

In vitro reconstitution reveals cooperative mechanisms of adapter protein-mediated activation of phospholipase C- γ 1 in T cells

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Junya Wada¹, Udumbara Rathnayake¹, Lisa M. Jenkins², Avinash Singh³, Moosa Mohammadi³, Ettore Appella², Paul A. Randazzo¹, and Lawrence E. Samelson^{1,*}

From the ¹Laboratory of Cellular and Molecular Biology, and ²Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland, USA; ³Department of Biochemistry and Molecular Pharmacology, School of Medicine, Langone Medical Center, New York University, New York, New York, USA

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Activation of T cells upon engagement of the T cell antigen receptor rapidly leads to a number of phosphorylation and plasma membrane recruitment events. For example, translocation of phospholipase-C γ 1 (PLC- γ 1) to the plasma membrane and its association with the transmembrane adapter protein LAT and two other adapter proteins, Gads and SLP-76, are critical events in the early T cell activation process. We have previously characterized the formation of a tetrameric LAT-Gads-SLP-76-PLC- γ 1 complex by reconstitution *in vitro* and have also characterized the thermodynamics of tetramer formation. In the current study, we define how PLC- γ 1 recruitment to liposomes, which serve as a plasma membrane surrogate, and PLC- γ 1 activation are regulated both independently and additively by recruitment of PLC- γ 1 to phosphorylated LAT, by formation of the LAT-Gads-SLP-76-PLC- γ 1 tetramer, and by tyrosine phosphorylation of PLC- γ 1. The recently solved structure of PLC- γ 1 indicates that, in the resting state, several PLC- γ 1 domains inhibit its enzymatic activity and contact with the plasma membrane. We propose the multiple cooperative steps that we observed likely lead to conformational alterations in the regulatory domains of PLC- γ 1, enabling contact with its membrane substrate, disinhibition of PLC- γ 1 enzymatic activity, and production of the phosphoinositide cleavage products necessary for T cell activation.

The phospholipase C (PLC) family of enzymes contains 13 isozymes divided into six classes (β , γ , δ , ϵ , η , and ζ) (1–3). These proteins are central to metazoan cellular function. Signaling pathways regulated by PLC control cellular activation, proliferation and differentiation, and mutations in these enzymes can be oncogenic or lead to autoimmunity. Recruitment and activation of PLC occur at the plasma membrane or at internal membranes where its phosphoinositide substrates are located. The very well-studied cleavage of these substrates leads to the production of diacylglycerol and inositol

phosphates that in turn serve as second messengers, activate a host of enzymes directly and *via* the elevation of intracellular calcium (4, 5).

Activation and regulation of most of the PLC isozymes are mediated by activated G proteins (6). However, the γ isoforms of the enzyme, phospholipase C γ 1 and C γ 2, differ structurally and in their mode of activation (7). They contain a modular region defined by a combined nSH2-cSH2-SH3 domain that lies between a split pleckstrin homology (PH) and enzymatic domains in the enzyme. Recently, elegant structural studies demonstrated that this region lies on top of a classic phospholipase enzymatic domain, which is in a closed, auto-inhibited resting state. Upon activation of the enzyme by targeted tyrosine phosphorylation of a critical regulatory residue, the authors of that study proposed that conformational changes occurred, allowing movement of the modular adapter region and leading to disinhibition of the enzyme and thus its activation (8).

Phospholipase C- γ 1 (PLC- γ 1) activation has been extensively studied in the context of receptor tyrosine kinases (RTK) such as those for epidermal and fibroblast growth factors (9–11). Upon ligand binding to the extracellular domains of these receptors, the receptors dimerize and their cytoplasmic tyrosine kinase domains are activated by transactivation. These activated enzymatic regions trans-autophosphorylate other cytosolic tyrosines within the RTK. Specific phosphorylated tyrosines within the cytosolic domain of the RTK are then bound by signaling proteins including PLC- γ 1, which contain SH2 or other protein tyrosine-binding domains. Some of these recruited molecules are subsequently phosphorylated by the RTK and propagate critical downstream signaling pathways.

PLC- γ 1 isoforms are also central to lymphocyte activation, but in the T and B lymphocytes, PLC- γ 1 is not recruited to the antigen receptor or their associated cytosolic protein tyrosine kinases. Instead, the enzymes are recruited to tyrosine phosphorylated adapter proteins. In the case of T cells activated by antigenic peptide bound to a molecule encoded within the major histocompatibility complex, the integral membrane adapter protein linker for activation of T cells (LAT) is a critical early substrate for the protein tyrosine kinase (PTK),

* For correspondence: Lawrence E. Samelson, samelsonl@helix.nih.gov.

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ZAP-70, activated at the T cell antigen receptor (TCR) (12, 13). Specific phosphorylated tyrosine residues of LAT then bind many SH2-domain-containing signaling proteins and adapter molecules (14, 15). These include PLC- γ 1, which binds LAT through its N-terminal SH2 domain, and the adapter protein Gads (Grb2-related adapter protein downstream of Shc) (16). In the T cell cytoplasm, a large fraction of Gads is constitutively bound to the adapter protein, SLP-76 (Src homology 2 (SH2) domain containing leukocyte protein of 76 kDa), which is thus recruited as a heterodimer with Gads to LAT (17–21). While bound to LAT, adjacent PLC- γ 1 and Gads-SLP-76 dimers also interact *via* the PLC SH3 domain and a poly-proline region of SLP-76, thus forming a tetrameric complex (LAT-Gads-SLP-76-PLC- γ 1) (22). PLC- γ 1 recruited to the plasma membrane within this complex is activated by tyrosine phosphorylation of PLC- γ 1 at Y783 by the PTK ITK (interleukin-2-inducible T cell kinase), which is also recruited to the LAT-based complexes (23, 24).

We have previously reported the reconstitution of the LAT-Gads-SLP-76-PLC- γ 1 tetrameric complex *in vitro* using soluble recombinant and synthesized proteins (25). In that study we employed isothermal titration calorimetry and analytical ultracentrifugation to define the thermodynamics of tetrameric complex formation. We found that *in vitro* the tetrameric complex is unstable, as a fraction of the added PLC- γ 1 dissociated from the other components. Moreover, formation of the complex must overcome a significant entropic penalty. We hypothesized that the instability of the complex enables dynamic regulation of its function in the cellular milieu. In the current study, we focused on the functional consequences of the formation of this complex. We again used an *in vitro* approach with recombinant and synthesized proteins, but we reconstituted the complex to address the functional consequences of tetrameric complex formation at the surface of liposomes comprised of lipids found in the plasma membrane of T lymphocytes. These experiments model the manner in which PLC- γ 1 is recruited and activated at the plasma membrane of activated T cells. We determined that formation of the tetrameric complex and tyrosine phosphorylation of PLC- γ 1 independently increase the activity of PLC- γ 1 in this setting.

Results

For our functional studies of activation of PLC- γ 1 in the context of the LAT-Gads-SLP-76-PLC- γ 1 tetrameric complex, we made protein constructs similar to those we used previously. PLC- γ 1 was nearly full-length with just the C-terminal 71 residues removed to prevent aggregation (26). The adapter protein LAT was prepared without its transmembrane domain but instead with a hexa-His tag at the N-terminus. Only two of the LAT intracellular tyrosines (Y132 and Y171) were present. These sites were extrinsically phosphorylated *in vitro*. The full-length adapter Gads was modified at its N-terminus by addition of a Halo-tag to enhance solubility (27). Only the tyrosine- and proline-rich domains (103–258aa) of the multi-domain SLP-76 adapter were used. Techniques for protein

purification, *in vitro* phosphorylation, and confirmation of phosphorylation stoichiometry are described in the Experimental procedures section. The domain structures of these proteins are depicted in Figure 1A. The purity and concentration of the recombinant proteins (Fig. S1A) were assessed as described in the Experimental procedures section.

Upon T cell activation, cytosolic PLC- γ 1 is recruited to the inner leaflet of the plasma membrane where it interacts with its substrate phosphoinositide, phosphatidylinositol 4, 5-bisphosphate (PIP2). To model this interaction, we used large unilamellar vesicles (LUVs), which are also known as liposomes, comprised primarily of lipids that make up the major components of the plasma membrane: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and cholesterol. These four components together comprised 80% of the total lipid in the LUVs. Additionally, we added phosphatidylinositol 4,5-bisphosphate (PIP2), the substrate of PLC- γ 1, and the Ni-ion-chelating lipid (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl]; DGS-NTA). This latter reagent enabled binding to Hexa-His-tagged proteins in our assays. The LUVs we made ranged in size from 0.3 to 0.6 μ m (Fig. S1B).

