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Activated PLC- γ 1 is Catalytically Induced at LAT but Activated PLC- γ 1 is Localized at both LAT- and TCR-Containing Complexes

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Abstract

Phospholipase C- γ 1 (PLC- γ 1) is a key regulator of T cell receptor (TCR)-induced signaling. Activation of the TCR enhances PLC- γ 1 enzymatic function, resulting in calcium influx and the activation of PKC family members and RasGRP. The current model is that phosphorylation of LAT tyrosine 132 facilitates the recruitment of PLC- γ 1, leading to its activation and function at the LAT complex. In this study, we examined the phosphorylation kinetics of LAT and PLC- γ 1 and the cellular localization of activated PLC- γ 1. We observed that commencement of the phosphorylation of LAT tyrosine 132 and PLC- γ 1 tyrosine 783 occurred simultaneously, supporting the current model. However, once begun, PLC- γ 1 activation occurred more rapidly than LAT tyrosine 132. The association of LAT and PLC- γ 1 was more transient than the interaction of LAT and Grb2 and a pool of activated PLC- γ 1 translocated away from LAT to cellular structures containing the TCR. These studies demonstrate that LAT and PLC- γ 1 form transient interactions that catalyze the activation of PLC- γ 1, but that activated PLC- γ 1 resides in both LAT and TCR clusters. Together, this work highlights that our current model is incomplete and the activation and function of PLC- γ 1 in T cells is highly complex.

Keywords

TCR; LAT; PLC- γ 1; Phosphorylation kinetics; Grb2

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1. Introduction

T cells are critical for the adaptive immune response to pathogens but dysfunctional T cells are linked to the initiation and progression of multiple human diseases. The primary stimulatory signal for T cells is the activation of the T cell antigen receptor (TCR). A central regulator of intracellular signals downstream of the TCR is the phospholipase PLC- γ 1 [1, 2]. Upon TCR activation, PLC- γ 1 is phosphorylated on tyrosine 783, leading to its enhanced enzymatic activity [3, 4]. The enzymatic function of PLC- γ 1 is to cleave phosphatidylinositol 4,5 phosphate into inositol 1,4,5 phosphate (IP3) and diacylglycerol (DAG) [1, 2, 5]. IP3 stimulates IP3-sensitive calcium channels in the endoplasmic reticulum, resulting in the influx of calcium into the cytoplasm of the T cell and ultimately, the activation of the calcium sensitive transcription factor NFAT. DAG activates several intracellular signaling proteins including RasGRP and members of the PKC family. PLC- γ 1 is required for T cell development, TCR-induced signaling and the function of both mature T cells and regulatory T cells [6]. In support of its critical role in TCR-mediated signaling, the widely used non-selective activators of T cell function, PMA and ionomycin, mimic the activation and function of PLC- γ 1.

The current model of activation of PLC- γ 1 downstream of the TCR is that receptor stimulation results in the phosphorylation of the adaptor protein linker for activation of T cells or LAT. Phosphorylated LAT tyrosine 132 directly binds to the N-terminal SH2 domain of the phospholipase PLC- γ 1 [7-10]. The recruitment of PLC- γ 1 to the LAT complex requires LAT tyrosines 171 and/or 191 [7, 9, 10] due to weak, SH3 domain-mediated interactions between PLC- γ 1 and SLP-76 and/or multiple SLP-76 interacting proteins, including c-Cbl and Vav [11-13]. Once bound to LAT, PLC- γ 1 is phosphorylated on tyrosine 783 by the tyrosine kinase Itk, which associates with the LAT complex via binding to SLP-76 [14]. Once phosphorylated, PLC- γ 1 continues to associate with the LAT complex and the enzymatic activation of PLC- γ 1 is enhanced. This results in the LAT-based activation of PKC and RasGRP, calcium influx and stimulation of NFAT DNA binding activity [1, 2]. At the same time, LAT tyrosines 171, 191 and 226 directly interact with the central SH2 domains of the related adaptor proteins, Grb2, Gads and Grap [7, 8, 10, 15]. LAT tyrosine 132 is clearly important for T cell differentiation and function. Mice with phenylalanine mutations of LAT tyrosine 132 have defects in TCR-induced signaling and calcium influx, but the mice develop a progressive CD4⁺ T cell lymphoproliferative disease [16, 17]. The lymphoproliferative disease is due to decreased production of T regulatory T cells [18, 19] and a compensatory increase in Erk activation in CD4⁺ T cells [20].

