFP	This report
Dajumin-GFP	Marker of the contractile vacuole	G418^r	AX2	Gabriel *et al.* (1999)
Dajumin-GFP/Vmp1$^-$	Marker of the contractile vacuole in *vmp1*$^-$	G418^r; BS^r	Dajumin-GFP	This report

nonmature autophagosomes containing nondigested cytoplasm and organelles (Otto *et al.*, 2003, 2004). This observation suggests a possible implication of Vmp1 in macroautophagy.

Vmp1 Is an Endoplasmic Reticulum Protein in Dictyostelium

We next cloned the entire *Dictyostelium vmp1* gene fused to GFP as a reporter for subcellular localization studies. The expression of the composite gene was driven by the constitutive actin 15 promoter. Mutant cells were transformed with the construct and the resulting strains fully complemented the phenotype in growth, development and osmoregulation (Figure 5, A and B). Complementation of the phenotype strongly suggests that the fused protein is functional. The complemented strain was used for confocal studies to determine the subcellular localization of the protein. The GFP fluorescence pattern as shown in Figure 5C, suggested that the protein was localized in internal membranes. We therefore used several markers of intracellular compartments for colocalization by immunofluorescence. Although a prominent defect in the mutant is related with the CV and the endosomal pathway (see below), Vmp1 did not colocalize with V-ATPase (VatA), a CV/endosomal protein (Clarke *et al.*, 2002), or with the endosomal marker P80 (Supplemental Figure 1). However, as shown in Figure 5D complete colocalization was observed with protein disulfide isomerase (PDI), a typical marker of the ER (Monnat *et al.*, 1997). Moreover, a strong GFP fluorescence labeled the nuclear envelope as can be seen in Figure 5D surrounding several nuclei stained with DAPI.

We next wanted to determine whether the expression of a mammalian Vmp1 in *Dictyostelium* was able to complement the mutant strain. The complete rat Vmp1 was cloned by RT-PCR and placed in frame with GFP in a similar construct as the one described for the *Dictyostelium* protein. This construct was transformed in wild type, and the mutant strains and stable transformants were isolated. As expected by the high conservation of the amino acid sequence, the mammalian protein was also localized in the endoplasmic reticulum in both strains (Supplemental Figure 2). Interestingly, growth in axenic media and development were almost completely recovered as well as the resistance to hypoosmotic conditions as described in Supplemental Figure 2. These results strongly suggest that the mammalian protein is functionally similar to the *Dictyostelium* protein.

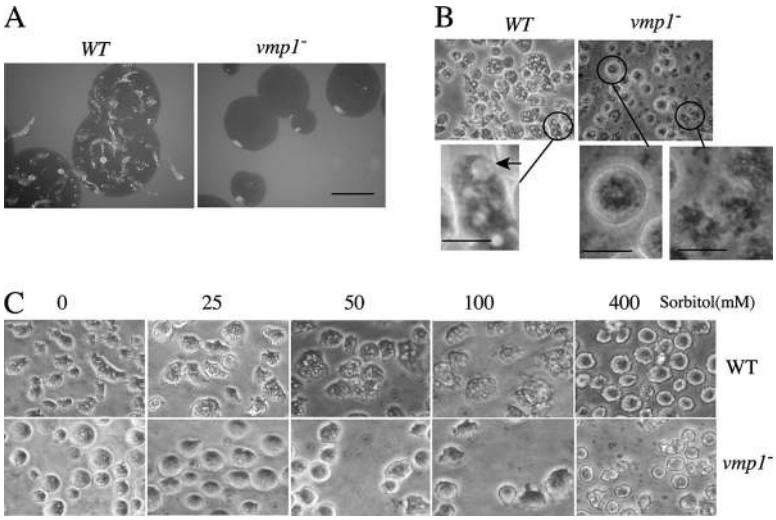

Figure 2. Vmp1 mutant cells are defective in growth, development and osmoregulation. (A) Wild type and *vmp1*$^-$ cells were cultivated in association with *Klebsiella aerogenes* in SM plates. The clearing plaques in the mutant are slightly smaller than those of the WT and do not show any sign of aggregation and development. Bar, 1 cm. (B) Cells were taken from the growing zones of *Klebsiella*-SM plates and incubated in water. After ~30 min, phase contrast photographs were taken with a Nikon Eclipse microscope. Wild type showed extensive vacuolization. A magnified image shows a contractile vacuole before discharging water outside the cell. The mutant cells were round and had a flat appearance with few vacuoles. Some of the mutant cells showed evidence of cell lysis. Magnified images show a mutant cell and cell debris. Bar, 10 μm. (C) Wild-type and mutant cells were incubated at different concentrations of sorbitol to study their response to osmotic pressure. Cell rupture and morphology were gradually recovered in the mutant as the osmotic pressure increased. Bar, 10 μm.

