Structural Insights into Activation of Phosphatidylinositol 4-Kinase (Pik1) by Yeast Frequentin (Frq1)*

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Yeast frequentin (Frq1), a small N-myristoylated EF-hand protein, activates phosphatidylinositol 4-kinase Pik1. The NMR structure of Ca2+-bound Frq1 complexed to an N-terminal Pik1 fragment (residues 121–174) was determined. The Frq1 main chain is similar to that in free Frq1 and related proteins in the same branch of the calmodulin superfamily. The myristoyl group and first eight residues of Frq1 are solvent-exposed, and Ca2+ binds the second, third, and fourth EF-hands, which associate to create a groove with two pockets. The Pik1 peptide forms two helices (125–135 and 156–169) connected by a 20-residue loop. Side chains in the Pik1 N-terminal helix (Val-127, Ala-128, Val-131, Leu-132, and Leu-135) interact with solvent-exposed residues in the Frq1 C-terminal pocket (Leu-101, Trp-103, Val-125, Leu-138, Ile-152, and Leu-155); side chains in the Pik1 C-terminal helix (Ala-157, Ala-159, Leu-160, Val-161, Met-165, and Met-167) contact solvent-exposed residues in the Frq1 N-terminal pocket (Trp-30, Phe-34, Phe-48, Ile-51, Tyr-52, Phe-55, Phe-85, and Leu-89). This defined complex confirms that residues in Pik1 pinpointed as necessary for Frq1 binding by site-directed mutagenesis are indeed sufficient for binding. Removal of the Pik1 N-terminal region (residues 8–760) from its catalytic domain (residues 792–1066) abolishes lipid kinase activity, inconsistent with Frq1 binding simply relieving an autoinhibitory constraint. Deletion of the lipid kinase unique motif (residues 35–110) also eliminates Pik1 activity. In the complex, binding of Ca2+-bound Frq1 forces the Pik1 chain into a U-turn. Frq1 may activate Pik1 by facilitating membrane targeting via the exposed N-myristoyl group and by imposing a structural transition that promotes association of the lipid kinase unique motif with the kinase domain.

In animal cells and yeast (1, 2), phosphoinositides mediate selective recruitment of proteins to membranes (3–6) and serve as precursors for intracellular second messengers (7–9). Phosphoinositide biosynthesis begins with phosphorylation of the myo-inositol headgroup of phosphatidylinositol (PtdIns)3 at the D-4 position by PtdIns 4-kinase (ATP:1-phosphatidyl-1-D-myo-inositol 4-phosphotransferase, EC 2.7.1.67) (10–12). The first PtdIns 4-kinase to be purified (13), and the corresponding gene cloned (14), was Pik1 from the yeast Saccharomyces cerevisiae. Pik1 is an essential gene required for vesicular trafficking in the late secretory pathway (15, 16), for nuclear functions (17), and possibly cytokinesis (18). Pik1-like isoforms are conserved in metazoans (10, 11, 19).

Yeast frequentin (Frq1), a 22-kDa Ca2+-binding protein, copurifies with Pik1 and is essential for its optimal activity (20). The site where Frq1 docks on Pik1 was localized to a region (residues 121–174) that lies far upstream of the catalytic domain (residues 792–1066) (21). Mammalian frequentin also interacts with Pik1 (22), and frequentin may regulate PtdIns 4-kinase activity in animal cells (23–25). Ca2+-dependent activation of PtdIns 4-kinase by frequentin may be especially important in neurons because modulation of phosphoinositide synthesis by intracellular Ca2+ controls exocytosis (26) and is involved in synaptic plasticity (27).

Frq1 and other frequentins belong to the neuronal calcium sensor (NCS) branch of the calmodulin superfamily, which includes recoverin and neurocalcin (28–31). These proteins are small (≤25 kDa) and characterized by a consensus signal for N-terminal myristoylation and four EF-hand Ca2+-binding sites (Fig. 1). We have shown previously that, at saturation, Frq1 binds only three Ca2+ (32). Frq1, which is itself essential for the viability of yeast cells (20), associates with membranes in a manner that depends on both the N-myristoyl group and conformational changes induced upon Ca2+ binding, suggesting that Frq1, like other NCS proteins, may possess a Ca2+-myristoyl switch (32). Indeed, prior work indicated that N-myristoylation of Frq1 is important (but not essential) for stimulating both the catalytic activity (20) and the membrane recruitment of Pik1 (17).

Three-dimensional structures for Frq1 and other NCS proteins have been determined by x-ray crystallography (23, 33–37) and NMR spectroscopy (32, 38–40). The structure of

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The atomic coordinates and structure factors (code 2JU0) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: PtdIns, phosphatidylinositol; Frq1, yeast frequentin; HSQC, heteronuclear single quantum coherence; HMOC, heteronuclear multiple quantum coherence; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; r.m.s.d., root mean square deviation; PDB, Protein Data Bank; LKU, lipid kinase unique; NCS, neuronal calcium sensor.
Fqr1 in solution revealed that calcium is bound at EF-2, EF-3, and EF-4, and the overall main chain fold is similar to that seen previously for Ca$^{2+}$-bound forms of recoverin, neurocalcin, GCAP2, and KChIP1. The four EF-hands form two domains packed in a globular arrangement that contrasts with the dumbbell-shaped arrangement of EF-hand domains seen in calmodulin and troponin C (41, 42). A striking feature of the NCS structures is a solvent-exposed hydrophobic groove formed by residues Phe-22, Trp-30, Phe-34, Phe-48, Ile-51, Tyr-52, Phe-55, Phe-85, and Leu-89 in the N-terminal domain of Fqr1 that are invariant in all other NCS proteins (Fig. 1). The corresponding hydrophobic residues in GCAP2, recoverin, and KChIP1 have been implicated previously in target recognition (35, 40, 43, 44).

