Recruitment and activation of PLC γ 1 in T cells: a new insight into old domains

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Engagement of the T-cell antigen receptor leads to recruitment of phospholipase C γ 1 (PLC γ 1) to the LAT-nucleated signaling complex and to PLC γ 1 activation in a tyrosine phosphorylation-dependent manner. The mechanism of PLC γ 1 recruitment and the role of PLC γ 1 Src homology (SH) domains in this process remain incompletely understood. Using a combination of biochemical methods and real-time fluorescent imaging, we show here that the N-terminal SH2 domain of PLC γ 1 is necessary but not sufficient for its recruitment. Either the SH3 or C-terminal SH2 domain of PLC γ 1, with the participation of Vav1, c-Cbl and Slp76, are required to stabilize PLC γ 1 recruitment. All three PLC γ 1 SH domains are required for phosphorylation of PLC γ 1 Y783, which is critical for enzyme activation. These novel findings entailed revision of the currently accepted model of PLC γ 1 recruitment and activation in T lymphocytes.

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Introduction

Ligation of the T-cell antigen receptor (TCR) initiates a cascade of molecular events leading to T-cell activation, cellular proliferation and cytokine production. Proximal events that immediately follow TCR engagement include activation of protein kinases and phosphorylation of multiple enzymes and adaptor molecules (Burack *et al*, 2002; Samelson, 2002). The phosphorylation of LAT, a lipid raft-associated adaptor protein, creates docking sites for Src-homology 2 (SH2) domain-containing proteins (Zhang *et al*, 1998). Slp76, another adaptor protein phosphorylated following TCR engagement (Wu and Koretzky, 2004), is recruited to LAT through its constitutive interaction with a small adaptor protein Gads (Liu *et al*, 2001). The LAT–Gads–Slp76 complex creates a platform for the recruitment of multiple signaling

molecules, including phospholipase C γ 1 (PLC γ 1), the adaptors Grb2 and Nck, the Rho-family GTPase exchange factor Vav, the adaptor with ubiquitin-ligase activity c-Cbl, and the Tec-family kinase Itk (Zhang *et al*, 1998, 2000; Bunnell *et al*, 2000; Lewis *et al*, 2001; Tybulewicz *et al*, 2003; Wu and Koretzky, 2004).

Recruitment and activation of PLC γ 1 is a key step in the T-cell activation process triggered by the TCR (Desai *et al*, 1990; Bonvini *et al*, 2003). The activated enzyme hydrolyzes phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-trisphosphate (IP $_3$), which stimulates the release of Ca $^{2+}$ from intracellular stores, and diacylglycerol, which activates protein kinase C and RasGRP-dependent signaling pathways (Katan, 1998; Ebinu *et al*, 2000; Rhee, 2001). The increase in intracellular free Ca $^{2+}$ concentration triggered by IP $_3$ plays a crucial role in the induction of numerous T-cell activation-associated responses (Desai *et al*, 1990).

Two forms of PLC γ have been identified. PLC γ 1 is ubiquitously expressed, whereas PLC γ 2 is limited to certain cell types primarily of hematopoietic lineage. T cells express predominantly the PLC γ 1 form (Katan, 1998). A common structural feature of all PLCs is a split catalytic domain comprised of two conserved subdomains. In PLC γ , these subdomains are separated by a regulatory region, which includes two SH2 domains followed by a single Src-homology 3 (SH3) domain (Katan, 1998; Rhee, 2001). PLC γ 1 contains at least five potential sites of tyrosine phosphorylation. Among them only Y775 and Y783, located between the C-terminal SH2 (SH2C) domain and the SH3 domain, are essential for enzyme activation *in vivo* (Irvin *et al*, 2000; Rhee, 2001; Serrano *et al*, 2005).

Despite the vital function of PLC γ 1 in T cells and the ubiquitously pivotal role of PLCs in signal transduction in general, the mechanism of PLC γ 1 activation following TCR engagement is incompletely understood. Several proteins participating in formation of the TCR-proximal signaling complex have been implicated in the regulation of PLC γ 1 activity. Impaired Ca $^{2+}$ mobilization has been reported in Jurkat cell lines deficient in LAT (Finco *et al*, 1998; Zhang *et al*, 2000), Slp76 (Yablonski *et al*, 1998), Vav1 (Cao *et al*, 2002), as well as in c-Cbl-deficient (Naramura *et al*, 1998) or Vav-deficient thymocytes (Reynolds *et al*, 2002) and Vav-deficient (Costello *et al*, 1999) or Itk-deficient T cells (Liu *et al*, 1998; Schaeffer *et al*, 1999). LAT has been identified as a primary docking site for PLC γ 1 following TCR engagement (Zhang *et al*, 2000; Zhu *et al*, 2003). The recruitment of PLC γ 1 to LAT occurs through specific binding of the N-terminal SH2 (SH2N) domain of PLC γ 1 to the phosphorylated Y132 (pY132) of LAT (Stoica *et al*, 1998; Zhang *et al*, 2000). Mutations of either SH2N of PLC γ 1 or Y132 of LAT abrogated PLC γ 1–LAT association and inhibited PLC γ 1 phosphorylation and activation (Stoica *et al*, 1998; Irvin *et al*, 2000; Zhang *et al*, 2000; Lin and Weiss, 2001; Paz *et al*, 2001; Zhu *et al*, 2003). No other *in vivo* binding partners for either LAT-pY132 or the SH2N domain of PLC γ 1 have been identified in T cells to date.