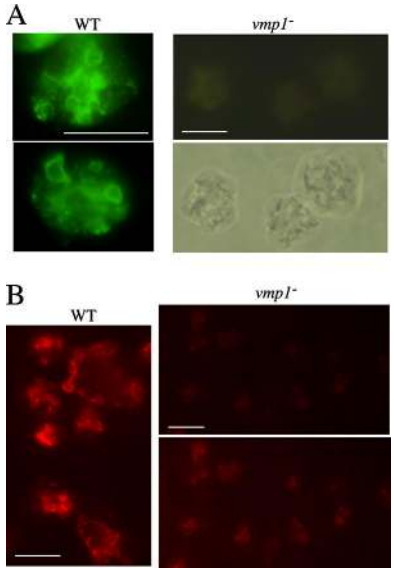

Figure 3. Analysis of the contractile vacuole activity. (A) *vmp1* was disrupted by homologous recombination in a strain expressing Dajumin fused to GFP, a marker of the CV network in *Dictyostelium*. WT and mutant cells were fixed and observed in a Nikon fluorescence microscope. Two representative cells of WT showed the expected pattern. However, fluorescence was barely detected in the mutant (top). Bottom, bright field image of the cells. (B) Immunofluorescence using Rh50, a CV-specific antibody coupled to Alexa 546, was performed in wild-type and mutant cells. A field containing several cells is shown for WT and the mutant. Although some staining was detected in the mutant, the intensity and the size of the vacuoles were reduced. The mutant top panel shows a photograph taken under the same intensity and contrast than the one shown for WT at the left. The bottom panel is the same picture with increased contrast to reveal the weak staining. Bars, 10 μm.

Vmp1 Is Required for ER Integrity and Membrane Traffic-dependent Processes

Although Vmp1 is present in the endoplasmic reticulum, we have shown evidence of defects in the CV. Because the ER is the starting point of the secretory pathway, we hypothesized that a primary defect in the structure of the ER, generated by the loss of Vmp1, could affect the biogenesis and the function of other organelles as a result of defective membrane traffic. To test this hypothesis, we analyzed the structure of the ER and the Golgi apparatus, endocytic trafficking, and the kinetics of protein secretion in the *vmp1*$^{-}$ mutants. We found acute alterations in most of these processes, which are summarized in Figure 6.

The ER in *Dictyostelium*, as observed by immunofluorescence microscopy with the PDI marker, has a typical tubulovesicular structure in WT (Figure 6A). Remarkably, it showed a fragmented appearance in the mutant suggesting a role for Vmp1 in the maintenance of its normal membrane structure. To visualize the Golgi apparatus in the mutant cells, Vmp1 was disrupted in a strain expressing Golvesin fused to GFP, which specifically labels this organelle (Schneider *et al.*, 2000). As displayed in Figure 6B, whereas wild-type cells usually showed fluorescence in a single and restricted area close to the nucleus, most of the mutant cells showed dispersed and fragmented areas of fluorescence.

The endocytic pathway in *Dictyostelium* is primarily dedicated to nutrition, and it depends, as in the rest of eukaryotes, on membrane traffic processes. We have studied the time course of endocytosis and exocytosis as a measure of the functional activity of endocytic organelles. Macropinocytosis as determined by the internalization of fluorescent markers was dramatically affected in the mutant (Figure 6C). Moreover, the staining of F-actin with phalloidin-rhodamine in wild-type and mutant cells showed a clear difference in the presence of crown-like structures (data not shown). Crowns are actin structures located in macropinosomes marking the early stages of macropinocytosis (Hacker *et al.*, 1997). There were almost no such structures in the mutant (data not shown). Exocytosis was difficult to evaluate because the cells accumulated much less fluorescent marker than the wild type shown in Figure 6D. Even so, the slope of the secretion graph at any point was also reduced in the mutant. As anticipated by the ability of the mutant cells to grow in association with bacteria, phagocytosis of fluores-