Although previous studies have identified a potential mechanism for the TCR-induced phosphorylation of PLC- γ 1, there is evidence that the regulation of the induction of PLC- γ 1 at LAT may be more complex than initially thought. LAT tyrosine 132 has significantly delayed TCR-induced phosphorylation kinetics compared to LAT tyrosine 191 in both Jurkat E6.1 T cells and isolated peripheral blood T cells [21]. This suggests that the phosphorylation of PLC- γ 1 binding site on LAT may be differentially regulated compared to other sites. However, these experiments have not addressed the effects of slower LAT tyrosine 132 phosphorylation kinetics on the activation of PLC- γ 1. In this study, we have used a combination of quantitative techniques to address this question in more depth. We observed that the initial activation of PLC- γ 1 is linked to the phosphorylation of LAT tyrosine 132, but that the phosphorylation of PLC- γ 1 subsequently occurs much faster than this site on LAT. Additionally, activated PLC- γ 1 forms a transient interaction with LAT and a substantial portion of the cellular pool of phosphorylated PLC- γ 1 translocates away from LAT to the TCR complex. These data show that the current model that the TCR-mediated activation and enzymatic function of PLC- γ 1 occurs entirely while bound to LAT tyrosine

132 is incorrect. Instead, the regulation of the function of PLC- γ 1 upon TCR stimulation is more complex than the current model.

2. Materials and Methods

2.1 Materials

RPMI 1640, L-glutamine, and penicillin/streptomycin were purchased from GIBCO/Invitrogen and the Fetal Bovine Serum (FBS) was obtained from HyClone, Atlas and Atlanta Biologicals. Complete protease inhibitor tablets were acquired from Roche. The Criterion Precast Polyacrylamide gels were obtained from Bio-Rad and the polyvinylidene difluoride, recombinant ZAP-70 and phospho-tyrosine 4G10 antibody were purchased from Millipore. The phospho-LAT tyrosine 132 antibody, phospho-LAT tyrosine 191 antibody and phospho-PLC 1 tyrosine 783 antibody were acquired from BioSource International. The phospho-PLC- γ 1 tyrosine 783 antibody and pan PLC- γ antibody were obtained from Cell Signaling Technology. The phospho-LAT-226 and pan PLC- γ was purchased from BD Biosciences Pharmingen. The pan PLC- γ antibody, pan Grb2 antibody, pan GFP and protein A/G Plus agarose were purchased from Santa Cruz Biotechnology. The GAPDH antibody was purchased from Biodesign International and the Actin antibody was acquired from Chemicon International. The pan LAT antibody was purchased from BioLegend. The Cell Line Nucleofector Kit V was acquired from Amaxa Biosystems.

2.2 Cell transfections

Jurkat E6.1 were grown to a concentration of $3-5 \times 10^5$ cells/ml in complete RPMI (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin). Before transfection, the T cell lines were washed with RPMI 1640 without supplements and resuspended to a concentration of 1×10^8 cells/ml. Plasmids encoding YFP alone, a YFP fusion protein with full length human Grb2 (Lawrence Samelson, NCI/NIH) or a YFP fusion protein with bovine PLC- γ 1 (amino acids 545-855) produced by PCR directed cloning were transfected into Jurkat E6.1 T cells. The cells were transfected using the Nucleofector Kit V from Amaxa Biosystems using the manufacturer provided protocol.

2.3 Cell stimulation

Jurkat E6.1 were grown to a concentration of $3-5 \times 10^5$ cells/ml in complete RPMI. Before treatment, the T cell lines were washed with RPMI 1640 without supplements and resuspended to a concentration of 5×10^7 cells/ml. The cells were then treated with various concentrations of anti-TCR antibody (OKT3) for time points described in the figures. The cells were then lysed using an excess of 2X sample buffer (20mM Tris (pH8.0), 2 mM EDTA, 2 mM Na_3VO_4 , 20 mM DTT, 2% SDS, and 20% glycerol). The samples were heated at 95°C for 4 minutes and sonicated to reduce the viscosity of the solution.

For plate bound stimulations, the 12-well plates were exposed to 1 μ g/mL anti-TCR antibody (OKT3) in PBS and were incubated for 4 hours at 37°C. The plates were washed three times with PBS and then used as described below. The Jurkat E6.1 T cells were grown to a concentration of $3-5 \times 10^5$ cells/ml in complete RPMI. The cells were washed with RPMI 1640 without supplements and resuspended to a concentration of 1×10^7 cells/ml. The cells were plated onto anti-CD3 antibody-treated wells for various time points. The cells were lysed using an excess of 2X sample buffer. The samples were heated at 95°C for 4 minutes and sonicated to reduce the viscosity of the solution.