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On the other hand, the SH2C domain of PLC γ 1 is substantially less selective than the SH2N domain. A chimeric protein consisting of GST fused to the SH2C domain is able to precipitate a range of phosphorylated proteins from activated Jurkat T cells (Stoica *et al*, 1998; Irvin *et al*, 2000; Bonvini *et al*, 2003). However, it is still unclear which of these proteins interact with PLC γ 1 via its SH2C domain *in vivo*. The loss-of-function mutation of the SH2C domain (R694K) did not affect PLC γ 1 interaction with LAT and was reported to have a mild to negligible effect on the PLC γ 1 phosphorylation in Jurkat T cells (Stoica *et al*, 1998; Irvin *et al*, 2000). Yet, expression of SH2C-mutated PLC γ 1 failed to reverse the impaired IL-2 transcriptional activation in Jurkats expressing low levels of PLC γ 1 (Irvin *et al*, 2000) or rescue IP $_3$ production in PLC γ -deficient B cells (DeBell *et al*, 1999). The active conformation of PLC γ 1 involves intramolecular association of the SH2C domain with phosphorylated Y783 (pY783) (Poulin *et al*, 2005). Without this association the enzyme is only moderately active. Thus, it has been suggested that, although required for the PLC γ 1 activity, the SH2C domain is dispensable for PLC γ 1 recruitment and phosphorylation (Irvin *et al*, 2000; Bonvini *et al*, 2003).

The SH3 domain of PLC γ 1 has been shown to interact constitutively with c-Cbl in T cells (Rellahan *et al*, 2003). Several studies have demonstrated that the SH3 domain of PLC γ 1 can also bind other proline-rich domain-containing proteins, including Slp76 (Yablonski *et al*, 2001), Sos (Kim *et al*, 2000), Itk (Perez-Villar and Kanner, 1999) and PIKE (Ye *et al*, 2002). The significance of these interactions and their occurrence *in vivo* following TCR engagement remains unclear. Mutation of the SH3 domain has been reported to have no effect on PLC γ 1 phosphorylation (DeBell *et al*, 1999). Moreover, in Jurkat cells expressing low levels of endogenous PLC γ 1, the SH3-mutated PLC γ 1 was able to recover impaired IL-2 transcriptional activation more efficiently than wild-type (WT) PLC γ 1 (Irvin *et al*, 2000). It has been hypothesized that the SH3 domain is dispensable for PLC γ 1 phosphorylation and may negatively regulate PLC γ 1 activity in T cells (Irvin *et al*, 2000; Bonvini *et al*, 2003; Rellahan *et al*, 2003).

To summarize, the currently accepted model of PLC γ 1 regulation in T cells postulates that the SH2N domain of PLC γ 1 is both necessary and sufficient for its recruitment and phosphorylation following TCR engagement, whereas the SH2C and SH3 domains of PLC γ 1 are dispensable for this purpose. Our current study contradicts both these propositions. Using a combination of biochemical methods and real-time fluorescent imaging, we show here that the SH2N domain of PLC γ 1 is necessary but not sufficient for its recruitment to the LAT-nucleated complex. Furthermore, all three SH domains of PLC γ 1 are required for the efficient phosphorylation and activation of PLC γ 1 in T cells. In addition, the results of this study contribute new information on the role of other signaling proteins in the process of PLC γ 1 activation. Thus, we propose a different model of PLC γ 1 regulation in T cells that can account for both our new findings and previously reported data.

Results

To visualize the recruitment of PLC γ 1 following TCR stimulation, we have expressed full-length PLC γ 1 fused to the monomeric version of yellow fluorescent protein (Zacharias

et al, 2002) (PLCwt-YFP) in WT Jurkat E6 T cells. Our imaging analysis was based on a previously published technique (Bunnell *et al*, 2002). In this protocol, T cells were dropped onto a surface coated with a stimulatory monoclonal antibody binding the TCR. This resulted in TCR clustering and recruitment of multiple signaling molecules to the points of contact with the stimulatory surface (Bunnell *et al*, 2002; Barda-Saad *et al*, 2005). Following the initial engagement, the cells spread out on the planar surface over a period of 2–3 min for the Jurkat cells and up to 30 min for human peripheral blood lymphocytes (Bunnell *et al*, 2002; Barda-Saad *et al*, 2005). Similar clusters of signaling molecules have recently been observed upon T-cell contact with lipid bilayers containing antigen–MHC complexes (Campi *et al*, 2005).

Stimulation of E6 cells stably expressing PLCwt-YFP resulted in formation of PLC γ 1 clusters within seconds of the initial contact. The cluster formation continued during the cell spreading, then gradually diminished with eventual dissipation within 4–6 min after the beginning of the process (Figure 1A; Supplementary video 1). To evaluate whether the clustering of PLCwt-YFP represented its recruitment to signaling complexes, we have created a Jurkat E6 cell line expressing both PLCwt-YFP with either LAT or Slp76 fused with the Cerulean variant of cyan fluorescent protein (Rizzo *et al*, 2004) (LAT-CFP and Slp76-CFP, respectively). It has been shown previously that upon cell stimulation, LAT and Slp76 form clusters that colocalize with the sites of tyrosine phosphorylation, with the TCR and with other molecules recruited to the TCR-proximal signaling complex (Bunnell *et al*, 2002; Barda-Saad *et al*, 2005; Campi *et al*, 2005). As is evident from Figure 1B and C, the PLCwt-YFP clusters colocalize completely with LAT-CFP and Slp76-CFP, indicating that the PLCwt-YFP clustering represents recruitment of PLC γ 1 to the LAT-nucleated signaling complex.