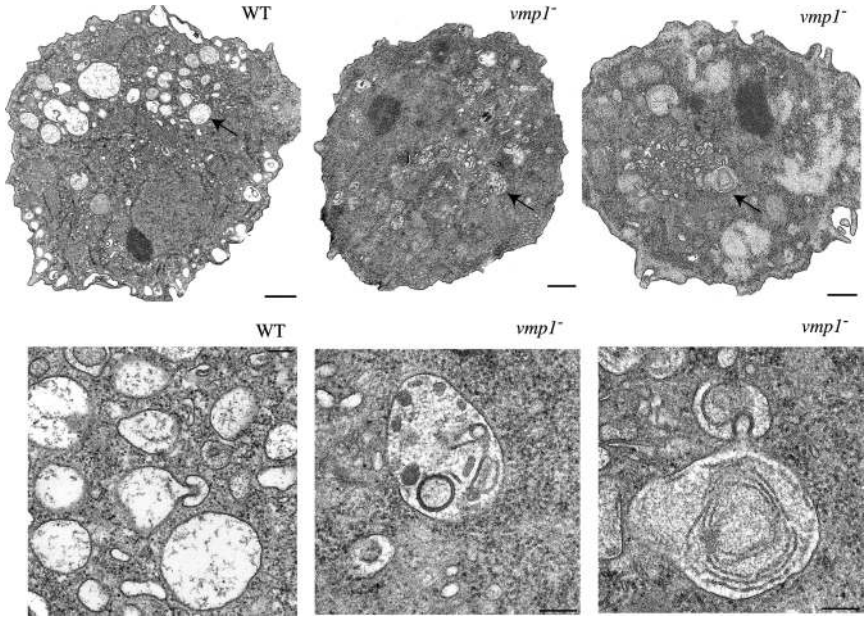

Figure 4. Electronic microscopy of WT and mutant cells. The arrows aim to the magnified area that is shown below. Mutant cells show accumulation of electrodense vacuoles enclosing large granular and membranous material. Bars, 1 μm (top); 200 nm (bottom).

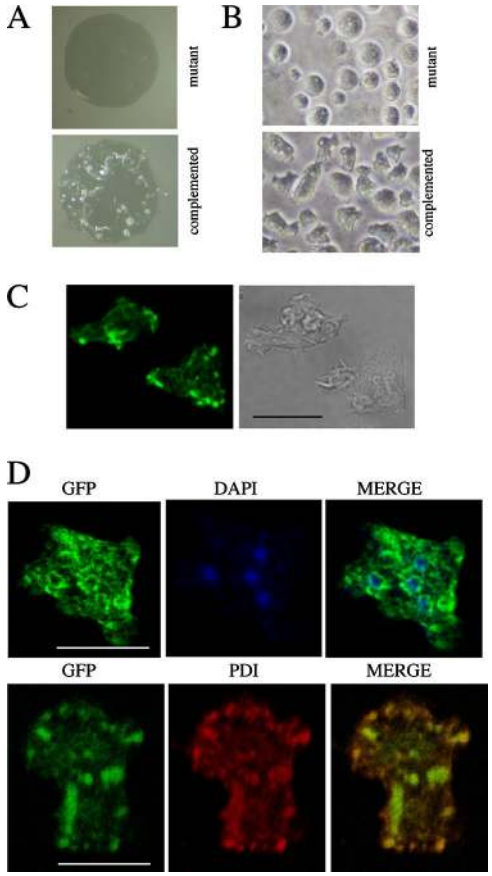

Figure 5. Vmp1 is an endoplasmic reticulum protein in *Dictyostelium*. *Dictyostelium vmp1* gene was fused to GFP and transformed into the *vmp1*$^-$ mutant. The transformant strain showed a WT phenotype with respect to development, osmoregulation and growth. (A) Mutant and complemented strains were grown in SM plates in association with *K. aerogenes*. (B) The strains were incubated in water for 30 min, and the complemented strain showed a normal response to hypoosmotic conditions as can be seen by the irregular shape of the cells and the presence of vacuoles. (C) Complemented cells were fixed and the fused Vmp1-GFP protein was visualized by confocal microscopy. Left, GFP fluorescence Right, bright field of the same cells. Bar, 10 μm. (D) The complemented strain expressing the fusion Vmp1-GFP protein was stained with DAPI (top) or treated for immunodetection of the endoplasmic reticulum marker PDI (bottom). The merged images show the presence of GFP fluorescence in the nuclear envelopes and colocalization with PDI. Bars, 10 μm.

cent beads was observed in the mutant, although at lower rate than in WT (Figure 6E).

Protein secretion is a process dependent on membrane traffic. Therefore, we wanted to determine a possible role of Vmp1 in the kinetics of secretion of AprA, a protein secreted by a conventional mechanism (ER–Golgi transit) that regulates *Dictyostelium* growth (Brock and Gomer, 2005). Wild-type and mutant cells were set in fresh HL5 media, and aliquots were taken at the indicated times. The amount of AprA secreted to the media was analyzed by Western blot (Figure 6F). A rapid accumulation of the protein in the extracellular media was observed in wild type. However, very little protein was detected in the mutant media suggesting a defect in protein secretion. The presence of intracellular AprA was also determined as a control.

