An intramolecular interaction within the lipid kinase Fab1 regulates cellular phosphatidylinositol 3,5-bisphosphate lipid levels

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ABSTRACT Phosphorylated phosphoinositide lipids (PPIs) are low-abundance signaling molecules that control signal transduction pathways and are necessary for cellular homeostasis. The PPI phosphatidylinositol (3,5)-bisphosphate (PI(3,5)P₂) is essential in multiple organ systems. PI(3,5)P₂ is generated from PI3P by the conserved lipid kinase Fab1/PIKfyve. Defects in the dynamic regulation of PI(3,5)P₂ are linked to human diseases. However, few mechanisms that regulate PI(3,5)P₂ have been identified. Here we report an intramolecular interaction between the yeast Fab1 kinase region and an upstream conserved cysteine-rich (CCR) domain. We identify mutations in the kinase domain that lead to elevated levels of PI(3,5)P₂ and impair the interaction between the kinase and CCR domain. We also identify mutations in the CCR domain that lead to elevated levels of PI(3,5)P₂. Together these findings reveal a regulatory mechanism that involves the CCR domain of Fab1 and contributes to dynamic control of cellular PI(3,5)P₂ synthesis.

INTRODUCTION Phosphatidylinositol (3,5)-bisphosphate (PI(3,5)P₂) is a low-abundance signaling lipid (Dove et al., 1997; Whiteford et al., 1997), which constitutes 0.05–0.1% of total phosphoinositide lipids (Bonangelino et al., 2002; Duex et al., 2006a). That levels of this lipid are dynamically regulated suggests a role in adaptation to external stimuli and a role in the maintenance of cellular homeostasis (Dove et al., 1997; Sbrissa et al., 1999; Duex et al., 2006a; Bridges et al., 2012; Zolov et al., 2012).

PI(3,5)P₂ is generated from phosphatidylinositol 3-phosphate (PI3P) by the conserved lipid kinase Fab1 (PIKfyve in mammals; Gary et al., 1998; Zolov et al., 2012). Fab1/PIKfyve is regulated via a protein complex with at least three proteins in mammalian cells—PIKfyve, Vac14, and Fig4—and five proteins in Saccharomyces cerevisiae—Fab1, Vac14, Fig4, Vac7, and Atg18 (Botelho et al., 2008; Jin et al., 2008). Little is known about how these proteins regulate Fab1.

Yeast Fab1 and mammalian PIKfyve are approximately fourfold longer than other phosphatidylinositol 5-kinases (Rao et al., 1998). The Fab1 kinase domain lies at the C-terminus and comprises less than one-fifth of the protein (Figure 1A). The N-terminal FYVE domain (Figure 1A) binds PI3P (Burd and Emr, 1998; Sbrissa et al., 1999), but the functions of additional conserved regions remain largely unknown. Fab1 residues 819–1550 encompass the conserved cysteine-rich (CCR) and chaperone-containing TCP1 (CCT) domain (Figure 1A). Based on sequence similarity between this region and the GroEL chaperonin, the CCR and CCT domains were proposed to associate with regulatory proteins (Efe et al., 2005; Micheli et al., 2006; Botelho et al., 2008). The CCT domain is essential for the interaction of yeast Fab1 with the critical scaffold protein Vac14 (Botelho et al., 2008; Jin et al., 2008). Specific point mutations in the CCT domain were identified that result in greatly reduced PI(3,5)P₂ levels (Botelho et al., 2008). However, the mechanisms that impair PI(3,5)P₂ production in these CCT mutants are unknown.

Here we provide insight into a function of the CCR domain. We report that the CCR domain contacts a C-terminal portion of Fab1, including the kinase domain, and that this interaction regulates
Fab1 PI3 5-kinase activity. Point mutations in both the kinase and CCR domains result in elevation of PI(3,5)P₂ levels. Furthermore, dominant-active mutations in the kinase domain impair a physical interaction between the kinase and CCR domains.

