Dynamic association of the PI3P-interacting Mon1-Ccz1 GEF with vacuoles is controlled through its phosphorylation by the type 1 casein kinase Yck3

Gus Lawrence*a, Christopher C. Brown*a, Blake A. Flood*a, Surya Karunakaran*a, Margarita Cabrera*b, Mirjana Nordmann*b, Christian Ungermann*b, and Rutilio A. Frattia

ABSTRACT Maturation of organelles in the endolysosomal pathway requires exchange of the early endosomal GTPase Rab5/Vps21 for the late endosomal Rab7/Ypt7. The Rab exchange depends on the guanine nucleotide exchange factor activity of the Mon1-Ccz1 heterodimer for Ypt7. Here we investigate vacuole binding and recycling of Mon1-Ccz1. We find that Mon1-Ccz1 is absent on vacuoles lacking the phosphatidic acid phosphatase Pah1, which also lack Ypt7, the phosphatidylinositol 3-kinase Vps34, and the lipid phosphatidylinositol 3-phosphate (PI3P). Interaction of Mon1-Ccz1 with wild-type vacuoles requires PI3P, as shown in competition experiments. We also find that Mon1 is released from vacuoles during the fusion reaction and its release requires its phosphorylation by the type 1 casein kinase Yck3. In contrast, Mon1 is retained on vacuoles lacking Yck3 or when Mon1 phosphorylation sites are mutated. Phosphorylation and release of Mon1 is restored with addition of recombinant Yck3. Together the results show that Mon1 is recruited to endosomes and vacuoles by PI3P and, likely after activating Ypt7, is phosphorylated and released from vacuoles for recycling.

INTRODUCTION

Eukaryotic cells are compartmentalized by membrane-bound organelles, leading to the specific localization of proteins and enzymatic reactions. Cargo is packaged into vesicles at donor organelles and transported to acceptor membranes, culminating in a fusion event that is regulated by organelle-specific Rab GTPases, their effectors, and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. It was discovered that organelles can mature from early to late stages, characterized in part by sequential recruitment and activation of Rab proteins (Rivera-Molina and Novick, 2009). In a general model it is now believed that Rab GTPase guanine nucleotide exchange factors (GEFs) regulate Rab delivery to specific membranes (Gerondopoulos et al., 2012). In the endocytic pathway, early endosomes recruit and activate Rab5/Ypt21 through the function of the GEF Vps9/Rabex-5 (Hama et al., 1999; Esters et al., 2001). GTP-bound Rab5 is the active form of the protein and can recruit early endosome-specific effectors, including the tethering factor EEA1 and rabaptin-5 (Stenmark et al., 1995; Christoforidis et al., 1999; McBride et al., 1999). Rabaptin-5 inhibits the GTPase activity of Rab5, prolonging the “on” state of Rab5 (Rybin et al., 1996). In Caenorhabditis elegans, Rab5-positive endosomes recruit SAND-1/Mon1, the GEF for the late endosome and lysosome-specific Rab7. Recruitment of SAND-1 occurs in a phosphatidylinositol 3-phosphate (PI3P)-dependent manner (Poteryaev et al., 2010). SAND-1 physically interacts with the Rab5 GEF Rabex-5, leading to its displacement. Subsequently, Rab5 hydrolyzes GTP to become the inactive GDP-bound form, which is then lost from the endosome. However, the constitutively active Rab5Q79L mutant is resistant to SAND-1-mediated inactivation, indicating that...
Rab5 must hydrolyze GTP before it is removed from endosomes. Others have shown that SAND-1 physically interacts with Rab7, leading to its recruitment to maturing phagosomes (Kinchen and Ravichandran, 2010). In addition, mutations in SAND-1, Ccz1, or Rab7 lead to an arrested maturation characterized by the retention of high levels of Rab5 and PI3P on phagosomes containing apoptotic corpses.

In Saccharomyces cerevisiae the Ypt7 GEF was initially believed to be the Vps39 subunit of the heterohexameric homotypic fusion and vacuole protein-sorting (HOPS) complex (Wurmser et al., 2000; Ostrowicz et al., 2010). However, Rytka and colleagues later reported that Ypt7 GEF activity was controlled by the protein Ccz1 (Kucharczyk et al., 2001). More recent studies demonstrated that the Mon1-Ccz1 dimer is the definitive GEF for Ypt7 (Nordmann et al., 2010), thus nicely explaining the intriguing in vivo data obtained in C. elegans (Kinchen and Ravichandran, 2010; Poteryaev et al., 2010). Initially, the Mon1-Ccz1 complex was found to function in various autophagy pathways (Wang et al., 2002). In addition, deletion of either Mon1 or Ccz1 leads to vacuole fragmentation, a hallmark of defective vacuole homeostasis. Vacuoles lacking Mon1 or Ccz1 are blocked in vitro homotypic fusion (Wang et al., 2003). The defect in fusion was mapped to the Ypt7-dependent tethering stage, and deleting Mon1 or Ccz1 prevented the interaction between HOPS and SNAREs. Of interest, it was observed that Mon1 was released from vacuoles through an ATP-dependent mechanism. Furthermore, released Mon1 displayed an electrophoretic mobility shift, as detected by immunoblotting, suggesting that Mon1 undergoes posttranslational modification as part of the release mechanism (Wang et al., 2003).

