Cellular Stress in Acute Pancreatitis

2

J.L. Iovanna

Introduction

Acute pancreatitis is the most frequent disease of the pancreas. The spectrum of acute pancreatitis can range from mild edematous to severe necrotizing [1]. One of the most interesting hypotheses for the pathogenesis of the disease is that autodigestion of the gland occurs when hydrolytic enzymes (e.g., trypsinogen, chymotrypsinogen, proelastase, and prophospholipase A) are unduly activated within the pancreas rather than into the intestinal lumen [2, 3]. Activated enzymes, especially trypsin, not only digest pancreatic and peripancreatic tissues but can also activate other zymogens, such as proelastase and prophospholipase A. The active enzymes then digest cellular membranes and lead to edema, interstitial hemorrhage, vascular damage, coagulation necrosis, fat necrosis, and parenchymal cell necrosis.

Living organisms respond at the cellular level to stress or pathological aggression by altering the normal pattern of protein synthesis [4–8]. That change is characterized by a dramatic induction of stress proteins with concomitant inhibition of the normal array of cellular proteins. Stress proteins are not novel components of the stressed cells since most of them are expressed to some level in cells grown under normal conditions [9, 10]. Because most attacks of acute pancreatitis are mild and self-limiting, it is possible that the pancreatic cells respond with a rapid adaptation of their phenotype, which eventually stops the progression of pancreatitis. However, in 10–20% of cases, a severe disease with multiple local and systemic complications develops [11]. In these cases, we can speculate that the pancreatic defense mechanisms fail to protect the gland and therefore the organism. Studies in animals and humans, performed during the acute phase of pancreatitis, demonstrated that the content and secretion of pancreatic enzymes, which are potentially harmful, were generally reduced, as part of a defense mechanism. Conversely, other genes were strongly activated during the acute phase of the disease [12, 13]. Therefore, like the liver [14] and other

J.L. Iovanna
INSERM U.624, Stress Cellulaire, 163 Avenue de Luminy, CP 915, 13288 Marseille Cedex 9, France
e-mail: iovanna@marseille.inserm.fr

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organs [6, 7], the pancreas exposed to acute stress seems to trigger a stringent emergency program that helps the gland fight the aggression and, consequently, protects the organism from the deleterious effects of pancreatitis.

The aim of our research is to characterize at the molecular level the pancreatic emergency program set up in response to pancreatitis. We developed a strategy in which the phenotype of the pancreas with acute pancreatitis was established by characterization of a large number of its transcripts. Such a complementary DNA (cDNA) collection represents a reservoir from which transcripts involved in the emergency response can be identified on the basis of their expression patterns. In this report, we describe a novel membrane protein, named "vacuole membrane protein 1" (VMP1), that is strongly and rapidly induced in pancreas during acute pancreatitis.

Cloning the Rat VMP1 Messenger RNA

A cDNA library of 4.1×10^5 clones was constructed from the polyadenylated RNA fraction purified from a rat pancreas with acute pancreatitis. From this cDNA library, 1,536 randomly selected clones were partially sequenced and the resulting sequences were compared with the GenBank database. Among these clones, 256 could not be related to any sequence in the database. Expression of these messenger RNAs (mRNAs) during acute pancreatitis was systematically analyzed by northern blot. Expression analysis of clone 10F5 showed an interesting pattern. This mRNA is strongly and rapidly activated after the induction of experimental acute pancreatitis. The VMP1 mRNA codes for a protein of 406 amino acids, with a theoretical p*I* of 6.28. The predicted molecular mass is 45,901 Da. Analysis of the deduced VMP1 primary structure revealed the presence of six transmembrane helices which are typical of a transmembrane protein.

VMP1 mRNA Expression During the Course of Acute Pancreatitis

Pancreatic RNA was obtained from rats at different times after induction by caerulein of experimental acute pancreatitis. Northern blot analysis with the VMP1 cDNA probe revealed a very low level of expression at time zero. Significant induction was observed after 30 min. It was maximal after 1 h and remained activated during the whole study (18 h). Also, high levels of VMP1 mRNA were expressed during taurocholate-induced pancreatitis. In situ hybridization was performed to identify which cell type expressed the VMP1 transcript in pancreas. Pancreatic tissues from control and caerulein-treated rats were hybridized with the digoxigenin-labeled antisense VMP1 RNA. A strong labeling was observed in the acinar cells. By contrast, VMP1 remained undetectable in the islets of Langerhans, ducts, inflammatory infiltrate, and stromal tissue in pancreas. Pancreas from control rats showed no signal.

