The Pancreatitis-induced Vacuole Membrane Protein 1 Triggers Autophagy in Mammalian Cells*

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Autophagy is a degradation process of cytoplasmic cellular constituents, which serves as a survival mechanism in starving cells, and it is characterized by sequestration of bulk cytoplasm and organelles in double-membrane vesicles called autophagosomes. Autophagy has been linked to a variety of pathological processes such as neurodegenerative diseases and tumorigenesis, which highlights its biological and medical importance. We have previously characterized the vacuole membrane protein 1 (VMP1) gene, which is highly activated in acute pancreatitis, a disease associated with morphological changes resembling autophagy. Here we show that VMP1 expression triggers autophagy in mammalian cells. VMP1 expression induces the formation of ultrastructural features of autophagy and recruitment of the microtubule-associated protein 1 light-chain 3 (LC3), which is inhibited after treatment with the autophagy inhibitor 3-methyladenine. VMP1 is induced by starvation and rapamycin treatments. Its expression is necessary for autophagy, because VMP1 small interfering RNA inhibits autophagosome formation under both autophagic stimuli. VMP1 is a transmembrane protein that co-localizes with LC3, a marker of the autophagosomes. It interacts with Beclin 1, a mammalian autophagy initiator, through the VMP1-Atg domain, which is essential for autophagosome formation. VMP1 endogenous expression co-localizes with LC3 in pancreas tissue undergoing pancreatitis-induced autophagy. Finally, VMP1 stable expression targeted to pancreas acinar cell in transgenic mice induces autophagosome formation. Our results identify VMP1 as a novel autophagy-related membrane protein involved in the initial steps of the mammalian cell autophagic process.

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The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; VMP1, vacuole membrane protein 1; LC3, light-chain 3; siRNA, small interfering RNA; 3-MA, 3-methyladenine; GST, glutathione S-transferase; GFP, green fluorescent protein; mTOR, mammalian target of rapamycin; Atg, autophagy-related domain; RFP, red fluorescent protein.
triggers autophagy, even under nutrient-replete conditions. VMP1 interacts with Beclin 1 through its hydrophilic C-terminal region, which we named Atg domain because it is essential for autophagosome formation. We also show that VMP1 co-localizes with the microtubule-associated protein 1 light chain 3 (LC3) in vacuole membranes in pancreas tissue undergoing pancreatitis-induced autophagy. Finally, we found that VMP1 expression promotes the formation of LC3-positive vacuoles when specifically targeted to the pancreas acinar cells in transgenic mice.

EXPERIMENTAL PROCEDURES

Mammalian Cell Lines, Transfections, and Treatments—Human HeLa, 293T and MCF7, and mouse NIH3T3 (ATCC) cell lines were used. Low-Beclin 1 levels in MCF7 cells were verified by Western blot. Cells were transfected using FuGENE-6 reagent (Roche Applied Science). Plasmids were pcDNA4-V5-His (Invitrogen) and pEGFP-N1 (Clontech), containing full-length rat VMP1 cDNA (NM_138839) (22) or VMP1ΔAB subcloned within the HindIII and BamHI restriction sites. Full-length human Beclin 1 cDNA (NM_003766) was subcloned into pECFP-C1 (Clontech) within the EcoRI and SalI or into pcDNA3 (Invitrogen) within EcoRI and Apal restriction sites. pRFP-C1 containing full-length rat LC3 was kindly provided by Dr. Maria I. Colombo (Universidad Nacional de Cuyo, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina).

Cells were also transfected using Oligofectamine (Invitrogen)-mediated transfer with siRNA pre-designed for human VMP1 mRNA using 5’-GGCAGAAUAAUGUCUCUG-GUGtt-3’, as sense, and 5’-CACAGGCCAAUUUCUCUG-CCTt-3’, as antisense (Ambion ID32935) and 24 h later, cells were subjected to autophagy stimuli for an additional 6 h period. For autophagy inhibition, cells were treated with 10 mM 3-methyladenine (3-MA) (Sigma) 2 h before and during co-transfection with pcDNA4-VMP1 and pRFP-LC3. The efficacy of 3-MA treatment was verified on starved cells (data not shown). For lysosomal hydrolase inhibition, cells were treated with E64d (10 µg/ml) for 4 h before processed. Autophagy was induced by amino acid/serum-deprived medium using Earle’s balanced salt solution (Invitrogen) or 55 µM rapamycin (Calbiochem) treatments. Fluorescence was observed using a fluorescence microscope Nikon Eclipse 200 (Plan100×), an inverted fluorescence microscope Olympus GX71, a confocal microscope Nikon Eclipse C1 (Plan40×/0.95 and Plan60×/1.40), or an inverted confocal microscope Olympus FV1000 (PLAPO N/1.42).