To assess the role of its SH domains in the process of PLC γ 1 recruitment, we have created Jurkat E6 cell lines expressing PLC γ 1-YFP conjugates bearing the previously described loss-of-function point mutations in the SH3 domain (SH3*-YFP), the SH2C domain (SH2C*-YFP) or the SH2N domain (SH2N*-YFP) (Stoica *et al*, 1998; Irvin *et al*, 2000). The mutation of the SH2N domain abrogated the TCR-induced clustering of PLC γ 1 (Figure 1D), consistent with the notion that the SH2N domain serves as a binding site for LAT and, as such, is necessary for PLC γ 1 recruitment to the LAT-nucleated complex.

The mutations of either the SH3 or the SH2C domain did not affect the clustering of PLC γ 1 (Figure 1D). It has been suggested that these domains may negatively regulate PLC γ 1 activity, possibly through binding of c-Cbl, and as such may be responsible for the dissipation of the clusters (Stoica *et al*, 1998; Bonvini *et al*, 2003; Rellahan *et al*, 2003). Therefore, disruption of both these domains might enhance or prolong the clustering of PLC γ 1. We have created an additional PLC γ 1-YFP variant bearing mutations in both the SH3 and the SH2C domains (SH3*SH2C*-YFP). Surprisingly, however, SH3*SH2C*-YFP completely failed to cluster, similar to SH2N*-YFP (Figure 1D). The clusters were undetectable at all stages of cell spreading, suggesting that the inhibition of clustering was absolute and not relative to the duration of TCR stimulation. Similar results were obtained using primary murine CD4⁺ T cells transiently expressing the PLC γ 1-YFP forms mentioned above (Figure 1E).

Figure 1 Visualization of PLC γ 1 recruitment to signaling clusters in Jurkat E6 cells. (A) Jurkat E6 cells expressing PLCwt-YFP were seeded on stimulatory coverslips. Images obtained 0.5, 2 and 5 min into the spreading process are shown. The size bar corresponds to 5 μ m. (B) Jurkat E6 cells expressing LAT-CFP and PLCwt-YFP were seeded on stimulatory coverslips. Images obtained 1.5 min into the spreading process are shown. The size bar corresponds to 5 μ m. (C) Same as (B) for cells expressing Slp76-CFP and PLCwt-YFP. (D) Jurkat E6 cells expressing mutant PLC γ 1-YFP conjugates as indicated were seeded on stimulatory coverslips. Images obtained 1–1.5 min into the spreading process are shown. The size bar corresponds to 5 μ m. (E) Murine primary CD4⁺ T cells expressing PLC γ 1-YFP conjugates as indicated were seeded on stimulatory coverslips and fixed 5 min into the spreading process. The size bar corresponds to 5 μ m.

To confirm the results obtained using the imaging technique, we evaluated the interaction of the various PLC γ 1-YFP forms with LAT by precipitating the YFP conjugates with an anti-GFP serum and blotting with an antibody recognizing the phosphorylated Y132 residue (pY132) of LAT. Because maximal PLC γ 1 phosphorylation is observed by 1 min after stimulation under similar conditions (Houtman *et al*, 2005), 1-min stimulation was chosen for this and other biochemical experiments in this study. In agreement with the results described above, phospho-LAT co-precipitated with the clustering conjugates—PLCwt-YFP, SH3*-YFP and SH2C*-YFP (Figure 2A and B). However, no co-precipitation of LAT was observed with the nonclustering SH2N*-YFP and SH3*SH2C*-YFP (Figure 2A and B). This result further sup-

ports the conclusion that the observed clusters of PLC γ 1-YFP (Figure 1) represent recruitment of PLC γ 1 to LAT and that the concomitant mutation of both the SH3 and SH2C domains block this recruitment. These results indicate that contrary to the currently accepted model, the SH2N domain alone is not sufficient for the PLC γ 1 recruitment to the LAT-nucleated cluster. Although the SH3 and the SH2C domains, taken separately, are dispensable for the PLC γ 1 recruitment, at least one of them is required in combination with the SH2N domain to maintain the association of PLC γ 1 with activated LAT.

It has been reported previously that mutations of either the SH3 or SH2C domains had little or no effect on the general tyrosine phosphorylation of PLC γ 1 (Stoica *et al*, 1998; DeBell

Figure 2 Effect of SH domain mutations on PLC γ 1 recruitment and phosphorylation. (A) Jurkat E6 cells (1×10^7) expressing the indicated PLC γ 1-YFP variants were either stimulated with C305 for 1 min or left unstimulated. The cells were lysed and PLC γ 1-YFP conjugates were precipitated with anti-GFP. The precipitates were resolved on SDS-PAGE and probed with anti-phospho-LAT-pY132 (upper blot). The same membrane was stripped and probed with anti-GFP (lower blot). The data are representative of three independent experiments. (B) Quantitative analysis of blots obtained in experiments represented by (A). The data represent densitometry of LAT-pY132 bands as a percentage of band intensity obtained in cells expressing PLCwt-YFP. Each bar represents an average over four independent experiments. Error bars indicate s.e. (C) Jurkat E6 cells expressing the indicated PLC γ 1-YFP variants were either stimulated with OKT3 for 1 min or left unstimulated and lysed. The lysates were resolved on SDS-PAGE and probed with either anti-phospho-PLC γ 1-pY783 (upper blot) or anti-PLC γ 1 (lower blot). The data are representative of five independent experiments. (D) Quantitative analysis of blots obtained in experiments in (C). The data represent densitometry of PLC γ 1-YFP-pY783 bands as a percentage of endogenous PLC γ 1-pY783 band intensity in corresponding lanes. The results were normalized to account for the relative amounts of PLC γ 1-YFP and endogenous PLC γ 1 in each cell line. Each bar represents an average over five independent experiments. Error bars indicate s.e.