In a previous study, we showed that deletion of PAH1 blocked the recruitment of Ypt7, the HOPS subunit Vps39, the phosphatidylinositol (PI) 3-kinase Vps34, and its product PI3P to the vacuoles (Sasser et al., 2012). Pah1 is a soluble phosphatidic acid phosphatase that generates diacylglycerol during the fusion reaction. PI3P is produced by Vps34 in vacuolar fractions and plays important roles in the formation of membrane microdomains, SNARE function, and actin dynamics (Schu et al., 1993; Boeddindinghaus et al., 2002; Fratti et al., 2004; Thorngren et al., 2004; Karunakaran et al., 2012). Taking the results together, we hypothesized that Pah1 activity leads to the recruitment of the Mon1-Ccz1 complex and maturation of vacuoles via PI3P, culminating in Ypt7 recruitment. In this study, we show that Mon1-Ccz1 is excluded from pah1Δ vacuoles and that Mon1-Ccz1 associates with vacuoles in a PI3P-dependent manner. Of importance, we find that Mon1 is released from vacuoles after phosphorylation by the casein kinase Yck3 as part of a putative recycling mechanism.

**RESULTS**

**Mon1 and Ccz1 are absent from pah1Δ vacuoles**

In a previous study, we found that deletion of PAH1 causes severe deficiency of multiple factors to vacuole membranes (Sasser et al., 2012). The PI 3-kinase Vps34, the HOPS subunit Vps39, and the Rab GTPase Ypt7 were severely depleted or completely absent from pah1Δ vacuoles. Because Vps39 and Vps34 are linked to the early endosome Rab5/Vps21 and the late endosome/lysosome Rab7/Ypt7, we further investigated the effect of Pah1 on Rab conversion. In C. elegans, the recruitment of the Rab7 GEF SAND-1 to late endosomes required the presence of PI3P (Poteryaev et al., 2010), even though PI3P depletion did not affect Mon1-Ccz1 localization to endosomes in Drosophila tissues (Yousefian et al., 2013). Because pah1Δ vacuoles lack Vps34 and its product PI3P, we examined whether the membrane association of the SAND-1 homologue Mon1 would be altered on these mutant vacuoles. Using isolated vacuoles from wild-type and pah1Δ yeast strains, we found that Mon1 was largely absent from pah1Δ vacuoles (Figure 1A). As expected, the Mon1-interacting Ccz1 was also absent from pah1Δ vacuoles. Probing for Nyv1 served as a loading control.
We next asked whether the addition of purified HOPS or Mon1-Ccz1 would rescue the fusion defect of pah1Δ vacuoles. In Figure 1B, we show in vitro fusion reactions of pah1Δ vacuoles in the presence or absence of purified HOPS and Mon1-Ccz1. We find that Mon1-Ccz1 alone does not affect the fusion of pah1Δ vacuoles. This is likely due to the absence of Ypt7 on isolated vacuoles. However, the addition of purified HOPS to pah1Δ reactions can stimulate fusion at 20-40 nM by twofold. This suggests that HOPS has the ability to tether vacuoles in the absence or at reduced levels of Ypt7. The ability of HOPS to stimulate the fusion of pah1Δ vacuoles is likely due to tethering vacuoles through interactions with SNAREs and other phosphoinositides (Stroupe et al., 2006).

The recruitment of Mon1-Ccz1 to endosomes is believed to lead to inactivation of the early endosome Rab Vps21, followed by recruitment and activation of Ypt7. However, the physical exchange of these two Rabs is difficult to directly observe. Other studies showed that overexpression of BLOC-1, which recruits the Vps21 GTPase-activating protein (GAP) Msb3 to endosomes, does not lead to the loss of the early Rab (John Peter et al., 2013). In addition, deletion of MSB3 causes accumulation of Vps21-GTP and its effector Vps8 at the vacuole along with Ypt7, indicating that loss of Vps21 is not required for acquisition of Ypt7 (Lachmann et al., 2012). Similarly, cells harboring the active Vps21Q66L mutant, which is enriched on vacuoles, causes a shift of Mon1 to the vacuole fraction. Along with the exchange of Rabs, it is believed that class C core vacuole/endosome tethering (CORVET) complex, an early endosome-tethering complex, would be exchanged for the late endosomal–tethering complex HOPS (Peplowska et al., 2007). CORVET and HOPS share a core class C heterotetramer composed of Vps11, Vps16, Vps18, and Vps33. The CORVET complex also contains Vps3 and Vps8 as Rab-binding subunits, whereas HOPS contains Vps39 and Vps41, respectively. Thus we imagined that lack of Mon1 and Ypt7 on pah1Δ vacuoles would be accompanied by accumulation of Vps21 and CORVET subunits. To test this, we examined in vivo localization of fluorescently tagged proteins. In Figure 1C, we show that deletion of PAH1 leads to redistribution of various factors from punctate staining to dispersed cytoplasmic staining. Vps21 and the CORVET subunits Vps3 and Vps8 show decreased punctate staining in pah1Δ cells relative to wild-type yeast, indicating that sorting to endosomes is altered. We originally predicted that pah1Δ vacuoles would be enriched in Vps21 and CORVET subunits. Instead, we find that Vps21 and CORVET are missing from the vacuole and reduced on endosomes. This suggests that Pah1 might play a role throughout the endolysosomal pathway. When GFP-Mon1 distribution was examined in vivo, we observed a decrease in punctate staining, illustrating that the PAH1 deletion had negative effects on endosomes, as well as on vacuoles.

