The Fab1/PIKfyve Phosphoinositide Phosphate Kinase Is Not Necessary to Maintain the pH of Lysosomes and of the Yeast Vacuole

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**Background:** Based on qualitative pH probes, loss of Fab1/PIKfyve was thought to impair vacuolar/lysosomal acidification.

**Results:** Using several quantitative assays, vacuoles/lysosomes remained acidic in Fab1/PIKfyve-inhibited cells in a V-ATPase-dependent manner.

**Conclusion:** Fab1/PIKfyve is not necessary to maintain the steady-state vacuolar/lysosomal pH.

**Significance:** Contrary to current thought, we propose a model in which Fab1/PIKfyve is dispensable to maintain vacuolar/lysosomal acidification.

Lysosomes and the yeast vacuole are degradative and acidic organelles. Phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P\textsubscript{2}), a master architect of endolysosome and vacuole identity, is thought to be necessary for vacuolar acidification in yeast. There is also evidence that PtdIns(3,5)P\textsubscript{2} may play a role in lysosomal acidification in higher eukaryotes. Nevertheless, these conclusions rely on qualitative assays of lysosome/vacuole pH. For example, quinacrine, an acidotropic fluorescent base, does not accumulate in the vacuoles of \textit{fab1} yeast. Fab1, along with its mammalian ortholog PIKfyve, is the lipid kinase responsible for synthesizing PtdIns(3,5)P\textsubscript{2}. In this study, we employed several assays that quantitatively assessed the lysosomal and vacuolar pH in PtdIns(3,5)P\textsubscript{2}-depleted cells. Using ratiometric imaging, we conclude that lysosomes retain a pH < 5 in PIKfyve-inhibited mammalian cells. In addition, quantitative fluorescence microscopy of vacuole-targeted pHluorin, a pH-sensitive GFP variant, indicates that \textit{fab1}\textsubscript{Δ} vacuoles are as acidic as wild-type yeast. Importantly, we also employed fluorimetry of vacuoles loaded with DCFDA, a pH-sensitive dye, to show that both wild-type and \textit{fab1}\textsubscript{Δ} vacuoles have a pH < 5.0. In comparison, the vacuolar pH of the V-ATPase mutant \textit{vph1}\textsubscript{Δ} or \textit{vph1}\textsubscript{Δ} \textit{fab1}\textsubscript{Δ} double mutant was 6.1. Although the steady-state vacuolar pH is not affected by PtdIns(3,5)P\textsubscript{2} depletion, it may have a role in stabilizing the vacuolar pH during salt shock. Overall, we propose a model in which PtdIns(3,5)P\textsubscript{2} does not govern the steady-state pH of vacuoles or lysosomes.

Lysosomes are a degradative powerhouse in cells; they are enriched in the hydrolytic enzymes that digest nutrients, cellular debris, and even engulfed pathogens (1, 2). In addition, numerous channel and transporter proteins are embedded in the lysosomal membrane that control the transport of small molecules like amino acids, sugars, and ions into and out of the lysosome (1, 3–5). Hence, lysosome dysfunction can build up debris and impair cell metabolism, leading to a variety of lysosomal storage diseases (3). The yeast vacuole is the analogue of the lysosome, serving as the main degradative storage and detoxification compartment in yeast (6). Because of its functional similarities to the lysosome and its easy manipulation, the yeast vacuole is an excellent model system for studying lysosomal regulation and function.

Both lysosomes and vacuoles are highly acidic, which is crucial for proper lysosomal/vacuolar functions. The acidic pH is required for the delivery and activation of hydrolytic enzymes in the lysosome (6, 7). In addition, the H\textsuperscript{+} electrochemical gradient across the lysosomal membrane drives the transport of amino acids, ions, and metals into and out the lysosome/vacuolar lumen (5, 8, 9). This H\textsuperscript{+} gradient is achieved by the vacuolar-type H\textsuperscript{+}-ATPase (V-ATPase), a highly conserved multisubunit enzyme that pumps H\textsuperscript{+} from the cytosol into the vacuolar lumen by coupling to ATP hydrolysis. The V-ATPase consists of a peripheral (V\textsubscript{p}) complex, the site of ATP hydrolysis, and an integral (V\textsubscript{o}) complex that forms the proton pore (10–12). The mammalian subunit \textit{α} (Vph1p in yeast), a subunit of the V\textsubscript{o} complex, is important for connecting the V\textsubscript{p} and V\textsubscript{o} on the membrane (12, 13). ATP hydrolysis by the V\textsubscript{o} complex drives the rotation of the V\textsubscript{o} complex and allows the translocation of protons across the vacuolar membrane (13–17).

