Molecular Interactions of Yeast Frequentin (Frq1) with the Phosphatidylinositol 4-Kinase Isoform, Pik1*

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Frq1, a 190-residue N-myristoylated calcium-binding protein, associates tightly with the N terminus of Pik1, a 1066-residue phosphatidylinositol 4-kinase. Deletion analysis of an Frq1-binding fragment, Pik1-(10–192), showed that residues within 80–192 are necessary and sufficient for Frq1 association in vitro. A synthetic peptide (residues 151–199) competed for binding of [35S]Pik1-(10–192) to bead-immobilized Frq1, whereas shorter peptides (164–199 and 174–199) did not. Correspondingly, a deletion mutant, Pik1Δ152–191, did not co-immunoprecipitate efficiently with Frq1 and did not support growth at elevated temperature. Site-directed mutagenesis of Pik1-(10–192) suggested that recognition determinants lie over an extended region. Titration calorimetry demonstrated that binding of an 83-residue determinant to Frq1 is largely entropic, and even a non-myristoylated Frq1(G2A,C15A) double mutant rescued the inviability of frq1Δ cells. This study defines the segment of Pik1 required for high affinity binding of Frq1.

Recognition that phosphoinositides and inositol phosphates are key regulators of many processes in eukaryotic cells has brought increased attention to the enzymes that regulate the synthesis and turnover of these molecules (reviewed in Refs. 1–3). Of particular interest are the enzymes responsible for producing the various polyphosphoinositides situated on the cytosolic face of cellular membranes, which initiate several different signaling pathways by serving as highly specific recognition determinants for the selective recruitment of proteins to membranes (reviewed in Refs. 4–7) and as the precursors for several intracellular second messengers (reviewed in Refs. 8–10). The first committed step in the synthesis of the polyphosphoinositide, phosphatidylinositol 4,5-bisphosphate, is considered to be ATP-dependent phosphorylation of the hydrophilic myo-inositol head group of phosphatidylinositol (PtdIns)1 at the d-4 position by PtdIns 4-kinase (ATP:1-phosphatidyl-1D-myoinositol 4-phosphotransferase, EC 2.7.1.67) (reviewed in Refs. 11–13). The resulting product, PtdIns 4,5(P2), can be phosphorylated on the d-5 position by PtdIns(4)P 5-kinase to generate PtdIns(4,5)P2, which can be phosphorylated on the d-3 position by yet other lipid kinases, and the phosphoinositides so generated can be converted to other species by specific phosphatases and phospholipases (reviewed in Refs. 14–17).

The first PtdIns 4-kinase to be purified to homogeneity from any organism (18), and to have the corresponding gene cloned (19, 20), was Pik1 from the yeast Saccharomyces cerevisiae. Thereafter, a second isoform, Stt4, which is the product of a discrete gene, was described (21). Absence of either Pik1 or Stt4 is lethal, and overproduction of each protein cannot compensate for absence of the other, indicating that these enzymes participate in distinct cellular processes and generate discrete pools of PtdIns(4)P that are essential for yeast cell viability. Indeed, subsequent work has shown that, together, Pik1 and Stt4 account for all of the PtdIns(4)P generated in the yeast cell (22) and that Pik1 is required for vesicular trafficking in the late secretory pathway (23, 24) and perhaps for cytokinesis (20), whereas Stt4 plays roles in cell wall integrity, maintenance of vacuole morphology, and aminophospholipid transport from the endoplasmic reticulum to the Golgi (25–27). The presence of Pik1- and Stt4-like isoforms is also conserved in metazoans (11, 12, 28).

We have shown previously that Frq1, a small calcium-bind-
ing protein, co-purifies with Pik1 and is required for optimal activity of the enzyme (29). Frq1 is the yeast ortholog of a protein called frequenin, first described in Drosophila (30), and referred to as neuronal-calumel-sensor-1 (NCS-1) in mammalian cells. Members of a large subfamily of small, EF-hand-containing, calcium-binding proteins that includes frequentin (31–34) are characterized by a consensus signal for N-terminal myristoylation and four Cys2-His2-binding sites (of which the first and, in some cases, the fourth or another contain substitutions that make them non-functional). We have shown previously that Frq1 binds three Cys2-His2 (35). Available evidence indicates that frequentin/NCS-1 may also modulate PtdIns 4-kinase activity in animal cells (36, 37).

Frq1, which is itself essential for the viability of yeast cells (29), associates with membranes in a manner that depends on both the N-myristoyl group and conformational changes induced upon Cys2-His2 binding (35). Thus, in addition to its stimulation of enzymatic activity, Frq1 may contribute to the optimal function of Pik1 by assisting with its membrane recruitment, because Pik1 itself lacks any obvious membrane-targeting motifs. Indeed prior work indicated that N-myristoylation of Frq1 is important, but not essential, for its function (29). In some Cys2-His2-binding proteins, in addition to the N-terminal myristoyl group, palmitoylation of a cysteine residue near the N terminus is also required for efficient membrane association (38, 39). Frq1 has only two Cys residues, one is near its N terminus and the other buried in the interior (35).

In this study, as a prelude to structural analysis to determine at atomic resolution how Frq1 recognizes Pik1, we have applied several independent methods to determine the affinity and stoichiometry of the Frq1-Pik1 interaction, used different approaches to delineate the sequences in Pik1 responsible for high affinity binding of Frq1 and utilized both biochemical and genetic techniques to explore the role, if any, of S-palmitoylation in the function of Frq1.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions—** Yeast strains used in this study are listed below in Table I. Standard rich (YP) and synthetic complete (SC) media (40) were supplemented with carbon sources (either 2% Glc, or 2% Gal/0.2% Suc, as indicated) and with appropriate nutrients for the selection and maintenance of plasmids. Yeast was cultivated at 30°C, unless otherwise noted. Conventional methods for DNA-mediated transformation and other genetic manipulations of yeast cells were used (41).

**Plasmid Construction—** Plasmids were constructed using standard methods for the manipulation of recombinant DNA (42). Escherichia coli DH5 invites (43) was used for routine manipulation and propagation of plasmids. Unless otherwise indicated, all PCR reactions were performed using Pfu DNA polymerase (Stratagene, La Jolla, CA). All recombinant plasmids were verified by dye-oxy chain termination sequencing.

Fragments of the N terminus of Pik1 were tagged with a C-terminal His10 tag and expressed in E. coli, as follows. pET23d–PIK1(10–192) was constructed by inserting via blunt-end ligation the HindIII fragment of PIK1 into pET23d (Novagen, WI) that had been cleaved with NcoI and EcoRI. pET32a–PIK1(10–192, ΔN17–79) was produced by cleaving pET32a–PIK1(10–192) with EcoRI and NcoI and retaining the plasmid after filling in the recessed 3'-ends using Klenow fragment of E. coli DNA polymerase I. pET32a–PIK1(10–163) and pET32a–PIK1(10–125) were generated by PCR amplification of pET32a–PIK1(10–192) using as primers spanning the NcoI site of PIK1 and oligonucleotides introducing a NcoI site 3' to the sequences encoding, respectively, either codon 163 or codon 125 of the PIK1 open-reading-frame. The resulting PCR fragments were ligated into pET32a–PIK1(10–192) that had been cleaved with NcoI and NolI. An XbaI–XhoI fragment of pET32a–PIK1(10–192) was inserted into pET28a–PIK1(10–192) using as primers the XbaI–NcoI cleaved with the same enzymes, to generate the cloning intermediate, Litmus28–PIK1(10–192), Litmus28–PIK1(10–192, P181A,V183A) and Litmus28–PIK1(10–192, R188A,R189A,) that had been cleaved with EcoRI and XhoI and inserted into the corresponding sites of pET23d–PIK1(10–192), yielding pET23d–PIK1(10–192, R188A,R189A,) and pET23d–PIK1(10–192, L175A,P181A,V183A) that had been cleaved with EcoRI and XhoI and ligated with a PCR product carrying the appropriate substitutions and a XhoI site 3' to sequences encoding residue 192 of Pik1, all introduced by site-directed mutagenesis, was cleaved with EcoRI and XhoI and inserted into the corresponding sites of pET23d–PIK1(10–192), yielding pET23d–PIK1(10–192, R188A,R189A,) and pET23d–PIK1(10–192, L175A,P181A,V183A) that had been cleaved with EcoRI and XhoI and ligated with a PCR product carrying the appropriate substitutions to produce E154A, N155A, and V158A) that had been cleaved with EcoRI and PstI and ligated with a PCR product carrying the appropriate substitutions to produce N155A, V156A, and P158A) cleaved with PstI and PstI. pET23d–FRQ1 has been described previously (29, 39).