et al, 1999; Irvin *et al*, 2000). Yet, these findings did not correlate well with the functional impact of the mutations on downstream signaling. PLC γ 1 contains several tyrosine residues that can be phosphorylated upon stimulation. However, only two of them, Y783 and the recently discovered Y775, are necessary for enzyme activation (Irvin *et al*, 2000; Rhee, 2001; Serrano *et al*, 2005). Therefore, we have assessed the effect of the SH domain mutations on phosphorylation of these critical tyrosine residues using specific antibodies binding these phosphorylated sites—anti-pY783 (Figure 2C and D) and anti-pY775 (not shown). The ability to distinguish the endogenous PLC γ 1 and the PLC γ 1-YFP conjugates as separate bands on Western blots provided a clear advantage for analyzing these results. Comparison between the level of

phosphorylation of the YFP conjugates with that of the endogenous PLC γ 1 in the same cells allowed more accurate assessment of the effect produced by the mutants and prevented skewed results because of a possible general effect of the mutants on the stimulation. The results clearly demonstrate (Figure 2C and D) that upon stimulation PLCwt-YFP is phosphorylated to the same extent as endogenous PLC γ 1, ruling out the possibility of YFP interfering with phosphorylation. However, the loss-of-function mutation in either of the three SH domains strongly inhibits phosphorylation of Y783. Moreover, despite the fact that SH3*-YFP and SH2C*-YFP are recruited to the LAT-nucleated signaling complex, Y783 phosphorylation in these mutants is inhibited more severely than that in SH2N*-YFP, which is not recruited (Figure 2C and D). Virtually identical phosphorylation pattern was obtained using anti-pY775 (not shown). Note that the mutations did not interfere with the ability of the proteins to act as a substrate for tyrosine kinases, as pervanadate stimulation induced Y783 phosphorylation in all PLC-YFP conjugates at levels comparable with those obtained in endogenous PLC γ 1 (Supplementary Figure S1). These results suggest that both the SH3 and SH2C domains are indispensable for the efficient phosphorylation and activation of PLC γ 1 and that PLC γ 1 recruitment to the signaling complex alone is not sufficient for this purpose.

The deleterious effect of the SH domain mutations on recruitment and activating phosphorylation of PLC γ 1 might be explained by disruption of PLC γ 1 interactions with other signaling proteins normally mediated by these domains. Precipitation of PLCwt-YFP from the stimulated E6 cells reveals four clear bands of tyrosine-phosphorylated proteins co-precipitating with PLC γ 1 (Figure 3A, second lane). The 36–38 kDa band has already been identified as LAT (Figure 2A). The other three bands correspond in sizes to Slp76, Vav1 and c-Cbl, all of which have been implicated in the PLC γ 1 regulation in T cells (Naramura *et al*, 1998; Yablonski *et al*, 1998; Costello *et al*, 1999; Cao *et al*, 2002; Reynolds *et al*, 2002; Rellahan *et al*, 2003). Application of antibodies specific to Slp76, phosphorylated Vav1 (pVav1) or phosphorylated c-Cbl (pc-Cbl) confirmed the identity of these bands (Figure 3B). Mutation of any of the SH domains abrogated co-precipitation of PLC γ 1 with Slp76 (Figure 3B). Consistent with the previously reported constitutive interaction between PLC γ 1 and c-Cbl mediated via the SH3 domain of PLC γ 1 (Rellahan *et al*, 2003), co-precipitation of these two proteins was completely blocked in the PLC γ 1 mutants lacking a functional SH3 domain (Figure 3B and C). Precipitation of Vav1 was substantially inhibited with either SH3*-YFP or SH2C*-YFP and was undetectable with either SH2N*-YFP or SH3*SH2C*-YFP (Figure 3B and D). These results suggest that the SH domains of PLC γ 1 mediate its interactions with Slp76, Vav1 and c-Cbl. The loss of these interactions might account for the impaired recruitment and phosphorylation of the SH domain mutants.

Co-precipitation does not necessarily indicate direct interaction between the protein molecules. Therefore, we have applied the fluorescence resonance energy transfer (FRET) technique to address this issue. As FRET can occur between fluorescent species separated no further than ~ 10 nm, detection of FRET between two protein molecules strongly suggests their direct interaction. Unfortunately, we have not been able to detect FRET between full-length PLC γ 1-YFP and either

Figure 3 Co-precipitation of PLC γ 1-YFP variants with various signaling proteins. (A) Jurkat E6 cells (1×10^7) expressing PLCwt-YFP were either stimulated for 1 min with C305 or left unstimulated and lysed. PLCwt-YFP was precipitated with anti-GFP. The precipitates (three left lanes) and a sample from stimulated whole-cell lysate WCL (right lane) were resolved on SDS-PAGE and probed with anti-phosphotyrosine 4G10 antibody. NC lane represents a control experiment performed without cell lysate. All lanes were from the same gel. The three left lanes were obtained at equal exposure times. WCL lane was obtained at a shorter exposure time. HC, heavy chain of Ig; LC, light chain of Ig. The data are representative of three independent experiments. (B) Jurkat E6 cells (1×10^7) expressing the indicated PLC γ 1-YFP variants were either stimulated with C305 for 1 min or left unstimulated. The cells were lysed and PLC γ 1-YFP conjugates were precipitated with anti-GFP. The precipitates were resolved on SDS-PAGE and probed with anti-phospho-c-Cbl-pY774 (top blot). The same membrane was stripped and probed with anti-GFP (second from top blot). In a different experiment, the precipitates were resolved on SDS-PAGE and probed consecutively with anti-phospho-Vav1-pY160, anti-Slp76 and anti-GFP (lower blots from top to bottom). NS, nonspecific band. The data are representative of at least four independent experiments. (C) The data represent densitometry of phospho-c-Cbl bands from experiments in (B) as a percentage of band intensity obtained in cells expressing PLCwt-YFP. Each bar represents an average of five independent experiments. Error bars indicate s.e. (D) Same as (C) for phospho-Vav1 bands. Each bar represents an average of four independent experiments.

