CHARACTERIZATION OF PHOSPHOLIPASE C-β3 COMPLEXES BY X-RAY CRYSTALLOGRAPHY AND SINGLE PARTICLE ELECTRON CRYO-MICROSCOPY

Angeline M. Lyon1,2,3, Somnath Dutta1,3, Georgios Skiniotis1,3, John J. G. Tesmer1,2,3
1Life Sciences Institute and the Departments of 2Pharmacology and 3Biological Chemistry, University of Michigan, Ann Arbor, MI, United States

1. ABSTRACT
The effector enzyme phospholipase C-β (PLCβ) is activated by extracellular hormonal signals via direct interactions with the heterotrimeric G protein Gαq. Despite the discovery of this signaling pathway over 20 years ago, the molecular mechanisms governing regulation of PLCβ have only recently begun to be elucidated. We recently solved the structure of full-length PLCβ3 in complex with activated Gαq, which included the characteristic C-terminal coiled-coil domain of PLCβ that is essential for maximum activity and membrane association. However, the number of inter- and intramolecular interactions formed by this extended domain within the crystal lattice presented difficulties in interpretation. To deconvolute the crystal structure, we determined the architecture and conformational states of the Gαq–PLCβ3 complex in solution through electron cryo-microscopy (cryo-EM) and a multiple 3D reference-supervised classification scheme. This hybrid X-ray crystallography/cryo-EM approach identified two conformations of the distal CTD with respect to Gαq and the PLCβ3 catalytic core that occur in solution, providing novel insights into how this domain contributes to regulation of basal activity, membrane association, and activation by Gαq.

2. INTRODUCTION
Phospholipase C-β (PLCβ) enzymes hydrolyze the inner membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to generate the second messengers inositol-1,4,5-triphosphate (IP3) and diacylglycerol. IP3 binds to IP3 receptors, increasing intracellular Ca2+. Diacylglycerol remains membrane associated and, together with increased Ca2+, activates protein kinase C. PLCβ enzymes are characterized by low basal activity and are dramatically stimulated by direct interactions with the heterotrimeric G protein Gαq [1, 2]. The Gαq-PLCβ signaling pathway is essential for normal cardiovascular function, with dysregulation resulting in cardiac arrhythmias, hypertrophy, and heart failure [3-6].
The first insights into the structure of PLCβ came from crystal structures of the catalytic core [8, 9], which consists of a highly compact and globular structure stabilized by extensive intramolecular interactions between an N-terminal PH domain, four tandem EF hand repeats, a catalytic TIM barrel-like domain which houses the active site, and a C2 domain (Figure 1). This is the minimal fragment that retains the ability to hydrolyze PIP₂, however it has greatly reduced basal activity and cannot be activated by Gα₉. Consequently, these structures do not provide much insight into the regulation of the enzyme. The PLCβ subfamily is characterized by a ~400 amino acid C-terminal extension (Figure 1), which is required for maximum basal and Gα₉-stimulated activity, membrane association, Gα₉ binding, and maximum PLCβ-stimulated GTP hydrolysis (GAP activity) on Gα₉ [10-17]. Structural insights into this extension were first obtained in the crystal structure of the isolated C-terminal ~300 amino acids from turkey PLCβ, termed the distal C-terminal domain (CTD). The distal CTD forms an unusual ~130 Å long extended coiled-coil structure comprised of three long, mostly antiparallel helices bridged by two shorter helices. This segment crystallized as a homodimer, and the oligomeric state seemed consistent with its elution volume by size exclusion chromatography [18]. A potential membrane binding surface on the distal CTD was identified by the sequence conservation of three clusters of basic residues along one face of each monomer, generating a highly polarized surface. Mutation of these clusters decreases association with membranes, the particulate fraction of cells, or lipid vesicles [12-15]. Despite these insights, it remained unclear whether or how the C-terminal extension interacts with the PLCβ catalytic core and/or Gα₉.