Multicopy (2-μm, DNA-based) URA3-marked plasmids, Yepl352–FRQ1, Yepl352–GAL–FRQ1, and Yepl352–FRQ1(Gal) (29) have been described previously (29). Yepl352–GAL–FRQ1(Gal) was cleaved by excising the corresponding fragment from pET32a–FRQ1(Gal) (29) and inserting it into pET23d–GAL-44. Yepl352–FRQ1(Gal) was generated by replacing a HindIII fragment of pET32a–FRQ1(Gal) with a PCR product, carrying the appropriate mutations to encode C15A, that was digested with the same enzymes. The low copy (CEN-based) URA3-marked plasmid, pRS316–FRQ1(Gal) (29), was generated by inserting an EcoRI fragment from Yepl352–FRQ1(Gal) into pRS316–FRQ1 (29). Construction of the TRP1-marked CEN plasmids, pRS14–PIK1, pRS14–GAL10, and pRS14–GAL–mycPIK1 is described elsewhere (45). The cloning intermediate, Litus28–PIK1, was produced by inserting a BamHI–SacI fragment containing the entire coding sequence of PIK1, excised from pRS14–PIK1, into Litus28. Litus28–PIK1(Δ152–191) was generated by insertion of a PCR-derived HindIII fragment, encoding residues 10–151 of Pik1, into Litus28–PIK1(Δ152–191) that had been cleaved with HindIII. pRS14–PIK1(Δ152–191) was produced by subcloning the EcoRI fragment of Litus28–PIK1(Δ152–191) into pRS14–PIK1. Inserting the BamHI–SacI fragment of pRS14–PIK1(Δ152–191) into pRS14–PIK1(Δ152–191) generated the pRS14–GAL10, which had been cleaved with SacI and BamHI, yielding pBAT4–FRQ1. A PCR fragment was amplified from pET23d–PIK1(10–192) using appropriate primers to introduce a Smal site 5' to the coding sequence and a HindIII site 3' to codon 192 of the Pik1 open-reading frame, cleaved with Smal and HindIII, and ligated into pBAT4 that had been cleaved with the same enzymes, generating pBAT4–PIK1(10–192). "S-Labeled proteins were produced by coupled in vitro transcription and translation in the presence of [35S]Met and [35S]Cys (PerkinElmer Life Sciences, Boston, MA) using the TNT coupled reticulocyte lysate system (Promega, Madison, WI), according to the manufacturer's instructions. Translation mixtures were performed in 15 μl, at 30°C, 4°C at 4°C in a microcentrifuge. If not used immediately, the resulting supernatant fractions were mixed with an equal volume of glycerol and stored at −20°C.

**Bacterial Expression and Purification of (His)10-tagged Proteins—** The Pik1 and Frq1 constructs containing a C-terminal His10 tag were expressed in E. coli strain BL21 (Novagen, WI) that had been transformed with pET23d–PIK1(10–192) using as primers the NcoI cleaved with the same enzymes, to generate the cloning intermediate, Litmus28–PIK1(10–192), Litmus28–PIK1(10–192, P181A,V183A) and Litmus28–PIK1(10–192, R188A,R189A,) that had been cleaved with EcoRI and XhoI and ligated with a PCR product carrying the appropriate substitutions and a XhoI site 3' to sequences encoding residue 192 of Pik1, all introduced by site-directed mutagenesis using a commercial kit (QuickChange, Stratagene, La Jolla, CA), according to the manufacturer's instructions. The XbaI–XhoI fragments from Litus28–PIK1(10–192, P181A,V183A) and Litus28–PIK1(10–192, L175A,P181A,V183A) were generated by exchanging the T7fl fragment in Litus28–PIK1(10–192) with T7fl-digested PCR prod-
was grown in SC-Raf lacking uracil at 30 °C to mid-exponential phase and induced by addition of Gal (2% final concentration). After incubation for 6 h at 30 °C, cells were collected by centrifugation and resuspended in an equal volume of distilled water. The cell suspension was frozen by drying in liquid nitrogen, and the resulting pellets were crushed with a pestle in a precooled mortar. The resulting frozen cell powder was dissolved in lysis buffer (5 mM imidazole, 145 mM NaCl, 50 mM Na-PO4 (pH 7.5); 20-mU/1 liter yeast culture) containing a mixture of protease inhibitors (Complete™, Promega, Madison, WI). The crude lysate was clarified by centrifugation at maximum rpm in a microcentrifuge at 4 °C for 15 min and then at 72,000 × g for 90 min in a LS–80 ultracentrifuge (Beckman-Coulter Inc., Fullerton, CA). The resulting supernatant fraction was then applied to a Ni2+-saturated NTA-agarose beads (Qiagen, Hilden, Germany) that had been pre-saturated with three volumes of lysis buffer. After washing with 10 bed volumes of lysis buffer and 6 volumes of wash buffer (20 mM imidazole, 145 mM NaCl, 50 mM Na-PO4 (pH 7.5)), the bound Frq1-His6 was eluted with 3 volumes of elution buffer (120 mM imidazole, 145 mM NaCl, 50 mM Na-PO4 (pH 7.5)). The eluate was concentrated by ultracentrifugation through an anisotropic membrane (3-kDa cut-off, Centricon YM-3, Amicon, Beverly, MA) until a final concentration of 0.5 μM/ml was reached. To confirm identity and purity, the resulting fraction was resolved by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue and by immunoblotting.

In Vitro Protein Binding Assays—Prior to use, Ni2+-saturated NTA-agarose beads used in protein binding and peptide competition experiments were pre-blocked by incubation for 30 min in 8 volumes of buffer A (10 mM imidazole, 100 mM NaCl, 1 μg/ml CaCl2, 1 mM dithiothreitol, 50 mM Tris-HCl (pH 7.4)) containing 5 mg/ml ovalbumin at room temperature. All in vitro binding assays were carried out at 4 °C. Radiolabeled Frq1, prepared by coupled in vitro transcription and translation, was mixed with an equal volume of a slurry of pre-blocked Ni2+-saturated NTA-agarose beads in buffer A and incubated on a roller drum for 30 min. The beads and any nonspecifically bound radioactivity were removed by brief sedimentation in a microcentrifuge, and the resulting pre-cleared supernatant fraction was collected. Aliquots (400 μl) of the pre-cleared fraction were mixed either with 40 μl of pre-blocked Ni2+-saturated NTA-agarose beads for buffer A, as a control, or with Ni2+-saturated NTA-agarose beads on which had been immobilized Pik1-(10–192)-His6 or its deletion derivatives (30 μg of protein/40 μl of beads) and incubated for 1 h on a roller drum. The beads were collected by centrifugation for 15 s in a microcentrifuge and washed three times with buffer A. Bound proteins were eluted from the beads in 50 μl of buffer A containing 300 mM imidazole, and samples of the resulting eluate were resolved by SDS-PAGE on a 12% gel and visualized by autoradiography.

