The activation loop of PIP5K functions as a membrane sensor essential for lipid substrate processing

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Phosphatidylinositol 4-phosphate 5-kinase (PIP5K), a representative member of the phosphatidylinositol phosphate kinase (PIPK) family, is a major enzyme that biosynthesizes the signaling molecule P(4,5)P2 (phosphatidylinositol 4,5-bisphosphate) in eukaryotic cells. The stringent specificity toward lipid substrates and the high sensitivity to the membrane environment strongly suggest a membrane-sensing mechanism, but the underlying structural basis is still largely unknown. We present a nuclear magnetic resonance (NMR) study on a peptide commensurate with a PIP5K’s activation loop, which has been reported to be a determinant of lipid substrate specificity and subcellular localization of PIP5K. Although the activation loop is severely disordered in the crystal structure of PIP5K, the NMR experiments showed that the largely unstructured peptide folded into an amphipathic helix upon its association with the 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) micellar surface. Systematic mutagenesis and functional assays further demonstrated the crucial roles of the amphipathic helix and its hydrophobic surface in kinase activity and membrane-sensing function, supporting a working model in which the activation loop is a critical structural module conferring a membrane-sensing mechanism on PIP5K. The activation loop, surprisingly functioning as a membrane sensor, represents a new paradigm of kinase regulation by the activation loop through protein-membrane interaction, which also lays a foundation on the regulation of PIP5K (and other PIPKs) by membrane lipids for future studies.

INTRODUCTION

Using phosphatidylinositol phosphates (PIPs) as substrates, phosphatidylinositol phosphate kinases (PIPKs) exclusively biosynthesize phosphatidylinositol 4,5-bisphosphate [P(4,5)P2] and phosphatidylinositol 3,5-bisphosphate [P(3,5)P2], two lipid signaling molecules crucial for many biological processes (1). On the basis of substrate specificity, the PIPK family is further divided into three subfamilies: phosphatidylinositol 4-kinase (PIP5K, type I PIPK) phosphorylates phosphatidylinositol 4-phosphate [P(4)P] into P(4,5)P2, phosphatidylinositol 5-phosphate 4-kinase (PIP4K, type II PIPK) produces the same product but with phosphatidylinositol 5-phosphate [P(5)P] as the preferred substrate, and FYVE finger-containing phosphoinositide kinase (PIKFyve, type III PIPK) converts phosphatidylinositol 3-phosphate [P(3)P] into P(3,5)P2, a very-low-abundance lipid signaling molecule that is essential in intracellular vesicle trafficking. Because the enzymes exclusively produce P(4,5)P2 and P(3,5)P2, PIPKs are involved in numerous physiological and pathological processes (2–4) and have been proposed to be drug targets for cancer, chronic pain, diabetes, and inflammation (5–10).

PIPK5 is a stringent lipid kinase that processes only lipid substrates, because it cannot catalyze phosphate-transferring reaction toward any known soluble compound commensurate with the head group of individual lipid substrates. The activity of PIP5K can be significantly modulated by the membrane lipid composition, and the phosphatidic acid is a known activator of PIP5K (11). These facts strongly suggest the presence of a membrane-sensing mechanism for PIP5K. The highly superimposable crystal structures of the kinase domains of PIP5K and PIP4Ks revealed a canonical protein kinase fold with a large and flattened surface, which was proposed to associate with membrane surface (12, 13). This unique membrane-binding model has been further supported by mutagenesis and functional studies (14). According to this model, the catalytic site faces toward the membrane surface, and a short segment, called activation loop, in the proximity of the catalytic site would directly associate with the membrane (Scheme 1A). Previous biochemical studies have discovered that swapping the activation loops between PIP4K and PIP5K led to not only swapped substrate specificity but also altered subcellular distribution (15, 16), demonstrating the pivotal role of the activation loop of PIP5K in membrane interaction. Because it is exchangeable among different PIPKs, the activation loop is likely to be a structurally independent module with certain α-helical content as indicated by secondary structure prediction (Scheme 1B). Unfortunately, in all the available crystal structures of PIPKs (12, 13), this functionally critical segment is severely disordered, suggestive of either the presence of multiple conformations or being intrinsically unstructured. We reason that the activation loop is not structured and is thus not solved in crystal structures, unless it associates with the membrane surface. We further hypothesize that the activation loop is a critical module responsible for membrane sensing through conformational changes induced by membrane association.

