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The yeast vacuolar Rab GTPase Ypt7p has an activity beyond membrane recruitment of the HOPS/Class C Vps complex

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Synopsis

A previous report described lipid mixing of reconstituted proteoliposomes made using lipid mixtures that mimic the composition of yeast vacuoles. This lipid mixing required SNARE proteins, Sec18p and Sec17p (yeast NSF¹ and α -SNAP), and the HOPS/Class C Vps complex, but not the vacuolar Rab GTPase Ypt7p. The present study investigates the activity of Ypt7p in proteoliposome lipid mixing. Ypt7p is required for lipid mixing of proteoliposomes lacking cardiolipin. Omission of other lipids with negatively charged and/or small headgroups does not cause Ypt7p dependence for lipid mixing. Yeast vacuoles made from strains disrupted for cardiolipin synthase (CRD1) fuse to the same extent as vacuoles from strains with functional CRD1. Disruption of CRD1 does not alter dependence on Rab GTPases for vacuole fusion. It has been proposed that HOPS complex recruitment to membranes is the main function of Ypt7p. However, Ypt7p is still required for lipid mixing even when the concentration of HOPS complex in lipid-mixing reactions is adjusted such that cardiolipin-free proteoliposomes with or without Ypt7p bind equal amounts of HOPS. Ypt7p therefore must stimulate membrane fusion by a mechanism in addition to HOPS-membrane recruitment. This is the first demonstration of such a stimulatory activity – that is, beyond bulk effector recruitment – for a Rab GTPase.

Keywords

Rab GTPase; Ypt7p; HOPS/Class C Vps complex; membrane fusion; biochemical reconstitution; cardiolipin

Introduction

Rab GTPases regulate intracellular membrane budding [1], transport [2], and docking [3]. Rab effectors, which interact with GTP-bound Rab proteins, constitute a diverse group of proteins and multiprotein complexes [4]. The activities of many of these effectors have been identified – mediating intermembrane interactions, for example [5] – but the role of the Rab GTPases themselves in performing these activities is not known. A recent study suggested that the primary function of the yeast vacuolar Rab GTPase Ypt7p is to recruit its effector,

¹Abbreviations used: NSF, N-ethylmaleimide sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; HOPS, homotypic fusion and protein sorting; Vps, vacuole protein sorting; DAG, 1-palmitoyl-2-oleoyl-sn-glycerol; POPA, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine; PI, L- α -phosphatidylinositol; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidyl-L-serine; PI(3)P, L- α -phosphatidylinositol-3-phosphate, PI(4,5)P₂, L- α -phosphatidylinositol-4,5-bisphosphate; DPPE, 1,2-Dipalmitoyl-sn-glycero-3-phosphatidylethanolamine; ANOVA, analysis of variance; GAP, GTPase activating protein.

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the HOPS/Class C Vps complex [6], to membranes [7]. Here, it is shown that Ypt7p has a stimulatory activity in addition to HOPS complex recruitment for membrane fusion.

Proteoliposome lipid mixing that requires SNARE proteins, the SNARE disassembly catalysts Sec18p and Sec17p (yeast NSF and α -SNAP, respectively), and the HOPS complex has been reported [8]. These proteoliposomes were made from a lipid mixture resembling the lipid composition of yeast vacuoles; this mixture includes 1.6 mole percent cardiolipin [8, 9]. In a follow-up study, Ypt7p-dependent lipid mixing was observed in proteoliposomes with reduced cardiolipin content relative to the original vacuole-mimic lipid mixture [10]. This result raised the question of whether cardiolipin affects Rab dependence for lipid mixing and membrane fusion.

The effect of systematically varying proteoliposome cardiolipin content was therefore investigated. Proteoliposomes made without cardiolipin are completely dependent on Ypt7p for lipid mixing. Omission of other lipids with small and/or negatively charged headgroups does not have this effect. Cardiolipin is not required for fusion of purified yeast vacuoles *in vitro*, and vacuoles lacking cardiolipin do not have an altered requirement for Rab GTPases for fusion. HOPS complex binds to proteoliposomes lacking both cardiolipin and Ypt7p – i.e., proteoliposomes that cannot fuse – though to a lesser extent than to proteoliposomes with Ypt7p. However, when the concentration of HOPS complex in lipid-mixing reactions is varied such that the same amount of HOPS is recruited to proteoliposomes with or without Ypt7p, lipid mixing remains dependent on Ypt7p. HOPS complex binding to membranes is therefore insufficient for proteoliposome lipid mixing. Thus, Ypt7p plays a role beyond HOPS recruitment in membrane fusion. This is the first report of such a stimulatory activity for a Rab GTPase.