PLC enzymatic activity was determined using a colorimetric HPLC assay described by Mayr and colleagues (28, 29). In this assay, the phosphoinositol products produced by PLC- γ 1 enzymatic cleavage of PIP2 are fractionated by anion exchange HPLC and detected by a transition metal (yttrium) and complexometric dye [(4-(2-pyridylazo)resorcinol) (PAR)]-based detection system. One of the PIP2 cleavage products, inositol-3-phosphate (IP3), competes with the yttrium ion for binding to PAR and thus decreases the absorbance measured at 546 nm wavelength (Fig. S2A). Generation of a standard curve using an IP3 standard in this assay demonstrated linearity and a measurable range of 1000 to 10,000 pmol of substrate (Fig. S2B).

To test the functionality of our recombinant PLC- γ 1 in this assay, the enzyme was titrated and added to the LUVs. IP3 production was linear with PLC- γ 1 concentration to 100 nM. We compared the addition of an inactive PLC- γ 1 mutant, which has two substitutions from histidine to alanine in the catalytic domain (H335, H380 > A335, A380). Incubation with this inactive form of PLC- γ 1 resulted in no IP3 production at 2000 nM (Fig. 1B). These results indicated that both the reconstitution system and the enzymatic assay worked correctly. They also indicated that concentrations of 20 to 50 nM of PLC- γ 1 would be optimal for the following experiments.

One of our underlying hypotheses is that a major function of the *in vivo* formation of the tetrameric LAT-Gads-SLP-76-PLC- γ 1 complex is to bring PLC- γ 1 to the plasma membrane for more efficient contact with its substrate, PIP2. To model binding of PLC- γ 1 to the plasma membrane, we incubated PLC- γ 1 without or with the other components of the tetramer to LUVs and then used ultracentrifugation to determine the percentage of PLC- γ 1 bound to the LUV membranes using image scanning, as described in the Experimental procedures section. As an initial test of the hypothesis, we

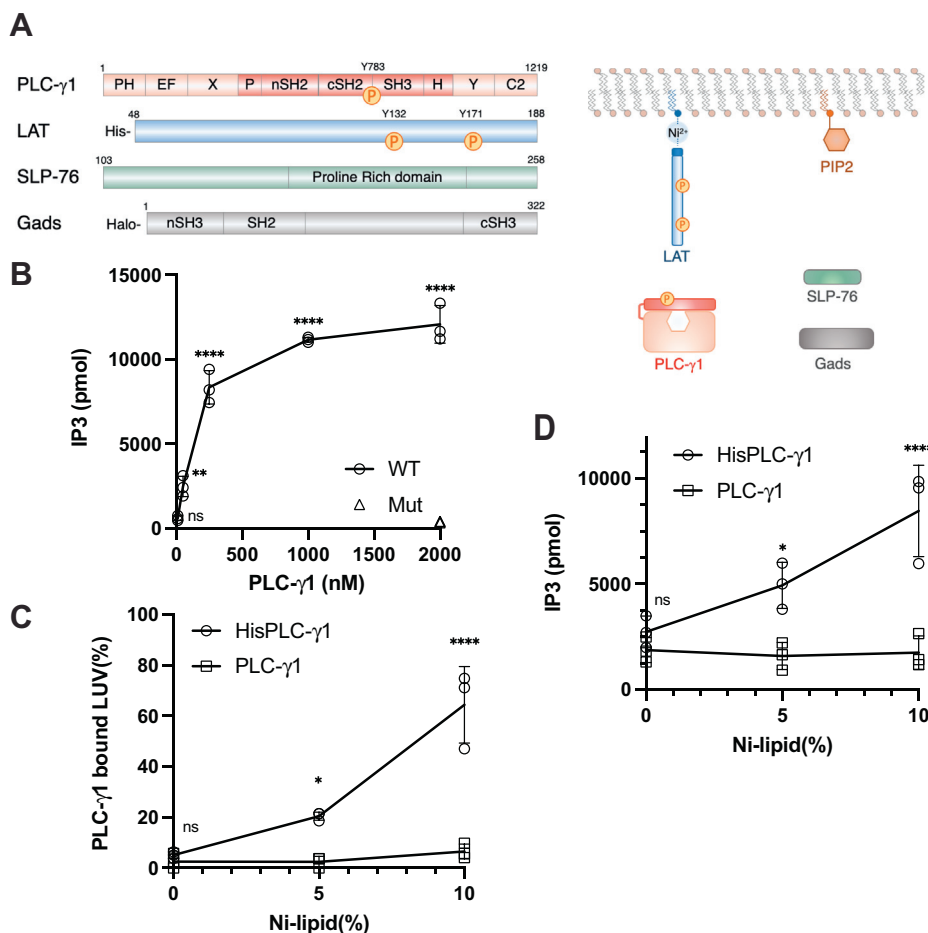


Figure 1. Domain organization of the recombinant proteins used in the study and tests of the *in vitro* reconstitution system. A, functional domains and phosphorylation sites are depicted in bar models. Relevant phosphorylation sites are depicted. His- or Halo-tags added at the N-termini of LAT and Gads are indicated. The cartoons depict the *in vitro* reconstitution system in which the membrane of the liposome contains DGS-NTA(Ni) (lipid in blue) and PIP2 (lipid in brown). The His-tagged, doubly phosphorylated LAT (LAT protein in blue) is moored at the surface due to the interaction between the His-tag and the Ni²⁺ ion in DGS-NTA(Ni). B, PLC- γ 1 titration for PIP2 hydrolysis using wild type (WT; circle) or an enzymatically dead PLC- γ 1 mutant (Mut; triangle). The protein concentration of added PLC- γ 1 WT is 10, 50, 250, 1000, and 2000 nM. Data were analyzed by one-way ANOVA. Wild-type proteins were compared with 2000 nM of the mutant PLC- γ 1 using Dunnett's multiple comparison post-hoc test. C, titration of PLC- γ 1 lipid binding comparing His-tagged PLC- γ 1 (HisPLC- γ 1; circle) or untagged PLC- γ 1 (PLC- γ 1; square). The protein concentration of added PLC- γ 1 is 100 nM. Following two-way ANOVA, binding of the His-tagged and untagged protein were compared at each Ni-lipid concentration using Bonferroni's multiple comparison post-hoc test (Table S1). D, titration of PLC- γ 1 enzyme activity comparing His-tagged PLC- γ 1 (His-PLC- γ 1; circle) or untagged PLC- γ 1 (PLC- γ 1; square). The protein concentration of added PLC- γ 1 is 50 nM. Data were analyzed as in C (Table S1). The data shown in B–D are the means \pm S.D. from three independent experiments [$*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; ns, not significant].

determined the effect of direct recruitment of PLC- γ 1 to the LUVs by adding an 8xHis-Tag to the N-terminus of PLC- γ 1 and comparing binding of nontagged and tagged versions of PLC- γ 1 to LUVs. The amount of His-PLC- γ 1 bound to LUV was 5% in the absence of the Ni-lipid (DGS-NTA(Ni)) and was similar to binding of PLC- γ 1 without the His tag. Increasing the content of Ni-lipid of the LUV had little or no effect on binding of PLC- γ 1 without the His tag, but increased binding of His-PLC- γ 1, reaching 60% of total PLC bound to LUVs with 10% Ni-lipid. (Fig. 1C and Table S1). The His tag on PLC- γ 1 and the Ni-lipid were synergistic, increasing enzymatic activity of His-PLC- γ 1 two- or three-fold over the same titration (Fig. 1D and Table S1). Enzymatic activity of the non-His-tagged PLC- γ 1 was not detectably affected by increasing the percent of the Ni-lipid in the LUVs. These results indicate that increased membrane recruitment of PLC- γ 1, facilitated by

binding of the His-tagged version to Ni-lipids in the LUV, itself enhanced PLC- γ 1-mediated PIP2 cleavage.

