The Structural Basis for Calcium Inhibition of Lipid Kinase PI4K II α and Comparison With the Apo State

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Summary

PI4K II α is a critical enzyme for the maintenance of Golgi and is also known to function in the synaptic vesicles. The product of its catalytic function, phosphatidylinositol 4-phosphate (PI4P), is an important lipid molecule because it is a hallmark of the Golgi and TGN, is directly recognized by many proteins and also serves as a precursor molecule for synthesis of higher phosphoinositides. Here, we report crystal structures of PI4K II α enzyme in the apo-state and inhibited by calcium. The apo-structure reveals a surprising rigidity of the active site residues important for catalytic activity. The structure of calcium inhibited kinase reveals how calcium locks ATP in the active site.

Key words

Lipid kinase • Calcium • Phosphatidylinositol • Crystal structure

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Introduction

Phosphatidylinositol 4-phosphate (PI4P) is an important regulatory molecule that helps to define Golgi and trans-Golgi network (TGN) and serves as a precursor for higher phosphoinositides (Cauvin and Echard 2015, Tan and Brill 2014). Phosphoinositides also serve as specific markers for intracellular membranes that are more or less specifically recognized by phosphoinositide binding domains such as PH domains (Hammond and Balla 2015, Boura and Hurley 2012). In humans, PI4P is

synthesized by four phosphatidylinositol 4-kinases (PI4Ks) divided into two groups. We have two type II PI4Ks (PI4K II α and PI4K II β , also known as PI4K2A and PI4K2B) and two type III PI4Ks (PI4K III α and PI4K III β also known as PI4KA and PI4KB) (Boura and Nencka 2015, Clayton *et al.* 2013). Type II PI4Ks are inhibited by calcium whereas type III kinases are sensitive to wortmannin, an inhibitor of PI3 kinases (Downing *et al.* 1996). The Golgi localized PI4K II α is responsible for the synthesis of ~50 % of total PI4P and together with PI4K III β is important for Golgi related trafficking (Jovic *et al.* 2012, Jovic *et al.* 2014). Notably, some lipids important in Parkinson's or Gaucher's disease such as ceramide or glucosylceramide are transported in PI4P dependent fashion (Waugh 2015, Dornan *et al.* 2016).

Until recently no structures of PI4Ks were available at all. It was only in the last few years that structural knowledge of PI4K biology began to emerge. The structures of PI4K II α and II β were solved in a nucleotide-bound complex (Baumlova *et al.* 2014, Zhou *et al.* 2014, Klima *et al.* 2015) and the structure of PI4K III β was solved in complex with the small GTPase Rab11 and an unspecific lipid kinase inhibitor Pik93 (Burke *et al.* 2014). The structural insight confirmed bioinformatics-based predictions (Brown and Auger 2011) that type II PI4Ks adopt an atypical lipid kinase fold where only the C-lobes bear structural similarity to other lipid kinases and the N-lobes resemble the N-lobes of a protein kinase. On the other hand, structural analysis of PI4K III β revealed a typical kinase fold very similar to PI3Ks. Importantly, PI4K III β is considered a potential

drug target against plus-RNA viruses (Altan-Bonnet and Balla 2012). The synthesis of highly specific inhibitors and structural basis of their function was described recently (Mejdrova *et al.* 2015), as was the structural mechanism of PI4K III β recruitment and activation by the Golgi resident ACBD3 protein (Klima *et al.* 2016).

For most kinases the structure of the apo-state is very difficult to obtain as a ligand stabilizing the kinase domain is critical for successful crystallization experiments. This missing structural information is necessary to better understand co-factor recognition by PI4K II α . Therefore, it would also be useful for inhibitor design. Previously, to obtain crystals of PI4K II α with ATP, we had to remove the intrinsically disordered regions that impede crystallization (Rozycki and Boura 2014) and replace a flexible loop that is palmitoylated in cells with T4 lysozyme (Baumlova *et al.* 2014). Now we took advantage of the available crystals with ATP and we used them for the first round of microseeding using conditions without a nucleotide. After further optimization we obtained diffraction quality crystals of PI4K II α with no ligand bound. In this study we present the structure of PI4K II α in the apo-state. To our surprise, we demonstrate that most of the residues in the active site have the same conformation as when bound to ATP with the exception of the G-loop, a region that binds ATP phosphate groups.

