

Dysfunction of phospholipase C γ in immune disorders and cancer

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Highlights:

- New evidence for disease-linked mutations in phospholipase C γ (PLC γ)
- Molecular mechanism of dysfunction involves impact on auto-inhibition

Abstract

The surge in genetic and genomic investigations over the past five years resulted in many discoveries of causative variants relevant to disease pathophysiology. Although phospholipase C enzymes have long been recognized as important components in intracellular signal transmission, it is only recently that this approach highlighted their role in disease development through gain-of-function mutations. In this review, we describe these new findings, linking the phospholipase C γ family to immune disorders and cancer, and illustrate further efforts to elucidate the molecular mechanisms that underpin their dysfunction.

Phospholipase C (PLC) enzymes back under the spotlight

PLC enzymes form key elements in signal transmission networks, linking almost all types of cell-surface receptors to downstream components that are involved in the regulation of a variety of cellular functions [1-5]. In mammals, six families of PLCs (PLC β , γ , δ , ϵ , η and ζ) fulfill this role, each characterized by a distinct set of regulatory links. The PLC γ family (PLC γ 1 and PLC γ 2, encoded by the genes *PLCG1* and *PLCG2*, respectively) transmits signals triggered by many growth factors, antigens, and other extracellular stimuli to regulate both essential functions in multiple cell types, and specific responses of particular specialized cells from the immune system (Box 1). Ubiquitously expressed PLC γ 1 is mainly activated downstream of growth factor stimulation, including stimulation by platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF). PLC γ 2, being predominantly expressed in haematopoietic cells, is activated by immune cell receptors such as B-cell and Fc receptors. The exception is T-cell receptor activation, which is linked to PLC γ 1, not PLC γ 2. PLC γ enzymes are mainly activated through tyrosine phosphorylation by receptor and non-receptor kinases. As in all other PLC families, the main signaling output is generation of the second messengers inositol 1,4,5-trisphosphate (IP $_3$, or InsP $_3$) and diacylglycerol (DAG), from phosphatidylinositol 4,5-bisphosphate (PIP $_2$, or PtdIns(4,5)P $_2$). The released IP $_3$ binds to IP $_3$ receptors on the endoplasmic reticulum resulting in Ca $^{2+}$ release into the cytoplasm. DAG remains in the membrane and activates some isozymes of protein kinase C (PKC), other kinases and GTPase regulating proteins. PIP $_2$ itself has functions through interactions with proteins such as ion channels and components of the actin-cytoskeleton [6]; therefore, PLC enzymes could also impact these processes by changing local concentrations of PIP $_2$.

Different aspects of PLC-mediated signalling have been covered in a number of excellent reviews that focus, for example, on the catalytic mechanism [7], regulatory interactions and physiological roles of PLC families [1-5] or on structural and mechanistic aspects of regulation [8-10]. In this review, we highlight recent experimental evidence related to the dysfunction of PLC γ enzymes and links to disease, based on genetic and genomic analysis of animal models and patients; we further discuss the molecular mechanisms that underpin dysfunction. These recent findings, supporting a connection between PLC γ variants harbouring key, gain-of-function mutations and disease development, provide a new perspective for the role of PLC enzymes in various disorders and underscore their importance as therapeutic targets.

Mutations in PLC γ enzymes and disease development

The discovery of putative disease-causing mutations in PLC γ enzymes (Tables 1 and 2) reflects a surge in genetic and genomic investigations that has been facilitated by the development of massively parallel, high-throughput, next-generation sequencing [11, 12]. Germline mutations in PLC γ have emerged as the cause of certain immune disorders, and somatic mutations have been identified as drivers of some types of cancer.

PLC γ mutations in immune diseases

The first link between PLC γ 2 and immune diseases was established in the context of a large-scale ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis screen, in which gain-of-function mutations were identified (Table 1). Two mouse strains with limb defects, abnormal limb 5 and 14 (Ali5 and Ali14) were found to harbour single amino acid replacements in PLC γ 2, leading to severe autoinflammatory disease [13-15].

More recently, genetic analyses revealed the role of PLC γ 2 in dominantly inherited human immune disorders (Table 1). Germline deletion mutations were identified in three families with multiple cases of complex immune disease, designated as PLAID (*PLCG2*-associated antibody deficiency and immune dysregulation) [16]. These mutations, resulting in constitutive PLC activation, diminished downstream signalling. Both Ca²⁺ flux and extracellular signal-regulated kinase (ERK) phosphorylation were reduced in B cells. NK cells showed reduced Ca²⁺ flux and reduced degranulation, while Ca²⁺ flux in T-cells was normal. These downstream effects were unexpected

given the gain of enzymatic function; however, they might be the result of local PIP₂ depletion or of feedback inhibition leading to down-regulation of distal signalling pathways. This complex downstream signalling output is also reflected by the presence of both autoimmune disorder and immune dysfunctions in patients with these mutations. Moreover, reduced temperatures alter the signalling downstream of PLC γ 2 at the cellular level in these patients, triggering degranulation of mast cells; this results in the condition known as cold-induced urticaria.

Another genetic study of an inherited immune disease identified S707Y substitution in PLC γ 2; the associated disease was designated as APLAID (autoinflammatory PLAID) [17]. Discovery of the causal mutation was not amenable to linkage analysis and required whole exome sequencing of the germline of individuals from the affected family (Table 1). APLAID patients do not suffer from cold-induced urticaria, and autoinflammatory disease is more pronounced than immune dysfunction. In contrast to cells with PLC γ 2 deletions, the S707Y substitution not only leads to a gain of enzymatic function but also to increased downstream signalling, specifically Ca²⁺ flux and ERK phosphorylation in B cells [17]. Possibly, the smaller increase in PLC γ 2 activity, relative to the PLAID-causing deletion, that is caused by the point mutation might not be sufficient to trigger a negative feedback mechanism. One common manifestation for both disorders is the absence of class-switched memory B-cells; however, this is probably the result of distinct downstream signalling effects.