LAT is the transmembrane adapter primarily involved in recruiting PLC- γ 1 and the Gads-SLP-76 dimer to the plasma membrane due to the PLC- γ 1 N-SH2 domain binding to LAT pY132 and the Gads SH2 domain binding either LAT pY171 or pY191. We next determined whether His-tagged-diphospho-LAT (pLAT) containing pY132 and pY171 bound to LUVs containing Ni-Lipids. Titration of Ni-lipids indicated that His-tagged pLAT binding neared maximal binding of 75 to 80% with LUVs containing 2.5% Ni-lipid (Fig. 2A). We then tested whether pLAT bound to LUVs in this manner was sufficient to enhance PLC- γ 1 binding to LUVs. For this experiment, we compared LUV made with and without the addition of Ni-lipids. As previously determined in Figure 1B, PLC- γ 1 itself showed low level binding to the LUVs without or

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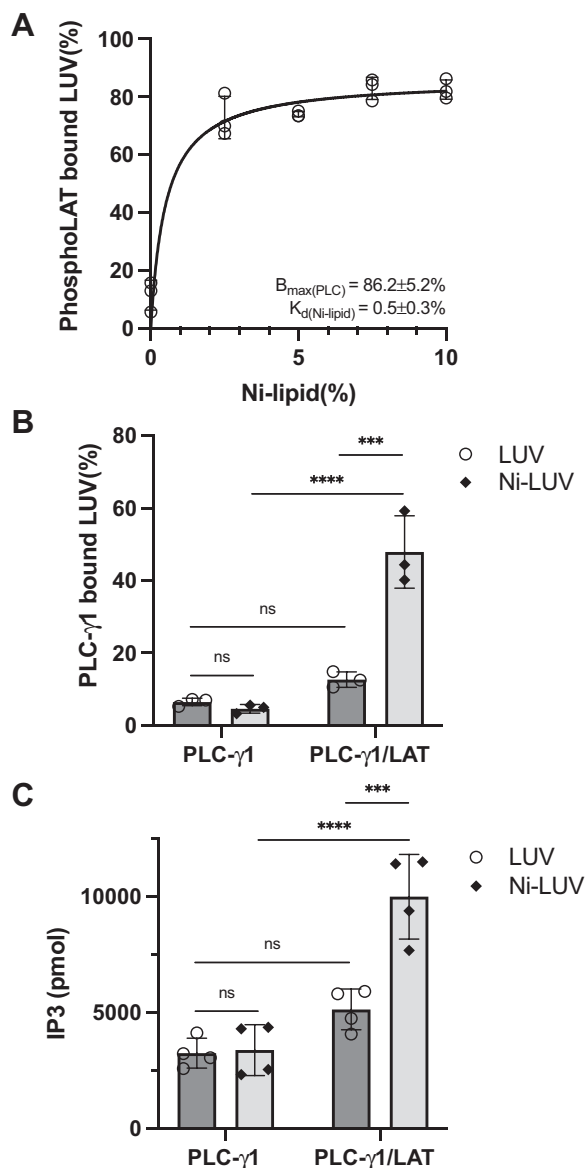


Figure 2. His-tagged LAT bound to Ni-linked lipids enhances membrane recruitment and enzymatic activity of PLC- γ 1. A, titration of lipid binding of doubly phosphorylated LAT. The protein concentration of LAT is 1 μ M. The data were fit to one-site specific binding equation in Prism. B, lipid binding of untagged PLC- γ 1 to LUVs loaded with or without DGS-NTA(Ni) [Ni-LUV (square) or LUV (circle)] comparing addition of PLC- γ 1 alone (PLC- γ 1) to addition of PLC- γ 1 and doubly phosphorylated His-tagged LAT (PLC- γ 1/LAT). The protein concentration of PLC- γ 1 is 100 nM, and LAT is 500 nM. Following two-way ANOVA, all groups were compared using Tukey's multiple comparison post-hoc test (Table S2). C, PIP₂ hydrolysis with the LUV loaded with or without DGS-NTA(Ni) [Ni-LUV (square) or LUV (circle)] comparing addition of PLC- γ 1 alone (PLC- γ 1) and PLC- γ 1 with doubly phosphorylated His-tagged LAT (PLC- γ 1/LAT). The protein concentration of PLC- γ 1 is 50 nM, and LAT is 250 nM. Data were analyzed as in B (Table S2). The data shown are the means \pm S.D. from three (A and B) or four (C) independent experiments. [* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; ns, not significant]. LUV, large unilamellar vesicle; PLC- γ 1, phospholipase-C γ 1.

with Ni-lipids added to the LUVs. Addition of His-pLAT to form a LAT-PLC- γ 1 complex modestly increased pLAT-PLC- γ 1 binding to LUVs without Ni-lipids. His-pLAT and Ni-lipids synergistically increased PLC- γ 1 binding (Fig. 2B and Table S2, significant interaction, $p = 0.0003$). Consistent with the

binding results, the pLAT-PLC- γ 1 complex showed a modest increase in enzymatic activity over baseline in the presence of LUVs without Ni-lipids. However, Ni-lipids and His-pLAT synergistically increased IP₃ release indicating that PLC- γ 1 recruitment to the LUV was required for optimal PLC- γ 1 activity (Fig. 2C and Table S2, significant interaction by two-way ANOVA, $p = 0.0019$).

To further confirm that PLC- γ 1 binding to LUVs and enzymatic activity were dependent on binding to phosphorylated LAT molecules, we performed a titration of non-phosphorylated *versus* phosphorylated His-tagged LAT added to the Ni-LUVs (Fig. 3A and Table S3). PLC- γ 1 binding approached saturation at 500 nM pLAT, and there was no binding of PLC- γ 1 to nonphosphorylated LAT. Under the same conditions, we then showed that PLC- γ 1 cleavage of the PIP₂ substrate was linearly dependent on pLAT concentration up to 100 nM (Fig. 3B and Table S3). In previous biochemical studies, the primary LAT-binding site of the PLC- γ 1 N-SH2 domain has been shown to be LAT pY132 (30). To confirm the interaction in our *in vitro* system, we prepared pLAT peptides containing a Y > F mutation at either Y132 or Y171, in addition to the doubly phosphorylated LAT peptide. As predicted, compared with the binding to the doubly phosphorylated LAT peptide, the Y132F mutation reduced binding of PLC- γ 1, whereas the Y171F mutation had no effect on PLC- γ 1 binding. Enzymatic activity followed the binding results. There was a slight increase in activity in the presence of Y132F, which followed the slight increase in PLC binding to this peptide over baseline (Fig. 3, C and D). Statistical analysis revealed no significant difference in the binding or hydrolysis results in which the 132F LAT mutant protein or no LAT at all was added to the assay. However, the increase in activity in PLC- γ 1 bound to the pYLAT171F was comparable to the activity of PLC- γ 1 bound to the doubly phosphorylated LAT peptide (Fig. 3D). These results confirm that binding of PLC- γ 1 to pYLAT132 is the primary way by which PLC- γ 1 is recruited to the membrane and activated.

The binding characteristics of the other components of the tetrameric complex, Gads and SLP-76, and the effect of such binding on PLC- γ 1 activity were then evaluated. In coprecipitation studies, the SH2 domain of Gads has been shown to interact with either LAT pY171 or pY191 residues. For simplicity of phosphopeptide synthesis, we have focused on tyrosine 171. Mutation of LAT from tyrosine to phenylalanine at Y171, but not at Y132, where PLC- γ 1 interacts, reduced binding of Gads in our LUV binding assay, as expected (Fig. 4A). Binding to LUVs of various combinations of the four components needed to form the tetrameric complex was then compared (Table 1 and Supporting Table for Table 1). The marked increase in PLC- γ 1 binding to LUVs in the presence of pLAT was observed again. The addition of Gads and/or SLP-76, which together form a dimer, each showed little binding to the LUVs and the addition of PLC- γ 1 to Gads and SLP-76 also did not induce binding of any of these three molecules. However, the addition of phosphorylated hexa-His-LAT (pLAT) to the LUVs enabled binding of the Gads-SLP-76 dimer *via* the Gads SH2 domain. Addition of Gads, SLP-76