The V-ATPase is regulated by a number of different mechanisms, one of which is believed to be phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P\textsubscript{2})\textsuperscript{3} (18, 19). PtdIns(3,5)P\textsubscript{2} is a low abundance phosphoinositide found mainly on the vacuolar membrane in yeast and on endolysosomes in higher eukaryotes.

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\textsuperscript{3}The abbreviations used are: PtdIns(3,5)P\textsubscript{2}, phosphatidylinositol 3,5-bisphosphate; ANOVA, analysis of variance; cDCFDA, 5-(and-6)-carboxy-2′,7′-di-chlorofluorescein diacetate; CCCP, carbonyl cyanide m-chlorophenylhydrazone; CMAC, 7-amino-4-chloromethylcoumarin; ConA, concanamycin A; DMSO, dimethyl sulfoxide; ILV, intraluminal vesicle; V-ATPase, vacuolar-type H\textsuperscript{+}-ATPase.
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(18, 20, 21). PtdIns(3,5)P$_2$ is involved in a variety of cellular functions including controlling lysosome/vacuole size, membrane recycling, and ion transport (22–25).

Deletion of genes involved in the synthesis of PtdIns(3,5)P$_2$, including FAB1, which encodes the phosphatidylinositol 3-phosphate 5-kinase, impairs the vacuolar accumulation of quinacrine, a fluorescence base that generally accumulates in acidic compartments (25–28). This led to the belief that PtdIns(3,5)P$_2$ is required to maintain an acidic vacuolar lumen. In fact, recently, PtdIns(3,5)P$_2$ was suggested to bind to the V$_0$ complex to stabilize the assembly of the V-ATPase and membrane recycling, and ion transport (22–25).

In this study, we measured the lysosomal and vacuolar pH to better understand the role of PtdIns(3,5)P$_2$ in lysosome/vacuolar acidification. To determine the vacuolar pH, we employed the pH-sensitive fluorescent dye 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate (cDCFDA), and super-ecliptic pHluorin. cDCFDA was used previously to measure the vacuolar pH in Cryptococcus neoformans by fluorimetry (33). In addition, the super-ecliptic pHluorin, a pH-sensitive GFP variant, can be targeted to the vacuolar lumen to query the pH status of yeast vacuoles (34). We also utilized ratiometric imaging of lysosomes labeled with FITC-dextran to quantitate the lysosomal pH (35). Using these methods, we revealed that yeast vacuoles lacking PtdIns(3,5)P$_2$ were as acidic as wild-type cells. Similarly, both control and PIKfyve-abated mammalian cells exhibited similarly acidic lysosomes.

EXPERIMENTAL PROCEDURES

Media and Reagents—7-Amino-4-chloromethylcoumarin (CMAC), cDCFDA, FITC-dextran, LysoTracker DND-99, fetal bovine serum, Hanks’ balanced salt solution, and DMEM were purchased from Life Technologies. Quinacrine, nigericin, and monensin were purchased from Sigma-Aldrich. Yeast media and nutrients were from Biobasic (Toronto, Ontario, Canada). Concanamycin A, concanavalin A, and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were purchased from BioShop (Burlington, Ontario, Canada). Apillimod was purchased from Toronto Research Chemicals. MF4 was a kind gift from Dr. Kevan Shokat (UCSF).

Yeast Strains—The strains used in this study are listed in Table 1. We employed the PCR-based gene deletion method described in Ref. 36, using F1 and R1 primer plasmid-specific sequences, to generate vph1Δ in the SEY6210 background. DNA for gene deletions was generated using the Phusion high-fidelity DNA polymerase (New England Biolabs). Yeast transformation was performed according to standard procedures using the lithium acetate method, and homologous recombination was confirmed by PCR. Mating was used to generate fab1Δ vph1Δ in the SEY6210 background, and fab1Δ Mup1-pHluorin and vph1Δ Mup1-pHluorin.

TABLE 1

| S. cerevisiae strains employed in this study | Strain name | Genotype | Source |
|-------------------------------------------|-------------|----------|--------|
| SEY6210 | MATa his3Δ200 trplΔ S. Emr |   |
| SEY6211 | MATa leu2Δ-3,112 ura3Δ-52 lys2Δ-801 trplΔ S. Emr |   |
| fab1Δ | SEY6210; fab1ΔΔ H9262 |   |
| SRY13 | SEY6210; att89A-HIS3 | S. Emr |
| JG145 | SEY6210; vac1ΔΔ-TRP1 | S. Emr |
| SHY4 | SEY6210; vph1ΔΔ TRP1 | This study |
| SHY1 | SEY6210; fab1ΔΔ H9262 | This study |
| BWT3818 | SEY6210; Mup1-pHluorin::KAN | B. Wendland |
| SHY2 | SEY6210; fab1ΔΔ H9262 | This study |
| Mup1-pHluorin::KAN |  |
| SHY3 | SEY6210; vph1ΔΔ TRP1 | This study |
| Mup1-pHluorin::KAN |  |
| BY4741 | Mat a fab1ΔΔ leu2ΔΔ met15Δ0 | B. Andrews |
| ura3Δ0 |  |
| BY4741 | Mat a fab1ΔΔ leu2ΔΔ met15Δ0 | B. Andrews |
| ura3Δ0 met15Δ0 |  |

Cell Culture—The RAW264.7 (RAW) macrophage-like cell line was maintained in DMEM supplemented with 5% fetal bovine serum (Wisent Inc., Quebec, Canada) at 5% CO$_2$ and 37 °C.