Vmp1 Is Necessary for the Transition from Growth to Development

As shown in Figures 2 and 7A, *vmp1*$^-$ mutant cells were unable to aggregate in association with bacteria or on nitrocellulose filters soaked with PDF. We have previously determined that mutant cells, in contrast to their response in water, remained viable in PDF buffer and showed no signs of cell lysis (data not shown). Nevertheless, no aggregation of mutant cells was observed when PDF was supplemented with sorbitol (50 and 100 mM), suggesting that the deficiency in initiating development was not due to their osmosensitivity (data not shown). What aspects of the phenotype could account for such block in development? It is well known that the transition from growth to development in *Dictyostelium* is dependent on the activation of a specific gene expression pattern involving many genes that are regulated by extracellular signals induced by starvation (Kessin, 2001). Among them there are genes coding for the synthesis and relay of cAMP signaling that are essential for aggregation. We studied by Northern blot the expression pattern during early development of some of these markers. As shown in Figure 7B, the level of expression of the cAMP receptor Car1 and the adenylyl cyclase ACA were barely detected in the mutant. The absence of expression of any of these genes would be sufficient to account for the lack of aggregation as seen in the mutant. Discoidin I, in contrast, is a developmentally regulated lectin whose expression depends on cell density. The level of expression during early development was also strongly reduced in the mutant. As a control of constitutive expression, *patA*, a gene coded for a P-type ATPase, was used (Moniakis *et al*., 1995).

In *Dictyostelium*, the expression of these early developmental markers and discoidin are dependent on several secreted proteins during growth and starvation such as prestarvation response factor and conditioned medium factor (Clarke and Gomer, 1995) among others. Therefore, the observed impairment in the starvation response could be due to an abnormal cell response to these extracellular proteins, or to the absence of those signals as a result of abnormal protein secretion, as observed for AprA during growth, or more likely the combination of these two possibilities. Mixing experiments of mutant cells with different proportions of WT were unable to rescue mutant development, suggesting a cell autonomous defect (data not shown). Nevertheless, we undertook a proteomic approach to identify possible differences in the pattern of secreted proteins. Conditioned media was obtained by incubation of cells in starvation during 7 h. The media were then washed free of cells by centrifugation, and the secreted proteins were analyzed by SDS-PAGE electrophoresis. A direct comparison of the protein pattern between wild type and the mutant is shown in Figure 8. Most of the protein bands were present in both media with small differences in abundance. However, some striking differences were observed. Some protein bands were present in wild type but absent in the mutant and more interestingly there were bands present in the mutant that were barely detectable in wild type. This result might indicate a more complex scenario than just the lack of certain protein complement in the mutant suggesting an aberrant regulation of protein secretion.

We next tried to identify the differential protein bands by MALDI-MS/MS. Three different proteins were successfully identified (Figure 8). α-Mannosidase precursor (DDB0201569) and a cysteine proteinase (DDB0219654) were present in WT conditioned media but almost absent in the mutant. These two proteins are secreted by conventional mechanisms (ER/