LAT-CFP or Slp76-CFP, although direct interaction between PLC γ 1 and LAT is well documented (Stoica *et al*, 1998; Zhang *et al*, 2000). This may be due to the size and structure of PLC γ 1, which places YFP at a greater distance from the interacting proteins than is allowed for FRET. Therefore, we have created a truncated version of PLC γ 1-YFP (PLCs-YFP) by removing the C-terminal part of the PLC γ 1 catalytic domain, thereby placing YFP closer to the SH domains of PLC γ 1.

Figure 4 demonstrates that PLCs-YFP is recruited to the signaling clusters similarly to PLCwt-YFP. Furthermore, a FRET signal has been detected between PLCs-YFP and either

LAT-CFP, c-Cbl-CFP, Vav1-CFP or Slp76-CFP (Figure 4A–D) suggesting direct interaction between these proteins. Note that FRET between PLCs-YFP and c-Cbl-CFP, in contrast to the other proteins, is not limited to the clusters, which is likely due to the constitutive nature of the PLC γ 1 and c-Cbl interaction. Interestingly, the C-terminus labeled Slp76 (Slp76-CFP) exhibited substantially lower FRET efficiency with PLCs-YFP than its N-terminus labeled counterpart (CFP-Slp76) (Figure 4D and E). This sensitivity of the observed FRET efficiency to the structural variations within the protein complex suggests that the obtained FRET signal reflects actual protein interactions and is not merely due to high protein density within the cluster. Predictably, no significant FRET was observed between LAT-CFP and PLCs-YFP mutated at the SH2N domain of PLC γ 1 (Figure 4F).

To further evaluate the role of Slp76 and Vav1 in PLC γ 1 recruitment, we expressed PLC γ 1-YFP conjugates in cells deficient in either of these proteins. Expression of PLCwt-YFP in the Slp76-deficient Jurkat J14 cells revealed strong inhibition of PLC γ 1 recruitment to the signaling clusters (Figure 5A). This result was unexpected, as normal co-precipitation of PLC γ 1 with LAT in the J14 cells has been previously shown (Yablonski *et al*, 1998). In the course of an independent study, we have discovered that the expression of c-Cbl in the J14 cells used in our experiment is substantially reduced compared to those observed in E6 cells. To assess whether the reduced level of c-Cbl can account for the impaired PLC γ 1 recruitment, we have created a J14 cell line expressing c-Cbl-CFP (J14-Cbl) at a level similar to the level of c-Cbl in E6 cells (Supplementary Figure S2). Reconstitution of J14 cells with c-Cbl restored normal clustering of PLC-YFP and SH2C*-YFP (Figure 5B). Colocalization of PLC γ 1 and c-Cbl clusters is worth noting. However, c-Cbl-CFP was unable to rescue the clustering of SH3*-YFP (Figure 5B) observed in the WT Jurkats (Figure 1D). These results suggest that the SH3-mediated interaction of PLC γ 1 with c-Cbl (Rellahan *et al*, 2003) stabilizes PLC γ 1 recruitment to the signaling complex. Although reconstitution of the J14 cells with c-Cbl restored recruitment of PLC γ 1, it was unable to rescue PLC γ 1 activity (Figure 5C). Yet, reconstitution of J14 cells with Slp76 (J14-Slp) restored calcium mobilization (Figure 5C) and PLCwt-YFP clustering (not shown) in these cells. Therefore, in the presence of Slp76, c-Cbl is either redundant for PLC γ 1 activation or the low levels of c-Cbl present in J14 cells are sufficient to maintain its functionality under these conditions.

To differentiate between these two possibilities, we have transiently expressed PLCwt-YFP in CD4⁺ T cells derived from c-Cbl knockout (KO) mice. Contrary to the results obtained in WT CD4⁺ T cells (Figure 1E), PLCwt-YFP clustering was abolished in c-Cbl-deficient T cells, despite normal TCR stimulation as indicated by multiple phospho-tyrosine clusters (Figure 5D). In addition, it has been shown previously that calcium elevation in T cells from c-Cbl KO mice is strongly inhibited (Chiang *et al*, 2004). These results together indicate that c-Cbl at some level is required for PLC γ 1 recruitment and activation.

Impaired calcium mobilization has also been demonstrated in Vav1-deficient T cells (Costello *et al*, 1999; Cao *et al*, 2002; Reynolds *et al*, 2002). However, the general tyrosine phosphorylation of PLC γ 1 was not affected by Vav1 deficiency in Jurkat T cells (Reynolds *et al*, 2002).