Next we were interested in the mechanism of inhibition by calcium ions. We were able to obtain calcium and ATP containing crystals. Our structural analysis provides atomic insight into calcium ions inhibition of PI4K II α .

Materials and Methods

Protein expression and purification

The proteins were expressed and purified using previously developed protocols (Boura *et al.* 2010, Nemecek *et al.* 2013). Briefly, the proteins were expressed in *E. coli* strain BL21 Star. After affinity purification on Ni-NTA resin (Machery-Nagel) the solubility tag (6xHis-GB1) was removed by TEV protease and the proteins were further purified using Superdex 200 column (GE Healthcare). Proteins were concentrated to 8 mg/ml and stored at -80 °C until needed.

Kinase luminescence assay

The commercial ADP-Glo assay (Promega) was

used according to previously published protocol (Tai *et al.* 2011). Briefly, reactions were carried out in a total volume of 5 μ l to contain 100 nM PI4K II α (wt or mutants), 50 μ M PI in kinase buffer (20 mM Tris pH 7.5; 5 mM MgCl₂; 0.2 % Triton-X; 0.1 mg/ml BSA; 2 mM DTT) with and without 10 mM calcium chloride. The reaction was carried out for 60 min at RT (295 K) and the amount of hydrolyzed ATP was measured according to the manufacturer's protocol using TECAN infinite M 1000 plate reader.

Crystallographic analysis

Diffraction quality crystals of the apo-state enzyme were obtained by microseeding into a Morpheus screen (Gorrec 2009) based condition (12.5 % w/v PEG 1000, 12.5 % w/v PEG 3350, 12.5 % v/v MPD, 0.1 M bicine/Trizma base pH 8.5). The calcium bound crystals grew in similar conditions supplemented with 30 mM calcium chloride. The complete datasets were collected at the BESSY 14.1 MX beamline using Pilatus 6M detector (Mueller *et al.* 2012). Crystals of the apo-state diffracted to 3 Å, calcium bound crystals diffracted to 2.4 Å and they both belonged to the P2₁2₁2 spacegroup. Data were scaled and integrated in XDSAPP (Krug *et al.* 2012) and were cut at CC_{1/2}=50 %. The structures were solved by molecular replacement using the structure of ATP bound PI4K II α (pdb code 4PLA) as the search model. The structure was refined in Phenix (Adams *et al.* 2010) and Coot (Emsley *et al.* 2010) to R_{work, apo}=24.64 % and R_{free, apo}=28.89 % and to R_{work, Ca}=21.92 % and R_{free, Ca}=25.77 % and to good stereochemistry as summarized in Table 1.

Results

The overall conformation of the kinase, as expected, did not change in either case (Fig. 1). The kinase has N- and C-lobes with the active site in-between. The T4 lysozyme that improved solubility of the recombinant protein and the quality of crystals.

Prior to our study, only structures with a nucleotide or nucleotide analogue MD59 (Dejmek *et al.* 2015) were available. We compared the apo-active site to the active site bound to ATP (pdb code 4PLA) and to the active site bound to MD59 (pdb code 4YC4). Analysis of the apo-state and ATP bound state (Fig. 2A) revealed surprising rigidity of the active site residues important for catalytic activity (S134, V150, K152, Q261, and I345).

Table 1. Statistics of crystallographic data collection and refinement.