RESULTS AND DISCUSSION

Dominant-active mutations in the Fab1 kinase domain reveal a potential regulatory region

Several dominant-active Fab1 alleles with multiple point mutations were previously identified; however, in most cases, the causative point mutation was not determined (Duex et al., 2006b). Several of the mutations are at residues conserved between yeast and humans (Figure 1A). Ten of 11 alleles harbor at least one mutation at the C-terminal end of the kinase domain (Figure 1B, light green; Duex et al., 2006b). Intriguingly, the C-terminal mutations cluster on a single surface of a homology-based model of the kinase domain. Within this surface, three of the residues mutated in dominant-active alleles—T2250, Q2253, and R2264—reside on a predicted, solvent-exposed α-helix (Figure 1C).

On the basis of this model, we generated and characterized seven single–point mutation candidates. Six were mutations present in the original dominant-active alleles, whereas R2257D also resides on the putative solvent-exposed helix. In most cases, fragmentated vacuoles correlate with elevated PI(3,5)P₂ levels, whereas mutations that negatively affect PI(3,5)P₂ levels lead to enlarged vacuoles (Duex et al., 2006b). Each of the seven mutants exhibited fragmentated vacuoles indicative of elevated levels of PI(3,5)P₂ (Figure 1A and Supplemental Figure S1A). These results support the model that this predicted surface of the kinase domain functions in the regulation of Fab1.

We directly measured PI(3,5)P₂ levels of two alleles—Fab1-R2257D and Fab1-T2250A. In yeast, PI(3,5)P₂ levels transiently change upon introduction into hyperosmotic media (Dove et al., 1997). Within 5 min, PI(3,5)P₂ levels increase >15-fold, plateau for 10 min, and then rapidly return to basal levels (Duex et al., 2006a). Under basal conditions, both Fab1-T2250A and Fab1-R2257D display elevated PI(3,5)P₂ levels compared with wild type (WT; Figure 2B). In addition, in response to a hyperosmotic stimulus, both mutants elevate PI(3,5)P₂ levels and continue to display elevated levels at the 30-min time point compared with WT (Figure 2B). This indicates that the C-terminal portion of the kinase domain acts to control both basal levels of PI(3,5)P₂ and production of PI(3,5)P₂ in response to an extracellular stimulus.

The dominant-active mutation Fab1-T2250A elevates PI(3,5)P₂ levels independently of the PI(3,5)P₂ phosphatase Fig4

Fab1 exists in a complex with its opposing phosphatase, Fig4 (Botelho et al., 2008; Jin et al., 2008). Fig4 catalyzes PI(3,5)P₂ turnover (Rudge et al., 2004) and only functions within the Fab1-Vac14-Fig4 complex (Duex et al., 2006b; Jin et al., 2008). Thus a Fab1 mutation that alters association of Fig4 with the Fab1-Vac14 complex could contribute to elevation of PI(3,5)P₂ via a defect in turnover of PI(3,5)P₂ rather than an increase in intrinsic Fab1 kinase activity. To test this possibility, we analyzed PI(3,5)P₂ levels in cells expressing either Fab1-WT or Fab1-T2250A in a fig4Δ mutant. Under both basal and hyperosmotic shock conditions, Fab1-T2250A exhibits higher levels of PI(3,5)P₂ relative to Fab1-WT even in the absence of Fig4 (Figure 2C). Thus Fab1-T2250A displays higher levels of PI(3,5)P₂ relative to Fab1-WT independently of Fig4 function.

The dominant-active mutations in the kinase region impair an interaction with the Fab1 CCR domain