PLC γ mutations in cancer

PLC γ enzymes contribute to some oncogenic signalling pathways through signal transduction downstream of receptor and non-receptor tyrosine kinases. It is therefore conceivable that aberrant PLC γ activity may contribute to tumour development. Initial studies indicated that higher expression levels of PLC γ 1 occurred in a variety of malignancies such as breast or colon cancer [18, 19]. More recently, acquired (somatic), driver mutations in the *PLCG* genes have been identified by massively parallel sequencing in angiosarcoma, cutaneous T-cell lymphoma (CTCL), and in chronic lymphocytic leukaemia (CLL) (Table 2). Within each tumour type, recurrent missense mutations of a specific residue were found, which is the hallmark of activating driver mutations [20-22]. Thus, PLC γ 1/2 mutations are likely to activate the respective enzymes, although the distal downstream functional consequences of specific mutations probably vary. Initial data also suggest that calcium responses and proximal signalling (such as phosphorylation of a subset of intracellular targets) could also be commonly up-

regulated [21, 22]. In angiosarcoma, a vascular tumour with endothelial differentiation, activating *PLCG1* mutations are likely to result in overactive angiogenesis growth factor signaling. In CTCL and CLL activation of PLC γ enzymes would be predicted to enhance T-cell receptor and B-cell receptor signaling, respectively, thereby promoting tumour growth. An important clinical implication of *PLCG1/2* mutations is that they may represent a resistance mechanism to inhibitors of tyrosine kinases. This is exemplified by the case of *PLCG2* mutations in CLL, which were found in patients who developed resistance to the tyrosine kinase inhibitor, ibrutinib [22]. An interesting detail of the mutational data on PLC γ 1 in human tumours is that the mutations may show tumour type specificity. Although the overall number of cases that have been studied to date are low, for PLC γ 1 it appears that R707 is mutated in angiosarcoma whereas S345 is mutated in CTCL [20, 21]. If confirmed in larger studies, such tumour type specificity would indicate that despite both occurring in the same gene and being activating, these mutations are non-interchangeable and modulate function of PLC γ 1 enzyme in different ways. Ongoing efforts to characterize cancer genomes will define the extent to which *PLCG1/2* mutations contribute to different types of human cancer, beyond angiosarcoma, CTCL, and CLL. At the same time, mechanistic studies into PLC γ are required to elucidate changes in enzyme function in the context of specific diseases.

Structural and mechanistic aspects of PLC γ function and mechanisms of deregulation

A large number of studies related to regulatory interactions of PLC γ enzymes identified tyrosine phosphorylation of PLC γ as the major mechanism of activation. Some other, phosphorylation-independent mechanisms, such as activation of PLC γ 2 by Rac, were also revealed (Box 1). However, it was only recently that sufficient structural information became available that would allow understanding of deregulation at the molecular level (Figures 1 and 2).

Structural basis of auto-inhibition and activation of PLC γ proteins

Understanding the molecular mechanisms underlying auto-inhibition and activation of PLC γ isoforms has been an intense area of research for over 20 years. Early work, focussing on the differences between PLC γ , δ and β regulation, determined that PLC γ signalling lay downstream of receptor tyrosine kinases (RTKs) [23]. Consequently, recruitment and subsequent phosphorylation of PLC γ by RTKs was shown to be crucial for activation. Later reports provided evidence that

phosphorylation on tyrosine 783 (Y⁷⁸³), located within the region specific to PLC γ enzymes (γ specific array or γ SA; Figure 1), was both required and sufficient for PLC γ activation. Further, Y⁷⁸³ phosphorylation created an intramolecular binding site for the C-terminal src-homology 2 (cSH2) domain [24]. The main role of the N-terminal src-homology 2 (nSH2) domain is to engage RTK. In the specific case of the FGF receptor (FGFR), the nSH2 binds to the receptor through canonical phosphotyrosine pY and through secondary sites [25] leading to an exquisitely specific interaction. As well as playing a crucial role in the activation of PLC γ , the cSH2 domain was shown to be critical for maintaining auto-inhibition [26-28]. In addition, the “split” pleckstrin homology (spPH) domain has been reported to contribute to maintenance of PLC γ in the auto-inhibited state in the absence of phosphorylation [26, 28].

Despite these advances, the precise molecular basis of auto-inhibition and activation and importantly the amino acid residues crucial for these phenomena were unknown due to the lack of critical structural information, in particular related to the full-length PLC γ molecule and unique γ SA (depicted in Figure 1A, top panel). Generating an X-ray crystallographic structure proved challenging due to the inherent flexibility in various regions of the protein. Nevertheless, other techniques are available that have provided insight into the structural arrangements of the γ SA and its interactions with the core. For instance, a combination of nuclear magnetic resonance (NMR), small angle X-ray scattering (SAXS), crystallography (of fragments of the protein), isothermal titration calorimetry (ITC) and modelling were utilised to study PLC γ 1 [29]. Previous studies that highlighted the sole importance of Y⁷⁸³ in PLC γ 1 activation were confirmed. Furthermore, NMR spectroscopy showed that the spPH, nSH2 and src-homology three (SH3) domains made few interactions with each other in the γ SA. By contrast, comparing the cSH2 spectrum with the γ SA spectrum suggested that there are dynamic interactions between cSH2 and the other domains and linkers within γ SA. Upon further analysis of tandem γ SA fragments (cSH2-spPH), compared to isolated domains, it was clear that cSH2 and spPH have a transient interaction.

SAXS, a method that provides low-resolution structures of proteins in solution, was performed on the γ SA, a number of tandem domains within the γ SA and various tagged and complexed constructs [29]. Taking into account the SAXS and NMR data, a model was generated representing a possible average structural

arrangement of the γ SA, in which the spPH and cSH2 occupy the central lobe with nSH2 and SH3 peripheral to them (Figure 1A, bottom panel).