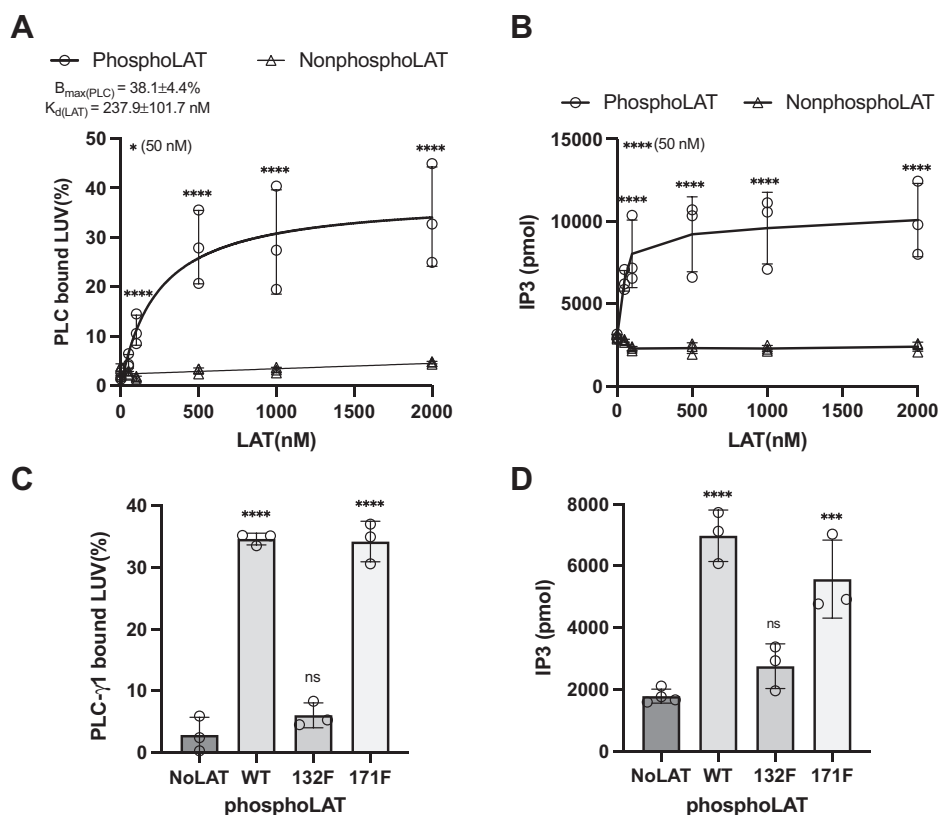


Figure 3. Phosphorylation of LAT at Y132 is critical for the recruitment and activation of PLC- γ 1. *A*, titration of lipid binding of PLC- γ 1 with doubly phosphorylated His-tagged LAT (PhosphoLAT; *circle*) or Non-phosphorylated His-tagged LAT (NonphosphoLAT; *triangle*). The protein concentration of PLC- γ 1 is 100 nM, and LAT is 0, 50, 100, 500, 1000, and 2000 nM. The data were fit to either one-site specific binding (PhosphoLAT) or straight line (NonphosphoLAT) in Prism. To normalize variance and reduce difference in variance between conditions, data were log-transformed prior to analysis by two-way ANOVA. Bonferroni's multiple comparison post-hoc test was used to compare binding of PLC- γ 1 at each concentration of LAT (Table S3). *B*, titration of PIP2 hydrolysis with doubly phosphorylated His-tagged LAT (PhosphoLAT; *circle*) or Non-phosphorylated His-tagged LAT (NonphosphoLAT; *square*). The protein concentration of PLC- γ 1 is 50 nM, and LAT is 0, 50, 100, 500, 1000, and 2000 nM. Data were analyzed as in *A* (Table S3). *C*, lipid binding of PLC- γ 1 without LAT protein (NoLAT), with doubly phosphorylated His-tagged LAT (WT), with singly phosphorylated His-tagged LAT containing a Y132F mutation and pY171 (132F), or with singly phosphorylated His-tagged LAT containing pY132 and a Y171F mutation (171F). The protein concentration of PLC- γ 1 is 100 nM, and LAT is 500 nM. Data were analyzed by one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test, comparing WT, 132F and 171F to the NoLAT condition. *D*, PIP2 hydrolysis of PLC- γ 1 without LAT protein (NoLAT), with doubly phosphorylated His-tagged LAT (WT), with singly phosphorylated His-tagged LAT containing a Y132F mutation and pY171 (132F), or with singly phosphorylated His-tagged LAT containing pY132 and a Y171F mutation (171F). The protein concentration of PLC- γ 1 is 50 nM, and LAT is 250 nM. Data were analyzed as in *C*. The data shown in *A–D* are the means \pm S.D. from three independent experiments. [$*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; ns, not significant]. PLC- γ 1, phospholipase-C γ 1.

and PLC- γ 1 also resulted in binding of all three to the pLAT bound to the LUV membrane. Notably, the addition of the Gads-SLP76 dimer did not increase PLC- γ 1 recruitment to LUVs. In the assay of PLC- γ 1 activity on LUVs bearing pLAT, the addition of either Gads or SLP-76 individually had little effect (Fig. 4, *B* and *C*), but added together were synergistic, increasing activity over twofold (Fig. 4C and Table S4, significant interaction by two-way ANOVA, $p = 0.0077$). This latter result is consistent with the conclusion that formation of the tetrameric complex at the membrane optimizes PLC- γ 1 activation.

Full activation of PLC- γ 1 enzymatic activity requires phosphorylation on one or more tyrosine residues with Y783 the most studied site (26). Phosphorylation on this residue is postulated to induce conformational changes in PLC- γ 1 so that the enzyme becomes disinhibited and thus activated. When activation of PLC- γ 1 occurs while bound to a receptor tyrosine kinase (RTK) such as the epidermal growth factor receptor (EGFR) or the fibroblast growth receptor (FGFR), the

intracellular domain of the RTK phosphorylates the Y783 residue on a PLC- γ 1 molecule also bound to that or an adjacent RTK domain. In activated T cells, tyrosine phosphorylation of PLC- γ 1 is mediated by ITK, a TEC-family PTK, which is also recruited to LAT-based signaling complexes by binding LAT-bound SLP-76 (31).

We used a soluble recombinant tyrosine kinase domain from the FGFR to phosphorylate PLC- γ 1 to determine whether phosphorylated PLC- γ 1 molecules demonstrated enhanced activity in our reconstitution system (10). We determined that in an *in vitro* kinase reaction using the FGFR kinase domain, PLC- γ 1 became maximally phosphorylated on Y783 within an hour (Fig. 5A). Phosphorylated or non-phosphorylated PLC- γ 1 molecules were then tested in our PLC- γ 1 binding and enzymatic assays in the presence or absence of pLAT. We saw no effect of PLC- γ 1 phosphorylation on the ability of PLC- γ 1 to bind the LUVs in the absence or presence of pLAT (Fig. 5B). In the enzymatic assay, binding of nonphosphorylated PLC- γ 1 to pLAT increased enzymatic

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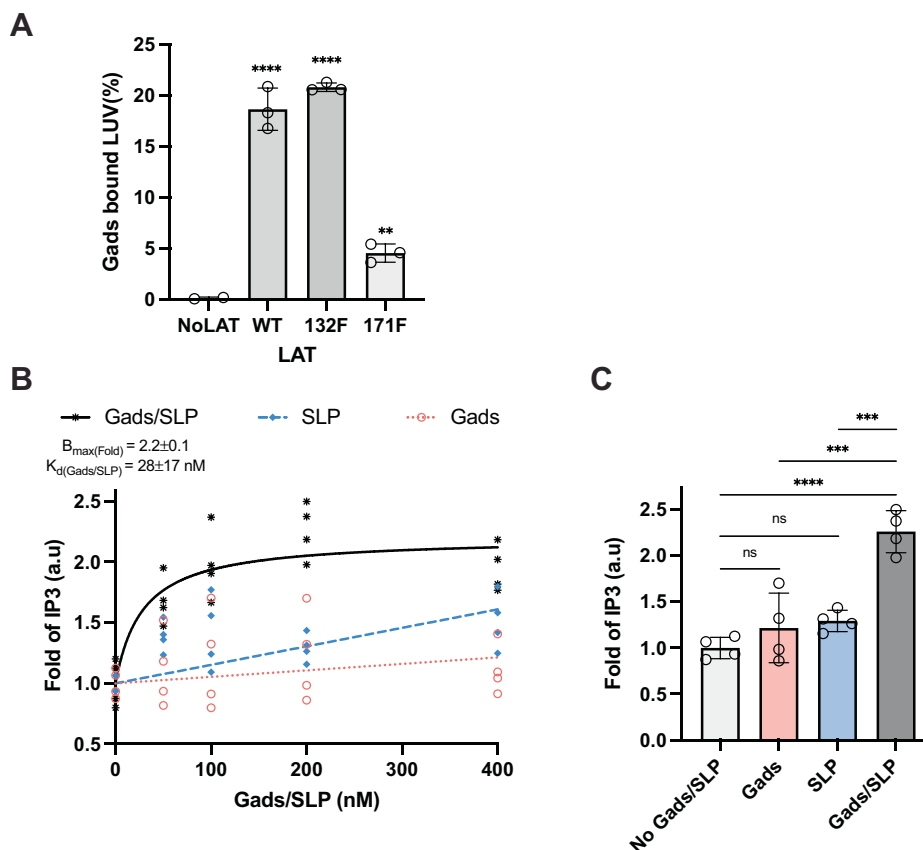


Figure 4. Gads and SLP-76 in combination enhance the enzymatic activity of PLC- γ 1. A, lipid binding of Gads without LAT (NoLAT), with doubly phosphorylated His-tagged LAT (WT), with singly phosphorylated His-tagged LAT containing a Y132F mutation and pY171 (132F), or with singly phosphorylated His-tagged LAT containing pY132 and a Y171F mutation (171F). The protein concentration of Gads is 200 nM, and LAT is 1000 nM. Following one-way ANOVA, WT 132F and 171F were compared to NoLAT using Dunnett's multiple comparison post-hoc test. B, concentration titration of Gads and/or SLP-76 for PIP2 hydrolysis in the presence of PLC- γ 1 and doubly phosphorylated His-tagged LAT (Gads; red circle, SLP-76; blue square, Gads and SLP-76; black star). The amount of PIP2 hydrolysis is normalized by the data without Gads/SLP-76. The protein concentration of PLC- γ 1 is 20 nM, LAT is 100 nM, and Gads and/or SLP-76 is 0, 50, 100, 200, and 400 nM. The data were fit to either one-site specific binding equation (Gads/SLP) or a straight line (Gads and SLP) in Prism. C, PIP2 hydrolysis of PLC- γ 1 and doubly phosphorylated His-tagged LAT with 200 nM of Gads and/or SLP-76 in Figure 4B. Following two-way ANOVA, all groups were compared with Tukey's multiple comparison post-hoc test (Table S4). The data are the means \pm S.D. from three (A) or four (B and C) independent experiments. [$*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; ns, not significant]. PLC- γ 1, phospholipase-C γ 1.

