The Vac14p–Fig4p complex acts independently of Vac7p and couples PI3,5P_2 synthesis and turnover

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Phosphoinositide-signaling lipids function in diverse cellular pathways. Dynamic changes in the levels of these signaling lipids regulate multiple processes. In particular, when *Saccharomyces cerevisiae* cells are exposed to hyperosmotic shock, PI3,5P_2 (phosphatidylinositol [PI] 3,5-bisphosphate) levels transiently increase 20-fold. This causes the vacuole to undergo multiple acute changes. Control of PI3,5P_2 levels occurs through regulation of both its synthesis and turnover. Synthesis is catalyzed by the PI3P 5-kinase Fab1p, and turnover is catalyzed by the PI3,5P_2 5-phosphatase Fig4p. In this study, we show that two putative Fab1p activators, Vac7p and Vac14p, independently regulate Fab1p activity. Although Vac7p only regulates Fab1p, surprisingly, we find that Vac14 regulates both Fab1p and Fig4p. Moreover, Fig4p itself functions in both PI3,5P_2 synthesis and turnover. In both the absence and presence of Vac7p, the Vac14p–Fig4p complex controls the hyperosmotic shock–induced increase in PI3,5P_2 levels. These findings suggest that the dynamic changes in PI3,5P_2 are controlled through a tight coupling of synthesis and turnover.

Introduction

Phosphoinositides are required in cellular events such as signal transduction, cytoskeletal rearrangements, and membrane trafficking. Phosphoinositides function by recruiting protein effectors to specific membrane domains. Most phosphoinositides bind to multiple effectors. This enables cells to control multiple functions simultaneously by regulating the levels of a single phosphoinositide isomer. Therefore, tight control of phosphoinositide levels is critical for the ability of a cell to orchestrate diverse signaling pathways.

The most recently discovered phosphoinositide, PI3,5P_2 (phosphatidylinositol [PI] 3,5-bisphosphate), is required for signaling pathways that invoke a response within the endomembrane system. For example, PI3,5P_2 is important for insulin-induced trafficking of the glucose transporter GLUT4 to the plasma membrane (Ikonomov et al., 2002; Berwick et al., 2004). In addition, increases in PI3,5P_2 levels accompany a cellular response to UV irradiation (Jones et al., 1999) and EGF stimulation (Tsujita et al., 2004). A similar increase in PI3,5P_2 is observed in yeast and plant cells in response to acute increases in environmental osmolarity (Dove et al., 1997; Meijer et al., 1999; Zonia and Munnik, 2004).

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Abbreviations used in this paper: SC, synthetic complete; PI, phosphatidylinositol; PI3,5P_2, PI 3,5-bisphosphate.

PI3,5P_2 levels are also important for constitutive membrane traffic to the lysosome. Mammalian EGF receptor trafficking to the lysosome is impaired in cells that overexpress the PI3,5P_2 3-phosphatase mytotubularin (Tsujita et al., 2004). In addition, *Salmonella enterica* secrete a phosphoinositide phosphatase (SopB) that decreases host PI3,5P_2 levels (Hernandez et al., 2004) and diverts maturing phagosomes away from the lysosomal degradation pathway (Scott et al., 2002; Vergne et al., 2003).

In *Saccharomyces cerevisiae*, the levels of PI3,5P_2 under normal conditions are low (Dove et al., 1997; Whiteford et al., 1997)—20-fold lower than the other detectable phosphoinositides PI3P, PI4P, and PI4,5P_2 (Bonangelino et al., 2002). Yeast mutants that cannot produce PI3,5P_2 have grossly enlarged vacuoles. Moreover, the vacuoles are not acidified properly despite the proper localization of the vacuolar ATPase (Bonangelino et al., 1997; Gary et al., 1998). Furthermore, some proteins that are normally transported to the lumen of the vacuole through multivesicular bodies are mislocalized to the limiting membrane of the vacuole (Odorizzi et al., 1998; Shaw et al., 2003; Dove et al., 2004). Vesicular traffic out of the vacuole is also compromised in the absence of PI3,5P_2 (Bryant et al., 1998; Dove et al., 2004). These observations demonstrate that PI3,5P_2 controls multiple vacuolar functions.

The low levels of PI3,5P_2 in *S. cerevisiae* increase dramatically when cells are acutely exposed to hyperosmotic

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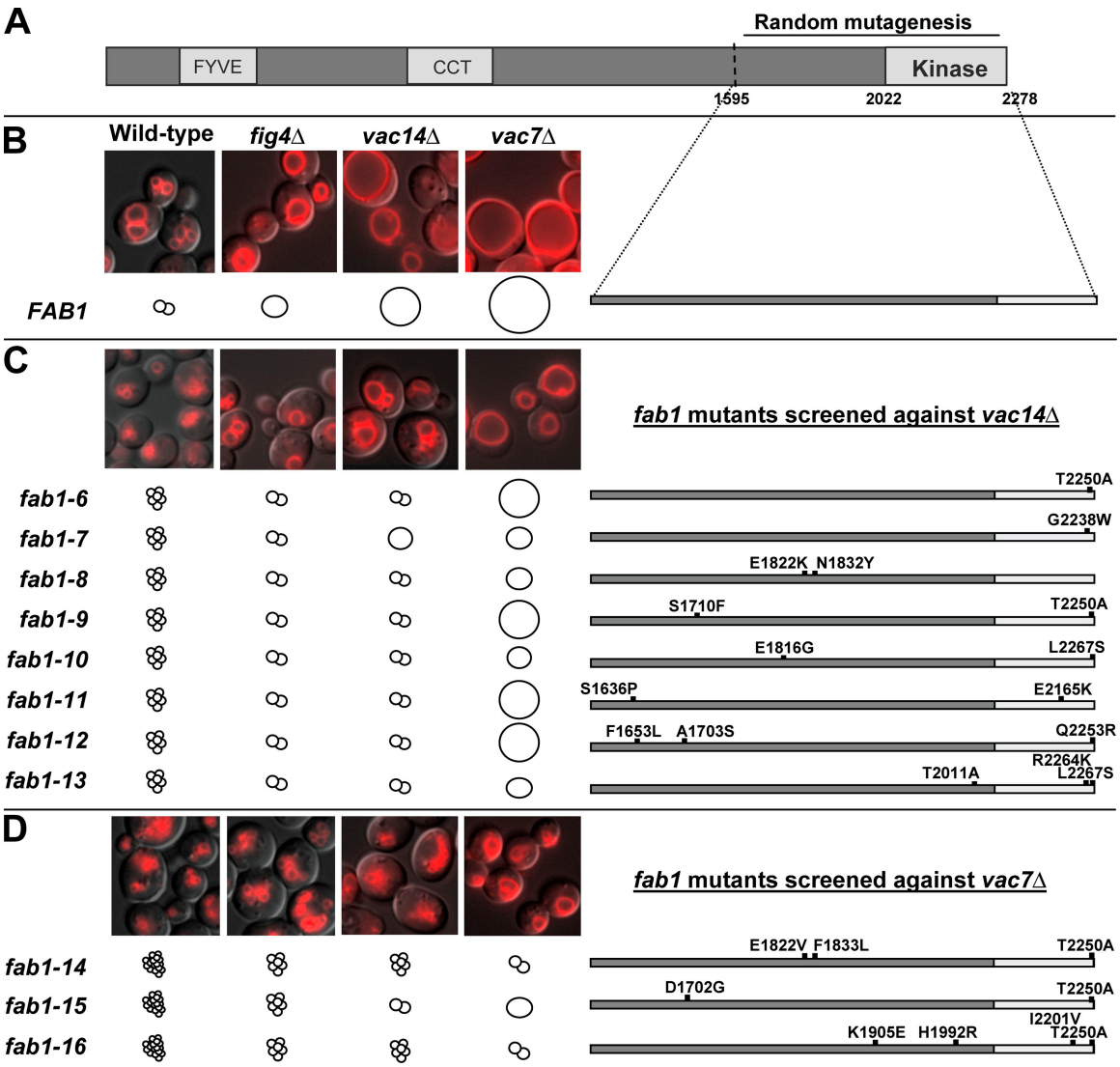


Figure 1. **Selected point mutants in the Fab1p kinase domain or a region just proximal to the kinase domain result in suppression of vacuole volume defects in *vac7*Δ and/or *vac14*Δ.** (A) Schematic of Fab1p. *FAB1* was subjected to random mutagenesis in the region encoding the kinase domain. Mutants were screened in *vac7*Δ or *vac14*Δ cells for their ability to restore normal growth at 37°C and normal vacuole volume. (B) Wild-type (LWY7235), *fig4*Δ (LWY6474), *vac14*Δ (LWY5177), and *vac7*Δ (LWY2054) cells were labeled with FM4-64 to visualize vacuole membranes. Schematics under the images depict vacuole volume and approximate number of lobes. Representative fields shown. (C) *fab1-6* through *fab1-13* mutants were isolated for their ability to suppress defects in *vac14*Δ cells. Images of the *fab1-6* mutant. (D) *fab1-14* through *fab1-16* mutants were isolated for their ability to suppress defects in *vac7*Δ cells. Images of the *fab1-14* mutant.

conditions (Dove et al., 1997). Within 5 min, there is a 20-fold increase in $PI3,5P_2$ levels (Duex et al., 2006). These elevated levels are briefly maintained, but by 30 min, $PI3,5P_2$ levels return to their normal, low levels.