### Mon1 requires PI3P for its retention on vacuoles

Other investigators showed that the Mon1 homologue SAND-1 requires the presence of PI3P for its recruitment to endosomal membranes (Poteryaev et al., 2010). Because pah1Δ vacuoles lack the PI 3-kinase Vps34, as well as newly synthesized PI3P, we hypothesized that the absence of Mon1 from pah1Δ vacuoles was due in part to its inability to bind vacuoles in the absence of PI3P. To examine the role of PI3P in Mon1 binding to vacuoles, we used wild-type vacuoles that produce PI3P and performed release assays using the high-affinity PI3P ligand FYVE or the PI3P 3-phosphatase MTM-1 (Gillooly et al., 2000; Taylor et al., 2000). Fusion reactions were treated with buffer or dose curves of FYVE or MTM-1. After incubation on ice for 5 min, the membranes and soluble fractions were separated by centrifugation and probed by immunoblotting for Mon1. We found that Mon1 was displaced from the vacuole membrane fraction by the FYVE domain in a dose-dependent manner (Figure 2A), suggesting that Mon1 binding required PI3P. Similarly, we found that treatment with MTM-1 caused a dose-dependent release of Mon1 (Figure 2B). We confirmed that our recombinant MTM-1 was active by using a malachite green phosphatase assay as described by others (Carter and Karl, 1982; Maehama et al., 2000; Figure 2C). We next used purified yeast actin to examine whether the release of Mon1 seen earlier was due to the role of PI3P or nonspecific protein excess. We found that adding exogenous actin did not result in the release of Mon1, indicating that the effects of FYVE and MTM-1 were not due to an effect of excess protein in the reaction (Figure 2D). Together these experiments show that like SAND-1 in C. elegans, the yeast homologue Mon1 requires PI3P for stable association with membranes. This is consistent with in vitro studies showing that purified Mon1 binds to liposomes containing PI3P (Cabrera et al., 2014).

In a previous study we found that treating vacuoles with the drug propranolol inhibited fusion by inhibiting the PA phosphatase activity of Pah1 (Sasser et al., 2012). Because deletion of PAH1 resulted in the exclusion of Mon1 (Figure 1A), as well as of Vps34, we tested whether propranolol would lead to an increase in Mon1 release from vacuoles. Figure 2E shows a dose–response curve of propranolol and Mon1 release. These concentrations of propranolol were previously shown to inhibit fusion (Sasser et al., 2012). We observed a significant increase in Mon1 release in a dose-dependent concentration of propranolol. This suggested that newly formed PI3P might stabilize the interaction between Mon1 and the vacuoles. However, propranolol could also have indirect effects that result in Mon1 release independent of PI3P synthesis. Vacuoles acquire PI3P by two modes. PI3P is made on endosomes (Gillooly et al., 2000), which can traffic to the vacuole and transfer the lipid upon fusion. In addition, vacuole-associated Vps34 makes new PI3P on the vacuole surface during the homotypic fusion reaction (Thorngren et al., 2004). To ask whether new rounds of PI3P synthesis play a role in Mon1 association with vacuoles, we treated fusion reactions with the PI 3-kinase inhibitor LY294002. We found that treating vacuoles with LY294002 had a minimal effect on Mon1 retention (Figure 2F), suggesting that the starting levels of PI3P were sufficient for Mon1 vacuole binding. This is consistent with the notion that Mon1 arrives at the vacuole via endolysosomal trafficking. This is also consistent with previous findings showing that inhibiting PI 3-kinase activity with wortmannin had no effect on membrane fusion (Boeddinghaus et al., 2002).

To further examine the role of PI3P in vacuole maturation, we compared the protein profile of vps34Δ and wild-type vacuoles. We found that vps34Δ vacuoles were severely depleted in many components of the fusion machinery, including the SNAREs Vam3 and Vam7, the HOPS subunits Vps11 and Vps18, and Mon1, Ccz1, and Ypt7 (Figure 2G). However, not all proteins were depleted on vps34Δ vacuoles, as shown by the wild-type levels of Sec18 and actin. Although this mutant shared several protein deficiencies versus pah1Δ vacuoles, it should be noted that vps34Δ vacuoles were more severely affected. This is consistent with the fact that pah1Δ was not identified as a vacuolar protein sorting mutant.

### Mon1 is released from the vacuole after phosphorylation

Previously, Klionsky and colleagues found that Mon1 was released from the membrane after the addition of ATP (Wang et al., 2003). To further characterize the observed release reaction, we performed time-course experiments to monitor the release of Mon1 from vacuoles. Fusion reactions were incubated at 27°C from 0 to 60 min, after which the membranes were separated from the supernatant by...
panied the release of Mon1, as expected for the heterodimer. As
accumulated in the supernatant over time (Figure 3A). Ccz1 accom-
 centrifugation. We found that very little Mon1 was present in the
supernatant at the beginning of the fusion reaction, and that Mon1
was a result of its activity during the fusion process and not due to membrane
lysis. Actin served as loading control for both pellet and supernatant fractions.

To identify the requirements for Mon1 release during fusion, we used a panel of
cell-characterized inhibitors that block fu-
sion at various stages of the pathway. We used anti-Sec17 immunoglobulin G (IgG) to
inhibit the priming stage. Ypt7-dependent
tethering was inhibited with anti-Ypt7 IgG or
the GTPase-activating factor Gyp1. Docking
was inhibited with antibodies against the
SNARE Vti1 or the HOPS subunit Vps11. Re-
actions were incubated for 0 or 60 min, and
released Mon1 was collected by centrifuga-
tion. We found that the untreated control
released Mon1 in a time-dependent man-
ner, whereas blocking SNARE priming with
anti-Sec17 reduced Mon1 release between
the two time points (Figure 3B). This sug-
gests that Mon1 release is linked to the start
of the fusion cascade. Blocking Vti1 had lit-
tle effect on Mon1 release, suggesting that
SNARE pairing occurs after Mon1 activity is
completed. Of interest, blocking Ypt7 di-
rectly with antibody or converting it to the
GDP-bound state with Gyp1 accelerated
Mon1 release. We suspect that the antibody
directly inhibited the interaction between
Mon1 and Ypt7. We also found that block-
ing HOPS with anti-Vps11 antibody acceler-
ated release. This was likely due to dispro-
sion of direct interaction between Mon1 and
the HOPS complex (Wang et al., 2003;
Nordmann et al., 2010; Poteryaev et al.,
2010). In fact, the effects of anti-Ypt7 and
Gyp1 could also be linked to disturbing the
HOPS–Mon1 interaction, as previous re-
ports showed that both the antibody and
GAP resulted in the release of HOPS from
the membrane (Eitzen et al., 2000; Price et
al., 2000; Brett et al., 2008).

