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Structural analysis of phosphatidylinositol 4-kinase IIIβ (PI4KB) – 14-3-3 protein complex reveals internal flexibility and explains 14-3-3 mediated protection from degradation in vitro

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ABSTRACT

Phosphatidylinositol 4-kinase IIIβ (PI4KB) is responsible for the synthesis of the Golgi and trans-Golgi network (TGN) pool of phosphatidylinositol 4-phospahte (PI4P). PI4P is the defining lipid hallmark of Golgi and TGN and also serves as a signaling lipid and as a precursor for higher phosphoinositides. In addition, PI4KB is hijacked by many single stranded plus RNA (+RNA) viruses to generate PI4P-rich membranes that serve as viral replication organelles. Given the importance of this enzyme in cells, it has to be regulated. 14-3-3 proteins bind PI4KB upon its phosphorylation by protein kinase D, however, the structural basis of PI4KB recognition by 14-3-3 proteins is unknown. Here, we characterized the PI4KB:14-3-3 protein complex biophysically and structurally. We discovered that the PI4KB:14-3-3 protein complex is tight and is formed with 2:2 stoichiometry. Surprisingly, the enzymatic activity of PI4KB is not directly modulated by 14-3-3 proteins. However, 14-3-3 proteins protect PI4KB from proteolytic degradation in vitro. Our structural analysis revealed that the PI4KB:14-3-3 protein complex is flexible but mostly within the disordered regions connecting the 14-3-3 binding site of the PI4KB with the rest of the PI4KB enzyme. It also predicted no direct modulation of PI4KB enzymatic activity by 14-3-3 proteins and that 14-3-3 binding will not interfere with PI4KB recruitment to the membrane by the ACBD3 protein. In addition, the structural analysis explains the observed protection from degradation; it revealed that several disordered regions of PI4KB become protected from proteolytical degradation upon 14-3-3 binding. All the structural predictions were subsequently biochemically validated.

1. Introduction

Phosphatidylinositol 4-kinase IIIβ (PI4KB or PI4K IIIβ) produces, together with PI4K2A, the Golgi pool of phosphatidylinositol 4-phosphate (PI4P) (Boura and Nencka, 2015; Clayton et al., 2013). PI4P serves as a signaling molecule, and, additionally, as a precursor for higher phosphoinositides (Balla, 2013; Tan and Brill, 2014). PI4Ks and their product, PI4P, have also been implicated in human diseases such as cancer, Gaucher disease, several neural disorders and degeneration of spinal cord axons (Jovic et al., 2012; Jovic et al., 2014; Simons et al., 2009; Waugh, 2012, 2015). Additionally, PI4KB was identified as an essential host factor for several human viruses including poliovirus, hepatitis C virus, coxsackievirus B3, enterovirus 71, and Aichi virus and is, thus, considered a potential target for antiviral therapy (Altan-Bonnet and Balla, 2012; Berger et al., 2009; Dornan et al., 2016; Greninger et al., 2012; Sasaki et al., 2012; van der Schaar et al., 2013). Subsequently, highly specific inhibitors of PI4KB were developed (Mejdrova et al., 2015, 2017; Rutaganira et al., 2016; Sala et al., 2016) including fluorescent inhibitors (Humpolickova et al., 2017). Additionally, the crystal structures of PI4KB and other PI4K isozymes became available (Baumlova et al., 2014, 2016; Burke et al., 2014; Klima et al., 2015). Given the importance of this enzyme and its product, the PI4P lipid, in human physiology, the PI4KB enzyme should be tightly controlled within the cell. Indeed, several mechanisms were reported. PI4KB is a soluble cytoplasmic enzyme with no membrane binding properties yet it phosphorylates phosphatidylinositol embedded within the membrane. Therefore, it must be recruited to the target (Golgi) membrane. Recently, the Golgi resident protein ACBD3 was shown to be the major factor that recruits PI4KB to membranes and increases its enzymatic activity (Klima et al., 2017, 2016; McPhail et al.,

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2017). Another protein that has been reported to control the PI4KB enzymatic activity in the cell is the 14-3-3 protein (Hausser et al., 2005, 2006).

