Diaacylglycerol and Its Formation by Phospholipase C Regulate Rab- and SNARE-dependent Yeast Vacuole Fusion*

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Although diaacylglycerol (DAG) can trigger liposome fusion, biological membrane fusion requires Rab and SNARE proteins. We have investigated whether DAG and phosphoinositide-specific phospholipase C (PLC) have a role in the Rab- and SNARE-dependent homotypic vacuole fusion in *Saccharomyces cerevisiae*. Vacuole fusion was blocked when DAG was sequestered by a recombinant C1b domain. DAG underwent ATP-dependent turnover during vacuole fusion, but was replenished by the hydrolysis of phosphatidylinositol 4,5-bisphosphate to DAG by PLC. The PLC inhibitors 3-nitrophenol and U73122 blocked vacuole fusion in *vitro*, whereas their inactive homologues did not. Plc1p is the only known PLC in yeast. Yeast cells lacking the *PLC1* gene have many small vacuoles, indicating defects in protein trafficking to the vacuole or vacuole fusion, and purified Plc1p stimulates vacuole fusion. Docking-dependent Ca\(^{2+}\) efflux is absent in *pla1Δ* vacuoles and was restored only upon the addition of both Plc1p and the Vam7p SNARE. However, vacuoles purified from *pla1Δ* strains still retain PLC activity and significant 3-nitrophenol-resistant PLC activity associated with vacuoles. We have purified a recombinant C1b domain. DAG underwent ATP-dependent turnover during vacuole fusion, but was replenished by the hydrolysis of phosphatidylinositol 4,5-bisphosphate to DAG by PLC. The PLC inhibitors 3-nitrophenol and U73122 blocked vacuole fusion in *vitro*, whereas their inactive homologues did not. Plc1p is the only known PLC in yeast. Yeast cells lacking the *PLC1* gene have many small vacuoles, indicating defects in protein trafficking to the vacuole or vacuole fusion, and purified Plc1p stimulates vacuole fusion. Docking-dependent Ca\(^{2+}\) efflux is absent in *pla1Δ* vacuoles and was restored only upon the addition of both Plc1p and the Vam7p SNARE. However, vacuoles purified from *pla1Δ* strains still retain PLC activity and significant 3-nitrophenol-resistant PLC activity associated with vacuoles.

Regulated membrane fusion is essential for transport of proteins and lipids between subcellular compartments and for the maintenance of organelle identity in eukaryotic cells. Fusion events require proteins and lipids that are conserved from yeast to mammals. Among these are SNAREs and Sec1/Munc18 family proteins, Sec18/N-ethylmaleimide-sensitive factor and Sec17/α-SNAP and the Ypt/Rab GTPases and their effectors proteins. Lipids, including phosphoinositides and sterols, also play crucial roles.

Yeast vacuoles are used to study membrane fusion mechanisms. The homotypic fusion of yeast vacuoles, reconstituted in *vitro*, occurs in three stages: priming, docking, and bilayer fusion and contents mixing (1, 2). During priming, Sec18p (N-ethylmaleimide-sensitive factor) disassembles cis-SNARE complexes containing the three Q-SNAREs (Vti1p, Vam7p, and Vam3p) and an R-SNARE (Nvy1p or Ykt6p). Priming releases Sec17p (α-SNAP) and a soluble SNARE, Vam7p, from vacuoles. The docking stage begins with tethering, which is regulated by the small Rab GTPase Ypt7p. Tethering is followed by the accumulation of fusion factors into a vacuole membrane microdomain termed the “vertex ring,” which surrounds the closely apposed membranes of the tethered vacuoles (3). The final step of docking is the formation of SNARE complexes in trans between apposed vacuoles. Docking requires the HOPS/VpsC complex, composed of the subunits Vps11p, Vps16p, Vps18p, Vps33p, Vps39p, and Vps41p. HOPS fulfills several functions. It serves as a nucleotide exchange factor for Ypt7p (4). HOPS also binds to Ypt7p-GTP and is thus a Ypt7p effector (5). The Vps33p subunit of HOPS is a Sec1/Munc18 family protein that allows HOPS to interact with the SNARE complexes. *trans*-SNARE interactions trigger a Ca\(^{2+}\) efflux from the vacuole lumen (6). The late stages of fusion may require additional factors such as the Vv1p, a subunit of the V-ATPase (7, 8), the Vtc complex (9), the armadillo repeat protein Vac8p (10, 11), and actin and actin assembly regulators (12).