The clustering of dominant-active mutations to a conserved surface region of the Fab1 kinase domain led us to hypothesize that these
mutations disrupt a protein–protein interaction that modulates kinase activity. As an initial test, we assayed for intramolecular interactions between the kinase domain and other regions of Fab1 using the yeast two-hybrid (Y2H) test (Figure 3A). The kinase region (residues 1723–2278; Kinase, Figure 1A) interacts with residues 1181–1585 (CCT-CCR-L2-L3; Figure 1A), which also includes the CCT domain (residues 538–1917) and the L2 and L3 domains (residues 1373–1917) do not interact with the CCR domain. In contrast, the CCT domain (residues 538–1085) and the L2 and L3 domains (residues 1373–1917) do not interact with the kinase region. Furthermore, neither the kinase domain nor the CCR domain interacts with other members of the Fab1 complex: Vac14, Fig4, Vac7, or Atg18 (Figure 3, B and C). These data suggest that the Fab1 kinase region and CCR domain interact with each other, allowing copurification through two sequential affinity columns: amylose resin followed by glutathione–S-transferase (GST)–CCR fusion proteins (Figure 4A). Expression of either construct alone in E. coli yielded no soluble protein (unpublished data); however, as determined by SDS–PAGE and Western blot analysis, coexpression yielded soluble polypeptides for both the kinase region and CCR domain (Figure 4B). The recombinant polypeptides bound to each other, allowing copurification through two sequential affinity columns: amylase resin followed by glutathione–S-transferase, which bind alternatively to His-MBP-kinase and GST-CCR, respectively (Figure 4B). This provides in vitro evidence for a direct interaction between these domains.

That the kinase and CCR domains interact directly and that individual point mutations in the kinase region both enhance Fab1 kinase activity and impair the Y2H interaction between these domains suggest that the CCR domain inhibits Fab1 kinase activity through direct contact with the kinase region. Of note, another phosphoinositide kinase is hypothesized to be regulated via a protein–protein contact on the kinase domain (Budovskaya et al., 2013). Thus the interaction between the adaptor-binding domain and the kinase domain (Huang et al., 2013) is likely intramolecular.

To assess further the interaction between the CCR domain and kinase region, we expressed the CCR domain and kinase regions in Escherichia coli. Owing to the instability of multiple S. cerevisiae Fab1 constructs tested, we turned to the thermophilic fungus Chaetomium thermophilum. Recombinant proteins from this organism are often more stable (Amlacher et al., 2011; Baker et al., 2015), and C. thermophilum Fab1 has a similar domain architecture to both S. cerevisiae Fab1 and human PIKfyve (Figure 4A). Of importance, the CCR domain and kinase region of Fab1, including residues that are mutated in S. cerevisiae dominant-active Fab1 alleles, are conserved in C. thermophilum (Supplemental Figure S2). We generated histidine (His) maltose-binding protein (MBP)–kinase and glutathione–S-transferase (GST)–CCR fusion proteins (Figure 4A). Expression of either construct alone in E. coli yielded no soluble protein (unpublished data); however, as determined by SDS–PAGE and Western blot analysis, coexpression yielded soluble polypeptides for both the kinase region and CCR domain (Figure 4B). The recombinant polypeptides bound to each other, allowing copurification through two sequential affinity columns: amylase resin followed by glutathione–S-transferase, which bind alternatively to His-MBP-kinase and GST-CCR, respectively (Figure 4B). This provides in vitro evidence for a direct interaction between these domains.

That the kinase and CCR domains interact directly and that individual point mutations in the kinase region both enhance Fab1 kinase activity and impair the Y2H interaction between these domains suggest that the CCR domain inhibits Fab1 kinase activity through direct contact with the kinase region. Of note, another phosphoinositide kinase is hypothesized to be regulated via a protein–protein contact on the kinase domain (Budovskaya et al., 2002; Rostislavleva et al., 2015). The PI3-kinase p110α exhibits an intramolecular interaction between the adaptor-binding domain and the kinase domain (Huang et al., 2007, 2008). Oncogenic mutations in either region of p110α lead to elevated kinase activity (Zhao and Vogt, 2008) and were suggested to dissociate this interaction (Zhao and Vogt, 2008). Here we characterize a regulatory intramolecular interaction in Fab1. That this mode of regulation is seen in at least two lipid kinases makes it tempting to speculate that other phosphatidylinositol kinases and perhaps many protein kinases have similar types of regulation.
Dominant-active mutations in the CCR region of Fab1 indicate a regulatory role for this domain