To test this hypothesis, in this work, we conducted nuclear magnetic resonance (NMR) studies on an isolated peptide commensurate with the activation loop region of a PIP5K from zebrafish (zPIP5Kα). Here, we show that the largely unstructured peptide underwent a marked conformational change upon association with the 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) micellar surface and folded into an amphipathic helix. Further mutagenesis and activity assays revealed the crucial roles of the hydrophobic surface of the amphipathic helix in the kinase activity and membrane-sensing function of PIP5K. On the basis of these findings, we propose that the activation loop acts as a membrane sensor conferring PIP5K absolute specificity toward the lipid substrates and high sensitivity to the membrane environment, providing a new perspective on the mechanism of PIP5K activation and regulation by membrane lipids.
RESULTS

The isolated activation loop peptide can be prepared for NMR studies

A 21-residue peptide (AL peptide) corresponding to the activation loop of zPIP5Kα was expressed as an MBP (maltose-binding protein) fusion protein in Escherichia coli. The high yield of the fusion protein (up to 100 mg of protein per liter of culture) enabled us to prepare enough purified AL peptide for structural studies. The AL peptide released from the fusion protein has an additional three residues at the N terminus derived from the tobacco etch virus (TEV) cleavage site (fig. S1A). The 15N, 13C-double-labeled peptide was prepared for NMR characterization. Similar to human PIP5K isoforms (I1), zPIP5Kα exhibits clear preference toward Pl(4)P with unsaturated acyl chains (fig. S1B). Accordingly, acyl chain sensitivity appears to be a common feature among PIP5Ks from different species, and our study on the AL peptide from zPIP5Kα would likely reveal a general mechanism for PIP5K activation and regulation by membrane lipids.

The AL peptide is intrinsically unstructured in the absence of membrane mimics

The AL peptide in solution renders clean NMR spectra in which the resonances of backbone amides are well dispersed, as shown in the 1H–15N heteronuclear single-quantum coherence (HSQC) spectrum (Fig. 1A). The assignment of backbone resonances is achieved through standard triple-resonance NMR experiments, and the presence of residual structures in the peptide is revealed by chemical shift indices (CSIs) (17) and medium-range nuclear Overhauser effect (NOE) patterns. Although the 13Ca CSIs are rather small, indicating that, overall, the AL peptide is largely unstructured, the continuous positive values of 13Ca CSI and the concomitant negative values of 1Hα CSIs for the S392-A395 segment suggest a helical conformation in this region (Fig. 1C). The presence of helical conformation is further confirmed by the medium-range NOE pattern (fig. S2A). Together, the activation loop intrinsically adopts two rapidly exchanged conformations: an unstructured conformation and a partially folded conformation with a helical turn at S392-A395. According to 13Ca CSIs, the population of the unstructured conformation is much larger than that of the partially folded one, which may be less than 20% (18).

The AL peptide folds into an amphipathic helix in the presence of membrane mimics

To mimic the membrane environment, we mixed DHPC, a short-chain phospholipid commonly used in the studies of membrane proteins, with the AL peptide. When the concentration of DHPC (1%, w/v) is just about its critical micelle concentration (CMC) (0.7%, w/v), no significant conformational changes of the AL peptide were observed, as monitored by 1H–15N HSQC spectral comparison (fig. S3). However, in the presence of 7.5% DHPC with a significant amount of DHPC micelles being formed, the 1H–15N HSQC spectrum of the AL peptide changed completely (Fig. 1B).

To our surprise, it turned out that two major conformations, noted as conformations I and II hereafter, coexist with roughly equal populations because there are two sets of strong resonances with similar intensities for individual residues (Fig. 1B). For conformation I, in comparison to that without DHPC, significant changes in amide resonances are among residues E390 and W393-H398 with averaged differential chemical shifts of amides (19), ∆av(HN), larger than 0.1 parts per million (Fig. 1E). In addition, although the general CSI patterns are essentially the same as those without detergent, the 13Ca CSIs for W393-L396 are three to four times larger on average (Fig. 1D). A very similar medium-range NOE pattern remains in this region and is absent in the rest of the region (fig. S2B). Together, both CSIs and NOEs strongly suggest that the partially folded conformation in the absence of DHPC is stabilized by DHPC micelles, likely because of a direct association with the micellar surface through the α-helical segment (W393-L396), as indicated by the large ∆av(HN) values in this region but negligibly small values in the rest.