PLC γ activation through the interaction between cSH2 and pY⁷⁸³ has been known for some time, having been established through various lines of evidence. However, the crystal structure of phosphorylated tandem nSH2-cSH2, showing that residues 781 to 790 bound in cis to the canonical cSH2 pY peptide binding pocket, was obtained only recently [29]. The crucial pY⁷⁸³ interacts with three arginine residues, R⁶⁷⁵, R⁶⁹⁴ and R⁶⁹⁶. Also, V⁷⁸⁴, A⁷⁸⁶ and P⁷⁸⁸ make van der Waals contacts with the cSH2 surface and bury approximately 620Å² of solvent accessible surface. A second crystal structure was later reported of the isolated cSH2 domain bound in trans to a PLC γ 1 derived phosphopeptide [30]. Interestingly, ITC experiments performed with the apo nSH2-cSH2, the phosphorylated nSH2-cSH2 and FGFR1^{pY766} (phosphorylation at tyrosine 766 creates a binding site for PLC γ) showed that the affinity of the nSH2 domain for FGFR1 decreased upon engagement of the pY⁷⁸³ linker in the cSH2 binding site [29]. The decreased affinity of phosphorylated γ SA and full length PLC γ for FGFR1 was also reported using a pull-down assay. The intriguing possibility that phosphorylated PLC γ has a lower affinity for RTK than unphosphorylated PLC γ suggests that many PLC γ molecules can be phosphorylated by a single RTK. This would lead to greater signal amplitude than if the PLC γ remained engaged with the RTK.

Confirmation that the cSH2 is the domain in γ SA directly involved in auto-inhibition came from NMR titration experiments [29]. The PLC-core from PLC γ was added independently into ¹⁵N labelled domains of nSH2, cSH2 and spPH. An interaction could be measured only between the core and the cSH2 domain and this could be reversed through subsequent addition of the pY⁷⁸³ linker. Importantly, this approach also revealed which surface of the cSH2 domain is in contact with the catalytic core of PLC γ and showed that this surface overlaps with the binding surface of the pY⁷⁸³ linker. ITC with mutated cSH2 and/or core polypeptides confirmed that the following residues are important at the cSH2/core interface: R748, R753, N728, S729 and D1019. Introducing substitutions in these residues into full length PLC γ and assaying for PLC activity in cells also showed that disrupting the cSH2/core interface imparted increased basal activity and increased EGF stimulated activity compared to wild type protein [29]. Therefore, a clearer picture of the molecular basis of auto-inhibition and activation emerges from these data (Figure 1B). In the basal state, the low PLC γ

catalytic activity is maintained through a specific interaction between the cSH2 domain and residues surrounding the active site. Phosphorylation at Y783 creates a binding site for the cSH2 domain that is of greater affinity than its binding site at the core. With the cSH2 occlusion removed from the active site the protein can now access its substrate in the membrane.

Structural basis of dysfunction

The structural basis of auto-inhibition and activation provides a useful framework for interpretation of the effects of various mutations and brings together observations from mouse models and human diseases, including immune disorders and cancer (Figure 2). Given that the cSH2 domain was found to form an autoinhibitory interface with the catalytic domain, it is not surprising that the partial or complete deletion of this domain, as it is the case in PLAID patients, leads to a constitutively active PLC γ 2 enzyme (Figure 2). The mechanism of auto-inhibition also explains some of the more subtle disease-associated mutations; for instance the S707Y substitution found in APLAID patients, disturbs the autoinhibitory interface between cSH2 and the catalytic domain, which was confirmed by NMR and ITC studies [29]. The effect of this mutation on the enzymatic activity is less pronounced than for cSH2 deletions. This S707Y mutation is also an example of a point mutation that was found in both immune disease and cancer; recently, some patients with ibrutinib-resistant CLL were found to harbour this mutation, either in isolation or in combination with other PLC γ 2 mutations and/or a C481S mutation in Bruton's tyrosine kinase, the target of ibrutinib [22]. Our understanding of the functional effect of these mutations can also be transferred to a recently discovered PLC γ 1 point mutation that results in the substitution R707Q in angiosarcoma. R707Q (not to be confused with S707Y in PLC γ 2) is located within the beta-sheet of the cSH2 domain. A destabilization of cSH2 structure, leading to a gain of enzymatic function by a similar mechanism, seems likely [20]. The PLC γ 2 substitution R665W, found in ibrutinib-resistant CLL samples, is also located in the cSH2 domain [22]. Residue R665 in PLC γ 2 (equivalent to PLC γ 1 R687) is spatially close to PLC γ 1 residue R707 mutated in angiosarcoma. However, it is more surface-exposed than the latter; therefore, it is not yet clear how its activating effect occurs.

Other mutations directly affecting PLC γ autoinhibition affect the ridge region of the catalytic domain (Figure 2). For instance, mice with the ALI5 phenotype harbour the substitution D993L in PLC γ 2, which leads to only a small increase in basal PLC γ

activity; however, the activity following stimulation is greatly enhanced compared to the wild type [13, 15]. ITC, NMR and surface plasmon resonance experiments suggest that the reason for the higher activity in cells is the disruption of the autoinhibitory interface between the catalytic domain and the cSH2 domain, combined with enhanced membrane recruitment [15, 29]. The PLC γ 1 S345F substitution, found in CTCL samples, is also located in the ridge region of the catalytic domain. Furthermore, based on a previous structural study, it was predicted that the specific region around this residue forms the autoinhibitory interface between cSH2 and the catalytic domain [29]. It can be inferred that the PLC γ 1 S345F substitution is likely to disrupt the autoinhibitory interface in a similar fashion to the immune disease-causing PLC γ 2 D993L substitution in ALI5 mice.