Figure 1. Crystal structure of the Gα₉–PLCβ₃ complex. The PLCβ₃ catalytic core is shown in cyan, the proximal CTD in green, and the distal CTD in purple. Activated Gα₉ is shown as a gray surface, with the switch regions involved in nucleotide and effector enzyme binding shown in yellow. Regions of PLCβ₃ that directly contact with Gα₉ are shown in magenta. The inset diagram shows the primary structure of PLCβ₃ colored as in the cartoon.
Recently, the crystal structure of G\(\alpha_q\) in complex with a longer PLC\(\beta_3\) C-terminally truncated variant (PLC\(\beta_3\)-Δ887) revealed the primary sites for G\(\alpha_q\) binding and GAP activity. Surprisingly, the most critical G\(\alpha_q\) binding site resides in the first ~20 amino acids of the C-terminal extension, immediately following the C2 domain. These residues form a helix-turn-helix that binds the effector binding site of G\(\alpha_q\) [19]. Immediately following the G\(\alpha_q\) binding site is a helix that forms an autoinhibitory interaction with the PLC\(\beta\) catalytic core in the absence of G\(\alpha_q\) [20]. These two elements together form what is termed the proximal CTD. The PLC\(\beta\) catalytic core also contributes to interactions with G\(\alpha_q\), via loops connecting the TIM barrel and C2 domains and joining the third and fourth EF hands. The latter loop is required for GAP activity and makes extensive interactions with the active site of G\(\alpha_q\) [19] (Figure 1). However, this structure lacked the distal CTD, which is connected to the proximal CTD by a non-conserved linker region of 28–61 amino acids, depending on the human PLC\(\beta\) isoform [19, 20].

The findings that the C-terminal extension is comprised of discrete functional domains and that the distal CTD is dispensable for G\(\alpha_q\)-mediated activation led to a re-evaluation of the function of the distal CTD. The primary role for the distal CTD was proposed to be membrane association [10, 11, 19], but this hypothesis fails to fully account for numerous reports that the distal CTD is also required for maximum basal and G\(\alpha_q\)-stimulated activity and for high affinity interactions with G\(\alpha_q\) [7, 13, 15-17, 20]. To clarify the roles of the distal CTD in G\(\alpha_q\) activation, we solved the crystal structure of full-length human PLC\(\beta_3\) in complex with activated G\(\alpha_q\). This structure revealed the fold of the distal CTD in the context of a fully functional signaling complex. However, the distal CTD could not be unambiguously assigned to a specific PLC\(\beta_3\) core molecule due to the disordered CTD linker. Furthermore, the extended nature of the distal CTD allowed it to make numerous interactions with different conserved regions of G\(\alpha_q\) and the PLC\(\beta_3\) core within the crystal lattice, making identification of physiologically relevant interfaces difficult. To deconvolute the crystal structure and identify functionally important interactions, we turned to single particle electron cryo-microscopy to determine which of these interfaces might occur in solution. Using an innovative approach involving multiple 3D reference-supervised classification, we were able to determine the solution architecture of the ~180 kDa G\(\alpha_q\)-PLC\(\beta_3\) complex and identify two interactions that occur in crystals and in solution.

3. CRYSTALLOGRAPHIC STUDIES OF THE G\(\alpha_q\)-PLC\(\beta_3\) COMPLEX

3.1. Complex formation and characterization

The interaction between G\(\alpha_q\) and PLC\(\beta\) is of high affinity, with reported EC\(_{50}\) values between 1–400 nM [7, 19-22]. This interaction is dependent on the activation state of G\(\alpha_q\), as only the activated GTP-bound state of G\(\alpha_q\) interacts with PLC\(\beta\). Activation of G\(\alpha_q\) in vitro is difficult, as it does not readily bind GTP on its own, and the robust GAP activity of PLC\(\beta_3\) would catalyze the hydrolysis of any GTP bound to G\(\alpha_q\). An alternative approach is to incubate G\(\alpha_q\) with GDP and AlF\(_4^–\), resulting in the formation of a GTP hydrolysis transition-like state that facilitates interaction with effectors, including PLC\(\beta\) [23-26]. Following G\(\alpha_q\) activation, G\(\alpha_q\) and PLC\(\beta_3\) were incubated at a 1.2:1 molar ratio, and the resulting complex was isolated by size exclusion chromatography.