It is likely that the transient increase in $PI3,5P_2$ provides protection against osmotic changes in the environment. For example, vacuole volume decreases dramatically as $PI3,5P_2$ levels increase (Bonangelino et al., 2002; Duex et al., 2006). $PI3,5P_2$ may also regulate channels that release ions, other osmolytes, and water from the vacuole into the cytoplasm (Thumm, 2000).

In *S. cerevisiae*, Fab1p is the sole kinase that generates $PI3,5P_2$ from a pool of PI3P (Yamamoto et al., 1995). Overexpression of *FAB1* does not lead to an increase in $PI3,5P_2$ levels (Gary et al., 1998). This suggests that Fab1p activity is tightly regulated. In fact, two putative activators of Fab1p have been identified: Vac7p and Vac14p. Like *fab1*Δ cells, *vac7*Δ and *vac14*Δ cells have no detectable $PI3,5P_2$ under basal conditions. Moreover, in response to hyperosmotic shock, *vac7*Δ and *vac14*Δ cells are defective in producing the 20-fold increase in $PI3,5P_2$ levels observed in wild-type cells (Gary et al., 1998; Bonangelino et al., 2002; Duex et al., 2006). Furthermore, human Vac14 was recently shown to physically associate with mammalian Fab1 (PIKfyve), and overexpression of human Vac14 increased the PI3P 5-kinase activity of PIKfyve (Sbrissa et al., 2004). These observations support the hypothesis that Vac7p and Vac14p regulate $PI3,5P_2$ levels through the activation of Fab1p. Neither Vac7p nor Vac14p have identifiable conserved domains, and it is not yet known how they regulate $PI3,5P_2$ levels.

Fig4p is a known $PI3,5P_2$ 5-phosphatase (Rudge et al., 2004). Thus, our recent observation that *fig4*Δ cells are defective in the hyperosmotic shock–induced increase in $PI3,5P_2$ levels was surprising (Duex et al., 2006). It raised the possibility that Fig4p also functions as an activator of Fab1p. Moreover, Fig4p forms a complex with Vac14p (Dove et al., 2002; Gary et al., 2002; Rudge et al., 2004), suggesting that Vac14p may regulate Fig4p or that Fig4p may regulate Vac14p. Therefore, we sought to determine the roles of Vac7p, Vac14p, and Fig4p in regulation of the lipid kinase activity of Fab1p and determine the potential roles of Vac14p and Vac7p in regulation of the lipid phosphatase activity of Fig4p.

In this study, we identify specific mutations in two distinct regions of Fab1p that enhance its activity. The resulting hyperactive Fab1p mutants produce elevated levels of $PI3,5P_2$ under basal conditions and respond normally to hyperosmotic shock when Vac7p, Vac14p, and Fig4p are present. In their absence, the Fab1p mutants are still able to produce detectable levels of $PI3,5P_2$ but have lost their ability to respond to hyperosmotic shock. These hyperactive Fab1p mutants allowed us to individually test the roles of the putative Fab1p activators in the regulation of $PI3,5P_2$ levels. We found that Vac7p and Vac14p independently regulate $PI3,5P_2$ levels. We also show that Vac14p is absolutely required for Fig4p-mediated turnover of $PI3,5P_2$ after hyperosmotic shock. Lastly, we show that Fig4p point mutants defective in $PI3,5P_2$ turnover are simultaneously defective in hyperosmotic shock–induced elevation of $PI3,5P_2$ levels, suggesting a direct role for Fig4p in both $PI3,5P_2$ synthesis and turnover. The ability of Fig4p and Vac14p to act in both the synthesis and turnover of $PI3,5P_2$ may be key to the ability of cells to acutely regulate transient changes in $PI3,5P_2$ levels.

Results

Fab1p mutants that suppress *vac14*Δ phenotypes

In vivo, Fab1p cannot produce $PI3,5P_2$ in the absence of either Vac7p or Vac14p. Furthermore, in the absence of Fig4p, there is a dramatic defect in $PI3,5P_2$ elevation after hyperosmotic shock. Therefore, to analyze the roles of Vac7p, Vac14p, and Fig4p as potential activators of Fab1p, Fab1p mutants that function in the absence of these proteins were sought. These mutants would enable us to study the individual roles of Vac7p, Vac14p, and Fig4p in controlling $PI3,5P_2$ levels and may also reveal regulatory regions within the Fab1p protein. A Fab1p mutant that is partially active in the absence of Vac7p had been previously identified (Gary et al., 2002) but had multiple mutations that were distributed throughout two thirds of the protein. Thus, we isolated new Fab1p mutants with single amino acid changes.

Random mutagenesis was performed on the region of *FAB1* that encodes the kinase domain (<30% of the total gene; Fig. 1 A). *FAB1* constructs subjected to mutagenesis were transformed into *fab1*Δ*vac14*Δ cells (Fig. 1 B). Mutant alleles that exhibited wild-type growth rates at 37°C and suppressed the large vacuole volume of *vac14*Δ cells were sequenced (Fig. 1 C). Two of these mutant alleles, *fab1-6* (T2250A) and *fab1-7* (G2238W), were each the result of a single amino acid substitution of highly conserved residues in the COOH-terminal end of the kinase domain. Notably, all previously reported kinase domain mutations had a deleterious effect on Fab1p function (Gary et al., 1998; Friant et al., 2003).

A second type of informative mutant (e.g., *fab1-8*) had mutations exclusively in the region proximal to the kinase domain. Both sets of mutants also displayed reduced vacuole volume in *fig4*Δ, wild-type, and, to a lesser extent, *vac7*Δ cells (Fig. 1 C). The identification of these mutants supports a hypothesis that specific residues in the kinase domain and the region proximal to the kinase domain are involved in the up-regulation of Fab1p kinase activity.

Fab1p mutants that suppress *vac7*Δ phenotypes

The Fab1p mutants identified above restored normal vacuole volume in *vac14*Δ cells but had much less effect in *vac7*Δ cells (Fig. 1 C). There are two possibilities as to why the mutants suppressed *vac14*Δ better than *vac7*Δ mutants. First, *vac7*Δ cells have a greater defect in the production of $PI3,5P_2$. Therefore, the basal PI3P 5-kinase activity of these Fab1p mutants may be increased enough to restore normal $PI3,5P_2$ levels and vacuole volume in *vac14*Δ cells but not in *vac7*Δ cells. Alternatively, the mutants may specifically bypass Vac14p function but not Vac7p function. Therefore, we performed an additional screen for Fab1p mutants that could restore normal vacuole volume in *vac7*Δ cells. We randomly mutagenized *fab1-6* to ensure that new mutations would be obtained. New mutations might specifically bypass *vac7*Δ defects or may be more general and bypass *vac7*Δ defects while further bypassing *vac14*Δ defects.

The screen yielded mutants in this latter category. The mutants contained the original *fab1-6* kinase domain mutation plus mutations in the region proximal to the kinase domain (Fig. 1 D). These Fab1p mutants led to a decrease in vacuole volume in both *vac7*Δ and *vac14*Δ cells as well as wild-type and *fig4*Δ cells. This suggests that specific mutations in either domain result in Fab1p molecules with elevated kinase activity.

Mutations in each domain were additive. For example, the most active mutant (*fab1-14*) contained mutations in both the kinase domain and in the region proximal to the kinase domain. The kinase domain mutation (T2250A) was identical to that of *fab1-6*. The proximal region mutated residues (E1822V/F1833L) were nearly identical to those of a proximal region mutant, *fab1-8* (E1822K/N1832Y). In fact, a $fab1^{E1822V/F1833L}$ mutant and a $fab1^{E1822K/N1832Y}$ mutant (*fab1-8*) had identical suppression of *vac7*Δ and *vac14*Δ vacuole volume defects (not depicted). In addition, suppression of vacuole volume defects by *fab1-14* was equal to that of *fab1-6* and *fab1-8* combined (Fig. 1, C and D).