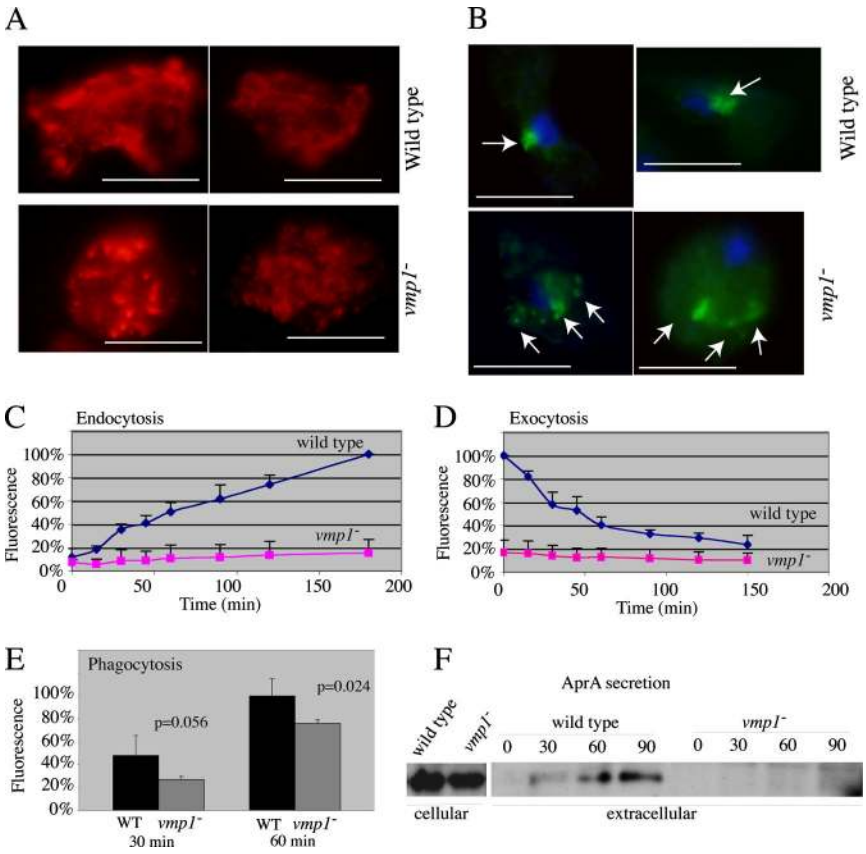

Figure 6. Characterization of defects in other membrane-traffic dependent processes. (A) Antibody label of protein disulfide isomerase (PDI), a marker of the endoplasmic reticulum. Photographs were taken in a fluorescence microscope and two representative cells of each strain are shown. Mutant cells showed fragmented endoplasmic reticulum. Bar, 10 μm. (B) *vmp1* was disrupted in a strain expressing Golvesin-GFP, a marker of the Golgi apparatus. The morphology of the Golgi apparatus was observed by fluorescence microscopy in the mutant and the parental strain. The Golgi apparatus in the mutant seemed disorganized. (C) Endocytosis assay. Wild-type and mutant cells were incubated in the presence of a soluble fluorescent marker. At the times indicated, the internal cellular fluorescence was determined. The mean of three independent experiments and the SD is shown. (D) Exocytosis assay. Cells were first preloaded with the marker and the decrease in internal fluorescence was measured at the times indicated. The mean of three independent experiments, and the SD is shown. (E) Phagocytosis assay. WT and mutant cells were exposed for the indicated times to fluorescent beads. Fluorescence was expressed as arbitrary units. The mean of four independent experiments is shown and the significance of differences is indicated by the p value. Bars show the SD. (F) Protein secretion assay. Wild-type and mutant cells were incubated in HL5 for the indicated times and the secreted AprA was analyzed in the media by Western blot using a specific antibody. The cellular extract was also analyzed as a control.

Golgi), reinforcing the data obtained by AprA (Pannel *et al*., 1982; Wood and Kaplan, 1985). Interestingly, a band overrepresented in the mutant media was identified as 70-kDa hear shock protein (Hsp70) (DDB0219654), a chaperone that has been described to play an additional role as an extracellular protein secreted by nonconventional mechanisms (Mambula *et al*., 2007; Multhoff, 2007). Although a comprehensive proteomic analysis would be necessary to fully characterize the defects in mutant conditioned media, our findings suggest that Vmp1 is also required for normal protein secretion during starvation.

DISCUSSION

Analysis of Vmp1 in Dictyostelium Reveals a Complex Function for This New Protein

Vmp1 is a conserved eukaryotic protein that seems to be lost in the fungi lineage during evolution. Consequently, *Dictyostelium* is one of the simplest genetically tractable model systems to address its function. This is the first loss-of-function mutant described for this gene in an experimental system, and Figure 9 shows a summary of the defects observed in the mutant in the context of the secretory pathway.

Our results show that Vmp1 is an ER protein required to maintain the structure of this organelle. Despite its location, it is involved in a wide range of membrane traffic-dependent processes such as organellar biogenesis and protein secretion. The morphology of the ER itself, the Golgi apparatus and the CV are compromised in the mutant as well as the maturation of autophagosomes and the function of the endocytic pathway. Protein secretion during growth and starvation is also dependent on Vmp1. It is conceivable that a primary defect in the ER, which is the starting point of the secretory pathway, could have an impact on other processes, which are directly dependent on the correct trafficking of protein and membrane components. This is not without precedent. For example, defective ER-resident proteins CLN6 and CLN8 are responsible for lysosomal dysfunctions (Kyttala *et al*., 2006). Secretion of extracellular matrix is impaired when CopII coat component Sec 23a is mutated as a result of defective transport from the ER (Lang *et al*., 2006).

We have found that some of these defects such as the disorganization of ER and Golgi are gradually aggravated over time as the cells remain in HL5 axenic culture. Cells in these conditions are likely to be starved due to the observed defects in nutrient uptake, suggesting that starvation might aggravate those phenotypes. However, other defects such as osmosensitivity and the onset of development do not seem to depend on the growth conditions. Cells taken directly from SM-plates, where they are feeding on bacteria to almost normal rates did not survive hypoosmotic conditions and were unable to initiate multicellular development.

