Supplementary Information - Results and Discussion

Membrane binding mode of PI4K IIα. In our replica exchange Monte Carlo simulations we identified a second group of solutions where the kinase binds the membrane only loosely. As shown in Fig. S3B, in our simulations the W^{359} residue always remains close to the membrane, and only weakly fluctuates above the z=2nm plane. In contrast, R^{275} in helix H5 exhibits much larger variations in the distance to the membrane. Here, we observe altering periods when it is in close contact with the membrane, and periods when it is well-separated from the membrane. This observation suggests that the kinase may fluctuate between different membrane-bound states. Indeed, visual inspection of the simulation configurations revealed two major groups of configurations: One group corresponds to a state in which the kinase is tightly bound to the membrane as shown in Fig. 3A. The other group corresponds to a state in which the kinase is loosely bound to the membrane as depicted in Fig. S3C. In the "tight" configuration, the ATP molecule points directly towards the lipid bilayer, and the kinase is bound to the membrane by several spatially distinct regions, which include the segment between P^{164} and D^{182}, the lateral hydrophobic pocket, and groups of residues in helices H5 and H7. In the "loose" state, the ATP binding site is exposed to the solution and away from the membrane, and the kinase is bound to the membrane only by the P^{164}-D^{182} segment and the lateral hydrophobic pocket. This variability in positioning could in principle allow for an effective exchange of ADP for ATP subsequent to the transfer of phosphate to the inositol ring of the lipid. We also note that in the "loose" state, the protein has more configurational freedom and, thus, larger entropy. In the "tight" state, on the other hand, the interaction energy dominates.

Possible ligands of the hydrophobic pocket. As mentioned before, sterically speaking the hydrophobic pocket could accommodate the head group of several phospholipids, including the inositol ring of PI and cholesterol (Fig. S2). However, the proximity of the hydroxyl groups of an inositol ring to the hydrophobic side chains of F^{364} and W^{359} would make such interaction energetically unfavorable. Another lipid implied in the control of PI4K IIα palmitoylation and activity is cholesterol. A cholesterol molecule would sterically fit in the hydrophobic pocket and all interactions would be energetically plausible. However, a proof of cholesterol binding to the kinase has so far proven to be difficult as the enzyme precipitated in any buffer where cholesterol was at least partially soluble.

Supplementary Materials and Methods

Protein expression and purification. Wild type and mutant constructs were expressed in *E. coli* BL21 Star in ZY 5052 autoinduction media [1]. The temperature was lowered to 18°C after they reached an optical density of 0.8 (OD 600 nm). Selenomethionyl derivatives were prepared in the same way in PASM-5052 media [1]. The proteins were affinity purified using Ni-NTA resin (QIAGEN) and the affinity tag was removed by cleavage with TEV protease (18 hours, 4°C). The pseudo-wt protein and mutants (residues 76-479) were further purified by anion exchange chromatography Mono Q 5/50 GL column (GE Healthcare). The crystallographic construct (residues 76-467 with T4 insertion) was further purified by size exclusion chromatography at Superdex 200 column (GE Healthcare) in 30 mM Citrate, pH = 5.5, 200 mM NaCl, 3 mM 2-mercapthoethanol buffer. Proteins were concentrated to 5 mg/ml, flash frozen in liquid nitrogen and stored at -80°C until use.

Crystallization and data collection. Before setting up crystallization drops, PI4K IIα was supplemented with ATP and $MgCl_2$ (final concentration of 5 mM and 2 mM, respectively).. Crystals of the wt PI4K IIα grew in two days at 20°C in hanging drops consisting of a 1:1 mixture of the protein and a well solution (8% PEG 4000; 150 mM $(NH_4)_2SO_4$; 100 mM MES, pH = 6.5; 10 mM betaine). To generate construct suitable for anomalous diffraction data collections using selenomethionine (SeMet) crystals seven point mutations (I121M, C124A, I125M, C183A, L315M, I316M, C320A) were introduced to improve stability and anomalous signal of the SeMet crystals. SeMet crystals were prepared in sitting drops consisting of a 1:1 mixture of the protein and a well solution (6% PEG 8000; 20% ethylene glycol; 30 mM $MgCl_2$; 30 mM $CaCl_2$; 100 mM MES/imidazole pH = 6.5). Prior to freezing, native crystals were cryo-protected in the well solution supplemented with 20% glycerol. Most crystals were screened at the ESRF beamlines ID 14-4 and ID-23-1. Final datasets were collected from single frozen crystal at beamline 14-2 (native) and 14-1 (SeMet) operated by the Helmholtz-Zentrum Berlin (HZB) at the BESSY II electron storage ring [2]. All data were processed and scaled using the XDS package. The crystals belonged to the $P2_12_12$ spacegroup and diffracted to 2.77Å. The structure was solved using MR-SAD with T4 lysozyme used as the MR model in MolRep of the CCP4 package [3], A further 8 SeMet sites were found with Phaser [3], density modification was performed in Parrot [3] and initial model was build using Buccaneer [4]. The model was

manually improved using Coot [5] and Phenix [6]. The model was refined to to R_{work} = 21.15% and R_{free} = 25.11%. Structural figures were generated by using PyMol [7].