Under basal conditions, *fab1-6* and *fab1-14* mutants produce elevated levels of $PI3,5P_2$

The ability of isolated Fab1p mutants to suppress vacuole volume defects of *vac7*Δ and *vac14*Δ cells suggests that these Fab1p mutants produce elevated levels of $PI3,5P_2$. Indeed, the *fab1-6* (0.24 units) and *fab1-14* (0.68 units) mutants had

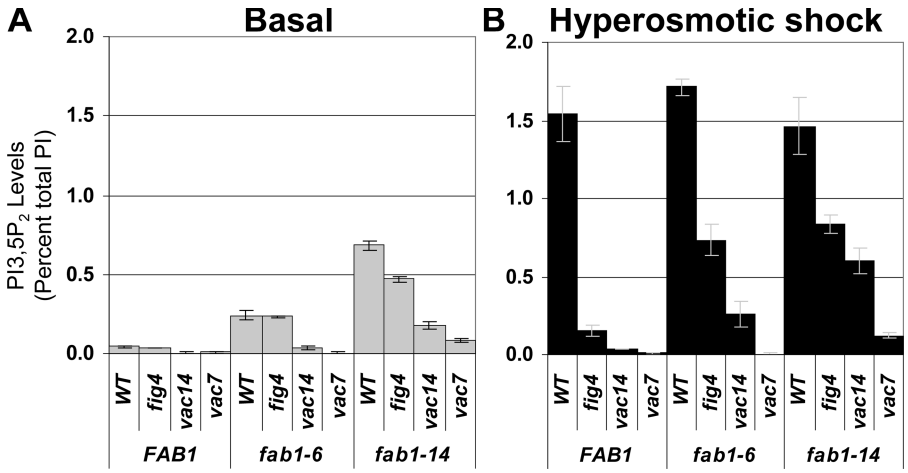


Figure 2. **The Fab1p mutants require Fig4p, Vac7p, and Vac14p for maximum production of PI3,5P$_2$.** *FAB1*, *fab1-6*, or *fab1-14* alleles were expressed in *fab1*Δ (LWY2055), *fab1*Δ*fig4*Δ (LWY7002), *fab1*Δ*vac14*Δ (LWY5956), and *fab1*Δ*vac7*Δ (LWY2046). Strains were labeled with [^{3}H]inositol for 12 h and were untreated (A) or treated (B) with 0.9 M NaCl for 10 min. Total cellular PI was deacylated and analyzed by HPLC. To normalize for the number of cells and [^{3}H]inositol incorporation, each value is shown as the percentage of total extracted [^{3}H]PI. Mean of three independent experiments. Error bars represent SD.

significantly higher PI3,5P$_2$ levels than wild-type *FAB1* (0.04 units; Fig. 2 A). The observation that *fab1-14* results in three times more basal PI3,5P$_2$ than *fab1-6* likely explains why *fab1-14* suppresses both *vac7*Δ and *vac14*Δ vacuole volume defects, whereas *fab1-6* suppresses only *vac14*Δ.

In the presence of *FAB1*, basal levels of PI3,5P$_2$ in *fig4*Δ cells are similar to wild type (Rudge et al., 2004; Duex et al., 2006), whereas basal levels of PI3,5P$_2$ in *vac7*Δ or *vac14*Δ cells are significantly lower (Bonangelino et al., 2002). These relative differences between the strains were also present when either *fab1-6* or *fab-14* was expressed as the sole copy of *FAB1* in *fig4*Δ, *vac14*Δ, or *vac7*Δ cells (Fig. 2 A). In each case, substitution of *FAB1* with *fab1-6* resulted in a modest increase in steady-state levels of PI3,5P$_2$, whereas substitution of *FAB1* with *fab1-14* resulted in a larger increase in PI3,5P$_2$ levels. These findings suggest that neither mutant specifically bypasses the requirement for Vac7p, Vac14p, or Fig4p under basal conditions.

Although the *fab1-6* and *fab1-14* alleles result in increased Fab1p PI3P 5-kinase activity, our data do not indicate whether the increased activity is a result of changes in the catalytic activity of Fab1p itself or whether the mutations affect modulators of Fab1p activity. For example, the Fab1p mutants presented in this study may have increased PI3P 5-kinase activity because of a reduction in negative regulation by Atg18p. The observation that *atg18*Δ cells have extremely high levels of PI3,5P$_2$ led to the proposal that Atg18p negatively regulates Fab1p (Dove et al., 2004; Efe et al., 2005). Furthermore, Atg18p has also been reported to bind Fab1p in the region proximal to the kinase domain, which is the site of mutations in *fab1-14* (Georgakopoulos et al., 2001).

Hyperosmotic shock–induced elevation in the levels of PI3,5P$_2$ requires Vac7p, Vac14p, and Fig4p

We tested whether the *fab1-6* or *fab1-14* mutants produced even higher levels of PI3,5P$_2$ after hyperosmotic shock. Indeed, in a wild-type background, both mutants responded to hyperosmotic shock (Fig. 2 B). Unexpectedly, the mutants produced the same level of PI3,5P$_2$ as wild-type *FAB1*. This was surprising because under basal conditions, the *fab1-6* and *fab1-14* alleles had elevated levels of PI3,5P$_2$ compared with wild-type *FAB1*. Perhaps these Fab1p mutants are already partially activated under basal conditions and cannot be further activated beyond a specific maximum. Alternatively, the regulation of Fab1p under basal conditions may be different than the activation of Fab1p after hyperosmotic shock. Another alternative is that there may be regulated inhibition of Fab1p activity to maintain specific levels of PI3,5P$_2$ at specific times. Along similar lines, perhaps regulated turnover of PI3,5P$_2$ prevents the levels from exceeding a maximum. It should also be noted that the levels of PI4P and PI4,5P$_2$ did not change significantly with the expression of *fab1-6* or *fab1-14* when compared with *FAB1* (not depicted).

In the absence of Vac7p, Vac14p, or Fig4p, neither *fab1-6* nor *fab1-14* achieved the normal levels of PI3,5P$_2$ induced by hyperosmotic shock (Fig. 2 B). Furthermore, the relative differences in hyperosmotic shock–induced levels of PI3,5P$_2$ observed when *fab1-6* or *fab1-14* were expressed in *vac7*Δ, *vac14*Δ, or *fig4*Δ mutants parallel those observed under basal conditions. Specifically, when these mutant alleles are expressed in *vac7*Δ cells, they are most defective in the elevation of PI3,5P$_2$ levels; when expressed in *vac14*Δ cells, they are moderately defective; and when expressed in *fig4*Δ cells, they are least defective in the elevation of PI3,5P$_2$ levels.

A previous study showed that mouse Fab1 (PIKfyve) PI3P 5-kinase activity is increased upon its dephosphorylation (Sbrissa et al., 2000). Based on this information, we tested the hypothesis that the *fab1-6* allele (*fab1*^{T2250A}) is more active because it prevents a possible inhibitory phosphorylation at the conserved Thr2250 residue. We generated a *fab1*^{T2250D} mutant to mimic inhibitory phosphorylation and observed that the *fab1*^{T2250D} mutant had elevated levels of PI3,5P$_2$ that were almost identical to that of the *fab1*^{T2250A} mutant before and after hyperosmotic shock (unpublished data). Therefore, it is unlikely that the threonine at position 2,250 regulates the activity of Fab1p via its phosphorylation/dephosphorylation.

In the absence of Vac7p, Vac14p, and Fig4p, the *fab1-14* mutant is not activated by hyperosmotic shock

A *vac7*Δ strain containing the hyperactive *fab1-14* mutant exhibited a small increase in PI3,5P$_2$ levels after hyperosmotic shock (Fig. 2, A and B). Likewise, when the *fab1-14* mutant

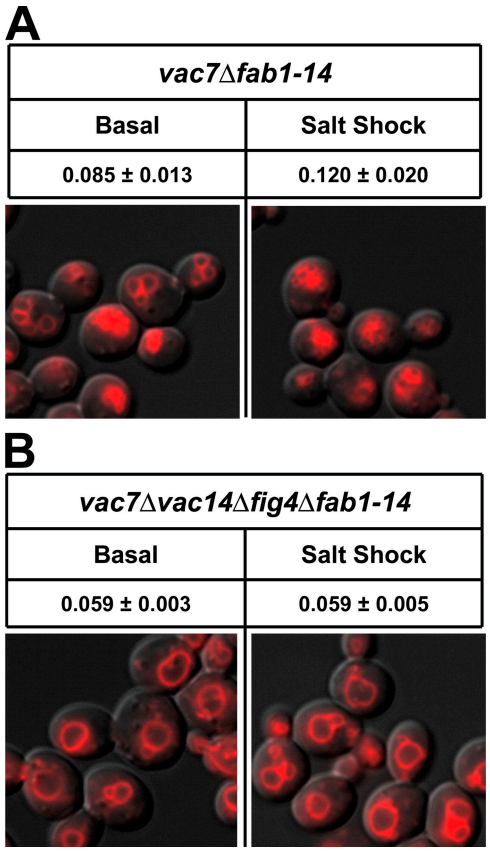


Figure 3. **In the absence of Vac7p, Vac14p, and Fig4p, the *fab1-14* mutant shows no elevation of PI3,5P_2 levels or decrease in vacuole volume after hyperosmotic shock.** PI3,5P_2 levels and vacuole morphology were determined for *vac7Δfab1-14* (LWY7179) (A) and *vac7Δvac14Δfig4Δfab1-14* (LWY7197) (B) cells before and after a 10-min incubation in 0.9 M NaCl. Each data point is a mean of three independent experiments. Representative fields shown.