Vmp1 Subcellular Localization and Its Functional Implications

Several lines of evidence suggest that Vmp1 is an ER-resident protein in *Dictyostelium*. We have shown that the fused protein Vmp1-GFP is localized in the endoplasmic reticulum and no colocalization has been observed with CV or endosomal markers. The fused protein showed full complementation of the phenotype when transformed in the mutant strain, suggesting that the protein must be in a functional conformation and also localizes to in the correct cellular compartment. Moreover, a knockin strain was obtained by

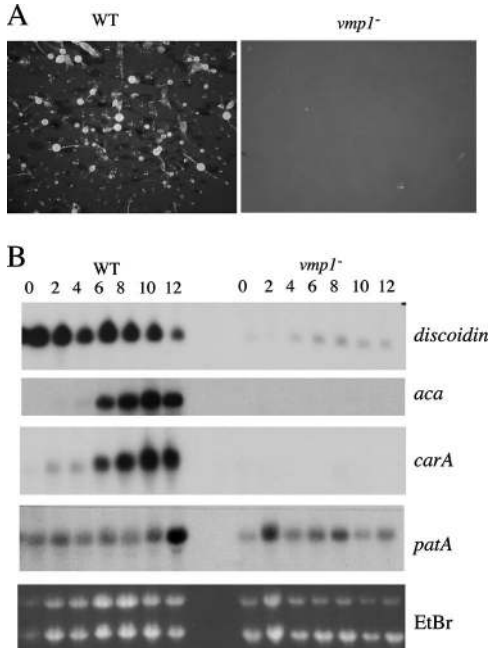

Figure 7. Developmental gene expression in *vmp1*$^{-}$ mutant. (A) WT and mutant cells were deposited in nitrocellulose filters for development and photographs of a representative experiment were taken after 30 h. No aggregation was observed in the mutant. (B) RNA was isolated form cells developed on nitrocellulose filters for the indicated times, transferred to nylon membranes and hybridized to radioactive probes. The same blot was stripped and hybridized several times. The expression of the lectin Discoidin, the adenylyl cyclase ACA, and the cAMP receptor Car 1 was greatly reduced. The expression of P-type ATPase (PatA), which is not developmentally regulated, was used as a control. The staining of ribosomal RNAs by ethidium bromide is shown at the bottom.

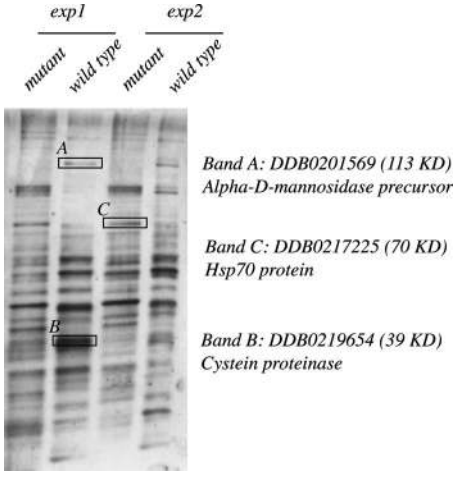

Figure 8. Conditioned medium and protein identification by MALDI-MS/MS. Wild-type and mutant cells were washed free of nutrients and resuspended in PDF for 4 h in shaking culture. Cells were then sedimented by centrifugation and the conditional media analyzed by SDS-PAGE. The gel was silver stained, and the protein pattern was compared. Two independent experiments are shown. Differential bands were cut and analyzed by MALDI-MS/MS. Three bands were successfully identified with a Mascot score of 1.1E-6 (band A), 5.6E-10 (band B), and 7.0E-6 (band C).

single crossover (Charette *et al*., 2006), in which a unique copy of the fused protein was under the control of the endogenous promoter (Supplemental Figure 3). This strain is expected to give rise to a level of expression that is similar to wild-type cells. In fact, the intensity of fluorescence was lower than that obtained in the overexpressor strains (data not shown). Even then, the localization was found in the ER as determined by colocalization with PDI (data not shown).

The amino acid sequence of *Dictyostelium* Vmp1 showed a conserved KKXX-like ER-membrane retention signal at the C terminus as detected by the PSORT program (Nakai and Horton, 1999), resulting in a K-NN prediction 66,7% for ER, which is confirmed by our results. In this connection, an ER localization for Vmp1 has also been described in *Drosophila* (Bard *et al*., 2006) and also in a proteomic study in *Arabidopsis* (Dunkley *et al*., 2006).

Regarding cell localization, the scenario in mammalian cells seems to be more complex. Vmp-1 has been reported to be localized in different subcellular compartments and these results suggest that it may play different roles. The first report by Dusetti *et al*., 2002 localized Vmp1 as a fused protein with GFP in vacuoles, the area of the ER, and the Golgi apparatus. Unfortunately, colocalization analysis with cell markers was not performed in this study to determine the precise localization. The expression of this Vmp1-GFP–fused protein induced vacuolization and death in Cos7 cells (Dusetti *et al*., 2002). Interestingly, our results have shown that a mammalian Vmp1 expressed in *Dictyostelium* also has an ER localization. Remarkably, this construct also complemented the mutant phenotype, suggesting correct subcellular targeting and functional conservation of the protein.