The Y495C substitution in PLC γ 2, which is found in mice with the ALI14 phenotype, leads to symptoms similar to those observed for ALI5 mice [14, 15]. Y495 is located in the beta-sheet in the spPH domain, which only in PLC γ 2 binds Rac. In cells expressing Y495C variant, basal and Rac or EGFR-stimulated PLC γ 2 activity was found to be increased. This observation, in combination with NMR and ITC studies, illustrates that this substitution does not change the interaction between spPH and Rac, but rather destabilizes the γ SA and thereby contributes to overcome auto-inhibition and facilitate activation [31]. It is possible that other cSH2-spPH interaction contacts could have a role in this context [29]. The recently discovered PLC γ 1 S520F substitution in CTCL and to some extent also the PLC γ 2 L845F substitution in ibrutinib-resistant CLL are spatially close to the ALI14 substitution, Y495C (Figure 2). This suggests that these cancer-associated substitutions could both be activating PLC γ 1 and PLC γ 2 by destabilizing γ SA, and consequently relieving auto-inhibition, as was found to be the case for the ALI14 substitution.

Deletions and single amino acid substitutions in PLC γ 1 and PLC γ 2 identified so far directly affect enzyme activity as a common functional outcome, further supporting genetic evidence for the link between these mutations and disease development. Based on these findings, PLC γ enzymes should be further considered as a potential therapeutic target.

Pharmacological inhibitors of PLC activity

Selective small molecules or other selective probes are critical in elucidating physiological and aberrant functions of specific proteins in cells and whole

organisms. Furthermore, such compounds serve as candidates for drug development. Specific pharmacological inhibitors and promising drug compounds have now been developed for many signalling components of “core processes” operating in signal transmission within cellular networks. PLC enzymes, however, notably lack not only potential drug molecules but, it appears, there is not even a reliable, direct small molecule inhibitor. Based on structural insights and a detailed understanding of the catalytic mechanism of PIP₂ hydrolysis [7], PLC proteins are not intrinsically intractable. The main limitations in inhibitor development have been related to a lack of suitable high-throughput screening, difficulties of generating chemical probes based on the PIP₂ substrate and insufficient evidence linking changes in PLC function with disease development. As discussed further in Box 2, all these problems are now being addressed.

Recent advances in studies of PLC enzyme, however, also highlighted some new important points that should be considered in future developments of PLC inhibitors. The new findings support diverse, and sometime opposing roles of different PLC isoforms, in particular in cancer [20-22, 32-37], and indicate that the generation of isozyme-specific inhibitors is highly desirable. Nevertheless, recent studies of the gain-of-function mutations that affect auto-inhibitory surfaces (Figure 2) would imply that active site of these variants can be more exposed and accessible, suggests the possibility that more general PLC inhibitors could also be effective.

Concluding remarks

The emergence of pathogenic PLC γ 1 and PLC γ 2 mutations in human cancer and autoimmune diseases may herald the discovery of further examples of human disease that is driven by aberrant PLC γ 1 or PLC γ 2 function. Despite the high structural and mechanistic overlap, PLC γ 1 and PLC γ 2 are relevant in very different medical conditions. All mutated sites linked to human diseases are conserved between PLC γ 1 and PLC γ 2. Furthermore, all identified mutations appear to be gain-of-function by destabilising the regulatory γ SA and/or by disrupting the autoinhibitory interface between γ SA and the catalytic domain. Studies of relevant murine models and patients, together with structural insights, will help to understand new mutations occurring in very different contexts. Further research, covering molecular mechanisms, animal models of disease and specific and potent PLC inhibitors, will

be needed as the next step towards full elucidation of the role of PLC γ enzymes in various disorders.

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TABLES

Table 1. PLC γ mutations in immune disorders

Table 2. PLC γ mutations in cancer

FIGURES

Figure 1. Structural insights in PLC γ regulation.

A) Linear (top) and 3-D representation (bottom) of domains that comprise PLC γ isoforms. The protein is organized into two regions: the PLC-core, shared by PLC families, and the array of domains specific for PLC γ family (γ SA). Each domain within the PLC-core [an N-terminal pleckstrin homology (PH) domain, EF-hands, triosephosphate isomerase (TIM)-barrel like fold and a C2 domain] and γ SA [“split” PH domain (spPH), two src-homology two (SH2) domains (nSH2 and cSH2) and one src-homology three (SH3) domain] is represented in a different colour. In linear representation (top), to emphasize two regions in PLC γ family, the PLC-core and γ SA domains are shown along separate axis. Proposed structural arrangements within these two regions are shown as surface and ribbon representations (bottom panels). Bottom left, an SAXS- and NMR-based model of a likely dynamically averaged conformation of the γ SA in the non-phosphorylated state. Each domain (shown in a different colour as in top panel) is represented as a surface. The cSH2 domain is also illustrated as a ribbon representation and the region shown to be involved in auto-inhibition is indicated. The interaction interfaces of Rac with the spPH domain of PLC γ 2 and of tyrosine kinases with the nSH2 are indicated. Bottom right, a model of the core domains (excluding the PH domain, based on PDB: 2ZKM) is shown as surface structures with the TIM-like barrel also represented by a ribbon. The position of IP $_3$ – representing the headgroup of the PIP $_2$ substrate- is modelled into the TIM-like barrel (based on crystal structure of another isozyme; PDB: 1DJX), showing the site of catalysis. The surface involved in autoinhibition is also indicated.

B) Molecular basis of PLC γ autoinhibition and activation. The autoinhibited (inactive, left panel) form of the PLC γ molecule is represented using the same domain colours as in the above structural representations. The TIM-like barrel and cSH2 domains are shown juxtaposed in the likely orientation that confers autoinhibition on the unphosphorylated molecule. Subsequent phosphorylation by tyrosine kinases on Y⁷⁸³ leads to interruption of the autoinhibitory interface through binding of the cSH2-SH3 linker to the canonical binding site on the cSH2 surface (right panel). This event leads to a change in the conformation of the γ SA with respect to the PLC-core that reveals access to the active site so that catalysis can proceed.

Figure 2. Molecular mechanisms of PLC γ deregulation.

A) Locations of disease-relevant amino acid substitutions identified in PLC γ 1 or PLC γ 2 are indicated by red arrows. In addition to point mutations, deletions affecting the cSH2 domain have also been described. All mutations are activating. Surface areas of the TIM-like barrel (catalytic domain) and cSH2 domain form the autoinhibitory interface (marked by a red line).