Vacuole Staining and Hyperosmotic Shock—Yeast cultures were grown overnight in synthetic complete (SC) medium (yeast nitrogen base without nitrogen and ammonium sulfate, sodium glutamate, and 2% glucose supplemented with appropriate amino acids) to an A$_{600}$ of ~0.6. Cells were washed and resuspended in SC medium buffered to pH 7.5. Vacuoles were stained with 200 μM quinacrine for 10 min at 26 °C in 100 mM Hepes-KOH, pH 7.5. Cells were washed and resuspended in 25 μl of ice-cold 100 mM Hepes-KOH, pH 7.6, with 2% glucose. For CMAC staining, vacuoles were labeled with 100 μM CMAC in the dark for 15 min at room temperature in SC medium. Cells were washed and resuspended in 25 μl of SC medium. For cDCFDA staining, vacuoles were labeled with 50 μM cDCFDA for 1 h at 26 °C, washed, and resuspended in SC medium, pH 7.5. For osmotic shock, after cDCFDA staining, cells were exposed to 0.9 M NaCl for 10 min followed by fluorimetric measurement immediately after and for every 5 min over a period of 25 min.

Fluorimetry—About 10 ml of yeast cells was continuously grown in SC medium to A$_{600}$ of 0.6. About 2 million cells were then incubated in 100 μl of yeast calibration medium (50 mM MES, 50 mM Hepes, 50 mM KCl, 50 mM NaCl, 0.2 mM ammonium acetate, 10 mM Na$_2$PO$_4$, and 10 mM 2-deoxyglucose, pH 7.5) for 4 min without CCCP to obtain the basal fluorescence intensity. Subsequently, individual aliquots of 2 million cells were incubated for 4 min in yeast calibration medium containing 50 μM CCCP and set to pH 4.5–7.5. Fluorescence intensity was measured immediately. When necessary, cells were treated with 1 μM concanamycin A (ConA) for 1 h before calibration. Fluorescence intensity was measured with a fluorimeter (Hitachi F-2500) using an excitation scan between 400 and 520 nm and emission at 535 nm. The samples were measured in triplicate. Importantly, this assay is independent of absolute fluorescence intensity because each sample is internally calibrated; in other words, the basal fluorescence and calibration curves for each
condition are all derived from a single population of cells labeled with cDCFDA. GraphPad Prism 6 software was used to calculate a calibration curve as a function of the measured values relative to the pH values of the calibration buffer. The data were then fitted to a sigmoidal dose-response curve for each sample. Statistical analysis is based on a minimum of three independent experiments.

Microscopy—Fluorescence, bright field, or differential interference contrast images of quinacrine, CMAC, and cDCFDA-labeled cells were obtained with a DM5000X Leica epifluorescence microscope. Time lapse imaging was captured using an Olympus IX81 quorum spinning disk microscope with PerkinElmer Volocity software. For ratiometric imaging, images were captured using a DM-IRB Leica epifluorescence microscope with MetaFluor software (MDS Analytical Technologies). The fluorescence intensity of FITC-labeled lysosomes and Mup1-pHluorin-labeled vacuoles was analyzed with ImageJ 1.47v.

Mup1-pHluorin and Imaging Analysis—Yeast culture were grown overnight in synthetic medium deficient in methionine to an A600 of ~0.5. Methionine was added to the yeast culture at a concentration of 20 μg/mL and incubated for 2 h at 26 °C. Cells were then washed and resuspended in SC medium buffered to pH 7.5. Cells were seeded onto concanavalin A-coated glass coverslips for microscopy or ratiometric imaging analysis. Fluorescence intensity was measured until the signal was stable, and then 50 μM CCCP was added to the samples. The cells were allowed 10 min to equilibrate to media at pH 7.5. The fluorescence intensity was again measured until the signal was stable. Statistical analysis is based on 300–500 cells over a minimum of three independent experiments.