A second report by the same group several years later further defined the vacuolization induced by Vmp1 expression as autophagy in mammalian cells (HeLa293T and NIH3T3) (Ropolo *et al*., 2007). Autophagy is a degradation process of cytoplasmic cellular components that is essential as a survival mechanism during starvation (Klionsky and Emr, 2000). Vmp1 colocalized with LC3, a marker of the autophagosomes and induces autophagosome formation in pancreas acinar cells in transgenic mice (Ropolo *et al*., 2007). The endogenous expression of Vmp1 in HeLa cells was very low and only detectable under conditions inducing autophagome formation such as starvation. In this case, Vmp1 is detected in punctuate structures by immunofluorescence consistent with the autophagosome vesicle formation. It is possible as stated by the authors that a low nondetected basal Vmp1 expression could be related with other physiological processes (Ropolo *et al*., 2007).

Interestingly, a recent report showed that Vmp1 was located in the plasma membrane playing a role in cell–cell contact (Sauermann *et al*., 2008). We have never found *Dictyostelium* Vmp1 in the plasma membrane at the vegetative stage. We also checked the localization pattern during development (finger stage) to determine whether some localization was found in cell–cell contacts. However, the protein remained exclusively at the ER (data not shown).

Together, these results suggest that Vmp1 might be located in the ER in lower organisms, including the social amoebae, insects, and plants, in which it could play a basic role in membrane traffic as we have shown in *Dictyostelium*. In mammalian cells, Vmp-1 may play more diverse roles in different organelles including autophagosome vesicles and the plasma membrane. Whether Vmp1 has a specific role in the ER of mammalian cells as suggested by our results in *Dictyostelium* remains to be investigated.

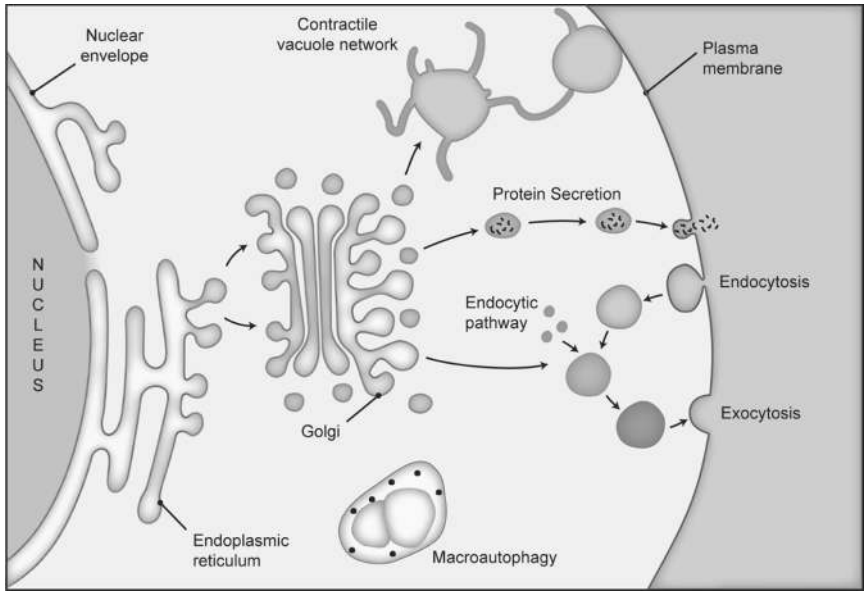

Figure 9. Summary of Vmp1-dependent processes. The amino acid sequence of the encoded protein contains several putative transmembrane domains suggesting that Vmp1 is an integral membrane protein located in the ER. Despite its localization, the loss of this gene in *Dictyostelium* leads to defects in several membrane traffic-dependent processes, including the biogenesis of the CV, the structure of the Golgi apparatus, and the pattern of macroautophagy vacuoles. In contrast, the endocytic pathway, which in *Dictyostelium* is essential for nutrition, is also affected. Endocytosis and exocytosis is severely impaired in the mutant. Protein secretion during growth and probably also during starvation is also defective.

Vmp1 Is Required for Osmoregulation, an Essential Process in Soil Microorganisms

The survival in hypoosmotic conditions is essential for single-celled organisms. These organisms, including *Dictyostelium*, have developed a complex network of vesicles and tubules known as the CV. If CV function is compromised as a result of a general defect in membrane trafficking, the expected phenotype would be dramatic. The cells would not be able to cope with water influx and may burst as a consequence of cell swelling. This is precisely what occurs in the absence of Vmp1 in *Dictyostelium*. A similar phenotype have been reported for a *Dictyostelium* mutant in the AP-1 clathrin-adaptor (Lefkir *et al*., 2003). AP-1 complex participates in vesicle transport from the *trans*-Golgi network to endocytic compartments. This mutant showed a severe growth defect and delayed development and most importantly the biogenesis of the contractile vacuole was compromised. This is another example of a defect in organelle biogenesis as a consequence of defective membrane traffic.