The nucleotide-binding state of Ypt7
affects Mon1 release
Because Rab GTPases undergo conforma-
tional changes when interacting with GEFs,
we hypothesized that release of Mon1 was
tied to conformational changes in Ypt7
upon binding GTP. To test this, we used
point mutations of Ypt7 that affect GTP/
GDP binding (Eitzen et al., 2000). Based on
conserved residues in the Rab family, the
Ypt7Q68L mutant is believed to be locked in the GDP-bound stage, maintaining an active state. The Ypt7T22N mutant is believed be a
nucleotide empty mutant remaining in the inactive state. We used
reactions containing wild-type (WT) or mutant Ypt7 and examined
Mon1 release by immunoblotting. We hypothesized that Mon1 is

FIGURE 2: Mon1-Ccz1 interacts with vacuolar membranes in a PI3P-dependent manner. Fusion
reactions containing WT vacuoles were incubated with buffer or a dose curve of GST-FYVE (A)
or MTM-1 (B) for 5 min on ice. The reactions were separated into membrane (bound) and
supernatant (released) fractions and resolved by 10% SDS–PAGE. Immunoblot analysis was
carried out using antisera against Mon1. (C) Quantitation of MTM-1 activity using a malachite
green phosphatase activity assay. Calf intestinal phosphatase (CIP) was used as control. (D) As a
control for Mon1 release in the presence of exogenous proteins, fusion reactions were incubated
with a dose curve of purified yeast actin. Mon1 binding was determined by immunoblotting.
Actin served as loading control for the membrane (Eitzen et al., 2000; Brett et al., 2008).

centrifugation. We found that very little Mon1 was present in the
supernatant at the beginning of the fusion reaction, and that Mon1
accumulated in the supernatant over time (Figure 3A). Ccz1 accom-
ppanied the release of Mon1, as expected for the heterodimer. As
controls, we probed for the release of HOPS subunit Vps41, actin,
We also examined the release of Ypt7T22N were nearly devoid of the protein (Figure 4D). This is consistent with the exclusive association of an effector complex such as Ccz1 and the HOPS subunit Vps41 and found that vacuoles containing GTP-Ypt7Q68L by GDP dissociation inhibitor (GDI) and by enhanced vacuole association. This could be due to inhibited delivery of Mon1p distribution was examined as described in A.

Recycled from the vacule after Ypt7 activation and that a block in activation would alter Mon1 membrane release. We found that vacuoles containing Ypt7T22N released more Mon1 relative to WT organelles (Figure 4, A and B). In contrast, vacuoles harboring Ypt7Q68L did not release Mon1 even after 60 min of incubation. These data indicate that release of Mon1 is directly tied to conformational changes that Ypt7 undergoes during nucleotide exchange. Our findings are consistent with previous studies by Horazdovsky and colleagues that showed augmented binding between the inactive Rab5 homologue Vps21S21N and its GEF Vps9 (Hama et al., 1999). These data are also in keeping with the enhanced binding of Rab5S34H with its GEF Rabex-5 (Zhu et al., 2009). Taken together, it is apparent that the conformation of Rab GTPases affects interactions with their respective GEFs. As a control, we probed for the presence of Ypt7 on these vacuoles (Figure 4C). We found that both mutations of Ypt7 resulted in a reduction in vacuole association. This could be due to inhibited delivery of GTP-Ypt7S22N by GDP dissociation inhibitor (GDI) and by enhanced GDI removal of GDP-Ypt7T22N. We also examined the release of the HOPS subunit Vps41 and found that vacuoles containing Ypt7T22N were nearly devoid of the protein (Figure 4D). This is consistent with the exclusive association of an effector complex such as HOPS with the Rab-GTP form (Brocker et al., 2012). Others also showed reduced binding of HOPS subunits with these Ypt7 mutants (Eitzen et al., 2000; Brett et al., 2008) and Ccz1 (Kucharczyk et al., 2001).

Mon1 is phosphorylated during release

It was previously observed that the soluble form of Mon1 migrated more slowly on SDS–PAGE gels relative to the membrane-bound pool of Mon1 (Wang et al., 2003). We also observed a shift in the previous experiments using 10% SDS–PAGE gels and posited that the observed electrophoretic mobility change might be due to protein phosphorylation. To further distinguish the shift in gel migration, we resolved vacuole extracts using 6% SDS–PAGE. Figure 5A shows that a second, higher–molecular weight Mon1 band appeared only in the presence of ATP, suggesting that Mon1 was phosphorylated during the fusion reaction. On the basis of comparisons with the membrane release data presented in Figure 3, we hypothesize that Mon1 modification occurs before membrane release. To test the effect of Mon1 modification on membrane association, we performed membrane fractionation assays and found that the supernatant contained only the upper Mon1 band, indicating that phosphorylation might trigger release from vacuoles (Figure 5B). In addition, release of Mon1 from the membrane only occurred in the presence of ATP, further suggesting that protein modification leads to the dissociation from the vacuole. Of interest, modified Mon1 was also present in the pellet fraction. Thus modification of Mon1 is linked but not sufficient to trigger its release from the membrane, indicating that other factors are involved. To determine whether the phosphorylation shift was truly due to phosphorylation, we treated reactions with either buffer or calf intestinal phosphatase (Figure 5C). We found that using the phosphatase downshifted the higher–molecular weight band.

Previous studies showed that exogenous recombinant Vam7 can bypass a block in SNARE priming by anti-Sec17 IgG (Merz and Wickner, 2004; Thorngren et al., 2004). To determine whether fusion is required for Mon1 phosphorylation, we blocked fusion reactions with anti-Sec17 IgG in the presence or absence of Vam7 for 0 or 60 min. Control reactions show that Mon1 phosphorylation only occurred in the presence of ATP (Figure 5D). The addition of anti-Sec17 in the presence of ATP did not affect phosphorylation of Mon1, indicating that SNARE priming was not required for its modification. The addition of Vam7, which bypasses the anti-Sec17 block to stimulate fusion, did not alter the amount of phosphorylated Mon1.