We report here the NMR-derived structure of Fqr1 in solution bound to a fragment (residues 121–174) corresponding to the Fqr1-docking site in Pik1 (21), hereafter referred to as Pik1-(121–174). This is the first atomic resolution structure of a Ca$^{2+}$-myristoyl switch protein bound to a lipid kinase target protein. The structure reveals that Pik1-(121–174) forms two antiparallel α-helical segments that interact with bipartite binding sites on the surface of Fqr1. In essence, binding of Fqr1 generates a U-turn in the Pik1 polypeptide, shedding considerable light on how Fqr1 may potentiate the activity of this enzyme. Moreover, the structure of Fqr1 bound to Pik1-(121–174) is somewhat different from the target complexes of other EF-hand proteins, like recoverin (40), calmodulin (45), KChIP1 (35, 36), calcineurin B (46), and troponin C (47), and thus provides new insights about the molecular basis of target recognition specificity in this class of regulatory proteins.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**—To prepare recombinant Fqr1 uniformly labeled with nitrogen-15 and/or carbon-13, Fqr1 tagged with a C-terminal His$_6$ tract was expressed in *Escherichia coli* strain BL21(DE3) carrying a derivative of the pET23d vector (Novagen) harboring the FQR1 coding sequence, constructed as described previously (20) and grown in M9 minimal medium containing $[^{15}$N]$\text{NH}_4\text{Cl}$ and $[^{13}$C$_6$]glucose, according to well established procedures (48–50). Labeled Fqr1 was purified from the soluble fraction of bacterial cell lysates using Ni$^{2+}$-chelate affinity chromatography on a nitrotriacetate resin (Qiagen), according to the manufacturer’s instructions. Peak fractions were then applied to an anion-exchange column (50-ml bed, DEAE-Sepharose, GE Healthcare) equilibrated in buffer A (2 mM CaCl$_2$, 20 mM Tris-HCl, pH 7.4) and eluted with a linear salt gradient (0–0.4 M KCl) at a flow rate of 2 ml min$^{-1}$ over the course of 180 min. Peak fractions were concentrated to 5 ml and subjected to size-exclusion chromatography (Sephacryl S-100, GE Healthcare) in buffer B (1 mM dithiothreitol, 2 mM CaCl$_2$, 50 mM HEPES, pH 7.4). Final purity was greater than 98%, as judged by SDS-PAGE.

A functional polypeptide fragment of Pik1 (residues 121–174, named Pik1-(121–174)) uniformly labeled with nitrogen-15 and/or carbon-13 and tagged with a C-terminal His$_6$ tract was expressed in *E. coli* strain BL21(DE3)-RIL (Stratagene) carrying the pET23d vector (Novagen) harboring the Pik1-(121–174) coding sequence (21) grown in M9 medium containing $[^{15}$N]$\text{NH}_4\text{Cl}$ and $[^{13}$C$_6$]glucose (48–50). Labeled Pik1-(121–174) was isolated from the insoluble fraction of bacterial cell lysates dissolved in 8M urea buffer and purified using Ni$^{2+}$-chelate affinity chromatography on a nitrotriacetate resin (Qiagen), according to the manufacturer’s instructions. Peak fractions were then dialyzed extensively against 4 liters of 25 mM sodium acetate (pH 5.0) to remove urea. After dialysis, the Pik1-(121–174) polypeptide remained soluble at pH 5.0 and was concentrated about 10-fold to a final concentration of 1 mM used in NMR experiments.
The Frq1-Pik1-(121–174) complex used in NMR experiments was prepared by slowly mixing a relatively dilute solution of Ca^{2+}-bound Frq1 (0.05 mM Frq1 in 2 mM CaCl₂ and 5 mM dithiothreitol at pH 7.0) to 1 eq of Pik1-(121–174) (0.05 mM Pik1 in 5 mM sodium acetate, 2 mM CaCl₂, and 5 mM dithiothreitol at pH 5.0). The dilute preparation was then concentrated 20-fold to generate a final sample used in NMR studies (0.3 ml of 1 mM Frq1-Pik1-(121–174) complex).

**NMR Spectroscopy**—Samples for NMR analysis consisted of 15N-labeled or 13C/15N-labeled Frq1 bound to unlabeled Pik1-(121–174) (1.0 mM) in 0.3 ml of a 95% H₂O, 5% [2H]H₂O solution containing 5 mM sodium acetate and 2 mM CaCl₂ (pH 5.0). Reverse-labeled samples (i.e. 15N- or 13C/15N-labeled Pik1-(121–174) bound to 1 eq of unlabeled Frq1) were also prepared for some of the NMR experiments. All NMR experiments were performed at 40 °C on a Bruker DRX-500 or DRX-600 spectrometer equipped with a four-channel interface and triple resonance probe with triple axis pulsed field gradients and DRX-600 spectrometer equipped with an Ultrashield Bruker magnet, a three-channel interface, and cryo-probe with z-axis pulsed field gradients. The 1H-15N HSQC spectra (see Fig. 2) were recorded on a sample of 15N-labeled Frq1 bound to unlabeled Pik1-(121–174) (Fig. 2A) and 15N-labeled Pik1-(121–174) bound to unlabeled Frq1 (Fig. 2B) in 95% H₂O, 5% H₂O. The number of complex points and acquisition times were as follows: 256, 180 ms (15N(F1)); and 512, 64 ms (1H(F2)). The 13C(F1)-edited, 13C(F3)-filtered NOE-HMQC spectra (see Fig. 2C) were recorded on a sample of unlabeled Frq1 protein bound to 13C-labeled Pik1-(121–174) (51) as well as 13C-labeled Frq1 bound to unlabeled Pik1-(121–174) (data not shown). Intermolecular NOE experiments were performed as described previously (52). Stereospecific assignments of chiral methyl groups of valine and leucine were obtained by analyzing 1H-13C HSQC experiments performed on a sample that contained 10% 13C labeling in either Frq1 or Pik1-(121–174) (53). All triple resonance experiments were performed, processed, and analyzed as described (54, 55) on a sample of 13C/15N-labeled Frq1-Pik1-(121–174) complex (in 95% H₂O, 5% H₂O) with the following number of complex points and acquisition times: HNCO (15N(F1) 32, 23.7 ms; 13CO(F2) 64, 42.7 ms; 1H(F3) 512, 64 ms); CBCACONNH (15N(F1) 32, 23.7 ms, 1H(F2) 64, 42 ms, 1H(F3) 384, 64 ms); and, HBHAACONNH (15N(F1) 32, 23.7 ms, 1H(F2) 64, 42 ms, 1H(F3) 512, 64 ms).

