Lipids and lipid domains of the yeast vacuole

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Abstract

The membrane raft has been a focus of intensive research for the past two decades. Liquid-ordered domains form in artificial liposomes containing sterol and saturated lipids, but their presence in living cell membranes has been controversial. The yeast vacuole is exceptional in that micron-sized raft-like domains form in the stationary phase and under several other conditions. The sterol content of the vacuole in the log phase is much lower than that of liposomes showing liquid-ordered domains, suggesting that sterols may need to be supplied to the vacuole for the raft-like domain formation. We will discuss how lipids and lipid domains are organized in the vacuolar membrane and examine whether evidence is strong enough to conclude that the observed micron-sized domains are rafts.

Abbreviations list

ILV, intralumenal vesicle; IMP, intramembrane particle; L_d, liquid-disordered; L_o, liquid-ordered; LD, lipid droplet; MVB, multi-vesicular body; NPC, Niemann-Pick type C; NVJ, nuclear–vacuolar junction; PI(3)P, phosphatidylinositol 3-phosphosphate; $PI(3,5)P_2$, phosphatidylinositol 3,5-bisphosphate; PI(4)P, phosphatidylinositol 4-phosphosphate; V-ATPase, vacuolar-type H^+-ATPase; vCLAMP, vacuole–mitochondria patch

Introduction

Membrane domains enriched with specific lipids may form via mechanisms involving protein–lipid and lipid–lipid interactions. The raft hypothesis proposes that sterols and saturated lipids, such as sphingolipids, make clusters and generate membrane domains of a higher order than surrounding membrane areas, to which proteins having affinity concentrate. Micron-sized domains showing the property of liquid-ordered (*Lo*) phase were observed in giant unilamellar liposomes [1] and giant plasma membrane vesicles [2]. No such domain, however, has been observed in live cell samples, although the plasma membrane of mammalian cells, which contains abundant cholesterol and sphingolipids, was scrutinized using a variety of microscopic methods [3]. Thus, it is now generally thought that rafts in living cell membranes have only a short life span (e.g., within milliseconds) and are too small to be resolved by diffraction-limited microscopy [4]. Various conditions that do not exist in artificial membranes, such as presence of abundant membrane proteins and continuous perturbation of membranes (by endocytosis and exocytosis, for example), are thought to preclude formation of stable micron-sized rafts in living cell membranes.

In contrast to the above explanation, the vacuolar membrane of budding yeast (*Saccharomyces cerevisiae*) shows formation of micron-sized domains, which seem to have *Lo* phase-like properties. The observation is intriguing, especially considering that the sterol content of the vacuolar membrane in log-phase yeast is much lower than that of the mammalian plasma membrane. In this article, we will summarize what is known about lipids and lipid domains of the vacuole and discuss whether and how *Lo*-like domains form.

Lipids in the vacuole membrane

The vacuole corresponds to the lysosome in mammalian cells and harbor a variety of digestive enzymes in an acidic internal milieu [5, 6]. Biochemical analysis of subcellular fractions showed that the vacuole contains only a low level of ergosterol and other sterols [7-9]; the ratio of ergosterol to proteins (mg/mg) and that of ergosterol to phospholipids (mol/mol) in the vacuole were reported to be 1/8 and 1/18 of those of the yeast plasma membrane, respectively [9]. The phospholipid composition is also vastly different between the vacuole and the plasma membrane of yeast: the vacuole contains phosphatidylcholine most abundantly (46.5%), followed by phosphatidylethanolamine,

phosphatidylinositol, and phosphatidylserine, whereas the plasma membrane contains phosphatidylserine most abundantly (33.6%) [9]. The percentage of saturated fatty acids in phospholipids is significantly lower in the vacuole than in the plasma membrane [7]. Additionally, microscopic methods showed the presence of phosphatidylinositol 3-phosphate [PI(3)P] [10, 11], phosphatidylinositol 3,5-bisphosphate [$PI(3,5)P_2$] [12], and phosphatidylinositol 4-phosphate [PI(4)P] [13]. For sphingolipids, the vacuole contains inositolphosphorylceramide most, whereas the plasma membrane is relatively enriched with more complex mannosylinositolphosphorylceramide and mannosyldiinositolphosphorylceramide [14].