Synthetic peptides corresponding to Pik1-(174–199), Pik1-(164–199), and Pik1-(151–199) were prepared by standard solid phase peptide synthesis (using Fmoc chemistry) on an automated synthesizer (Model ABI 431A, PerkinElmer Life Sciences-Applied Biosystems, Foster City, CA) and purified by high-performance liquid chromatography (Model ABI 431A, PerkinElmer Life Sciences-Applied Biosystems, Foster City, CA) and incubated at 4 °C with either 2 μM Frq1-His6, or either Pik1-(151–199) or Pik1-(1–110). The resulting mixtures were subjected to SDS-PAGE. The species corresponding to [35S]Pik1-(151–192) (10 μCi) was produced primarily in inclusion bodies, which were solubilized using 8 M guanidine hydrochloride (40%) and purified using Ni2+-saturated NTA-agarose chromatography, essentially as described above. Samples for NMR analysis were prepared by dissolving [15N-labeled Frq1 (0.4 mM) with various amounts (0, 1, or 2 molar equivalents) of Pik1-(110–192) or the synthetic peptide, Pik1-(151–192), in 0.5 ml of a 95% H2O/5% [1H15N]H2O solution containing 10 mM imidazole (pH 6.7), 10 mM [1H15N]dithiothreitol, and either 1 mM EDTA (Ca2+-free) or 5 mM CaCl2 (Ca2+-bound). All NMR experiments were performed at 37 °C on a Model DRX-500 or DRX-600 NMR spectrometer (Bruker Instruments, Billerica, MA) equipped with a four-channel interface and a triple-resonance probe with triple-axis pulsed field gradients as described before (35). The NMR spectra were processed and analyzed as described previously (50).

Fluorescence Spectroscopy—The effect of Pik1 peptides on the intrinsic tryptophan fluorescence emission of Frq1, excited at 290 nm, was measured (at 300–420 nm) using a SPEX fluorometer (Jobin Yvon Inc., Edison, NJ). Neither Pik1-(151–199) nor Pik1-(110–192) contain any tryptophan, and they do not contribute any fluorescence under these conditions. Titrations were performed using 5 μM Frq1 in 2 ml of 50 mM HEPES (pH 7.5), 0.1 mM KCl, 1 mM CaCl2, 1 mM dithiothreitol at 25 °C. Various amounts (0, 0.5, 1, 1.5, 2.0, 3.0, and 4.0 molar equivalents) of either Pik1-(151–199) or Pik1-(110–192) were injected into the sample cuvette containing the Frq1 solution, and fluorescence emission spectra were recorded after each addition.

Isothermal Titration Calorimetry—Binding of Ca2+-Fyrq1 to either Pik1-(151–199) or Pik1-(110–192) was measured by isothermal titration calorimetry (51) using a MicroCal VP-ITC MicroCalorimeter (Microcal Inc., Northampton, MA). Frq1 and Pik1-(110–192) protein samples were dialyzed against a buffer containing 10 mM HEPES (pH 7.4), 5 mM CaCl2, and 1 mM dithiothreitol. Lyophilized Pik1-(151–199) was dissolved in the same buffer used for the dialysis of Frq1. Experiments were performed at 25 °C. Into the sample cell containing a solution (10 μM) of either Pik1-(151–199) or Pik1-(110–192) were injected a total of 270 μl of 58 μM Frq1 in 18 aliquots. Heats of dilution, determined by titrating Frq1 into same buffer alone, were subtracted from the raw titration data before data reduction and analysis. A single-site model was fit to the data using the background for Pik1-(151–192) to Pik1-(110–192) binding to Frq1. A two-site model was needed to describe the multiphasic interaction of Pik1-(110–192) with Frq1.

Preparation of Yeast Cell Extracts, Immunoprecipitation, and Immunoblot Analysis—Lysis conditions and centrifugation for the preparation of clarified yeast cell extracts for immunoprecipitation were as described previously (29). Samples (1 mg of total protein) of extract were diluted into lysis buffer (220-μl final volume) and mixed with 40 μl of a 1:1 (settled beads:buffer) suspension of protein G−/protein A-coupled agarose beads and the mixture was incubated on the roller drum for 45 min at 4 °C. Bead-bound immune complexes were collected by brief centrifugation in a microcentrifuge, washed four times with lysis buffer (1 ml each), resuspended in SDS-PAGE sample buffer, and solubilized by boiling in a water bath for 5 min. After removal of the beads by centrifugation, equal volumes of the resulting supernatant were collected, concentrated by SDS-PAGE and analyzed by immunoblotting with appropriate antibodies.

Analytical Size Exclusion Chromatography—Size exclusion chromatography was performed on a Superdex 200 column (Amersham Biosciences, Piscataway, NJ) using an fast protein liquid chromatography apparatus (650E Advanced Protein Purification System, Waters, Milford, MA) that was operated at a constant flow rate of 1 ml/min at 4 °C (pH 7.6) containing 150 mM NaCl. Protein standards (either High Molecular Weight Gel Filtration Calibration kit from Amersham Biosciences, Piscataway, NJ, or Gel Filtration Standard from Bio-Rad, Hercules, CA) were prepared according to the manufacturer’s specifications and loaded onto the column (90-ml bed volume). Fractions (0.75 ml) were collected and the protein concentration of each fraction was determined by Bradford method, as described above. The column void volume (V0) was assessed using blue dextran 2000. Kav values for each protein were calculated using the equation, Kav = (Vv − V0)/[(Vf − V0)], where V0 = 36 ml and Vf = 90 ml,
and plotted semilogarithmically against the corresponding molecular weight. To examine the apparent molecular weight of native Pik1-Frq1 complexes in yeast cell extracts, strain YPH499, transformed with pRS314-GAL-mycPik1, was grown to an optical density of 0.6 in SCraf-Trp at 30 °C and induced by addition of Gal (2% final concentration) and incubated for 1.5 h. Induced cells were collected by centrifugation, washed once with distilled water, resuspended in ice-cold 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and protease inhibitors (Complete™, Promega, Madison, WI). The washed cells were broken by 10 pulses (1 min each) of vigorous vortex mixing with glass beads. The resulting crude extract was clarified by centrifugation in a TL-100 tabletop ultracentrifuge (Beckman Coulter Inc., Fullerton, CA) at 49,000 × g for 30 min at 4 °C. The protein concentration of the clarified extract was determined, and 250 μl (2.5 mg of total protein) was loaded onto the gel filtration column. Fractions (0.75 ml) were collected, and protein was concentrated by precipitation with 10% trichloroacetic acid in the presence of 0.15% deoxycholate for 10 min at room temperature. The precipitates were dissolved in 30 μl of SDS-PAGE sample buffer, and the remaining acid was neutralized by addition of 5 μl of an unbuffered saturated solution of Tris. The resulting fractions were split into two, resolved by SDS-PAGE, and analyzed separately by immunoblotting with either anti-c-Myc or anti-Frq1 antibodies, respectively.

Mass Spectrometry—Mass measurements were performed by electrospray-ionization mass spectrometry using a Model 3000 ion trap mass spectrometer (Bruker Instruments, Billerica, MA). Prior to determining its mass spectrum, each peptide or protein was desalted by microbore reversed-phase high-performance liquid chromatography.

RESULTS

Residues 164–192 of Pik1 Are Necessary for the Binding of Frq1—We previously demonstrated that Frq1 associates tightly with Pik1 and that a fragment of the N-terminal domain of Pik1 comprising residues 10–192 is sufficient to mediate this interaction (29). We also noted before (19) that this segment of Pik1 contains a sequence element (residues 35–110), distinct from the catalytic domain per se, that is weakly conserved among PtdIns 3-kinase, PtdIns 4-kinases, and even more distantly related enzymes that appear to be protein kinases, such as the Tor proteins. This motif has been referred to subsequently as the “lipid kinase unique domain” (LUK) (12, 28, 53). Because Frq1 binds to the region of Pik1 that contains the LUK, we suggested that the motif itself might be the binding site for this regulatory protein (29).