2.4 Immunoprecipitation

The Jurkat E6.1 T cells were grown as described for the cell stimulation. Before treatment, the cells were washed with RPMI 1640 without supplements and resuspended to a

concentration of 5×10^7 cells/ml. The cells were then treated with anti-CD3 antibody for various time points and lysed using immunoprecipitation buffer (25 mM Tris (pH8.0), 150 mM NaCl, 1% Brij-97, 0.5% n-Octyl- γ -D-glucopyranoside, 5 mM EDTA, 1 mM Na_3VO_4 , complete protease inhibitor tablets). The cellular lysates were incubated on ice for 20 minutes and then centrifuged. The supernatants were rotated for 30 minutes with protein A/G plus agarose and then incubated at with protein A/G plus agarose and the respective immunoprecipitation antibody for 3 hours at 4°C. The beads were pelleted by centrifugation and then quickly washed 3-4 times with washing buffer (25 mM Tris (pH8.0), 150 mM NaCl, 1% Brij-97, 0.5% Octyl Glucoside, 5 mM EDTA). The immunoprecipitated proteins were eluted with 2X sample buffer and heated at 95°C for 4 minutes. Samples before immunoprecipitating antibodies were added and after the immunoprecipitating antibodies were removed were saved to determine the relative amount of cellular proteins that were isolated.

2.5 Immunoblotting

Cellular lysates and immunoprecipitates were separated by PAGE using 4-15% Criterion Precast polyacrylamide gels. The separated proteins were then transferred to polyvinylidene difluoride and the membrane was blocked for 1 hour at room temperature using TBST (10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% Tween-20) with 1% BSA or 5% milk. The membranes were incubated for 1 hour at room temperature or overnight at 4°C with the primary antibody diluted in TBST with 1% BSA or 5% milk, followed by 30 minutes of incubation at room temperature with the respective secondary antibody diluted in TBST with 1% BSA or 5% milk. The blots were then visualized by chemiluminescence, using FUJIFILM Image Reader LAS-3000 Lite. The anti-phospho-LAT 191, anti-phospho-LAT tyrosine 132, anti-phospho-LAT 226, anti-phospho-PLC- γ 1 tyrosine 783, anti-actin, anti-Grb2, anti-PLC- γ 1 and anti-GAPDH antibodies were specific, giving a single major band.

2.6 Analysis of immunoblotting

The intensity of the immunoblotting bands was determined using the gel plotting macro of ImageJ. The immunoblotting exposures were selected such that the maximal response from each individual experiment had approximately equal intensity. To decrease possible variations during the gel electrophoresis and immunoblotting, GAPDH or actin were used as a loading control for all the experiments, except for the immunoprecipitation studies. The measured intensity of each experimental timepoint was normalized to the level of actin or GAPDH expression using the formula (1) below:

$$\text{Normalized Intensity (NI)} = \frac{\text{Raw Intensity for Timepoint}}{\left(\frac{\text{Actin Raw Intensity for Timepoint}}{\text{Actin Raw Intensity of Weakest Timepoint}} \right)} \quad (1)$$

Upon normalization, the percent of control activation was calculated using formula (2) below:

$$\% \text{ of Max Activation} = \left(\frac{(\text{NI of Timepoint} - \text{NI of 0 Second Timepoint})}{(\text{NI of Timepoint with Max Activation} - \text{NI of 0 Second Timepoint})} \right) \times 100\% \quad (2)$$

The values for percent of maximal activation for each time point from three separate experiments were then averaged and the mean $\pm$ SEM was plotted using Graphpad Prism using the Boltzmann sigmoidal equation. The time of 50% activation and the 95% confidence interval for these values were derived from the Boltzmann equation fit of the data.

The normalization of the immunoprecipitation data was calculated using equation (3).

$$\% \text{ of Max Grb2 activation} = \left(\frac{(\text{Intensity of Timepoint})}{(\text{Intensity of Grb2 Timepoint with Max Activation})} \right) \times 100\% \quad (3)$$

The values for percent of maximal Grb2 activation for each time point from three separate experiments were then averaged and the mean $\pm$ SEM was plotted using Graphpad Prism using the Boltzmann sigmoidal equation.

The relative expression of PLC- γ 1 and Grb2 in Jurkat E6.1 T cells was calculated using equation (4)

$$\text{Ratio of PLC}\gamma\text{1:Grb2} = \left(\frac{\left(\frac{\text{Endo}_{\text{Grb2}}}{\text{YFP}_{\text{Grb2}}} \right) \times \text{YFP}_{\text{Grb2}}}{\left(\frac{\text{Endo}_{\text{PLC}\gamma\text{1}}}{\text{YFP}_{\text{PLC}\gamma\text{1}}} \right) \times \text{YFP}_{\text{PLC}\gamma\text{1}}} \right) \quad (4)$$

Where Endo Grb2 is density of the endogenous Grb2 band from the pan Grb2 immunoblot, YFP Grb2 is density of the YFP-Grb2 fusion protein band from the pan Grb2 immunoblot, Endo PLC- γ 1 is density of the endogenous PLC- γ 1 band from the pan PLC- γ 1 immunoblot, YFP PLC- γ 1 is density of the YFP-PLC- γ 1 fusion protein band from the pan PLC- γ 1 immunoblot, YFP Grb2 is the density of the YFP-Grb2 fusion protein band from the pan YFP immunoblot and YFP PLC- γ 1 is density of the YFP-PLC- γ 1 fusion protein band from the pan YFP immunoblot.