Replica Exchange Monte Carlo simulations. To simulate physical interactions of the kinase with the membrane, we used a coarse-grained model equipped with a transferable energy function that has been developed to simulate proteins both on membranes and in solution [8]. In the framework of this model, amino acid residues are represented as spherical beads centered at the C-alpha atoms. The interactions of the residue beads with the membrane are described by statistical amino-acid dependent potentials and Debye-Hückel-type electrostatics. A detailed description of the model can be found in [8]. Here, the lysozyme in the crystal structure was replaced with the physiological LCCPCCF sequence. To mimic the attachment of the four palmitoylated Cys residues to the lipid membrane, we imposed soft harmonic potentials on the z-coordinates of the C-alpha atoms of these Cys residues relative to the membrane surface located at z=2nm. The 19-residue segment between P^{164} and D^{182} was simulated as a flexible loop (as part of it was replaced by T4 lysozyme in our crystal structure and the residues in the vicinity could have been affected by the fusion) with appropriate amino-acid dependent bending, stretching and torsion potentials. Also the two loops disordered in the structure (segments between R^{232} and R^{250}, and between D^{319} and P^{338}) were simulated as flexible. The remaining part of the protein was simulated as a rigid body based on the crystal structure. We performed replica exchange Monte Carlo (MC) simulations with 10^8 MC sweeps, followed by 10^7 sweeps of equilibration. In the course of the simulations, configurations of the kinase were saved every 10^4 MC sweeps, which gave us an ensemble of 10^4 configurations for further analysis.

To characterize the relevant interactions of the kinase with the membrane, we computed the probabilities of individual residues being inserted into the membrane, i.e., the probabilities that the residue beads are below the z=2nm plane, see Fig. S3A. This analysis shows that the kinase regions that localize in the membrane most often are the segment between Pro^{164} and Asp^{182} (which includes the LCCPCCF motif), and the lateral hydrophobic pocket. Other residues that frequently interact with the membrane are localized in helices H5 and H7. To further investigate this observation, we selected several residues in the membrane-interacting regions of the kinase, and analyzed their distance to the z=2nm membrane plane as a function of simulation progression, see Fig. S3B. This analysis revealed two groups of membrane-bound configurations, as described in Results and Discussion (Fig. 3A and Fig. S3C).

Docking studies. The 3D structures of the docked molecules were built using ACD/ChemSketch 12.01 (www.acdlabs.com) and the geometry was optimized with MOPAC2012 (www.OpenMOPAC.net) using PM7 method. The necessary format conversions were performed using OpenBabel [9]. The preparation of the pdbqt files was done by standard procedure using AutoDock Tools 1.5.6. The docking runs were performed in AutoDock Vina using the default scoring function. Docking of the ligands (cholesterol, inositol and inositol-1-phosphate) into the lateral hydrophobic binding pocked was performed in 30 × 28 × 32 Å search space centered at 42.9, 9.5, -24.0 Å and exhaustiveness 100 [10]. Docking of inositol-1-phosohate into the putative PI binding pocket was done in 18×18×18 Å search space centered at -16.6, -13.1, 24.5 with exhaustiveness 100 and two flexible residues Leu^{272} and Leu^{437}, the model with the most probable orientation of phosphate group was chosen for presentation.

In vitro Soluble PI4KIIα Kinase Assay. COS-7 cells transiently transfected with PI4KIIα-mRFP constructs were lysed in ice-cold lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.25% deoxycholate, 1% Nonidet P-40, 1 mM Na3VO4, 1 mM dithiothreitol, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Soluble fraction was then incubated for 1 h with streptavidin-conjugated Dynabeads (Life Technologies, Carlsbad, CA) prebound with biotin-conjugated anti-mRFP antibody (Rockland, Gilbertsville, PA). Samples were washed five times with cold lysis buffer supplemented with 1 M LiCl and protease inhibitors, followed by three washes in the kinase assay buffer, containing 40 mM Tris–HCl, pH 7.5, 20 mM MgCl2, 1 mM EGTA, 0.2% Triton X-100, 0.5 mM DTT and 0.1% bovine serum albumin (BSA). PI4KIIα-bound Dynabeads were then incubated in 200 μL of kinase assay buffer supplemented with ultrapure ATP (0.3 mM final concentration) and PI (0.8 mM final concentration) for 1 h. Reactions were stopped with the addition of 200 μL of ADP-Glo reagent (Promega, Madison, WI), incubated for 40 min 400 μL of Kinase Detection Reagent (Promega) and read on Tristar2 plate reader (Berthold, Bad Wildbad, Germany). In case of purified recombinant proteins the assay was done in the same way with modifications. Reactions were carried out in a total volume of 5μl in the 384-well plate format by mixing three stock solutions: 2μl of PI4KIIalpha or PI4KIIalpha carrying mutation (final concentration 85 nM) in kinase buffer (20mM TRIS pH 7,5; 5mM MgCl2; 0,2% Triton-X; 0,1 mg/mlBSA; 2mM DTT), 1μl of PI in kinase buffer (50