A model in which the CCR domain inhibits Fab1 kinase activity predicts that hyperactivation of Fab1 can be achieved through mutation of the CCR domain. To test this prediction, we performed random mutagenesis of the CCR domain and screened for colonies that bypass deletion of the Fab1 activator Vac7 (Supplemental Figure S3A). We isolated and sequenced 21 mutants representing seven unique alleles (Figure 1B, dark green). These mutants displayed fragmented vacuoles when expressed in WT or fab1Δ cells, indicative of elevated PI(3,5)P₂ levels (Figure 5A and Supplemental Figure S3B). We directly measured PI(3,5)P₂ levels for 3 of the CCR mutants (Fab1-Q1419R, Fab1-D1486N, and Fab1-T1491A) and found that each displayed a twofold to threefold elevation of PI(3,5)P₂ compared with wild type (Figure 5B). The identification of hyperactive mutations in the CCR domain is consistent with an inhibitory role for this domain in regulating Fab1 kinase activity.

We further tested whether, as with dominant-active mutations in the kinase region, these mutations in the CCR domain disrupted the Y2H interaction of the CCR domain with the kinase region; none of these mutants did (Figure 5C). It is therefore unclear whether these CCR domain mutations enhance Fab1 kinase activity through the same mechanism as the dominant-active mutations identified in the kinase region. If the mechanisms are related, release of an inhibitory interaction between the CCR domain and kinase region may involve loss of key contacts rather than complete disruption of this interaction (Figure 5D). Alternatively, the enhanced kinase activity of the CCR mutants may be due to an independent mechanism, for instance, through enhanced association with additional positive regulators of Fab1.

These studies reveal that the Fab1 CCR domain regulates Fab1 kinase activity, and that this regulation at least in part, involves a physical interaction between these domains. Posttranslational modifications may reversibly stabilize or disrupt an inhibitory binding interaction between the CCR domain and the kinase region (Figure 5D) with or without the involvement of additional regulatory binding partners.

Structure–function studies that rely on loss-of-function mutants are often difficult to interpret: these mutations often impair protein folding or stability. These studies show that the generation and characterization of dominant-active mutations provide an effective approach to determine the function of specific protein domains. Characterization of dominant-active Fab1 alleles 1) identified a regulatory region on the putative surface of the C-terminal Fab1 kinase domain, which contains regulatory residues conserved among Fab1 proteins, and 2) identified an intramolecular interaction between this surface of the kinase domain and the upstream CCR domain (Figure 5E). Analogous genetic approaches with other genes that encode large proteins may be similarly informative. For instance, other proteins within the lipid kinase family, such as Stt4...
and Pik1, have several domains of unknown function (Foti et al., 2001; Audhya and Emr, 2002; Strahl and Thorner, 2007; Baird et al., 2008). If dominant-active alleles could be generated, this would suggest that negative regulation occurs. Moreover, this type of mutant screen has the potential to reveal residues that are critical to the negative regulation of these kinases.

MATERIALS AND METHODS

Strains, plasmids, and media

Strains are listed in Supplemental Table S1. Strains were grown in either yeast extract/peptone/glucose (YEPD) or synthetic complete (SC) minimal medium. Plasmids are listed in Supplemental Table S2. Point mutations were generated as previously described (Weiner et al., 1993; Sawano and Miyawaki, 2000).

Fluorescence microscopy

Yeast cells were grown in the appropriate SC medium to an OD400 of 0.5 and then were labeled with FM4-64 (Vida and Emr, 1995). Images were acquired using the DeltaVision RT Restoration Microscopy System (Applied Precision, Issaquah, WA).