2.7 Imaging of the cellular localization of signaling molecules

Imaging was performed using a Leica AM TIRF MC imaging system as previously described (Cite). Briefly, Jurkat E6.1 T cells were activated for 5 minutes on glass chamber slides coated with 10 μ g/ml of anti-TCR antibody (OKT3). Cells were fixed with 3% paraformaldehyde and permeabilized with 0.25% Triton X-100. After blocking, cells were antibody stained, followed by incubation with secondary antibodies. Images were acquired using Leica AF software and processed using Fiji software package. Co-localization was analyzed using the Coloc 2 plug-in for the Fiji software package. To assess the percentage of co-localization, 30 cells were randomly selected from each of the two independent experiments and the Pearson's correlation coefficient for each cell was determined. Statistical significance was determined using Student t tests in Graphpad Prism.

3. Results

3.1 The phosphorylation of PLC- γ 1 tyrosine 783 is rapid compared to LAT tyrosine 132 in TCR-stimulated T cells

Upon phosphorylation, LAT tyrosine 132 binds to PLC- γ 1, leading to the phosphorylation and activation of PLC- γ 1 [2]. A previous study has shown that the phosphorylation kinetics of LAT tyrosine 132 is slower than LAT tyrosine 191 in stimulated Jurkat E6.1 T cells and isolated peripheral blood T cells [22]. However, this study did not thoroughly examine the effects of the delayed phosphorylation of LAT tyrosine 132 on the activation of PLC- γ 1. In order to address this question, we activated Jurkat E6.1 T cells with soluble or plate-bound stimulatory anti-TCR antibody and compared the phosphorylation kinetics of LAT tyrosines 132 and 191 to PLC- γ 1 tyrosine 783 using phospho-specific antibodies. To evaluate the kinetics of phosphorylation of the different tyrosines, the percentage of maximal activation for each time point was determined after normalizing to the expression of a control protein.

The rigorous data normalization allows the comparison of the phosphorylation kinetics of different tyrosines on both the same protein and other proteins [21].

As expected, we observed that LAT tyrosine 132 had substantially delayed phosphorylation compared to LAT tyrosine 191 in T cells stimulated with high doses of soluble anti-TCR (Figure 1A and 1B), low doses of soluble anti-TCR (Figure 2A and 2B) or low doses of plate-bound anti-TCR (Figure 2C and 2D). This strongly suggests that the delayed phosphorylation of LAT tyrosine 132 is not an artifact of high dose stimulation of the TCR but instead is an intrinsic feature of LAT activation. Using the same samples, the phosphorylation kinetics of PLC- γ 1 tyrosine 783, a site previously shown to be required for PLC- γ 1 activation in human T cells [3], was examined. The initiation time of PLC- γ 1 tyrosine 783 phosphorylation closely matched LAT tyrosine 132 in T cells activated with various doses of soluble and plate-bound anti-TCR (Figure 1 and Figure 2). The initial phosphorylation of both sites was substantially slower than the start of LAT tyrosine 191 phosphorylation (Figure 1 and Figure 2). However, upon commencement, the phosphorylation of PLC- γ 1 tyrosine 783 was much more rapid than LAT tyrosine 132 and in the later times of activation it more resembled LAT tyrosine 191 (Figure 1 and Figure 2). This observation is highlighted by the fact that the time of 50% maximal stimulation of PLC- γ 1 tyrosine 783 was not significantly different that LAT tyrosine 191 in all three methods of stimulation, while the time of 50% activation of both of these sites was significantly different that LAT tyrosine 132 (Figure 3).

In order to characterize the de-phosphorylation kinetics of LAT and PLC- γ 1 in human T cells, Jurkat E6.1 T cells were activated for up to 30 minutes using a high dose of anti-TCR antibody. By 5-10 minutes after induction, LAT tyrosines 132 and 191 and PLC- γ 1 tyrosine 783 had visibly decreased levels of phosphorylation compared to the peak of phosphorylation at approximately 2 minutes (Figure 1A and 1C). The kinetics of de-phosphorylation of LAT tyrosines 132 and 191 were similar, with a decrease in the level of phosphorylation with near complete de-phosphorylation by 15-20 minutes (Figure 1A and 1C). These data indicate that, unlike the initial phosphorylation kinetics, there appears to be no differences in the de-phosphorylation kinetics of LAT tyrosine 132 compared to LAT tyrosine 191. In contrast, the de-phosphorylation kinetics of PLC- γ 1 tyrosine 783 was much slower than LAT tyrosines 132 and 191 (Figure 1A and 1C). PLC- γ 1 tyrosine 783 had substantial phosphorylation at 30 minutes suggesting that the de-phosphorylation of PLC- γ 1 tyrosine 783 is partially decoupled from the de-phosphorylation of LAT. Together, these data show that the phosphorylation of LAT tyrosine 132 appears to control the initiation of PLC- γ 1 phosphorylation. However, upon commencement, the phosphorylation kinetics of LAT tyrosine 132 and PLC- γ 1 tyrosine 783 rapidly diverge. Additionally, the timing of de-phosphorylation of LAT tyrosines 132 and 191 is similar but diverges from the kinetics of PLC- γ 1 tyrosine 783 de-phosphorylation.