Induction of VMP1 mRNA Expression in Stressed Kidney

To see whether VMP1 induction was specific to the injured pancreas, we monitored its expression in the postischemic kidney. In our experimental model, the left kidney of the rats was subjected to 30-min ischemia, the right kidney being used as control, and expression of VMP1 mRNA was studied 16 h later. VMP1 mRNA expression is induced by ischemic treatment. VMP1 mRNA expression was low in the right (control) kidney, but strong in the left (ischemic) kidney. Pancreas of the same animals showed no VMP1 mRNA induction.

VMP1 Expression Induced Vacuole Formation

The intracellular localization of VMP1 was assessed by transfecting Cos7 cells with a vector allowing expression of an enhanced green fluorescent protein (EGFP)-tagged VMP1 protein and direct monitoring of EGFP fluorescence. Figure 1 shows that VMP1 is located in the Golgi apparatus and the endoplasmic reticulum area, and that its expression induces formation of vacuoles and this is why we named the protein "vacuole membrane protein 1" (VMP1). The same fusion plasmid was transfected into other cell lines with similar results. The forced expression of VMP1 induces vacuole formation in mammalian cells and its endogenous expression correlates with vacuole formation in pathological pancreatic tissue [15]. Therefore, we thought that VMP1 could be involved in the autophagic process and we tested the hypothesis that VMP1 expression triggers the formation of autophagosomes in mammalian cells.

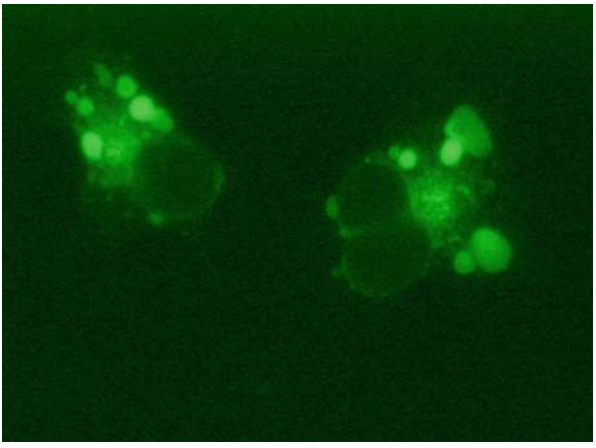

Fig. 1 Forced expression of the vacuole membrane protein 1 (VMP1)–enhanced green fluorescent protein (EGFP) fusion protein in Cos7 cells. Cos7 cells were transfected with the plasmid pEGFP–VMP1 using FuGENE reagent. VMP1 expression was evidenced by direct green fluorescence of the VMP1–EGFP fusion protein. Fluorescence microscopy images show that VMP1–EGFP expression induced cytoplasmic vacuole formation

What Is Autophagy ?

Autophagy is an evolutionarily preserved degradation process of cytoplasmic cellular constituents, which serves as a survival mechanism in starving cells [16–18]. This catabolic process is involved in the turnover of long-lived proteins and other cellular macromolecules, and might play a protective role in development, aging, cell death, and defense against intracellular pathogens [19–22]. Autophagy is also associated with nonapoptotic type II cell death, also called "autophagic degeneration" [17, 23–25]. Recently, autophagy was described to be required for apoptotic cell clearance during embryonic development [26]. By mostly morphological studies, autophagy has been linked to a variety of pathological processes. Early reports of autophagy in human disease include the ultrastructural autophagic features described in pancreas from human pancreatitis [27]. Furthermore, autophagy has been linked to neurodegenerative diseases and tumorigenesis, which highlights its biological and medical importance [19, 28, 29].