Specific “regulatory lipids” are also essential for vacuole fusion. Ergosterol, a yeast sterol, is required for priming (13). At least two phosphoinositides are involved in vacuole docking. Phosphatidylinositol 3-phosphate (PI(3)P) is recognized by the Phox homology domain of Vam7p to facilitate the rebinding of this SNARE to the vacuole (14, 15). Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\(_2\)) regulates priming and docking (16), although its functions have been unknown. Theses lipids are required for the enrichment of fusion factors in the vertex ring during docking. Enzymes that generate or modify these lipids are therefore likely to play essential roles in vacuole fusion. Cells deleted for any of the genes for ergosterol biosynthesis have fragmented vacuoles (13), which often indicate a fusion defect. Vacuoles are also fragmented in *vps43Δ* cells that lack phosphatidylinositol (PI) 3-kinase activity (17), and vacuoles isolated from spheroplasts pretreated with the PI 3-kinase inhibitor wortmannin are incompetent for fusion in *vitro* (15). PI(4,5)P\(_2\) participates in the formation of the vertex ring microdomain and may serve as a membrane anchor for fusion catalysts such as those that remodel actin (18) or as a biosynthetic precursor of other lipids that are needed for fusion. PI(4,5)P\(_2\) is synthesized in yeast through the sequential phosphorylation of PI by the PI 4-kinase Stt4p and the PI(4)P 5-kinase Mss4p (19). PI(4,5)P\(_2\) is hydrolyzed to diaacylglycerol.

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The abbreviations used are: SNAREs, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; α-SNAP, α-soluble N-ethylmaleimide-sensitive factor attachment factor; HOPS, homotypic fusion and vacuole protein sorting complex; PI, phosphatidylinositol; PI(3)P, phosphatidylinositol 3-phosphate; PI(4,5)P\(_2\), phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; PLC, phosphoinositide-specific phospholipase C; GFP, green fluorescent protein; HA, hemagglutinin; PIPES, 1,4-piperazinediethanesulfonic acid; rVam7p, recombinant Vam7p; NBD, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))-1,4-piperazinediethanesulfonic acid.

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(DAG) and IP₃ by phosphoinositide-specific phospholipase C (PLC). We refer here to all phospholipase C activities as PLC, whereas PLC1 encodes the only known yeast PLC. DAG is a hydrophobic lipid of intrinsic negative curvature that promotes the fusion of bilayer membranes (20). Biophysical studies have shown that DAG promotes the fusion of synthetic vesicles by altering the physical properties of a membrane lipid bilayer, modifying its fluidity, or inducing lateral phase separations and transitions (21). DAG may fulfill these roles in biological membrane fusion events as well. DAG has been implicated in several membrane trafficking events. It promotes Ca²⁺-induced fusion of chromaffin granules with other membranes (22) and facilitates exocytosis of amylase from parotid gland secretory granules (23). DAG also has an essential role in protein transport from the yeast Golgi complex (24).

We now report that DAG and PLC, which hydrolyzes PI(4,5)P₂ to DAG, are required for vacuole fusion, suggesting that the requirement of PI(4,5)P₂ for vacuole fusion may reflect an essential role of DAG. DAG is produced by PLC on yeast vacuoles and is consumed in an ATP-dependent manner. There is only one known PLC, encoded by PLC1, in Saccharomyces cerevisiae (25–27). Yeast cells lacking the PLC1 gene have a fragmented vacuole phenotype, which often indicates a vacuole fusion defect. However, vacuoles isolated from plcΔ cells still retain PLC activity and exhibit reduced but meaningful fusion, indicating that there is an additional gene, structurally unrelated to PLC1, encoding PLC activity. These studies establish that DAG regulation through PLC is required for Rab- and SNARE-dependent biological membrane fusion.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Genetic Modifications—**BJ3505 (Matα ura3-52 trp1Δ101 his3Δ200 lys2-801 gal1Δ (gal3 can1) trp1Δ1Δ6R pep4Δ:His3) (28) and DKE6281 (Matα ura3-52 leu2-3,112 trp1Δ101 his3Δ200 lys2-801 sec23Δ pho8Δ:TRP1) (29), or pho8Δ::neo and pep4Δ::neo derivatives of BY4742 (Matα his3Δ1 leu2Δ200 lys2Δ200 ura3Δ0) were used for the vacuole fusion reaction. BJ3505 and BY4742 pho8Δ::neo were used to generate YJY1 (BJ3505 plcΔ::URA3) and YJY2 (BY4742 pho8Δ::neo plcΔ::URA3), respectively. BY4742 pho8Δ::neo was used to generate YJY3 (BY4742 plcΔ::plc::RS403-GFP-PLC1) or YJY4 (BY4742 plcΔ::plc::RS403-3·HA-PLC1). Briefly, the integrating vector pRS403 containing the green fluorescent protein (GFP) module (30) or three tandem in-frame hemagglutinin (HA) modules fused in-frame to the 5′-end of the PLC1 gene, flanked by its native promoter and part of the downstream region, was transformed into strain BY4742 and transformants were selected on yeast extract/peptone/2% agar plates. Transformants were confirmed by PCR and Western blotting. BJ4549 (Matα ura3-52 trp1Δ1 his3Δ200 lys2-801 leu2Δ200 gal1Δ (gal3 can1) trp1Δ1Δ6R pep4Δ:His3 GAL1) (28) was transformed with pFF137 in which PLC1 was placed under the control of the GAL1 promoter (a generous gift from Dr. Jeremy Thorner, University of California, Berkeley, CA) (25) to generate YJY11. To generate a lipase-inactive mutant of PLC1, His³ and Asn³⁹⁸ were changed to Ala⁶⁰⁵ and Ala³⁸⁹ (31) using a QuickChange site-directed mutagenesis kit (Stratagene) with TGCATCTTCAGCTGCTACTTATTTATTGGGGAAC and GTTCTCCCAATAAAAAATAGCAGCTGGAAGATGCA. The mutations were confirmed by creation of a new PvuII site (underlined) and by complete loss of PLC activity of the purified mutant protein. The wild-type PLC1 gene or the lipase-inactive mutant of the PLC1 gene was subcloned into the pRS18 vector (32).

