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TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways

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ABSTRACT

During reticulocyte maturation, some membrane proteins and organelles that are not required in the mature red cell are lost. Several of these proteins are released into the extracellular medium associated with the internal vesicles present in multivesicular bodies (MVBs). Likewise, organelles such as mitochondria and endoplasmic reticulum are wrapped into double membrane vacuoles (i.e., autophagosomes) and degraded via autophagy. Morphological, molecular, and biochemical studies have shown that autophagosomes fuse with MVBs forming the so-called amphisomes, a prelysosomal hybrid organelle. SNAREs are key molecules of the vesicle fusion machinery. TI-VAMP/VAMP7 and VAMP3/cellubrevin are two v-SNARE proteins involved in the endocytic and exocytic pathways. We have previously shown that in the human leukemic K562 cells, Rab11 decorates MVBs and it is necessary for fusion between autophagosomes with MVBs. In the present report, we present evidence indicating that VAMP3 is required for the fusion between MVBs with autophagosomes to generate the amphisome, allowing the maturation of the autophagosome, but it does not seem to be involved in the next step, i. e., fusion with the lysosome. On the other hand, we demonstrate that VAMP7 is necessary for this latter event, allowing the completion of the autophagic pathway. Furthermore, VAMP7 and ATPase NSF, a protein required for SNAREs disassembly, participate in the fusion between MVBs with the plasma membrane to release the internal vesicles (i.e., exosomes) into the extracellular medium.
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1. Introduction

K562 is a cell line derived from a patient who had chronic myeloid leukemia, and these cells are an excellent tool to study multivesicular bodies (MVBs) formation and exosome secretion [1,2]. During erythroid maturation, some plasma membrane proteins are selectively sorted by inward budding and the subsequent pinching off of the endosomal membrane into the luminal space, leading to the formation of multivesicular bodies (MVBs), which accumulate internally small membrane vesicles (60–80 nm). These intraluminal vesicles once released into the extracellular medium by fusion of MVBs with the plasma membrane are known as exosomes [3–8].

The main physiological pathway for degradation of intracellular macromolecules in eukaryotic cells is autophagy [9,10]. This intracellular process plays a key role in the turnover of long-lived proteins, RNA, cytoplasmic macromolecules, and also organelles [11–13]. This degradation process of superfluous organelles has been also associated with red blood cell maturation [14–16]. During autophagy, parts of the cytoplasm and organelles are encapsulated in double membrane vacuoles called autophagosomes, which subsequently fuse with lysosomes to degrade the incorporated material (for a review, see Shintani and Klionsky [17]). Several reports have shown the convergence between the endocytic and autophagic pathways. It has been visualized by electron microscopy that MVBs deliver their endocytosed markers to the early autophagosomes where the two pathways meet [18]. Although the molecular machinery that participates in trafficking and convergence between both autophagy and MVBs pathway has not been fully uncovered, some of the proteins involved in this event have been recently elucidated [19]. Moreover, in a recent publication, it has demonstrated that isolated autophagic vacuoles can fuse, in vitro, with both early and late endosomes [20].

Genetic studies in yeast have shown that the combined action of a group of class E (Vps) proteins termed ESCRT I, II, and III (endosomal sorting complexes required for transport) is necessary for MVB biogenesis. Many of these Vps proteins are recruited from the cytoplasm to the endosomal membrane in a sequential form [21–24]. Another important factor that regulates and coordinates the biogenesis of MVB is Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), which is able to bind ubiquitylated proteins [25–29]. Interestingly, recent publications have shown that autophagy degradation is abrogated in cells depleted for ESCRT subunits or in cells overexpressing a mutant of CHMP2B (charged multivesicular body protein 2B/chromatin-modifying protein 2B), which caused an accumulation of ubiquitin–protein aggregates [30,31], establishing a connection

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between autophagy and MVBs at the molecular level (for a review, see Fader and Colombo and Rusten and Simonsen [32,33]).

Protein trafficking in the endocytic and secretory pathway requires a series of events including cargo selection and vesicle budding at the donor organelle, followed by transport, docking, and fusion of vesicles with its proper target organelle. Yeast and mammalian cells appear to share highly conserved machinery that participates in protein sorting and membrane fusion. The main model for targeting of the cargo to a suitable destination is focused in the role of the SNARE complex, which postulates that during neuronal exocytic events, an interaction between VAMP/synaptobrevin (v-SNARE) and syntaxin (t-SNARE) molecules provides the specificity required [34]. This complex also needs a stable interaction with an additional t-SNARE (SNAP-25), which forms a complex with syntaxin. SNARE proteins contain a specific region (SNARE motifs) that contribute to the formation of a highly stable four-helix bundle, termed the SNARE complex. Each SNARE motif contributes to the formation of this four alfa-helix bundle, which are all aligned in parallel [34–36]. The folding of this bundle is thought to drive membrane to the fusion event. Initially, the SNARE complex has a *trans*-configuration, where the SNARE proteins involved in the formation of the complex are localized in different membranes. Subsequently, the complete SNARE complex temporally coincides with membrane fusion, resulting in the formation of a *cis*-complex because all of the SNARE proteins are localized to the same membrane. Finally, the ternary *cis*-complex is disassembled by the chaperone ATPase NSF (*N*-ethyl-maleimide-sensitive factor) in conjunction with SNAPs (soluble *N*-ethyl-maleimide attachment proteins) proteins [34,37].