Lysosomal Labeling—RAW cells were grown on glass cover-slips in DMEM supplemented with 10% fetal bovine serum and 5% CO2 at 37 °C. Cells were pulsed with 2 mg/mL FITC-dextran for 1 h and chased for 1 h at 37 °C. Cells were then treated with 200 nM M4, 20 nM apilimod, 1 μM concanamycin A, or an equivalent volume of DMSO for 10 min. Pellets were washed with 1 ml of ice cold 0.1 M EDTA and resuspended in 50 μL of 4.5% perchloric acid (v/v) on ice for 15 min, scraped, and pelleted at 12,000 × g for 10 min. Pellets were washed with 1 mL of ice cold 0.1 M EDTA and resuspended in 50 μL of water. Phospholipids were deacylated with 500 μL of methanol/40% methylamine/1-butanol (45.7% methanol:10.7% methylamine:11.4% 1-butanol (v/v)) for 50 min at 53 °C. Samples were vacuum-dried and washed twice by resuspending them in 300 μL of water and drying. The dried samples were then resuspended in 450 μL of water, extracted with 300 μL of 1-butanol/ethyl ether/ethyl formate (20:4:1), vortexed for 5 min, and centrifuged at 12,000 × g for 2 min. The bottom aqueous layer was collected and extracted twice more. The aqueous layer was vacuum-dried and resuspended in 50 μL of water. Equal counts of 3H were separated by HPLC (Agilent Technologies) through an anion exchange 4.6 × 250-mm column (Phenomenex) with a flow rate of 1 mL/min and subjected to a gradient of water (buffer A) and 1 M (NH4)2HPO4, pH 3.8 (adjusted with phosphoric acid) (buffer B) as follows: 0% B for 5 min, 0 to 2% B for 15 min, 2% B for 80 min, 2 to 10% B for 20 min, 10% B for 30 min, 10 to 80% B for 10 min, 80% B for 5 min, and 80 to 0% B for 5 min. The radiolabeled eluate was detected by β-RAM 4 (LabLogic) with a 1:2 ratio of eluate to scintillant (LabLogic) and analyzed using Laura 4 software. Each of the phosphoinositides was normalized against the parent phosphatidylinositol peak.

Statistical Analysis—Experimental values are given as the mean of a minimum of three independent experiments and include standard error of the mean (S.E.). The population size is indicated in the text or figure legends. Comparisons between groups were made by Student’s t test or using an ANOVA test followed by Tukey’s post hoc test as appropriate.

RESULTS

Lysosomes Remain Acidic in PIKfyve-inhibited Cells—Lysosomes depend on their highly acidic milieu for optimal degradative capacity and to drive molecular transport across its membrane. Therefore, it is important to understand the mechanisms that establish and maintain lysosomal acidification. The role of PtdIns(3,5)P2 in controlling lysosomal acidification in mammalian cells remains unclear.

To better address this issue, we employed RAW macrophages as a model cell line given the importance of lysosomes in eliminating pathogens. RAW macrophages were treated for 1 h with 20 nM apilimod, a potent PIKfyve antagonist (38). Importantly, we limited PIKfyve inhibition to 1 h to avoid any non-specific, indirect effects of prolonged PIKfyve abatement. First, we used myo-[2-3H]inositol labeling and HPLC-coupled flow...
scintillation to show that 20 nM apilimod treatment was sufficient to cause a ~80% reduction in PtdIns(3,5)P₂ levels relative to control cells and a concurrent increase in PtdIns(3)P, consistent with previous work (Fig. 1A and Ref. 38). The loss of PtdIns(3,5)P₂ coincided with extensive vacuolation as observed previously (Fig. 1B and Ref. 39).

Subsequently, cells were exposed to LysoTracker to label acidic compartments. In control cells, LysoTracker labeled punctate structures (Fig. 1B and supplemental Movie S1). In contrast, LysoTracker decorated the limiting membrane of vacuoles induced by PIKfyve inhibition (Fig. 1C). In addition, LysoTracker also associated with what appeared to be intraluminal vesicles (ILVs) within the swollen lysosomes (Fig. 1C). These ILVs moved freely within the vacuoles as depicted by live cell, time-resolved imaging (Fig. 1C, arrowheads, and supplemental Movie S2). Notably, swollen lysosomes were never homogeneously filled with LysoTracker. We observed similar results when cells were treated with 200 nM MF4, a distinct PIKfyve antagonist (30) (data not shown). Overall, our data suggest that swollen lysosomes in PIKfyve-inhibited cells are still acidic.