**Plasmids—**3-Nitrocoumarin (a generous gift from Dr. Enzo Martegani, Università di Milano, Milan, Italy) and 7-hydroxy-3-nitrocoumarin (a generous gift from Dr. Dhiren R. Thakker, University of North Carolina, Chapel Hill, NC) were dissolved in Me₂SO. U73322 and U73343 were purchased from Calbiochem and dissolved in Me₂SO. FM₄-64 (Molecular Probes, Inc., Eugene, OR) was dissolved in Me₂SO; 4 μM FM₄-64 was used to visualize vacuoles in yeast cells. The Cib domain was produced as a recombinant GST fusion protein in Escherichia coli (33) and dialyzed against 125 mM KCl and 20 mM PIPES-KOH (pH 6.8). The Cib domain labeled with the fluorophore Alexa 488 (Molecular Probes, Inc.) was generated according to the manufacturer’s protocol. Anti-Sec18p antibody and anti-Sec17p antibody were prepared as described (34). Gd1Δ (35), recombinant Vam7p (Vam7Δ7p) (6), and anti-Vam7p (6), anti-Vam3p (36), anti-Vam6p (37), anti-Pep4p (38), anti-GFP (39), and anti-Vps33p (39) antibodies were prepared as described. His₆-Sec18p was purified as described (34) with an additional gel filtration step on an S300 HR column (Amersham Biosciences) in 20 mM PIPES-KOH (pH 6.8), 200 mM sorbitol, 125 mM KCl, 5 mM MgCl₂, 2 mM ATP, 2 mM dithiothreitol, and 10% glycerol. Recombinant Pho8p (IB₂) was purified as described (40). His₆-Plc1p was prepared as described (25) except that strain YJY11 was used and the overexpression of His₆-Plc1p was induced with 2% galactose in yeast extract/peptone medium instead of synthetic dextrose medium lacking uracil.

**Vacuole Isolation and In Vitro Fusion Assay—**For in vitro fusion, vacuoles were isolated from yeast strains BJ3505 and DKE6281 unless indicated otherwise (29). Standard fusion reactions (30 μl) contained 3 μg of vacuoles lacking the protease Pep4p, 3 μg of vacuoles from cells without Pho8p, reaction buffer (125 mM KCl, 5 mM MgCl₂, 20 mM PIPES-KOH (pH 6.8), and 200 mM sorbitol), 1 mM ATP, 40 mM creatine phosphate, 0.5 mg/ml creatine kinase, 10 μM coenzyme A, and 4.5 μM recombinant Pho8p (IB₂) (29). Fusion assays were incubated for 90 min at 27 °C, and fusion was measured by assaying alkaline phosphatase (41).

**PLC Assay and Thin Layer Chromatography—**A simple PLC assay was developed utilizing a fluorescent PI(4,5)P₂ substrate and TLC by modifying an assay for phosphoinositide phosphatase (42). DI-C₅-NBD-PI(4,5)P₂ (0.3 μg, Echelon Research) was incubated with 50 μM purified PLC1, 1 μM of isolated vacuoles in PLCl buffer (50 mM Hepes-HCl (pH 7.2), 100 mM NaCl, 5 μM CaCl₂, and 0.5 mM bovine serum albumin) for 30 min at 30 °C. Reactions were terminated by the addition of 100 μl of acetone and then evaporated to dryness in a SpeedVac evaporator set on low heat. The dried reaction products were resuspended in 10 μl of methanol/2-propanol/glacial acetic acid (5:5:2) and spotted onto a glass-backed TLC plate (K6 silica gel, 60 Å, 20 × 20 cm). The TLC plate was developed in chloroform/methanol/glacial acetic acid/water (46:15:17:4.8) and air-dried. Fluorescent lipids were visualized using a Typhoon PhosphorImager 8600 (Amersham Biosciences).

**Calcium Efflux Assay—**Cα²⁺ efflux assays were performed as described for fusion, but with 10 μg rather than 6 μg of vacuoles from BJ3505 or YJY1 (BJ3505 plcΔ::URA3) in 30-μl reactions. Aequorin containing assays were performed as described previously (43). Samples were analyzed in 96-well low protein binding conical bottom plates (Nunc, Rochester, NY) in a luminometer (Molecular Devices). IGOR Pro 4 (WaveMetrics) and JMP (SAS Institute) were used for data analysis. Calibration was done using buffered Ca²⁺-EGTA standard solutions (Molecular Probes, Inc.) as described previously (6).