activity about twofold, as shown previously (Fig. 3B). However, phosphorylated PLC- γ 1 recruited to membrane-bound phospho-LAT demonstrated enhanced enzymatic activity to a level about fourfold higher than that of free PLC- γ 1 in the assay

(Fig. 5C). Both the presence of pLAT and the phosphorylation of PLC- γ 1 each induced a statistically significant enhancement of PLC- γ 1 enzymatic activity. However, the phosphorylation of PLC- γ 1 without inclusion of pLAT did not result in a

Table 1

Lipid binding properties of Gads and SLP-76 in combinations with PLC- γ 1 and/or LAT

Assayed protein(s)	PLC- γ 1	LAT	Gads	SLP-76
PLC- γ 1	4.6 \pm 0.6	-	-	-
PLC- γ 1, LAT	44.2 \pm 2.8 [#]	86.6 \pm 4.7	-	-
Gads	-	-	1.0 \pm 0.6	-
SLP-76	-	-	-	1.3 \pm 1.3
LAT, Gads	-	90.1 \pm 1.4	26.3 \pm 1.5 ^{&}	-
LAT, SLP-76	-	83.2 \pm 3.3	-	1.5 \pm 0.6 ^{ns(s)}
Gads, SLP-76	-	-	6.5 \pm 3.1	2.8 \pm 2.8
PLC- γ 1, Gads, SLP-76	2.4 \pm 1.0 ^{ns(p1)}	-	7.9 \pm 4.4	1.5 \pm 1.5
LAT, Gads, SLP-76	-	87.0 \pm 1.4	31.4 \pm 9.9 ^{ns(g), %}	24.0 \pm 3.7 ^{~@}
PLC- γ 1, LAT, Gads, SLP-76	37.9 \pm 1.3 ^{ns(p2)}	84.3 \pm 1.2	42.8 \pm 9.8	25.3 \pm 3.6

(% of protein binding to LUV)

LAT in this experiment is His-tagged and doubly phosphorylated at Y132 and Y171. The protein concentration of PLC- γ 1 is 100 nM, and the other proteins are 500 nM. The data shown in the table are the means \pm S.D. from three independent experiments. Each column was analyzed by one-way ANOVA.

Specific comparisons were made using the Bonferroni's multiple comparison post-hoc test. Differences are indicated by the following symbols: #, $p < 0.0001$, comparison with PLC- γ 1; ns(p1), not significant, comparison with PLC- γ 1; ns(p2), not significant, comparison with PLC- γ 1, LAT; &, $p < 0.05$, comparison with Gads; ns(g), not significant, comparison with LAT, Gads; %, $p < 0.05$, comparison with Gads, SLP-76; ns(s), not significant, comparison with SLP-76; ~, $p < 0.001$, comparison with LAT, SLP-76; @, $p < 0.001$, comparison with Gads, SLP-76. Summary of the statistical analysis is shown in Supporting Table for Table S1.

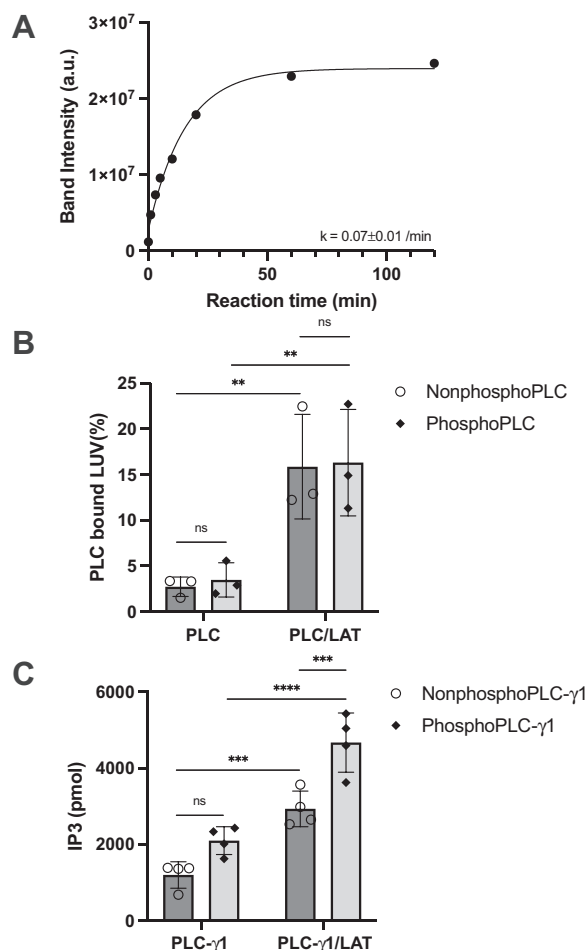


Figure 5. Phosphorylation of PLC- γ 1 at Y783 enhances the enzymatic activity of PLC- γ 1. *A*, time-course of *in vitro* phosphorylation at Y783 in PLC- γ 1. The data were fit to one-phase association in Prism. k is a rate constant (/min). a.u. is arbitrary unit. The data shown in *A* is representative of four independent experiments. *B*, lipid binding of PLC- γ 1 with PLC- γ 1 added alone (PLC- γ 1) or with doubly phosphorylated His-tagged LAT (PLC- γ 1/LAT). The PLC- γ 1 is either phosphorylated (PhosphoPLC- γ 1; square) or non-phosphorylated (NonphosphoPLC- γ 1; circle). The protein concentration of PLC- γ 1 is 100 nM, and LAT is 500 nM. Data were log-transformed to normalize and decrease differences in variances between conditions prior to analysis by two-way ANOVA. The indicated conditions were compared using Bonferroni's multiple comparison post-hoc test (Table S5). *C*, PIP₂ hydrolysis of PLC- γ 1 with PLC- γ 1 added alone (PLC- γ 1) or with doubly phosphorylated His-tagged LAT (PLC- γ 1/LAT). The PLC- γ 1 is either phosphorylated (Phospho-PLC- γ 1; gray) or non-phosphorylated (Nonphospho-PLC- γ 1; black). The protein concentration of PLC- γ 1 is 50 nM, and LAT is 250 nM. Data were analyzed as in *B* except they were not log-transformed because variances were similar between groups. The data shown are the means \pm S.D. from three (*B*) or four (*C*) independent experiments. [$*$, $p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; ns, not significant]. PLC- γ 1, phospholipase-C γ 1.

statistically significant elevation in a post-hoc test (Table S5). The effects of the combination of recruitment of PLC- γ 1 to the LUV *via* phospho-LAT and the phosphorylation of the enzyme on enzymatic activity were additive, suggesting independent mechanisms (Table S5, significant effect of each factor but no significant interaction when analyzed by two-way ANOVA).

Finally, we studied the combined effect of forming the tetrameric complex on the LUV and phosphorylating the critical regulatory tyrosine residue Y783 on PLC- γ 1. As shown above, the amount of PLC- γ 1 recruited to the LUVs *via*

binding pLAT again was the same whether or not Gads and SLP-76 were also present or whether PLC- γ 1 was phosphorylated at Y783 (Fig. 6A). Consistent with results shown in previous figures, PLC- γ 1 activity increased in the presence of pLAT and with the addition of Gads and SLP-76. Importantly, under each of these conditions, the prior phosphorylation of PLC- γ 1 at Y783 enhanced PLC- γ 1 activity still further, inducing tenfold higher enzymatic activity compared with nonphosphorylated PLC- γ 1 alone (Fig. 6B). The effects were additive (no significant interaction by two-way ANOVA, Table S6), suggesting independent activation mechanisms. Based on these results, we conclude that tetrameric complex formation and PLC- γ 1 phosphorylation can independently and additively enhance PLC- γ 1 enzymatic activity.

Discussion

Studies over the past two decades have documented the early events that follow T cell receptor (TCR) engagement (12–15). Activation of protein tyrosine kinases at the TCR leads in seconds to phosphorylation of critical protein substrates such as LAT and the consequent formation of signaling complexes nucleated at that adapter molecule. Coimmunoprecipitation studies and reconstitution of LAT-deficient T cells with LAT mutants defined the sites of interactions of multiple proteins at LAT. Similar studies were undertaken to define binding sites and binding partners for the molecules recruited to LAT. In this fashion, it became clear that following tyrosine phosphorylation of LAT, the adapter molecules Gads and SLP-76, and the enzyme phospholipase C- γ 1 formed a quaternary complex or tetramer. Further analysis has demonstrated that other molecules with relevance for phospholipase C- γ 1 activation also bind SLP-76 during activation. These include the adapter Nck and the enzymes Vav and ITK, with ITK important for activation of PLC- γ 1 in T cells. Moreover, many other proteins bind LAT upon activation, such as Grb2, which in turn binds to the enzymes SOS and Cbl. Grb2 might compete with Gads for binding at phosphorylated LAT (32–34).