Yck3p phosphorylates Mon1p

Studies show that the casein kinase Yck3 phosphorylates the fusion regulators Vps41 and Vam3 (LaGrassa and Ungermann, 2005; Brett et al., 2008; Cabrera et al., 2009). Because this kinase is linked to the fusion machinery, we examined whether Yck3 phosphorylated Mon1 during fusion. To this aim, we used vacuoles from yck3Δ yeast and monitored the relative mobility shift of Mon1. We found that Mon1 remained as a single low–molecular weight form on yck3Δ vacuoles (Figure 6A). As a control, we also monitored Vps41 in these experiments and found that it was not modified on yck3Δ vacuoles, which is in keeping with previous studies. In comparison, wild-type vacuoles exhibited phosphorylation of Mon1 and Vps41. To confirm that Yck3 was directly modifying Mon1, we used recombinant hexahistidine (His6)-Yck3 in complementation experiments. Here yck3Δ vacuoles were incubated in fusion reactions that were treated with buffer or exogenous His6-Yck3. We found that adding His6-Yck3 restored the phosphorylation pattern of Mon1 during fusion, indicating that the kinase directly modified Mon1 during fusion (Figure 6B). In addition, we fractionated these reactions and found that Mon1 was not released from yck3Δ vacuoles and that Mon1 release was restored in the presence of His6-Yck3. Together these data strongly indicate that modification and release of Mon1 from vacuoles...
FIGURE 4: The nucleotide-binding state of Ypt7 affected the distribution of Mon1 and binding of HOPS. Fusion reactions were performed using vacuoles that harbor WT, Ypt7Q68L, or Ypt7T22N and incubated for 60 min at 27°C. After incubation the reactions were separated into membrane and soluble fractions as described. Membrane and supernatant fractions were examined by immunoblotting using antibodies against (A, B) Mon1, (C) Ypt7, and (D) Vps41. (A) A representative immunoblot of Mon1 distribution at 0 or 60 min of incubation using the indicated strains. (B–D) Quantitations of three experiments. Data represent mean values ± SEM.

during fusion was due to Yck3 function. It is also important to note that phosphorylated Mon1 was present on the membrane fraction of wild-type vacuoles. This suggests that phosphorylation is not the only cause of membrane release. It is likely that other step(s) occur after Mon1 phosphorylation that leads to the ultimate release of the protein. To examine whether Yck3 directly phosphorylated Mon1, we performed an in vitro assay using purified Mon1 and recombinant Yck3. Figure 6C shows that Yck3 directly phosphorylates purified Mon1 in the absence of other vacuolar components. Note that the amount of in vitro–phosphorylated Mon1 is reduced in comparison to what is seen using whole-vacuolar reactions. One possibility for the difference could be due to conformational changes that occur on the membrane in the presence of Ypt7, HOPS, and PI3P. This is only a hypothesis and will be tested in future studies. Finally, we asked whether inhibiting Ser/Thr kinases during the fusion reaction would alter release of Mon1. We performed fusion reactions in the presence of increasing amounts of staurosporine, a broad-spectrum protein kinase inhibitor. We found that increasing amounts of staurosporine correlated with increased retention of Mon1 on the membrane fraction (Figure 6D). This is in keeping with our other experiments showing that protein phosphorylation is linked to Mon1 release from the vacuole.

To further examine the link between Mon1 phosphorylation and membrane association, we generated Mon1 mutants that eliminated Yck3 phosphorylation sites (NetPhos, www.cbs.dtu.dk/services/NetPhos/; Scansite, http://scansite.mit.edu/). In one strain, the Mon1 residues S35, T38, T39, S130, S135, and S138 were mutated to Ala (Mon1-6A). A second mutant contained phosphomimetic mutations of the same residues changed to Asp (Mon1-6D). Mutations of these sites to Ala or Asp abolished the mobility shift of Mon1 during fusion reaction, which was identical to the effect of deleting YCK3 (Figure 7A). Because the mutations blocked the electrophoretic shifts seen with phosphorylation, we next asked whether membrane release was also affected. These experiments were performed in the presence or absence of ATP and observed after 0 and 60 min. As seen before, deletion of YCK3 blocked Mon1 phosphorylation as well as release (Figure 7B). Similarly, Mon1-6A and Mon1-6D remained associated with the vacuoles (Figure 7, C and D). This further demonstrates that Yck3-dependent phosphorylation is required for the release of Mon1 during the fusion reaction. The lack of release of Mon1-6D likely indicates that these phosphomimetic mutations were insufficient in duplicating the phospho-Mon1 phenotype.

To further characterize the Mon1 mutations, we first examined the stability of Mon1 mutants in heterodimers with Ccz1 and found that there was no change in heterodimer formation (Figure 7E). In addition, we performed fusion experiments in which wild-type vacuoles were treated with Gyp1 to convert Ypt7 to the GDP-bound state and to block fusion. Next we added purified Mon1-Ccz1, Mon1-6A-Ccz1, or Mon1-6D-Ccz1 to reactivate Ypt7 and restore fusion. We found that both Mon1 mutants were able to counteract GAP activity and restored fusion as well as the wild-type heterodimer (Figure 7F). This suggests the phosphorylation of Mon1-Ccz1 does not affect the activity of the complex. Finally, we examined vacuole morphology and found that neither mutation affected the vacuole phenotype (Figure 7G). Together these data further support the notion that Mon1 phosphorylation likely occurs downstream of its activity on Ypt7 to ensure release of Mon1-Ccz1 from vacuoles.