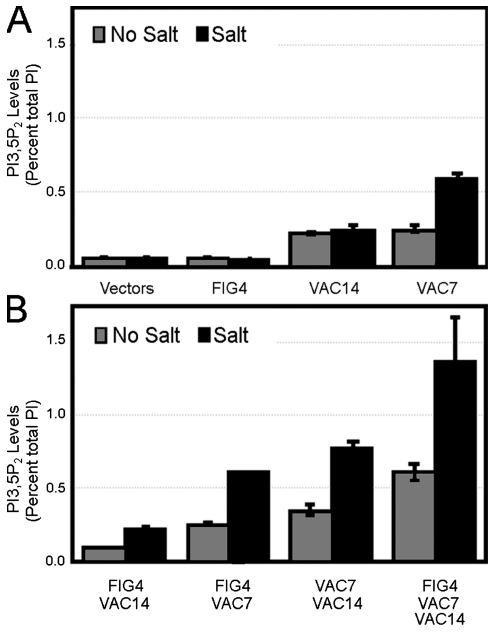


Figure 4. **Maximum elevation of PI3,5P_2 levels requires Vac7p, Vac14p, and Fig4p.** (A) Overexpression of *VAC14* or *VAC7* increases the levels of PI3,5P_2 under basal conditions. *VAC7* alone increases the levels of PI3,5P_2 in response to hyperosmotic shock. In a *vac7Δvac14Δfig4Δfab1-14* (LWY7197) strain, each individual activator was expressed from a multicopy plasmid. PI3,5P_2 levels were determined before and after a 10-min incubation in 0.9 M NaCl. Mean of three independent experiments. (B) Coexpression of *FIG4* and *VAC14* increases PI3,5P_2 levels in response to hyperosmotic shock. Simultaneous expression of all three putative Fab1p activators was performed in *vac7Δfab1-14* (LWY7179). Error bars represent SD.

was integrated into the *FAB1* chromosomal locus of a *vac7Δ* strain, hyperosmotic shock induced a modest increase in PI3,5P_2 levels (0.08–0.12 units) and a corresponding decrease in vacuole volume (Fig. 3 A). These results suggest that a second mechanism of elevating PI3,5P_2 exists that is independent of Vac7p. This other mechanism may involve the Vac14p–Fig4p complex. To test this possibility, we simultaneously deleted Vac7p, Vac14p, and Fig4p and measured the levels of PI3,5P_2. The *vac7Δvac14Δfig4Δfab1-14* strain produced low but detectable levels of PI3,5P_2 under basal conditions (Fig. 3 B). This is in contrast with wild-type *FAB1*, where PI3,5P_2 cannot be detected in the absence of Vac7p, Vac14p, and Fig4p. Notably, hyperosmotic shock of the *vac7Δvac14Δfig4Δfab1-14* strain did not induce an increase in the levels of PI3,5P_2 or a decrease in vacuole volume (Fig. 3 B). These observations strongly suggest that a hyperosmotic shock–induced increase in the levels of PI3,5P_2 requires Vac7p, Vac14p, and/or Fig4p.

Vac7p and Vac14p independently increase the levels of PI3,5P_2

To determine whether Vac7p, Vac14p, or Fig4p alone elicit an increase in the levels of PI3,5P_2, we individually expressed each gene in the *vac7Δvac14Δfig4Δfab1-14* strain. Because the localization of Vac14p and Fig4p are partially dependent on each other (Rudge et al., 2004; Duex et al., 2006), each gene was overexpressed to mitigate potential problems in their localization. Overexpression of *FIG4* in the *vac7Δvac14Δfig4Δfab1-14* strain had no effect on the levels of PI3,5P_2 before or after hyperosmotic shock (Fig. 4 A). This indicates that Fig4p alone cannot elevate the levels of PI3,5P_2. Overexpression of *VAC14* resulted in a 4.5-fold increase in the basal levels of PI3,5P_2. This suggests that Vac14p can elevate levels of PI3,5P_2 in the absence of Vac7p and Fig4p. After hyperosmotic shock, there was no further increase in PI3,5P_2 levels. Overexpression of *VAC7* resulted in a 4.5-fold increase in the basal levels of PI3,5P_2. Moreover, after hyperosmotic shock, there was a further increase in PI3,5P_2 levels. This suggests that Vac7p can elevate levels of PI3,5P_2 in the absence of Vac14p and Fig4p. Moreover, it also suggests that the majority of the hyperosmotic shock–induced elevation of PI3,5P_2 levels occurs via Vac7p.

A maximal increase in the levels of PI3,5P_2 requires both Vac7p and the Vac14p–Fig4p complex

To test whether the potential regulators act in concert with each other, we overexpressed pairwise combinations of *VAC7*, *VAC14*, and *FIG4* in the *vac7Δvac14Δfig4Δfab1-14* strain. When *FIG4* and *VAC14* were overexpressed simultaneously,

the basal levels of $PI3,5P_2$ were lower than when *VAC14* was expressed alone (Fig. 4 B). This is likely caused by the ability of the Vac14p–Fig4p complex to turn over $PI3,5P_2$ under basal conditions. Upon hyperosmotic shock, the levels of $PI3,5P_2$ increased. This is in contrast to the overexpression of Vac14p or Fig4p alone where hyperosmotic shock had no effect on the levels of $PI3,5P_2$ (Fig. 4 A). This finding suggests that the Vac14p–Fig4p complex plays a role in the hyperosmotic shock–induced increase in $PI3,5P_2$ levels. This postulate is also supported by the observation of a Vac7p-independent hyperosmotic shock–induced increase in $PI3,5P_2$ levels in *vac7*Δ*fab1-14* cells (Fig. 3, A and B). Analysis of a Fig4p point mutant is also consistent with a role of the Vac14p–Fig4p complex in the hyperosmotic shock–induced increase in $PI3,5P_2$ levels (see the last section of Results).

When *VAC7* and *VAC14* were overexpressed simultaneously, the levels of $PI3,5P_2$ were the sum of the levels observed with *VAC7* or *VAC14* alone. This is consistent with the hypothesis that Vac7p and Vac14p independently elevate the levels of $PI3,5P_2$. The highest levels of $PI3,5P_2$ observed under both basal and hyperosmotic shock conditions occurs when all three regulators were overexpressed simultaneously. This further supports the hypothesis that all three proteins play important roles in the elevation of $PI3,5P_2$ levels.

All of the aforementioned results support a model in which Vac7p and Vac14p independently increase $PI3,5P_2$ levels. Under basal conditions, Vac7p and Vac14p play an equal role. After hyperosmotic shock, Vac7p is the main protein required to elevate $PI3,5P_2$ levels. In addition, after hyperosmotic shock, Fig4p is important for the elevation of $PI3,5P_2$ levels and requires Vac14p for this function.

The Vac14p–Fig4p complex is required for turnover of $PI3,5P_2$

Analysis of *vac14*Δ cells revealed that after hyperosmotic shock, there is a delay in the turnover of $PI3,5P_2$ (Fig. 5 A). This delay is similar to that observed in *fig4*Δ cells where the levels of $PI3,5P_2$ at 30 min after hyperosmotic shock are the same or even higher than the levels achieved at 10 min. This delay in the turnover of $PI3,5P_2$ can also be observed when the *fab1-14* allele was expressed in *vac14*Δ or *fig4*Δ cells (Fig. 5 B). These findings clearly demonstrate that in either a *vac14*Δ or *fig4*Δ mutant, there is a delay in the turnover of $PI3,5P_2$. Therefore, both Vac14p and Fig4p are required for the hyperosmotic shock–induced turnover of $PI3,5P_2$.