Because LysoTracker provides only a qualitative indication of lysosomal pH, we used FITC, a ratiometric pH-sensitive fluorochrome, to quantitatively measure lysosomal pH by ratiometric imaging (35). Lysosomes were labeled by pinocytosis of FITC-labeled dextran. Because PtdIns(3,5)P₂ may be involved in vesicular trafficking to lysosomes (40, 41), we first labeled lysosomes with FITC-dextran before treatment with MF4 or apilimod. Cells were then imaged and analyzed by ratiometric
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Mup1-pHluorin Suggests That fab1Δ Vacuoles Are Acidic—We next examined the pH status of the enlarged vacuoles in fab1Δ yeast strains. We expected a defect in acidification given that past reports showed that quinacrine does not accumulate in the vacuoles of PtdIns(3,5)P2-deficient yeast (27, 28). We first employed the super-eclipitic pHluorin, a GFP variant in which fluorescence is potently quenched at low pH (34). pHluorin can be targeted to the lumen of yeast vacuoles by fusing it to Mup1 (34). Mup1 is a methionine transporter that localizes to the plasma membrane under low levels of methionine (34). However, excess extracellular methionine triggers the endocytosis of Mup1 followed by sorting into multivesicular bodies and trafficking to the vacuole for degradation (42). Consequently, by using the Mup1-pHluorin chimera, pHluorin accumulates in the vacuole in the presence of methionine (Ref. 34 and Fig. 3A).

To observe the pH status of vacuoles in PtdIns3,5P2-deficient yeast, fab1Δ Mup1-pHluorin and vph1Δ Mup1-pHluorin were generated. In the wild type, fab1Δ, and vph1Δ, there was a dim pHluorin-associated fluorescence signal in CMAC-labeled vacuoles, suggesting that pHluorin was being quenched by acidification (Fig. 3A). To better evaluate the vacuolar pH in these strains using pHluorin, we then measured its fluorescence intensity before and after alkalinization with the proton ionophore CCCP in media at pH 7.5. CCCP quickly dissipates the pH gradient and forces the vacuolar pH to equilibrate to the pH of the medium (43, 44). Wild-type, fab1Δ, and vph1Δ cells all exhibited an increase in the fluorescence intensity of vacuole-targeted pHluorin after alkalinization (Fig. 3A). But strikingly, when we quantified the ratio of fluorescence intensity after and before alkalinization, both wild type and fab1Δ showed similar-fold increases in fluorescence intensity, which were not significantly different from each other (6.1 ± 1.9 and 5.5 ± 0.67, respectively). By contrast, there was only a 3.4 ± 0.63-fold increase in pHluorin-based fluorescence in vph1Δ vacuoles (Fig. 3B). These data convey that fab1Δ and wild-type vacuoles are similarly acidic, whereas vph1Δ vacuoles are more alkaline.

Accumulation of Quinacrine and cDCFFDA in Yeast Vacuoles—The Mup1-pHluorin assay produced an unexpected result, that is, fab1Δ and wild-type vacuoles appear to be similarly acidic. To ensure that our fab1Δ strain was not altered, we stained yeast vacuoles with quinacrine. As stated previously, quinacrine is a fluorescent dye that is thought to accumulate in the vacuole upon protonation. Therefore, quinacrine is often used to report on vacuolar acidification. Previous work had shown that quinacrine fails to accumulate in vacuoles in cells depleted for PtdIns(3,5)P2 (25–28). Consistent with the literature, we confirmed that quinacrine failed to accumulate in vacuoles of fab1Δ, vph1Δ, and of the fab1Δ vph1Δ double mutant stained with CMAC, a vacuolar probe (Fig. 4A). In contrast, wild-type vacuoles, also identified by CMAC, were enriched in quinacrine (Fig. 4A).

To better understand the apparently contradictory data provided by the Mup1-pHluorin and the quinacrine assays, we employed an alternative method to quantitatively measure the vacuolar pH. The pH-sensitive fluorescent base cDCFFDA had been shown previously to accumulate in and to measure the pH...
of vacuoles in *C. neoformans* (33). Therefore, we explored whether cDCFDA would be suitable to measure the pH of vacuoles in *Saccharomyces cerevisiae*. Foremost, we showed here that cDCFDA can accumulate in the vacuoles irrespective of apparent vacuolar acidification; cDCFDA accumulated in vacuoles identified by CMAC staining in wild-type, *fab1Δ*, and *fab1Δ vph1Δ* yeast cells (Fig. 4B).

**Quantitative pH Assays Indicate That *fab1Δ* Vacuoles Are Acidic**—To quantify the vacuolar pH, cells were labeled with cDCFDA in SC medium. From a single pool of labeled cells, an aliquot was used to measure the baseline fluorescence intensity of cDCFDA by fluorimetry in SC medium at pH 7.5, recording the peak fluorescence intensity. After reading the baseline fluorescence, subsequent aliquots from the same culture were then treated with calibration buffer containing CCCP for 4 min to force the vacuolar pH to equilibrate to known media pH (pH 4–7), and the peak fluorescence intensity was recorded (Fig. 5A). These values were used to generate a standard curve of fluorescence intensity versus pH, which was then fit to a sigmoidal curve to extrapolate the baseline vacuolar pH for each strain. This method avoids the need for ratiometric fluorimetry, as each reading is an average of millions of cells and because calibration is internally controlled for each sample, *i.e.* the baseline and calibration readings use the same population of cells.