Consistent with the low ergosterol content, the vacuolar membrane in log phase shows properties of non-raft, liquid-disordered (L_d) phase. Compared with the plasma membrane, which contains abundant ergosterol and sphingolipids, lipid mobility estimated by measuring the fluorescence anisotropy with trimethylammonium diphenylhexatriene was higher [9], whereas the membrane thickness estimated using electron microscopy was lower [7].

Lipid transport to the vacuole

Lipid synthesis is not likely to occur in the vacuole except for $PI(3,5)P_2$, so lipids in the vacuolar membrane need to be supplied by vesicular and non-vesicular pathways (Figure 1). Major vesicles coming in to the vacuole are multi-vesicular bodies (MVB), the AP-3 vesicles, and autophagosomes, including the cytoplasm-to-vacuole (Cvt) vesicles. For sterol, which is necessary for L_o domain formation, mammalian data suggest that the limiting membrane of MVB and autophagosomes contains only low levels of sterol [15, 16], whereas the intralumenal vesicles (ILVs) of MVB are enriched with sterol in both yeast and mammalian cells [15, 17]. Ergosterol released from digested ILVs is thought to be transferred to the vacuolar membrane by the Niemann-Pick type C (NPC) protein homologs, Ncr1 and Npc2 [17]. Contribution of AP-3 vesicles and even less well-characterized Vid vesicles to vacuolar lipids is not known [18, 19].

Two membrane contact sites, the nucleus–vacuole junction (NVJ) and the vacuole–mitochondria patch (vCLAMP), are engaged in non-vesicular lipid transport. Ltc1/Lam6, a StART-like domain-containing protein showing sterol transfer activity *in vitro*, distributes in NVJ and mediates transport of ergosterol to the vacuolar membrane

[20, 21]. Osh1, a member of the oxysterol-binding protein, is likely to exchange ergosterol and PI(4)P at NVJ [22]. NVJ also contains Nvj2, a synaptotagmin-like-mitochondrial-lipid binding protein, which is implicated for ergosterol transport [23], and Mdm1/Snx13 and Nvj3, which may play some role in lipid transfer [24]. vCLAMP is thought to be an apparatus to transport lipids from the ER to mitochondria across the vacuolar membrane [25, 26]. The effect of vCLAMP on vacuolar lipids, especially ergosterol, is currently unclear.

Vacuolar membrane domains in the stationary phase

When nutrients in a medium are exhausted, yeast enters the post-diauxic phase and then the stationary phase, during which a metabolic shift from fermentation to respiration occurs [27]. In accordance with the change, vacuoles fuse each other, forming a large vacuole that occupies a large portion of the cell. Because of the size, the spherical shape, and a lucent lumen, the vacuole can be clearly observed under differential interference contrast or phase contrast microscopes. The large vacuole in stationary phase yeast is no longer a smooth sphere but often takes a polyhedral shape. An early electron microscopic study investigated the morphological change using freeze-fracture and showed that intramembrane particles (IMPs) that largely represent transmembrane proteins are lined along the edge of polygons, thus making the polygonal face portion IMP-deficient [28].

Toulmay and Prinz revisited the phenomenon and showed that Vph1, a multispanning protein constituting the vacuolar ATPase complex, takes a similar polygonal distribution as IMPs (Figure 2). They also showed that filipin, a fluorescent polyene antibiotic binding to sterol, labels the Vph1-deficient area of the vacuolar membrane, whereas FAST diI, a carbocyanine dye bound to an unsaturated lipid (18:2) that preferentially assimilates to the L_d phase [29], is enriched in the complementary Vph1-positive area [30]. Together with the observation that inhibition of ergosterol synthesis or extraction of sterol suppressed the biased distribution of Vph1, the Vph1-deficient membrane area was suggested to be a raft-like L_o domain [30]. More recently, the Vph1-deficient domains were shown to merge upon collision with each other and to also form and dissolve reversibly upon temperature changes, further supporting the idea that the domain is an L_o membrane induced by phase separation [31].