Because dimerization was posited as a mechanism for G\(\alpha_q\)-dependent activation of PLC\(\beta\) [18], an understanding of the quaternary structure of the complex was essential. Analysis of the G\(\alpha_q\)-PLC\(\beta_3\) complex by a standard method such as size exclusion chromatography is
complicated by the fact that the flexibly tethered distal CTD and its extended shape could significantly change the hydrodynamic radius of PLCβ3. Indeed, the calculated molecular weight of PLCβ3 is 139 kDa, but based on its elution volume it has an apparent molecular weight of ~180 kDa, which is the same as the calculated molecular weight of the Gαq–PLCβ3 complex. To address this problem, we used multi-angle light scattering (MALSA), as it requires relatively small amounts of sample (~200 µg total protein) and yields shape-independent molecular weight estimates. By this method, the experimentally determined molecular weight of PLCβ3 was found to be 152 kDa, much closer to its calculated molecular weight, whereas the molecular weight of the Gαq–PLCβ3 complex was determined to be 181 kDa. Thus, binding of Gαq does not seem to induce dimerization [7].

3.2 Challenges in crystallization and data collection.

Crystals of the Gαq–PLCβ3 complex appeared within 3–4 d at 4 °C. The crystals were long and thin, with average dimensions of 160 x 20 x 10 µm. Initial diffraction was observed to ~3.3 Å, but rapid decay ultimately limited the data to 4.0 Å. Efforts to increase the crystal size and diffraction quality were hampered by the instability of full-length PLCβ3. The distal CTD undergoes proteolysis over time, and several previous PLCβ crystal structures were obtained by proteolysis of what was initially presumed to be full-length enzymes [20]. In order to ensure crystals were set with the maximum amount of full-length protein possible, PLCβ3 was purified within 6-8 hours and maintained at 4 °C or on ice throughout the preparation. Immediately following formation and purification of the Gαq–PLCβ3 complex, crystal trays were set at 4 °C. Despite these efforts, crystals analogous to the Gαq–PLCβ3-Δ887 truncation complex [19] were also observed, sometimes alongside crystals containing full-length PLCβ3.

Due to the small size of the crystals, their relatively high solvent content (65%), and their radiation sensitivity, a third-generation synchrotron source was essential for data collection. The LS-CAT and GM/CA beamlines were used for all diffraction experiments, as the small size of their beam at the sample (20 and 5 µm, respectively) allowed us to take multiple sweeps of data from each rod while reducing background scattering. This was particularly important in this case because individual crystals were significantly nonisomorphous with others. The larger size and flexibility of the complex (~180 kDa) likely led to differences in the unit cell constants (between 89–92 Å x 184–190 Å x 287–294 Å). Indeed, despite collecting over 20 data sets, data from only two crystals could ultimately be merged to generate a relatively complete diffraction data set [7].

3.4 Difficulties in interpreting the Gαq–PLCβ3 crystal structure.

The structure of the Gαq–PLCβ3 complex provided a somewhat convoluted snapshot of the structure and interactions of the distal CTD in the context of a fully functional signaling complex (Figure 1). The Gαq–PLCβ3 complex crystallized as an asymmetric dimer containing two copies of the Gαq–PLCβ3 core complex, but only one copy of the distal CTD. To verify that we had crystallized intact PLCβ3, we used SDS-PAGE analysis of the crystal contents and confirmed they only contained full-length PLCβ3. Thus only one of the distal CTDs formed crystal contacts that gave rise to observable electron density. The observed distal CTD forms multiple interactions with Gαq and the PLCβ3 catalytic core in the crystal lattice. The fact that the other distal CTD in the asymmetric unit is not observed suggests that if these contacts are of physiological relevance, they are likely transient in nature. Further complicating interpretation was that the distal CTD is connected to the PLCβ3 core by a 56-residue non-conserved and
flexible linker, which was disordered in the crystal structure. Thus, it was not possible to unambiguously assign the distal CTD to a specific PLCβ3 molecule in the crystal lattice.