B) Mutations in the spPH domain. Three amino acid substitutions, Y495C and L845F in PLC γ 2 and S520F in PLC γ 1, are listed; for amino acid substitutions in PLC γ 2 the corresponding wild type residues in PLC γ 1 are shown below in brackets. All residues affected by mutations are indicated in structural representation using numbering corresponding to PLC γ 1 sequence. All mutations are located at (or close to) the cSH2-spPH interaction interface, distinct from the interface between spPH and Rac. They are likely to destabilize the γ SA region of the protein. The structural model has been created from a combination of spH structures (PDB: 2FJL and 2W2X) and the γ SA model by aligning the spPH domains of these models.

C) Mutations in the cSH2 domain. Two amino acid substitutions in PLC γ 2 (E665W and S707Y) and one in PLC γ 1 (R707Q) are listed; for amino acid substitutions in PLC γ 2 the corresponding wild type residues in PLC γ 1 are shown below in brackets. All residues affected by mutations are indicated in structural representation (PDB: 4FBN) using numbering corresponding to PLC γ 1 sequence. The S707Y substitution activates by disrupting the autoinhibitory interface with the catalytic domain. R707Q is not located at the interface, but within the beta sheet. This substitution might lead to disruption of the structure and thereby also release autoinhibition. E665W is

spatially close to R707Q and part of the protein surface. Other residues, considered to be a central part of the autoinhibitory interface between the cSH2 and catalytic domain, are also shown.

D) Mutations in the catalytic domain. Two amino acid substitutions (D993G in PLC γ 2 and S345F in PLC γ 1) are listed; for the amino acid substitution in PLC γ 2 the corresponding wild type residues in PLC γ 1 is shown below in brackets. Both residues affected by mutations are indicated in structural representation using numbering corresponding to PLC γ 1 sequence. The substituted residues are located at the proposed autoinhibitory interface. For D993G, it was demonstrated that this interface is disrupted as a consequence of the mutation; therefore both mutations are likely to activate PLC γ by disrupting autoinhibition. Following activation, they may also facilitate interaction with the cellular membrane.

BOXES

Box 1. Domain organization of PLC γ enzymes and their roles in signal transduction.

PLC γ 1 and PLC γ 2 have the same domain organisation and share high sequence similarity across all domains [4, 5, 8] (Figure 1A). They incorporate a core set of domains (PLC-core): an N-terminal pleckstrin homology (PH) domain, EF-hands, triosephosphate isomerase (TIM)-barrel like fold and a C2 domain. Uniquely for PLC γ , the linker between the two-halves (X and Y boxes) of the TIM-barrel is highly structured and referred to as the γ specific array (γ SA). γ SA consists of a “split” PH domain (spPH), two src-homology two (SH2) domains (nSH2 and cSH2) and one src-homology three (SH3) domain. While the PLC-core contains TIM-barrel with the enzymatic function, the γ SA is largely involved in interaction with regulatory proteins.

PLC γ 1 and PLC γ 2 are activated downstream of receptor (RTK) and non-receptor tyrosine kinases [38, 39]. Stimulation via RTKs is best documented for PLC γ 1 (Figure 1B, left). Action of non-receptor tyrosine kinases has been best defined in the context of activation of immune cell (T-cell, B-cell and Fc) receptors associated with multi-protein complexes. Most immune cell receptors activate PLC γ 2; the regulatory interactions defined in B-cells are shown in Figure 1B, right. More recently it has been discovered that PLC γ 2 is also activated by the small GTPase Rac [40, 41].

Figure 1. Similarity between PLC γ enzymes and regulatory links in different physiological settings

A) For each indicated domain shared by PLC γ 1 and PLC γ 2 the similarity is shown as a percentage. Sequence similarity was calculated based on alignment (using MUSCLE, EMBL-EBI tool).

B) Different regulatory interactions of PLC γ downstream of receptor tyrosine kinases (RTKs) and immune cell receptors.

Left panel: Engagement of growth factor (GF) by RTKs creates activated receptors (pRTKs). Phosphorylated tyrosines on pRTKs become binding sites for SH2 domain-containing proteins such as PLC γ 1 that are recruited and subsequently phosphorylated by the kinase.

Right panel: Antigen engagement of the B-cell receptor (BCR) leads to phosphorylation by the Lyn non-receptor tyrosine kinase within and outside specific (ITAM) motifs. This is followed by recruitment of adaptor molecules (BLNK) and further non-receptor tyrosine kinases (Syk). Phosphorylated BLNK acts as a scaffold for PLC γ 2 where Syk can then phosphorylate and activate the phospholipase. Furthermore, Btk is recruited to the membrane environment by parallel pathways and can also phosphorylate PLC γ 2. Finally, activated Rac can bind and stimulate PLC γ 2 by a phosphorylation independent mechanism (dotted arrow).

Box 2. Developments of pharmacological inhibitors that target PLC enzymes.

Historically, the first attempts to develop pharmacological inhibitors against PLC were focused on the development of PIP₂-based chemical probes that could be used as a substrate in screens and as lead compounds. Despite the initial difficulties, some promising, fluorogenic substrate analogues were reported [42-45]. Paralleling this work, compounds that were structurally unrelated to PIP₂ were identified as potential PLC inhibitors. Among those, aminosteroid U73122 has been the most frequently used. However, it was difficult to directly and strictly link the effects of such inhibitors to PLC inhibition for functional readouts in cells. Furthermore, a number of reports on U73122 suggested it had other targets, including calcium pumps and unrelated enzymes regulating lipid metabolism [46-51]. It has also been shown that treatment of purified PLC isoforms with U73122 *in vitro* had surprisingly diverse effects on activity, including increased activity of PLC γ [52]. These observations highlighted the need to reevaluate these early inhibitors and generate new, specific compounds against PLC isoforms.