The low content of ergosterol and saturated lipids in the log phase vacuole membrane does not favor formation of an L_o membrane. In the stationary phase, however, the vacuolar domain formation and lipophagy, i.e., engulfment of lipid droplets (LDs) by the vacuolar membrane, proceed in a feed-forward manner, indicating that ergosterol released from digested LDs is inserted to the vacuolar membrane and supports continual L_o domain formation [32]. We showed an increase of ergosterol in the stationary phase vacuole and further found that Ncr1 and Npc2 are essential for the increase of ergosterol, domain formation, and lipophagy [17]. The domain formation was deficient in yeast lacking the core autophagy machinery or several other proteins [30, 32], and in many of these cases, Ncr1 and Npc2, which normally distribute in the membrane and the lumen of the vacuole, respectively, showed mislocalization [17], suggesting that the NPC protein-mediated pathway plays a dominant role for transporting ergosterol to the vacuolar membrane in the stationary phase.

Along with the increase of ergosterol content, acyl chains of phosphatidylcholine in the vacuolar membrane were reported to be more saturated and longer in the stationary phase than in log phase [33]. Thus, the vacuolar membrane in stationary phase yeast has a lipid composition consistent with L_o domain formation. Considering the lipid composition alone, other membranes, including the mammalian plasma membrane, may be also opt for L_o domain formation. However, micron-sized domains have been observed only in the stationary phase vacuole so far.

The reason that micron-sized L_o domain occurs in the vacuolar membrane, but not in other membranes, remains unknown, but there may be several possible factors to explain the uniqueness of the vacuole. The first factor is the relative paucity of membrane perturbation. Endocytosis and autophagy decrease in stationary phase yeast [34], suggesting that vesicular trafficking to the vacuole may be far less frequent than that of the mammalian plasma membrane. Second is a low protein-to-phospholipid ratio in the vacuolar membrane [7]. This ratio counts the V-ATPase complex that has a large peripheral V_1 sector (ca. 500 kDa) and exists in large numbers; thus, if only transmembrane proteins are counted, the protein-to-phospholipid in the vacuolar membrane may be much lower than in other membranes. Third, ergosterol is more efficient than cholesterol in promoting the lipid-ordered state [35]. This difference between sterols may explain why the stationary phase vacuole does not show the domain separation when ergosterol is substituted with cholesterol [30].

The L_o domain in the stationary phase vacuole was shown to be enriched with an EGO complex component, Gtr2, and an inverse BAR protein, Ivy1, that interacts with the EGO complex [30]. TORC1 and the SEAC subcomplex, which inhibits TORC1 signaling, also localize to a Vph1-deficient domain induced under a different setting, suggesting that the domain may function to inactivate TORC1 [36]. Atg6 and Atg14, constituents of PI 3-kinase complex I, were also shown to distribute in the L_o domain of the vacuole, but its functional meaning is not known [32, 37].

The L_o domain in the stationary phase vacuole is engaged in lipophagy by adhering closely to LDs and bulging toward the lumen [17]. Ivy1, an I-BAR protein, localizes to the L_o domain [38], but whether it is indispensable for the process is not known. On the other hand, the L_o domain may have an endogenous propensity for inward budding [39]. In the stationary-phase vacuole, however, only the L_o domain adhering to a cargo shows bulging toward the lumen and its area expands considerably to enclose a LD of more than 0.5 μm in diameter [17]. This result suggests the presence of an inductive mechanism: that is, tight binding of an L_o domain with a cargo imposes inward curvature, which then recruit Npc2 preferentially, facilitating further expansion of the domain. It is not clear whether a similar L_o domain-mediated process is involved in lipophagy that occurs under different conditions [37, 40, 41].