MATERIALS AND METHODS

Reagents

Recombinant Ypt7p [7, 10], HOPS complex [11], Vam3p [8], Vti1p [8], Nyv1p [8], Vam7p [12], Sec17p [12], his₆-Sec18p [13], Gyp1-46 [10], and I₂^B [14] were prepared as described. Phosphoinositides were from Echelon Research, ergosterol was from Sigma-Aldrich, fluorescent lipids were from Molecular Probes/Invitrogen, and all other lipids were from Avanti Polar Lipids.

Proteoliposome preparation and lipid-mixing assay

Proteoliposomes were prepared as described [8], using a 1:1000 protein:lipid molar ratio for SNAREs and Ypt7p. When Ypt7p was omitted, an equivalent volume of Ypt7p buffer [7] was used in its place. The “complete” vacuole-mimic lipid mixture was 42 mol% (donor) or 44 mol% (acceptor) POPC, 18 mol% soy PI, 4.4 mol% POPS, 2 mol% POPA, 1.6 mol% bovine heart cardiolipin, 8 mol% ergosterol, 1 mol% DAG, 1 mol% ea. di-C₁₆ PI(3)P and PI(4,5)P₂, and 1.5 mol% ea. Rhodamine-DPPE and NBD-DPPE (donor) or 1 mol% Dansyl-DPPE (acceptor). For proteoliposomes with non-standard lipid composition, when the amount of any lipid was increased or decreased, POPC levels were adjusted correspondingly. Lipid concentrations were estimated using NBD and rhodamine or dansyl fluorescence.

Lipid mixing was measured using NBD dequenching [15]. Lipid-mixing reactions were performed in 384-well plates (Corning no. 3676). 4 μ l acceptor and 0.5 μ l donor proteoliposomes (both at 2 mM lipids) were mixed and diluted, on ice, with 6.5 μ l RB150 (20 mM NaHEPES pH 7.4, 150 mM NaCl, 10% v/v glycerol), 2 μ l 10 mM Na₂ATP in RB150, and 2 μ l 60 mM MgCl₂ in RB150, then incubated at 27°C for 10 minutes.

Recombinant Sec17p, Sec18p, and HOPS complex, mixed and diluted with RB150 to 5 μ l, were then added at room temperature to final concentrations of 1 μ M, 2 μ M (monomer), and 40 nM, respectively. When HOPS was omitted, an equivalent volume of HOPS buffer [11] was used. Master mixes were routinely used for proteoliposome and protein solutions. Reactions were incubated at 27°C for 30 minutes with measurement of NBD fluorescence (460 nm λ_{ex} , 538 nm λ_{em}) every 30 seconds. 2 μ l 10% w/v Thesit were then added and fluorescence was measured every 30 seconds for 10 minutes at 27°C.

NBD fluorescence as a percentage of final fluorescence was calculated by subtracting fluorescence at t=0, dividing by the difference between fluorescence after 10 minutes in the presence of Thesit and fluorescence at t=0, and multiplying by 100.

For representation of total lipid mixing as the sum of all the NBD fluorescence readings in a reaction, the minimum fluorescence (as a percentage of final fluorescence) for that reaction was subtracted from each fluorescence value (as a percentage of final fluorescence) for that reaction, and these corrected values were then added.

Yeast strain construction

The His3MX6 cassette from pFA6-His3MX6 [16] was amplified by PCR using primers designed for disruption of CRD1 [17]. This PCR product was gel-purified (Qiagen QIAquick Gel Extraction Kit) and transformed [18] into BY4742 *pep4 Δ ::kanMX6* and BY4742 *pho8 Δ ::kanMX6* (ATCC). Transformants were screened initially for temperature sensitivity [19] and disruption of CRD1 was confirmed by PCR [17].