Importantly, we first assessed the effect of extracellular pH on the vacuole pH measured by cDCFDA fluorimetry by labeling cells in SC medium at pH 5.5 or 7.5 for 1 h. There was no observable difference in the apparent vacuolar pH in wild-type cells labeled in medium at pH 5.5 or 7.5 (data not shown). Therefore, all measurements were done with cells labeled in medium at pH 7.5. Strikingly, the steady-state vacuolar pH in *fab1Δ* cells was comparable to the wild type (pH 4.9 ± 0.1 and 4.9 ± 0.2, respectively, Fig. 5B), whereas a V-ATPase mutant, *vph1Δ*, had a more alkalinized vacuolar pH (pH 6.1 ± 0.2; Fig. 5B), which was significantly different from both the wild-type and *fab1Δ* cells. Importantly, the vacuolar pH of *fab1Δ vph1Δ* double mutant was 6.1 ± 0.2, comparable with *vph1Δ* (Fig. 5B). Because our assay produced similar vacuolar pH values for both the *vph1Δ* and *fab1Δ vph1Δ* strains, this suggests that cDCFDA fluorescence was not being quenched by other factors altered in *fab1Δ* cells that might lead to a falsely acidic reading of *fab1Δ* vacuoles. Lastly, the strains that we used in this study were based on the SEY6210 genetic background. Thus, we also measured the vacuolar pH in the BY4741 background and found that there was no significant difference between the wild type and *fab1Δ* (pH 4.9 ± 0.2 and 5.2 ± 0.4; Fig. 5C). This suggests that the genetic background did not account for the unexpected acidic vacuoles in *fab1Δ* identified by our assays.

We also tested whether the vacuolar pH remained intact upon deletion of *VAC14* and *ATG18*, two key regulators of the PtdIns(3,5)P₂ pathway. In *vac14Δ* cells, active Fab1 protein complexes fail to assemble, reducing PtdIns(3,5)P₂ levels to 10% of the wild type (27, 28, 45, 46). In comparison, *atg18Δ* cells exhibit a 5–10-fold increase in the levels of PtdIns(3,5)P₂ (47). Upon measuring the vacuolar pH, we observed no significant differences in the vacuolar pH between these strains and those of wild-type and *fab1Δ* cells (Fig. 5B). Thus, the steady-state vacuolar pH seems unaffected in cells deficient in or with elevated PtdIns(3,5)P₂ levels.

**The Acidification of *fab1Δ* Vacuoles Requires V-ATPase Activity**—Our data strongly indicate that *fab1Δ* vacuoles are not defective in acidification. It remained possible that vacuole acidity in *fab1Δ* yeast was independent of V-ATPase activity. To test for this possibility we treated *fab1Δ* cells with ConA, a potent V-ATPase antagonist. Importantly, both wild-type and *fab1Δ* cells treated with ConA displayed an alkalinized vacuolar pH (pH 5.8 ± 0.2 and 5.9 ± 0.1, respectively; Fig. 6). This is consistent with our measurements obtained for *vph1* and
Depletion of PtdIns(3,5)P_2 has been thought to alkalinize the yeast vacuole and, in some instances, to alkalinize lysosomes in higher eukaryotes (26, 29, 31, 32, 41). However, these studies employed qualitative assays that did not probe the actual pH of the yeast vacuole and of lysosomes. In contrast, we have provided quantitative evidence that suggests that the luminal pH of mammalian lysosomes and of the yeast vacuole remains acidic in PtdIns(3,5)P_2-deficient cells.

**The Lysosomal pH in PIKfyve-inhibited Mammalian Cells—**

Up to this point, the requirement for PIKfyve in maintaining the acidic lumen of lysosomes in higher eukaryotes has remained unclear. For instance, there is no apparent difference in acridine orange accumulation between control and MF4-treated mammalian cells (30). In contrast, others have suggested that cells treated with YM201636, another PIKfyve inhibitor, have reduced LysoTracker accumulation in swollen lysosomes (31).

Here, we show that LysoTracker does indeed decorate swollen lysosomes, indicating that they are still acidic. However, LysoTracker fluorescence was not present throughout the lumen of the swollen lysosomes, as might be expected. Instead, LysoTracker adhered to ILVs within and with the limiting membrane of swollen lysosomes. Others have reported this LysoTracker behavior in PIKfyve-inhibited RAW cells, although they did not emphasize this effect. Although we cannot rule out that this LysoTracker behavior is specific to PIKfyve-inhibited cells, we speculate that LysoTracker also adheres to ILVs and to the limiting membrane of lysosomes in control cells; however, this is not resolvable because “normal” lysosomes are difficult to distinguish when observed by light microscopy. We also speculate that because LysoTracker does not decorate the luminal space of swollen lysosomes, this may lead to a misinterpretation of the acidic state of lysosomes in PIKfyve-inhibited cells.