Autophagy is characterized by sequestration of bulk cytoplasm and organelles in double-membrane vesicles called "autophagosomes," which eventually acquire lysosomal-like features. During autophagy, an isolation membrane (herein referred to as "autophagosomal membrane") forms as a preautophagosomal structure, invaginates as a cup-shaped structure, and sequesters cytoplasmic constituents, including mitochondria, endoplasmic reticulum, and ribosomes. The edges of the membrane fuse to form a double-membrane or multimembrane structure, known as the "autophagosome," or "autophagic vacuole." The outer membrane of the autophagosome fuses with the lysosome to deliver the inner membranous vesicle to the lumen, thus forming the autolysosome. The final degradation step takes place within these structures, where lysosomal hydrolases digest the luminal content of the autophagic vacuole to turn it into recyclable breakdown products [30, 31].

Autophagy is inhibited by a serine threonine protein kinase originally recognized as a target of rapamycin and therefore named "TOR" [16, 32], which is inhibited under starvation-induced autophagy. The progression of the autophagy is sensitive to the phosphatidylinositol 3-kinase (PI3K) inhibitors such as 3-methyladenine (3-MA), with the target being the class III PI3K [33]. Most of the detailed molecular mechanistic work on autophagy has been carried out in the yeast *Saccharomyces cerevisiae* as a cellular response for survival during nutrient-limited conditions [34]. Autophagosome formation is mediated by a set of evolutionarily conserved autophagy-related proteins (Atg proteins) [32]. One of the best-defined Atg proteins is microtubule-associated protein 1 light chain 3 (LC3). LC3, the mammalian homolog of yeast Atg8, undergoes complex C-terminal proteolytic and lipid modifications, upon which it translocates from the cytosol to the autophagosomal membrane [35–37]. The recruitment and proper localization of critical Atg complexes to the autophagosomal membrane is mediated by a lipid kinase complex formed by VPS34 and autophagy-specific subunits such as Atg6, which is eventually followed by the translocation of Atg8/LC3 to the autophagosomal membrane [38]. In mammalian cells, the initial step of autophagic process is controlled by Beclin 1 [39], the mammalian ortholog of the yeast Atg6. Beclin 1 is a Bcl-2 interacting protein [18, 40, 41] that promotes autophagosome formation when it functions as part of a complex with hVsp34, the class III PI3K [42]. Subcellular distribution to a membrane structure and generation of phosphatidylinositol 3-phosphate by the Beclin 1–class III PI3K complex is thought to be important in mediating

the localization of other autophagy proteins to autophagosomal membranes. However, despite the advances in understanding autophagy, autophagosome formation in mammalian cells is a complex process and neither the molecular mechanism leading to its formation nor all the implicated genes have been fully elucidated [43, 44].

VMP1 Is an Autophagy-Involved Protein

To find out if VMP1 triggers autophagy, we transfected HeLa cells with the expression plasmid pcDNA4–VMP1, which codes for the VMP1–V5 fusion protein. Cells were cultured in nutrient-replete conditions and fixed in glutaraldehyde 24 h later so we could perform transmission electronic microscopy. We observed that cells expressing VMP1 showed multiple autophagic features. We found 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) [45, 46]. Autophagy features did not differ from those obtained in rapamycin, a well-known inductor of authophagy, treated cells. The same morphological features were obtained when 293T or AR42J pancreatic acinar cells were transfected with the VMP1 expression plasmid. During autophagy, the precursor LC3 is cleaved by the proteolytic enzyme atg4 (LC3-I) and then undergoes C-terminal lipid modifications (LC3-II), and translocates from the cytosol to the autophagosomal membrane [36, 47] as expected. With some limitations [48], LC3 is currently used as a specific marker of autophagy [49]. To confirm the extent and specificity of VMP1 autophagosome induction, we first immunostained pcDNA4–VMP1-transfected cells with a specific LC3 antibody and we observed the signal of endogenous LC3 in punctate structures. Then, we investigated LC3-I and LC-II forms by western blot analysis and we found induction of LC3 with increased LC3-II form signal in pcDNA4–VMP1-transfected cells as expected. Since intra-autophagosomal LC3-II is degraded by lysosomal proteases, we blocked its proteolysis using the lysosomal protease inhibitor E64d [50] and we found the enhancement of LC3-II signal in VMP1-expressing cells. In another series of experiments, HeLa, 293T, and AR42J pancreatic acinar cells cultured under nutrient-replete conditions were concomitantly transfected with an expression plasmid encoding for the red fluorescent protein (RFP)–LC3 fusion protein and pcDNA4–VMP1 or pcDNA4-empty plasmids. We found the recruitment of LC3 fluorescence fusion protein in punctate structures in VMP1-transfected cells in contrast to the diffuse RFP–LC3 fusion protein signal observed in control cells. Finally, we investigated the potential for inhibiting the pathway with an agent well documented to inhibit autophagy such as the PI3K inhibitor 3-MA [33]. Although 3-MA was reported to have side effects apart from inhibiting autophagy [24], the inhibition of LC3 recruitment is due to its ability to inhibit autophagy. HeLa, 293T, and AR42J pancreatic acinar cells were treated with 3-MA before the cotransfection with pRFP–LC3 and pcDNA4–VMP1 expression plasmids and the percentage of RFP–LC3 cells with punctate staining was low and almost the same as that observed in pcDNA4-empty transfected cells. 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 [51]. 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 assay and of VMP1 protein expression by western blot analysis. The expression of VMP1 mRNA and that of VMP1 protein are activated under starvation. VMP1 expression was evident after 2 h of treatment. Moreover, we localized VMP1 in punctate structures by immunofluorescence. To confirm the above-described findings, we induced autophagy by pharmacological means. 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 [52] through a downstream mechanism, which is still unknown. Since mTOR can be inhibited by rapamycin, this compound is routinely used as a pharmacological agent to induce autophagy. We treated several cells with rapamycin and, using reverse transcription PCR, western blot analysis, and immunofluorescence, we found that mTOR inhibition induces VMP1 expression as expected. Thus, extracellular-stimuli-induced autophagy activates VMP1 expression. 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 a starvation standard protocol or rapamycin treatment. 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 fluorescent fusion protein. These findings demonstrate that VMP1 expression is required for autophagosome formation.