3.2 PLC- γ 1 has a lower LAT-binding stoichiometry than Grb2

The experiments described above show that the phosphorylation kinetics of LAT tyrosine 132 and PLC- γ 1 tyrosine 783 diverge once the phosphorylation of LAT tyrosine 132 is initiated. This suggests that the interaction of PLC- γ 1 with LAT may be more complex than previously appreciated. Therefore, we examined the TCR-induced binding of LAT with PLC- γ 1, the ligand for LAT tyrosine 132 was compared to the adaptor protein Grb2, a high affinity ligand for LAT tyrosines 171, 191 and 226. For these experiments, T cells were stimulated for various times with soluble anti-TCR antibody and Grb2 and PLC- γ 1 were immunoprecipitated with specific antibodies. The amount of phosphorylated LAT in the PLC- γ 1 and Grb2 immunoprecipitates was assessed by immunoblotting with anti-phosphotyrosine antibody.

As seen in Figure 4, the interaction of PLC- γ 1 with phosphorylated LAT was visible after 30 seconds of stimulation with the maximal association of PLC- γ 1 with phosphorylated LAT occurring by 60 seconds. Although the phosphorylation levels of LAT tyrosine 132 (Figure 1) and PLC- γ 1 (Figure 1 and Figure 2) increased between 60 and 120 seconds, there was little change in the amount of phosphorylated LAT that associates with PLC- γ 1 during this time (Figure 3). In contrast, there was a visible interaction between Grb2 and phosphorylated LAT beginning at 10 seconds with maximal levels of binding occurring by 60 seconds (Figure 4). Interestingly, the amount of phosphorylated LAT bound to Grb2 substantially increased after 30 seconds whereas there was little increase in the interaction of LAT with PLC- γ 1 during this timeframe (Figure 4). By 120 seconds, at least 10-fold more phosphorylated LAT was bound to Grb2 than PLC- γ 1 (Figure 4). Similar results were observed when using a sub-maximal dose of anti-TCR antibody (data not shown).

There are several experimental artifacts that could result in the clear differences in the levels of LAT binding between PLC- γ 1 and Grb2. First, the difference in binding stoichiometry may be due to the intrinsic affinity of Grb2 and PLC- γ 1 for phosphorylated LAT tyrosines. However, previous biophysical studies have shown that these proteins have similar binding affinity for their individual binding sites on LAT [23], suggesting that variations in the intrinsic affinity of these proteins for their binding sites on LAT do not drive the observed differences. Second, disparity in the relative cellular expression of these proteins may account for the observed differences in LAT-binding stoichiometry. To test this possibility, Jurkat E6.1 T cells were transfected with YFP fusion proteins of PLC- γ 1 and Grb2. The expression of these fusion proteins was then compared to endogenous PLC- γ 1 and Grb2 and each other to determine the relative cellular amount of these proteins. Using this method, PLC- γ 1 and Grb2 have a 1:1 molar ratio in Jurkat E6.1 T cells (Figure 5A). Thus, the increased levels of binding between Grb2 and LAT do not appear to be due to differences in the expression levels of PLC- γ 1 and Grb2 in T cells. Finally, the antibodies used for these studies could more efficiently isolate Grb2 than PLC- γ 1. This does not appear to be the case since approximately 75% of the total cellular PLC- γ 1 was immunoprecipitated, whereas approximately 45% of the total cellular Grb2 was pulled down (Figure 5B). This suggests that disparity in the binding of LAT to Grb2 compared to PLC- γ 1 could even be larger than 10-fold since more cellular PLC- γ 1 was immunoprecipitated than Grb2.

3.3 Phosphorylated PLC- γ 1 localizes to both LAT- and TCR-containing clusters

The observations that 1) the phosphorylation kinetics of PLC- γ 1 is different from LAT tyrosine 132, 2) the de-phosphorylation PLC- γ 1 is partially decoupled from LAT and 3) PLC- γ 1 has less stable binding to LAT compared to Grb2 suggests that PLC- γ 1 has a transient and highly dynamic interaction with LAT. Previous microscopy studies have shown that PLC- γ 1 is localized with LAT-containing clusters [12], but no studies to date have examined the cellular localization of PLC- γ 1 upon its phosphorylation and activation. Because of this, the recruitment of tyrosine 783 phosphorylated PLC- γ 1 to TCR- or LAT-mediated clusters was assessed using confocal microscopy. Initially, SLP-76 deficient Jurkat J14 T cells that stably express SLP-76-YFP [24] or E6.1 T cell that stably express ZAP-70-YFP [25] were stimulated with anti-CD3 antibody-coated glass coverslips. The cellular localization of phosphorylated PLC- γ 1 tyrosine 783 was then assessed by immunofluorescence using a phosphospecific antibody. This pattern was compared to the localization of functional LAT-mediated microclusters containing SLP-76-YFP or TCR-induced clusters containing ZAP-70-YFP. Unexpectedly, PLC- γ 1 phosphorylated on tyrosine 783 strongly colocalized with TCR/ZAP-70-YFP-containing clusters but had substantially less overlap with SLP-76/LAT containing clusters (Supplemental Figure 1).