**RESULTS**

DAG enhances the fusion of liposomes by changing their surface properties (21), suggesting that DAG may also fulfill this function for biological membrane fusion. DAG becomes enriched in vertex rings during yeast vacuole fusion and regulates the vertex enrichment of other lipids and of proteins such as Ypt7p, SNAREs, and HOPS.² We have tested the role of DAG in membrane fusion by examining the effect of DAG ligands and the need for DAG production from PI(4,5)P₂. We first examined whether DAG is required for vacuole fusion, using an in vitro fusion assay and a specific DAG ligand, the C1b domain from mammalian protein kinase CβII (33). We assayed the homotypic fusion of vacuoles by mixing vacuoles from two strains. One strain has the vacuolar luminal protease Pep4p, but is deleted for the gene encoding the Pho8p phosphatase. The other strain lacks the protease Pep4p and therefore accumulates catalytically inactive pro-Pho8p. During incubation with ATP, fusion allows the Pep4p protease to gain access to pro-Pho8p, converting it to the active form, which is assayed colorimetrically. The recombinant C1b domain inhibited the fusion reaction (Fig. 1), indicating that DAG is involved in vacuole fusion.

**PI(4,5)P₂, which is hydrolyzed by PLC to DAG and inositol 1,4,5-triphosphate (IP₃), is required for early stages of vacuole fusion** (16). PI(4,5)P₂ may be needed as a substrate for PLC to produce DAG. To examine whether PLC activity is required for vacuole fusion, we employed two specific PLC inhibitors, 3-nitrocoumarin and U73122. 3-Nitrocoumarin inhibits the PLC activity of Ptc1p in vitro with a Ki of 57.6
increasing concentrations of GST-C1b.

on isolated yeast vacuoles (see Fig. 8).

action of one or more PLCs. This is supported by the finding

A greater loss of vacuole surface DAG was seen in the presence

of 40 M, whereas its structural analogue U73343 did not

inhibit (Fig. 2B). U73122 also inhibited the activity of puri-

fied His<sub>6</sub>-Plc1p (Fig. 2C) and the PLC activity that is present

on isolated yeast vacuoles (see Fig. 8E below).

We developed an assay of vacuole surface DAG to determine

whether it is dynamically altered during in vitro fusion reactions. C1b labeled with the Alexa 488 fluorophore was incubated with vacuoles, and the vacuole-bound Alexa 488-labeled C1b was assayed by sedimenting the organelle. During a standard fusion reaction, there was an ATP-dependent loss of vacuole surface DAG (Fig. 3A, circles, + ATP, and squares, − ATP). A greater loss of vacuole surface DAG was seen in the presence of the PLC inhibitor U73122 (Fig. 3B, bar 3 versus bar 2), suggesting that the DAG pool was being replenished by the action of one or more PLCs. This is supported by the finding that the addition of purified His<sub>6</sub>-Plc1p increased the level of accessible DAG (Fig. 3B, bars 4 and 5). DAG is thus synthesized by PLC on isolated vacuoles and undergoes further ATP-dependent modification or internalization.

Vacuole fusion occurs in three stages. Priming, initiated by ATP-dependent Sec18p action, is a prerequisite for docking and fusion (48). To determine the stage at which PLC functions during vacuole fusion and whether PLC function is also regulated by the priming factor Sec18p, the reaction was arrested by blocking priming using either anti-Sec18p (Fig. 4A) or anti-Sec17p (Fig. 4B) antibody. Although anti-Sec18p antibody blocked the reaction (Fig. 4A, bar 3), fusion could be restored after a 30-min incubation by the addition of excess Sec18p (bar 10) as reported (49). Without anti-Sec18p antibody, substantial docking and even fusion occurred during a 30-min incubation (bar 16), but the presence of anti-Sec18p antibody prevented priming, and thereby docking and fusion, during this period (bar 9). Therefore, upon reversal of an anti-Sec18p antibody block with Sec18p, fusion reactions were still fully sensitive to docking inhibitors such as anti-Ypt7p (bar 11), anti-Vam3p (bar 12), or anti-Vps33p (bar 13) antibody. However, fusion reactions had acquired substantial resistance to 3-nitrocoumarin or U73122 during a 30-min incubation with anti-Sec18p antibody (bars 14 and 15), showing that PLC function is not regulated by Sec18p action. To further determine when PLC functions during vacuole fusion, we exploited the recent observation that rVam7p can bypass the requirement for Sec17/18p-mediated priming (6, 50). Fusion reactions were arrested by anti-Sec17p antibody (Fig. 4B, bar 4) and at least partially restored after a 30-min incubation by the addition of rVam7p (bar 9). This fusion reaction was largely resistant to U73122 (bar 11), although it was fully sensitive to anti-Vam3p antibody, a docking inhibitor (bar 10), or to the C1b domain (bar 12), suggesting that PLC function is largely completed before rVam7p-driven docking, but that DAG, a product of PLC function, is still needed during or after docking. These experiments show that the production of DAG by PLC is an early event that is not regulated by Sec18p and that DAG generated at earlier stages is required for the later stages of vacuole fusion.

S. cerevisiae has only one known PLC, encoded by PLC1 (25–27). Wild-type yeast cells have one to three large vacuoles (Fig. 5A), but deletion of the PLC1 gene caused striking vacuole fragmentation (Fig. 5B), which may reflect a defect either in vacuole fusion or in biosynthetic trafficking to the vacuole (17). Introduction of the PLC1 gene into plc1Δ cells restored the normal vacuole morphology (Fig. 5C). A lipase-inactive mutant of PLC1 failed to restore the normal vacuole phenotype (Fig. 5D), suggesting that the enzymatic activity of Plc1p is required for normal vacuole structure in vivo.