Vacuole purification and fusion assay

Yeast were grown at 30°C to mid-logarithmic phase and vacuoles were purified as described [20].

Vacuole fusion assays were performed in 20 mM PIPES-KOH pH 6.8, 200 mM sorbitol, and contained 3 μ g (protein) of BY4742 *pep4 Δ ::kanMX6* or BY4742 *pep4 Δ ::kanMX6 crd1 Δ ::his3MX6* vacuoles, 3 μ g (protein) of BY4742 *pho8 Δ ::kanMX6* or BY4742 *pho8 Δ ::kanMX6 crd1 Δ ::his3MX6* vacuoles, 0.8 μ M I₂^B, 1 mM Mg₂ATP, an ATP regenerating system [20], 15 μ M CoA lithium salt, 125 mM KCl, 5 mM MgCl₂, 50 nM recombinant Vam7p, and 230 nM recombinant his₆-Sec18p. Total reaction volume was 30 μ l. In experiments using inhibitors, uninhibited reactions were buffer-controlled. Reactions were incubated on ice or at 27°C for 90 minutes, then developed as described [20]. Absorbance at 400 nm was measured and converted to units of alkaline phosphatase using a standard curve of o-nitrophenol [20].

RESULTS

A recent study suggested a link between cardiolipin content and Ypt7p dependence for proteoliposome lipid mixing [10]. Proteoliposomes therefore were made using the “complete” vacuole-mimic lipid mixture (see Experimental Procedures), but with twice the standard cardiolipin level (3.2 mol%), the standard level (1.6 mol%), half the standard level (0.8 mol%), or no cardiolipin. These proteoliposomes (and all the proteoliposomes used throughout this study) bore the vacuolar SNARE proteins Vam3p, Vam7p, Vti1p, and Nyv1p, and were made either with or without Ypt7p. Lipid mixing was measured using NBD fluorescence dequenching (see Experimental Procedures). Representative traces of NBD fluorescence as a function of time are shown in Figures 1a-d. As a measure of overall lipid mixing for a particular reaction condition, mean sums (for n=3 independent proteoliposome preparations) of all the dequenching values from a reaction are also reported

in Figure 1e. At 3.2 mol% cardiolipin, robust Ypt7p-independent proteoliposome lipid mixing is observed in the presence of HOPS complex, and Ypt7p provides only a modest stimulation (Figure 1a and 1e). At 1.6 mol% cardiolipin, less lipid mixing is observed without Ypt7p, and Ypt7p stimulates lipid mixing to a greater extent (Figure 1b and 1e). This result has been reported previously [10]. When half the standard level of cardiolipin is present, i.e. 0.8 mol%, still less Ypt7p-independent lipid mixing is observed (Figure 1c and 1e). This result has also been described [10]. Finally, when cardiolipin is not present, no lipid mixing is observed without Ypt7p, whereas robust lipid mixing takes place when Ypt7p is present (Figure 1d and 1e). Initial rates of lipid mixing are much lower in the absence of cardiolipin (compare Figure 1b and 1d), but overall lipid mixing is only slightly reduced (Figure 1e). Thus, lipid mixing of proteoliposomes lacking cardiolipin is completely dependent on the presence of Ypt7p.

Is this effect specific to cardiolipin? Cardiolipin has a negatively charged headgroup and can adopt a conical shape characteristic of lipids with small headgroups [21]. The complete vacuole-mimic lipid mixture also contains phosphatidic acid (POPA), which has a small, negatively charged headgroup, and diacylglycerol (DAG), which has a small, neutral headgroup (see Experimental Procedures). Proteoliposomes made using vacuole-mimic mixtures lacking DAG or POPA efficiently mix lipids even in the absence of Ypt7p (Figure 2a; compare open circles and triangles with open diamonds, and 2b; compare bars 7 and 11 with bar 3). Proteoliposomes with Ypt7p but without POPA or DAG also mix lipids as efficiently as proteoliposomes made from the complete vacuole-mimic mixture and bearing Ypt7p (Figure 2a; compare filled circles and triangles with filled diamonds, and 2b; compare bars 8 and 12 with bar 4). These results show that POPA and DAG, i.e. lipids with small and/or negatively-charged headgroups, do not play the same role as cardiolipin in proteoliposome lipid mixing.