In contrast, conformation II appears very different from that without DHPC, as indicated by the large ∆av(HN) values throughout the peptide (Fig. 1E). Both 13Ca and 1Hα CSIs strongly indicate an extended helical conformation starting from the N terminus all the way to L397, which is reinforced by medium-range NOEs all over this region (fig. S2C). S392 clearly lacks the corresponding medium-range NOEs with K394 and A395, implying that the extended helix may not be straight but kinked at S392. The helix wheel analysis reveals that the formed helix in conformation II is an amphipathic helix with a hydrophobic surface on one side and a highly positively charged surface on the
Fig. 1. NMR characterization of the AL peptide. $^{1}$H–$^{15}$N HSQC spectra of the AL peptide in the absence (A) and presence (B) of 7.5% DHPC. Spectra were recorded at 10°C on a Bruker AVANCE 900-MHz NMR instrument. The assignment of backbone amides for individual residues is indicated by one-letter notations. The two conformations, I and II, with DHPC in (B) are denoted without and with a parenthesis for each residue, respectively. The inset in each spectrum is the side-chain resonance of residue W393. The $^{13}$C$\alpha$ and $^{1}$H$\alpha$ CSIs of the AL peptide without DHPC (C) and that for the two conformations, I and II, respectively, in the presence of 7.5% DHPC (D) are shown below the corresponding spectra. The profiles of averaged differential chemical shifts of backbone amides, $\Delta\delta$(NH), of the peptide for conformations I and II in the presence of DHPC micelles with respect to that without DHPC are given in (E). (F) Helical wheel presentation of the predicted amphipathic helix of the AL peptide with hydrophilic residues in black letter notations, positively charged residues in blue, negatively charged residues in red, and other residues in green. (G) The proposed folding status of the AL peptide in the absence (left) and presence (right) of DHPC micelles. In the absence of DHPC micelles, the AL peptide is largely unstructured, rapidly exchanging with a small population of the partially folded peptide. Upon DHPC micelle association, the AL peptide folds into two slowly exchanged conformations (I and II), and conformation I appears to be a stable intermediate derived from the partially structured conformation in the absence of the DHPC micelles. The red and black ribbons represent $\alpha$ helices and random coils, respectively. (H) CD spectra of the peptide in the absence of detergent (black curve), with 5% (w/v) DHPC (blue), and with 5% (w/v) DMPC/DHPC at a molar ratio of 1 (red).
opposite side, with E390 and S392 being at the boundary (Fig. 1F). Accordingly, the AL peptide in conformation II likely associates with the surface of the DHPC micelles through hydrophobic interactions while exposing the positively charged surface to the bulky solvent. This model is further supported by linewidth analysis (table S1): For conformation II, the resonances corresponding to the V386-H398 segment, but not to H391 and S392, have clear broader linewidths compared to those in the absence of DHPC micelles, strongly suggesting that the helical region of the AL peptide directly associates with, though weakly, the DHPC micelles that have a much larger molecular weight (≈20,000) and thus slower tumbling rates; in contrast, for conformation I, the residues with broader linewidths are restricted to the region of S392-H398, supporting an association with the DHPC micelles through the limited helical region (W393-L396), as revealed by CSIs (Fig. 1D) and the Δν (HN) (Fig. 1E) data. Because there are no species corresponding to conformation II in the absence of the DHPC micelles, we conclude that micelle-associated conformation I is a stable folding intermediate that is slowly converted into conformation II, which associates with the micellar surface through a much larger hydrophobic interface (Fig. 1G). Consistent with the results derived from the NMR experiments, the helical content of the AL peptide increased markedly when 5% DHPC was added, as demonstrated by the negative peak at 222 nm in circular dichroism (CD) spectra (Fig. 1H). Furthermore, we observed an even higher helical content in the presence of DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine)/DHPC mixed micelles (5%, w/v; DMPC/DHPC molar ratio of q = 1), indicating that the AL peptide undergoes further conformational changes when the membrane-mimic environment is more analogous to the authentic cell membrane.