Transmission Electron Microscopy—Cells were fixed without being brought into suspension and processed for transmission electron microscopy by standard procedures. Grids were examined under a Carl Zeiss C-10 electron microscope (Laboratorio Nacional de Investigación y Servicios en Microscopía Electrónica, University of Buenos Aires).

Percentage of RFP-LC3 Cells with Punctate Staining—The number of cells with punctate staining per 100 fluorescent RFP-LC3 transfected cells was determined in three independent experiments. To quantify, the number of fluorescent cells with punctate staining was counted in six random fields representing 100 fluorescent cells and expressed as the mean ± S.D. of combined results. We consider an RFP-LC3 cell to have punctate staining when all the red fluorescence is present as punctate and no diffused protein remains.

Recombinant GST-VMP1 Peptides and His<sub>6</sub>-Beclin 1 Protein—VMP1 hydrophilic peptides were amplified from pcDNA4-VMP1 by PCR and subcloned into pGEX-5X-2 (Amersham Biosciences) to obtain GST-VMP1 peptides. Peptides were expressed in Escherichia coli BL21 and purified by glutathione affinity chromatography (Amersham Biosciences). Beclin 1 cDNA was obtained from HeLa cell total RNA extracts and cloned into pQE31 (Qiagen). His<sub>6</sub>-Beclin 1 was expressed in E. coli M15 and purified using nickel-nitrilotriacetic acid-agarose beads (Qiagen).

Membrane Isolation—Cells were washed and homogenized with a motor driven Teflon pestle homogenizer in ice-cold SBE buffer (250 mM sucrose, 1 mM EGTA, 10 mM Hepes/KOH, pH 7.5) containing protease inhibitors. Homogenates were centrifuged twice at 500 × g for 15 min. The resulting supernatants were centrifuged at 100,000 × g for 1 h. Membrane pellets were resuspended in ice-cold SBE buffer and treated with 1% Nonidet P-40 (Nonidet P-40, 0.2 M Na<sub>2</sub>CO<sub>3</sub>, pH 11.0, or 1.5 M NaCl. All steps were performed at 4 °C. The membrane pellets were obtained by centrifugation, and retained proteins were separated by SDS-PAGE. Western blot analysis was performed using as secondary antibody a peroxidase-labeled IgG antibody provided with the ECL-kit (Amersham Biosciences). Immunoblotting was performed using the ECL kit and membranes were exposed to a Kodak BioMax film.

Pulldown Assays—HeLa cells transfected with the pcDNA4-VMP1, pcDNA4-VMP1ΔAB, and pcDNA4-empty plasmids were lysed by sonication and solubilized with 1% Triton X-100, followed by a 30-min centrifugation at 100,000 × g. The supernatants containing His-tagged proteins were incubated with nickel-nitrilotriacetic acid-agarose beads (Qiagen) for 2 h at 4 °C, extensively washed with a buffer containing 5 mM imidazole, and eluted with a buffer containing 120 mM imidazole. Proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. In another experiment His<sub>6</sub>-Beclin 1 produced in E. coli was incubated in nickel-nitrilotriacetic acid-agarose beads with the purified GST-VMP1 peptides.

Co-immunoprecipitation Assays—For the co-immunoprecipitation assays, the supernatants from lysates of pcDNA4-VMP1-transfected or rapamycin-treated cells were incubated with anti-Agarisent to protein A-Sepharose<sup>TM</sup> CL-4B beads (Amersham Biosciences) for 2 h at 4 °C and washed extensively. Bound proteins were eluted with 100 mM glycine-HCl, pH 2.5, precipitated by 5% trichloroacetic acid, washed with ice-cold acetone, and resuspended in a SDS sample buffer.

Antibodies—Polyclonal rabbit antisera to VMP1 against the peptide MAQSYAKRIQQRNLSEKTK (residues 386–406) was obtained and used at 1:100 dilution. Polyclonal goat anti-LC3, goat anti-Beclin 1, rabbit anti-GST, monoclonal mouse anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal mouse anti-V5 (Invitrogen) antibodies were used according manufacturer. Donkey anti-rabbit and anti-goat Alexa Fluor 488 and 590, and rabbit anti-mouse Alexa Fluor 590 (Molecular Probes) antibodies were used for immunofluores-
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cence. Peroxidase-labeled anti-rabbit, anti-mouse, and anti-goat IgG antibodies were used for Western blot according Amersham Biosciences.