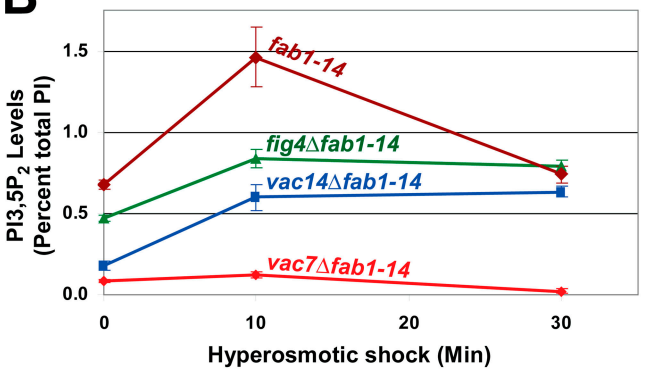
The Fig4p sequence is similar to known lipid phosphatases. Moreover, Fig4p functions in vitro as a $PI3,5P_2$ 5-phosphatase (Rudge et al., 2004). These findings, combined with the aforementioned analysis, strongly suggests that the rapid turnover of $PI3,5P_2$ after hyperosmotic shock is the result of the Fig4p-dependent conversion of $PI3,5P_2$ to PI3P.

The requirement for Vac14p in hyperosmotic shock–induced turnover of $PI3,5P_2$ was surprising because Vac14p was first proposed to be an activator of Fab1p. More recently, Vac14p had also been shown to interact with Fig4p (Dove et al., 2002; Rudge et al., 2004). Here, we find that the levels of Fig4p are greatly diminished in a *vac14*Δ mutant (Fig. 5 C). This suggests

A

Hyper-osmotic shock (Min)	$PI3,5P_2$ Levels		
	Wild-type	*fig4*Δ	*vac14*Δ
0	0.08 ± 0.02	0.08 ± 0.01	0.005 ± 0.001
10	2.10 ± 0.20	0.39 ± 0.00	0.026 ± 0.000
20	0.58 ± 0.11	0.50 ± 0.01	0.041 ± 0.015
30	0.13 ± 0.02	0.38 ± 0.02	0.034 ± 0.022
60	0.07 ± 0.01	0.12 ± 0.02	0.022 ± 0.010

B



C

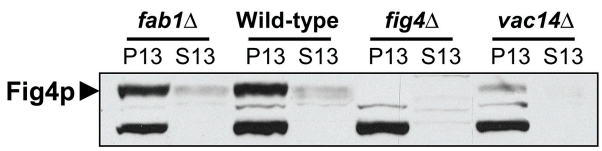


Figure 5. ***fig4*Δ and *vac14*Δ mutants are defective in hyperosmotic shock–induced turnover of $PI3,5P_2$.** (A) *vac14*Δ and *fig4*Δ cells have a defect in $PI3,5P_2$ turnover after hyperosmotic shock. Data was compiled from Duex et al. (2006). (B) *fig4*Δ and *vac14*Δ cells with *fab1-14* as the sole copy of *FAB1* have higher levels of $PI3,5P_2$. Thus, defects in hyperosmotic shock–induced $PI3,5P_2$ turnover can be readily detected. *fab1-14* was expressed in *fab1*Δ (LWY2055), *fab1*Δ*fig4*Δ (LWY7002), *fab1*Δ*vac14*Δ (LWY5956), and *fab1*Δ*vac7*Δ (LWY2046) cells. Strains were treated with 0.9 M NaCl for 0, 10, or 30 min, and the levels of $PI3,5P_2$ were determined. Data are the means of three independent experiments. Error bars represent SD. (C) Fig4p levels are reduced in *vac14*Δ cells. *fab1*Δ (LWY2055), wild-type (LWY7235), *fig4*Δ (LWY6474), and *vac14*Δ (LWY5177) cells were lysed in the absence of detergent, and extracts were separated into membrane (P) and soluble (S) fractions by centrifugation at 13,000 *g*. The P13 fraction contains large organelles such as the vacuole, Golgi, ER, and nucleus. Western blot analysis was performed using rabbit anti-Fig4p. Equal protein was loaded in each set of P13 and S13 fractions. Data is representative of three independent experiments.

that Vac14p may play a role in the turnover of $PI3,5P_2$ either by stabilization and/or regulation of Fig4p.

The observation that hyperosmotic shock–induced $PI3,5P_2$ levels eventually decrease even in the absence of Fig4p suggests that there are other pathways that degrade $PI3,5P_2$. Five other proteins (Sac1p, Sjl1p, Sjl2p, Sjl3p, and Inp54p) are predicted to have phosphoinositide 5-phosphatase activity. However, defects in $PI3,5P_2$ turnover have not been observed in the corresponding knockout strains. Only when multiple genes are knocked out are elevated $PI3,5P_2$ levels observed

(Gary et al., 2002; Stefan et al., 2002; Parrish et al., 2004). Furthermore, none of these phosphoinositide phosphatases localize to the vacuole membrane, which is the site of $PI3,5P_2$ synthesis and function. Although it is likely that at least some of these proteins can degrade $PI3,5P_2$, none of them are likely to have a primary role in the rapid turnover of $PI3,5P_2$ after hyperosmotic shock.

The *vac7Δfab1-14* strain produced very little $PI3,5P_2$ in response to hyperosmotic shock, but these levels decreased at 30 min (Fig. 5 B). This suggests that Vac7p is not required for the rapid turnover of $PI3,5P_2$.

Fig4p requires Vac14p to function in the hyperosmotic shock–induced turnover of $PI3,5P_2$

We used the *vac7Δvac14Δfig4Δfab1-14* strain to further test the function of Vac14p and Fig4p in the hyperosmotic shock–induced turnover of $PI3,5P_2$. Simultaneous overexpression of *FIG4*, *VAC7*, and *VAC14* in the *vac7Δvac14Δfig4Δfab1-14* strain resulted in the hyperosmotic shock–induced turnover of $PI3,5P_2$ (Fig. 6 A). Overexpression of *FIG4* alone resulted in no turnover of $PI3,5P_2$ (Fig. 6 B). Similarly, overexpression of *VAC14* alone resulted in no turnover of $PI3,5P_2$. However, simultaneous overexpression of *FIG4* and *VAC14* resulted in the turnover of $PI3,5P_2$. This supports the hypothesis that the Vac14p–Fig4p complex functions in the turnover of $PI3,5P_2$ after hyperosmotic shock.

When *VAC7* was overexpressed alone in the *vac7Δvac14Δfig4Δfab1-14* strain, a modest decrease in $PI3,5P_2$ levels was observed (Fig. 6 C). However, the amount of turnover (0.19 units) was less than half the amount (0.41 units) observed when *FIG4*, *VAC7*, and *VAC14* were overexpressed (Fig. 6 A). The observed turnover is likely the result of a Fig4p-independent mechanism (discussed in the previous section).

Simultaneous overexpression of *VAC7* and *FIG4* had a rate of $PI3,5P_2$ turnover that was similar to *VAC7* alone. Simultaneous overexpression of *VAC7* and *VAC14* also had a rate of $PI3,5P_2$ turnover that was similar to *VAC7* alone. These data suggest that Fig4p is the major enzyme responsible for the rapid turnover of hyperosmotic shock–induced $PI3,5P_2$ levels and that Fig4p stability and function require Vac14p.