DISCUSSION

Nucleotide exchange of Rab GTPases is an integral part of the progression or maturation of organelles. In the early-to-late endosome maturation pathway, the exchange of early-endosomal Rab5/Vps21 for late-endosomal Rab7/Ypt7 depends on the acquisition of Mon1-Ccz1, a heterodimer that functions as the GEF for Ypt7 (Nordmann et al., 2010). Others have found that recruitment of Mon1/SAND-1 interrupts activation of Rab5, allowing for its removal while Rab7 is recruited to the transitioning endosome (Kinchen and Ravichandran, 2010; Poteryaev et al., 2010; Gerondopoulos et al., 2012). In SAND-1–knockdown cells, Rab5 remains active, leading to enlarged endosomes. In C. elegans this mechanism depends on the regulatory lipid PI3P, and depletion of the lipid leads to inhibition of organelle maturation (Poteryaev et al., 2010). In a previous study we found that deletion of the phosphatidylic acid phosphatase Pah1 causes arrest in vacuole maturation that is characterized by exclusion of Ypt7 (Sasser et al., 2012). This is accompanied by reduction in the PI 3-kinase Vps34 and its product PI3P. In addition, the HOPS subunit Vps39, which is linked to Mon1-Ccz1 GEF activity (Nordmann et al., 2010), is sharply reduced relative to the other five HOPS subunits. Thus we hypothesize that Pah1 creates the environment that is favorable for Mon1-Ccz1 activity.

In this study we examined our hypothesis and monitored the membrane retention of Mon1 during vacuole fusion. In our experiments, we found that the amount of Mon1-Ccz1 was severely reduced on pah1Δ vacuoles, suggesting that Pah1 activity indirectly...
affects Mon1-Ccz1 recruitment to vacuoles. This further suggests that Pah1 indirectly interferes with Rab conversion and the transition from early to late endosomes. Part of the regulation of Mon1 binding was due to lack of PI3P on vacuoles. We further characterized the connection between Mon1 vacuole binding and PI3P by competing Mon1 from the vacuoles with the high-affinity ligand FYVE or modifying the lipid with the phosphatase MTM-1. PI3P not only stabilizes Mon1 and its interaction partner Ccz1 on membranes, but it also stimulates Mon1-Ccz1 GEF activity (Cabrera et al., 2014). The link between GEF activity and phosphoinositides is not unique to the vacuole model. Others have shown that the Sec4 GEF Sec2 functions in Rab cascade in a PI4P-regulated manner (Mizuno-Yamasaki et al., 2010).

FIGURE 5: Mon1 is phosphorylated before release. (A) Fusion reactions were incubated for the indicated times at 27°C and resolved on 6% SDS–PAGE and probed for Mon1 and Ypt7 by immunoblotting. (B) Reactions were performed as described and separated into membrane and supernatant fractions before being resolved by 6% SDS–PAGE. Mon1p mobility was examined by immunoblot. Actin served as a loading control for pellets and supernatants. (C) A 60-min reaction was performed as described and treated with buffer or calf intestinal phosphatase (CIP) before immunoblotting. Actin served as a loading control for pellets and supernatants. (D) The role of fusion activity on Mon1 was examined by blocking fusion with anti-Sec17 IgG. Inhibited reactions were bypassed by the addition of recombinant Vam7 in the indicated lanes. Controls lacking anti-Sec17 were performed in the presence or absence of ATP to detect the electrophoretic mobility shift of modified Mon1. After incubation at 27°C, the membranes and supernatants were separated, and the membrane-bound fraction of Mon1 was detected by immunoblotting. Asterisk Indicates the cross reaction of IgG heavy chain.

FIGURE 6: Yck3 phosphorylates Mon1 during the fusion reaction. (A) Fusion reactions were performed using vacuoles purified from WT and yck3Δ strains. The fusion reactions were incubated at 27°C for the indicated times and processed for immunoblotting using antibodies against Mon1 and Vps41. (B) Fusion reactions containing yck3Δ vacuoles were performed as described in the presence of buffer or 6 μM recombinant His6-Yck3. After incubation the reactions were separated into membrane and supernatant fractions and processed for immunoblotting of Mon1p. Vam3 distribution was used as a control for the separation of soluble and membrane-bound proteins. (C) In vitro phosphorylation assays of purified Mon1 and recombinant Yck3 were run in the presence or absence of ATP. Phosphorylated Mon1 was detected by immunoblotting. (D) To test whether inhibiting protein kinase activity during the fusion reaction would affect Mon1 distribution, staurosporine was added at the indicated concentrations. After incubation, the membrane-bound fraction of Mon1 was examined by immunoblotting.
While investigating Mon1 binding to membranes, we found that Yck3 phosphorylated Mon1 and that its modification leads to release of phospho-Mon1 from the membrane. The release of Mon1 was blocked when Yck3 was absent or when the casein kinase phosphorylation sites on Mon1 were mutated. Although little is known about the regulation of GEF activity, there are several reports that show how GEF phosphorylation affects function. For example, during hyphal growth of the opportunistic pathogenic yeast Candida albicans the Rab Sec4 is activated by its GEF, Sec2. Localization of Sec2 to the tip of polarized hyphal growth requires phosphorylation by the cyclin-dependent kinase Cdc28 (Bishop et al., 2010). Others have shown that in Mast cells the RhoA GTPase is activated by the Src family kinase Fyn to inhibit Trio activity (Degeer et al., 2013). The inability of Fyn to phosphorylate Trio leads to continued Rac1 function and dysregulated axon outgrowth.