Although these initial studies suggested that activated PLC- γ 1 preferentially interacted with TCR-containing clusters, the fact that SLP-76 or ZAP-70 were overexpressed and that

different variants of the Jurkat T cell line were used could have introduced unforeseen artifacts. To confirm these studies, we examined the colocalization of activated PLC- γ 1 with TCR- or LAT-containing clusters using total internal reflection fluorescence (TIRF) microscopy [26]. This technique allows for the excitation of fluorophores within 150 nm of the plasma membrane, which reduces background noise from the body of the cell and allows for examination of the formation of LAT and TCR clusters at the plasma membrane. For these studies, Jurkat E6.1 T cells were stimulated with anti-CD3 antibody-coated glass coverslips [27] and the cellular localization of various proteins were examined by immunofluorescence using antibodies specific for each protein. We examined these events after 5 minutes of TCR stimulation because the phosphorylation of both LAT and PLC- γ 1 were rapidly changing at this time point. The Pearson's correlation coefficient for a pair of proteins in an individual cell was calculated using NIH ImageJ, allowing for the comparison of co-localization of two proteins in multiple cells. Initially, the ability of TIRF microscopy to discern LAT clusters vs. TCR clusters was examined. LAT clusters, as assessed using an anti-LAT antibody, had little visible overlap with TCR containing clusters (Figure 6A), whereas the TCR markedly co-localized with its principal ligand ZAP-70 (Figure 6B). There was a significantly higher average Pearson coefficient for the co-localization of the TCR with ZAP-70 compared to the overlap between the TCR and LAT (Figure 6E). These data show that distinct TCR and LAT clusters can be identified in the membrane of T cells using TIRF microscopy.

The localization of phosphorylated PLC- γ 1 tyrosine 783 was then assessed by immunofluorescence and compared to the localization of LAT or TCR clusters. Phosphorylated PLC- γ 1 had substantial co-localization with both LAT (Figure 6C) and TCR (Figure 6D) microclusters. However, a substantial portion of phosphorylated PLC- γ 1 did not co-localize with either TCR or LAT individually suggesting strongly that activated PLC- γ 1 resides in multiple cellular pools. The Pearson coefficient for the co-localization of phosphorylated PLC- γ 1 with LAT and phosphorylated PLC- γ 1 with the TCR were similar to the co-localization of the TCR and ZAP-70, but significantly different than the overlap of the TCR and LAT (Figure 6E). These data contradict the current model that phosphorylated PLC- γ 1 exclusively resides at the LAT complex but shows instead that activated PLC- γ 1 can be found at both LAT- or TCR-containing microclusters.

4. Discussion

In this study, we found that LAT tyrosine 132 had delayed phosphorylation kinetics compared to LAT tyrosine 191 in T cells stimulated with varying doses of soluble and plate-bound anti-TCR antibody. This strongly suggests that the delay in LAT tyrosine 132 phosphorylation is an intrinsic feature of the *in vivo* activation of LAT. In conjunction with previous work [21], our study shows that the phosphorylation of LAT tyrosine 132 is differentially regulated compared to other LAT tyrosines. This led us to address the question of what is the effect of the slower phosphorylation kinetics of LAT tyrosine 132 on the activation of PLC- γ 1. The initial phosphorylation of LAT tyrosine 132 and PLC- γ 1 tyrosine 783 occurred simultaneously in stimulated T cells but these events are delayed compared to the Grb2 binding site of LAT tyrosine 191. This observation is supported by the elegant work of Huse and coworkers, who used a photoactivatable peptide ligand to precisely control the activation of the TCR [28]. In this study, the authors observed that Grb2-containing clusters formed first, followed later by calcium influx and DAG production, both products of PLC- γ 1 activation [28]. However, we observed that after the initiation phase the phosphorylation kinetics of LAT tyrosine 132 and PLC- γ 1 tyrosine 783 rapidly diverge, with the later phases of PLC- γ 1 phosphorylation having similar kinetics to LAT tyrosine 191. This shows that PLC- γ 1 requires the phosphorylation of LAT tyrosine 132 for activation but upon commencement, the phosphorylation of PLC- γ 1 occurs rapidly. This

observation indicates that LAT tyrosine 132 functions as a catalyst for the activation of PLC- γ 1, where phosphorylation is followed by disassociation to allow for the interaction/activation with another PLC- γ 1 molecule (Figure 7)