The effect of cardiolipin on yeast vacuole fusion was next examined. Cardiolipin synthase (Crd1p) generates cardiolipin from one molecule of phosphatidyl-CMP and one molecule of phosphatidylglycerol [22]. CRD1 was therefore disrupted in yeast strains containing disruptions of either PHO8, which encodes the alkaline phosphatase proenzyme, or PEP4, which encodes the vacuolar protease needed for Pho8p maturation (see Experimental Procedures). Vacuoles were then prepared from these doubly-disrupted strains, and vacuole fusion was assayed *in vitro* [20]. Vacuoles from strains lacking Crd1p fused as efficiently as vacuoles from strains with Crd1p (Figure 3a).

To assess whether cardiolipin influences the requirement for Rab GTPases for vacuole fusion, vacuoles from “wild-type” strains and from “ Δ crd1” strains were incubated with Gyp1-46, a catalytically-active fragment of the Rab GTPase-activating protein Gyp1p [23] that inhibits fusion of yeast vacuoles *in vitro* [24]. Gyp1-46 inhibits fusion of vacuoles from wild-type and Δ crd1 strains to the same extent (Figure 3b). Therefore, cardiolipin is not required for vacuole fusion, nor does it influence the degree of vacuoles’ dependence on Rab GTPases for fusion.

The ability of the HOPS complex to bind to proteoliposomes in the presence or absence of cardiolipin and of Ypt7p was then investigated. Proteoliposome lipid-mixing reactions were subjected to centrifugation in sucrose density step gradients, proteoliposomes were harvested from the top interfaces of the gradients, and HOPS complex binding was measured by quantitative immunoblotting. HOPS binds to proteoliposomes in the absence of Ypt7p whether or not cardiolipin is present (Figure 4a, compare bars 2 and 4 with bar 5; $p < 0.0001$ for both comparisons by one-way ANOVA). Nevertheless, Ypt7p increases HOPS-membrane binding. For proteoliposomes with cardiolipin, this stimulation is modest (roughly 40%) though statistically significant (Figure 4a, compare bars 1 and 2; $p = 0.03368$

by one-way ANOVA). For proteoliposomes without cardiolipin, however, Ypt7p increases HOPS complex recruitment 2.1-fold (Figure 4a, compare bars 3 and 4; $p=0.00205$ by one-way ANOVA).

Thus, cardiolipin- and Ypt7p-free proteoliposomes bind HOPS complex and yet lack the ability to fuse. Still, Ypt7p-free proteoliposomes clearly bind HOPS to a lesser extent than proteoliposomes with Ypt7p (Figure 4a). If no-Ypt7p proteoliposomes bound the same amount of HOPS complex as proteoliposomes with Ypt7p, would the proteoliposomes without Ypt7p then engage in lipid mixing? To address this question, lipid-mixing reactions were prepared at several concentrations of HOPS complex: 80, 40, 20, and 10 nM for proteoliposomes with Ypt7p, and 160, 80, 40, and 20 nM for Ypt7p-free proteoliposomes. (All reactions used cardiolipin-free proteoliposomes.) HOPS binding and lipid mixing were measured for each concentration of HOPS (Figure 4b). Lower overall lipid mixing levels compared to Figure 1 are likely due to the use of different preparations of HOPS, Sec17p, and Sec18p for these experiments. Proteoliposomes without Ypt7p did not mix lipids even at the highest observed level of HOPS recruitment – about 18 nM – while proteoliposomes with Ypt7p mixed lipids (albeit at a sub-maximal level) even at a membrane-associated HOPS concentration of 4.8 nM (Figure 4b). At equivalent levels of bound HOPS, therefore, Ypt7p-positive proteoliposomes engage in robust lipid mixing while proteoliposomes without Ypt7p show no observable lipid mixing.