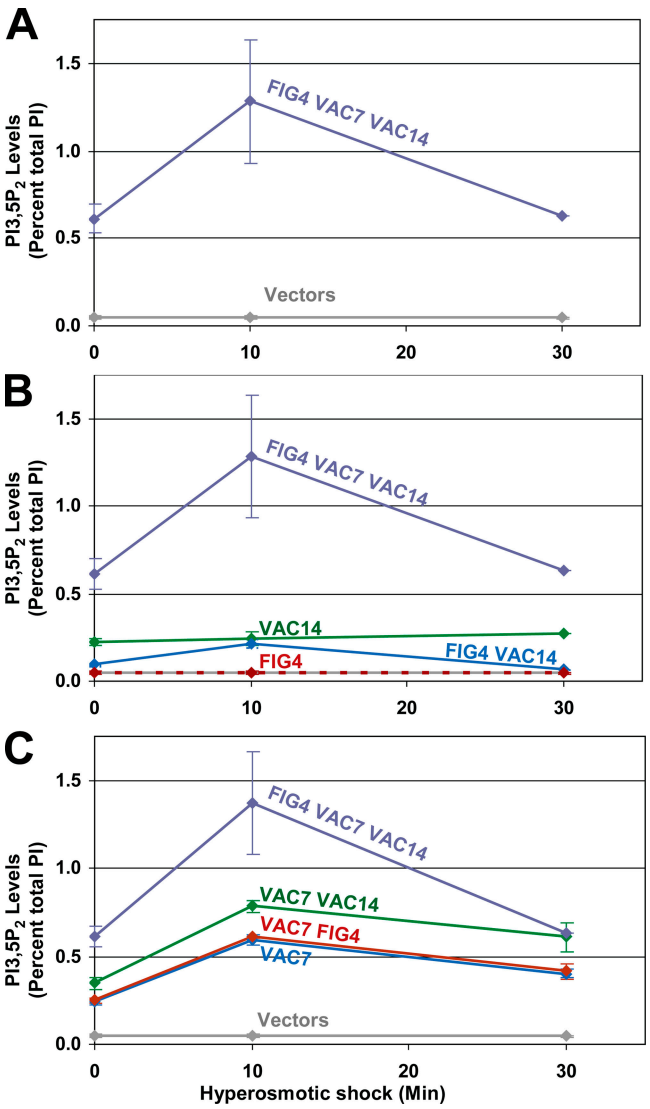


Figure 6. **Fig4p function requires Vac14p.** (A–C) *VAC7*, *VAC14*, and *FIG4* were overexpressed alone and in combination in *vac7Δvac14Δfig4Δfab1-14* (LWY7197). All three genes were simultaneously overexpressed in *vac7Δfab1-14* (LWY7179). $PI3,5P_2$ levels were determined before and after treatment with 0.9 M NaCl. Mean of three independent experiments. Error bars represent SD.

The *fig4*^{G519R} phosphatase mutant is partially defective in both hyperosmotic shock–induced $PI3,5P_2$ elevation and turnover

Perturbation in the localization of Vac14p in a *fig4Δ* strain (Rudge et al., 2004; Duex et al., 2006) makes it difficult to directly assess a role for Fig4p in the hyperosmotic shock–induced elevation and turnover of $PI3,5P_2$. Therefore, we used a Fig4p point mutant in which the localization of Vac14p is normal (Fig. 7 A). The *fig4*^{G519R} point mutant (Gary et al., 2002) is defective in $PI3,5P_2$ 5-phosphatase activity in vitro (Rudge et al., 2004). Therefore, we tested the in vivo levels of $PI3,5P_2$ in a *fig4*^{G519R} mutant. When compared with wild-type *FIG4* (Fig. 7 B), basal levels of $PI3,5P_2$ in a *fig4*^{G519R} mutant are elevated (Fig. 7 C). This observation is consistent with a defect in $PI3,5P_2$ 5-phosphatase activity. However, *fig4*^{G519R} appears to retain some lipid phosphatase activity. At 30 min after hyperosmotic shock, *fig4*^{G519R} cells have degraded 0.5 units of $PI3,5P_2$ compared with 0.0 units in *fig4Δ* cells (Fig. 7 D) and 1.3 units in *FIG4* cells.

In an attempt to produce a Fig4p mutant with no phosphatase activity, we generated a second Fig4p mutant, *fig4*^{D469N}. Based on a similar mutation in Sac1p (Stock et al., 1999), the *fig4*^{D469N} mutant was predicted to have no lipid phosphatase activity. Moreover, the D469 residue is conserved in all Sac1 domain–containing proteins, and the residue lies within the conserved CX_5RT catalytic site of lipid (Maehama and Dixon, 1998) and protein (Stone and Dixon, 1994) phosphatases. The *fig4*^{D469N} mutant behaved nearly identically to the *fig4*^{G519R} mutant (unpublished data). Both mutants had elevated levels of

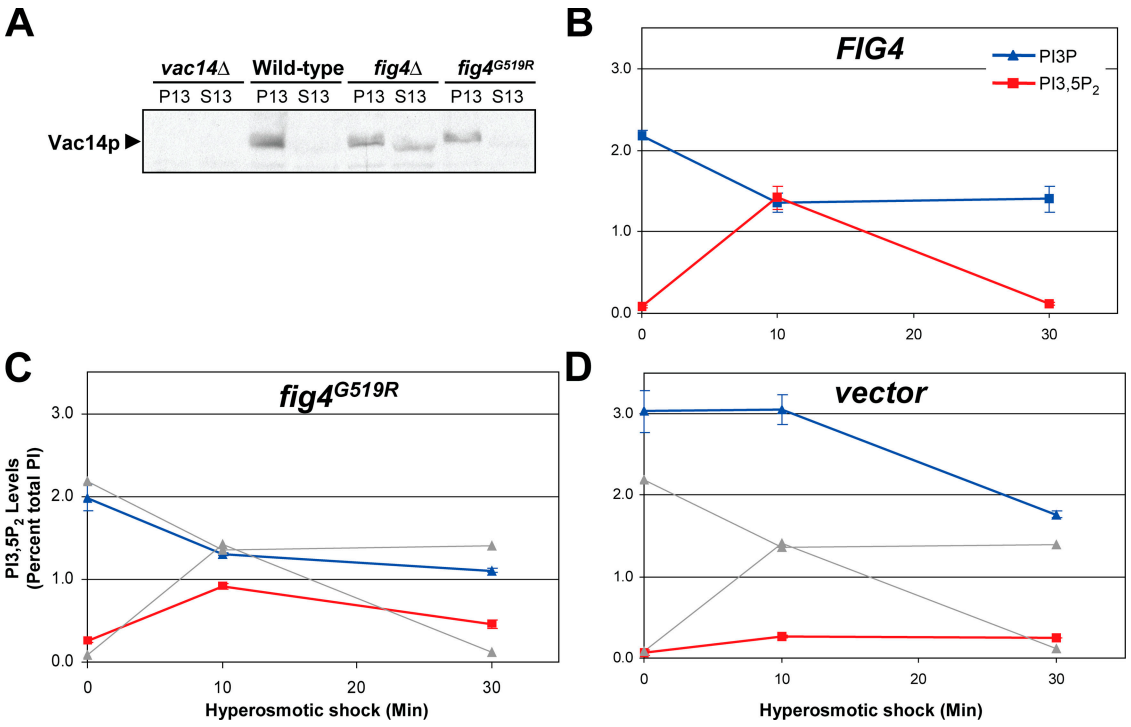


Figure 7. **Elevation and turnover of PI3,5P$_2$ is partially defective in the *fig4*^{G519R} mutant.** (A) Vac14p localization is normal in the *fig4*^{G519R} mutant. *vac14*Δ (LWY5177), wild-type (LWY7235), *fig4*Δ (LWY6474), and *fig4*Δ cells expressing *fig4*^{G519R} were lysed; extracts were spun at 13,000 *g* to generate membrane (P) and soluble (S) fractions. Equal protein was loaded between each P13 and S13 fraction. Western blot analysis was performed using goat anti-Vac14p. Data are representative of three independent experiments. (B–D) The *fig4*^{G519R} mutant is partially defective in both elevation and turnover of PI3,5P$_2$. PI3,5P$_2$ levels after exposure to 0.9 M NaCl for the indicated amount of time in *fig4*Δ cells expressing *FIG4*, *fig4*^{G519R}, or vector alone. In C and D, *FIG4* data are shown in gray. Mean of three independent experiments. Error bars represent SD.

PI3,5P$_2$ under basal conditions, a decreased rate of hyperosmotic shock–induced turnover of PI3,5P$_2$, and a defect in the elevation of PI3,5P$_2$ in response to hyperosmotic shock (~65% of wild type). Notably, the extent of the partial defect observed in PI3,5P$_2$ turnover parallels the extent of the partial defect in PI3,5P$_2$ elevation. These observations suggest that the Sac1 phosphatase domain of Fig4p is important for both an increase in PI3,5P$_2$ levels and their turnover.

Discussion

The dramatic and transient change in PI3,5P$_2$ levels after hyperosmotic shock raises the question of how acute changes in phosphoinositides are regulated. In this study, we investigated the putative PI3,5P$_2$ regulators Vac7p, Vac14p, and Fig4p to determine the role each plays in PI3,5P$_2$ synthesis and turnover.