In support of this model, we also observed that the interaction of PLC- γ 1 with LAT is transient. In contrast to the highly stable Grb2/LAT complex, the interaction of PLC- γ 1 with LAT was less stable and occurred slower than the Grb2 association. The ability of PLC- γ 1 to transiently interact with LAT could be linked to its unique association with the LAT complex. The recruitment of PLC- γ 1 to the LAT complex requires a SH3 domain-mediated interaction between PLC- γ 1 and SLP-76 and/or multiple SLP-76 interacting proteins, including c-Cbl and Vav [11-13]. Additionally, the expression of PLC- γ 1 is not required for the stability of LAT-mediated microclusters [24]. This suggests that the formation of a PLC- γ 1/LAT complex requires a high affinity SH2 domain-mediated interaction of PLC- γ 1 with LAT and secondary association that requires SLP-76-mediated complex. Interestingly, a recent study has shown that phosphorylation of PLC- γ 1 tyrosine 783 results in the binding of the C-terminal SH2 domain of PLC- γ 1 to this site. This appears to weaken the affinity of the N-terminal SH2 domain for its phosphorylated ligands [29]. This suggests a model where PLC- γ 1 is recruited to LAT via phosphorylated LAT tyrosine 132 and a stabilizing SH3-domain-mediated interaction. Once at LAT PLC- γ 1 is subsequently phosphorylated on tyrosine 783, which reduces the ability of the N-terminal SH2 domain to interact with LAT tyrosine 132 (Figure 7).

Finally, we observed that a portion of PLC- γ 1 phosphorylated on tyrosine 783 is not found at LAT-containing clusters but instead is located at TCR-containing clusters. The phosphorylated PLC- γ 1 found at LAT is partially composed of recently phosphorylated PLC- γ 1 yet to disassociate from LAT, but could also contain a pool of activated PLC- γ 1 that is functional at LAT. Also, we cannot rule out the possibility that activated PLC- γ 1 is found at cellular regions that are not assessed by TIRF microscopy. The translocation of PLC- γ away from sites of activation has also been observed for the EGF receptor, where Matsuda and coworkers found that the PLC- γ 2 was recruited to activated EGF receptor, but later portions of the recruited pool of PLC- γ 2 translocated to a vesicular structure that did not contain the EGF receptor [30]. These data clearly argue against the current model that phosphorylated PLC- γ 1 exclusively localizes to LAT-containing clusters. Instead, they suggest that a substantial proportion of activated PLC- γ 1 is found at cellular structures distinct from LAT microclusters. Whether this pool of PLC- γ 1 is functional and how it regulates T cell function is still an open question. Together, these observations show that commencement LAT tyrosine 132 phosphorylation initiates PLC- γ 1 activation, but that the LAT/PLC- γ 1 interaction is catalytic. PLC- γ 1 binds the LAT complex long enough to be activated but then disassociates and translocates to the TCR, LAT or other cellular structures (Figure 7).

This leaves an important question: what is the physiological role of the delayed phosphorylation of LAT tyrosine 132 and PLC- γ 1 activation? One possibility is that it is key to the ability of the TCR to discriminate between agonist peptide-bound MHC molecules and antagonist-associated or null peptide-associated MHC molecules. This hypothesis flows from the “kinetic proofreading model” [31] that has been expanded upon by several groups [32-37]. This model suggests that small differences in the interaction times between the TCR and various peptide-bound MHC complexes translate into large differences in downstream signaling, due a multi-step activation of intracellular signaling pathways. The data presented here fits well with this hypothesis. Essentially, the processive phosphorylation of LAT acts like a molecular stopwatch by measuring the duration of TCR activation. Only after a sufficient period of TCR activation will LAT become phosphorylated at tyrosine 132, resulting in the rapid, catalytic stimulation of PLC- γ 1. In

support of this idea, mice with phenylalanine mutations of LAT tyrosine 132 have severe autoimmune disease [16, 17], strongly suggesting that the dysregulated phosphorylation of this site can potentially lead to autoimmune diseases in humans. This suggests that the complex regulation of PLC- γ activation may play a critical role in the suppression of inappropriate T cell activation and autoimmunity.

5. Conclusions

The work presented in this manuscript alters the current model for how the activation and function of PLC- γ 1 is regulated upon TCR induction. We observed that 1) the timing of the initiation of LAT Y132 and PLC- γ phosphorylation is similar, 2) PLC- γ 1 is rapidly activated compared to LAT Y132, 3) PLC- γ 1 forms transient dynamic interaction with LAT compared to Grb2, and 4) activated PLC- γ is localized at both TCR and LAT microclusters. These data suggest a new model where LAT tyrosine 132 serves as a catalyst for the phosphorylation of PLC- γ 1, but that activated PLC- γ 1 functions at multiple cellular regions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

PLC	γ 1-Phospholipase C- γ 1
TCR	T cell receptor
IP3	inositol 1,4,5 phosphate
DAG	diacylglycerol

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Highlights

- Timing of the initiation of LAT Y132 and PLC- γ phosphorylation is similar
- The phosphorylation kinetics PLC- γ 1 rapidly diverge from LAT Y132
- PLC- γ 1 forms transient interaction with LAT compared to Grb2
- Activated PLC- γ is localized at both TCR and LAT microclusters

Figure 1.