Interpreting the structure depended on identifying which contacts made by the distal CTD represented functional interactions that occurred in vivo. Analysis of the crystal contacts of the distal CTD identified three potentially relevant interactions between the distal CTD, Gαq and the PLCβ3 core based on the amount of buried surface area, the sequence conservation of the residues involved, and prior biochemical studies. The first interface is between a conserved hydrophobic surface of the distal CTD, which formed part of the dimer interface in the turkey distal CTD structure [18], and conserved hydrophobic side chains in the N-terminal helix of Gαq, burying ~850 Å². The hydrophobic residues on the distal CTD are on the opposite face of the domain than the basic regions, which would allow the distal CTD to interact simultaneously with both the membrane and the N-terminus of Gαq. Furthermore, in this interaction the palmitoylated N-terminus of Gαq [27] would be able to engage the membrane, which could optimize the orientation of the complex at the membrane for maximum enzymatic activity. The second interaction is between the distal CTD and the PLCβ3 catalytic core, burying ~1000 Å² surface area. This interface principally involves the backbone atoms of residues within the distal CTD on the same face of the domain as the basic regions thought to mediate membrane association. These residues interact with a highly conserved ridge of hydrophobic amino acids adjacent to the PLCβ3 active site that are believed to insert into the membrane and anchor the active site during catalysis. If this interaction occurs in solution, it could inhibit both basal activity and membrane association, as both the active site and positively charged surface are mutually sequestered from the membrane. The third interaction observed is between the distal CTD and the Ras-like domain of Gαq, burying ~850 Å² surface area. This interface is formed primarily by hydrophobic interactions between the distal CTD and an extended loop from Gαq, however, the residues involved are not as conserved as those in the other interfaces. This interaction places the distal CTD in close proximity to the nucleotide binding pocket of Gαq, which would conceivably allow for increased GAP activity when the distal CTD is present [16, 17, 28], possibly by increasing the affinity of the Gαq–PLCβ3 complex [20]. However, enhanced affinity could also be accomplished by the first interaction between the distal CTD and the N-terminus of Gαq.

4. SINGLE PARTICLE ELECTRON CRYO-MICROSCOPY OF THE Gαq–PLCβ3 COMPLEX

4.1 Single particle reconstructions of the Gαq–PLCβ3 complex

The distal CTD from a single PLCβ3 molecule cannot simultaneously form all the interactions observed in the crystal lattice. Thus, we sought to employ an orthogonal structural approach in order to identify which inter- and intramolecular interactions within the Gαq–PLCβ3 crystal lattice occur in solution. Single particle electron cryo-microscopy (cryo-EM) can be used to determine the solution architecture of macromolecular complexes and identify distinct conformational states in a population of single particles. However, the relatively small size of the Gαq–PLCβ3 complex (~180 kDa) poses challenges in obtaining cryo-EM images with adequate contrast to facilitate accurate projection alignment for single particle analysis. This problem was partially resolved by recording Gαq–PLCβ3 cryo-EM images on a CCD with a TEM equipped with a field emission gun under an acceleration voltage of 120 kV, an operational mode with increasing popularity (Figure 2) [29-32]. The CCD image recording characteristics (point spread function and detective quantum efficiency) are improved at 120 kV, compared to the more
commonly used 200 or 300 kV, with significantly better particle contrast in images obtained at defocus values ranging from -1.5 to -3.5 μm [33]. In the case of the Gαq–PLCβ3 complex, the improved contrast was essential for correct alignment of the small particle projections. For single particle analysis, a total of 40,124 particle projections were extracted from cryo-EM images and processed with a pixel size of 4.48 Å. In preliminary 3D reconstruction tests, a low-resolution-filtered 3D volume of the atomic structure of Gαq–PLCβ3-Δ887, which lacks the distal CTD, was used as the initial reference structure for multiple rounds of cryo-EM projection matching and 3D reconstructions. The resulting 3D map showed limited agreement with the reference model and no clear density for the distal CTD. Instead, several noise-like densities were observed about the central density of the 3D map. Assuming that the particle projections were not completely misaligned, this result suggested that the distal CTD either adopts random conformations with respect to the Gαq–PLCβ3 core or forms multiple discrete conformations, which are averaged out upon combining all the particle projections in this simplistic approach. The next challenge was sorting the particle projections in an unbiased manner that would allow us to determine whether distinct conformational states exist within the full-length Gαq–PLCβ3 population. Therefore, a multiple 3D reference-supervised classification scheme was implemented.