**The hydrophobic surface of the amphipathic helix is essential for lipid substrate processing**

The amphipathic helix shown in conformation II is induced by DHPC micelles, raising a hypothesis that the helical surface of the activation loop represents a functional conformation that is essential for PIP5K activity. To test this hypothesis, we performed systematic mutagenesis on the hydrophobic residues in the activation loop because these residues constitute the hydrophobic surface and are thus supposed to interact with membrane per se (Fig. 2A). The activity assays were conducted in 5% DMPC/DHPC bicelles (q = 1), which support a strong activity of zPIP5Kα. Proline and glycine residues are known helix breakers; the former introduces a kink in the helix, whereas the latter generally destabilizes the helical structure. Proline scanning on the hydrophobic residues revealed that introducing a kink at L389 or W393 markedly reduced activities toward both lipid substrates, PI(4)P and PI(5)P, whereas the mutations at L396 and L397 had much greater detrimental effects on the activity toward PI(5)P, an unfavorable substrate of PIP5K (Fig. 2B). Glycine mutations had no significant effects on PI(4)P processing but affected PI(5)P processing differently: L389G markedly attenuated the activity, whereas the activities of L385G, V386G, and W393G increased significantly. Accordingly, the activation loop may adopt distinct conformations to accommodate different substrates. Together, it appears that L389 and W393, which are right in the middle of the amphipathic helix, play important roles in both substrate processing and recognition. To further explore the function of the hydrophobic surface of the amphipathic helix, L389 and W393 were mutated into glutamate and arginine, respectively. Because of the salt bridge between E389 and R393, the helical structure of the L389E/W393R double mutant should be preserved according to secondary structure prediction by PSIPRED (Psi-blast–based secondary structure prediction) (20). However, the double mutation did convert the hydrophobic surface into a more hydrophilic one. As expected, the activity of the double mutant decreased markedly, and a more radical mutant with four hydrophobic residues replaced by charged residues completely abolished the activity of PIP5K. Collectively, these data indicate that the structure of the amphipathic helix within the activation loop and its hydrophobic surface are crucial for PIP5K function.

**Disrupting the hydrophobic surface of the amphipathic helix within the activation loop affects the membrane-sensing function of PIP5K**

It has been reported that the activity of PIP5K can be markedly modulated by fatty acid composition of the lipid substrates and phosphatidic acid (11). Our study reveals that PIP5K activity is also sensitive to the size/curvature of the membrane mimics. In the effort of cocrystallization of PIP5K with lipid substrates dissolved in detergents (including Triton X-100, n-dodecyl-β-d-maltoside, decyl β-D-maltopyranoside, octyl β-D-glucopyranoside, Fos-Choline-12, and DHPC), we found that the activity of PIP5K was almost completely
abrogated at a detergent concentration of higher than 1× CMC. Remarkably, we also found that increasing the DMPC/DHPC molar ratio \(q\) greatly stimulated the activity of PIP5K (Fig. 3A). Because the total lipid concentrations were kept the same (5%, w/v) and because both DMPC and DHPC have the same head group, we believe that the increased activity is due to the sensitivity of PIP5K to the surface properties of the mixed micelles. For instance, the small-sized DHPC micelles have a higher surface curvature at a low \(q\) value, whereas the larger mixed micelles, with more DMPC incorporated into the DHPC micelles at a high \(q\) value, have a lower surface curvature or even a flat surface at the central region of the bicelles. Because the AL peptide undergoes markedly conformational changes upon association with membrane mimics, as revealed by both NMR and CD experiments (Fig. 1), we further reason that the activation loop is primarily responsible for the sensitivity to the altered membrane surface environment. In support of this idea, we compared the activation of the wild-type PIP5K by DMPC with that of the L389E/W393R double mutant PIP5K, in which the hydrophobic surface in the activation loop is partially disrupted by charged residues. As shown in Fig. 3B, although the mutant PIP5K can still be activated by adding DMPC, the activation curve is significantly right-shifted, indicating an impaired membrane-sensing function.