Since Plc1p might support vacuole structure either directly or indirectly, e.g. through biosynthetic trafficking to the vacuole, we characterized its presence and functions on purified vacuoles. Vacuoles were isolated from strains in which the PLC1 gene was replaced on the chromosome with a gene encoding GFP-Plc1p or HA-Plc1p under the control of the native PLC1 promoter. Vacuoles were analyzed by SDS-PAGE and immunoblotted with anti-GFP or anti-HA antibody. GFP-Plc1p (Fig. 6, upper panel) or HA-Plc1p (data not shown) was detected in purified vacuole extracts, although neither was enriched in vacuoles as was the vacuolar protease Pep4p (Fig. 6, lower panel). Although our vacuoles were purified ~30-fold from the cell lysate, Plc1p was present on vacuoles at the same relative abundance as in the total cell extract, making it unlikely that it is simply present because of cytosolic contamination.

Low concentrations of purified His<sub>6</sub>-Plc1p stimulated in vitro vacuole fusion (Fig. 7), although it inhibited at higher concentrations. The inhibition may be due to depletion of P(t(4,5)P<sub>2</sub>, which may have more than one role in vacuole fusion (16), or through production of excess DAG, which has an inhibitory effect on the membrane fusion of synthetic vesicles (51). Heat-treated His<sub>6</sub>-Plc1p had neither stimulatory nor inhibitory activity (data not shown).

To examine whether Plc1p is essential for vacuole fusion in vitro, we generated plc1Δ pep4Δ and plc1Δ pho8Δ strains, isolated vacuoles from these double deletion strains, and assayed their fusion. Surprisingly, vacuoles from plc1Δ strains were competent for in vitro fusion, although the fusion was not as efficient as for wild-type vacuoles (2–50% as efficient; 17% average from nine independent experiments with successive vacuole preparations) (Fig. 8A, bars 3 and 4). This reduced fusion of plc1Δ vacuoles was not restored by the addition of purified Plc1p (Fig. 8A, bar 6), suggesting that PLC1 deletion may affect the biosynthetic delivery or stability of other essential fusion catalysts. Indeed, plc1Δ vacuoles showed modest reductions in their levels of several SNAREs (Fig. 8B). The fusion of plc1Δ vacuoles could be restored by...
FIG. 2. PLC inhibitors block vacuole fusion, whereas their structural analogues do not. Standard fusion reactions were performed for 90 min at 27 °C in the presence of increasing concentrations of 3-nitrocoumarin (3NC; black bars) or 7-hydroxy-3-nitrocoumarin (7OH-3NC; gray bars) (A) or U73122 (black bars) or U73343 (gray bars) (B). U, units. Also shown is the inhibition of the PLC activity of His6-Plc1p by U73122 (C). NBD-Pl(4,5)P2 (13.3 μM) and 50 nM His6-Plc1p (second and third lanes) were incubated for 30 min at 30 °C in the absence (second lane) or presence (third lane) of 80 μM U73122. The reactions were spotted onto a TLC plate, which was developed as described under “Experimental Procedures.” Fluorescent lipids separated on the TLC plate were analyzed by a Typhoon PhosphorImager 8600.

FIG. 3. Quantitative analysis of vacuole surface DAG. A, vacuole fusion reactions were performed at 27 °C for indicated times in the presence (closed circles) or absence (closed squares) of ATP. After incubation, vacuoles were transferred to ice, labeled with 9.1 μM Alexa 488-labeled C1b for 30 min, centrifuged at 13,000 × g for 15 min at 4 °C, resuspended in 30 μl of 125 mM KCl and 20 mM PIPES-KOH (pH 6.8), and solubilized with 100 μl of 4 mM polidocanol. Equal amounts of polidocanol were added to the 30-μl supernatant fractions. Samples were analyzed using an ISS K2 fluorometer (ISS Inc., Champaign, IL) with excitation at 488 nm and recording emission at 519 nm. Surface DAG levels are expressed as the percent of Alexa 488-labeled C1b bound to membranes. B, vacuoles were incubated under standard fusion conditions on ice (bar 1) or at 27 °C for 90 min without inhibitor (bar 2) or in the presence of 80 μM U73122 (bar 3) or His6-Plc1p (bars 4 and 5) and assayed for exposed DAG by binding Alexa 488-labeled C1b as described for A.
the addition of excess rVam7p (Fig. 8A, bar 8), consistent with the SNARE deficiency and with the recent report that rVam7p-mediated “bypass” fusion is largely resistant to PLC inhibitors (50). Since DAG is required for vertex enrichment of three vacuolar Q-SNAREs (Vam3p, Vti1p, and Vam7p) and for vacuolar association of soluble Vam7p, one of the roles of DAG in vacuole fusion may be to promote SNARE complex formation at the vertex ring. By driving SNARE assembly by mass action, high levels of rVam7p may reduce the requirement for DAG. This idea was further corroborated by studying the ability of rVam7p to restore fusion to C1b-treated vacuoles (Fig. 8C). Vacuole fusion (Fig. 8C, bar 8) was almost completely blocked by 30 μM C1b (bar 2). The addition of rVam7p after a 25-min incubation with C1b partially restored fusion (bars 3–6), whereas rVam7p did not stimulate fusion in reactions that had not received C1b (bars 8–12). The fusion of plc1Δ vacuoles was still sensitive to anti-Sec17p, anti-Sec18p, or anti-Vam7p antibody or to the PLC inhibitor U73122 (Fig. 8D). Although Plc1p may contribute to vacuole fusion, it is apparently not essential. However, the sensitivity of the fusion of plc1Δ vacuoles to U73122 suggests that some PLC activity is still required. There may be another PLC whose activity is required during vacuole fusion in vitro in the absence of Plc1p. The existence of another PLC is strengthened by the observation that vacuoles purified from a plc1Δ strain retain substantial PLC activity (Fig. 8E, lane