The triple resonance and NOE spectra measured above were analyzed to determine secondary and tertiary structure in Frq1-Pik1-(121–174) complex. The chemical shift index (see Ref. 56 for detailed description), 3J\textsubscript{HNH} coupling constants, and NOE connectivity patterns for each residue were analyzed and provided a measure of the overall secondary structure. Small 3J\textsubscript{HNH} coupling constants (\(<5 Hertz\)), strong NOE connectivities (\(\text{NN}(i,i+1)\) and \(\text{aNN}(i,i+3)\)), and positive chemical shift index are characteristic of residues in an \(a\)-helix. Conversely, large 3J\textsubscript{HNH} coupling constants (\(>8 Hertz\)), strong \(\text{aNN}(i,i+1)\) and weak \(\text{NN}(i,i+1)\) NOE connectivities, and negative chemical shift index are characteristic of residues in a \(\beta\)-strand. The results of the secondary structure analysis of Frq1-Pik1-(121–174) complex are summarized schematically in Fig. 1.

\[ ^{13}N{^1}H\] NOE data were measured using two-dimensional 15N-1H HSQC-based experiments as described previously (57). Saturation was carried out with a series of 120° 1H pulses separated by 5-ms delays applied during the interscan delay (3 s).

**Structure Calculation**—Backbone and side chain NMR resonances of Frq1-Pik1-(121–174) complex were assigned as described previously (55). Structure calculations were performed using the YASAP protocol within X-PLOR (58), as described previously (59). A total of 2300 interproton distance constraints were obtained as described (55) by analysis of 13C- and 15N-edited NOE-HSQC spectra (100 ms mixing time) of 13C,15N-labeled Frq1 bound to unlabeled Pik1-(121–174) and 13C,15N-labeled Pik1-(121–174) bound to unlabeled Frq1. In addition to the NOE-derived distance constraints, the following additional constraints were included in the structure calculation: 18 distance constraints involving Ca\(^{2+}\) bound to loop residues 1, 3, 5, 7, and 12 in each EF-hand motif (EF-2, EF-3, and EF-4); 170 distance constraints for 85 hydrogen bonds; and 222 dihedral angle constraints. Fifty independent structures were calculated, and the 20 structures of lowest energy were selected. The average total and experimental distance energies were 4730 and 61 kcal mol\(^{-1}\). The average root mean square deviations (r.m.s.d.) from an idealized geometry for bonds and angles are 0.0068 Å and 2.05°. None of the distance and angle constraints were violated by more than 0.40 Å or 4°, respectively.

**Deletion Analysis of Pik1**—To construct Pik1-(Δ34–110), Pik1-(Δ8–760), and Pik1-(Δ16–833), DNA fragments of PIK1 carrying these respective internal deletions were generated by PCR with appropriate primers and pRS316-GAL1prom-GFP-Pik1 as the template. The resulting PCR products were incorporated in place of the normal PIK1 open reading frame in pRS316-GAL1prom-GFP-Pik1, which had been linearized by cleavage with MfeI, via homologous recombination-mediated gap-repair (60) in yeast strain BY4743. Plasmid DNA was recovered from the resulting Ura\(^+\) transformants, amplified in *Escherichia coli*, and sequenced to verify production of the proper in-frame deletions. The control plasmid, pRS314-GAL1prom-GFP-Pik1, has been described before (17). Standard methods for DNA-mediated transformation, sporulation, tetrad dissection, and other genetic manipulations of yeast cells were used (61). In *vitro* lipid-kinase assays and immunoblot analysis of proteins were done essentially as described before (17).

**RESULTS**

**Preparation and Characterization of the Frq1-Pik1-(121–174) Complex**—We showed previously that Frq1 interacts with Pik1 in a localized region (residues 121–174) that is necessary and sufficient for Pik1 activation by Frq1 (21). A stoichiometric complex of Frq1 bound to Pik1-(121–174) at saturating Ca\(^{2+}\) is soluble and stable under NMR conditions. The Frq1-Pik1-(121–174) complex is monomeric in solution with a total molecular mass of \(\sim 30\) kDa, as judged by dynamic light scattering analysis and size-exclusion chromatography. The binding energetics for the Frq1-Pik1-(121–174) complex were
quantified using isothermal titration calorimetry, as described previously (21). Analysis of the isothermal titration calorimetry data revealed that complex formation occurs with a stoichiometry of 1:1, a dissociation constant of $K_d = 100 \text{nM}$, and enthalpy of $+$7 kcal/mol, suggesting that binding is largely entropy-driven. Entropically driven binding is consistent with hydrophobic intermolecular interactions and/or a protein conformational change.