14-3-3 proteins are a highly conserved family of acidic regulators present in every eukaryotic organism from yeast to humans that have been reported to play a role in many diverse cellular processes ranging from signal transduction to carcinogenesis and apoptosis (Aghazadeh and Papadopoulos, 2016; Morrison, 2009). 14-3-3 proteins act in multiple diverse pathways because they regulate more than 300 binding partners that function in many cellular processes (de Boer et al., 2013; Obsilova et al., 2008a; Uhart and Bustos, 2014). 14-3-3 proteins recognize their binding partners upon phosphorylation of a specific serine or threonine residue although several binding partners that do not need to be phosphorylated have been described as well (Obsilova et al., 2014). Upon binding, they induce a specific molecular switch in their binding partners that can modulate their enzymatic activity (both up or down) (Ganguly et al., 2001; Lambeck et al., 2012), induce a conformational change (Rezabkova et al., 2011; Rezabkova et al., 2010), protect from dephosphorylation (Dent et al., 1995; Kacirova et al., 2015; Thorson et al., 1998) or change their subcellular localization as was reported for the FOXO4 transcription factor (Boura et al., 2010; Obsilova et al., 2005). PI4KB enzyme can be phosphorylated by protein kinase D (PKD) (Hausser et al., 2005) which was reported to protect PI4KB from dephosphorylation *in vivo* leading to increased PI4P production (Hausser et al., 2006). Here, we report on the structural basis for PI4KB regulation by 14-3-3 proteins. Surprisingly, we show that 14-3-3 does not directly increase the PI4KB enzymatic activity, however, we confer that it does protect the kinase from enzymatic degradation *in vitro* and that binding to 14-3-3 does not interfere with PI4KB membrane recruitment by ACBD3 protein.

2. Results and discussion

2.1. Biophysical characterization of PI4KB:14-3-3 protein complex

The interaction of PI4KB with 14-3-3 in a phosphorylation dependent manner is well documented in living cells (Hausser et al., 2005, 2006) and also the structure of yeast 14-3-3 protein Bmh1 with PI4KB derived phosphopeptide was solved recently (Eisenreichova et al., 2016). However, the PI4KB:14-3-3 protein complex formation was never shown *in vitro*. We used purified recombinant PI4KB (phosphorylated and non-phosphorylated) and 14-3-3ζ to characterize the PI4KB:14-3-3 protein complex *in vitro*. First we performed pull-down experiments and, as expected, only phosphorylated PI4KB was able to pull down 14-3-3 (Fig. 1A).

Next, we investigated the PI4KB:14-3-3 protein complex using analytical ultracentrifugation (AUC). Sedimentation velocity (SV) measurements revealed that PI4KB alone can dimerize with an apparent dissociation constant of 3 μM (Fig. 1B). Two peaks with weight average sedimentation coefficients (sw) of 4.3 S and 5.4 S corresponding to the monomer and dimer can be observed in the distribution. The dimerization is not affected by phosphorylation (Fig. 1C). As expected, continuous distribution of sedimentation coefficients – c(S) – of 14-3-3 protein showed a single peak with a sw of 3.6 S, which corresponds to a Mw of 56 kDa and is consistent with a dimeric-fold of the 14-3-3 protein family (Fig. 1D). Consistent with the pull-down experiments, non-phosphorylated PI4KB showed no significant interaction with 14-3-3, whereas phosphorylated PI4KB formed a stable complex with 14-3-3 (Fig. 1D). Analysis of SV data revealed that the binding stoichiometry of PI4KB:14-3-3 complex is preferentially 2:2 (dimeric 14-3-3 protein binds two molecules of PI4K). However, experiments with excess of 14-3-3 protein showed that the complex with 2:1 stoichiometry (dimeric 14-3-3 protein binds one molecules of PI4K) can also be formed (Fig. 1E). Although AUC estimates of molecular weights from complex protein mixtures are rather inaccurate because only an average friction coefficient is obtained during fitting in this case the theoretical and AUC estimated Mw correspond nicely (Fig. 1F).