To test this hypothesis directly and to begin to define the minimal region in Pik1 responsible for Frq1 binding, we constructed three deletion derivatives of the Frq1-binding Pik1-(10–192) fragment. One deletion (Δ31–79) removed the most conserved core of the LUK motif; the other two deletions were truncations that removed 29 residues (Δ164–192) and 67 residues (Δ126–192), respectively, from the C-terminal end (Fig. 1A). Full-length Pik1-(10–192) and each of the three deletions were tagged with a C-terminal His6 tag, expressed in and purified from E. coli, and immobilized on Ni2+-saturated NTA-agarose beads. Solubilization and analysis by SDS-PAGE followed by staining with Coomassie Brilliant Blue demonstrated that equivalent amounts of each construct were affixed to the beads (Fig. 1B, lower panel). The beads were then incubated with [35S]Frq1, prepared by coupled in vitro transcription and translation, washed, and subjected to SDS-PAGE, and the amount of radiolabeled Frq1 bound was analyzed by autoradiography. As observed previously using unlabeled Frq1 (29), Pik1-(10–192) bound radiolabeled Frq1 avidly, and there was little or no nonspecific binding to empty beads (Fig. 1B, upper panel). Strikingly, the 49-residue deletion lacking the heart of the LUK motif showed no detectable diminution in its ability to bind [35S]Frq1. In marked contrast, even the shortest (29-residue) C-terminal truncation completely ablated Frq1 binding. This result suggested that residues in the region 164–192 of Pik1 are necessary for the high affinity binding of Frq1. Moreover, these data indicated that the LUK motif does not mediate the interaction between Pik1 and Frq1.

FIG. 1. In vitro binding of [35S]Frq1 to immobilized Pik1-(10–192)-(His)6, and derived mutants. A, schematic diagram of Pik1 (top bar) and, in an expanded view, the N-terminal fragment (10–192) fragment (second bar) and an internal deletion mutant and two C-terminal truncation mutants derived from Pik1-(10–192). The LUK domain (residues 35–110) and the C-terminal catalytic domain (residues 792–1066) are also indicated. B, Pik1-(10–192)-(His)6 and the three derived mutants shown in A were expressed in bacteria, purified, bound to Ni2+-saturated NTA-agarose, and incubated with [35S]Frq1 prepared by in vitro translation. Bound-immunoreactivity was solubilized by boiling in SDS-gel sample buffer, resolved by SDS-PAGE, and detected by autoradiography (upper panel). To verify purity and equivalent loading of Pik1-(10–192) and the three derived mutants, bead-bound proteins were subjected to SDS-PAGE and visualized by staining with Coomassie Brilliant Blue dye (lower panel).

Residues 151–199 of Pik1 Are Sufficient for Binding to Frq1—To determine if the region of Pik1 from 164 to 192, or some sub-domain of it, was sufficient for Frq1 binding, we used an approach involving competition by synthetic peptides corresponding to different portions of Pik1 (Fig. 2A). For these binding experiments, the arrangement was reversed. In this case, [35S]Pik1-(10–192), prepared by coupled in vitro transcription and translation, was incubated with either empty Ni2+-saturated NTA-agarose beads (as a control for nonspecific binding) or the same beads coated with purified Frq1-His6, preincubated in the absence or the presence of increasing concentrations (1 nM to 20 μM) of the competing peptides. We found that the 36-residue peptide corresponding to residues 164–199 was unable to block the binding of [35S]Pik1-(10–192) to immobilized Frq1 (Fig. 2B), and, not surprisingly, a shorter 26-residue peptide, residues 174–199, was also ineffective (data not shown). Thus, although residues 164–192 of Pik1 are necessary for Frq1 binding, they are not sufficient. Indeed, we found that a longer 49-residue peptide, corresponding to residues 151–199, was able to prevent the binding of [35S]Pik1-(10–192) to Frq1 in a dose-dependent manner (Fig. 2B). Hence, we conclude that the Frq1-binding site
includes residues in Pik1 upstream of position 164.

The results from three independent trials measuring the ability of the 49-residue peptide (151–199) to compete for the binding of \(^{35}\text{S}\)Pik1-(10–192) to bead-immobilized Frq1-His\(_6\) were quantitated, after correcting for the minimal background binding of \(^{35}\text{S}\)Pik1-(10–192) to empty beads at each peptide concentration (Fig. 2C). The resulting data were then normalized to the amount of radioactivity bound in the absence of peptide and fitted to the following equation: 

\[
\frac{[\text{Frq-Pik} + \text{Pep}][\text{Frq-Pik} - \text{Pep}]}{[\text{Frq-Pik} + \text{Pep}]} = \frac{a}{b + c + (1 + [\text{Pep}]/K_c),}
\]

where \(K_c\) is the apparent dissociation constant for peptide binding, Frq-Pik + Pep is the amount of radiolabeled Pik1-(10–192) bound to Frq1 at a given peptide concentration and Frq-Pik – Pep is the amount of radiolabeled Pik1-(10–192) bound to Frq1 in the absence of peptide and \(a\), \(b\), and \(c\) are arbitrary constants. Assuming a stoichiometry for binding of \(^{35}\text{S}\)Pik1-(10–192) to immobilized Frq1-His\(_6\) of 1:1, \(K_c\) for binding of the 49-residue peptide (151–199) to Frq1 was 1.1 ± 0.5 \(\mu\text{M}\).

**Structural Characterization of Frq1 Target Complexes Using NMR** — NMR spectroscopy was used to examine whether any conformational changes occurred in Frq1 upon binding to either the 49-residue synthetic peptide (151–199) or to a somewhat larger 83-residue fragment of Pik1, Pik1-(110–192), prepared by expression in and purification from bacterial cells (see “Experimental Procedures”). Two-dimensional NMR experiments (\(\text{H}^1\)-\(\text{N}\) HSQC) were performed on samples of uniformly \(^{15}\text{N}\)-labeled Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms of Pik1, prepared as described previously (35), in the presence and absence of saturating amounts of either the 151–199 peptide or the Pik1-(110–192) fragment. Initial attempts to prepare NMR samples of Frq1 complexed with the 151–199 peptide were unsuccessful, because simple addition of the peptide to a solution of Frq1 at a concentration (0.4 mM) sufficient for NMR analysis resulted in irreversible denaturation and aggregation. In contrast, when a dilute solution of Ca\(^{2+}\)-bound Frq1 (20 \(\mu\text{M}\)) was added slowly to an equal volume of a dilute solution of the Pik1 peptide or Pik1 fragment (20 \(\mu\text{M}\)), a soluble complex was produced that could then be concentrated more than 20-fold to yield a stably soluble sample adequate for NMR studies. This approach was only successful in preparing complexes with Ca\(^{2+}\)-bound Frq1. Mixtures of Ca\(^{2+}\)-free Frq1 with the Pik1 peptide or Pik1 fragment were much less soluble and, hence, were not characterized further.

Two-dimensional NMR (\(\text{H}^1\)-\(\text{N}\) HSQC) spectra for Ca\(^{2+}\)-bound Frq1 in the absence and presence of Pik1-(110–192) are shown in Fig. 3. The spectrum of Ca\(^{2+}\)-bound Frq1 alone (Fig. 3A) was analyzed previously (35). Free Ca\(^{2+}\)-bound Frq1 contains fewer peaks than the expected number of amide groups in the protein (180 versus 220), apparently because some of the amide resonances have extremely weak intensity and therefore escape detection. Moreover, the wide range of NMR peak intensities suggested that Ca\(^{2+}\)-bound Frq1 exists as a somewhat heterogeneous population of species. In agreement with this conclusion, dynamic light scattering measurements performed on the sample of free Ca\(^{2+}\)-bound Frq1 used for the NMR analysis was indicative of significant polydispersity, suggest-