**DISCUSSION**

Recently, it was shown that PtdIns(3,5)P_2 stimulates V-ATPase assembly during hyperosmotic shock, although the authors did not measurevacuolar pH (29). Salt shock is known to cause a 5–10-fold increase in PtdIns(3,5)P_2 levels (20). Thus, we next tested whether salt shock might disturb the vacuolar pH in a Fab1-dependent manner. First, we observed a mild alkalinization of the vacuoles in both wild-type and fab1Δ cells exposed to 0.9 M NaCl over a period of 25 min (Fig. 6D). Interestingly, this alkalinization was not significant until 10 min post-salt shock in wild-type cells (Fig. 6D). By contrast, fab1Δ cells suffered a greater rate of alkalinization induced by salt shock, becoming significantly different immediately after the hyperosmotic stress (Fig. 6D). Thus, it appears that salt shock can disturb the vacuole pH and that PtdIns(3,5)P_2-deficient cells are more prone to this effect than wild-type cells. This is supportive of the notion that PtdIns(3,5)P_2 may play a role in stabilizing the V-ATPase during salt shock, as suggested previously (29).

Overall, using several quantitative assays, we report here that PIKfyve and Fab1 inactivation does not cause a defect in the steady-state pH of lysosomes and yeast vacuoles, respectively. This conclusion suggests that the role of PtdIns(3,5)P_2 in vacuolar acidification is more nuanced than thought previously.
Either way, acidotropic dyes like LysoTracker and acridine orange do not quantitatively assess pH. To resolve this caveat, we employed ratiometric imaging, which provides accurate and absolute pH values. Thus, using ratiometric imaging, we conclusively showed that lysosomes in control and PIKfyve-hindered cells retained a pH level of 4.8 to 4.9.

Overall, our results best support a model in which PIKfyve does not play a direct role in maintaining the acidic pH of mammalian lysosomes. Nevertheless, there are a few caveats to our study. First, it remains possible that chronic loss of PIKfyve (as in a gene knock-out of PIKfyve) may indirectly lead to less acidic lysosomes. However, there is no significant difference in this ratio between wild-type and fab1Δ cells. Second, drug-treated mammalian cells retain small levels of PtdIns(3,5)P2 that may suffice to maintain acidic lysosomes. Nevertheless, there are a few caveats to our study. First, it remains possible that chronic loss of PIKfyve (as in a gene knock-out of PIKfyve) may indirectly lead to less acidic lysosomes. However, there is no significant difference in this ratio between wild-type and fab1Δ cells. Second, drug-treated mammalian cells retain small levels of PtdIns(3,5)P2 that may suffice to maintain acidic lysosomes. Yet, at these low levels of PtdIns(3,5)P2, many other defects are observed including lysosome swelling, phagosome maturation arrest, cytokine production impairment, and trafficking inhibition (38, 39, 51). Third, we do not exclude the possibility that extremely enlarged lysosomes may be more prone to alkalinization, as suggested elsewhere (31). Lastly, our observations may not apply to all mammalian cells or eukaryotic models.

The Vascular pH in fab1Δ Yeast Cells—Yeast vacuoles depleted of PtdIns(3,5)P2 fail to accumulate quinacrine (25–27), leading to the conclusion that these vacuoles are not acidic. However, our data suggest otherwise. First, we targeted the super-ecliptic pHluorin to the lumen of vacuoles. Both the wild-type and fab1Δ vacuoles displayed dim pHluorin-associated fluorescence after the addition of methionine. Strikingly, forced equilibration of the vacuole to pH 7.5 caused a similar large increase in fluorescence signal in control and fab1Δ vacuoles. This suggests that the fab1Δ vacuoles were as acidic as the wild-type cells. In contrast, vph1Δ cells exhibited a lesser increase in pHluorin fluorescence, consistent with a more alkaline vacuolar pH in these cells. Second, we employed cDCFDA-based fluorimetry to measure the vacuolar pH in S. cerevisiae by modifying a method used to quantify the vacuolar pH in C. neoformans (33). Consistent with the Mup1p-pHluorin assay, the vacuolar pH of wild-type and fab1Δ cells was indistinguishable at pH 4.9. In addition, different levels of PtdIns(3,5)P2 present in fab1Δ, vac1Δ, and atg18Δ showed no effect on vacuolar pH.