**NMR Structural Analysis of the Frq1-Pik1-(121–174) Complex**—The $^1$H-$^15$N HSQC NMR spectrum of $^{15}$N-labeled Frq1 exhibited spectral changes that saturated upon the addition of 1 eq of unlabeled Pik1-(121–174) (Fig. 2A), confirming that Frq1 binds to Pik1-(121–174) in a 1:1 complex under NMR conditions. The $^1$H-$^15$N HSQC spectrum of $^{15}$N-labeled Frq1 in the complex exhibited the expected number of amide resonances (190) with uniform intensity, indicating that the complex is structurally homogeneous and stably folded. Pulsed field gradient NMR diffusion studies (62) confirmed that the complex is monomeric under NMR conditions. Sequence-specific assignments for the amide peaks are indicated in Fig. 2A. More than 96% of the amide resonances were assigned except those of Ser-60, Thr-91, Lys-100, Asn-184, and Leu-185 that could not be assigned because of chemical shift degeneracy and/or undetectable NMR intensities. The amide chemical shifts of many residues of Frq1 in the complex are similar to those of free Frq1 (32), suggesting that the overall main chain structure of Frq1 in the complex (discussed below) is similar to that of free Frq1 (32) and mammalian NCS-1 (23) determined in the absence of target.

A reverse labeled sample containing $^{15}$N-labeled Pik1-(121–174) bound to unlabeled Frq1 allowed us to selectively probe $^1$H-$^15$N HSQC NMR spectra of Pik1-(121–174) in the complex. The NMR spectrum of Pik1-(121–174) exhibited a subset of sharp resonances clustered near the middle of the spectrum at 8.0 ppm and a separate group of broader peaks (Fig. 2B). The sharp resonances were assigned to residues 138–154 of Pik1-(121–174). The sharpness of the peaks, narrow chemical shift dispersion, and low heteronuclear NOE values ($<0.6$, see supplemental Fig. 1) all suggest that these residues are largely unstructured. The remaining peaks exhibited much greater chemical shift dispersion and, higher heteronuclear NOE values and were assigned to residues 125–136 and 156–169, which form two separate $\alpha$-helices in Pik1-(121–174), as deduced from analyses of $J$-coupling, chemical shift index, and sequential NOE patterns (see “Experimental Procedures” and supplemental Fig. 1 and Fig. 1B).

Our analysis of the NMR data for the Frq1-Pik1-(121–174) complex (see “Experimental Procedures”) permitted the assign-
ment of more than 94% of all NMR resonances and NOE data, which then served as the basis for determining the structural constraints in Table 1. Differential isotope labeling of the complex and analysis of isotope-filtered NOE experiments (52, 55) enabled the selective probing of 89 intermolecular NOEs involving Frq1 residues located less than 5 Å away from residues of Pik1-(121–174) at the binding interface. Structures derived from the NMR data are illustrated in Figs. 3 and 4 (atomic coordinates are available from RCSB Protein Data bank, code 2JU0). The overall structure is a 1:1 complex of Frq1 and Pik1-(121–174) with overall dimensions of 46 Å (length) by 28 Å (height) by 31 Å (depth). The final NMR-derived structures (20 lowest energy structures out of a total of 50) were superimposed, and the r.m.s.d. relative to the mean structure was calculated to be 0.55 Å for main chain atoms and 1.2 Å for all heavy atoms in regions of regular secondary structure (see supplemental Fig. 2 and Table 1). The average main chain structure of the complex in solution is represented as a ribbon diagram in Fig. 3A.

The structure of Frq1 in the complex (Fig. 3) is similar to what has been seen previously for Ca$^{2+}$-bound Frq1 in the absence of any target (32) and other Ca$^{2+}$-bound NCS proteins (2.0 Å r.m.s.d. for the EF-hand regions). An important difference is that the C-terminal region of Frq1 (residues 179–186) is structurally disordered in the complex, in contrast to a well defined C-terminal helix observed for free Frq1 (32) and other NCS proteins (23, 33, 35, 63). The unmyristoylated N-terminal region of Frq1 (residues 2–8) is solvent-exposed and structurally disordered, as it is in all other Ca$^{2+}$-bound NCS proteins and in contrast to the highly sequestered N-terminal region of Ca$^{2+}$-free myristoylated recoverin (38). A total of 10 α-helices and 4 β-strands are observed in Frq1 as follows: H1 (residues 8–16), H2 (residues 25–35), H3 (residues 45–55), H4 (residues 62–72), H5 (residues 82–91), H6 (100–108), H7 (residues 118–129), H8 (residues 133–138), H9 (residues 145–155), H10 (residues 168–177), S1 (residues 42–44), S2 (residues 79–81), S3 (residues 115–117), and S4 (residues 163–165) (Fig. 1A). Frq1 contains two domains comprising four EF-hands as follows: EF1 (Fig. 1A green) and EF2 (salmon) are linked and form the N-terminal domain; likewise, EF3 (Fig. 1A cyan) and EF4 (yellow) are linked and form the C-terminal domain. The interface between the two domains is established by interactions between EF2 and EF3 and forms a noticeable cleft (see Fig. 3A, lower panel). Each EF-hand consists of a helix-turn-helix structure similar to the structure of Ca$^{2+}$-occupied EF hands seen in previous structures of calmodulin (41), troponin C (42), and recoverin (33, 39). The four EF hands are arranged in a tandem array and, overall, form a globular structure with a concave solvent-exposed groove lined by two separate hydrophobic patches (highlighted yellow in Fig. 3B). These two hydrophobic surfaces represent bipartite binding sites on Frq1 that interact with separate regions of Pik1-(121–174). A short α-helix