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**Fig. 2. Competition by synthetic peptides for binding of \(^{35}\text{S}\)Pik1-(10–192) to immobilized Frq1-(His)\(_6\).** A, schematic representation of three synthetic peptides spanning the Frq1-binding segment of the N-terminal region of Pik1. B, \(^{35}\text{S}\)Pik1-(10–192), prepared by *in vitro* translation (input, lane 1), was incubated with either empty Ni\(^{2+}\)-saturated NTA-agarose beads, in the absence (lane 2) or the presence (lane 3) of synthetic peptide, or with the same beads coated with Frq1-His\(_6\), in the absence (lane 4) and at the indicated concentrations of synthetic peptide (lanes 5–9). Bead-bound radioactivity was solubilized by boiling in SDS-gel sample buffer, resolved by SDS-PAGE, and detected by autoradiography (upper panel). Competition by the 151–199 peptide (upper panel) and the 164–199 peptide (lower panel) are shown. C, results from three independent trials of competition by the 151–199 peptide for binding of \(^{35}\text{S}\)Pik1-(10–192) to Frq1-His\(_6\) were quantitated using a PhosphorImager (Amersham Biosciences, Sunnyvale, CA) and corrected for the low nonspecific binding of the probe to ovalbumin-blocked Ni\(^{2+}\)-saturated NTA-agarose beads at each peptide concentration used. The values obtained were then normalized to the signal observed in the absence of any competing peptide and fit, assuming a stoichiometry of \(^{35}\text{S}\)Pik1-(10–192) binding to Frq1-His\(_6\) of 1:1, to the following equation: 

\[
\text{Relative Frq1(10–192) Bound} = \frac{[\text{Frq-Pik} + \text{Pep}]/[\text{Frq-Pik} - \text{Pep}]}{[\text{Frq-Pik} + \text{Pep}]} = \frac{a}{b + c + (1 + [\text{Pep}]/K_c),}
\]

where \(K_c\) is the apparent dissociation constant for peptide binding, Frq-Pik + Pep is the amount of radiolabeled Pik1-(10–192) bound to Frq1 at a given peptide concentration and Frq-Pik – Pep is the amount of radiolabeled Pik1-(10–192) bound to Frq1 in the absence of peptide and a, b, and c are arbitrary constants.
ing that Ca²⁺-bound Frq1 consists of a heterogeneous mixture of monomer, dimer, and higher oligomeric species under the concentration conditions required for NMR.

In marked contrast, the NMR spectrum of Ca²⁺-bound Frq1 in the presence of a saturating amount of Pik1-(110–192) (Fig. 3B) looked quite different from that of free Ca²⁺-bound Frq1, suggesting that Pik1 binding induces conformational changes in Frq1. The NMR spectrum of Ca²⁺-bound Frq1 in the presence of the 151–199 peptide looked essentially identical to that of the Frq1-Pik1-(110–192) complex (data not shown). The spectrum of each complex exhibits significantly sharper peaks compared with those in the spectrum of Ca²⁺-bound Frq1 alone, suggesting, first, that Frq1 is monomeric in each complex. Second, in the spectrum of the complex, some new peaks are observed that are not seen in the spectrum of free Ca²⁺-bound Frq1 (compare Fig. 3, A and B). The total number of observable peaks in the spectra of the complexes of Ca²⁺-bound Frq1 with either the 151–199 peptide or the Pik1-(110–192) fragment are now very close to the expected number of amide resonances (218 versus 220). Third, the intensities of all peaks in the spectra of the complexes are much more uniform than those of free Ca²⁺-bound Frq1, suggesting that both complexes are stable and homogeneous. In agreement with this conclusion, dynamic light scattering measurements performed on the sample of Ca²⁺-bound Frq1 at a saturating concentration of Pik1-(110–192) that was used for NMR analysis indicated a monodisperse scattering profile with a molecular mass of ~35 kDa, consistent with a monomer of Ca²⁺-saturated Frq1 (22.1 kDa) bound to one molecule of the His₆-tagged 85-residue Pik1-(110–192) fragment (10.0 kDa). Finally, the changes in the NMR spectrum of Ca²⁺-bound Frq1 induced in the presence of Pik1-(110–192) reached saturation upon the addition of 1 molar equivalent of the Pik1-(110–192) complex (data not shown), consistent with formation of a high affinity peptide:Frq1 complex with a stoichiometry of 2:1. Moreover, the NMR spectrum of Ca²⁺-bound Frq1 in the presence of 1 molar equivalent of the 151–199 peptide (data not shown) resembled the composite that would be expected from equal parts of the spectra (Fig. 3, A and B) for free and fully complexed Ca²⁺-bound Frq1. Thus, it seems that in the presence of 1 molar equivalent of the 151–199 peptide, half of the Frq1 molecules are peptide-bound and half are unbound. Furthermore, these observations indicate that the population of peptide-bound Frq1 molecules exchanges with the unbound species only very slowly, such that the dissociation rate must be slower than the time scale of NMR chemical shift, as expected for a high affinity complex. On this basis, we estimate that the dissociation constant for binding of the 151–199 peptide to Ca²⁺-bound Frq1 in solution must be in the nanomolar range.

**Pik1-Frq1 Interaction Monitored by Fluorescence Spectroscopy**—The 190-residue Frq1 polypeptide contains just two tryptophan residues (Trp-30 and Trp-103). The fluorescence emission of Trp is very sensitive to its surrounding chemical environment and, hence, provides a well-documented method for probing structural changes in a protein (54). Hence, we used the change in the intrinsic Trp fluorescence of Frq1 as an independent means both to monitor the effects of and to quantitate the binding of Frq1 to the Pik1-derived fragment and the synthetic peptide (see Fig. S1 in the Supplemental Material). Neither the Pik1-(110–192) fragment nor the 151–199 peptide contain any Trp; as expected, the fluorescence spectra of neither the Pik1-(110–192) fragment alone nor the 151–199 peptide alone exhibited any significant emission over the range of wavelengths examined. The fluorescence emission spectrum of free Ca²⁺-bound Frq1 exhibited a maximum at 340 nm, as observed before (35). Addition of a saturating concentration of either the Pik1-(110–192) fragment or the 151–199 peptide
increased the emission intensity by 30% and caused the emission maximum to shift very slightly toward the blue. These results indicate that, upon binding of the Pik1 sequences, conformational changes in Frq1 occur that cause one or both of its Trp residues to become more constrained and/or to enter a less solvent accessible and more non-polar environment. The emission intensity of Frq1 increased linearly with the addition of the Pik1-(110–192) fragment in the range from 0 to 1 molar equivalents, and no further increase was observed when more than 1 equivalent was added. Thus, in agreement with the conclusions drawn from the NMR and light scattering analysis, the stoichiometry of the complex of Pik1-(110–192) with Ca$^{2+}$-bound Frq1 is 1:1. In contrast, the emission intensity of Frq1 increased linearly with the addition of the 151–199 peptide in the range from 0 to 2 molar equivalents, and no further increase of intensity was observed when more than 2 molar equivalents of peptide were added. Again, this result agrees with the conclusions drawn from the NMR and light scattering analysis, which indicated that the stoichiometry of the complex of the 151–199 peptide with Ca$^{2+}$-bound Frq1 is 2:1.

Energetics of Pik1-Frq1 Interaction—As yet another independent means to examine the association of Frq1 with its apparent binding site in Pik1, we used isothermal titration calorimetry, which also permitted assessment of the energetics of the binding interaction between Ca$^{2+}$-bound Frq1 and either the Pik1-(110–192) fragment (Fig. 4, A and B) or the 151–199 peptide (Fig. 4, C and D). These calorimetric titrations were conducted at 25 °C in 10 mM HEPES (pH 7.4). In both cases, the heat change was endothermic, indicating that the binding reaction is largely entropically driven, most consistent with desolvation of one or both partners and a hydrophobic interaction between them. After correction for the heats of dilution (see "Experimental Procedures") and normalization, the concentration dependence of the absorbed heats were plotted for the interaction of Ca$^{2+}$-bound Frq1 with the Pik1-(110–192) fragment (Fig. 5B) and with the 151–199 peptide (Fig. 5D). Once the ratio of Ca$^{2+}$-bound Frq1 to the Pik1-(110–192) fragment exceeded 1:1, the heat no longer changed, again consistent with a one-to-one complex. However, the binding of Frq1 to Pik1-(110–192) was multiphasic and was fit to a two-site model, yielding dissociation constants ($K_{D1} = 62 \text{nM}$ and $K_{D2} = 200 \text{nM}$) and positive (non-favorable) enthalpies of binding ($\Delta H_1 = +5.4 \text{kcal/mol}$ and $\Delta H_2 = +30 \text{kcal/mol}$) for the two sites, respectively. The multiphasic binding of Ca$^{2+}$-bound Frq1 to the Pik1-(110–192) fragment might be explained, in part, by the fact that we observed, using dynamic light scattering, that the starting solution of the Pik1-(110–192) fragment alone displayed some polydispersity. This analysis indicated that more than 25% of the free Pik1-(110–192) fragment existed in a pre-aggregated form, which might account for the highly endothermic and poorer affinity phase of binding we observed ($K_{D2} = 200 \text{nM}$ and $\Delta H_2 = +30 \text{kcal/mol}$). By contrast, the bulk of the population of the Pik1-(110–192) molecules was monomeric and presumably accounts for the major, higher affinity phase of binding ($K_{D1} = 62 \text{nM}$ and $\Delta H_1 = +5.4 \text{kcal/mol}$). The binding of Ca$^{2+}$-bound Frq1 to the 151–199 peptide appeared homogeneous and was fitted to a one-site model, yielding a $K_{D} = 140 \text{nm}$, and a $\Delta H$ for binding = +9.5 kcal/mol. In these titrations, the heat no longer changed when the ratio of Frq1 to the 151–199 peptide reached 0.5, again consistent with a complex containing two peptides bound per Frq1 molecule.