4.2 Conformational analysis of the Gαq–PLCβ3 complex by cryo-EM.

In our multiple 3D reference-supervised classification, four low-resolution-filtered 3D volumes were generated corresponding to four different interactions of the distal CTD observed in the crystal structure: 1) with the hydrophobic ridge of the PLCβ3 catalytic core; 2) with the Gαq Ras-like domain; 3) with the N-terminal helix of Gαq; and 4) with the EF hands of the PLCβ3 core. The last interaction involves flexible residues that are not conserved, and was included as a negative control. The 40,124 particle projections were classified into four populations based on their maximum cross correlation to reprojections of each of the four crystal structure-based references. Each of the four projection sets was then independently used to generate a 3D reconstruction using as a reference the same low-resolution-filtered 3D volume of the Gαq–PLCβ3-Δ887 core complex, which lacks the distal CTD. By using this approach, the validity of the reconstruction scheme was evaluated and any possible bias for the position of the distal CTD was eliminated. Two of the resulting 3D reconstructions, representing approximately 30% of the particle projections each, showed very good agreement with their initial reference structures derived from the crystal lattice, corresponding to the distal CTD interactions with the PLCβ3 catalytic core and with the N-terminal helix of Gαq, with indicated resolutions of 21 Å and 19 Å, respectively (Figure 2). The other two projection sets failed to reproduce good quality reconstructions with clear density for the distal CTD, suggesting that either they do form as stable of interactions in solution or that the remaining ~40% of particle projections have the distal CTD domain in random orientations with respect to the Gαq–PLCβ3 core. Using site directed mutagenesis and biochemical assays, we subsequently demonstrated that disruption of the interaction between the distal CTD and the N-terminus of Gαq diminished maximal catalytic activity, and that the flexibility of the distal CTD with respect to the rest of the complex was essential for Gαq activation. However, for technical reasons we were unable to test whether the interaction of the distal CTD with the hydrophobic ridge is important via site-directed mutagenesis.
5. CONCLUSIONS

The structure of full-length PLCβ3 in complex with Gαq provided new insights into the overall configuration of this signaling complex and the molecular basis for regulation mediated by its distal CTD. A combined approach of X-ray crystallography, using data derived from state-of-the-art third-generation synchrotron beam lines, and single particle cryo-EM studies was essential to identify which interactions observed in the crystal structure occurred in solution. This hybrid approach was enlightening with respect to the idea that individual domains of a signaling protein could be involved in multiple transient interactions that may be relevant in distinct stages.
of a catalytic cycle. Thus one “snapshot” of the structure, either by X-ray crystallography or a non-guided cryo-EM single particle reconstruction, is insufficient to characterize the complexity of an enzyme’s conformational states and the various roles of its domains and interaction surfaces. In this study, the interaction between the distal CTD and the hydrophobic ridge of the PLCβ3 catalytic core may contribute to the regulation of basal activity by physically occluding the enzyme active site, and help partition PLCβ3 into membrane-bound and cytosolic populations. The interaction between the distal CTD and the N-terminal helix of Goq was found to be essential for maximum Goq-stimulated activity. We propose that this interaction may also aid in optimizing the orientation of the PLCβ3 active site at the membrane with the assistance of the N-terminal palmitoyl groups of Goq. A future challenge will be to assess which interactions of the distal CTD are formed when the complex is associated with a membrane surface where its substrate is found, a question that is quite difficult to address with X-ray crystallography alone, but possible with the assistance of emerging cryo-EM approaches.

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7. REFERENCES