Renewed efforts to target PLC enzymes are underway. Several novel inhibitors were identified using virtual high-throughput screening based on the 3D-structure of the PLC active site [53]. Significantly, ongoing efforts to develop a second generation of PIP₂-based chemical probes are also promising [54-56]. In particular, soluble, fluorogenic analogs will continue to greatly facilitate high-throughput screening to identify highly specific and more potent PLC inhibitors [56]. These PIP₂ analogues could also guide the generation of inhibitory compounds that bind to the conserved PLC active site.

As documented in this review, significant progress has been made in providing supporting data for the involvement of PLC γ enzymes in disease development where in many cases current treatments remain ineffective. This to a considerable degree addresses one of the previously raised issues of insufficient evidence linking changes in PLC function with diseases in need of new treatments.

Table 1. PLC γ mutations related to immune disease

Protein names	Mutation	Domain	Occurrence of mutation	Subjects	Clinical manifestations	Activity ^a : IP ₃ , Ca ²⁺ , distal ^a	Anti-bodies	T and B cells	Ref.
PLC γ 2	D993G	TIM-barrel	murine mutation from ENU mutagenesis ("ALI5")	mouse line	autoinflammation: inflammatory skin lesions (with inflammatory infiltrate), arthritis, keratitis, glomerulonephritis (ICECPGN), autoantibodies; other: growth retardation.	↑, ↑, o	IgM↑, some IgG↑	T cells↑, B cells↓	[11,13]
PLC γ 2	Y495C	spPH	murine mutation from ENU mutagenesis ("ALI14")	mouse line	autoinflammation: inflammatory skin lesions (with inflammatory infiltrate), arthritis; other: metabolic defects, reduced male fertility.	↑, ↑, o	IgM↑, some IgG↑	T cells↑, B cells↓	[12,13]
PLC γ 2	Δ(646-685)	cSH2	inherited mutation	27 patients (3 families)	PLAID. autoinflammation: cold-induced urticaria, granulomatous skin disease, autoimmune disease, allergic disease; autoantibodies; immune deficiency: recurrent sinopulmonary infection, common variable immunodeficiency.	↑, ↑, ↓	IgM↓, IgA↓, IgE↑	class-switched memory B cells↓	[14]
PLC γ 2	Δ(686-806)	cSH2 / SH3							
PLC γ 2	S707Y	cSH2	de novo and inherited mutation	2 patients (1 family)	APLAID. autoinflammation: inflammatory skin lesions (with inflammatory infiltrate), arthralgia, keratitis, enterocolitis, interstitial pneumonia; immune deficiency: recurrent sinopulmonary infection, cellulitis	↑, ↑, ↑	IgM↓, IgA↓	NK T-cells↓, class-switched memory B cells↓	[15]

^aActivity of PLC γ variants was assessed either in cells harboring the mutation or in transfected cells; accumulation of IP₃ or of Ca²⁺ were used as a direct readout of PLC activity and further downstream events referred as "distal"; up and down arrows indicate increase or decrease. o: no data available.

Table 2. PLC γ mutations related to cancer

Protein names	Mutation	Domain	Occurrence of mutation	Activity ^a : IP ₃ , Ca ²⁺ , distal ^a	Co-mutations	Ref.
PLC γ 1	R707Q	cSH2	secondary angiosarcoma	o, o, o	PTPRB	[17]
PLC γ 1	S345F	TIM barrel	cutaneous T-cell lymphoma	o, o, ↑		[18]
PLC γ 1	S520F	spPH	cutaneous T-cell lymphoma	o, o, ↑		[18]
PLC γ 2	R665W	cSH2	Ibrutinib resistance in CLL	o, ↑, ↑		[19]
PLC γ 2	L845F	spPH	Ibrutinib resistance in CLL	o, ↑, ↑	R665W, S707Y, BTK: C481S	[19]

^aActivity of PLC γ variants was assessed either in cells harboring the mutation or in transfected cells; accumulation of IP₃ or of Ca²⁺ were used as a direct readout of PLC activity and further downstream events referred as "distal"; up and down arrows indicate increase or decrease. o: no data available.