Size Determination of Native Pik1-Frq1 Complexes by Size Exclusion Chromatography—Despite the finding that the Pik1-(110–192) fragment formed a 1:1 complex with Ca$^{2+}$-bound Frq1, the fact that the smaller 151–199 peptide could form 2:1 complexes with Ca$^{2+}$-bound Frq1, raised the possibility, albeit remote, that in vivo Frq1 might serve to bridge two Pik1 molecules and thereby promote formation of enzyme dimers. To
determine if Pik1 and Frq1 form stable complexes in cell extracts and to estimate their apparent molecular mass, analytical size exclusion chromatography was performed on extracts of yeast cells (strain YPH499) overexpressing an epitope-tagged derivative of Pik1, which is otherwise quite an inabundant protein (18). For this purpose, the yeast cells carried a low copy number (CEN) plasmid expressing from the Gal-inducible GAL1 promoter full-length Pik1 containing an in-frame N-terminal c-Myc epitope. This construct is able to fully complement the inviability of a pik1Δ mutant, even when cells are propagated under conditions (Glc as the carbon source) that repress the propagation of endogenous Pik1 in vitro (lanes 3, 6, and 9) or, as a control, pre-immune serum from the same rabbit (lanes 1 and 4), or with mouse c-Myc mAb 9E10 (lanes 5, 7, and 8). The resulting immune complexes were washed, solubilized, resolved by SDS-PAGE, and visualized by immunoblotting with the anti-c-Myc mAb.

Site-directed Mutagenesis of the Frq1 Binding Region in Pik1—Comparison of Pik1 to the sequences of type III PtdIns 4-kinases from other organisms indicated that the region of Pik1 that is necessary and sufficient for Frq1 binding (residues 151–192) shows weak, but detectable, conservation. To determine whether any of the most conserved residues are critical for Frq1 binding, we generated derivatives of the Frq1-binding fragment, Pik1(10–192)-(His)6, carrying a variety of site-directed mutations. These mutants included two double mutants, Pik1(10–192; F181A,V183A) and Pik1(10–192; R188A,R189A), a triple mutant, Pik1(10–192; L175A,P181A,V183A), and a quadruple mutant Pik1(10–192; E154A,N155A,V156A,P158A). The resulting constructs were expressed in and purified from E. coli, immobilized on Ni2+-saturated NTA-agarose beads, and their ability to bind [35S]Frq1 in vitro was examined, as described above (see Fig. 1). None of the four mutants proteins showed any significant reduction in their capacity to bind radiolabeled Frq1 under these conditions (data not shown). In two of the mutants, charged or polar side chains (Glu-154 and Asn-155, and Arg-188 and Arg-189, respectively) were replaced with the hydrophobic residue, Ala. Likewise, all of the other substitutions replaced more bulky hydrophobic side chains with the less bulky, but nevertheless non-polar, Ala residue. Thus, all of the mutations, although perturbing individual residues, did not dramatically change the overall hydrophobic character of this region. The fact that Frq1 binding was not affected by these alterations provides a further indication that the association of Frq1 with Pik1 is largely a hydrophobic interaction, fully consistent with the data from calorimetry (Fig. 4) and fluorescence spectroscopy (see Fig. S1 in the Supplemental Material). Moreover, preliminary NMR analysis of the NOE patterns for the complex of 15N-labeled Pik1-(110–192)-(His)6, and fluorescence spectroscopy (see Fig. S1 in the Supplemental Material).

Deletion of the Frq1-binding Site Compromises Pik1 Function in Vivo—In contrast to the substitution mutations, truncations of Pik1-(10–192) did greatly impair Frq1 binding (see Fig. 1). Therefore, to determine whether the region of Pik1 found to be necessary and sufficient for Frq1 binding in vitro is also required for association of Frq1 with Pik1 in vivo and plays a role in the physiological function of this enzyme, we generated a mutant allele, pik1Δ(152–191), in which a small
(40-residue) internal deletion removes the apparent Frq1-binding site.

Before testing its phenotype, we wanted to confirm that the *pik1(Δ152–191)* mutation does not adversely affect the catalytic activity of the enzyme *per se* but does compromise the ability of Frq1 to bind to Pik1. For this purpose, we prepared extracts from yeast cells expressing from the GAL promoter on *CEN* plasmids versions of either wild-type Pik1 or Pik1(152–191) tagged at the N terminus with the c-Myc epitope, or, as a negative control, untagged Pik1(152–191). These extracts were then subjected to immunoprecipitation with either anti-Frq1 antibodies or, as a control, with pre-immune serum from the same rabbit. The resulting immune complexes were resolved by SDS-PAGE, transferred to filters, and the amount of Pik1 that co-immunoprecipitated determined by immunoblotting with anti-Myc mAb. Samples of the same extracts were also directly immunoprecipitated with the anti-Myc mAb to determine the level of production of the tagged Pik1 proteins, and to examine their activity. First, the c-Myc epitope tag allowed for highly sensitive, specific, and background-free determination, because no signal was observed after immunoprecipitation with anti-Myc mAb. Samples of the same extracts were processed for PtdIns 4-kinase catalytic activity, by methods that allowed for highly sensitive, specific, and background-free detection, because no signal was observed after immunoprecipitation with anti-Myc mAb. Samples of the same extracts were also directly immunoprecipitated with the anti-Myc mAb to determine the level of production of the tagged Pik1 proteins, and to examine their activity. First, the c-Myc epitope tag allowed for highly sensitive, specific, and background-free detection, because no signal was observed after immunoprecipitation with anti-Myc mAb.

To examine the consequences of defective Frq1 binding on the function of Pik1 in *vivo*, a heterozygous *pik1Δ::LEU2/PIK1* diploid strain (YES10; Table I) was transformed with a *CEN* vector, pRS314-myc-*pik1(Δ152–191)*, expressing Pik1(Δ152–191) under the control of the GAL1 promoter, or with the same vector expressing wild-type *myc*-Pik1, or the same empty vector (as a control). The transformants were then allowed to sporulate, and the resulting tetrads were dissected on medium containing Gal as the carbon source and grown at 30 °C. As expected, the diploids transformed with the empty vector only yielded tetrads in which two spores (*pik1*Δ+) were viable and two spores (*pik1Δ::LEU2*) were inviable (see Fig. S3 in the Supplemental Material), indicating that the absence of Pik1 function is lethal, as shown before (19). In contrast, the diploids transformed with the vector expressing normal Pik1 yielded many

tetrads in which three or all four spores were viable, indicating that the plasmid-borne copy of *PIK1* was able to rescue the inviability of the *pik1Δ::LEU2* spores. Under the same conditions, the diploids transformed with the vector expressing *pik1(Δ152–191)* also yielded tetrads in which three or four spores were viable. Thus, when overexpressed from the strong inducible GAL1 promoter, Pik1(Δ152–191) was able to complement the *pik1Δ::LEU2* null mutation. Nevertheless, one way to determine if a gene product is not operating optimally is to test its ability to function under conditions of stress. Indeed, we found that *pik1Δ::LEU2* spores expressing Pik1(Δ152–191) as the sole source of the enzyme were compromised in their growth at 36 °C and unable to grow at all at 37 °C, whereas *pik1Δ::LEU2* spores expressing normal Pik1 from the same vector grew robustly at both temperatures (data not shown). Thus, even when highly overexpressed, Pik1(Δ152–191) is not fully functional under the stressful condition of elevated temperature, presumably because its efficient association with Frq1 is required for its optimal function.