DISCUSSION

Here, data are presented that indicate a role for Ypt7p in membrane fusion in addition to – but certainly not to the exclusion of – the promotion of HOPS recruitment to membranes. This study exploits the effect of the lipid cardiolipin on the degree to which proteoliposomes require the yeast vacuolar Rab GTPase Ypt7p for lipid mixing. Proteoliposomes lacking cardiolipin are completely dependent on Ypt7p for lipid mixing (Figure 1). However, proteoliposomes without both cardiolipin and Ypt7p – that is, proteoliposomes that are unable to fuse – do not undergo lipid mixing even at levels of membrane-bound HOPS complex that result in lipid mixing in the presence of Ypt7p (Figure 4b). Thus, Ypt7p is required for lipid mixing regardless of the amount of HOPS bound to membranes. It is therefore concluded that Ypt7p has an activity in addition to HOPS complex recruitment for membrane fusion. This is the first report of such a stimulatory activity for a Rab GTPase.

Previous work has shown that HOPS-Ypt7p interactions are critically important for HOPS-membrane association. When YPT7 is deleted, HOPS localization at the vacuole is severely impaired [25]. Moreover, the casein kinase Yck3p phosphorylates the Vps41p subunit of the HOPS complex [26] on an “amphipathic lipid packing sensor” motif [27], abrogating the ability of this motif to interact with highly curved membranes [28]. Phosphorylation of Vps41p sharply – but not completely – reduces direct HOPS-membrane binding in the absence of Ypt7p and gives rise to Ypt7p-dependent proteoliposome lipid mixing [7]. These results have led to the proposal that the primary activity of Ypt7p is to promote HOPS-membrane associations [7]. The data presented here are fully consistent with a vital role for Ypt7p in recruiting the HOPS complex to membranes (Figure 4a), but they also demonstrate an additional, stimulatory activity for Ypt7p (Figure 4b). Finally, yeast deleted for both YCK3 and YPT7 have fragmented vacuoles, indicative of a defect in vacuole fusion [29]. Thus, Ypt7p must be needed *in vivo* for an activity in addition to its ability to counteract functionally the effect of Yck3p phosphorylation of Vps41p (i.e. blocking direct HOPS-membrane interaction).

If Ypt7p acts beyond bulk HOPS complex recruitment to membranes, what is this activity? One possibility is that Ypt7p nucleates a lipid-protein microdomain that is necessary for

fusion. Activation of Ypt7p has been proposed to trigger enrichment of fusion catalysts and PI(3)P at the site of vacuole fusion [30, 31]. This enrichment may be carried out by the HOPS complex, which has direct affinities for SNAREs and phosphoinositides as well as Ypt7p [5, 6]. In this scenario, the HOPS complex binds simultaneously to Ypt7p and to SNAREs and/or lipids, organizing them into a fusion-competent microdomain.

Other Rab GTPase effectors have been proposed to act as microdomain nucleators. Rabaptin-5, a Rab5 effector, also binds to a Rab5 guanine nucleotide exchange factor, Rabex-5, and may thus contribute to a positive feedback loop that establishes a high local concentration of active Rab5 [32]. Early Endosomal Antigen 1, another Rab5 effector that binds directly both to PI(3)P and to SNAREs [32], may then concentrate these factors at the site of fusion. The exocyst complex, an effector for the yeast exocytic Rab GTPase Sec4p [33], contains subunits that bind to the t-SNARE Sec9p [34], phosphoinositides [35, 36], Rho family GTPases [37], lethal giant larva proteins [38], and Sec1p [39]. The exocyst may determine the site of polarized exocytosis by simultaneously binding some or all of these factors.

The observation that proteoliposomes without cardiolipin require Ypt7p for lipid mixing (Figure 1) has usefully permitted the conclusion reached in this study, namely that bulk HOPS complex-membrane binding is insufficient for membrane fusion. However, cardiolipin is unlikely to play a role in physiological membrane fusion, since vacuoles made from yeast strains lacking cardiolipin synthase can fuse (Figure 3a). Moreover, yeast strains lacking cardiolipin synthase have normal vacuole morphology [19]. Cardiolipin has been included in the standard “complete” vacuole-mimic lipid mixture described previously [8] because cardiolipin was found in a mass spectrometry study of vacuolar membrane lipid composition [9]. Cardiolipin is synthesized exclusively in the inner membranes of mitochondria [40]. Thus, its reported presence in purified vacuolar membranes is likely due to mitochondrial contamination or mitophagy [41].