To further clarify the activation mechanism of PIP5K by addition of DMPC to DHPC micelles, we collected \(^1\text{H}-^{15}\text{N}\) HSQC spectra of the AL peptide at different \(q\) values \((q = 0, 0.25, 0.5, \text{and} 1.0)\). As shown in Fig. 3C, the \(^1\text{H}-^{15}\text{N}\) HSQC spectra underwent significant changes as \(q\) increased. By overlapping the spectra and tracing the resonances, we assigned most of the resonances in the spectra except for those in crowded regions. Most of the resonances of conformation II were greatly shifted, significantly broadened, and eventually disappeared at \(q = 1\), whereas the resonances of conformation I were also markedly shifted but only moderately broadened at \(q = 1\). These data have at least two important implications. First, the AL peptide underwent conformational changes upon addition of DMPC, which is consistent with the results of the CD experiments (Fig. 1H). Second, significant broadening of the resonances of conformation II indicates that conformation II strongly prefers binding to the bicelles with a larger and more flattened surface (as more DMPC was added), whereas conformation I does not, further suggesting that conformation II is most biologically relevant. Therefore, both conformational changes (as indicated by large changes of chemical shifts) and stronger association with the membrane surface (as indicated by broadening of resonances for conformation II only) are contributive to the higher activity of PIP5K as DMPC content increased (Fig. 3A).

**DISCUSSION**

Different from inositol polyphosphate kinases [the closest homologs of PIPKs (21)] that can process both soluble and lipid substrates efficiently (22), PIP5K does not exhibit activity toward any inositol polyphosphates. The crystal structure and the proposed membrane-binding model common for PIPKs do not support the idea that PIP5K directly binds and recognizes the fatty acyl chains of the lipid substrates. Therefore, the absolute specificity toward lipid substrates strongly implies the presence of a membrane-sensing mechanism that strictly controls the activity of PIP5K. This work focuses on the activation loop of zPIP5Kα, which is severely disordered in the crystal structure, and our combined structural and functional studies strongly suggest that this short segment functions as a membrane sensor conferring a membrane-sensing mechanism on PIP5K.

Consistent with the membrane sensor hypothesis, the largely unstructured AL peptide undergoes marked conformational changes; it
folds into two roughly equally populated conformations upon addition of DHPC micelles. Both conformations represent membrane-bound forms: Conformation II contains an extended amphipathic helix, and both Δav(NH) differential chemical shifts and NMR linewidth analysis suggest that the helix associates with the surface of the DHPC micelles through hydrophobic interactions; conformation I, on the other hand, is most likely derived from the minor species in the absence of DHPC micelles and is stabilized through the association of the preexisting helical turn in the W393-L396 segment with the micellar surface, as suggested by the Δav(NH) data and linewidth analysis. Replacing the conserved hydrophobic residues on the amphipathic helix with helix-destabilizing amino acid residues (proline or glycine) revealed that L389 and W393 are critical in kinase activity and substrate recognition (Fig. 2). Because they are right in the middle of the amphipathic helix in conformation II, mutations on these two residues may lead to the greatest structural changes. Notably, the unstructured L389 in conformation I is not involved in membrane association according to the analysis of Δav(NH) data (Fig. 1E), and therefore, the critical role of L389 in kinase activity suggests that conformation II with the extended amphipathic helix is more likely to be biologically relevant than conformation I. The latter may represent a stable intermediate during the conformational changes necessary for kinase activation (Fig. 1G).

It is interesting that the activity of PIP5K varies markedly upon changing the DMPC/DHPC molar ratio (the q value) in the mixed micelles (Fig. 3). The largely suppressed activity in the presence of pure DHPC micelles can be greatly restored when the q value increases up to 1. Although both NMR and CD data with the isolated peptide showed that it folds into helical structures in the presence of detergent micelles, they may not necessarily represent the exact productive conformation required for kinase activity. For example, the amphipathic helix has an apparent kink at S392 (Fig. 1, E and G, and fig. S2C), which is likely to be an artifact due to the association of the AL peptide on the highly curved DHPC micellar surface. When the curvature of the mixed micellar surface is reduced along with the increased q value, the activity of PIP5K is restored gradually, accompanied by further conformational changes and better membrane association of the AL peptide (Fig. 3C). Finally, the L389E/W393R double mutant exhibited significantly attenuated sensitivity to the changes in the lipid composition, indicating that the activation loop is at least, if not the only, a major structural element conferring high membrane sensitivity.