Transient elevation in PI3,5P$_2$ levels are primarily caused by its increased synthesis

Steady-state levels of PI3,5P$_2$ are dependent on both its rate of synthesis and turnover. In the studies reported here, the large increases in the levels of PI3,5P$_2$ observed after hyperosmotic shock or in the presence of the activated Fab1p mutants were accompanied by a decrease in the levels of PI3P (Fig. 7 B). Decreases in PI3P could be caused by the activation of Fab1p and a corresponding depletion of its substrate or caused by the inhibition of a PI3,5P$_2$ 5-phosphatase and a corresponding loss in the production of its product. Strains that are defective in normal hyperosmotic shock–induced PI3,5P$_2$ elevation, including *fab1*Δ in which no PI3,5P$_2$ is produced, have up to 35% higher levels of PI3P than wild-type cells (Fig. 7 D). This observation strongly suggests that the steady-state levels of PI3P are caused by its synthesis from PI rather than from the turnover of PI3,5P$_2$. Also, in wild-type cells under basal conditions, the levels of PI3,5P$_2$ are 20-fold lower than the levels of PI3P, making it unlikely that the turnover of PI3,5P$_2$ is a significant source of PI3P. These results are most consistent with the hypothesis that the increases in PI3,5P$_2$ levels and concomitant decreases in PI3P levels that occur during hyperosmotic shock are caused by increased Fab1p activity rather than the inhibition of a PI3,5P$_2$ 5-phosphatase.

Vac7p likely elevates PI3,5P$_2$ via the activation of Fab1p

Our data support the hypothesis that Vac7p increases PI3,5P$_2$ levels via the activation of Fab1p. In the *vac7*Δ*vac14*Δ*fig4*Δ*fab1-14* strain, PI3P but not PI3,5P$_2$ levels are elevated by hyperosmotic shock. However, when *VAC7* is overexpressed in this strain, the levels of PI3,5P$_2$ are elevated after hyperosmotic shock, and there is a corresponding decrease in PI3P levels (unpublished data). These changes are most consistent with the hypothesis that Vac7p activates Fab1p.

Vac7p is the primary activator of Fab1p. Vac7p activates the hyperactive *fab1-14* mutant in the absence of Vac14p and

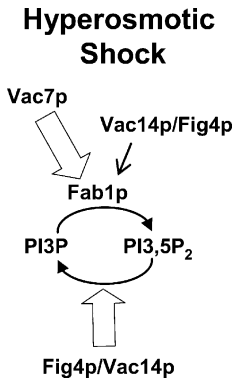


Figure 8. **Vac7p is the major activator of Fab1p during hyperosmotic shock.** Fig4p is the major enzyme required for hyperosmotic shock–induced turnover of PI3,5P_2. Vac7p and Vac14p independently activate Fab1p under basal conditions. After hyperosmotic shock, Vac7p further activates Fab1p. The Vac14p–Fig4p complex also activates Fab1p, but to a lesser extent than Vac7p. Maximum Fab1p activation requires all three Fab1p activators. Rapid turnover of PI3,5P_2 after hyperosmotic shock predominantly requires the Vac14p–Fig4p complex.

Fig4p. Furthermore, Vac7p activates Fab1p to a greater degree than Vac14p, Fig4p, or the Vac14p–Fig4p complex (Fig. 8). Notably, Vac7p homologues have only been observed in fungi. Perhaps there is a functional homologue of Vac7p in other eukaryotes that cannot be found through sequence comparison. Alternatively, in organisms that lack Vac7p, the conserved Vac14p–Fig4p complex may serve as the main regulator of Fab1p.

Vac14p-dependent elevation of PI3,5P_2 levels is independent of Vac7p

Overexpression of Vac14p alone in the *vac7*Δ*vac14*Δ*fig4*Δ*fab1-14* strain results in an increase in PI3,5P_2 levels. Overexpression of Vac14p and Vac7p together results in an increase in PI3,5P_2 levels that is equal to the sum of overexpression of either protein alone. Therefore, Vac14p and Vac7p function independently. Analysis of the Vac14p–Fig4p complex strongly suggests that Vac14p increases PI3,5P_2 levels via the activation of Fab1p (see the last section of Discussion). In addition, Vac14 physically associates with and increases the activity of mammalian Fab1p (PIKfyve; Sbrissa et al., 2004).

In contrast to Vac7p, the overexpression of Vac14p alone does not result in a further increase in PI3,5P_2 levels in response to hyperosmotic shock. Perhaps the *fab1-14* allele partially mimics the Vac14p-induced activation of Fab1p. Alternatively, Vac14p may require Fig4p for its function in hyperosmotic stress. Note that PI3,5P_2 levels increase in response to hyperosmotic shock when both Vac14p and Fig4p are present (Fig. 4).

The observation that Vac7p and the Vac14p–Fig4p complex independently increase PI3,5P_2 levels suggests that there may be multiple pathways for Fab1p activation. The Vac7p and Vac14p–Fig4p pathways may generate distinct pools of PI3,5P_2 or may be downstream of different signaling cascades.

It is tempting to speculate that Vac7p and Vac14p–Fig4p each bind to unique regions of Fab1p. Fab1p is a large protein (2,278 aa) and may contain multiple binding sites for multiple partners. Although the hyperactive mutants identified do not specifically bypass either Vac7p or Vac14p, only 30% of the protein was mutagenized. Perhaps Vac7p and/or Vac14p activate Fab1p through interaction with specific regions in the other 70% of the protein.

Fig4p requires Vac14p for its PI3,5P_2 5-phosphatase activity

Turnover of PI3,5P_2 after hyperosmotic shock is predominantly achieved through the activity of Fig4p, a PI3,5P_2 5-phosphatase. Surprisingly, Vac14p is required for the Fig4p-mediated turnover of PI3,5P_2. In *vac14*Δ cells, the rate of PI3,5P_2 turnover after hyperosmotic shock is decreased to the same degree as it is in *fig4*Δ cells. The defect in the turnover of PI3,5P_2 is unlikely caused by the reduced levels of Fig4p observed in the absence of Vac14p. When *FIG4* is overexpressed in a *vac7*Δ*vac14*Δ*fig4*Δ*fab1-14* strain, membrane-bound Fig4p levels are higher than membrane-bound Fig4p levels in a wild-type strain (unpublished data). Yet, the levels of PI3,5P_2 are identical in a *vac7*Δ*vac14*Δ*fig4*Δ*fab1-14* strain with or without the overexpression of *FIG4*. Thus, it appears that the defect in hyperosmotic shock–induced turnover of PI3,5P_2 observed in *vac14*Δ cells is likely caused by the absence of Vac14p. Further supporting the idea that Vac14p is a regulator of Fig4p is the observation that Vac14p has weak homology to the regulatory subunit of protein phosphatase 2A (PR65/A).

The Vac14p–Fig4p complex likely activates Fab1p

The most surprising outcome of our studies is the observation that Fig4p is required for the hyperosmotic shock–induced elevation of PI3,5P_2 levels. This elevation is likely the result of a Fig4p-dependent direct or indirect activation of Fab1p and not of the role of Fig4p in PI3,5P_2 turnover. First, if hyperosmotic shock–induced increases in the levels of PI3,5P_2 are caused by the inhibition of PI3,5P_2 turnover, then in the *fig4*Δ and *fig4*^{G519R} mutants, where turnover of PI3,5P_2 is already inhibited, one would expect normal elevation of PI3,5P_2. However, this is not observed. Second, the *fig4*^{G519R} mutant is partially defective in both the hyperosmotic shock–induced elevation of PI3,5P_2 and the corresponding decrease in PI3P that follows hyperosmotic shock (Fig. 7 C). Also note that in *fig4*Δ cells, basal levels of PI3P are 30% higher than wild type and remain high after hyperosmotic shock. This strongly suggests that production of PI3P is not perturbed in *fig4*Δ cells. The fact that the *fig4*Δ mutant has high levels of PI3P provides additional support for the model that turnover of PI3,5P_2 is not a significant source of PI3P production and that the defect in the decrease in PI3P levels is caused by a defect in the synthesis of PI3,5P_2.

We propose two models for how the Vac14p–Fig4p complex functions in the elevation of PI3,5P_2 levels after hyperosmotic shock. In all of these models, Vac14p functions as an activator of Fab1p under both basal and hyperosmotic conditions. In one model, Fig4p inhibits Vac14p activity under basal conditions and releases this inhibition upon hyperosmotic shock. This mechanism would require Fig4p to function under basal conditions as both a PI3,5P_2 phosphatase and an inhibitor

of Vac14p. In a second model, Fig4p either enhances Vac14p activity or directly activates Fab1p after hyperosmotic shock. It is tempting to speculate that Fig4p performs these functions as a protein phosphatase.

Phosphoinositide phosphatases may function as protein phosphatases. The mammalian proteins PTEN and myotubularin have protein phosphatase activity in vitro (Cui et al., 1998; Laporte et al., 1998; Ramaswamy et al., 1999) and phosphoinositide phosphatase activity both in vitro and in vivo (Maehama and Dixon, 1998; Myers et al., 1998; Blondeau et al., 2000; Taylor et al., 2000). Furthermore, the critical active site residues of tyrosine phosphatases, serine/threonine/tyrosine phosphatases, and phosphoinositide phosphatases share an active site CX_5RT/S motif. The Fig4p phosphatase point mutations presented in this study are located in or near this active site. Therefore, these mutations could affect both lipid and protein phosphatase activity.