We have taken a reductionist approach to characterization of the LAT-Gads-SLP-76- PLC- γ 1 heterotetramer. In our initial study, we prepared short (18–20 amino acid) peptides with the LAT sequence monophosphorylated at the central tyrosine, the proline-rich region of SLP-76 (amino acids 159–421), the binding domain fragment of PLC- γ 1 (SH2-SH2-SH3), and full-length Gads (30). Isothermal titration calorimetry (ITC) was used to define the K_ds of their various interactions. Notable findings included the high-affinity interaction of Gads and SLP-76 (19 nM), the specific 62 nM binding of the phosphopeptide containing pY132 with PLC- γ 1 *via* its N-SH2 domain, and the lack of a measurable binding affinity of SLP-76 with PLC- γ 1 at room temperature. This latter interaction was, however, demonstrated at 4 °C. Analytical ultracentrifugation demonstrated that an interaction of PLC- γ 1 with the Gads-SLP-76 complex occurred only when PLC- γ 1 was present at a superstoichiometric concentration. These studies thus provided basic binding information

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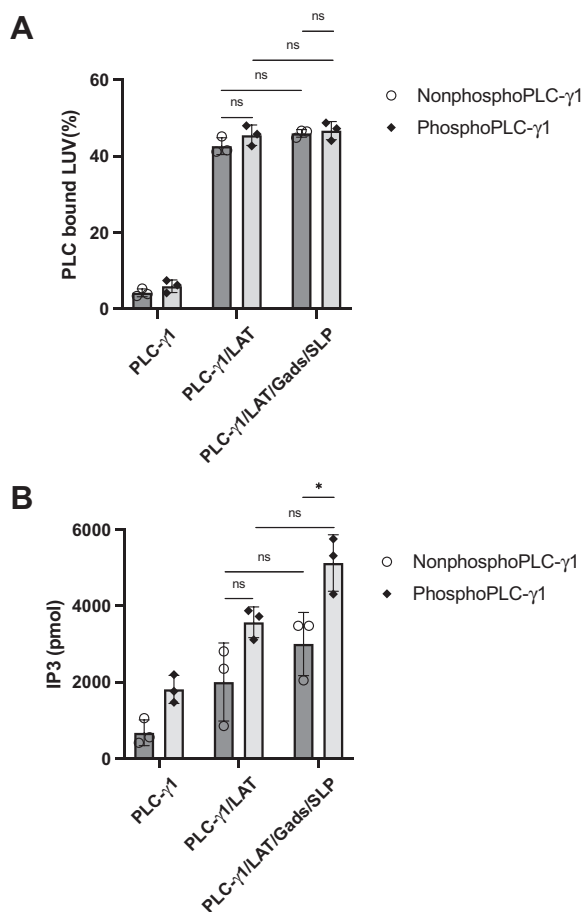


Figure 6. PLC- γ 1 phosphorylation and Gads-SLP76 binding additively enhance enzymatic activity of PLC- γ 1. A, lipid binding of PLC- γ 1 with PLC- γ 1 added alone (PLC- γ 1), PLC- γ 1 and doubly phosphorylated His-tagged LAT (PLC- γ 1/LAT), or with four added proteins (PLC- γ 1/LAT/Gads/SLP-76). The PLC- γ 1 is either phosphorylated (PhosphoPLC- γ 1; square) or non-phosphorylated (NonphosphoPLC- γ 1; circle). The protein concentration of PLC- γ 1 is 50 nM, and other proteins are 350 nM. Following two-way ANOVA, differences between the indicated groups were tested using Bonferroni's multiple comparison post-hoc test (Table S6). B, PIP₂ hydrolysis of PLC- γ 1 with PLC- γ 1 added alone (PLC- γ 1), PLC- γ 1 and doubly phosphorylated His-tagged LAT (PLC- γ 1/LAT), or with four added proteins (PLC- γ 1/LAT/Gads/SLP-76). The PLC- γ 1 is either phosphorylated (PhosphoPLC- γ 1; square) or non-phosphorylated (Nonphospho-PLC- γ 1; circle). The protein concentration of PLC- γ 1 is 30 nM, and other proteins are 210 nM. Data were analyzed as in A. The data shown in A and B are the means \pm S.D. from three independent experiments. [$*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; ns, not significant]. PLC- γ 1, phospholipase-C γ 1.

and in particular, showed that of the pairwise interactions between the four proteins, the SLP-76- PLC- γ 1 interaction was of low affinity.

More recently, we expanded our analysis to determine whether a tetrameric complex of LAT-Gads-SLP76- PLC- γ 1 could be reconstituted and formed *in vitro* (25). We used a doubly phosphorylated LAT peptide containing both a phosphotyrosine (Y132) for PLC- γ 1 binding and a phosphotyrosine for Gads binding (Y171). We prepared full-length Gads, SLP-76 lacking only the amino-terminal SAM domain, which is known to contribute to dimerization, and nearly full-length PLC- γ 1 lacking only 71 C-terminal residues, which contribute to aggregation (26, 35). Unique chromophoric labels were added to all but PLC- γ 1. These individual labels

enabled multicomponent AUC with which we demonstrated that each individual protein migrated as a single peak. Additionally, Gads-SLP76 migrated as a dimer, and when all proteins were subjected to AUC at a 1:1:1:1 ratio, a unique 8.2S peak appeared containing all the components and thus was compatible with the tetramer. An extensive thermodynamic analysis of the energetics of tetramer formation was also performed. Cooperative formation of the tetramer compensated for the entropic penalty associated with complex formation.

A 2.5 Å crystal structure of unphosphorylated, autoinhibited PLC- γ 1 was recently reported (8). The study demonstrated that core enzymatic domains in PLC- γ 1 have structural homology with those of other phospholipase-C isoforms. In this structure of the autoinhibited enzyme, the regulatory domains including N-SH2, C-SH2, and SH3 sit on top of the catalytic domain in a manner predicted to block its access to membrane substrate. Multiple studies have demonstrated that optimal activation of PLC- γ 1 requires phosphorylation of Y783, which is located between the cSH2 and SH3 domains (8, 26). The authors of the structural study postulate that phosphorylation at this site would induce binding of pY783 to the C-SH2 domain and thus disrupt association of this domain with the catalytic core region. This release would lead to a series of conformational changes and ultimately allow access of the catalytic domains to plasma membrane substrates. Similar conclusions about the role of phosphorylation of PLC- γ 1 at this site were discussed in another study in which cryo-electron microscopy was used for structure elucidation (36).

PLC- γ 1 enzymatic activity in T cells is mediated at the inner surface of the plasma membrane. To probe the functional effects of tetramer formation as well as the role of individual components, we generated liposomes or LUVs, the lipid composition of which reflected that of the plasma membrane. The PLC- γ 1 substrate phosphatidyl 4, 5-bisphosphate (PIP₂) was added to enable analysis of enzyme activity in this reconstitution assay. Recruitment of proteins to the LUV was enabled by incorporating nickel-conjugated lipids and adding a hexaHis tag to the protein that was targeted for direct LUV binding. PLC- γ 1 itself, brought to the LUV in this manner, was thus able to cleave PIP₂.

LAT is normally a transmembrane protein whose major function is to recruit various enzymes including PLC- γ 1 to the plasma membrane. The relevant portion of LAT, prepared as a phosphorylated peptide, was also brought to the LUV *via* the Ni-His coupling process. Phosphorylation of LAT at a site corresponding to LAT Y132 was required to efficiently bind PLC- γ 1 through its N-SH2 domain and thus recruit PLC- γ 1 to the LUV leading to enzymatic cleavage of PIP₂ to IP₃. PLC- γ 1 was recruited to the LUVs by phosphorylated LAT as efficiently as it was when PLC- γ 1 was tagged with the hexa His tag. The recruitment of PLC- γ 1 to the LUV by phospho-LAT enhanced PLC- γ 1 activity about twofold. Maximal *in vivo* activation of PLC- γ 1 requires at least one other phosphorylated LAT residue, shown in cellular studies to be one of three residues, Y171, 191 or 226 (14). We chose the Y171 site to simplify phosphopeptide synthesis. When phosphorylated this residue binds Gads *via* its SH2 domain and, as Gads and

SLP-76 exist as a heterodimer in the cell, that pair is recruited to this site. Neither Gads nor the Gads-SLP-76 dimer bound to phosphorylated LAT-induced PLC- γ 1 membrane binding. However, there was a 2.5-fold enhancement of PLC- γ 1 activity in the presence of the Gads-SLP-76 bound to LAT. Under these conditions, it is likely, as demonstrated previously, that there was some low-affinity interaction between SLP-76 and the PLC- γ 1 SH3 domain (25, 30). Thus, the formation of the LAT-Gads-SLP-76-PLC- γ 1 tetramer at the LUV potentially induced conformational changes in PLC- γ 1 resulting in its enhanced activity (Fig. 7). Our results suggest that it would be worth examining whether conformational changes in PLC- γ 1 leading to partial enzymatic activation occur upon its binding to activated growth factor receptor tyrosine kinases.