VMP1 Is an Autophagosomal-Membrane Integrated Protein

To analyze the role of this putative transmembrane protein in the molecular mechanism of autophagy, we hypothesized that VMP1 is an integrated protein of the autophagosomal membrane. We noticed that the VMP1–EGFP fusion protein remains in the vacuole membrane induced by its own expression in cells transfected with pEGFP–VMP1. Consequently, to find out whether endogenous VMP1 induced during autophagy remains as an integrated membrane protein, we performed subcellular fractioning of HeLa cells undergoing rapamycin-induced autophagy and investigated VMP1 in membrane preparations by western blot analysis. While the protein is not detectable in untreated cells, VMP1 is detected in membrane preparations of cells undergoing autophagy, and the signal persists when the cell lysate is treated with 1.5 M NaCl or is exposed to pH 11.0 before membrane fractioning. These results indicate that VMP1 functions as an integrated membrane protein in autophagic cells. Then, to investigate if VMP1 is an autophagosomal membrane protein, we analyzed whether VMP1 colocalizes with endogenous LC3 in the autophagosomes. We performed inmunofluorescence using the anti-LC3 antibody and anti-V5 antibody in HeLa cells transfected with VMP1–V5 expression

plasmid. We found a remarkable colocalization between VMP1–V5 fusion protein and endogenous LC3 in the VMP1-induced vacuoles. These results indicate that VMP1 is a transmembrane protein integrated into the autophagosomal membrane.