On the basis of these findings, we propose a membrane-sensing mechanism of PIP5K, as illustrated in Fig. 4. In the absence of membrane, PIP5K is in the inactive state because the activation loop is largely unstructured and the substrate-binding pocket is not fully assembled, making it either too loose or too shallow to grasp and keep the substrate in a proper orientation for catalysis. Alternatively, the unstructured activation loop may block substrate accessing to the catalytic site (23). The reversible association of PIP5K with the membrane surface, which is primarily driven by electrostatic interactions through the positively charged and flattened surface, induces the formation of the amphipathic helix within the activation loop, of which the hydrophobic surface transiently interacts with the hydrophobic core of the membrane. The hydrophobic interactions help anchor the activation loop onto the membrane with proper orientation, which is crucial to position the positively charged surface of the amphipathic helix toward the catalytic aspartate residue. This is a critical step because it renders the participation of these residues at the catalytic site in such a way that an intact substrate-binding pocket is assembled. The essential roles of these highly conserved and positively charged residues for kinase activity had been demonstrated in a previous mutagenesis study (16). Because the PIP5K substrates (PIPs) are negatively charged, the positively charged residues from the activation loop in the proximity of the catalytic site are most likely involved in recognizing the phosphate groups and orienting the head group of the lipid substrate precisely for efficient phosphate transfer reaction. As a result, the evolution of the activation loop into a membrane-associating amphipathic helix enables two critical processes, that is, membrane anchoring/sensing (as the amphipathic helices in many membrane-associating proteins) and substrate binding/processing (like in most kinases), coupled directly, which we believe is the molecular basis of PIP5K activation and regulation by membrane lipids. To our knowledge, this mechanism is fundamentally different from that of well-characterized membrane-associating enzymes using an amphipathic helix for membrane binding and activity regulation (24–28). For

![Fig. 4. Proposed membrane-binding model of the activation loop of PIP5K.](image-url)

In aqueous solution (left), the activation loop is largely unstructured (dotted line), leading to a fairly shallow substrate-binding pocket that cannot keep the substrate in a proper orientation for catalysis. Reversible association with membrane (right) markedly changes the conformation of the activation loop. The induced amphipathic helix in the activation loop is anchored on the membrane surface through hydrophobic interaction with the hydrophobic core of the lipid membrane, whereas the positively charged hydrophilic surface is presented toward the catalytic center (aspartate in red), leading to an intact substrate-binding pocket accommodating and properly orienting the head group of the lipid substrate for catalysis. The structure of zPIP5Ku (PDB code: 4TZ7) is shown in cartoon and surface modes. The amphipathic helix in the activation loop is depicted in cartoon mode with highlighted L389 and W393 in stick mode. The activation loop is linked to the structure of PIP5K through dotted lines.
example, an amphipathic helix within the M domain of cytidine triphosphate:phosphocholine cytidylyltransferase plays an auto-inhibitory role in the membrane-unbound state, whereas it activates the enzyme in a membrane-bound state most likely through a long-range allosteric effect (29). In addition, somehow similar to the antimicrobial peptides that fold into an amphipathic helix and disintegrate the membrane (30), the activation loop of PIP5K may also disturb the membrane integrity and facilitate binding of the head group of the lipid substrate. Consistent with this model, disrupting the hydrophobic surface of the amphipathic helix by mutagenesis not only markedly reduced kinase activity (Fig. 2) but also diminished membrane-sensing function (Fig. 3).

In summary, because the formation of the amphipathic helix absolutely depends on the presence of membrane and the orientation of the critical substrate-coordinating residues is conceivably affected by membrane properties, PIP5K exhibits a stringent specificity toward lipid substrates and is highly sensitive to the membrane environment. Because the amphipathic helix is predicted to be a common structural feature for all the PIPKs (Scheme 1), we therefore propose that this membrane-sensing mechanism is likely shared among other lipid kinases in the PIPK family. It is well known that the activation loop is a critical site for kinase activity regulation primarily through phosphorylation/ dephosphorylation (23). The surprising function of the PIP5K activation loop as a membrane sensor represents a new paradigm on kinase regulation through the unprecedented activation loop–membrane interaction, which lays a foundation on the regulation of PIPKs by membrane lipids for future studies.