![FIGURE 3. NMR-derived structures of the Frq1-Pik1-(121–174) complex (PDB code 2JU0). A, ribbon diagrams depicting the average main chain structure of 20 NMR-derived structures of the Frq1-Pik1-(121–174) complex viewed from the binding interface (top) and rotated 180° (bottom). The EF-hands and Pik1 helices are colored as in Fig. 1, and bound calcium ions are orange. B, space-filling models depicting the average main chain structure of 20 NMR-derived structures of the Frq1-Pik1-(121–174) complex. Exposed hydrophobic residues in the bipartite binding sites on Frq1 are highlighted in yellow, and Pik1 residues are shown in magenta.](image-url)
FIGURE 4. Detailed intermolecular interactions between Frq1 and Pik1-(121–174). A. Selected slices of $^{13}$C(F1)-edited/$^{13}$C(F3)-filtered NOESY-HMQC spectra of $^{13}$C-labeled Pik1-(121–174) bound to unlabeled Frq1. These spectra yielded information on the individual atoms of Frq1 located less than 5 Å away from Pik1 residues, Ala-157, Ala-159, and Val-161. B. Close-up view of the C-terminal helix of Pik1-(121–174) (magenta) with side chains in the N-terminal hydrophobic pocket of Frq1 (yellow). C. Close-up view of the N-terminal helix of Pik1-(121–174) (magenta) with side chains in the C-terminal hydrophobic pocket of Frq1 (yellow). For reasons of clarity, in this panel, the positions of Ala-104 and Val-128 in Frq1 have been omitted.
(H8) between EF3 and EF4 also makes contact with N-terminal residues of Pik1-(121–174).

The structure of Pik1-(121–174) in the complex adopts a conformation that contains two α-helices (residues 125–136 and 156–169) connected by a disordered loop (supplemental Fig. 1A, B). The N-terminal helix contains hydrophobic residues (Ala-128, Val-131, Leu-132, and Leu-135) that contact C-terminal residues of Frq1 (Leu-101, Trp-103, Val-125, Val-128, Leu-138, Ile-152, Leu-155, and Phe-169). The opposite face of the Pik1 N-terminal helix contains polar and positively charged residues (Arg-129, Arg-130, Asn-133, and Asn-134) that point outward toward the solvent. The C-terminal helix of Pik1 contains many hydrophobic residues (Val-156, Ala-157, Ala-159, Val-161, Met-165, and Met-167) that contact the N-terminal hydrophobic groove of Frq1 (Trp-30, Phe-34, Phe-48, Ile-51, Tyr-52, Phe-55, Phe-85, and Leu-89). The two helices of Pik1-(121–174) do not interact with one another or with the unstructured connecting loop. The helix-loop-helix structure of Pik1-(121–174) in the complex is highly stabilized by interactions with Frq1. Free Pik1-(121–174) in the absence of Frq1 in solution exhibits narrow chemical shift dispersion and a complete lack of any medium range and long range NOEs, indicating it is completely unstructured in the absence of Frq1.

**Intermolecular Interactions in the Frq1-Pik1-(121–174) Complex**—Analysis of isotope-filtered NMR NOESY data selectively probed those residues of Frq1 located less than 5 Å away from residues of Pik1-(121–174) situated at the binding interface (Fig. 4). Representative slices of three-dimensional 13C(F2)-edited, 1-13C(F2)-filtered NOESY-HMQC spectra of 13C-labeled Pik1-(121–174) bound to unlabeled Frq1 (Fig. 4A) reveal that Pik1 residues in the C-terminal helix (157–168) form contacts with Frq1 residues of EF-1 and EF-2 in the N-terminal hydrophobic groove. Most striking are hydrophobic contacts involving Ala-157, Ala-159, and Val-161 (Fig. 4B). These contacts and others were confirmed in reverse labeling isotope-filtered NMR experiments performed on 13C-labeled Frq1 bound to unlabeled Pik1-(121–174). Intermolecular contacts were also observed for N-terminal Pik1 residues (125–136) with EF3 and EF4 of Frq1 (Fig. 4C). However, the intermolecular NOE intensities for the N-terminal Pik1 residues were quite weak by comparison, perhaps because of conformational instability in this region. At lower temperatures and higher salt concentrations, the intermolecular NOE to the N-terminal helix of Pik1 were not discernible, whereas intermolecular NOEs involving the C-terminal helix of Pik1 (Fig. 4A) were quite strong under all conditions. The sharper NMR linewidths for the C-terminal resonances of Pik1 suggest that the C-terminal helix of Pik1 forms more stable contacts with Frq1 than does the N-terminal helix of Pik1, which seems to be significantly less stable.

The C-terminal helix of Pik1-(121–174) contains hydrophobic residues that make extensive contacts with aromatic and other hydrophobic side chains of Frq1 (Fig. 4B). Both side chain methyl groups of Val-156 (Pik1) are spatially quite close (<5 Å) to the aromatic side chains of Phe-34 and Phe-85 from Frq1. The β-methyl group of Ala-157 (Pik1) makes van der Waals contacts with the δ1- and γ2-methyl groups of Ile-51 and the aromatic ring protons of Phe-55. The β-methyl group of Ala-159 (Pik1) contacts both the δ1- and δ2-methyl groups of Leu-89 and aromatic ring protons (HH2 and HZ2) of Trp-30. The δ2-methyl group of Leu-160 (Pik1) contacts the δ1-methyl group of Ile-51 and aromatic ring of Phe-48 and Phe-85. The γ1-methyl group of Val-161 (Pik1) contacts the γ2-methyl of Ile-51 and the aromatic ring of Tyr-52 and Phe-55. The ε-methyl group of Met-165 (Pik1) is close to the aromatic ring of Tyr-52 and Phe-55, and the ε-methyl group of Met-167 (Pik1) is close to the aromatic ring of Trp-30. The intricate network of intermolecular hydrophobic interactions illustrated in Fig. 4B explains the high affinity and strongly endothermic binding of Frq1 to Pik1 (21). The intermolecular interactions at this site (Fig. 4B) are remarkably stable (up to 70 °C) and exhibit slow equilibrium exchange rate, consistent with high affinity binding.