The validity of these results is supported by two key observations: (i) the cDCFDA test reported a pH above 6 for both vph1Δ and fab1Δ vph1Δ mutants, making it unlikely that cDCFDA was reporting the behavior of another factor, like Ca2+, that might be altered in fab1Δ vacuoles and that might mask an alkaline vacuolar pH; and (ii) both wild-type and fab1Δ vacuoles became alkaline after V-ATPase inhibition. This result is also consistent with the fact that fab1Δ cells do not exhibit a vma phenotype, defined by the inability of V-ATPase mutants (vma) to grow in media with high CaCl2 and high pH (48).

Overall, we present our data as evidence that fab1Δ vacuoles are as acidic as wild-type cells.

Recent elegant work indicates that PtdIns(3,5)P2 binds to the V-ATPase and helps to stabilize the V1-V0 assembly in vitro and in vivo (29). In addition, PtdIns(3,5)P2 appears to stimulate the ATPase activity of the V-ATPase using in vitro assays (29). However, it is important to note that the authors did not measure the actual pH of the vacuoles. Although seemingly contradictory, we believe these observations are not mutually

FIGURE 6. Basal vacuolar acidification in PtdIns(3,5)P2-deficient cells is dependent on V-ATPase activity. A, the vacuolar pH for cells labeled with cDCFDA and treated with 1 μM ConA or vector alone (DMSO) for 1 h. For each condition, three different independent experiments were performed. The mean ± S.E. is shown. Using Student’s t test, there was a significant difference (*) between control (DMSO) and ConA-treated cells for either wild-type or fab1Δ cells (p < 0.01). B, overnight cultures grown in normal SC medium were cut back to 600 was measured each hour up to 8 h. Shown is a representative growth trace. C, the ratio between the doubling time of cells grown at pH 5.5 versus 7.5 from two independent experiments, each with three replicates. There was no significant difference in this ratio between wild-type and fab1Δ cells. D, the calibrated vacuolar pH for wild-type (n = 3) and fab1Δ (n = 3) cells before (−15 and −10 min) and after a 10-min exposure to 0.9 M NaCl (0 min) followed by pH measurements over 25 min at 5-min intervals. Using two-way ANOVA and Tukey’s post hoc test, there was a significant difference (⁎) relative to the resting pH (−15 and −10 min) in the vacuolar pH in fab1Δ starting at 0 min and for the wild type 10 min after salt exposure (p < 0.05).
exclusive with our data. First, we speculate that the vacuolar membrane is equipped with a pH-sensing protein that adjusts H⁺ efflux to offset reduced H⁺ influx, if indeed the V-ATPase is less active in the absence of PtdIns(3,5)P₂. Although the identity of such a sensor in the lysosomal/vacuolar membrane is not known, there are examples of pH-sensing proteins in other systems; the activity of NHE3, an epithelial sodium/proton exchanger, is responsive to cytosolic pH (52, 53); Rim21 appears to sense external pH to modulate the Rim101 pathway, responsible for yeast adaptation to environmental pH (54, 55); even the V-ATPase may sense luminal endosomal pH to control Arf6 and ARNO membrane recruitment (56, 57). Second, a dearth of PtdIns(3,5)P₂ may down-regulate specific channel or/and transporter activity that might reduce H⁺ efflux; as a precedent, PtdIns(3,5)P₂ gates TRPML1, a lysosomal Ca²⁺ channel (24). Third, PtdIns(3,5)P₂ may still control V-ATPase activity in response to a specific stimuli but be dispensable for steady-state conditions (29). Indeed, fab1Δ cells appeared to alkalinize more quickly than wild-type cells upon salt shock, consistent with this notion (Fig. 6D).

Lastly, despite an acidic vacuolar pH, quinacrine did not accumulate in fab1Δ vacuoles. There are at least two possibilities that might explain why quinacrine does not decorate fab1Δ vacuoles. First, quinacrine may require an unknown PtdIns(3,5)P₂-dependent factor to enter or stay in the vacuole. For instance, quinacrine is known to bind ATP and other nucleotides and has been used to label ATP-rich vesicles (58, 59). Second, both quinacrine and chloroquine are weak bases that have been used to treat malaria, and in fact, chloroquine is often used to dissipate lysosomal pH (49, 60–62). Given that quinacrine is a weak base, we speculate that fab1Δ vacuoles may be more sensitive to quinacrine-mediated alkalinization than wild-type vacuoles, perhaps mimicking other stress conditions like hyperosmotic shock.

Summary—Overall, the work presented here makes use of potent quantitative tools including ratiometric imaging, fluorimetry, and quantitative fluorescence microscopy to show that mammalian lysosomes and yeast vacuoles retain a pH < 5.0 in cells depleted of PtdIns(3,5)P₂, which is indistinguishable from control cells. We also showed that this is dependent on the V-ATPase. We argue that PtdIns(3,5)P₂ has no role in main-

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