Caerulein-induced Pancreatitis—Male Wistar rats weighing 200–250 g were used. Animals were housed with free access to food and water. Experiments were performed according to the standard ethical and legal guidelines of the Administración Nacional de Medicamentos, Alimentos y Tecnología Médica, University of Buenos Aires, and the European Union regulations for animal experiments. Pancreatitis was induced by seven intraperitoneal injections of caerulein (Sigma, 50 μg/kg) given at 1-h intervals; the rats were killed by decapitation at different times after the first injection. In one series of experiments, the pancreas were removed, homogenized in HEPES buffer, pH 7.4, containing protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, 5 μg/ml leupeptin, and 2.5 μg/ml aprotinin) and processed for Western blot analysis. In another series of experiments, the pancreas were removed, fixed in formalin buffer, and processed for immunofluorescence.

Transgenic Mice—The transgene cassette was made using the pBEG vector (25). The expression cassette contains the acinar-specific control region (−500 to +8) from the rat elastase I gene and the human growth hormone 3′-untranslated region (+500 to +2657). This construct was digested with BamHI, filled in, dephosphorylated, and ligated with rat VMP1-EGFP released from pEGFP-VMP1 plasmid. A 1940-kb HindIII/NotI fragment was isolated and used for microinjections into in-bred Friend virus B-type susceptibility zygotes. Genomic DNA was prepared and tested by Southern blot or PCR.

RESULTS

VMP1 Expression Triggers Autophagy—To know if VMP1 triggers autophagy, we transfected HeLa cells with the expression plasmid pcDNA4-VMP1, which codes for the VMP1-V5 fusion protein. The pcDNA4-empty plasmid was used in controls. Cells were cultured in nutrient and grown factor-replete conditions and fixed in glutaraldehyde 24 h later to perform transmission electronic microscopy. We found that cells expressing VMP1 showed multiple autophagic features. Fig. 1A shows cup-shaped structures, double-membrane structures containing cytoplasmic material (autophagosome like structure), as well as single-membrane structures containing cytoplasmic constituents at different stages of degradation (autolysosome-like structure) (26, 27). The same morphological features were obtained when 293T cells were transfected with the VMP1-expression plasmid, and they did not differ from those obtained in rapamycin-treated cells (data not shown).

During autophagy, the cytosolic form of LC3 (LC3-I) undergoes C-terminal proteolytic and lipid modifications (LC3-II) and translocates from the cytosol to the autophagosomal membrane (28, 29). LC3 is currently used as a specific marker of autophagy (30, 31). To confirm the extent and specificity of VMP1 autophagosome induction, we first immunostained pcDNA4-VMP1-transfected 293T cells with a specific LC3 antibody, and we observed the recruitment of endogenous LC3 in punctate structures consistent with autophagy (Fig. 1B). Then, we investigated LC3-I and -II forms by Western blot (Fig. 1C). We found induction of LC3 with increased LC3-II form signal in pcDNA4-VMP1-transfected cells (Fig. 1C, lane 1). Because intra-autophagosomal LC3-II is degraded by lysosomal proteases, we blocked its proteolysis using the lysosomal protease inhibitor E64d (32), and we found that LC3-II signal was enhanced in VMP1-expressing cells (Fig. 1C, lane 4). In another series of experiments HeLa, 293T, and NIH3T3 cells cultured under nutrient and grown factor-replete conditions...
were concomitantly transfected with an expression plasmid encoding for the RFP-LC3 fusion protein and pcDNA4-VMP1 or pcDNA4-empty plasmids. Fig. 1D shows the recruitment of LC3 fusion protein in punctate structures in VMP1-transfected cells in contrast to the diffuse RFP-LC3 fusion protein signal observed in control cells. We determine the percentage of fluorescent RFP-LC3 cells with punctate staining in three independent experiments per cell line as under “Experimental Procedures.” We found high recruitment of LC3 in VMP1-transfected cells. Finally, we investigated the potential for inhibiting the pathway with an agent well documented to inhibit autophagy. The progression of the autophagy is sensitive to the PI3K inhibitors such as 3-MA, with the target being the Class III PI3K (33). We treated cells with 3-MA before the co-transfection with pRFP-LC3 and pcDNA4-VMP1 expression plasmids, and we found that the percentage of RFP-LC3 cells with punctate staining was low and almost the same as the observed in pcDNA4-empty transfected cells (Fig. 1D). These results collectively demonstrate that VMP1 expression triggers autophagy in mammalian cells, even under nutrient-replete conditions.