Phosphoinositide lipid labeling and quantification

Yeast [3H]inositol labeling and total cellular phosphatidylinositol extraction, deacylation, and measurements were performed as previously described (Bonangelino et al., 2002; Duex et al., 2006a). Cells were grown in the appropriate SC medium to an OD600 of 0.5. Cells (0.2 OD600 U) were washed three times with SC medium lacking inositol and then used to inoculate 5 ml of medium lacking inositol and containing 50 µCi of myo-[3H]inositol. Cells were labeled for between 12 and 16 h of shaking at 24°C, harvested by centrifugation, and resuspended in 100 µl of inositol-free medium. For hyperosmotic shock, 100 µl of inositol-free medium with 1.8 M NaCl was added to the 100 µl of sample and then stopped via the addition of ice-cold 4.5% perchloric acid. For basal conditions, 100 µl of inositol-free medium was added, followed by the addition of ice-cold 4.5% perchloric acid. Cells were lysed on a Beadbeater (Biospec) for 2 min at room temperature, followed by resting 2 min on ice. This was repeated two more times. Cell extracts were centrifuged at 14,000 rpm for 15 min at room temperature, and pellets were washed with 100 mM EDTA, pH 8.0, and then resuspended in 50 µl of distilled deionized water. The lipids were decayed with treatment by methyleneamine, and then the samples were dried in a SpeedVac. Pellets were resuspended in 300 µl of distilled deionized water. After this, the samples were mixed with 300 µl of butanol/ethyl ether/formic acid ethyl ester (20:4:1), vortexed, and centrifuged at 14,000 rpm for 5 min, and then the aqueous phase was transferred to a fresh microcentrifuge tube. This sample extraction was performed twice. Samples were then dried on a SpeedVac, resuspended in 60 µl of distilled deionized water, and analyzed by high-performance liquid chromatography using an anion exchange, PartisphereSAX (Whatman), column, as previously described (Bonangelino et al., 2002). For comparison of phosphatidylinositol polyphosphate (PI) levels, the raw counts in each peak were expressed as a percentage of total phosphatidylinositol calculated from summation of the counts of the five detectable glycerol-inositol phosphate peaks (PI, PI3P, phosphatidylinositol 4-phosphate, PI(3,5)P2, and phosphatidylinositol 4,5-bisphosphate).

Fab1-TAP-tag pull down

A fig4Δ, FAB1-3xGFP strain was transformed with plasmids expressing Fab1-TAP and Fig4–Myc or empty vectors. We harvested 25 OD600 U of log-phase cells, lysed them in immunoprecipitation (IP) buffer (50 mM Tris, pH 7.5, 120 mM NaCl, 10 mM EDTA, 1 mM ethylene glycol tetracetic acid, 5 mM 2-glycerophosphate, 1× Roche Complete inhibitor cocktail, and 3 mM benzamidine) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 3 µg/ml leupeptin, 5 µg/ml aprotinin, and 18 µg/ml chymostatin, and removed debris by centrifugation for 5 min at 500 × g. The supernatant was mixed with 5% octyl-glucoside (Sigma-Aldrich) in lysis buffer for a final concentration of 0.5% octyl-glucoside and incubated for 30 min. Octyl-glucoside–solubilized lysate was cleared by spinning at 13,000 × g for 10 min. The supernatant was incubated for 1 h with 20 µl of immunoglobulin G (IgG) Sepharose beads (GE Healthcare). IgG beads were washed three times with 500 µl of IP buffer containing 0.5% octyl-glucoside. Bound protein was eluted by heating IgG beads with 25 µl of sample buffer (50 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) at 80°C for 5 min. SDS–PAGE and Western blot analysis were used to detect Fab1-TAP, Fab1-3xGFP, and Fig4–Myc.

Protein purification

Protein expression plasmids were generated using the pQlink plasmid vectors (Scheib et al., 2007). Proteins were expressed in E. coli BL21 star (DE3). Cells were grown in modified terrific broth (1.2% [wt/vol] tryptone, 2.4% [wt/vol] yeast extract, 4% glycerol, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5) with 0.05 mg/ml ampicillin at 37°C to an OD600 of 0.4–0.5. After 18 h of
induction with 0.2 mM isopropyl-β-D-thiogalactoside at 20°C, cells were harvested via centrifugation at 5000 × g for 15 min at 4°C. 

Cells were lysed via sonication in 1 g/10 ml of lysis buffer (25 mM NaPO₄, pH 7.5, 100 mM NaCl, 100 µl/100 ml Sigma-Aldrich protease inhibitor cocktail, one tablet/100 ml Sigma-Aldrich protease inhibitor cocktail). A pRS416-Fab1 plasmid was gapped with a Pflm1 restriction enzyme digest at 37°C overnight, which cut out a region encoding amino acids 1014–1575 of Fab1. The gapped plasmid was purified by agarose electrophoresis followed by DNA purification (Qiagen). After this, primers MJL299_PflM1_FWD (5-CATTTCTGTGGATAAGTTGGCTACG-3) and MJL300_PflM1_REV (5-CATGAGTCATAGATATACCTGTTCCAC-3) were used to reamplify this region along with 100 base pairs of overhang on either side of the cut site using Taq polymerase with the recommended buffer (Invitrogen). Pf1m1-gapped pRS416-Fab1 and the Taq-amplified gapped region were then cotransformed into an EMD Millipore) and eluted with wash buffer plus 25 mM reduced glutathione.