**Fig. 4.** PLC activity is independent of Sec18p function. A, fusion reactions were incubated on ice (bar 1) or at 27 °C (bar 2) for 90 min in the presence of 0.14 μM affinity-purified anti-Sec18p antibody (bar 3), 0.33 μM affinity-purified anti-Ypt7p antibody (bar 4), 0.2 μM affinity-purified anti-Vam3p antibody (bar 5), 32 μM affinity-purified anti-Vps33p antibody (bar 6), 125 μM 3-nitrocoumarin (3NC; bar 7), or 80 μM U73122 (bar 8). Other fusion reactions were incubated for 30 min in the presence (bars 9–15) or absence (bars 16–22) of 0.14 μM anti-Sec18p antibody. After 30 min at 27 °C, various inhibitors were added, followed by 0.18 μM His6-Sec18p alone (bars 9–15) or a mixture of 0.14 μM anti-Sec18p antibody and 0.18 μM His6-Sec18p (bars 16–22). Reactions were incubated at 27 °C for an additional 70 min before assaying for alkaline phosphatase. U, units. B, fusion reactions were performed at 27 °C for 90 min in the presence of 27 nm rVam7p (bar 3), 0.15 μM affinity-purified anti-Sec17p antibody and 27 nm rVam7p without inhibitor (bar 13) or with 0.2 μM anti-Vam3p antibody (bar 14), 80 μM U73122 (bar 15), or 25 μM C1b (bar 16). Another set of fusion reactions was incubated at 27 °C for 30 min in the presence of 0.15 μM affinity-purified anti-Sec17p antibody (bars 8–12) and then mixed with 0.2 μM anti-Vam3p antibody (bar 10), 80 μM U73122 (bar 11), or 25 μM C1b (bar 12). These anti-Sec17p antibody-blocked reactions (bars 8–12) were then rescued with 27 nm rVam7p and incubated for an additional 70 min. Bar 4 is a “no rescue” control.
Furthermore, the PLC activity present in \textit{plc1\Delta/H9004} vacuoles was inhibited by either U73122 or 3-nitrocoumarin (lanes 9 and 10). The reduced but measurable fusion shown in \textit{plc1\Delta/H9004} vacuoles is consistent with the vacuole phenotype of \textit{plc1\Delta/H9004} cells as Class B (17), reflecting a modest defect in vacuole fusion (52), instead of Class C, which is more severe. The unknown gene encoding the novel PLC activity found in \textit{plc1\Delta/H9004} cells must be structurally unrelated to \textit{PLC1}, as there are no other genes that show significant sequence homology to \textit{PLC1} in \textit{S. cerevisiae}.

In a complex reaction pathway, a step that is not rate-limiting may be severely diminished, whereas the overall reaction is far less compromised. In this regard, vacuoles exhibited a broad peak of net Ca$^{2+}$ efflux after ~40 min of the fusion reaction (Fig. 9A, closed circles). This Ca$^{2+}$ efflux has been shown to depend on \textit{trans}-associations of SNAREs (6) and thus was stimulated by the addition of rVam7p (closed squares) or inhibited by anti-Vam3p antibody (open circles). This peak of Ca$^{2+}$ efflux was also stimulated by His$_6$-Plc1p (closed triangles), and there was synergistic stimulation of Ca$^{2+}$ efflux when both Plc1p and rVam7p were added (closed diamonds). In all cases, the efflux of Ca$^{2+}$ was blocked by anti-Vam3p antibody (open diamonds), a characterized docking inhibitor (48), as well as anti-Vam3p antibody (data not shown).

\textbf{DISCUSSION}

Fusion between two lipid bilayer membranes is not a spontaneous event. It requires energy to overcome hydration repulsion between apposed membranes and to disrupt the nor-
FIG. 8. Plc1p is not essential for in vitro vacuole fusion. A, plcΔ vacuoles fuse in vitro. Fusion reactions were performed with PLC1 vacuoles (black bars) isolated from strains BJ3505 and BY4742 pho8Δ::neo or with plcΔ vacuoles (gray bars) isolated from strains YJY1 and YJY2. Reactions were incubated on ice (bars 1 and 2) or at 27 °C (bars 3 and 4) for 90 min in the presence of 0.35 M His6-Plc1p (bars 5 and 6) or 2.7 μM rVam7p (bars 7 and 8). U, units. B, immunoblot analysis of vacuoles isolated from BJ3505 or YJY1 (BJ3505 plcΔ::URA3). C, excess rVam7p partially bypasses the DAG requirement for vacuole fusion. Fusion reactions were incubated at 27 °C in the presence (bars 1–6) or absence (bars 7–12) of 30 μM C1b domain. After 30 min, rVam7p was added at the indicated concentrations. Reactions were incubated at 27 °C for an additional 70 min before assaying for alkaline