Figure 4 FRET analysis between PLC γ 1-YFP and various CFP-conjugated proteins. (A) Jurkat E6 cells expressing PLCs-YFP and LAT-CFP were seeded on a stimulatory coverslip and fixed 2 min into the spreading process. FRET efficiency values are presented in a pseudo-colored scale, with black color representing saturated pixels. The average value of FRET efficiency $\pm$ s.e. obtained for a given pair is shown. The size bar corresponds to 5 μ m. (B) Same as (A) for cells expressing PLCs-YFP and c-Cbl-CFP. (C) Same as (A) for cells expressing PLCs-YFP and Vav1-CFP. (D) Same as (A) for cells expressing PLCs-YFP and CFP-Slp76. (E) Same as (A) for cells expressing PLCs-YFP and Slp76-CFP. (F) Same as (A) for cells expressing PLCs-SH2N*-YFP and LAT-CFP.

Because the total tyrosine phosphorylation does not necessarily reflect phosphorylation of the critical Y783 residue, we have evaluated phosphorylation of PLC γ 1 in the Vav1-defi-

cient Jurkat J.vav cells using an anti-pY783 antibody. As can be seen in Figure 6A, phosphorylation of Y783 in J.vav cells is greatly reduced compared to E6.1 cells. Predictably, the

Figure 5 Effect of Slp76 and c-Cbl deficiencies on PLC γ 1 recruitment and activation. (A) Jurkat J14 cells expressing PLCwt-YFP were seeded on a stimulatory coverslip. An image obtained 1 min into the spreading process is shown. The size bar corresponds to 5 μ m. (B) Jurkat J14 cells expressing c-Cbl-CFP and an indicated PLC γ 1-YFP conjugate were seeded on stimulatory coverslips. Images obtained 1–1.5 min into the spreading process are shown. The size bar corresponds to 5 μ m. (C) Time course of the response in cytosolic calcium concentration obtained from the indicated cell lines after stimulation with OKT3. AU, arbitrary units. (D) CD4⁺ T cells from c-Cbl KO mice expressing PLCwt-YFP were seeded on a stimulatory coverslip, fixed 5 min into the spreading process and stained with anti-pY antibody. The size bar corresponds to 5 μ m.

calcium response in J.vav cells is strongly inhibited (Figure 6B).

Comparison between tyrosine-phosphorylated proteins that co-precipitate with PLC γ 1 in J.vav versus E6 cells reveals a conspicuous absence of a band corresponding to Slp76 in precipitate, obtained from the former (Figure 6C). Interestingly, the amount of pc-Cbl co-precipitated with PLC γ 1 in J.vav is substantially greater than that obtained in E6 cells. These results suggest that Vav1, and possibly Slp76, may compete with c-Cbl for PLC γ 1 binding.

Expression of PLCwt-YFP in J.vav cells demonstrated that in the absence of Vav1, PLC γ 1 was still recruited to the signaling clusters (Figure 6D). However, unlike the result we observed in WT Jurkats (Figure 1D), both the SH3*-YFP

and SH2C*-YFP failed to cluster in J.vav cells (Figure 6D). Taken together, these data suggest that Vav1 plays an important role in both recruitment and activation of PLC γ 1.

Discussion

Stimulation of the TCR leads to recruitment of PLC γ 1 to the TCR-proximal signaling complex, followed by PLC γ 1 phosphorylation and activation (Zhang *et al*, 1998, 2000; Bonvini *et al*, 2003). The hallmark of PLC γ 1 recruitment is its association with LAT through binding of the PLC γ 1 SH2N domain to pY132 of LAT (Stoica *et al*, 1998; Zhang *et al*, 2000). In this study, we show that the PLC γ 1 SH2N domain is not sufficient for the recruitment of the enzyme to the LAT-nucleated signaling complex. Although mutation of either the SH2C or SH3 domain of PLC γ 1 separately does not interfere with its binding to LAT, mutation of both of these domains abolishes PLC γ 1 recruitment and activation (Figures 1D and E and 2C and D).

These results suggest that although the SH3 and SH2C domains of PLC γ 1 do not bind directly to LAT, they can participate in the stabilization of the PLC γ 1–LAT association via other proteins in the LAT-nucleated signaling complex. These proteins can bind the PLC γ 1 SH3 and SH2C domains and bridge between PLC γ 1 and LAT. Either of the SH3 or SH2C domains can sufficiently stabilize the PLC γ 1–LAT association without participation of the other. However, functional elimination of both of them or, alternatively, mutation of two or all three distal pYs of LAT (Zhang *et al*, 2000; Lin and Weiss, 2001; Paz *et al*, 2001; Zhu *et al*, 2003), destroys the ‘bridge’ and abolishes PLC γ 1 association with LAT.

We also show that recruitment of PLC γ 1 to the LAT-nucleated complex does not necessarily result in PLC γ 1 activation. It has been proposed earlier that the SH3 and SH2C domains are dispensable for PLC γ 1 phosphorylation, because an application of a general anti-pY serum did not reveal a substantial decrease in the tyrosine phosphorylation of the SH3* and SH2C* mutants (Stoica *et al*, 1998; DeBell *et al*, 1999; Irvin *et al*, 2000; Bonvini *et al*, 2003). However, the data presented here provide evidence to the contrary. Using site-specific anti-pY antibodies, we show that phosphorylation of Y783, which is critical for PLC γ 1 activation, is strongly inhibited in the SH3* and SH2C* mutants (Figure 2C and D). Moreover, the Y783 phosphorylation of the SH2N* mutant, which is not recruited to LAT, appears to be more efficient than that of the SH3* or the SH2C* mutants despite seemingly normal recruitment of these two proteins to the signaling complex. Similar results were obtained for the Y775 site. Thus, in addition to their role in stabilization of the PLC γ 1–LAT association, the SH3 and SH2C domains are essential for efficient phosphorylation of Y783 and Y755. It is possible that the collaborative engagement of the SH3 and SH2C domains confers a conformational change exposing Y783 and Y755, otherwise not readily accessible for phosphorylation. Mutation in either the SH3 or SH2C domain results in a nonproductive recruitment of PLC γ 1. Yet, this recruitment may still be sufficient to induce phosphorylation of PLC γ 1 tyrosine residues other than Y783 or Y755, which may explain seemingly unaffected phosphorylation of the SH3* and SH2C* mutants observed with a general anti-pY serum (Stoica *et al*, 1998; DeBell *et al*, 1999; Irvin *et al*, 2000; Bonvini *et al*, 2003).