The N-terminal helix of Pik1-(121–174) interacts with hydrophobic side chains of Frq1 (Fig. 4C). The side chain methyl groups of Val-127 (Pik1) and Val-131 (Pik1) are less than 5 Å away from the δ1- and δ2-methyl groups of Leu-138 from Frq1. The γ1-methyl group of Val-131 (Pik1) contacts the δ1-methyl group of Ile-152 (Frq1) and γ1-methyl group of Val-125 (Frq1). The side chain methyl groups of Leu-132 (Pik1) contact both the δ1- and δ2-methyl groups of Leu-101 (Frq1) and β-methyl group of Ala-104 (Frq1) (data not shown). The δ1-methyl of Leu-135 (Pik1) contacts the aromatic ring of Trp-103 (HH2 and HZ2) and β-methyl of Ala-104. The δ2-methyl of Leu-135 (Pik1) contacts the γ1-methyl of Val-128 (Frq1) (data not shown). These intermolecular interactions, illustrated in Fig. 4C, contribute to the overall binding energy. However, the exchange broadening of NMR resonances associated with the N-terminal helix of Pik1-(121–174) suggests a relatively unstable interaction in this region (Fig. 4C), in contrast to the more stable interaction involving the Pik1 C-terminal helix (Fig. 4B). The intermolecular interactions depicted in Fig. 4, B and C, are believed to represent two distinct and independent binding sites because the two sites are separated spatially and do not interact structurally. Nonetheless, these two sites presumably act synergistically because the ligands with which they interact (the 11- and 14-residue α-helices in Pik1) are tethered covalently by a 20-residue spacer.

**Catalytic Activity of Pik1 Deletion Mutants**—A series of deletion constructs of Pik1 were analyzed to identify regulatory domains (Table 2). To examine catalytic competency relative to wild-type Pik1, a qualitative immune complex in vitro lipid kinase assay was used (13, 17). A deletion construct of Pik1 containing only the lipid kinase catalytic domain, Pik1-(Δ8–760), exhibits very low basal activity (Fig. 5, lane 3), just barely detectable above a negative kinase-dead control (lane 1). This

**Table 2**

| Pik1 mutant | Kinase activity* | Frq1 binding | Phenotype                |
|-------------|------------------|--------------|-------------------------|
| Pik1-(Δ8–760) | −/−              | No           | Lethal in pik1Δ         |
| Pik1-(Δ16–833) | −               | No           | Lethal in pik1Δ         |
| Pik1-(Δ34–110) | −/+             | ?            | Lethal in pik1Δ         |
| Pik1-(Δ152–191) | ++              | No           | Temperature-sensitive in pik1Δ |

*Symbols used are as follows: ++, 100% activity; +, ~80% activity; −/+, ~<5% activity; −, inactive.

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**Structure of Frequenin Bound to PtdIns 4-Kinase**
Structure of Frequentin Bound to PtdIns 4-Kinase

FIGURE 5. Catalytic activity of Pik1 internal deletion mutants. Lysates of cells expressing either GFP-Pik1-(Δ16–833) (lane 1), GFP-Pik1-(Δ34–110) (lane 2), GFP-Pik1-(Δ8–760) (lane 3), GFP-Pik1 (lane 4), mycPik1 (lane 5), untagged Pik1 (lane 6), or mycPik1-(Δ152–191) (lane 7) were subjected to immunoprecipitation using either anti-green fluorescent protein monoclonal antibody 7.1/13.1 (lanes 1–4) or anti-Myc monoclonal antibody 9E10 (lanes 5–7) and protein A/G-agarose beads. Samples of the bead-immobilized proteins were then assayed in vitro for their lipid-kinase activity (top panel) and subjected to immunoblot analysis to assess the amount of protein present (bottom panel). The asterisk marks a proteolytic degradation product of GFP-Pik1 (lane 4).

FIGURE 6. Genetic analysis of Pik1 internal deletion mutants. Heterozygous pik1Δ::KanMX4/PIK1 diploid cells overexpressing either GFP-Pik1 (panel I), GFP-Pik1-(Δ34–110) (panel II), GFP-Pik1-(Δ8–760) (panel III), or GFP-Pik1-(Δ16–833) (panel IV) were induced to sporulate and the resulting tetrads were dissected and germinated on galactose medium. In each panel, the four spore clones (A–D) of 12 representative tetrads are shown. The fact that only the two Pik1+ spores in any given tetrad (panels II–IV) are able to grow demonstrates that, unlike wild-type Pik1 (panel I), none of the mutants is able to support the growth of either of the two Pik1Δ spores and, thus, that removal of the N-terminal segment of Pik1, or of just its LKU motif alone, inactivates the function of Pik1 in vivo.