Phosphorylation kinetics of LAT and PLC- γ 1 in maximally activated T cells. A) Jurkat E6.1 T cells were stimulated with 10 μ g/mL of soluble anti-TCR antibody (OKT3) and the phosphorylation of LAT tyrosines 132 and 191, PLC- γ 1 tyrosine 783, and the expression levels of GAPDH were evaluated by immunoblotting. The percent of maximal activation for B) the initial or C) sustained phosphorylation kinetics of these sites were determined. The mean $\pm$ SEM of three separate experiments for each time point was plotted and fit using Graphpad Prism.

Figure 2.

Phosphorylation kinetics of LAT and PLC- γ 1 in T cells with low stimulatory conditions. Jurkat E6.1 T cells were stimulated with A) 1 μ g/mL of soluble or C) 1 μ g/mL plate bound anti-TCR antibody (OKT3). The phosphorylation of LAT tyrosines 132 and 191, PLC- γ 1 tyrosine 783, and the expression levels of GAPDH were evaluated by immunoblotting. The percent of maximal activation for B) the low dose soluble stimulation or D) low dose plate bound stimulation were determined. The mean $\pm$ SEM of three separate experiments for each time point was plotted and fit using Graphpad Prism.

Figure 3.

Time to 50% activation for activated T cells. The time of 50% activation were calculated from the data presented in Figure 1 and Figure 2 for the A) high dose soluble stimulation, B) low dose soluble stimulation and C) low dose plate bound stimulation. The calculated 50% time of activation $\pm$ 95% confidence interval of three separate experiments were calculated using Graphpad Prism. Statistical significance was determined using Graphpad Prism and shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4.

PLC- γ 1 and Grb2 have different LAT binding stoichiometries. A) Jurkat E6.1 T cells were stimulated with 10 μ g/ml of anti-TCR antibody (OKT3) for various time points and then lysed. PLC- γ 1 and Grb2 were immunoprecipitated with specific antibodies. The association of phosphorylated LAT with PLC- γ 1 and Grb2 was assessed by immunoblotting with anti-phosphotyrosine antibodies. The level of Grb2 and PLC- γ 1 in the immunoprecipitates was assessed by immunoblotting with specific antibodies. B) The percent of LAT binding for the maximal Grb2 timepoint were determined. The mean $\pm$ SEM of three separate experiments for each time point was plotted using Graphpad Prism.

Figure 5.

Relative expression of Grb2 and PLC- γ 1 in T cells and Immunoprecipitation efficiency. A) The expression of endogenous and YFP labeled Grb2 and PLC- γ 1 were assessed by immunoblotting with pan Grb2, pan PLC- γ 1 and pan YFP antibodies. The intensity of each band was then assessed by densitometry and the relative expression of Grb2 and PLC- γ 1 were determined as described in the Materials and Methods. B) The levels of Grb2 and PLC- γ 1 in the lysates before and after immunoprecipitation were determined by immunoblotting with pan Grb2 and pan PLC- γ 1 antibodies. The levels in the post IP lysates were then normalized to the amounts found in the pre IP lysate.

Figure 6.

Cellular localization of phosphorylated PLC- γ 1. Jurkat E6.1 T cells were stimulated for 5 minutes using anti-TCR (OKT3) coated glass coverslips. The localization of A) total LAT and TCR- ζ pY128, B) ZAP-70 pY319 and TCR- ζ pY128, C) PLC- γ 1 pY783 and LAT pY226 and D) PLC- γ 1 pY783 and TCR- ζ pY128 were determined by immunofluorescent TIRF microscopy using specific antibodies. Representative images of each along with the merge and DIC image are shown. E) The Pearson correlation coefficient for the colocalization of the proteins shown in images A)-D) were calculated using Fiji for two experiments with 30 cells measured for each experiment (60 cells total). The Pearson coefficients for the individual cells are shown with the mean represented by a dark line.

Statistical significance was determined using Graphpad Prism and shown as * $p < 0.05$ and *** $p < 0.001$.

Figure 7.

Two step model of TCR activation. A) During the initial activation event, LAT is phosphorylated on tyrosines 171, 191 and 226, allowing for the clustering of LAT via stable interactions with Grb2 complexes. B) Continued TCR activation leads to a second phase of activation where LAT tyrosine 132 is phosphorylated where PLC- γ 1 is catalytically phosphorylated. Activated PLC- γ 1 then translocates to TCR-, LAT-containing structures or other cellular regions.