Once the stoichiometry was known, we used microscale thermophoresis (MST) to determine the dissociation constant (K_d) of the PI4KB:14-3-3ζ protein complex. The capillaries were loaded with a mixture of the recombinant GFP-14-3-3ζ protein (100 nM) and phosphorylated or non-phosphorylated PI4KB kinase (a concentration range from 4 nM to 36 μM). The samples were excited with the 488 nm laser and the changes in thermophoresis were analyzed using the Monolith NT Analysis Software with the 1:1 stoichiometry model as suggested by AUC experiments yielding the apparent Kd of 175 ± 14 nM for the phosphorylated PI4KB while no interaction was observed for the non-phosphorylated PI4KB (Fig. 1G).

2.2. Structural characterization of PI4KB:14-3-3 protein complex

Crystallization trials using both full length and significantly truncated variants of PI4KB and 14-3-3 proteins have failed. We speculated that intrinsic flexibility and disorder of PI4KB could be the reason for the failed crystallization trials. The method of choice for analysis of large, flexible protein complexes is small angle X-ray scattering (SAXS) (Rozycki and Boura, 2014). Recently we co-developed the ensemble refinement of SAXS (EROS) method that is designed for structural characterization of flexible multi-protein complexes (Boura et al., 2011; Boura et al., 2012b; Rozycki et al., 2011) such as the membrane bending ESCRT system (Boura and Hurley, 2012; Rozycki et al., 2012). The EROS method requires structural (usually crystallographically- or NMR-derived) restrains, usually individual domains or subunits that are treated as rigid bodies. The crystal structures of PI4KB and 14-3-3 proteins are known (Burke et al., 2014; Liu et al., 1995). However, the details of 14-3-3 binding to the PI4KB phosphoserine 294 were not described before. In principle, homology modeling based on the known structure of Bmh1 (a yeast 14-3-3 homolog) with PI4KB-derived phosphopeptide (Eisenreichova et al., 2016) could be used. However, to be more accurate, we sought to solve the crystal structure of human 14-3-3ζ in complex with the PI4KB-derived phosphopeptide.

We mixed 14-3-3ζ with PI4KB-derived phosphopeptide (289-LKRTApSNPKV-298) and obtained crystals that diffracted to 2.1 Å resolution. The structure was solved by molecular replacement and refined to R_{work} = 18.79% and R_{free} = 23.11% as summarized in Table 1. The structure revealed the usual 14-3-3-fold – a dimer formed from two α-helical monomers (Fig. 2A). Each monomer is composed of nine α-helices that form a central phosphopeptide binding groove within each monomer. The density for the phosphopeptide was clearly visible upon molecular replacement. Compared to the previously solved structure of yeast 14-3-3 protein with PI4KB peptide (Eisenreichova et al., 2016) two additional residues (R291 and K290) were visible. However, we could not trace the first and last amino acid of the peptide (L289 and V298) suggesting that these two residues are already disordered. We conclude that they are part of the flexible linker that connects phosphoserine 294 (pS294) to the rest of the PI4KB. We also note that the segment 290-KRTApSNPK-297 that we could trace is missing (disordered or deleted) in all previous crystal structures of PI4KB (Burke et al., 2014; Fowler et al., 2016; Mejdrova et al., 2015, 2017). The PI4KB phosphopeptide is held in the 14-3-3 central binding groove by a series of hydrogen bonds. The phosphate group of $pS294^{PI4KB}$ interacts with sidechains of $R56^{14\text{-}3\text{-}3}$, $R127^{14\text{-}3\text{-}3}$, and $Y128^{14\text{-}3\text{-}3}$ while $K49^{14\text{-}3\text{-}3}$, $N173^{14\text{-}3\text{-}3}$, $N224^{14\text{-}3\text{-}3}$, and $W228^{14\text{-}3\text{-}3}$ form hydrogen bonds with the backbone of the PI4KB peptide (Fig. 2B). Importantly, the sidechain of residue $T292^{PI4KB}$ makes no contacts with the 14-3-3 protein suggesting that its mutation will not influence PI4KB binding to 14-3-3 protein.

With all the prerequisites in place, we utilized SAXS in combination with molecular dynamics simulations to reveal the structure of the PI4KB:14-3-3 protein complex. We collected the SAXS data using equimolar mixtures of phosphorylated PI4KB and 14-3-3ζ at different concentrations, which were at least three orders of magnitude larger than the dissociation constant of the PI4KB:14-3-3ζ complex. The