We have demonstrated before that, when *PIK1* is highly overexpressed, it is able to support the growth of *frq1Δ* cells, which are otherwise inviable when Pik1 is present at its normal level (29). In other words, Frq1 becomes completely dispensable when Pik1 is highly abundant. Therefore, we tested whether Pik1(Δ152–191) was still able to rescue the inviability of *pik1Δ::LEU2* cells when it was expressed at a lower level rather than at the high level that results from expression from the GAL1 promoter. For this purpose, we constructed a *CEN* plasmid, pRS314-*pik1(Δ152–191)*, that expressed Pik1(Δ152–191) from the native *PIK1* promoter and introduced it into the *pik1Δ::LEU2/PIK1* heterozygous diploid. Even at this lower level of expression, diploids expressing Pik1(Δ152–191) yielded tetrads with three and four viable spores at essentially the same frequency as diploids expressing normal Pik1 (Fig. 6A). However, as observed before, *pik1Δ::LEU2* cells expressing Pik1(Δ152–191) were unable to grow above 35 °C (Fig. 6B). Thus, under the moderate stress of elevated temperature, efficient association of Frq1 with Pik1 becomes essential for the function of the enzyme, regardless of its level of expression.

**Cys15 is Neither S-Palmitoylated in Vivo nor Required for Frq1 Function**—One apparent role for the association of Frq1 with Pik1 is to promote its association with membranes, and we have shown previously that the N-terminal myristoyl group of Frq1 is important, but not essential, for this function (29, 35). First, we found that, like wild-type Frq1, a mutant, Frq1(G2A), that cannot be and is not myristoylated is capable of rescuing the inviability of *frq1Δ* cells; however, unlike overexpression of wild-type Frq1, overexpression of Frq1(G2A) is unable to rescue the temperature-sensitive lethality of *pik1Δ11* cells (29). Second, we found that, like normal Frq1, Frq1(G2A) still associates with membranes, but does so much less efficiently than normal Frq1 in either the absence or presence of Ca2+ (35). One explanation for both of these observations is that Frq1 might carry a second lipophilic modification that partially compensates for the absence of the N-myristoyl group. Moreover, there are precedents for S-palmitoylation of Cys residues situated near the N terminus of other small Ca2+-
binding regulatory proteins (38, 39). Indeed, Frq1 has only two Cys residues, and although one (Cys-38) appears to be buried, based on our NMR-derived structural model (35), the other (Cys-15) is located near the N terminus. We took two independent approaches to address whether S-palmitoylation has any role in the function of Frq1.

First, we generated a Frq1(C15A) single mutant and found that its properties were indistinguishable from those of wild-type Frq1. Next, we generated a Frq1(G2A,C15A) double mutant and examined its ability to rescue the temperature-sensitive lethality of frq1-1ts cells (strain YKBH4, Table I) when expressed from the native FRQ1 promoter in a multicopy vector. The empty vector was unable to permit cell growth at the non-permissive temperature, as expected, whereas both normal FRQ1 and frq1(G2A,C15A) expressed from the same vector suppressed the temperature-sensitive phenotype of the frq1-1ts cells (Fig. 7A). Likewise, when expressed from the same vector, expression of both normal Frq1 and Frq1(G2A,C15A) was able to restore viability to otherwise inviable sporadic Pik1 from the native PIK1 promoter (middle panel), the same vector expressing Pik1(Δ152–191) from the native PIK1 promoter (right panel) and subjected to conditions that induce sporulation, and samples of the resulting tetrads (six to ten shown) were dissected. Viability of the four spores (A–D) was assessed at 30 °C on medium containing Gal as the carbon source. B, a representative Leu+ Trp+ spore expressing myc-Pik1(Δ152–191) (left) and a representative Leu+ Trp+ spore expressing normal Pik1 (right), obtained as in A, were tested for growth on SCGal-Leu-Trp at the indicated temperatures.

**Fig. 6.** Efficient association of Frq1 with Pik1 is required for growth at elevated temperature. A, a complementation test was used to assess the function of overexpressed Pik1(Δ152–191). Heterozygous pik1Δ::LEU2/PIK1 diploid strain YES10 (Table I) was transformed with either the empty TRP1-marked CEN vector (left panel), or the same vector expressing wild-type Pik1 from the native PIK1 promoter (middle panel), or the same vector expressing Pik1(Δ152–191) from the native PIK1 promoter (right panel) and subjected to conditions that induce sporulation, and samples of the resulting tetrads (six to ten shown) were dissected. Viability of the four spores (A–D) was assessed at 30 °C on medium containing Gal as the carbon source. B, a representative Leu+ Trp+ spore expressing myc-Pik1(Δ152–191) (left) and a representative Leu+ Trp+ spore expressing normal Pik1 (right), obtained as in A, were tested for growth on SCGal-Leu-Trp at the indicated temperatures.

![Image](https://example.com/image.png)

**C**

| Spore | Tetrad | Spore | Tetrad | Spore |
|-------|--------|-------|--------|-------|
|       | A      | B     | C      | D     |
| 1     | 1      | 2     | 3      | 4     |
| 5     | 6      | 7     | 8      | 9     |
| 10    |        |       |        |       |

**D**

|          | SCD / 32°C | SCD / 35°C | SCD / 37°C |
|----------|------------|------------|------------|
| Pik1 (Δ152–191) | wt         | Pik1 (Δ152–191) | wt         |
| Pik1 (Δ152–191) | wt         | Pik1 (Δ152–191) | wt         |

We showed previously using primarily genetic means that the sole essential target of the small Ca2+-binding protein, Frq1, in the yeast *S. cerevisiae* is the PtdIns-4-kinase, Pik1 (29). At the biochemical level, Frq1 is present in a stoichiometric amount in preparations of Pik1 (18) that were purified more than 25,000-fold by ammonium sulfate fractionation followed by chromatography on five different columns in buffers lacking Ca2+ and containing chelator (EDTA). Thus, Frq1 is constitutively bound to Pik1, even in the absence of Ca2+, and should be considered a non-catalytic subunit of the enzyme. Indeed, we demonstrated, using an assay in which the substrate (PtdIns) was displayed in detergent micelles, rather than in authentic biological membranes, that the presence of Frq1 is required for optimal activity of the enzyme (29). As judged by cell fractionation experiments and other methods, Frq1 associates with the membrane-containing particulate fraction in a manner that depends on both its N-terminal myristoyl group and Ca2+-induced changes in the protein (35). Thus, *in vivo*, Frq1 associated with Pik1 presumably also assists in targeting Pik1 to membranes in a Ca2+-dependent manner. In our prior work, based on both genetic and biochemical approaches, we showed that the region of Pik1 responsible for Frq1 binding seemed to
reside within the first ~200 residues of the protein. In this present study, we sought to further define the Frq1-binding site and to characterize the nature of this interaction in greater detail.

We generated deletions in an His$_6$-tagged 183-residue fragment of Pik1, Pik1-(10–192)-(His)$_6$, that, when immobilized on Ni$^{2+}$-saturated NTA-agarose beads, is able to capture [35S]Frq1 from solution with high affinity. Using this in vitro binding method, we found that the region from 164 to 192 was essential for the observed interaction, whereas the segment from residue 31 to 79 was totally dispensable. However, using competition assays with synthetic peptides, we found that the 164–192 region of Pik1 was not sufficient for Frq1 binding. A peptide corresponding to residues 164–199 was not able to prevent the binding of [35S]Pik1-(10–192) to bead-immobilized Frq1-His$_6$, whereas a longer peptide corresponding to residues 151–199 did compete. Thus, residues N-terminal to the 164–192 region are also important for high affinity binding of Frq1 to Pik1. It also appears that the segment corresponding to residues 80–109 is dispensable for the interaction of Frq1 with Pik1, because a smaller 83-residue fragment of Pik1, Pik1-(110–192), bound to Frq1 with high affinity and a 1:1 stoichiometry, as judged by NMR, intrinsic Trp fluorescence, titration calorimetry, and light scattering.