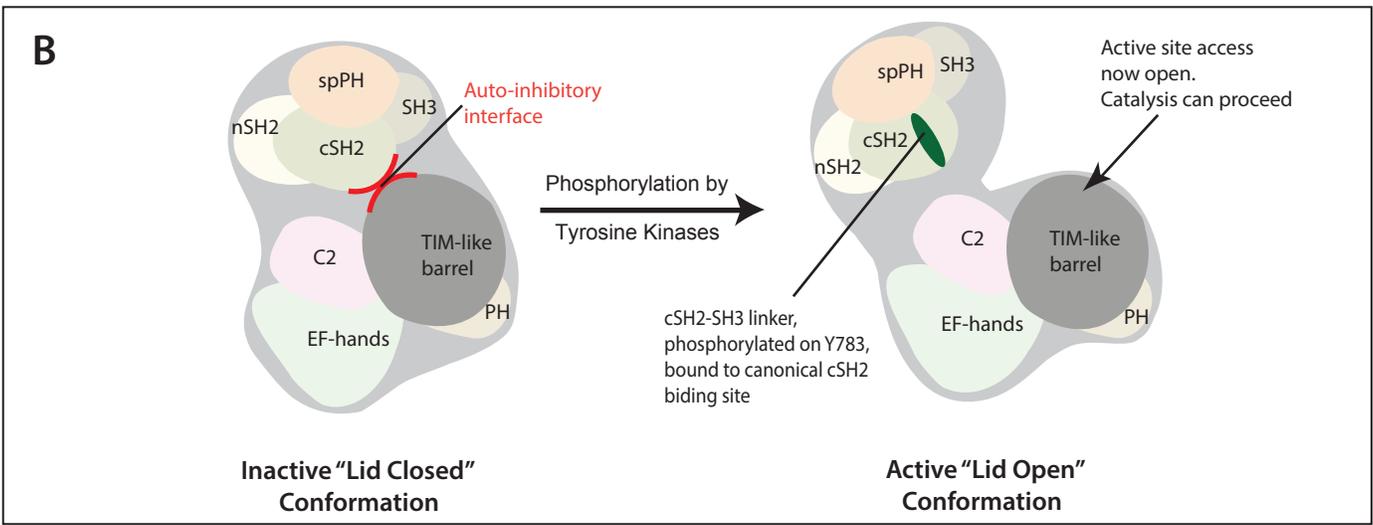
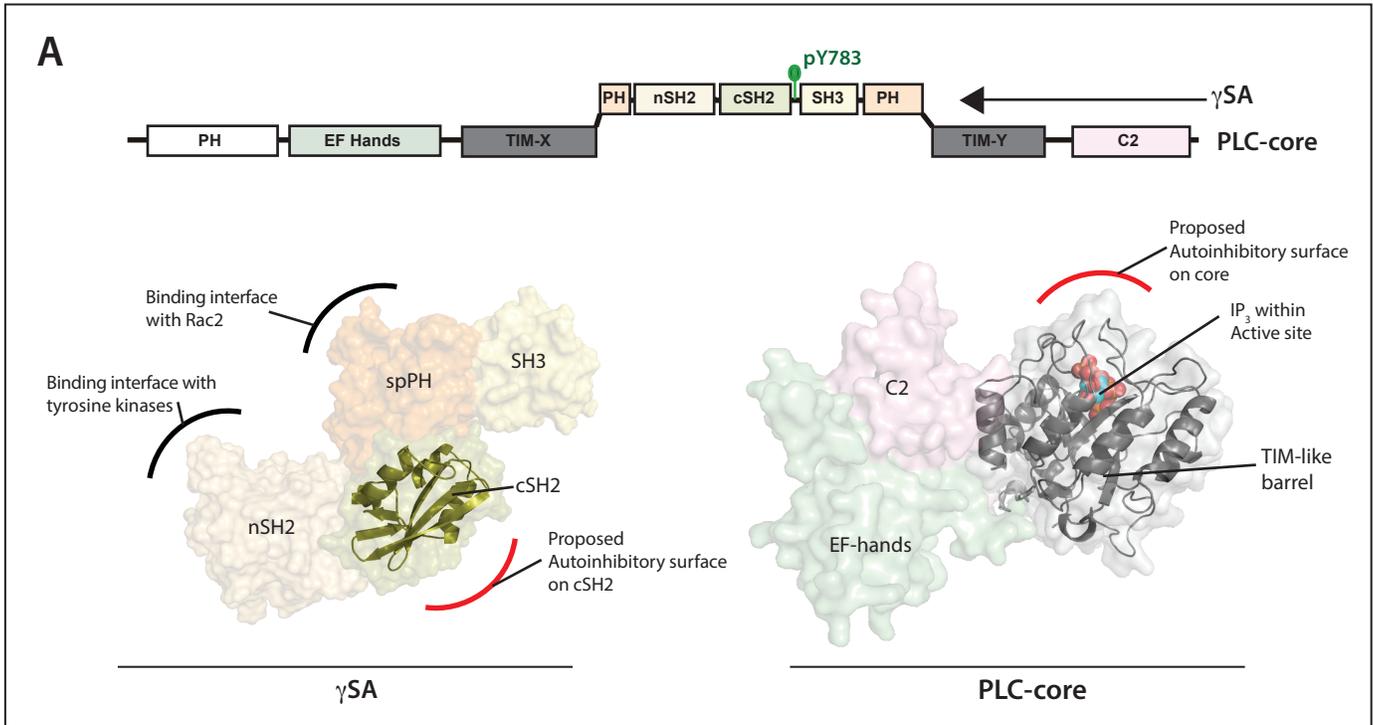