level of activity is much lower than that of wild-type Pik1 in the absence of Frq1 (Fig. 5, lane 4). Correspondingly, expression in yeast of the Pik1 catalytic domain alone, Pik1-(Δ8–760), does not complement the lethality of a pik1Δ mutation (Fig. 6). These results indicate that the lipid kinase domain of Pik1 by itself is nonfunctional both in vitro and in vivo, in contrast to the constitutive activation observed when the catalytic domains of many protein kinases are separated from their regulatory domains (64–67). A deletion construct of Pik1 that lacks only the Frq1-binding site, Pik1-(Δ152–191), does not bind Frq1, as demonstrated before (22). However, deletion of the Frq1-binding site Pik1-(Δ152–191) has almost no effect on basal catalytic activity; compare Pik1-(Δ152–191) to wild-type Pik1 (Fig. 5, lane 7 versus 5). Hence, activation of Pik1 by Frq1 does not involve relief of an autoinhibitory constraint, contrary to the mechanism by which calmodulin binding activates calmodulin-dependent protein kinases (65). Instead, the Pik1 catalytic domain by itself is inactive and requires interaction and stabilization by an auxiliary domain located within residues 8–760. Indeed, a deletion construct of Pik1 that lacks only the lipid kinase unique (LKU) motif (residues 35–110) has almost no activity compared with wild-type Pik1 (Fig. 5, compare lanes 2 and 4), and this deletion construct also does not complement a pik1Δ mutation (Fig. 6A). Taken together, these results indicate that the LKU motif and the catalytic domain are both essential for functional lipid kinase activity. In summary, it seems that Frq1 binding to Pik1 does not activate kinase activity by removing an autoinhibitory constraint but rather promotes interaction between the N-terminal LKU motif and the C-terminal catalytic domain.

DISCUSSION

We present here the atomic resolution structure of yeast frequentin (Frq1) bound to an N-terminal fragment of its target, a PtdIns 4-kinase isoform (Pik1). The overall main chain topology of Frq1 in the complex is similar to that of free Frq1 in solution (r.m.s.d. = 2.0 Å in EF-hand regions) and other NCS proteins (23, 33–36, 63). In the complex, the first 8 residues at the N terminus and last 10 residues at the C terminus of Frq1 are solvent-exposed and structurally disordered. We propose that the solvent-exposed N-terminal myristoyl group of Frq1 may help recruit Pik1 to membranes where it can encounter its substrate PtdIns. Three Ca2+ are bound to Frq1 at EF2, EF3, and EF4. The four EF-hands form an elongated groove on one side of the protein lined by two distinct hydrophobic patches that interact with two separate α-helical segments in Pik1-(121–174) (Fig. 3B). The intermolecular interactions between Frq1 and Pik1-(121–174) are mostly hydrophobic (Fig. 4). Most interestingly, Frq1 binding induces a U-shaped helix-loop-helix structure in Pik1-(121–174) that we propose permits functional lipid kinase activity by relieving an autoinhibitory constraint.

The sequence similarity of Frq1 and recoverin suggests that a Ca2+-myristoyl switch (i.e. Ca2+-induced extrusion of the N-terminal myristoyl group) (68, 69) by Frq1 might promote the targeting of Pik1 to membranes. Indeed, myristoylation of Frq1 enhances Pik1 activation (70). However, Frq1 binding to Pik1 does not require calcium or myristoylation, and Ca2+-binding deficient mutants of Frq1 and mammalian NCS-1 bind and activate Pik1 (20, 22). If neither Ca2+ nor myristoylation is essential for Frq1 binding to Pik1, then perhaps Frq1 does not possess a functional Ca2+-myristoyl switch. Indeed, we found...
previously that the N-myristoyl group of Frq1 remains solvent-exposed regardless of the Ca$^{2+}$ level (32) and that Frq1 binds to membranes in both the presence and absence of calcium (20). These observations suggest that the myristoyl group of Frq1 may remain extruded even in the Ca$^{2+}$-free state, in contrast to the sequestered myristoyl group observed in Ca$^{2+}$-free recoverin (39). These considerations likely explain the calcium-in-the-sequestered myristoyl group observed in Ca$^{2+}$-free Frq1 (32). The N-myristoyl group of Frq1 remains solvent-exposed regardless of the Ca$^{2+}$ level (32) and that Frq1 binds to membranes in both the presence and absence of calcium (20). These observations suggest that the myristoyl group of Frq1 may remain extruded even in the Ca$^{2+}$-free state, in contrast to the sequestered myristoyl group observed in Ca$^{2+}$-free recoverin (39). These considerations likely explain the calcium-in-the-sequestered myristoyl group observed in Ca$^{2+}$-free Frq1 (32).

Nonconserved residues of NCS proteins at the C terminus and immediately following EF3 (Fig. 1A) may be structurally important for explaining target specificity. The nonconserved C-terminal region of Frq1 (residues, 180–190) has NMR chemical shifts and $\delta_{NNH}$ indicative of an unstructured random coil in the target complex, in contrast to a well defined C-terminal helix seen in free Frq1 (32). The C-terminal helix in free Frq1 makes contact with residues in EF3 and EF4 (Leu-101, Ala-104, Met-121, Ile-152, Phe-169, and Ser-173) that also contact the N-terminal helix of Pik1-(121–174) in the complex (Fig. 4C). Therefore, the N-terminal Pik1 helix appears to substitute for and perhaps displace the C-terminal helix of Frq1, likely leading to the observed C-terminal destabilization in the complex. The corresponding C-terminal helix of KChIP1 is similarly displaced upon its binding to the Kv4.3 channel (36, 37) but not upon its binding to Kv4.2 (35). The C-terminal helix in recoverin forms a stable interaction with EF3 and EF4, enabling the C-terminal helix to perhaps serve as a built-in competitive inhibitor that would presumably block its ability to bind to targets like Pik1 and Kv4.3. This role for its C terminus may provide yet another reason why recoverin is unable to complement a frq1Δ mutation in yeast and could account for why the C-terminal sequences of NCS proteins are not well conserved (Fig. 1A). Another nonconserved region of Frq1 implicated in target specificity is the stretch between EF3 and EF4 (residues 134–146). This region of Frq1 adopts a short $\alpha$-helix in the complex that contacts the N-terminal helix of Pik1-(121–174). By contrast, the region between EF3 and EF4 is unstructured in many other NCS proteins (32, 34, 35, 63, 73).