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Homotypic vacuole fusion in S. cerevisiae is Ypt/Rab- and SNARE-dependent, but has also been shown to require specific regulatory lipids such as PI(4,5)P₂ (16), PI(3)P (15), and ergosterol (13). We have shown that DAG is also required for vacuole fusion. DAG and the other regulatory lipids become enriched in the vertex ring microdomain of docked vacuoles and are required for the assembly of selected fusion proteins into these vertex rings and thus for subsequent vacuole fusion. DAG present on vacuole membranes was both consumed and produced during normal vacuole fusion reactions (Fig. 3). Either the recombinant C1b domain (a ligand for DAG) or the PLC inhibitors 3-nitrocoumarin and U73122 (which prevent DAG production) blocked vacuole fusion. ET-18-OCH₃, a third PLC inhibitor (54), has been shown previously to block the vacuole fusion reaction (17). Although ET-18-OCH₃ inhibits PI 3-kinase such as wortmannin and LY-294002 (16) do not affect the fusion reaction, and the block of vacuole fusion by ET-18-OCH₃ is therefore likely due to inactivation of PLC. Moderate levels of purified His₆-Plc1p stimulated fusion, whereas high levels of added Plc1p inhibited vacuole fusion (Fig. 7). Such stimulation at low PLC levels and inhibition at high levels may be reminiscent of the DAG effects on fusion seen in liposome studies (51), where fusion is promoted by low DAG levels and inhibited by high DAG levels. These findings have been explained by the need for specific DAG concentrations to be produced at the correct time and place in the reaction pathway, i.e. during docking and (for vacuoles) at vertices, to promote non-bilayer lipid phases. Plc1p is found on vacuoles, and its absence through gene deletion results in a vacuole fragmentation phenotype in vivo and in substantial reduction, although not abolition, of the fusion of purified vacuoles in vitro. However, the ability of Vam7p, but not Plc1p, to restore fusion, combined with immunoblot analysis showing a general diminution of vacuolar SNARE levels, suggests that PLC1 deletion may inhibit fusion through lowered SNARE levels. We have also found that excess rVam7p can bypass the PLC requirement, as shown by the ability of rVam7p to restore fusion to 3-nitrocoumarin- or U73122-treated vacuoles (50). The ability of rVam7p to restore fusion to C1b-treated vacuoles suggests that a major function of DAG may be to help gather Ypt7p, PI(3)P, and the other SNAREs into vertices, where Ypt7p and PI(3)P can catalyze the assembly of the low concentrations of endogenous Vam7p into SNARE complexes. DAG can still be produced by plc1Δ vacuoles, although further studies will be needed to discover whether this represents a novel PLC activity or the sequential activity of enzyme pairs such as a phosphoinositide-specific phospholipase D (56) and a phosphatidic-acid phosphatase. The continued sensitivity to 3-nitrocoumarin and U73122 of plc1Δ vacuole fusion and of plc1Δ vacuole conversion of PI(4,5)P₂ to DAG suggests the existence of a second PLC. Although there is no homologue of PLC1 in yeast, the continued

![Graphical representation of Ca²⁺ release reactions](image)

**Fig. 9. Docking- and Plc1p-dependent Ca²⁺ efflux.** Standard Ca²⁺ release reactions (6) were performed with PLC1 vacuoles isolated from strain BJ3505 (A) or with plc1Δ vacuoles isolated from strain YJY1 (B). Reactions were initiated with ATP without additional proteins (Standard, closed circles) or in the presence of 2.7 μM rVam7p, 0.35 μM His₆-Plc1p, 2.5 μM Gdi1p, or 0.2 μM affinity-purified anti-Vam3p antibody alone or in the specified combinations.

[Further text and details about experimental procedures and findings related to DAG and PLC in yeast homotypic vacuole fusion, including the role of PLC1 and other regulatory lipids in membrane fusion processes, are discussed in the full text of the document.]

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**Note:** The text and figures provided are a synthesis of the information available in the full document, focusing on the key aspects of DAG and PLC's role in yeast homotypic vacuole fusion, as indicated by the references and experimental data cited.
sensitivity of the fusion of vacuoles from plc1Δ strains to 3-nitroso-coumarin and U73122 suggests that crucial active-site residues must be conserved in the second PLC. It is unclear why plc1Δ vacuoles show a strikingly synergistic requirement for the addition of both Plc1p and rVam7p to restore the normal dynamics of trans-SNARE pairing, assayed by Ca\textsuperscript{2+} efflux. In this regard, we have recently shown that, by driving SNARE complex assembly, rVam7p can bypass or substantially reduce the requirements for several fusion factors such as Ypt7p, ATP, Sec17p, and Sec18p (50). DAG may promote SNARE complex formation through promoting SNARE concentration in vertex rings; by driving SNARE assembly by mass action, high levels of rVam7p may reduce the requirement for DAG (Fig. 8C). This is further supported by the observations that bypass fusion that is driven by the addition of excess rVam7p is largely resistant to 3-nitroso-coumarin and U73122 (50) and that Cib prevented the enrichment of SNAREs in vertex rings.