Our results suggest that Vmp1 mutant cells contain very few contractile vacuoles as determine by transmission electron microscopy (TEM) and the use of CV markers. These markers (mainly Rh50) showed a weak staining, labeling few vacuoles in the mutant. This staining did not colocalize with ER or endosomes (data not shown). The reduced levels of these proteins might suggest that the components of the CV system are being degraded or synthesized in lower amounts because they do not seem to be accumulated in other organelles of the secretory pathway.

Mammalian cells lack the contractile vacuole system as they live in an osmotically controlled environment. Therefore, no such behavior is expected to occur by the loss of mammalian Vmp1.

A Possible Role of Vmp1 in Macroautophagy

It has been described that inhibition of Vmp1 expression by small interfering RNA blocks autophagosome formation in mammalian cells (Ropolo *et al*., 2007). Our results in *vmp1*$^-$ mutant in *Dictyostelium* might also support a role in autophagy. TEM images of mutant cells showed the accumulation of autophagic features similar to those described in macroautophagy mutants (Otto *et al*., 2003, 2004). Macroautophagy is induced by starvation and therefore it is probably activated in *vmp1*$^-$ mutant cells in axenic culture as a result of the observed defects in nutrient uptake. Thus, we cannot rule out the possibility that the observed accumulation of vacuoles with unprocessed cellular material might be due to an overactivation of autophagy in the mutant. Other possible explanation is a defect in autophagosome maturation. Such maturation involves dynamic membrane rearrangements and the fusion with lysosomal vesicles that makes the process dependent on the secretory pathway (Mizushima *et al*., 2002). Moreover, the ER itself might, in some cases, be the membrane source for autophagosome formation (Bernales *et al*., 2007; Yorimitsu and Klionsky, 2007). Nevertheless, *Dictyostelium* macroautophagy mutants do not show growth defects or osmotic sensitivity (Otto *et al*., 2003, 2004), suggesting that macroautophagy dysfunction does not account for the overall phenotype of *vmp1*$^-$ mutant. The precise role of Vmp1 in *Dictyostelium* macroautophagy will require further investigation.

Protein Secretion during Growth and Development Depends on Vmp1

We have shown that protein secretion during growth and development is affected by the loss of Vmp1. AprA is an autocrine proliferation repressor in *Dictyostelium*; therefore, a lower level of secretion of this specific factor would induce growth as described previously (Brock and Gomer, 2005). The *vmp1*$^-$ mutant, however, showed a severe growth defect in axenic media. This implies that AprA is not the only factor that is likely to be affected by a general dysfunction in protein secretion. Other unidentified growth-inducing factors may be absent and/or other additional defects may account for the lack of growth in shaking culture.

It is well known that secretion of autocrine factors is necessary for the transition from growth to development in *Dictyostelium* and that this transition is dependent on the onset of a specific gene expression program. We have shown that the expression of representative genes of this program is severely affected and the pattern of protein secretion is also different between wild type and the mutant. If the observed block in development is merely a consequence of the absence of these autocrine factors, we would expect a noncell

autonomous behavior in mixing experiments. However, when mutant cells were mixed with wild-type, mutant cells remained excluded from the aggregates when development was initiated (data not shown). This additional cell-autonomous defect is not surprising if taking into account the severity of the phenotype, which is not only restricted to protein secretion.

The pattern of proteins present in conditioned medium from wild type and the mutant Vmp1 is significantly different. This could be due to differences in protein secretion as well as differences in the expression levels of certain genes because the mutant response to starvation is impaired. Despite our efforts we have been able to identify only few of the differential protein bands. The secreted proteins enriched in the wild-type medium are representative of ER/Golgi-dependent secretory pathway. However, others such as Hsp70 are secreted by nonconventional secretion mechanisms (Nickel, 2005). In this case, Hsp70 was described to be internalized into endolysosmal vesicles by the activity of an ATP-binding cassette transporter, and these vesicles secrete their content by fusing with the plasma membrane (Mambula *et al*., 2007). The high levels of Hsp70 in the mutant supernatant might be related to the activation of stress mechanisms. However, RT-PCR analysis of the hsp70 mRNA levels did not show an induction of the expression of the gene in the mutant (data not shown).

In conclusion, the analysis of Vmp1 null-mutant in *Dictyostelium* has brought us a more comprehensive view of the intricate roles played by this new gene. Our results confirm some data obtained in other experimental systems with regard to the role of Vmp-1 in membrane traffic and protein secretion, but also reveal new functions of Vmp-1, including an essential role in organelle biogenesis and multicellular development.

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