Crystal	apo PI4K II α	Ca + ATP bound PI4K II α
Wavelength ($\text{\AA}$)	0.9796	0.9834
Resolution range ($\text{\AA}$)	48.97-2.80 (2.89-2.80)	48.95-2.28 (2.362-2.28)
Space group	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2
Unit-cell parameters ($\text{\AA}$, °)	a=80.01 b=101.52 c=78.14, $\alpha=\beta=\gamma=90$	a=78.88 b=102.03 c=78.93, $\alpha=\beta=\gamma=90$
Unique reflections	16220 (1522)	29715 (1409)
Multiplicity	7.07	7.3 (7.3)
Completeness (%)	99.22 (94.89)	99.93 (99.97)
Mean I/ σ (I)	8.38 (0.66)	11.16 (1.46)
CC _{1/2} *	48 %	54.2%
Wilson B factor ($\text{\AA}^2$)	70.5	44.9
R _{merge}	23.4 % (198.5 %)	13.2 % (136.9 %)
Reflections used for R _{free} (%)	5 %	5 %
R _{work}	24.64 %	21.92 %
R _{free}	28.89 %	25.77 %
No. of non-H atoms	3894	4228
R.m.s.d., bonds ($\text{\AA}$)	0.004	0.009
R.m.s.d., angles (°)	0.91	0.99
Ramachandran favored (%)	97	96
Ramachandran outliers (%)	0.21	0

* value used for data cut-off.

The whole glycine rich G-loop (130-136) was in a different conformation. However, this is expected as it binds the ATP phosphate groups. Probably the G-loop is highly flexible which is supported by its high B-factor (106.6) compared to the rest of the structure (76.5). The remarkable fact is that even the side chains of residues that do make hydrogen bonds with ATP (K152 and Q261) are in the same conformation as in the apo-structure (Fig. 2A). Comparison of the apo- and the MD59 bound states (Fig. 2B) revealed similar conformations of the G-loop. This is in agreement with the fact that the G-loop binds the phosphates of ATP which are not present in nucleotide analog MD59. The residues that contribute to MD59 binding are in the same conformation as residues that bind the ATP adenine ring and sugar (Klima *et al.* 2015). However, the conformation of the D346 residue is different. In the apo-state it resembles the conformation it has in the ATP bound state. PI4K II α also contains a lateral pocket within its C-lobe that is capable of ATP binding and its prosed physiological function is to position correctly position the kinase in respect to the lipid bilayer (Baumlova *et al.* 2014) as its surface exposed tryptophan residues were observed to be inserted to the lipid bilayer (Zhou *et al.* 2014). However, the comparison of ATP

bound and ligand bound lateral pocket did not reveal any conformational change (Fig. 2C).

Fig. 1. The overall fold of PI4K II α . The general fold is the same in the apo and liganded state. C-lobe is colored in cyan, N-lobe in orange and T4 lysozyme in gray. The superimposed liganded kinase (pdb code 4PLA) is colored in light blue and transparent.

Fig. 2. Comparison of the ATP binding sites. **A)** The apo and ATP bound active site. C-lobe is colored in cyan, N-lobe in orange and T4 lysozyme in gray. The superimposed ATP bound kinase (pdb code 4PLA) is in gray except for the ATP that is colored according its elements. **B)** The apo and MD59 bound active sites. C-lobe is colored in cyan, N-lobe in orange and T4 lysozyme in gray. The superimposed ATP bound kinase (pdb code 4YC4) is in red except for the ATP that is colored according its elements. The red arrow points to the flexible D346 residue. **C)** The apo and ATP bound lateral pocket. C-lobe is colored in cyan. The superimposed ATP bound kinase (pdb code 4PLA) is in gray except for the ATP that is colored according its elements.

Fig. 3. Structural and functional analysis of PI4K II α inhibition by calcium. **A)** The network of hydrogen bonds in the active site. C-lobe is colored in cyan, N-lobe in orange. The ATP is colored according to its elements. Calcium is shown as green spheres, water molecules as red spheres. The unbiased Fo-Fc map at 3 σ is shown for the calcium atoms. **B)** Detailed view of the active site. Colored as previous panel. **C)** The complex water network that holds the two calcium atoms in place. **D)** Mutagenesis analysis of the active site. The activity of each mutant was measured with and without 10 mM calcium ions and compared to the wt-enzyme.