The vacule lumen is an intracellular calcium store in yeast (57), as is the endoplasmic reticulum in mammalian cells. It is also possible that Plc1p induces or enhances Ca\textsuperscript{2+} efflux by generating IP\textsubscript{2} which triggers Ca\textsuperscript{2+} efflux in mammalian cells, as well as DAG. Indeed, Plc1p increased docking-dependent Ca\textsuperscript{2+} efflux (Fig. 9A, compare closed circles and closed triangles), and U73122 completely abolished Ca\textsuperscript{2+} efflux. However, this is unlikely, as yeast has no obvious homologue of the mammalian IP\textsubscript{3} receptor calcium channel proteins (58), and exogenous IP\textsubscript{3} did not affect docking-dependent Ca\textsuperscript{2+} efflux. We have not seen stimulation of vacuole fusion upon the addition of exogenous IP\textsubscript{3} and DAG.

Although a minor component of biological membranes, DAG is a key intermediate in lipid metabolism, cell signaling, and membrane fusion. DAG was identified as a cell fusions in classical studies (59). Since then, it has been implicated in membrane fusion in various systems. sn-1,2-DAG, but not sn-1,3-DAG, stimulates Ca\textsuperscript{2+} induced fusion of phosphatidylserine-containing vesicles (60). Treatment of phospholipid vesicles with phospholipase C, thereby forming DAG within the membrane, also causes vesicle fusion (61). Vesicles with phospholipase C, thereby forming DAG within the membrane, also causes vesicle fusion (61). Vesicles with phospholipase C, thereby forming DAG within the membrane, also causes vesicle fusion (61). Vesicles with phospholipase C, thereby forming DAG within the membrane, also causes vesicle fusion (61).

We previously showed that PI(4,5)P\textsubscript{2}, a precursor of DAG, is required for two early steps of vacuole fusion, priming and docking (16). Although all of the functions of PI(4,5)P\textsubscript{2} remain to be determined, it may promote the actin remodeling (18) that is required for vacuole fusion (12). The observation that excess Plc1p fully inhibited fusion (Fig. 7) may either reflect a role for PI(4,5)P\textsubscript{2} in processes such as actin reassembly during a late stage of vacuole fusion or indicate that excessive DAG can inhibit fusion, as reported in liposome fusion studies (51). PI(4,5)P\textsubscript{2} is also required as a source of DAG since PLC, which hydrolyzes PI(4,5)P\textsubscript{2} to generate DAG, is required for vacuole fusion. This idea is further supported by the observations that a PLC inhibitor could block DAG generation during fusion reactions and that His6-Plc1p increased DAG levels on the vacuolar membrane during the fusion reaction.

Plc1p is the only known PLC of S. cerevisiae. Vacuoles from plc1Δ are capable of in vitro fusion. However, this fusion is still sensitive to U73122, and these vacuoles still retain PLC activity (Fig. 5E, lane 8), implying that there is another PLC in S. cerevisiae. The unknown gene encoding the second PLC must be unrelated to PLC1 because there is no gene with significant sequence homology. In a previous report, PLC activity was not detected in PLC1 temperature-sensitive mutants at non-permissive temperature (62). We have detected the second PLC activity in a vacuole preparation from plc1Δ cells, whereas Yoko-o et al. used a high speed supernatant fraction from a cell lysate to detect PLC activity. If the second PLC is an integral or peripheral membrane protein specifically enriched on membranes, the soluble fraction may have little activity.

Our studies show that vacuolar DAG is generated from PI(4,5)P\textsubscript{2} by PLC activity, presumably including Plc1p, and is consumed by an ATP-dependent activity. DAG is needed for vacuole fusion. Each of the regulatory lipids (DAG, PI(3)P, PI(4,5)P\textsubscript{2}, and ergosterol) is interdependent with each other and with proteins (Ypt/Rab Ypt7p, the HOPS complex, and SNAREs) for the assembly of the vertex ring microdomain around the apposed membranes of docked vacuoles. DAG may contribute to the enrichment of Ypt7p, PI(3)P, and SNAREs such as Vam3p, Vti1p, and Nyv1p in vertex rings, allowing the vacuole re-association of soluble Vam7p and its incorporation into SNARE complexes. trans-SNARE pairing may trigger hemifusion, and the hemifusion intermediate may be resolved by the opening and expansion of fusion pore(s) around the vertex ring. As a lipid of negative curvature, DAG may also contribute to the formation of stalk and hemifusion intermediates. There is extensive documentation of the capacity of even a few molar percent of DAG to promote fusion in model membranes and of the promotion of fusion by PLC-mediated generation of DAG (21). Our present studies establish DAG and PLC as essential for the fusion of vacuoles, along with other lipids and with Rab GTPases, their effectors, and SNAREs.

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\[ \text{3 Y. Jun and W. Wickner, unpublished data.} \]