Does the casein kinase Yck3p play a role in mediating the Rab dependence of the fusion of the vacuoles from *crd1*Δ strains described here? Deletion of YCK3 results in vacuoles that fuse with decreased sensitivity to a Rab GAP [26]. Since fusion of the Crd1p-deficient vacuoles described here is sensitive to inhibition by a Rab GAP (Figure 3b), Yck3p must be present and active on “Δcrd1” vacuoles. It must be noted that the analysis of the Yck3p-deleted vacuoles used a different GAP (a fragment of Gyp7p) than that used in this study (a fragment of Gyp1p). However, Ypt7p is a substrate for both Gyp7p [42] and Gyp1p [43] and thus the Gyp1p sensitivity of cardiolipin-deficient vacuoles (Figure 3b) demonstrates that Yck3p retains activity on these vacuoles.

If cardiolipin is not involved in vacuole fusion (Figure 3 and ref. 19), why does it cause a bypass of the Ypt7p requirement for SNARE- and HOPS complex-dependent proteoliposome lipid mixing? In the presence of divalent cations such as Mg²⁺, which is present at 6 mM in the lipid-mixing reactions described here, cardiolipin can adopt nonbilayer configurations such as hexagonal phases [44]. These non-bilayer structures may be fusion intermediates [21]. Indeed, liposomes made from cardiolipin readily undergo fusion without lysis in the presence of Mg²⁺ [45]. Cardiolipin may therefore render proteoliposome membranes more likely to fuse. It should be noted that increasing proteoliposomes’ cardiolipin content above 1.6 mole percent does not increase overall lipid mixing in the presence of Ypt7p (Figure 1e). This could be because some other aspect of the reaction, such as SNARE or HOPS concentration, becomes limiting for lipid mixing of proteoliposomes with both high cardiolipin content and Ypt7p.

It should also be mentioned that a previous study described cardiolipin-free proteoliposomes that exhibited HOPS-dependent but Ypt7p-independent lipid mixing [46]. However, the reaction conditions used in that study were more conducive to membrane fusion than those used in this investigation: lower MgCl_2 concentrations (2 mM, vs. 6 mM in this study), and the presence of a short-chain diacyl phosphoinositide, di- $\text{C}_8\text{PI}(3)\text{P}$, that may have detergent properties [46]. In some cases, the previous study used Ypt7p- and cardiolipin-free but fusion-competent proteoliposomes that had much higher levels of POPA (18 mol%, vs. 2 mol% in this study) [46]. POPA increases membranes' propensity to fuse [47]. These fusion-promoting conditions are likely to have alleviated the need for Ypt7p for membrane fusion.

In summary, the work presented here demonstrates that the vacuolar Rab GTPase Ypt7p is required for proteoliposome lipid mixing irrespective of the amount of HOPS complex bound to the membrane (Figure 4b). Thus, Ypt7p stimulates membrane fusion by a mechanism in addition to its recruitment of the HOPS complex to membranes. Such a stimulatory activity has never before been reported for a Rab GTPase. This activating role may be nucleation of membrane microdomains replete in fusion factors such as SNAREs and phosphoinositides. It will be an interesting challenge to correlate simultaneous high local concentrations of multiple fusion catalysts with membrane fusion events; solution ensemble measurements are likely to be insufficient for such studies

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Figure 1.

Cardiolipin content influences Rab dependence for lipid mixing. **(a) – (d)** Proteoliposomes made using vacuole-mimic lipid mixtures (see Experimental Procedures) containing the indicated mole percentage of cardiolipin (POPC content was reduced or increased to compensate for non-standard levels of cardiolipin) were incubated in lipid-mixing reactions prepared as described in Experimental Procedures. NBD fluorescence as a percentage of total NBD fluorescence (see Experimental Procedures) for representative reactions is shown. Filled squares, + Ypt7p/+ HOPS; filled circles, no Ypt7p/+ HOPS; open squares, + Ypt7p/no HOPS; open circles, no Ypt7p/no HOPS. **(e)** Total lipid mixing, represented as the sum

of each normalized NBD fluorescence reading from 0 – 30 minutes (see Experimental Procedures), is plotted as a function of cardiolipin content. Symbols are the same as in a-d. Means of sums from n=3 independent reactions, each using a different proteoliposome preparation, are plotted; error bars represent standard deviations.