**VMP1 Expression Is Required for Extracellular Stimuli-induced Autophagy**—The autophagy trafficking pathway was first described as a cellular adaptation to starvation (34). To investigate whether starvation activates endogenous VMP1 expression, we developed a rabbit polyclonal anti-VMP1 antibody. We subjected HeLa cells to a standard starvation protocol (amino acid/serum-deprived medium) and then analyzed the time course of VMP1-mRNA expression by reverse transcription-PCR (Fig. 2A) and of VMP1-protein expression by Western blot (Fig. 2B). VMP1 expression was activated under starvation, and it was evident after 2-h treatment. Moreover, starvation-induced VMP1 expression was detected in punctate structures by immunofluorescence, whereas it was almost not detectable under nutrient-replete conditions (Fig. 2C). mTOR kinase plays a central role in the amino acid pool-sensing mechanism. In response to starvation, mTOR is inhibited, resulting in the induction of autophagy (35). Because mTOR can be inhibited by rapamycin, we treated HeLa cells with rapamycin as a pharmacological agent to induce autophagy. We found that mTOR inhibition induces VMP1-mRNA and VMP1-protein expression (Fig. 2, A–C). To establish whether VMP1 is required for autophagy, we reduced the expression of VMP1 using the small interfering RNA (siRNA) strategy. HeLa cells were transfected with VMP1-siRNA and then subjected to standard starvation protocol or rapamycin treatment. VMP1 expression was efficiently knocked down (Fig. 2, D and E). We found that autophagosome formation was almost completely inhibited in VMP1-siRNA cells under both treatments, as evidenced by the distribution of the RFP-LC3 fluorescence fusion protein (Fig. 2F). We analyzed three independent experiments per treatment, and we found that the percentage of RFP-LC3 cells with punctate staining in VMP1-siRNA transfected cells was highly reduced in comparison with those transfected with scramble siRNA (Fig. 2F). These findings show that VMP1 expression is required for extracellular stimuli-induced autophagy.

**VMP1 Remains as a Membrane Protein during Autophagy**—VMP1 primary structure predicts a transmembrane protein (22). GFP moiety folds properly and becomes fluorescent only if a membrane protein is stably inserted into the membrane (36). To verify if VMP1 remains in membrane structure we transfected cells with pEGFP-VMP1 expression plasmid, and cells were cultured in nutrient and grown factor-replete conditions. After 24 h the GFP fluorescence of the fusion protein VMP1-EGFP was observed in vacuole membranes induced by its own expression (Fig. 3A). We then analyzed whether VMP1 co-localizes with endogenous LC3 in the VMP1-induced vacuoles. We performed immunofluorescence using the anti-LC3 and anti-V5 antibodies in HeLa cells transfected with VMP1-V5 expression plasmid. We found a remarkable co-localization between VMP1-V5 fusion protein and endogenous LC3 in VMP1-induced vacuoles (Fig. 3B). To know if the endogenous

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**FIGURE 2.** VMP1 expression is required for extracellular stimuli-induced autophagy. A–C, HeLa cells were subjected to the standard starvation protocol or to rapamycin treatment, and the time course of VMP1 expression was analyzed by reverse transcription-PCR (primer pairs used were: VMP1 sense, 5’-TATGCGAAGAATCCGAGC-3’, and VMP1 antisense, 5’-GAGGGTGCTGCAATGATCATA-3’) (A) and Western blot (B). Immunofluorescence of VMP1 in starved, rapamycin-treated, and control HeLa cells was detected with a confocal microscope (C). D and E, reverse transcription-PCR (D) and Western blot (E) of VMP1 expression in HeLa cells transfected with VMP1-siRNA or scramble-siRNA (scble-siRNA) and subjected to the standard starvation protocol or to rapamycin treatment. VMP1 expression was efficiently reduced in these cells (>85%). F, HeLa cells transfected with pRFP-LC3 and VMP1-siRNA or scramble-siRNA and cultured under the standard starvation protocol or rapamycin treatment. LC3 redistribution was not observed in the VMP1-siRNA cells. The number of cells with punctate staining per 100 fluorescent RFP-LC3 transfected cells was determined as described under “Experimental Procedures” and expressed as the mean ± S.D. of combined results from three independent experiments (IB: Immunoblotting).
VMP1 also remains as a membrane protein during autophagy, we performed subcellular fractioning of HeLa cells undergoing rapamycin-induced autophagy and investigated VMP1 in membrane preparations by Western blot. Fig. 3C shows that, although the protein is not detectable in untreated cells, VMP1 is found in the membrane preparations from cells undergoing autophagy, and the signal persists when membrane fractions are treated with 1.5 M NaCl or exposed to pH 11.0. These results indicate that VMP1 functions as a membrane protein during autophagy.