Figure 6 Effect of Vav1 deficiency on PLC γ 1 recruitment and activation. **(A)** Jurkat E6 cells (two left lanes) and Jurkat J.vav cells (two right lanes) were either stimulated with OKT3 for 1 min or left unstimulated and lysed. The lysates were resolved on SDS-PAGE and probed with either anti-phospho-PLC γ 1-pY783 (upper blot) or anti-PLC γ 1 (lower blot). The data are representative of two independent experiments. **(B)** Time course of the response in cytosolic calcium concentration obtained from the indicated cell lines after stimulation with OKT3. AU, arbitrary units. **(C)** Jurkat E6 or J.vav cells (1×10^7) expressing PLCwt-YFP were either stimulated for 1 min with C305 or left unstimulated and lysed. PLCwt-YFP was precipitated with anti-GFP. The precipitates were resolved on SDS-PAGE and probed with anti-phosphotyrosine 4G10 antibody. The data are representative of at least two independent experiments. **(D)** Jurkat J.vav cells expressing an indicated PLC γ 1-YFP conjugate were seeded on stimulatory coverslips. Images obtained 1–1.5 min into the spreading process are shown. The size bar corresponds to 5 μ m.

We confirm here that, in addition to LAT, at least three tyrosine-phosphorylated proteins—Slp76, c-Cbl and Vav1—co-precipitate with PLC γ 1 from stimulated Jurkat T cells (Figure 3). This co-precipitation is affected by mutations in the PLC γ 1 SH domains (Figure 3B). The FRET data indicate that PLC γ 1 bind these proteins directly (Figure 4). Together with previously reported evidence (Finco *et al*, 1998; Liu *et al*, 1998; Naramura *et al*, 1998; Yablonski *et al*, 1998; Costello *et al*, 1999; Schaeffer *et al*, 1999; Zhang *et al*, 2000; Cao *et al*, 2002; Reynolds *et al*, 2002), these results suggest that Slp76, c-Cbl and Vav1 are the likely candidates for the proteins participating in PLC γ 1 activation within the LAT-nucleated signaling complex. It has been demonstrated that c-Cbl binds constitutively to the PLC γ 1 SH3 domain (Rellahan *et al*, 2003). In agreement with these findings, we show here that co-precipitation of PLC γ 1 with c-Cbl critically depends on the functional integrity of the PLC γ 1 SH3 domain (Figure 3B and C). Moreover, recruitment (Figure 5) and activation (Chiang *et al*, 2004) of PLC γ 1 is strongly inhibited in c-Cbl-deficient T cells. These data suggest that c-Cbl may facilitate and stabilize recruitment of PLC γ 1 through their constitutive SH3-dependent interaction.

It has been shown that Slp76 is essential for PLC γ 1 Y783 phosphorylation and activation (Yablonski *et al*, 1998; Gonen *et al*, 2005). We show here that mutation in any of the three SH domains of PLC γ 1 results in a complete loss of PLC γ 1-Slp76 co-precipitation (Figure 3B). Interestingly, the loss of

PLC γ 1-Slp76 co-precipitation remarkably coincides with inhibition of the PLC γ 1 Y783 phosphorylation (Figures 2C and 3B and elsewhere: Yablonski *et al*, 1998; Reynolds *et al*, 2002; Gonen *et al*, 2005). The N-terminal pY residues of Slp76 have been identified as a binding site for the SH2 domain of the Tec-family kinase Itk, which can phosphorylate PLC γ 1 (Bunnell *et al*, 2000; Lewis *et al*, 2001). Thus, the key function of Slp76 in PLC γ 1 activation may lie in the recruitment of Itk to the signaling complex, whereas the SH3 and SH2C domains of PLC γ 1 jointly participate in the formation of a productive interaction between PLC γ 1 and the Slp76-Itk pair.

The N-terminal pY residues of Slp76 also provide a binding site for Vav1 (Fang and Koretzky, 1999) with a possible formation of a Slp76-Itk-Vav1 complex (Dombroski *et al*, 2005). Our results suggest that Vav1 facilitates association between PLC γ 1 and Slp76, corroborating similar observations reported previously (Reynolds *et al*, 2002). Both the SH2C and SH3 domains of PLC γ 1 mediate PLC γ 1-Vav1 association. This function of Vav1 may underlie its indispensability for PLC γ 1 phosphorylation and activation (Figure 6A and B and elsewhere; Costello *et al*, 1999; Cao *et al*, 2002; Reynolds *et al*, 2002).