VMP1 Is a Beclin 1 Binding Membrane Protein

To obtain a mechanistic insight as to how VMP1 triggers autophagy, we analyzed its function in the molecular pathway of autophagosome formation. The initial steps of the autophagic process in mammalian cells are controlled by Beclin 1 [39], which promotes autophagosome formation when it works as a complex with the class III PI3K [33, 38, 53]. During autophagy the Beclin 1–class III PI3K complex, apparently originating from the *trans*-Golgi network [53], is thought to undergo subcellular distribution to the autophagosomal membrane, which eventually leads to the recruitment of autophagy proteins and the proper conjugation of LC3 to membrane phospholipids [42, 54]. However, the transmembrane protein of the autophagosomal membrane with which the Beclin 1–class III PI3K complex interacts remains elusive. We therefore investigated whether VMP1 interacts with Beclin 1. First, we analyzed if Beclin 1 localizes in the membrane of the VMP1-induced vacuoles. To this end, we concomitantly transfected 293T cells with pEGFP–VMP1, pRFP–LC3, and pCFP–Beclin 1 expression plasmids. We found a remarkable colocalization between VMP1, LC3, and Beclin 1 fluorescent fusion proteins, suggesting that Beclin 1 could attach to VMP1-induced vacuole membranes, which are marked by endogenous LC3 as autophagosomes. To determine if VMP1 is the autophagosomal membrane protein target to which Beclin 1 attaches to allow the initiation of autophagy, we studied whether VMP1 interacts with Beclin 1 in cells transfected with the VMP1–V5 expression plasmid by conducting coimmunoprecipitation experiments. We investigated whether endogenous VMP1 interacts with endogenous Beclin 1 in cells developing rapamycin-induced autophagy. Immunoprecipitates of either VMP1 or Beclin 1 prepared from Triton X-100 solubilized rapamycin-treated HeLa cells were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblotting with anti-Beclin 1 and anti-VMP1 antibodies. Endogenous Beclin 1 and VMP1 induced by rapamycin treatment were detected in immunoprecipitates using anti-VMP1 antibody. Interaction of endogenous VMP1 with endogenous Beclin 1 was also confirmed by immunoprecipitation using anti-Beclin 1 antibody. Similar results were found in starved cells. We then 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 cells with punctate staining was low and similar to the percentage found in starved and control MCF7 cells. These results collectively show that VMP1 interacts with Beclin 1 to trigger the autophagic process and suggest that VMP1–Beclin 1 interaction is involved in the molecular mechanism of autophagosome formation.

To further delineate the interaction between VMP1 and Beclin 1, we tested VMP1 hydrophilic domains for Beclin 1 binding. VMP1 is predicted to contain six transmembrane domains. Four recombinant glutathione *S*-transferase (GST)–VMP1 hydrophilic

peptides, VMP1(aa1–75), VMP1(aa187–247), VMP1(aa324–366), and VMP1(aa378–406), and full-length His6–Beclin 1 were produced in *Escherichia coli*. We incubated the recombinant His6–Beclin 1 fusion protein from *E. coli* lysates with the purified GST–VMP1 peptides and retained protein was eluted with imidazole. Eluates were separated by SDS-PAGE followed by immunoblotting with anti-Beclin 1 and anti-GST antibodies. We observed that only GST–VMP1(aa378–406) was found in the eluates, indicating that VMP1 interacts with Beclin 1 through the aa378–406 hydrophilic domain, which we named "autophagy-related domain" (AtgD). To obtain further molecular insights into the proposed function of VMP1 in autophagosome formation and to evaluate the relevance of VMP1–Beclin 1 interaction, we constructed pEGFP and pcDNA4 plasmids containing the VMP1ΔAtgD defective mutant, in which the VMP1(aa378–406) peptide was specifically deleted. First, we performed a pull-down assay using the VMP1ΔAtgD–V5 fusion protein. Lysates from HeLa cells transfected with pcDNA4-VMP1ΔAtgD were incubated with nickel nitrilotriacetic acid agarose beads and retained proteins were eluted with imidazole, separated by SDS-PAGE, and immunoblotted with anti-Beclin 1 or anti-V5 antibodies. We found no signal of endogenous Beclin 1 in the elution fraction from VMP1ΔAtgD–V5 fusion protein, whereas, as expected, control experiments showed that both VMP1 and Beclin 1 were detected in the elution fraction from VMP1–V5 fusion protein. Then, we performed subcellular fractioning of HeLa cells transfected with pcDNA4–VMP1ΔAtgD and investigated VMP1ΔAtgD–V5 in membrane preparations by western blot analysis using anti-V5 antibody. We found that VMP1ΔAtgD was detected in membrane preparations, and the VMP1 signal persisted in the membrane fraction even after 1.5 M NaCl or pH 11.0 treatments. These results indicate that the VMP1ΔAtgD defective mutant still functions as an integrated membrane protein. In other series of experiments, HeLa cells were transfected with pEGFP–VMP1 or pEGFP–VMP1ΔAtgD, which encodes the VMP1ΔAtgD–EGFP fluorescent fusion protein, and cells were cultured in nutrient-replete conditions for 24 h. Surprisingly, we found that cells expressing VMP1ΔAtgD—EGFP fluorescent fusion protein do not show the characteristic vacuolization observed in VMP1–EGFP-expressing cells. To evaluate if the Atg domain of VMP1 is required for autophagy, HeLa cells were concomitantly transfected with the expression plasmids encoding the RFP–LC3 and VMP1ΔAtgD–V5, or VMP1–V5 fusion proteins and cells were cultured in nutrient-replete conditions for 24 h. We found that VMP1ΔAtgD expression failed to trigger autophagy as it is evidenced by the diffuse distribution of the RFP–LC3 fluorescence fusion protein observed in pcDNA4–VMP1ΔAtgD transfected cells in contrast to the recruitment of the RFP–LC3 observed in the full-length VMP1-expressing cells. The percentage of RFP–LC3 cells with punctate staining in VMP1ΔAtgD-expressing cells was highly reduced in comparison with that in those expressing the full-length VMP1. The results described above show that the VMP1 Atg domain is essential for autophagosome formation and suggest that VMP1 is an autophagosomal membrane protein, which interacts with the Beclin 1 complex, allowing the initiation of the autophagic process.