In summary, our data provide evidence that Yck3 kinase affects fusion processes at vacuoles at multiple levels and, as shown here, is an important regulator for efficient recycling of the Mon1-Ccz1 complex from vacuoles.
 MATERIALS AND METHODS

Reagents

Reagents were dissolved in PS buffer (20 mM 1,4-piperazinediethanesulfonic acid [Pipes]-KOH, pH 6.8, 200 mM sorbitol). The recombinant proteins glutathione S-transferase (GST)-FYVE (Gillooly et al., 2000), His6-MTM-1 (Taylor et al., 2000), GST-Vam7 (Fratti et al., 2007; Fratti and Wickner, 2007), yeast actin (Karunakaran et al., 2012), His6-Yck3 (Hickey and Wickner, 2010), and His6-Gyp1-46 (Wang et al., 2003) were prepared as described and stored in PS buffer with 125 mM KCl. Mon1 and Ccz1 were purified from yeast as a heterodimer as described (Nordmann et al., 2010). HOPS was purified as described (Brocker et al., 2012). Antibodies against Sec17, Ypt7, Vti1, and Vps11 were prepared as described (Mayer et al., 1996; Mayer and Wickner, 1997; Price et al., 2000; Ungermann et al., 1999).

Strains

Vacuoles from BJ3505 and DKY6281 were used for fusion assays (Table 1; Haas et al., 1995). Tester strains lacking YCK3 or PAH1 were described previously (Hickey et al., 2009; Sasser et al., 2012). Strains containing YPT7 mutations were previously described (Eitzen et al., 2000). MON1 was deleted from strains using a kanMX cassette using PCR products amplified from pFA6-kanMX4 with homology flanking the MON1 coding sequence (Longtine et al., 1998). For complementation studies of mon1Δ, strains were transformed with 2μ plasmids encoding Mon1-6A or Mon1-6D to generate CUF609 and CUY6093. Transformants were selected on complete synthetic media lacking uracil. For fluorescence microscopy experiments, wild-type and pah1Δ strains were transformed with plasmids for the expression of green fluorescent protein (GFP)–Vps21 (1324), GFP-Mon1 (1381), Vps3-GFP (3098), or Vps8-GFP (3096; Table 2). Transformants were grown in selective media.

Vacuole isolation and in vitro vacuole fusion

Vacuoles were isolated by floatation as described (Haas et al., 1995). Standard in vitro fusion reactions (30 μl) contained 3 μg each of vacuoles from BJ3505 and DKY6281 backgrounds, fusion reaction buffer (20 mM PIPES-KOH, pH 6.8, 200 mM sorbitol, 125 mM KCl, and 200 mM sorbitol). The recombinant proteins glutathione S-transferase (GST)-FYVE (Gillooly et al., 2000), His6-MTM-1 (Taylor et al., 2000), GST-Vam7 (Fratti et al., 2007; Fratti and Wickner, 2007), yeast actin (Karunakaran et al., 2012), His6-Yck3 (Hickey and Wickner, 2010), and His6-Gyp1-46 (Wang et al., 2003) were prepared as described and stored in PS buffer with 125 mM KCl. Mon1 and Ccz1 were purified from yeast as a heterodimer as described (Nordmann et al., 2010). HOPS was purified as described (Brocker et al., 2012). Antibodies against Sec17, Ypt7, Vti1, and Vps11 were prepared as described (Mayer et al., 1996; Mayer and Wickner, 1997; Price et al., 2000; Ungermann et al., 1999).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJ3505</td>
<td>MATa pep4::His3 prb1Δ1.6R</td>
<td>Cabrera et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>his3–200 lys2–801 trp1Δ101 (gal3) ura3–52 gal2 can1</td>
<td></td>
</tr>
<tr>
<td>DKY6281</td>
<td>MATa pho8::TRP1 leu2–3 leu2–112 ura3–52 his3–Δ200 trp1Δ901 lys2–801</td>
<td></td>
</tr>
<tr>
<td>RFY17</td>
<td>BJ3505, pah1Δ::Kan’</td>
<td>Cabrera et al. (2013)</td>
</tr>
<tr>
<td>RFY18</td>
<td>DKY6281, pah1Δ::Kan’</td>
<td>Cabrera et al. (2013)</td>
</tr>
<tr>
<td>BY4741</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td></td>
<td>ura3Δ0</td>
<td></td>
</tr>
<tr>
<td>BY4732</td>
<td>MATa his3Δ200 leu2Δ0 met15Δ0</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td></td>
<td>trp1Δ63 ura3Δ0</td>
<td></td>
</tr>
<tr>
<td>BY4742</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0</td>
<td>Open Biosystems</td>
</tr>
<tr>
<td></td>
<td>ura3Δ0</td>
<td>(Huntsville, AL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BY4742 MATalpha his3Δ1 leu2Δ0</td>
<td>Open Biosystems</td>
</tr>
<tr>
<td></td>
<td>met15Δ0 ura3Δ0 vps3Δ4::KanMX6</td>
<td>(Huntsville, AL)</td>
</tr>
<tr>
<td>BJ3505</td>
<td>ypt7::YPT7T22N</td>
<td>Eitzen et al. (2000)</td>
</tr>
<tr>
<td>(Q68L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DKY6281</td>
<td>ypt7::YPT7T22N</td>
<td>Eitzen et al. (2000)</td>
</tr>
<tr>
<td>(Q68L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BJ3505</td>
<td>ypt7::YPT7T22N</td>
<td>Eitzen et al. (2000)</td>
</tr>
<tr>
<td>(T22N)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DKY6281</td>
<td>ypt7::YPT7T22N</td>
<td>Eitzen et al. (2000)</td>
</tr>
<tr>
<td>(T22N)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 1: Yeast strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>3096</td>
<td>pRS415-FpNOP1-VP58-GFP</td>
<td>Cabrera et al. (2013)</td>
</tr>
<tr>
<td>3098</td>
<td>pRS415-FpNOP1-VP53-GFP</td>
<td>Cabrera et al. (2013)</td>
</tr>
<tr>
<td>1381</td>
<td>pRS416-FpNOP1-GFP-MON1</td>
<td>This study</td>
</tr>
<tr>
<td>1324</td>
<td>pRS416-FpNOP1-GFP-VP521</td>
<td>This study</td>
</tr>
</tbody>
</table>

TABLE 2: Yeast plasmids used in this study.
5 mM MgCl₂, ATP-regenerating system (1 mM ATP, 0.1 mg/ml creatine kinase, 29 mM creatine phosphate), 10 μM CoA, and 283 nM inhibitor of protease B (lbz). Reactions were incubated at 27°C, and Pho8p activity was assayed in 250 mM Tris-Cl, pH 8.5, 0.4% Triton X-100, 10 mM MgCl₂, and 1 mM p-nitrophenyl phosphate. Fusion units were measured by determining the p-nitrophenol produced as min⁻¹ μg⁻¹ pep4Δ vacuole, and absorbance was detected at 400 nm.