Yeast two-hybrid assay

The indicated pGAD and pGBD plasmids (see Supplemental Table S2) were cotransformed into the yeast strain PJ69-4A as previously described (Jin et al., 2008). Transformants were plated onto SC-LEU-TRP, replica plated onto SC-LEU-TRP (control), SC-LEU-TRP-ADE-HIS+3AT (stringent test), and SC-LEU-TRP-ADE-HIS (test), and grown for 4–12 d at 24°C. Yeast strain PJ69-4A and the pGAD and pGBD vectors were described previously (James et al., 1996).

Coomassie stain and Western blot analysis

Samples were diluted from their original concentrations as follows. Total and soluble protein, 1:50 dilution; amylose elution and glutathione elution, 1:2 dilution. Samples were heated in sample buffer for 10 min at 70°C and run on a 10% SDS–polyacrylamide gel at 70 V until the dye front ran off the gel (2–3 h). Gels were stained with GelCode Blue Stain Reagent (Thermo Scientific) for 1 h at room temperature and then washed with water to destain and image. Images were processed using ImageJ, version 1.6.0_24 (National Institutes of Health).

For Western blot analysis, samples were run on an SDS–PAGE gel, followed by an 850 V-h transfer to polyvinylidene fluoride membrane. Membranes were blocked with 2.5% milk in Tris-buffered saline/Tween-20, probed with primary antibody, washed, probed with secondary antibody, and imaged on a Typhoon 9410 Molecular Imager (GE Amersham Molecular Dynamics). Images were processed using ImageJ, version 1.6.0_24. Antibody dilutions were as follows: anti-hexahistidine (552565; BD Pharimgen), 1:3000; anti–GST (901601; BioLegend), 1:3000; anti–GFP (Roche), 1:1000; anti-TAP (Pierce), 1:5000; and anti–Myc clone 9E10 (EMD Millipore), 1:1000.

Screen for dominant-active CCR domain alleles

This screen is adapted from a previously published dominant-active screen of the kinase domain of Fab1 (Duex et al., 2006b; Supplemental Figure S3A). A pRS416-Fab1 plasmid was gapped with a Pf1m1 restriction enzyme digest at 37°C overnight, which cut out a region encoding amino acids 1014–1575 of Fab1. The gapped plasmid was purified by agarose electrophoresis followed by DNA purification (Qiagen). After this, primers MJL299_PFLM1_FWD (5-CATTTCTGTGGATAAGTTGGCTACG-3) and MJL300_PFLM1_REV (5-CATTTCTGTGGATAAGTTGGCTACG-3) were used to reamplify this region along with 100 base pairs of overhang on either side of the cut site using Taq polymerase with the recommended buffer (Invitrogen). Pf1m1-gapped pRS416-Fab1 and the Taq-amplified gapped region were then cotransformed into an EMD Millipore) and eluted with wash buffer plus 25 mM reduced glutathione.

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exponentially growing culture of vac7Δ (LWW2054). One hundred fifty single colonies that grew faster than pRS416-Fab1 WT control were picked and stuck to single colonies. Of the original 150 mutants, 79 were able to grow at 37°C. These 79 candidate mutant Fab1 plasmids were extracted from yeast, amplified in E. coli, and retransformed into vac7Δ. Twenty-one candidate mutant plasmids of the original 150 rescued the 37°C vac7Δ growth defect. Visualization of the vacuole with FM4-64 and fluorescence microscopy indicated that all 21 mutant plasmids displayed a fragmented vacuolar phenotype in a fab7Δ mutant, indicative of elevated PI(3,5)P₂ levels. Sanger sequencing of candidate plasmids revealed eight unique alleles.

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