MATERIALS AND METHODS
Preparation of the AL peptide
To overexpress the AL peptide of zPIP5Kα, BL21 (DE3) plysS competent cells were transformed with a plasmid construct that carries the activation loop region (GHM380RLVKKLEHSWKALLHGDG401, in which the N-terminal three residues, GHM, were introduced after TEV digestion of the His-tag) with its N terminus fused to the C terminus of MBP and a His-tag plus a TEV cleavage site engineered between MBP and the peptide. The unlabeled peptide was produced in LB medium, whereas 15N- and 15N/13C-labeled samples in M9 minimal defined medium with 15N-ammonium chloride and U-13C-glucose served as sole sources for nitrogen and carbon atoms, respectively. To produce the unlabeled peptide, cell colonies from the agar culture plate containing ampicillin and chloramphenicol after incubating overnight at 37°C were collected and pooled into 1 liter of LB supplemented with the antibiotics. The LB rotary flask underwent vigorous shaking (220 rpm) at 37°C, and the culture was paused when optical density at 600 nm (OD600) reached ~0.65. The flask was then placed in a cold room (4°C) for half an hour, and the induction was initiated with 1 mM DTT, incubation continued overnight at 4°C. After the addition of 1 mM DTT, incubation continued overnight at 4°C. The TEV-digested peptide was eluted by centrifugation for 25 min using an Amicon centrifugal filter device (30,000 MWCO). The concentrated solution was transferred into a few 1.5-ml tubes, each containing 0.5-ml solution with the protein concentration at ~100 mM. Appropriate amounts of TEV protease, 0.5 mM EDTA, and 1 mM dithiothreitol (DTT) were added, and the volume of the final solution in each tube was 0.7 to 0.8 ml. The tubes were incubated on a roller at room temperature for 1 to 2 hours for TEV digestion. After the addition of 1 mM DTT, incubation continued overnight at 4°C. The TEV-digested peptide was eluted by centrifugation for 25 min using an Amicon centrifugal filter device (30,000 MWCO) and then subjected to another elution using a new Amicon centrifugal unit to eliminate residual MBP contamination. The peptide concentration was measured on a NanoDrop. The peptide solution was further concentrated through evaporation under vacuum, and the substitution for the NMR buffer was achieved through dialysis using small tubes with a 1000-MWCO membrane.

NMR measurement
The NMR samples contained 0.2 to 0.4 mM peptide solution in 100 mM NaCl and 20 mM phosphate buffer at pH 7.0 without detergent. The volume of each NMR sample was ~300 μl in a Shigemi tube, containing 5% D2O, 150 μM NaN3, and 50 μM 4,4-dimethyl-4-silapentane-1-sulfonic acid as inner NMR reference. All NMR data were collected on a Bruker AVANCE 900-MHz instrument equipped with a TCI cryoprobe. The optimum temperature for NMR measurements was found to be 10°C after recording a series of 1H–15N HSQC spectra at 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, and 35°C, based on the appearance of resonance and the linewidth. For the NMR sample in the absence of detergent, the backbone resonance assignment was accomplished through the analysis of three-dimensional (3D) HNCA and 15N-dispersed [1H–15N]-NOESY (nuclear Overhauser effect spectroscopy) spectra. For the peptide in the presence of 7.5% DHPC, two additional 3D data sets, HNCA and HNCOCA, were recorded to facilitate NMR assignment. The mixing time for the...
NOESY spectra was set to 200 ms. Data were processed using the program NMRPipe (31) and were analyzed using the program NMRView (32). More details of the NMR experiments are described in the Supplementary Materials.

CD measurements

The far-ultraviolet CD spectra were recorded on a Chirascan spectropolarimeter (Applied Photophysics) using a 1-mm path-length quartz cuvette at 20°C over 200 to 260 nm with a bandwidth of 1 nm. The scan speed was set with a response time of 3 s and a step resolution of 0.3 nm. The results represent the average of three scans. All spectra were background-corrected by subtracting corresponding blank spectra without the peptide. The peptide (final concentration, ~0.1 mM) was dissolved in a buffer containing 20 mM sodium phosphate (pH 7.0) and 100 mM NaCl.