Perhaps Fig4p functions as both a lipid phosphatase and protein phosphatase, dephosphorylating Vac14p or Fab1p to increase Fab1p activity. Notably, dephosphorylation of mammalian Fab1p increases its in vitro PI3P 5-kinase activity three to fourfold (Sbrissa et al., 2000). According to this model, cells lacking Fig4p phosphatase activity would have diminished Fab1p activation after hyperosmotic shock and defects in $PI3,5P_2$ turnover. This is precisely what is observed in the Fig4p phosphatase point mutants. Moreover, the defect in Fab1p activation ($\sim$35%) parallels the defect in $PI3,5P_2$ turnover ($\sim$30%).

In both models, the Vac14p–Fig4p complex regulates both $PI3,5P_2$ turnover and synthesis. Such dual regulation may provide a mechanism to control acute and transient changes in $PI3,5P_2$ levels. A similar model has been proposed for control of the onset of cytokinesis (Maddox and Oegema, 2003). In that case, the small GTPase RhoA is positively regulated by a complex containing the guanine nucleotide exchange factor Ect2 and negatively regulated by the GTPase-activating protein MgcRacGAP. Changes in the local concentration of RhoA, coupled with changes in the activities of MgcRacGAP and Ect2, establish the formation of a RhoA activity zone, thereby controlling the onset of cytokinesis (Minoshima et al., 2003; Bement et al., 2005). Tight coupling of the activation and inactivation of regulatory elements such as RhoGTPases, phosphoinositide kinases, and phosphatases is likely a common mechanism used to provide spatial and temporal control of signaling pathways.

Materials and methods

Strains, media, and vectors

Strains are listed in Table I. Strains were grown at 24°C in either YPD (yeast extract/peptone/glucose) or synthetic complete (SC) minimal media. All yeast plasmids were derived from the pRS400 series of vectors (Christianson et al., 1992).

Genetic manipulations

PCR reactions used PfuUltra HF (Stratagene). Restriction enzymes and buffers were obtained from New England Biolabs, Inc. For pRS415-*fig4*^{G519R}, a 660-bp BsaBI–NcoI fragment from pET-15b-*fig4-1* (Rudge et al., 2004) was cloned into BsaBI–NcoI-gapped pRS415-*FIG4*. The *fig4*^{D469N} mutant was generated by site-directed mutagenesis. A 542-bp ClaI fragment from pRS415-*FIG4* was cloned into pUC18. The GAC codon (aspartic acid) was changed to AAC (asparagine) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The ClaI fragment was cloned into a pUC18 plasmid containing the SacI fragment of *FIG4*. This mutated SacI fragment was then cloned into pRS415 to generate pRS415-*fig4*^{D469N}. The mutation was confirmed with DNA sequencing.

Table I. ***S.cerevisiae*** **strains used in this study**

Strains	Genotype	Source
LWY7217	*MATa leu2,3-112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Bonangelino et al., 1997
LWY7235	*MATa leu2,3-112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Bonangelino et al., 1997
LWY6725	LWY7235 × LWY7240	Duex et al., 2006
LWY2054	LWY7217 *vac7Δ::HIS3*	Bonangelino et al., 2002
LWY2055	LWY7217 *fab1Δ::LEU2*	Bonangelino et al., 2002
LWY5177	LWY7235 *vac14Δ::TRP1*	Bonangelino et al., 2002
LWY6474	LWY7235 *fig4Δ::TRP1*	Duex et al., 2006
LWY6538	LWY7235 *fig4Δ::TRP1 vac14Δ::TRP1*	This study
LWY7179	LWY2054 *vac7Δ::HIS3 fab1*$^{1\text{-}14}$*:KAN*	This study
LWY7197	LWY7235 *fig4Δ::TRP1 vac7Δ::HIS3 vac14Δ::TRP1 fab1*$^{1\text{-}14}$*:KAN*	This study

Labeling of yeast vacuoles

Log phase cells were labeled with FM4-64 as described previously (Duex et al., 2006). Salt treatment of labeled cells was performed by resuspending cells in media containing 0.45 M NaCl, which elicits the same changes in vacuole volume as 0.9 M NaCl. A large change in refractive index when using 0.9 M NaCl makes it difficult to achieve focused images on the microscope. Cells were viewed at room temperature on a microscope (Axioplan II; Carl Zeiss MicroImaging, Inc.) with a 100× NA 1.40 ∞/0.17 plan Apochromat objective using a TRITC filter. Images were captured with a camera (RT-Spot; Diagnostic Instruments) and analyzed with MetaMorph software (Universal Imaging).

Subcellular fractionation and Western blot analysis

Experiments were performed as described previously (Duex et al., 2006). Log phase cells were lysed in cytosol buffer, and the extracts were spun at 300 *g* to sediment unbroken cells. The supernatant was spun at 13,000 *g* for 10 min at 4°C. The supernatant (S13) was moved to a fresh tube, and the pellet (P13) was resuspended in cytosol buffer in an equal volume to the supernatant. Fractions were subjected to SDS-PAGE and transferred to nitrocellulose for Western blot analysis. Blots were probed with rabbit anti-Fig4p sera or goat anti-Vac14p sera (Bonangelino et al., 2002). HRP-conjugated secondary antibodies (Invitrogen) followed by SuperSignal Chemiluminescence (Pierce Chemical Co.) was used to visualize immunoreactive proteins.

Random mutagenesis of FAB1

FAB1 was PCR amplified from pRS416-*FAB1*, forward primer CTCAAACCGAAGAACGTCCG, reverse primer GGCACCCCAGGCTTTACAC (in pRS416), and standard Taq polymerase conditions (Boehringer). This produced a 3.5-kbp product that included 2,053 bp *FAB1* (encoding aa 1,595–2,278). The PCR product was cotransformed into a *fab1Δvac14Δ* strain with NheI–NotI-gapped pRS416-*FAB1* and transformants selected on SC-uracil plates. Transformants were replica plated onto SC-uracil plates containing 100 μM of the iron chelator bathophenanthroline disulfonic acid (Fluka). Wild-type colonies have normal growth rates and are white, whereas *fab1Δ*, *vac7Δ*, or *vac14Δ* cells are red. After a 36-h incubation at 37°C, large white colonies were selected as *vac14Δ* bypass candidates. Their ability to bypass the large vacuole morphology of *vac14Δ* cells was verified with FM4-64 labeling. Candidate plasmids were isolated and retransformed into *fab1Δvac14Δ* cells to confirm that a plasmid encoded a hyperactive Fab1p mutant. The mutagenized regions were sequenced. The same approach was used to screen for *fab1* mutants that bypass *vac7Δ*, except pRS416-*fab1-6* was used as the starting PCR template.

Chromosomal integration of the *fab1-6* and *fab1-14* hyperactive mutants

The kanamycin cassette integrated along with the *FAB1* mutations was PCR amplified from the yeast haploid knockout collection (Open Biosystems) using primers that had 45 bp of flanking sequence that would anneal to either the 3′ end of *FAB1* or the 3′ untranslated region of *FAB1*. This PCR product was transformed into wild-type yeast along with BsrGI-digested (partial) pRS416-*fab1-6* or pRS416-*fab1-14* and transformants selected on YPD supplemented with gentamycin. Plasmid preps from candidate transformants were performed, and the pRS416-*fab1:KAN* cassettes were verified by restriction digest and DNA sequence. Positive plasmids were digested with SwaI–NotI, and the resulting 3.5-kbp fragment was transformed into *vac7*Δ (LWY2054) and transformants selected on YPD supplemented with gentamycin. Proper integration of the *fab1:KAN* mutants was verified with PCR. Generation of the *fig4*Δ*vac7*Δ*vac14*Δ*fab1-14* mutant (LWY7197) was performed by mating *fig4*Δ*vac14*Δ (LWY6538) with *vac7*Δ*fab1-14* (LWY7179). Diploids were sporulated, and genotypes of the resultant tetrads were determined by the presence or absence of expected nutritional markers and PCR.

Inositol extraction

PI was extracted and analyzed as described previously (Duex et al., 2006). In brief, total PI was extracted from [^{3}H]inositol-labeled cells and deacylated with methylamine. The inositol head groups were then separated with HPLC, and radioactive counts were measured. Values for PI3,5P_2 are reported as the percentage of total PI extracted from each strain to normalize for [^{3}H]inositol incorporation, the number of cells, and the amount of extracted inositol injected into HPLC.

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