**VMP1 Is a Beclin 1-binding Protein**—To obtain a mechanistic insight as to how VMP1 triggers autophagy, we analyzed its function in the molecular pathway of the autophagosome formation. During autophagy Beclin 1-Class III PI3K complex (37) is thought to undergo subcellular distribution to a membrane structure, which eventually leads to the recruitment of autophagy proteins and the proper conjugation of LC3 to membrane phospholipids (19, 38). However, the transmembrane protein with which Beclin 1 complex interacts remains elusive. To investigate if VMP1 is a target membrane protein with which Beclin 1 interacts, we first analyzed if Beclin 1 localizes in the membrane of the VMP1-induced vacuoles. We concomitantly transfected 293T cells with pEGFP-VMP1, pRFP-LC3, and pECFP-Beclin 1 expression plasmids (results are shown in Fig. 4A). We found a remarkable co-localization between VMP1, LC3, and Beclin 1 fluorescent fusion proteins, suggesting that Beclin 1 could attach to VMP1-induced vacuole membranes. We concomitantly transfected 293T cells with pEGFP-VMP1, pRFP-LC3, and pECFP-Beclin 1 expression plasmids (results are shown in Fig. 4A). We found a remarkable co-localization between VMP1, LC3, and Beclin 1 fluorescent fusion proteins, suggesting that Beclin 1 could attach to VMP1-induced vacuole membranes. Then, we studied if VMP1 interacts with Beclin 1. We performed a pulldown assay using the VMP1-V5-His<sub>6</sub> fusion protein. Lysates prepared from HeLa cells transfected with pcDNA4-VMP1 were treated with Triton X-100 and incubated with nickel-agarose beads. After extensively washed, retained proteins were eluted with imidazole and separated by SDS-PAGE followed by anti-Beclin 1 or anti-V5 antibody immunoblotting. VMP1-V5 and Beclin 1 were found in eluates (Fig. 4B). Control experiments showed that neither Beclin 1 nor VMP1-V5 was detected in the elution fraction of pcDNA4-empty-transfected cells. In another series of experiments, lysates from pcDNA4-VMP1-transfected cells were incubated with the anti-Beclin 1 antibody. Controls were incubated with control serum. Immunoprecipitates were separated and immunoblotted for VMP1-V5 with anti-V5 antibody. VMP1-V5 was detected in Beclin 1 antibody-treated lysates but not in the controls (Fig. 4C). To analyze the physiological relevance of the VMP1-Beclin 1 interaction, we investigated whether endogenous VMP1 interacts with endogenous Beclin 1 in cells developing rapamycin-induced autophagy by co-immunoprecipitation experiments. Endogenous Beclin 1 and VMP1 induced by rapamycin treatment were detected in immunoprecipitates of Triton X-100-solubilized rapamycin-treated HeLa cells using anti-VMP1 antibody (Fig. 4D). Interaction of endogenous VMP1 with endogenous Beclin 1 was confirmed using anti-Beclin 1 antibody (Fig. 4E). Similar results were found in starved cells (data not shown). Then, we analyzed whether VMP1 expression is able to induce LC3 recruitment in autophagy-deficient, low-Beclin 1-expressing, MCF7 cells. We transfected MCF7 cells with pcDNA4-VMP1 expression plasmid and found that the percentage of RFP-LC3 transfected cells with punctate staining is low and similar to the percentage found in starved and control MCF7 cells (Fig. 4F), suggesting that VMP1-induced LC3 recruitment would be dependent on Beclin 1. Moreover, restoring Beclin 1 expression in MCF7 cells enables VMP1-induced LC3 recruitment (Fig. 4F). These results collectively show that VMP1 interacts with Beclin 1.

**VMP1-Atg Domain Is Essential for Beclin 1 Interaction and Autophagy Induction**—To further delineate the interaction between VMP1 and Beclin 1, VMP1 hydrophilic domains were tested for Beclin 1 binding. VMP1 406 amino acid primary structure is predicted to contain 6 transmembrane domains (22). Four recombinant GST-VMP1 hydrophilic peptides, VMP1-(1–75), VMP1-(187–247), VMP1-(324–366), and VMP1-(378–406) (Fig. 5A), and the full-length His<sub>6</sub>-VMP1 were produced in *E. coli*. We incubated in nickel-agarose bead recombinant His<sub>6</sub>-Beclin 1 fusion protein with each purified GST-VMP1 peptide in triplicate independent experiments. After extensively washed, retained proteins were eluted with imidazole and separated by SDS-PAGE followed by immunoblotting with anti-Beclin 1 and anti-GST antibodies. Fig. 5B shows that only GST-VMP1-(378–406) was found in eluates, indicating that VMP1 interacts with Beclin 1 by the amino acid 378–406 hydrophilic domain, which we named the autophagy-related (Atg) domain.