**Tandem affinity purification**

Tandem affinity purification (TAP) was performed as described (Puig et al., 2001). Three liters of culture were grown at 30°C to an OD₆₀₀ of ~4, and cells were harvested by centrifugation. Cells were lysed in buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid/NaOH, pH 7.4, 300 mM NaCl, 1.5 mM MgCl₂, 1× FY protease inhibitor mix (Serva, Heidelberg, Germany), 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (DTT). Lysates were centrifuged for 90 min at 100,000 × g, and supernatants were incubated with IgG Sepharose beads for 2 h at 4°C. Beads were isolated by centrifugation at 800 × g for 5 min and washed with 15 ml of lysis buffer containing 0.5 mM DTT. Bound proteins were eluted by tobacco etch virus (TEV) cleavage, and eluates were analyzed on SDS–PAGE. Next the TEV elution buffer was exchanged using NAP-5 columns (GE Healthcare, Piscataway, NJ) equilibrated with vacuole buffer containing 5 mM ethylene glycol tetraacetic acid and trichloroacetic acid (TCA)/phosphatidylethanolamine (PE)/PI3P with lipid concentrations of 27°C, and Pho8p activity was assayed in 250 mM Tris-Cl, pH 8.5, 0.4% Triton X-100, 10 mM MgCl₂, and 1 mM p-nitrophenyl phosphate. Fusion units were measured by determining the p-nitrophenol produced as min⁻¹ μg⁻¹ pep4Δ vacuole, and absorbance was detected at 400 nm.

**In vitro phosphorylation**

TAP-tagged Mon1 was incubated with 500 μl of equilibrated IgG Sepharose bead slurry (4°C, 2 h, nutating). After washing, the beads were incubated with fusion reaction buffer and incubated with recombinant Yck3 at 48 μg/ml as a final concentration. Reactions were incubated with ATP-regenerating system or buffer alone for 6 h at 30°C while nutating. Next beads were washed with TEV cleavage buffer. TEV (5 μg/ml) was added to the washed beads and incubated for 2 h at 4°C. Eluted proteins were mixed with 6 ml of calmodulin-binding buffer containing a final concentration of 3 mM CaCl₂, mixed with 300 μl of calmodulin Sepharose, and incubated for 1 h at 4°C. The beads where then washed with buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 0.1% Nonidet P-40 alternative, 10% glycerol). Bound protein was eluted with elution buffer containing 5 mM ethylene glycol tetraacetic acid and trichloroacetic acid precipitated. Dried protein was solubilized with SDS buffer and analyzed by immunoblotting.

**Preparation of small unilamellar liposomes**

The following lipid solutions were prepared: phosphatidylcholine (PC)/phosphatidylethanolamine (PE)/PI3P with lipid concentrations 500/50/50 mM, respectively, and PC/PE with lipid concentrations 500/50/50 mM, respectively. Lipids were transferred from concentrated stocks to glass test tubes and the solvents evaporated under a nitrogen stream. The test tubes were then run in a SpeedVac for 30 min and stored in a desiccator under vacuum overnight. The next day, the dried lipids were resuspended in M1 vaccine buffer (50 mM sodium acetate, 25 mM Bis-Tris, 25 mM Tris-Cl, pH 6.8, and 5% [vol/vol] glycerol) with vortexing and sonicated in a water bath until the solution was visibly clear.

**MTM1 phosphatase assay**

A portion of the recombinant His₂-MTM1–purified protein was boiled for 10 min and then cooled on ice. Each of the liposome solutions described previously was aliquoted into three 160-μl samples, and 10 μg of MTM-1, boiled MTM-1, or calf intestinal phosphatase was added to individual aliquots. Samples were then incubated and reactions stopped by boiling for 10 min. The lipid and protein components were pelleted by centrifugation and the supernatants decanted. These supernatants were then separated into 50-μl aliquots, and the free phosphatase levels measured by the malachite green phosphatase assay.

**Malachite green phosphatase detection**

Reactions (50 μl) and blanks were prepared and added to a 96-well plate. Next 10 μl of reagent A (42.8 mM Na₂MoO₄, 1.14 M HCl) was added to each well, mixed, and incubated at room temperature for 10 min. Then 10 μl of reagent B (0.042% [wt/vol] malachite green, 1% [wt/vol] polyvinyl alcohol) was added to each well, mixed, and incubated for 20 min at room temperature. To stop the reaction, each well received 10 μl of reagent C (7.8% H₂SO₄), mixed, and incubated for 10 min at room temperature. Free phosphate was measured at 630 nm, and its concentration was derived using a standard curve of P."


Ostrowicz CW, Brocker C, Ahlert F, Nordmann M, Lachmann J, Pепловская K, Defined subunit arrangement and Rab interactions are required for functionality of the HOPS tethering complex. Traffic 11, 1334–1346.

Pепловская K, Markgraf DF, Ostrowicz CW, Bange G, Ungermann C (2007). The CORVET tethering complex interacts with the yeast Rab5 homolog Vps21 and is involved in endo-lyosomal biogenesis. Dev Cell 12, 739–750.