Figure 2.

Ypt7p dependence for lipid mixing is specific to omission of cardiolipin. **(a)** Proteoliposomes, made using vacuole-mimic lipid mixtures with the indicated lipid omitted and replaced with POPC, were incubated in lipid-mixing reactions as described in Experimental Procedures. NBD fluorescence as a percentage of total NBD fluorescence for representative experiments is plotted. All reactions plotted here contained HOPS complex; for each proteoliposome variety, reactions lacking HOPS were performed and showed no detectable increase in NBD fluorescence, but for clarity are not shown. Open symbols, proteoliposomes without Ypt7p; filled symbols, proteoliposomes with Ypt7p. Squares, no cardiolipin; circles, no POPA; triangles, no DAG; diamonds, complete vacuole-mimic lipid

mixture. **(b)** Overall lipid mixing, represented as the sum of each normalized NBD fluorescence value from 0 – 30 minutes, is plotted for reactions using the indicated varieties of proteoliposomes, incubated with and without HOPS complex. Means of sums from three independent reactions, each using different proteoliposome preparations, are plotted; error bars represent standard deviations. Bars 1-4 and 13-16 show the same data as that plotted in Figure 1e.

Figure 3.

Purified yeast vacuoles do not require cardiolipin for fusion, and vacuoles lacking cardiolipin do not show an increased requirement for Rab GTPases for fusion. **(a)** Vacuoles from yeast strains with wild-type *CRD1* (“WT”) and from strains with *CRD1* disrupted (“Δcrd1”; see Experimental Procedures) were purified and assayed for fusion in vitro [20]. Reactions using the indicated vacuoles were incubated on ice or at 27°C as indicated. Fusion is represented as average units of alkaline phosphatase [20] for three independent reactions, each using different vacuole preparations; error bars represent standard deviations. **(b)** Vacuole fusion reactions were incubated with the indicated amounts of Gyp1-46 protein

[23], or with an equivalent volume of the Gyp1-46 buffer, for ten minutes on ice, then moved to 27°C or kept on ice for the duration of the reaction. Fusion levels were normalized by subtracting the “ice” value, then dividing by the difference between the uninhibited and “ice” values. Open symbols, “wild-type” vacuoles; filled symbols, “ Δ crd1” vacuoles. Average normalized fusion levels for three independent experiments, each using different vacuole preparations, are shown; error bars represent standard deviations.

Figure 4.

Ypt7p is required for lipid mixing regardless of the level of bound HOPS complex. **(a)** Proteoliposomes (donor only) made with and without cardiolipin, and with and without Ypt7p, were incubated in 5x scale lipid-mixing reactions (Experimental Procedures) for 20 minutes. Reactions were mixed with 100 μ l RB150 + 2 M sucrose in 7x20 mm tubes (Beckman no. 343775) and overlaid with 40 μ l each RB150 + 0.95 M and 0.9 M sucrose, then 20 μ l RB150. Reactions were centrifuged for 90 minutes at 50,000 rpm at 4°C in a Beckman TLS-55 rotor with inserts (Beckman no. 358615). Proteoliposomes (20 μ l) were harvested from the top interfaces, lipid concentrations were estimated using NBD and rhodamine fluorescence, and bound HOPS was estimated by SDS-PAGE and

immunoblotting for Vps33p, using a standard curve of pure HOPS complex. Average concentrations of bound HOPS for at least three independent experiments, each using different proteoliposome preparations, are shown; error bars represent standard deviations.

(b) Reactions containing nocardiolipin proteoliposomes were prepared using 80, 40, 20, or 10 nM HOPS complex for proteoliposomes with Ypt7p and 160, 80, 40, or 20 nM HOPS for proteoliposomes without Ypt7p. Lipid mixing and HOPS binding were measured as described above and in Experimental Procedures, respectively. Average overall lipid mixing and bound HOPS for each set of reactions (n=3) are shown; error bars represent standard deviations for both measurements. Filled squares, reactions with Ypt7p; open squares, reactions without Ypt7p.