PIPK5 preparation and kinase activity assay

zPIPK5α and the mutant proteins were prepared as previously reported (13). In brief, the expression vector (pET41b) harboring the gene of zPIPK5α (kinase domain, residues 49 to 431, synthesized in GenScript Inc. with an optimized codon for E. coli expression) was transformed into Rosetta 2 (DE3) Competent Cells (Novagen). When the OD600 of the culture in LB medium reached 0.4 at 37°C, the culture was cooled down on ice for half an hour, and overexpression protein in the supernatant was purified using Co2+-resin (Talon, Clontech). The OD600 of the culture in LB medium reached 0.4 at 37°C, the culture was cooled down on ice for half an hour, and overexpression protein was induced by 0.1 mM IPTG at room temperature for an additional 16 hours. The cells were harvested and suspended in a lysis buffer containing 50 mM sodium phosphate (pH 7.3), 300 mM NaCl, 5% glycerol, 0.5% Triton X-100 (American Bioanalytical), and EDTA-free protease inhibitor cocktail (Roche). The cells were lysed by sonication on ice and subjected to centrifugation (10,000 rpm for 30 min). The protein in the supernatant was purified using Co2+-resin (Talon, Clontech) and then applied to a size-exclusion column equilibrated with the gel filtration buffer containing 10 mM Hepes (pH 7.3), 300 mM NaCl, 5% glycerol, and 0.03% Triton X-100. The peak fraction was used for activity assay, and the protein concentration was measured using the Bradford method (Bio-Rad).

The kinase activity assay was modified from a previously reported approach (13). The following components were included in one reaction (50 μl): 100 ng of purified zPIPK5α, 100 mM tris-HCl (pH 7.4), 50 mM EGTA, 100 mM MgCl2, 20 μM ATP with 1 μCi [γ-32P]-ATP (PerkinElmer), and 10 μM diC16-P(4)P (Echelon Biosciences Inc.). The reaction was performed at room temperature for 1 hour and was stopped by the addition of lipid extraction solution containing chloroform, methanol, and HCl with a volume ratio of 3:3:3.7:0.1, as well as bovine follicular fluid (10 μg/ml). After vortexing for 20 s, the sample was centrifuged at 6000 rpm for 1 min, and the lower organic phase was collected, loaded, and separated on a thin-layer plate. The product of the reaction was quantified by a Storm 820 PhosphorImager (GE). To measure the activity of PIP5K in DMPC/DHPC bicelles, diC16-P(4)P (final concentration, 10 μM) was dissolved in 5% (w/v) DMPC/DHPC (q = 1) mixture by incubation for 30 min at room temperature following by three cycles of freeze-thaw treatment as described above. Before adding ATP to initiate the reaction, the purified PIP5K was incubated with the lipid substrate dissolved in DMPC/DHPC mixed micelles for 10 min at room temperature. The other processes were the same as described above. The results obtained in diC16-P(4)P vesicles are shown in Figure S2 and the activity measured in DMPC/DHPC bicelles (q = 1) is shown in Figure 2B.

To prepare DMPC/DHPC (both from Avanti Polar Lipids) mixed micelles, a bicele sample [q = 3 (15%, w/v)] was produced according to previously reported approaches (33, 34). In brief, DMPC powder was dissolved in DHPC solution with at least five cycles of freeze-thaw treatment until DMPC was completely dissolved and the solution was clear and homogeneous at low temperatures. In each cycle, the samples were vigorously vortexed for 1 min at room temperature and subjected to incubation at 42°C for 10 min, followed by freezing at ~80°C for 10 min and then thawing on ice. By mixing the bicele sample [q = 3; 15%, w/v] and DHPC (q = 0; 15%, w/v) with different ratios, the DMPC/DHPC mixed micelles were prepared, and the stock solutions were stored at ~80°C. The lipids were all dissolved in water.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/11/e1600925/DC1

REFERENCES AND NOTES


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The activation loop of PIP5K functions as a membrane sensor essential for lipid substrate processing
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