VMP1–EGFP Targeted to Pancreatic Acinar Cells in the Pancreas of Transgenic Mice Triggers the Formation of LC3-Positive Vacuoles

To gain in vivo insight into how stable expression of VMP1 is able to induce autophagy, we developed transgenic mice in which the pancreatic acinar cell specific elastase promoter drove VMP1–EGFP expression. Expression of the VMP1–EGFP transgenic fusion protein was detected by western blot analysis in the pancreas of transgenic mice, but not in the liver, kidney, spleen, heart, or lung as expected. Pancreatic tissue from VMP1–EGFP transgenic mice showed numerous vacuoles in acinar cells whose membranes were immunostained with anti-EGFP (Fig. 2). VMP1-induced vacuoles in pancreas from transgenic mice were assayed for LC3 by immunofluorescence using anti-LC3 and anti-GFP antibodies. We found that VMP1 colocalizes with endogenous LC3 in the membrane of VMP1-induced vacuoles, showing that stable expression of VMP1 in pancreatic tissue from transgenic mice is able to induce the formation of LC3-positive vacuoles. These data indicate that the in vivo expression of VMP1 triggers autophagosome formation and strongly support the in vitro findings.

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 [55–57]. Pancreatic tissue from rats treated with caerulein, a widely used experimental model of pancreatitis,

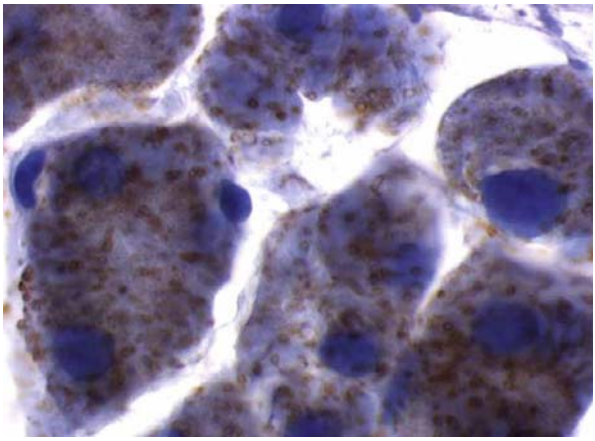

Fig. 2 VMP1–EGFP expression in the acinar cells of the pancreas of transgenic mice induces autophagic vacuoles. Immunohistochemistry of EGFP in pancreatic tissue from VMP1–EGFP mice (original magnification 100×) using mouse anti-EGFP and anti-mouse horseradish peroxidase antibodies

developed cytoplasmic vacuolization with ultrastructural features of autophagy [55, 56]. We analyzed the expression of the VMP1 protein by western blot in pancreas tissue during the development of the experimental pancreatitis and found the maximal VMP1 expression after 6 h of treatment. To determine 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, and anti-Beclin 1 antibodies. We found that endogenous VMP1 highly colocalizes with endogenous LC3 in vacuolated structures. This in vivo result confirms the autophagosomal localization of VMP1. Moreover, we found that endogenous VMP1 also colocalized with endogenous Beclin 1 in the vacuole membrane of the autophagic tissue, showing the vacuolar localization of Beclin 1 during in vivo induced autophagy. These results strongly support the findings obtained in vitro and suggest that the early expression of VMP1 could be related to the autophagy induction in tissue suffering from a pathological process.