μM final concentration), and the reaction was started by adding 2μl of ATP in kinase buffer (final concentration 100 μM). The reaction was carried out for 60 minutes at room temperature (22° C) and the amount of hydrolyzed ATP was measured according to the manufacturer's protocol (add 5 μl of ADP/GloTM Reagent to terminate the kinase reaction and deplete the remaining ATP, then add 10 μl of Kinase Detection Reagent to simultaneously convert ADP to ATP and allow the newly synthesized ATP to be measured using a luciferase/luciferin reaction). Luminiscence was measured using spectrophotometer TECAN infinite M 1000.

Supplementary Figure Legends

Supplementary Figure S1

A) **Superposition of PI4K IIα and ctkA.** The N-lobe and C-lobe of PI4K IIα are in orange respectively cyan. The *Helicobacter pylori* ctkA kinase domain is shown in pink.

B) **Alignment of selected type II PI4 kinases**. Secondary structure is annotated. Key residues forming the ATP binding site are highlighted in violet. Key residues forming the hydrophobic pocket are highlighted in blue. The catalytically important D^{308} residue is highlighted in red.

C) **Superposition of N-lobes of PI4K IIα and PI4K IIIβ.** The N-lobe of PI4K IIα is in orange and N-lobe of PI4K IIIβ is in pink. Topology plots in respective colors are shown for both lobes.

D) **Superposition of C-lobes of PI4K IIα and PI4K IIIβ.** The C-lobe of PI4K IIα is in cyan and N-lobe of PI4K IIIβ is in pink. Topology plots in respective colors are shown for both lobes.

Supplementary Figure S2

A) **Inositol docked in the hydrophobic pocket.** While sterically possible the hydroxyl groups of the inositol ring are in close proximity to the hydrophobic side chains of F^{364} and W^{359} making the interaction energetically unfavorable, which suggests that inositol is not the physiological ligand of the lateral C-lobe pocket. The protein backbone is shown in cartoon representation, N-lobe colored in orange, C-lobe in cyan. ATP and side chains of selected residues are shown in stick representation and colored according to elements. Oxygen is shown in red, nitrogen in blue,

phosphor in orange, kinase carbon atoms are colored according to domain assignment and inositol's carbons are in silver. Hydrogen atoms are not shown.

B) **Cholesterol docked in the hydrophobic pocket.** Cholesterol molecule sterically fits in the hydrophobic pocket and all interactions are energetically plausible. The color code is the same as in panel A).

Supplementary Figure S3

Analysis of the kinase configurations obtained in simulations

A) Probability that the individual residue beads are below the z=2nm membrane plane. The kinase regions that often localize in the membrane are helices H5 and H7, the hydrophobic pocket (hp), and the segment between K^{165} and F^{179}.

B) Distances of R^{275} (top), W^{368} (middle) and W^{359} (bottom) from the z=2nm membrane plane as functions of simulation progression.

C) A typical configuration corresponding to the "loose" state in which the kinase is tethered to the membrane only by the four palmitoyl groups, the segment between K^{165} and K^{172}, and the lateral hydrophobic pocket. N-lobe colored in cyan, C-lobe in orange, membrane-interacting segments in purple. ATP and W^{359} of the hydrophobic pocked shown as sticks colored according to elements as in Fig. 2. Palmitoyl groups are modeled and shown as black sticks.

Supplementary Figure S4

Effects of PI4KIIα mutations on subcellular localization of the kinase.

Confocal micrographs of live COS-7 cells expressing either the wild-type PI4KIIα-mRFP construct (upper right panel), or PI4KIIα-mRFP 4Cys mutant (the palmitoylated motif CCPCC mutated to SSPSS; upper left panel), solvent exposed (SE) hydrophobic loop mutant 332-WVVV-335 mutated to 332-SAAA-335 (lower left panel), or W^{359}A W^{368}A (lower right panel). Note the higher cytoplasmic fraction and plasma membrane localization of the 4Cys mutant, but no obvious effect on the distribution of the other mutant forms. Scale bars: 10 μm.

Supplementary Citations

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