Clostridial neurotoxins (NTs) carry a proteolytic activity that selectively cleaves defined SNARES. Hence, these toxins have been extensively used to demonstrate the involvement of NTs-sensitive SNAREs in vesicular transport (see review [38]). However, cumulative evidence indicates the participation of SNAREs insensitive to neurotoxins in specific transport events. It is believed that tetanus neurotoxin-insensitive VAMP proteins are involved in the constitutive exocytosis of neurons, since the neurotoxin completely inhibited the regulated neurotransmitter release but not neurite outgrowth [39,40]. Indeed, a VAMP tetanus toxin-insensitive, designated as TI-VAMP [41] or VAMP7 [42], was identified. TI-VAMP/VAMP7 has a long N-terminal extension (90 amino acids) compared with other VAMP family proteins. Interestingly, this N-terminal extension produces an inhibitory effect on the SNARE complex formation, and the expression of this domain inhibits neurite outgrowth in neurons and PC12 cells in culture [43]. VAMP7 is also believed to be involved in apical transport of constitutive vesicles in polarized epithelial cells, such as MDCK cells and CaCo-2 cells [41]. Moreover, it has been shown that TI-VAMP/VAMP7 is localized in late endosomes and is thought to function as a v-SNARE for endosomal vesicle trafficking to lysosomes [42,44]. On the other hand, it has been recently demonstrated that VAMP7 is involved in constitutive exocytosis in HSY cells [45]. Nevertheless, the physiological roles and distribution of TI-VAMP/VAMP7 in intracellular vesicle trafficking are still not clear.

VAMP3/cellubrevin has been postulated to be a v-SNARE for early and recycling endosomes and probably for constitutive exocytosis, but mice with a null mutation of this gene were normal in most endocytic and exocytic pathways including constitutive exocytosis [46,47]. Thus, the actual role of VAMP3 in both pathways is not completely understood.

In the present report, we provide evidence suggesting that VAMP7 is a v-SNARE that participates in the fusion between MVBs with plasma membrane to release exosomes into the extracellular medium. Furthermore, we demonstrate that VAMP7 is required for the last step of the autophagic process (i.e., fusion with the lysosomes). On the other hand, our results indicate that VAMP3 is necessary for the fusion of MVBs with autophagosomes to generate the hybrid organelle termed amphisome, allowing the maturation of the autophagosome.

2. Materials and methods

2.1. Materials

RPMI cell culture medium and fetal calf serum were obtained from Invitrogen Argentina S.A. (Buenos Aires, Argentina). *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (N-Rh-PE) was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Acetylthiocholine and 5,5′-dithio-*bis*(2-nitrobenzoic acid) were obtained from Sigma (Buenos Aires, Argentina). *N*-ethylmaleimide was obtained from Sigma (Buenos Aires, Argentina).

2.2. Plasmids

The pEGFP-LC3wt was kindly provided by Dr. Noboru Mizushima (Tokyo Medical and Dental University, Tokyo, Japan). The insert encoding the LC3 protein was subcloned into the red fluorescent protein vector (pRFP, kindly provided by Dr. Philip Stahl, Washington University, St. Louis, MO). Briefly, the insert from pEGFP-LC3wt was cut with the *Bgl*II and *Eco*RI restriction enzymes and subcloned in the corresponding restriction sites of pRFP vector. The pEGFPRab11 plasmids were used as previously described [48]. The insert corresponding to Rab11wt was also subcloned in the pRFP vector. pEGFPC3 encoding GFP-VAMP3, GFP-TI-VAMP, or GFP-Longin domain was kindly provided by Dr. Thierry Galli (Institut Jacques Monod, Paris, France) and have been described previously [43]. pCMV5-TeNT was kindly provided by Dr. Heiner Niemann (Hannover Medical School, Hannover, Germany) and Dr. Jochen Lang (Institut Européen de Chimie et Biologie, Bordeaux, France).

2.3. Cell culture and transfection

K562, an erythroleukemia cell line of human origin, was grown in RPMI supplemented with 10% FCS, streptomycin (50 μg/ml) and penicillin (50 U/ml). For some experiments, cells were incubated in starvation media EBSS (Earle's balanced salt solution). Stably transfected K562 cells overexpressing pEGFP (control) or pEGFP-Rab11wt-generated previously [48] were used. For some experiments, K562 cells were transfected with pRFP-LC3, the stable transfectants were selected with geneticin (0.5 mg/ml) and subsequently cloned. K562 cells were also transiently transfected with pEGFP-Rab11wt, pEGFP-VAMP3, pEGFP-VAMP7, pEGFP-NT-VAMP7, or cotransfected with pRFP-Rab11wt/pEGFP-VAMP3, pRFP-Rab11wt/pEGFP-VAMP7, pRFP-Rab11wt/ pEGFP-NT-VAMP7, and pRFP-LC3/ pEGFP-VAMP3, using DMRIE-C (Invitrogen Argentina S.A.), according to the manufacturer's instructions or by electroporation, following standard procedures.

2.4. Labeling with N-Rh-PE

The fluorescent phospholipid analog N-Rh-PE was inserted into the plasma membrane as previously described. Briefly, an appropriate amount of the lipid, stored in chloroform/methanol (2:1), was dried under nitrogen and subsequently solubilized in absolute ethanol. This ethanolic solution was injected with a Hamilton syringe into serum-free RPMI (<1% v/v) while vigorously vortexing. The mixture was then added to the cells, and they were incubated for 60 min at 4 °C. After this incubation period, the medium was removed, and the cells were extensively washed with cold PBS to get rid of excess unbound lipids. Labeled cells were cultured in complete RPMI medium under several conditions allowing the internalized lipid to reach the MVBs. After this incubation, cells were washed in PBS and immediately mounted on coverslips and analyzed by confocal microscopy.

To label endocytic compartments at different times after internalization, cells incubated for 60 min at 4 °C with N-Rh-PE as indicated above were washed, centrifuged for 2 min at 400×*g* and incubated in suspension at 37 °C, 5% CO_2. At different time points, the cells were

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