Discussion and Conclusions

We have previously reported that gene expression is strongly altered in pancreas during the acute phase of pancreatitis [13, 58]. These phenotypic changes could enable the pancreas to protect itself against an acute attack of pancreatitis or, conversely, participate in the pathophysiological mechanism of the disease. Therefore, identifying the genes involved in cell response to pancreatitis could lead to new strategies in the treatment of the disease. Using a systematic approach, we identified by sequencing its cDNA a new protein that we named VMP1. It was strongly expressed in the pancreas of rats suffering from acute pancreatitis. Our studies indicated that VMP1 is a transmembrane protein located at the Golgi apparatus and endoplasmic reticulum area and that its overexpression induces vacuole formation. Our functional studies present experimental evidence that the sole expression of VMP1 triggers autophagy, even under nutrient-replete conditions. 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 transient expression of RFP–LC3 fluorescent fusion protein. In all the experiments more than 85% of RFP–LC3 cells showed punctate staining. Consistent results were found when endogenous LC3 was assayed. Moreover, VMP1 induces processing of endogenous LC3-I to LC3-II, which is enhanced in the presence of hydrolase inhibitors. Although transiently overexpressed LC3 protein could be prone to aggregate in an autophagy-independent manner, the processing of the endogenous LC3-I to LC3-II was reported to be unaffected by the overexpression and remains a hallmark of autophagy [48, 49]. On the other hand, VMP1-expression-induced LC3 recruitment was inhibited by 3-MA, which, although not specifically, inhibits autophagy [24, 33]. Moreover, VMP1 is involved in the extracellular-stimuli-induced autophagy since 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. On the other hand, our results have shown that VMP1 is an integral protein of

the autophagosomal membrane. VMP1 remains in the membrane fraction of cells undergoing rapamycin-induced autophagy and it colocalizes with LC3 in the vacuole membrane. Finally, the transgenic mice for VMP1 expression targeted to pancreatic acinar cells allowed us to confirm, within a physiological setting, the results discussed above. The in vivo stable expression of VMP1 induces the formation of numerous vacuoles in acinar cells, where it colocalizes with endogenous LC3.

VMP1 is involved in the initial steps of the autophagic process. We have obtained experimental data indicating that VMP1 interacts with Beclin 1 and this interaction is essential for autophagosome formation. Both VMP1 and Beclin 1 endogenous proteins coimmunoprecipitated from rapamycin-treated cells. On the other hand, VMP1-induced autophagy is dependent on Beclin 1, since VMP1 expression fails to induce autophagy when it is expressed in autophagy-deficient, low Beclin 1 expressing MCF7 cells. VMP1–Beclin 1 interaction was confirmed using recombinant peptides. Beclin 1 directly interacts with the VMP1–AtgD peptide (aa378–406) and the VMP1ΔAtgD mutant failed to precipitate endogenous Beclin 1 in transfected cells. Our results also show that the VMP1 Atg domain is essential for the formation of autophagosomes. We found that the mutant protein VMP1ΔAtgD, which is also a transmembrane protein, does not induce autophagosome formation since it failed to promote vacuole formation and LC3 recruitment. These findings also suggest that the mechanism by which VMP1 induces autophagy is likely to involve the interaction of Beclin 1 with the VMP1–Atg domain. Beclin 1 is a haploinsufficient tumor-suppressor gene [18], and is involved in the autophagosome formation mediating the localization of other autophagy proteins to the autophagosomal membrane [42]. Beclin 1 activity seems to be dependent on its partners and its subcellular localization. Two major Beclin 1 interactors have been described affecting its autophagic activity, Bcl-2 and class III PI3K. While Bcl-2 inhibits Beclin 1-dependent autophagy and this complex was localized at the endoplasmic reticulum [41], the Beclin 1–kinase complex was localized in the *trans* Golgi network in nutrient-replete HeLa cells and was shown to be essential for early stages of autophagosome formation [53]. The data presented here show the colocalization of LC3 and Beclin 1 in VMP1-induced vacuoles. In contrast, this triple colocalization was abolished in VMP1ΔAtgD-expressing cells. Furthermore, we found endogenous VMP1–Beclin 1 colocalization in vacuole membranes when a pathological tissue undergoing autophagy was assayed. These findings support the hypothesis that the distribution of Beclin 1 to the autophagosomal membrane is required for autophagy progression. To our knowledge, there are few reports showing Beclin 1 autophagosomal membrane location during autophagy. Transfected Beclin 1 was found localized in the membrane of the autophagosome in starved HEK293 cells [59] and endogenous Beclin 1 was reported to colocalize with LC3 in dorsal root ganglion cells from diabetic rats [60]. It is thought that during autophagy, subcellular distribution of the Beclin 1 complexes from the *trans* Golgi network to an autophagosomal structure would require the interaction of Beclin 1 with an autophagosomal transmembrane protein. Our results suggest that VMP1 is an autophagosomal-integrated protein where Beclin 1 interacts to initiate autophagosome formation.