Revealingly, the 151–199 peptide also bound to Frq1 avidly, however, as judged by the same criteria (NMR, intrinsic Trp fluorescence, titration calorimetry, and light scattering), the stoichiometry of peptide:Frq1 binding was 2:1, unlike that of the Pik1-(110–192) fragment. Moreover, as judged by calorimetric measurements in solution, the association of Frq1 with the 83-residue Pik1-(110–192) fragment was tighter (apparent $K_d = 62$ nM) than its association with the 49-residue 151–199 peptide (apparent $K_d = 140$ nM). When measured by competition for the binding of [35S]Pik1-(10–192) to bead-bound Frq1-His$_6$, the $K_d$ for the 151–199 peptide was 1 μM. However, in this assay format and given that a tracer level of the radioactive probe was used, competition was only observed when the amount of peptide added approached that of the large excess of

![Diagram](https://example.com/diagram.png)
immobilized Frq1 present. Nevertheless, the 151–199 peptide may be missing residues upstream of 151, which are present in Pik1(110–192), that also contribute to maximal binding affinity.

The intriguing 2:1 stoichiometry observed for binding of the 151–199 peptide suggests that the Pik1-binding site in Frq1 may be, in some sense, bipartite. Indeed, preliminary HSQC spectra for [15N]Pik1-(110–192) bound to Ca2+-saturated Frq1 indicate that contact is made with two short segments in the Pik1 fragment of 12 residues (Phe-125 to Gln-136) and 13 residues (Ala-157 to Ala-169), respectively, both of which assume a helical conformation in the complex.4 The intervening 20 residues (Thr-137 to Val-156) appear to be unstructured4 and presumably form a loop that does not interact with Frq1. The 13-residue segment is comparable in length to the short helices and other sequence elements (e.g. IQ motif) recognized by the well-characterized Ca2+-binding regulatory protein, calmodulin (55). Furthermore, this 13-residue segment (APALVLSSMIMSA) is comprised exclusively of non-polar and uncharged residues, in agreement with the evidence we obtained from titration calorimetry and intrinsic Trp fluorescence that the association between Pik1(110–192) is primarily a hydrophobic interaction. An interaction between another myristoylated Ca2+-binding regulatory protein and a largely hydrophobic element in its target has recently been described. The "CIB" (calcium- and integrin-binding) protein binds tightly to a 15-residue α-helical sequence (LVLMWKGFPKKRN-) in the cytoplasmic tail of the integrin αIIb chain (56). This sequence bears some resemblance to the 13-residue Frq1-binding segment of Pik1.

We derived a model for the three-dimensional structure of Ca2+-bound yeast Frq1 based on NMR analysis in solution (35). A very similar structure for the Ca2+-bound form of its human ortholog, NCS-1, was determined by x-ray analysis and refined to 1.9-A resolution (36). Both structures revealed that frequency has two tightly folded domains that pack against each other to form an accessible crevice lined primarily with hydrophobic side chains. This hydrophobic cleft would seem the most likely site for binding of the 13-residue hydrophobic segment in the Frq1-binding region of Pik1. By contrast, the 12-residue segment in the Pik1(110–192) fragment (-FQVARIVNMLQ-) that also appears to associate with Frq1 contains both polar and charged residues, the most striking feature of which is a tandem pair of Arg residues (Arg-129 and Arg-130). We note that, although the 151–199 peptide lacks this basic hydrophilic sequence found in Pik1(110–192), there is a 12-residue segment at the C-terminal end of the 151–199 peptide that has some similarity (ESQGRQKAPVF-), including a tandem pair of Arg residues (Arg-188 and Arg-189). Thus, it is possible that the 151–199 peptide binds to Frq1 with a 2:1 stoichiometry because one copy of the peptide uses the 13-residue hydrophilic motif (Ala-157 to Ala-169) near its N-terminal to correctly occupy the corresponding hydrophobic pocket in Frq1, and another copy of the peptide uses the basic segment near its C-terminal end to artifactually occupy the site in Frq1 that would normally recognize the 12-residue hydrophilic motif (Phe-125 to Gln-136). Indeed, our site-directed mutagenesis experiments indicated that the Arg-188/Arg-189 pair is not required for the binding of Frq1 to Pik1(10–192), consistent with the view that normally it is the upstream Arg-129/Arg-130 pair that fulfills this function.

The fact that a single Frq1 was able to bind two 151–199 peptides raised the possibility that one role of Frq1 in vivo might be to promote dimerization of Pik1. However, the fact that the Pik1(110–192) fragment bound to Pik1 with a 1:1 stoichiometry made such a dimerization scenario unlikely. Nonetheless, there is precedent for protein dimerization being induced by the binding of small, EF-hand type, Ca2+-binding proteins, most notably the oligomerization of the SK class of potassium channels promoted by calmodulin binding (57). Hence, we examined the nature of the native Frq1-Pik1 complexes found in yeast cell extracts by size-exclusion chromatography. Consistent with the binding of Pik1(110–192) to Frq1 in vitro, we found that the bulk of the Pik1-Frq1 complexes in yeast cell extracts (Peak II) had an apparent molecular mass most consistent with a 1:1 complex. However, we did observe a fraction (Peak I) that contained both Pik1 and Frq1 that had a much larger apparent size. Because the extracts were prepared in the absence of detergent, and Frq1 has a propensity to interact with membranes, this fraction most likely represents the 1:1 Pik1-Frq1 complex associated with small vesicles or membrane fragments. On the other hand, we cannot rule out the possibility that this fraction indeed represents a higher order Pik1-Frq1 oligomer or a novel complex of Pik1-Frq1 that contains other tightly associated polypeptides.

We demonstrated previously that Frq1 lacking its N-terminal myristoyl group retains a residual capacity to associate with membranes in a Ca2+-dependent manner (35). This behavior could be due to greater exposure of previously buried hydrophobic side chains upon Ca2+-induced conformational change. Alternatively, however, it was possible that some other lipophilic substituent is also present in Frq1 that becomes more solvent exposed when Ca2+ binds to the protein. In other small, EF-hand type, Ca2+-binding proteins, such as a flagellar regulatory protein and a largely hydrophilic C-terminal end to artifactually occupy the site in Frq1 that would normally recognize the 13-residue hydrophilic motif (Phe-125 to Gln-136). Indeed, our site-directed mutagenesis of the most N-terminal Cys (Cys-15) did not compromise the function of Frq1 in vivo, nor did it exacerbate the effects of a mutation (G2A) that prevents N-myristoylation of Frq1. Therefore, S-palmitoylation is not involved in the physiological function of Frq1.

Taken together, our data support the conclusion that a single molecule of Frq1 docks onto a single molecule of Pik1 and does so by binding to a site that includes as its core a 13-residue hydrophobic sequence (Ala-157 to Ala-169). As expected if this region is critical for high affinity binding of Frq1 to Pik1, we found that a deletion mutation, pik1(Δ152–191), which removed the 13-residue hydrophobic motif, produces a protein that, in contrast to normal Pik1, co-immunoprecipitates very inefficiently with Frq1, yet retains catalytic activity. The lack of efficient Frq1 binding to Pik1 appears to compromise the function of the enzyme because, again in contrast to wild-type Pik1, Pik1(Δ152–191) was unable to support the growth of pik1Δ yeast cells at elevated temperature. We do not know if the binding modality we have defined for the Pik1-Frq1 interaction is conserved in the interaction of metazoan frequenins with PtdIns 4-kinase-β and/or any other targets. However, in collaborative studies, which will be described in greater detail elsewhere, we have delineated the site in yeast Pik1 bound by human NCS-1 using in vitro pull-down assays of the sort presented here and using the two-hybrid method in vivo. As observed for the interaction of Frq1 with Pik1, the most essential sequences to support the interaction of human NCS-1 with Pik1 fall between residues 145 and 172, which includes the

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4. J. B. Ames, unpublished results.
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13-residue hydrophobic sequence (Ala-157 to Ala-169). Furthermore, as also observed for Frq1 association with Pik1, residues in the region 100–144, which includes the 11-residue hydrophilic element (Phe-125 to Gin-136), contribute to the strength of the interaction between human NCS-1 and Pik1.  

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