We solved the structure of calcium bound PI4K II α to understand how calcium ions inhibit the enzyme at the atomic level. The structure revealed a complex network of hydrogen bonds formed between two calcium atoms, water molecules and residues within the active site (Fig. 3). We wanted to prepare a kinase that would not be inhibited by calcium because it would be a great tool to explore the physiological role of PI4K II α calcium inhibition. We

selected three residues that directly (N313, D346) or indirectly (E193) bind calcium (Figs 3B and 3C) but are not essential for kinase activity for mutagenesis analysis (Fig. 3D). Based on our structure we hypothesized that some of the mutants (N313A, D346A, D346E, E193A) could retain high kinase activity but would not be inhibited by calcium. However, only E193A mutant retained significant kinase activity (46 $\pm$ 2 %) but was still almost

entirely inhibited by calcium ($8\pm 5.3\%$) just as the wild type enzyme ($1\pm 2.4\%$).

Discussion

The motivation for this study was to compare the active sites of the PI4K II α kinase with and without a ligand and to understand the PI4K II α inhibition at the atomic level. Our data suggest that the active site of PI4K II α is relatively rigid and ready for the nucleotide – no induced fit is required besides the ATP phosphate binding G-loop. We identified the G-loop and aspartate D346 (Figs 2A and 2B) as the most flexible parts of the PI4K II α active site. D346 changes its conformation to form a hydrogen bond with the MD59 nucleotide analogue. Our data suggest D346 is the most flexible residue of the active site highlighting its importance for inhibitor design. It could be argued that conformational rigidity of the active site could be a crystal packing artifact. However, the active site is not directly involved in crystal packing and in the ADP bound structure published by Zhou and colleagues (2014) has the same conformation as in our previous ATP bound structure (Baumlova *et al.* 2014) despite different crystallization constructs and spacegroups. However, the conformation of the lateral C-lobe pocket is wholly crystal packing dependent. It has the same conformation in our previous ATP bound structure (Baumlova *et al.* 2014) and in the apo-state reported here (Fig. 2C) but it has a different conformation in the structure by Zhou and colleagues (2014) (different construct and spacegroup). The PI4K II α enzyme also contains a lateral pocket with surface exposed tryptophan residues that can be inserted into lipid bilayer, a process important for proper positioning of the enzyme (Zhou *et al.* 2014, Baumlova *et al.* 2014) that is also known to influence the biophysical properties of the lipid membrane (Hurley *et al.* 2010, Rozycki *et al.* 2012). However, our data suggest that the observed conformation of the lateral C-lobe pocket was governed by crystal contacts.

PI4K II α kinase activity is associated with synaptic vesicles (Guo *et al.* 2003) and synaptic vesicles are able to rapidly fuse in response to calcium (Jahn and Fasshauer 2012) implying possible role of calcium in

regulation of PI4K II α activity in respect to the synaptic vesicles fusion process. We solved the structure of calcium bound PI4K II α to gain further knowledge of its architecture. We discovered that the ATP γ -phosphate has a different conformation when calcium is bound (Fig. 3). In fact, calcium atoms are held in place by a complex water network, extending from the calcium mainly to α - and γ -phosphates of the ATP molecule and to residues Asp346, Asn313, Gln261 and Glu193 (Figs 3B and 3C). We speculate that the two calcium atoms lock the ATP molecule and prevent its hydrolysis thus effectively inhibiting the enzyme. To understand the physiological role of calcium in the regulation of PI4K II α an active enzyme not inhibited by calcium is needed. We prepared several mutants based on our structure (N313A, D346A, D346E, E193A). Unfortunately, neither mutant had the desired properties.

To summarize, our data reveal details about the architecture of PI4K II α active site that should be helpful for future inhibitor design. PI4K II α is a known host factor for several intracellular bacteria such as *Chlamydia trachomatis* or *Legionella pneumophila* (Pizarro-Cerda *et al.* 2007, Pizarro-Cerda *et al.* 2012, Moorhead *et al.* 2010, Weber *et al.* 2006) and thus considered as a potential drug target. When computational algorithms search the chemical space for suitable compounds we propose that all the residues in the active should be fixed with the exception of the G-loop and aspartate D346.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Pdb accession code

The coordinates have been deposited in PDB under ID code 5EUT (the apo-enzyme) and 5I0N (the calcium bound enzyme).

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