To obtain further molecular insights into VMP1 proposed function and to evaluate the relevance of VMP1-Beclin 1 interaction in the autophagosome formation, we constructed pEGFP and pcDNA4 plasmids containing the defective mutant VMP1<sup>ΔN158</sup>, in which the VMP1-(378–406) peptide was deleted. First, we performed a pulldown assay using the
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VMP1\(^{ΔAtg}\)/V5-His\(_6\) fusion protein. Lysates from HeLa cells transfected with pcDNA4-VMP1\(^{ΔAtg}\) were incubated with nickel-agarose beads, and retained proteins were eluted, separated, and immunoblotted with anti-Beclin 1 or anti-V5 antibodies. We found no signal of endogenous Beclin 1 in the eluted fraction (Fig. 5C), indicating that VMP1\(^{ΔAtg}\) was not able to interact with Beclin 1. Then, we performed subcellular fractioning of HeLa cells transfected with pcDNA4-VMP1\(^{ΔAtg}\) and investigated VMP1\(^{ΔAtg}\)-V5 in membrane preparations by Western blot using anti-V5 antibody. Fig. 5D shows that VMP1\(^{ΔAtg}\) is detected in membrane preparations, and the signal persisted in membrane fraction even after 1.5 M NaCl or pH 11.0 treatments. These results indicate that the defective mutant VMP1\(^{ΔAtg}\) still functions as a transmembrane protein. In another series of experiments, HeLa cells were transfected with the pEGFP-VMP1 or the pEGFP-VMP1\(^{ΔAtg}\) that encodes for the VMP1\(^{ΔAtg}\)-EGFP fluorescent fusion protein. Cells were cultured in nutrient and grown factor-replete conditions for 24 h. Surprisingly, we found that cells expressing VMP1\(^{ΔAtg}\)-EGFP did not form the characteristic vacuoles observed in VMP1-EGFP-expressing cells (Fig. 5E). To evaluate if the Atg domain of VMP1 is required for autophagy, HeLa cells were

FIGURE 4. VMP1 is a Beclin 1 binding membrane protein. A, 293T cells cultured under nutrient and growth factor-replete conditions for 24 h after transfection with pEGFP-VMP1, pRFP-LC3, and pECFP-Beclin 1. Fluorescence detected using the inverted confocal microscope shows remarkable colocalization of VMP1-EGFP, RFP-LC3, and ECFP-Beclin 1. An example of a cell with LC3-marked vacuole co-staining with VMP1 and Beclin 1 is shown in detail. B, pulldown assay using the VMP1-V5-His\(_6\) fusion protein. HeLa cells were transfected with pcDNA4-VMP1 or pcDNA4-empty plasmids. Lysates were incubated with nickel-agarose beads. After washes, eluted proteins were separated and subjected to Beclin 1 and V5 immunoblotting. The TCL lane contains equivalent amounts of the protein used for binding. C, co-immunoprecipitation assays. Lysates from HeLa cells transfected with the pcDNA4-VMP1 expression plasmid incubated with anti-Beclin 1 or control sera covalently bound to protein A-Sepharose beads. After washes, eluted proteins were separated and subjected to Beclin 1 and V5 immunoblotting. Data is representative of three independent experiments. D, MCF7 cells transfected with pRFP-LC3 and cultured under the standard starvation protocol (Unstarved) or concomitantly transfected with pRFP-LC3 and pcDNA4-VMP1 plasmids (VMP1) or with pRFP-LC3, pcDNA3-Beclin 1, and pcDNA4-VMP1 plasmids (VMP1 + Beclin 1). Control MCF7 cells (Unstarved) cultured under nutrient and growth factor-replete conditions. The number of cells with punctate staining per 100 fluorescent RFP-LC3 transfected cells was determined as described under "Experimental Procedures" and expressed as the mean ± S.D. of combined results from three independent experiments (TCL: total cell lysate; IP: immunoprecipitate; and IB: immunoblotting).
concomitantly transfected with expression plasmids encoding for RFP-LC3 and VMP1ΔAtg-V5 or VMP1-V5 fusion proteins, and cells were cultured in nutrient and growth factor-replete conditions. Fig. 5F shows that VMP1ΔAtg expression failed to trigger autophagy, as it is evidenced by the diffused distribution of the RFP-LC3 fluorescent fusion protein observed in pcDNA4-VMP1ΔAtg-transfected cells. The percentage of RFP-LC3 cells with punctate staining in VMP1ΔAtg-expressing cells was highly reduced in comparison with cells expressing the full-length VMP1. Moreover, VMP1ΔAtg expression did not induce the recruitment of Beclin 1, which remains diffuse in pEGFP-VMP1ΔAtg-transfected cells, as it is shown in Fig. 5G. The above described results show that VMP1-Beclin 1 interaction through the VMP1-Atg domain is essential for the autophagosome formation. Therefore, we propose that VMP1 is a membrane protein that interacts with Beclin 1 to trigger the autophagic process.