Pik1 binding to Frq1 is somewhat analogous to target binding seen in other NCS proteins, including recoverin and KChIP1 (Fig. 7). NCS proteins generally have an N-terminal domain of two EF-hands that form an exposed hydrophobic crevice that interacts with a helical segment of target proteins. In recoverin, the two N-terminal EF-hands form an exposed hydrophobic groove that interacts with a hydrophobic target helix from rhodopsin kinase (RK25) (40) (Fig. 7A). The N-terminal EF-hands of KChIP1 interact with a target-helix derived from the T1 domain of Kv4.3 channels (Fig. 7B) (35) and Kv4.3 channels (36). The orientation of the target helices bound to recoverin and KChIP1 are somewhat similar; the C-terminal end of the target helix is spatially close to the N-terminal helix of EF-1. By contrast, the Pik1 target helix binds to Frq1 in almost the exact opposite orientation (Fig. 7C). The N-terminal end of the Pik1 helix is closest to EF1 (Fig. 7C, green) in Frq1, whereas the C-terminal end of the RK25 target helix is closest to the corresponding region of recoverin. Residues Gly-33 and Asp-37 in Frq1, which are not conserved in recoverin, make important contacts with the Pik1 target helix and presumably assist in imposing the observed orientation of the helix. Thus, the requirement that the helix (in this case, from Pik1) must bind to Frq1 with a polarity opposite to that observed for the helices in other target-NCS family member complexes could clearly contribute to dictating the substrate specificity of frequenins, as compared with other NCS subtypes. Another important structural feature seen in the Frq1-Pik1 interaction is that two helical segments of the target are captured in the complex, whereas in the target complexes characterized for recoverin and KChIP1, only one helix.

FIGURE 7. Target binding to various EF-hand proteins. A, RK25 of rhodopsin kinase bound to recoverin (PDB code 2I94). B, Kv4.2N30 peptide derived from T1 domain of A-type voltage-gated K$^+$ channel bound to KChIP1 (PDB code 156C). C, Pik1-(121–174) peptide bound to Frq1 (PDB code 2JU0).
is bound. Therefore, selective substrate recognition by frequenins may be explained by two distinct and unique properties not shared by other NCS proteins as follows: orientation of the bound target helix and the number of target helices bound.

The structure of the Frq1-Pik1-(121–174) complex combined with our deletion and mutational analysis of Pik1 (Table 2) provides insight into the activation mechanism of Pik1 by Frq1 (Fig. 8). Our structure of the Frq1-Pik1 complex reveals that the N-terminal myristoyl group of Frq1 is exposed and thus able to recruit the Frq1-Pik1 complex to membranes where the enzyme can encounter inositol headgroups. Indeed, N-terminal myristoylation of Frq1 enhances Pik1 activation at least 2-fold (70), but myristoylation of Frq1 is not necessary for its activation of Pik1. Therefore, in addition to membrane recruitment, Frq1 must also influence the structure and activity of the lipid kinase catalytic domain in another way. One possibility was that Frq1 might activate the kinase domain indirectly by binding to an autoinhibitory domain in Pik1, analogous to activation of calmodulin-dependent protein kinases (65, 66). However, a deletion construct of Pik1 that retains only the catalytic domain, Pik1-(Δ8–760), has almost no detectable lipid kinase activity compared with full-length Pik1 (Fig. 5), and Pik1-(Δ8–760) does not complement a loss of Frq1 activity (Fig. 5 and Table 2), suggesting that the LKU motif (in addition to Frq1 binding) is essential for lipid kinase activity, possibly because the N-terminal LKU motif must interact with and activate the C-terminal lipid kinase domain. Second, consistent with this notion, the LKU motif is highly conserved in both PtdIns4-kinases and PtdIns3-kinases (11, 14, 19). Third, also consistent with this proposal, in structures of PtdIns3-kinase determined by x-ray crystallography, the LKU motif does seem to interact structurally with the catalytic domain (74–76).

Our structure of the Frq1-Pik1 complex (Figs. 3 and 4) suggests how Frq1 might modulate the interaction between the LKU motif and the catalytic domain (Fig. 8). Frq1 interacts with two antiparallel α-helices of Pik1 (colored magenta in Fig. 8), producing a U-turn structure that causes the N-terminal and C-terminal ends to point in the same direction. We propose that this U-turn structure in Pik1 might orient the N-terminal LKU motif in close proximity to the C-terminal catalytic domain. Given that association between the LKU motif and the catalytic domain has been observed in PtdIns3-kinase (76), and given the findings we report here for the PtdIns4-kinase Pik1, interaction of the LKU motif and the catalytic domain may be a general feature of all lipid kinases that contain such elements and are required for their catalytic activity. It is certainly plausible, in light of the structural information we obtained and as depicted in Fig. 8, that Frq1 binding to residues 121–174 in Pik1 (which are situated adjacent to the LKU motif) would achieve the goal of bringing the LKU motif in close proximity to the C-terminal catalytic domain by causing the conformational change that promotes this structural outcome. Future structural studies on the full-length Pik1 enzyme are now needed at the atomic level to further test this proposed activation mechanism and to define more rigorously the structural nature of the Pik1 active site.

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