In many cellular systems, PLC- γ 1 phosphorylation at its Y783 site is a critical activation event. In lymphocytes, this phosphorylation is mediated by a cytosolic protein tyrosine kinase of the Tec family, which in T cells is ITK (31). This kinase also is recruited to the LAT-based signaling complex where it interacts with SLP-76 and other bound proteins to ensure access to the PLC- γ 1 phosphorylation site. As mentioned above, this phosphorylation is likely to induce PLC- γ 1 conformational changes leading to release of enzyme domain inhibition and cleavage of the substrate. Instead of recruiting ITK to the tetrameric complex, as this would require several other additional proteins and phosphorylation events, we phosphorylated PLC- γ 1 protein exogenously *in vitro* with the kinase domain of the FGF receptor. PLC- γ 1 phosphorylated in this fashion did not show enhanced binding to the LUVs. However, it had greater activity than non-phosphorylated PLC- γ 1 when simply added to the LUVs, when it was added with phosphorylated LAT, and especially when added with phosphorylated LAT, Gads, and SLP-76.

What we describe is a highly complex, cooperative mechanism by which PLC- γ 1 activation is regulated in T cells. The process requires formation of an entropically disfavored tetrameric complex at the plasma membrane. As we have shown in this study, forced recruitment of PLC- γ 1 to the membrane has some effect on enzyme activation. However, the additional presence of the adapter proteins LAT, Gads, and SLP-76 within the tetrameric complex with PLC- γ 1 presumably induces additional conformational changes to enhance activation. We have not studied the addition of ITK and other accompanying adapter molecules to the complex, but assume that they too are recruited in a cooperative fashion. ITK in that state would phosphorylate a critical tyrosine in PLC- γ 1, and as postulated in Hajicek *et al.*, (8) this event would result in further conformational changes in the enzyme resulting in its maximal activation.

As noted above, we have taken a reductionist approach in our analysis of this tetrameric complex by using simplified versions of the four proteins of interest in this work. We have removed multiple components of these proteins: regions of SLP-76 and PLC- γ 1 that induce protein aggregation, additional phosphorylation sites on LAT that can enable LAT-mediated oligomerization (32), and the transmembrane and palmitoylation sites on LAT that regulate plasma membrane interaction. Additionally we note that PLC- γ 1 itself can contribute to protein-protein interactions (37). These proteins and protein domains mediate complex interactions that induce phase transitions and form higher-order condensates of great relevance to the signal transduction process (38). We trust that results that focus on both simple and complex systems inform each other and lead ultimately to a greater understanding of TCR-mediated signal transduction.

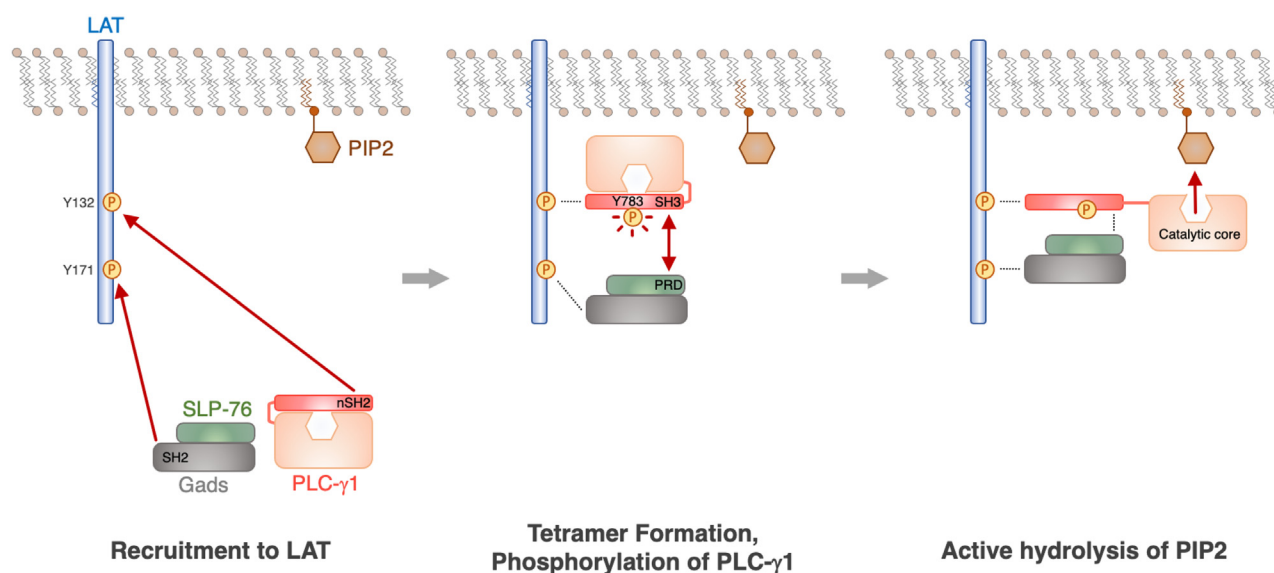


Figure 7. Model of PLC- γ 1 activation in T cell. The nSH2 domain of autoinhibited PLC- γ 1 (red and orange) is recruited to LAT (blue) at phosphoY132. The Gads-SLP-76 dimer (Gads; gray, SLP-76; green) is also recruited to LAT as the Gads SH2 domain binds pY171 (protein recruitment phase). The SH3 domain of PLC- γ 1 transiently interacts with the proline-rich domain (PRD) of SLP-76 forming a tetrameric complex (complex formation phase). Formation of the complex leads to a conformational change of PLC- γ 1 from the autoinhibited form to the activated form, allowing the catalytic core to be exposed and interact with the LUV surface enabling access to its substrate, PIP2 (brown) (conformational change phase). PLC- γ 1, phospholipase-C γ 1.

Cooperative activation of phospholipase C- γ 1 in T cells

Normally PLC- γ 1 recruitment and activation are a very rapid process as demonstrated by intracellular molecular imaging studies (39, 40). It is also likely to be highly regulated and dynamic as protein complex disassembly and protein dephosphorylation of components of the complex have been observed in previous studies (15). Much has been written about the kinetic proofreading model of T cell activation in which a series of sequential and contingent events must occur at the outset of T cell activation in order to ensure completion of the process (41–43). Formation of the LAT-Gads-SLP-76-PLC- γ 1 tetrameric complex, addition of more components to ensure PLC- γ 1 phosphorylation, and formation of the products of PLC- γ 1 activity on its lipid substrate can all be thought of as critical steps in this complex activation scheme.

Experimental procedures

Protein and peptide purification

Proteins were expressed in bacteria or insect cells and were purified using one or more of the following: Ni-affinity column (5 ml HisTrap HP column, GE Healthcare); GST-affinity resin (Glutathione resin, GenScript); ion-exchange column (1 ml Resource Q column, GE Healthcare); HaloTag-affinity resin (HaloTag Protein Purification Resins, Promega); and further purified by size-exclusion chromatography (SEC) using a Superdex-75 or Superdex-200 10/300 GI column (GE Healthcare) with SEC elution buffer (25 mM HEPES, 100 mM NaCl, 2 mM β -ME, pH 7.4). Protein purity was assessed by SDS/PAGE and then by using a ChemiDoc imager (Bio-Rad), and concentration was determined spectrophotometrically (Fig. S1A).

The purification procedures for PLC- γ 1 and Gads were described in Manna *et al.* (25). The cDNA of PLC- γ 1 (1–1219aa, Uniprot ID-P19174) or Gads (1–322aa, Uniprot ID-O89100) was amplified by PCR and cloned into the pHTN HaloTag CMV-neo vector (Promega). These HaloTagged cDNA fragments were inserted into the pDest vector, expressed in Sf9 insect cells, and harvested (Leidos Biomedical Research, Inc). Cells were lysed and disrupted by French press, and cell debris was removed by centrifugation. HaloTag-affinity resin was used for PLC- γ 1 purification and a Ni-affinity column was used for Gads purification. The HaloTag on PLC- γ 1 was cleaved away after purification. The HaloTag on Gads remained to enhance solubility. Both PLC- γ 1 and Halo-Gads were then purified by SEC.