**VMP1 Expression in Pancreas of Transgenic Mice Triggers the Formation of LC3-positive Vacuoles—**To have an in vivo insight on whether VMP1 stable expression is able to induce autophagy, we developed a transgenic mouse in which the pancreas acinar-cell-specific elastase promoter drove VMP1-EGFP expression. Expression of the VMP1-EGFP transgenic fusion protein was detected by Western blot in the pancreas of transgenic mice (Fig. 6A) but not in the liver, kidney, spleen, heart, or lung (data not shown). Pancreases from VMP1-EGFP transgenic mice showed numerous vacuoles in acinar cells whose membranes were immunostained with anti-GFP (Fig. 6B). VMP1-induced vacuoles in pancreas from transgenic mice were assayed for LC3 by immunofluorescence using anti-LC3...
VMP1 Localizes in the Membrane of Pancreatitis-induced Autophagic Vacuoles—Finally, we investigated whether VMP1 is involved in the autophagy during a pathological process using an experimental animal model of pancreatitis. Autophagy has been described as an early cellular event in human and experimental acute pancreatitis (12, 39, 40). Pancreas tissue from rats treated with caerulein, a widely used experimental model of pancreatitis, developed cytoplasmic vacuolization with ultrastructural features of autophagy (39, 40). We analyzed the expression of the VMP1 protein by Western blot in pancreas tissue during the development of the experimental pancreatitis.

Fig. 7A shows that acute pancreatitis induces VMP1 expression that was maximal after 6-h treatment. To know if VMP1 expression is related to the autophagic process in pancreas undergoing pancreatitis, we analyzed VMP1, LC3, and Beclin 1 in pancreas specimens from the animal model by immunofluorescence using anti-VMP1, anti-LC3, or anti-Beclin 1 antibodies. We found that, although VMP1 is not detectable in control pancreas tissue, pancreatitis-induced endogenous VMP1 was observed in control tissues. The experiments are representative of three independent experiments (IB: immunoblotting).

and anti-GFP antibodies. We found that VMP1 co-localizes with endogenous LC3 in the membrane of VMP1-induced vacuoles (Fig. 6C), showing that VMP1 in vivo stable expression in pancreas from transgenic mice is able to induce the formation of LC3-positive vacuoles. We also analyzed VMP1-EGFP and Beclin 1 using anti-GFP and anti-Beclin 1 antibodies. We found that VMP1 co-localizes with endogenous Beclin 1 in the VMP induced-vacuole membrane (Fig. 6D) showing the subcellular distribution of Beclin 1 to VMP1-induced vacuolar structures in pancreas from transgenic mice. These results suggest that VMP1-stable expression is able to trigger autophagosome formation in mammalian tissue.

VMP1 expression in pancreas of transgenic mice triggers the formation of LC3 positive vacuoles. A, pancreas was processed for Western blot analysis using anti-GFP antibody. VMP1-EGFP was expressed in the pancreas from transgenic mice, whereas no signal was observed in pancreas from wild-type mice (control). B, immunohistochemistry of GFP in pancreas tissue from control and VMP1-EGFP mice (original magnifications: ×40 and ×100) using mouse anti-GFP and anti-mouse-horseradish peroxidase antibodies. A vacuole with GFP immunostained is shown in detail. C and D, immunofluorescence using anti-GFP, anti-LC3, and anti-Beclin 1 antibodies in pancreas tissue from transgenic mice. Co-localization of VMP1-EGFP and LC3 (C) and VMP1-EGFP and Beclin 1 (D) were observed in vacuole membrane using the confocal microscope. The experiments are representative of three independent experiments (IB: immunoblotting).

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result suggests the autophagosomal localization of pancreatitis-induced VMP1. Moreover, we found that endogenous VMP1 also co-localized with endogenous Beclin 1 in acinar cell membranes of the autophagic tissue (Fig. 7C). These results strongly support the findings described in vitro and suggest that the early VMP1 expression during acute pancreatitis could be involved in the autophagy induced by the disease.

DISCUSSION

VMP1 Triggers Autophagy Even under Nutrient-replete Conditions—Our results have presented experimental evidence that shows that the expression of VMP1 triggers autophagy. VMP1 expression in cells cultured under nutrient-replete conditions presents multiple ultrastructural features of autophagy, including cup-shaped structures, autophagosomes, and autolysosome-like features. In addition, VMP1 induces the almost total recruitment of RFP-LC3 fluorescence protein. In all the experiments >85% RFP-LC3 fluorescent cells was found punctate staining. Consistent results were found when endogenous LC3 was assayed. Moreover, VMP1 induces processing of endogenous LC3-I to LC3-II, which enhances in presence of hydrolyase inhibitors. Although transiently overexpressed LC3 protein could be prone to aggregate in an autophagy-independent manner (41), the processing of the endogenous LC3-I to LC3-II was report to be not affected by the overexpression and remains as a hallmark of autophagy (30, 41). On the other hand, VMP1-induced LC3 recruitment was highly reduced by the autophagy inhibitor 3-MA (33). 3-MA was reported to have side-effects apart from inhibit autophagy (42), however, the inhibition of LC3 recruitment is due to its ability to inhibit autophagy. Moreover, VMP1 is involved in the extracellular stimuli-induced autophagy, because treatments currently used to trigger autophagy, such as starvation, and pharmacological mTOR inhibition, induce VMP1 expression. Furthermore, the knockdown of VMP1 expression abolishes starvation as well as rapamycin-induced autophagosome formation suggesting that VMP1 expression is required for autophagy. Our results have also shown that VMP1 remains in the membrane fraction of cells undergoing rapamycin-induced autophagy. VMP1-EGFP remains in the vacuole membrane induced by its own expression and VMP1-V5 fusion protein co-localizes with endogenous LC3 in the vacuole membrane, suggesting that VMP1 could be an integrated protein of the autophagosomal membrane. Finally, the transgenic mouse for VMP1 expression targeted to pancreatic acinar cells allowed us to confirm, within a physiological setting, the results discussed above. The in vivo stable VMP1 expression induces the formation of numerous vacuoles in acinar cells, where it co-localizes with endogenous LC3.