Domains induced by hyperosmotic stress

Hypertonic stress is known to induce a rapid increase of $PI(3,5)P_2$ [42] and fragmentation of the vacuole within approximately 10 min [43]. The vacuolar fragmentation is a process that requires $PI(3,5)P_2$ [44, 45], and Atg18, recruited to the vacuolar membrane through binding to $PI(3,5)P_2$ and PI(3)P, plays a critical role through its scission activity [46].

The vacuolar membrane undergoing fragmentation was shown to form IMP-deficient domains, in which Vph1 is also deficient, and that $PI(3,5)P_2$ as well as PI(3)P distribute in a higher density than in IMP-rich domains [12] (Figure 2). Formation of the IMP-deficient domain in hyperosmotic condition requires PI(3)P, whereas $PI(3,5)P_2$ and Atg18 are dispensable. However, in the absence of $PI(3,5)P_2$ or Atg18, the IMP-deficient membrane expands and generates aberrant structures [12].

The IMP-deficient membrane induced by hyperosmotic stress looks similar to the L_o domain in stationary phase, but whether the membrane contains rich ergosterol is not

known. However, the vacuolar membrane that constitutes NVJ is also IMP-deficient, expands drastically by hyperosmotic stress [12], and harbors Vac8, a palmitoylated protein resistant to detergent extraction [47], suggesting a possibility that rapid ergosterol transport to the vacuole might be occurring. Moreover, the electrochemical gradient that is increased by enhanced assembly of V_0 and V_1 sectors of V-ATPase under hyperosmotic stress [48] is thought to increase membrane order [49, 50]. Transmembrane osmosis that was shown to induce phase separation in giant unilamellar liposomes [51] may also work additively. A combination of these factors may be sufficient to induce L_o membrane formation under hyperosmotic stress, but experiments to directly examine lipids and lipid order may be necessary for confirmation.

Domains observed in other conditions

The Vph1-deficient domain also forms after 3 hours of glucose starvation, cycloheximide treatment, and weak acid stress [30] (Figure 2). The domain formation caused by glucose starvation and cycloheximide is suppressed in Ltc1/Lam6-deficient cells, whereas that in weak acid stress is not suppressed [21]. However, expression of an Ltc1/Lam6 mutant, which is made to distribute only in NVJ but not in ER–mitochondria contact, induces Vph1-deficient domain formation [21]. These results suggested that ergosterol transport performed by Ltc1/Lam6 at NVJ plays a critical role in forming the Vph1-deficient domains in many, but not all, instances.

Nitrogen starvation for several hours also induces the Vph1-deficient domain, which is deficient in IMP and executes lipophagy in a similar manner as in the stationary phase [17] (Figure 2). Domain formation and lipophagy in nitrogen starvation are suppressed in the absence of NPC proteins, indicating that they are also engaged in ergosterol transport to the vacuolar membrane under this condition [17]. The source of ergosterol to induce the vacuolar domain formation in nitrogen starvation is intraluminal vesicles of MVB, the formation of which are enhanced acutely under this condition [52].

The vertex ring that forms in vacuoles undergoing homotypic fusion is enriched with ergosterol as well as PI(3)P, phosphatidylinositol 4,5-bisphosphate, and diacylglycerol [53]. Vph1 is neither enriched nor excluded from the boundary membrane in fusing vacuoles in live cells, but when the homotypic fusion was

reconstituted *in vitro* in an acidic condition, Vph1 was excluded from the boundary membrane (although this *in vitro* result was observed after samples were cooled on ice) [54].