Figure 1

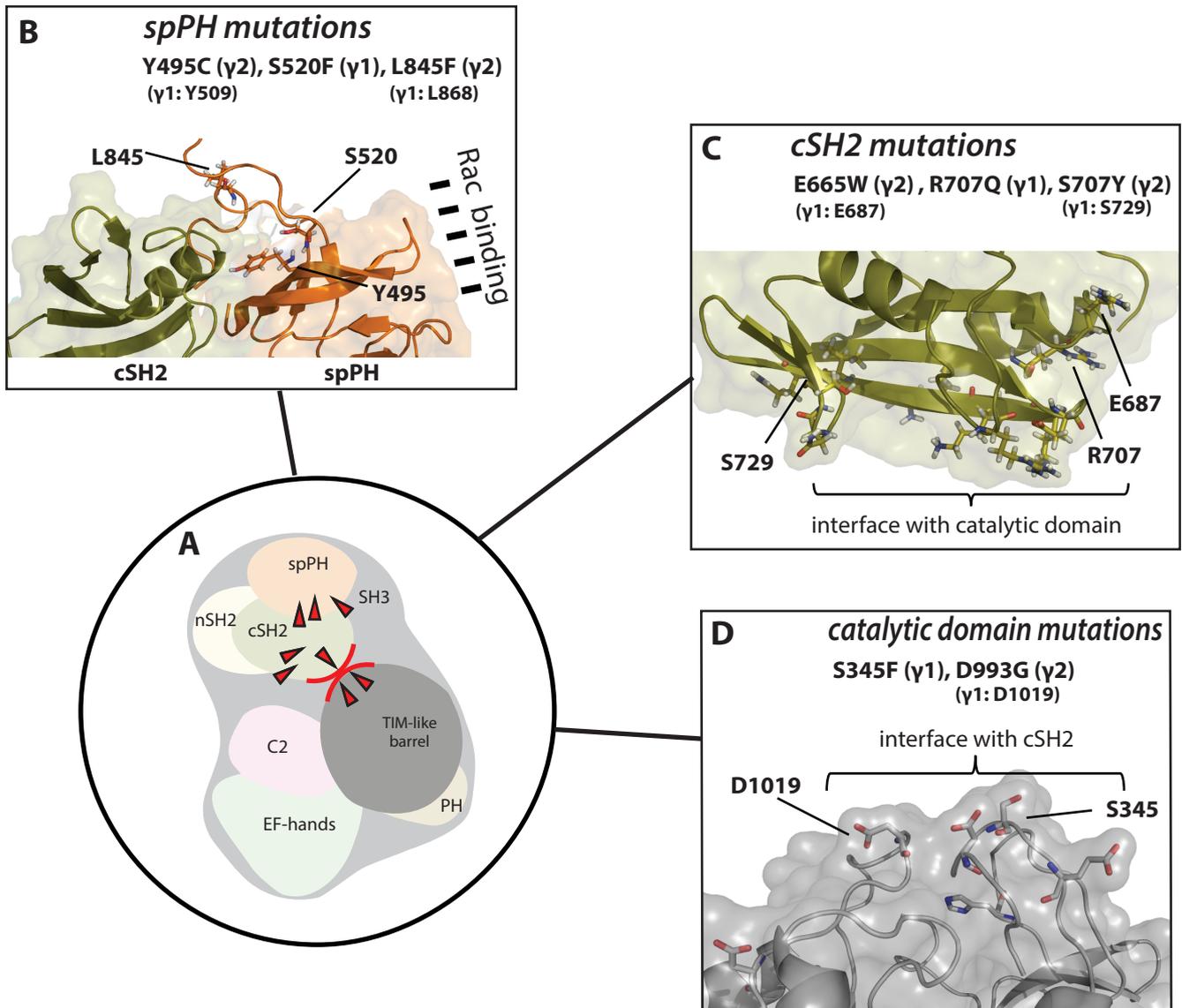
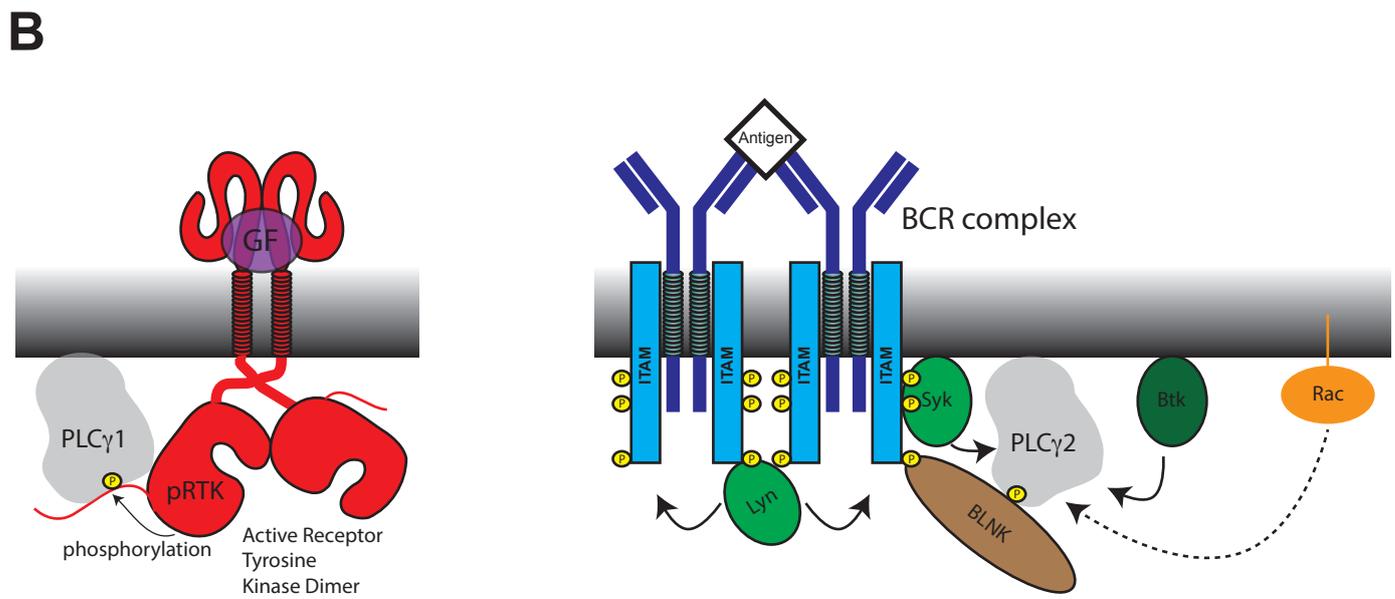
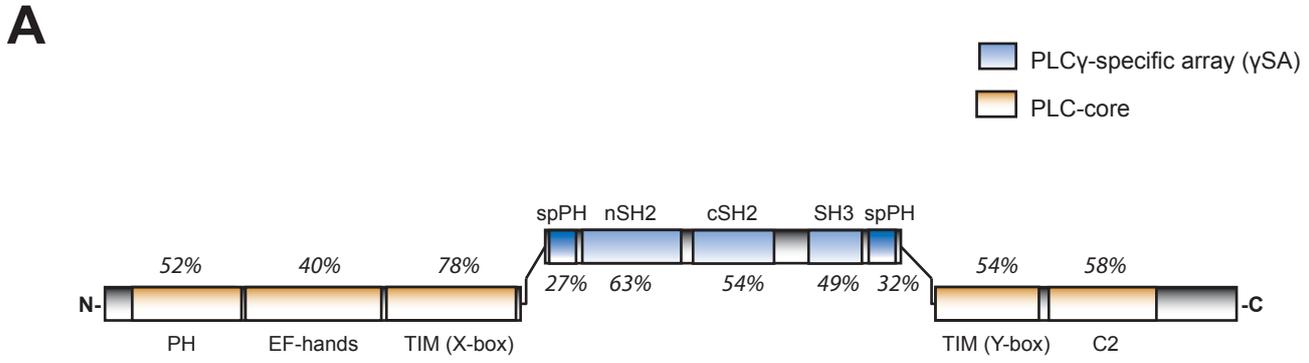


Figure 2



Box 1-
Figure I