VMP1 Functions as a Beclin 1-interacting Membrane Protein—We have presented experimental data indicating that VMP1 interacts with Beclin 1. We found the interaction of VMP1 with endogenous Beclin 1 in VMP1-expressing cells and the interaction of both endogenous proteins in rapamycin-induced autophagic cells. VMP1-Beclin 1 direct interaction was confirmed using recombinant peptides. Moreover, VMP1-Atg domain has proved to be essential for VMP1-induced autophagy, because VMP1ΔAtg expression failed to induce LC3 recruitment. Our data have also shown the co-localization of transient Beclin 1 and LC3 in VMP1-induced vacuoles, and this triple co-localization was abolished when cells were transfected with the defective mutant VMP1ΔΔ,Δ. On the other hand, VMP1 expression failed to induce autophagy when it was expressed in low-Beclin 1, MCF7 cells. These findings suggest that VMP1-induced autophagy probably involves the interaction with Beclin 1. Experimental animal models have shown VMP1-Beclin 1 co-localization. VMP1-EGFP co-localized with endogenous Beclin 1 in pancreas from transgenic mice, and both endogenous proteins co-localized in tissue undergoing pancreatitis-induced autophagy. Beclin 1 is a haploinsufficient tumor-suppressor gene (3), which is involved in the initial steps of autophagy. Several Beclin 1 interactions have been described affecting its autophagic activity (17, 18). Beclin 1-Class III PI3K complex was shown to be essential for early stages of autophagosome formation, mediating the localization of other autophagy proteins to the autophagosomal membrane (37). Our results allowed us to hypothesize that VMP1 might function as the transmembrane protein that interacts with the Beclin 1 to lead the localization of other autophagy proteins to the autophagosomal membrane in mammalian cells. So far, only two transmembrane proteins have been described in yeast to be autophagy-related, Atg9 (44) and Atg27 (45, 46). Mammalian Atg9 was also described, but its actual participation in the autophagosome formation remains to be determined (47–49). However, none of these transmembrane proteins has been reported to trigger autophagy or to interact with Atg6/Beclin 1. Moreover, VMP1 has no known homologues in yeast. To our knowledge, VMP1 would be the first transmembrane protein described to induce an autophagic process in mammalian cells. The finding, that VMP1, which has no known homologue in yeast, is able to start an autophagic process, supports the hypothesis that mammalian cells regulate autophagy in a different way.

In Vivo VMP1 Expression in Mammalian Tissue Is Involved in Autophagic Vacuole Formation—In this study two animal models of VMP1 expression support data obtained in the in vitro studies: (i) the VMP1 transgenic mouse, in which VMP1 expression was targeted to pancreatic acinar cells, and (ii) the experimental-induced acute pancreatitis in rats. The transgenic expression of VMP1 in mouse pancreas allowed us to show, within a physiological setting, that VMP1 expression is able to induce the autophagosome formation in mammalian cells. The in vivo stable VMP1 expression induced the formation of numerous vacuoles in acinar cells of transgenic mice. These vacuoles stain for endogenous LC3 by immunofluorescence suggesting autophagosomal membranes where VMP1-EGFP was found co-localized. These results strongly support those obtained when transient VMP1 expression was assayed in cell lines. In previous work, VMP1-mRNA basal expression was found in several rat tissues such as kidney (22). These observations are consistent with the basal autophagy found in the same tissue from the GFP-LC3 transgenic mouse (29). Although basal VMP1 expression could be related to other physiological processes, the relationship between GFP-LC3 transgenic mouse and VMP1 basal expression leads us to speculate that VMP1 expression might correlate to the presence of autophagy in mammalian tissue. Finally, results obtained in pancreas tis-
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VMP1 triggers autophagy in mammalian cells. The fact that this novel autophagy-related protein is induced by a pathological autophagic process might lead to further studies on the role of autophagy in cell response to disease.

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