The gene SLP-76 (103–258aa with Y113F and Y173F mutations; Uniprot ID-Q13094) was ordered from Integrated DNA Technologies, Inc with an N-terminus His₆-GST-TEV protease cleavage sequence. The DNA was PCR amplified, and the His₆-GST-TEV-SLP-76 (103–258aa) was inserted into pTE28a vector using BamHI and HindIII sites. The protein was expressed in Rosetta 2(DE3)pLysS (Novagen) cells. The cells were cultured in Luria-Bertani (LB) medium to an OD₆₀₀ of 0.4 to 0.6, and the protein expression was induced with 1 mM isopropyl- β -D1-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Harvested cells were resuspended in GST-binding

buffer (20 mM HEPES, 300 mM NaCl, 5 mM β -mercaptoethanol, and 5% glycerol, pH 7.4) supplemented with EDTA-free protease inhibitor tablet (Thermo Fisher Scientifics), and the cells were disrupted with French press. The cell lysate was incubated with the glutathione resin (GenScript) prewashed with GST-binding buffer, for 2 h at 4 °C. The resin was washed with five column volumes of GST-binding buffer and five column volumes of GST-binding buffer supplemented with 5 mM MgATP to remove DnaK contamination (44). The resin was washed again with five column volumes of GST binding buffer. Next the column-bound His₆-GST-TEV-SLP-76 protein was cleaved on column for 2 h at room temperature with His₆-TEV protease. The liberated SLP-76 protein and the His₆-TEV protease were eluted in the flowthrough after cleavage. The column was washed with GST-binding buffer and combined with the flowthrough from the previous step. EDTA-free protease inhibitor mixture was added to a final 2 \times concentration, and the cleaved protein fragment was purified by Ni-affinity chromatography using GST-binding buffer with 0 to 500 mM imidazole gradient. Taking the advantage of the low affinity of the cleaved product toward the Ni-affinity column despite the lack of His-tag, the target fraction of SLP-76 eluted after fractions of flowthrough. The eluted protein sample was further purified by SEC.

All LAT proteins were purified using bacterial expression systems. The cDNA for LAT (48–233aa; Uniprot ID-O43561–2) was amplified by PCR, and a triple His- and MBP-tagged fragment was inserted into the pET28 vector. The transformed Rosetta 2 (DE3) pLys (Novagen) cells were cultured in LB and treated with 1 mM IPTG for 3 h at 37°C. Cells were harvested and disrupted in lysis buffer (20 mM Hepes, 150 mM NaCl, 10 mM imidazole, 5 mM β -ME, 10% glycerol, 0.01% NP-40, pH 8.0) with a protease inhibitor tablet (Thermo Fisher). The cell supernatant was loaded onto a Ni-affinity column and eluted with lysis buffer containing 500 mM imidazole (pH8.0). The elution was diluted threefold with distilled water and then loaded onto an ion-exchange column pre-equilibrated in ion-exchange chromatography (IEX) binding buffer (20 mM Hepes, 100 mM NaCl, 2 mM β -ME, pH7.4). Multitagged LAT protein (His-MBP-His-LAT-His) was further purified using a NaCl gradient derived from IEX elution buffer containing 600 mM NaCl. The MBP tag and both His tags at the N- and C-termini were removed using TEV protease treatment for 4 h at 30 °C. Cleaved sample was loaded onto the same ion-exchange column again and purified as before the TEV treatment. For nonphosphorylated LAT, the elute was then applied for final SEC. In single-phosphorylated LAT, either tyrosine at 132 or 171 is substituted to phenylalanine.

To phosphorylate tyrosine residues in the LAT peptide, the protein was first concentrated up to >100 μ M with the reaction buffer (20 mM Hepes, 100 mM NaCl, 2 mM DTT, 15 mM ATP, 20 mM MgCl₂ pH7.4) and incubated for 24 h at 30 °C with active GST-ZAP70 (SignalChem). The phosphorylated protein was loaded onto an ion-exchange column, purified using a shallow NaCl gradient, and then was then subjected to

final SEC. Phosphorylation was confirmed by mass spectrometry.

Preparation of LUVs

All lipids were purchased from Avanti Polar Lipids. LUVs were prepared using a Mini extruder kit (Avanti Polar Lipids). The LUVs were comprised of molar ratios of 35% phosphatidylcholine (PC), 22.5% phosphatidylethanolamine (PE), 15% phosphatidylserine (PS), 15% phosphatidylinositol 4,5-bisphosphate (PIP₂), 7.5% cholesterol, and 5% 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl]; DGS-NTA(Ni). The lipids were mixed and then dried under a stream of nitrogen gas. The dried lipid film was hydrated with hydration buffer (either 25 mM Hepes, 100 mM NaCl, pH7.4 for the enzymatic assay or 25 mM Hepes, 20 mM NaCl, 200 mM sucrose, pH7.4 for the lipid binding assay) and then subject to five freeze/thaw cycles. The hydrated lipid suspension was then extruded ten times through a 0.4 μ m pore filter (Avanti Polar Lipids.) The size and shape of LUVs were confirmed by electron microscopy (Fig. S1B).

Lipid-binding assay

LUVs containing sucrose were prepared as above, and lipid binding was determined as described in Jian *et al.* (45, 46). Proteins were incubated with LUVs at 25 °C for 2 min in lipid-binding buffer (25 mM Hepes pH 7.4, 100 mM NaCl, 100 μ M CaCl₂). The sucrose-loaded LUVs were then precipitated by centrifugation at 75,000g for 10 min at 25 °C. The proteins bound to the vesicles were separated on SDS-PAGE and visualized by Stain-Free technology with a ChemiDoc imager (Bio-Rad). The density of the protein bands was quantified by densitometry using Image Lab 6.0.1 (Bio-rad).

Enzymatic assay

The proteins were mixed in reaction buffer (25 mM Hepes pH 7.4, 100 mM NaCl, 100 μ M CaCl₂) and preincubated at 30 °C, and the reaction was started by adding the LUVs in a final volume of 150 μ l. The reaction ran for 5 min and was stopped by adding 350 μ l of water and flash freezing the sample in liquid nitrogen. After thawing the sample on ice, the LUVs and proteins were removed by passing the sample through an Agilent Captiva EMR Lipid 1 ml cartridge one time and then a Macro Spin Column C18 (Harvard Apparatus) three times. The 450 μ l of eluted sample was flash-frozen again and lyophilized to a volume of 150 μ l for HPLC injection.

IP₃ was assayed by HPLC using a MiniQ PC 3.2/3 column (GE Healthcare) as described in Mayr *et al.* (28, 29). Two different buffers [Buffer A (0.2 mM HCl, 72 μ M YCl₃) and Buffer B (0.5 M HCl, 72 μ M YCl₃)] were employed to generate an HCl concentration gradient. The gradient was as follows: 0 to 3.9 min, 5% B; 3.9 to 4.6 min, 5 to 7% B; 4.6 to 10.7 min, 8 to 55% B; 10.7 to 16 min, 55 to 90% B; 16 to 17 min, 90% B; 17 to 19 min, 90 to 5% B; and 19 to 25 min, 5% B. The flow rate was 0.5 ml/min. Before analyzing the sample in each experiment, 1000 to 10000 pmol of Standard IP₃ (Sigma Aldrich)

was firstly injected to make a standard curve (Fig. S2). The elution was mixed in-line with PAR buffer (600 μ M 4-(2-pyridylazo)resorcinol, 1.8 M triethanolamine (pH 9.0 with HCl)). An absorbance at 546 nm from the solution after the postcolumn reaction was measured as the amount of IP₃ and quantified based on the standard curve. The quantification analysis was performed by Open Chrom (LABLIMATE) and then Image J.

In vitro phosphorylation of PLC- γ 1

In total, 2 μ M of PLC- γ 1 was incubated with 2 μ M of a FGFR kinase domain in phosphorylation buffer (20 mM HEPES (pH 7.4), 100 mM NaCl, 20 mM ATP, 20 mM MgCl₂). The FGFR kinase domain was provided from Dr Mohammad's lab at NYU and was purified by three-step purification through Ni-affinity column, ion-exchange column, and size exclusion column (10). In the time-course experiment to verify the maximum phosphorylation, the degree of phosphorylation of PLC- γ 1 at Tyr783 was measured by Western blot using anti-pPLC- γ 1 (#2821, Cell Signaling) and detected with anti-rabbit IgG-HRP (NXA934, Amersham GE)]. The chemiluminescent signal was detected by Chemidoc (Bio-Rad) and analyzed by Image Lab (Bio-Rad).

Statistics

All statistical analyses were performed using Prism9 software (Graph Pad). One-way or Two-way ANOVA analysis with post-hoc tests was applied as indicated. For experiments with differences in variance between groups (Figs. 3, A and B and 5B), data were log-transformed prior to ANOVA analysis and post-hoc tests. Data were fit to one-site binding and one-phase association equations provided in Prism9 where indicated.

Data availability

All data are contained with this article and in the supporting information.

Supporting information—This article contains supporting information.

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Cooperative activation of phospholipase C- γ 1 in T cells

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Abbreviations—The abbreviations used are: LUV, large unilamellar vesicle; PLC- γ 1, phospholipase-C γ 1; PTK, protein tyrosine kinase; RTK, receptor tyrosine kinases; TCR, T cell antigen receptor.

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