The source of the autophagosomal membrane remains to be elucidated [43, 44]. Two general models have been proposed: the membrane may be derived from a preexisting cytoplasmic organelle such as the endoplasmic reticulum, or it may be assembled from constituents at its site of genesis [43, 61]. The autophagosomal membrane is rich in lipids

and poor in proteins [62, 63]; therefore, it is difficult to determine its origin on the basis of protein content. So far, only two transmembrane proteins have been described in yeast to be autophagy-related, Atg9 [64] and Atg27 [65]. Atg9, as part of a functional complex [66], cycles between the mitochondria and the preautophagosomal structure and is thought to mediate the delivery of the membrane to the forming autophagosome. Mammalian Atg9 was also described, but its subcellular distribution is different and its actual participation in the autophagosome formation remains to be delineated [67–69]. In addition, none of these transmembrane proteins have been reported to interact with Beclin 1. Our results have identified VMP1 as an autophagosomal transmembrane protein required for autophagosome formation. The findings that VMP1 has no known homolog in yeast and that its expression is required to start the autophagic process, support the hyothesis that mammalian cells regulate autophagy in a different way and suggest VMP1 as a candidate protein to further explore the source of the autophagosomal membrane in pancreatic acinar cells during acute pancreatitis.

In this study two in vivo models of VMP1 expression were used to support the data obtained in the in vitro studies: the VMP1-transgenic mice, and the experimentally induced acute pancreatitis in rats. VMP1-transgenic mice, in which VMP1 expression was targeted to pancreatic acinar cells, show that the in vivo stable expression of VMP1 is able to induce autophagosome formation in acinar cells. These results support the findings obtained when transient VMP1 expression was assayed in cell lines. During experimental acute pancreatitis, autophagic morphology of the acinar cell has been reported to appear 3 h after pancreatitis induction and remains detectable for about 15 h [55, 70]. The data presented here show that the time course of VMP1 protein expression is consistent with that of the presence of autophagic morphology during experimental pancreatitis. Our results also show that VMP1 is localized in the membrane of the acinar cell autophagic vacuoles, since endogenous VMP1 highly colocalizes with endogenous LC3 in vacuolated structures in the pathological tissue. Moreover, endogenous Beclin 1 colocalizes with VMP1 in pancreatitis-induced vacuole membranes. These findings strongly support the hypothesis that VMP1 is an autophagosomal-integrated protein where Beclin 1 interacts.

Although autophagic morphology was described in human pancreatitis in 1980 [27], the physiopathological role of autophagy in pancreatitis has not been fully elucidated. Interestingly, no other morphological event resembling acute pancreatitis was observed in pancreatic tissue from transgenic mice, suggesting that VMP1-induced autophagy per se may not induce acute pancreatitis. In addition, GFP–LC3-transgenic mice subjected to starvation developed autophagy in pancreas without visible pancreatitis morphology, suggesting that autophagy might be a mechanism of degradation of secretory granules in starved pancreas [47]. Therefore, during acute pancreatitis, autophagy could be a defense mechanism, which activates as an early cellular response to the disease. On the other hand, VMP1 mRNA basal expression was found in several rat tissues such as kidney [71]. This observation is consistent with the basal autophagy found in the same tissue from the GFP–LC3 transgenic mice [47]. Although basal VMP1 expression could be related to other physiological processes, the relationship between GFP–LC3 transgenic mice and VMP1 basal expression leads us to speculate that the expression of VMP1 might correlate with the presence of autophagy in mammalian tissue. On the other hand, the fact that this novel autophagosomal transmembrane protein is involved in autophagy

during a pathological process will allow further studies on the role of autophagy in the cellular response to disease and would be of potential clinical relevance.

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