In many of the above instances, Vph1 is used as a sole marker of the vacuolar domain formation. Vph1 is a subunit of the vacuolar-type H^+-ATPase (V-ATPase) V_0 sector, and thus, when the expression is driven by an endogenous promoter, its distribution is likely to reflect that of the entire V_0 sector, a complex of integral membrane proteins that has a total molecular mass of about 250 kDa. The V_0 sector undergoes reversible assembly and disassembly with the peripheral V_1 sector, the process of which is controlled by several extracellular stimuli [55]: low glucose and low extracellular pH induce disassembly [56] [57] [58], whereas high salt causes assembly [48], probably through direct binding between $PI(3,5)P_2$ and Vph1 [59]. When assembled, distribution of the V_0 sector may be affected by actin that binds to the V_1 sector; requirement of microtubules for the V_0–V_1 disassembly suggests they might also bind to a V-ATPase component [60]. Curiously, ergosterol and sphingolipids are required for the V-ATPase activity [61] [62], and mammalian V-ATPase that has a structure similar to a yeast counterpart was shown to be concentrated in detergent-resistant membranes [63]. The discrepancy between this last observation and the preferential distribution of Vph1 in the L_d membrane in the stationary phase vacuole is not clear. Overall, considering that many factors can affect distribution of Vph1, exclusion of Vph1 alone may not be sufficient to judge the state of the membrane.

IMP deficiency also is not decisive evidence for L_o membrane. IMP is thought to represent most, if not all, transmembrane proteins [64], so that local deficiency of IMP may occur in a phase-separated membrane. With regards to the vacuolar membrane, however, in which the V-ATPase complex may be a major transmembrane protein, the same caution that was discussed above for Vph1 deficiency is necessary in using IMP deficiency as a criterion.

Concluding remarks

Various data indicate that the Vph1-deficient domain in the stationary phase vacuole is likely to be L_o membrane, whereas for domains observed in other conditions, more data on lipids and membrane state are necessary before drawing a conclusion. Notwithstanding, the yeast vacuole will continue to be a fascinating structure to analyze

physiological functions of micron-sized domains, which should involve proteins sensing the membrane nature and translating it for signaling [65]. It may also be interesting to study whether and why stable micron-sized domains are necessary for yeast survival, whereas transient and nanometer-sized domains appear to be sufficient for signaling in the mammalian plasma membrane [4]. We hope that technical advancements will clarify ambiguities and provide a clearer picture of the vacuolar membrane domains in the near future.

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Declarations of interest

The authors declare that there are no competing interests associated with this manuscript.

Author contribution statement

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Figures

Figure 1. Pathways that may transport sterol to the vacuolar membrane

Sterol released from LDs and ILVs of MVB is inserted to the vacuolar membrane by NPC proteins. Ltc1/Lam6 and Osh1 transport sterol from the nuclear membrane to the vacuole via NVJ. The contribution of other pathways to sterol transport has yet to be defined.

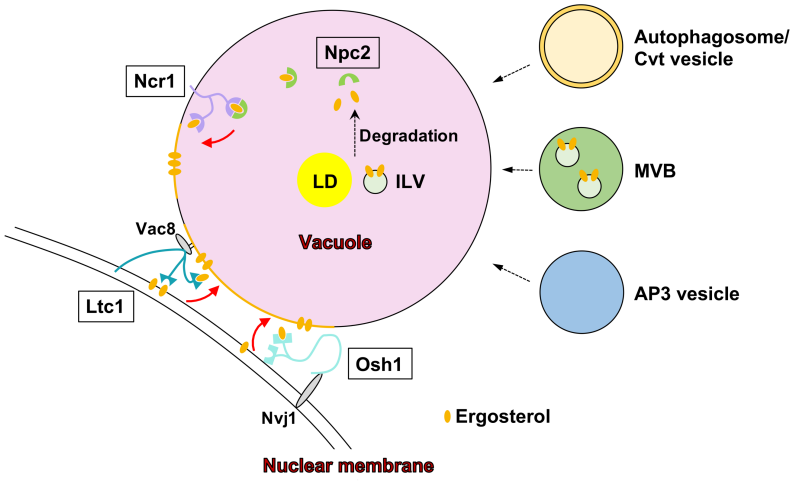

Figure 2. Vph1-deficient vacuolar domain

Several different conditions induce formation of Vph1-deficient domains in the vacuole. The domain is well characterized for the stationary phase yeast, but it is less so in other instances.