To summarize the data shown and cited above, we propose the following model for the recruitment and activation of PLC γ 1 in T cells (Figure 7). TCR engagement leads to recruitment of PLC γ 1 to the LAT-nucleated complex through binding

Figure 7 Schematic model of PLC γ 1 recruitment and activation in the LAT-nucleated signaling complex.

of the PLC γ 1 SH2N domain to the pY132 residue of LAT. However, this binding is not sufficient to maintain the PLC γ 1–LAT association. Either the SH3 or SH2C domain of PLC γ 1 must stabilize this association through binding to other proteins in the LAT-nucleated signaling complex. Vav1, c-Cbl and SLP76 bridge between the PLC γ 1 SH3 and SH2C domains and LAT. Although only one of these domains is required for stabilization of the PLC γ 1–LAT association, both the SH3 and SH2C domains together are necessary for an efficient presentation of PLC γ 1 Y783 and Y755 to the activating kinase, probably Ikk. Elimination of either of these domains or, alternatively, elimination of either SLP76 or Vav1 partially destabilizes the PLC γ 1–LAT complex and results in a nonproductive recruitment of PLC γ 1.

Materials and methods

Antibodies

Mouse anti-CD3 ϵ , anti-CD28, anti-CD4, human anti-CD3 HIT3a and anti-CD3 PE-Cy5 were purchased from Pharmingen. Anti-phosphotyrosine 4G10, anti-c-Cbl 7G10, goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP were from Upstate Biotechnology. Anti-LAT-pY132, anti-PLC γ 1-pY783, anti-Vav1-pY160 were from Biosource. Anti-SLP76 was from Antibody Solutions. Anti-c-Cbl-pY774 was from Cell Signaling Technology. Anti-PLC γ 1 was from Santa Cruz Biotechnology. Anti-GFP was from Roche. Anti-GAPDH was from Biodesign. Anti-PLC γ 1-pY775 was provided by Dr BL Rellahan (Division of Monoclonal Antibodies, US Food and Drug Administration, Bethesda, MD).

Expression vectors and plasmids

The bovine PLC γ 1 cDNA was provided by Dr E Bonvini (Division of Monoclonal Antibodies, US Food and Drug Administration, Bethesda, MD). The rat Vav1 cDNA was provided by Dr J Rivera (Section on Chemical Immunology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD). LAT-CFP, SLP76-CFP and c-Cbl-CFP were generated from previously described constructs (Donovan *et al*, 1996; Bunnell *et al*, 2002; Barda-Saad *et al*, 2005). The monomeric YFP plasmid was created from pEYFP-N1 (Clontech) by A206K substitution (Zacharias *et al*, 2002). The Cerulean CFP plasmid was created from pECFP-N1 (Clontech) by A206, S72A, Y145A and H148D substitutions (Zacharias *et al*, 2002; Rizzo *et al*, 2004). The resultant YFP and CFP variants were used to create all fluorescent conjugates by standard methods. Truncated PLC γ 1-YFP vector (PLC γ s-YFP) encodes for PLC γ 1_(1–933) fragment followed by YFP. Point mutations were introduced using a QuikChange II XL site-directed mutagenesis kit (Stratagene). The constructs were cloned

into either pMSCVhyg or pMSCVneo (Clontech) to create retroviral expression vectors. All constructs were verified using DNA sequencing.

Vector expression and generation of stable cell lines

Jurkat cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. The recombinant DNA was introduced into the cells using retroviral infection. The retroviral expression vectors and pVSV-G (Clontech) were cotransfected into GP2-293 packaging cells (Clontech) using Lipofectamine 2000 (Invitrogen). After 48 h, the virus-containing medium was removed from the packaging cells and mixed with Jurkat cells. The medium was replaced with the regular growth medium after 24 h and an appropriate selection medium was added 72 h post-infection. The cells were sorted and those expressing fluorescent conjugates at the levels comparable with the corresponding endogenous proteins in the WT Jurkats were collected and expanded. Protein expression was monitored at multiple time points using flow cytometry and Western blotting. TCR expression level was monitored using immunostaining with anti-CD3 PE-Cy5 followed by flow cytometry.

Transfection of murine T cells

Mice used in this study were on a C57BL/6 background. C-Cbl KO (Cbl^{−/−}) mice were described previously (Chiang *et al*, 2004). CD4⁺ T cells from lymph nodes were purified by magnetic bead separation as previously described (Sommers *et al*, 2005). Cells were transfected with PLC γ 1-YFP using AMAXA electroporator and AMAXA kit for primary murine T cells and used 48 h post-transfection.

Confocal microscopy

The spreading assays were performed as described previously (Bunnell *et al*, 2002; Barda-Saad *et al*, 2005). Briefly, chambered coverslips (LabTek) were coated overnight at 4°C with either the stimulatory antibody anti-CD3 ϵ HIT3a (10 μ g/ml) or mouse anti-CD3 ϵ , anti-CD28 and anti-CD4 (10 μ g/ml each). The cells were seeded onto coated coverslips containing imaging buffer (RPMI 1640 without phenol red, 10% fetal calf serum, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). Some cells were fixed at different time points with 2–4% paraformaldehyde in phosphate-buffered saline. Immunostaining was performed as described previously (Barda-Saad *et al*, 2005). Fluorescent images were acquired on a LSM510 confocal system (Carl Zeiss) using a $\times$ 63 Plan-Apochromat objective (Carl Zeiss). A hot air blower (Nevetec) was used to maintain live samples at 37°C, and fine adjustments were made with a digital probe monitoring the buffer temperature in the chamber. The acquired images were extracted with the LSM browser (Carl Zeiss), cropped and composed into figures with Adobe Photoshop. The images were pseudo-colored as following, CFP—cyan, YFP—yellow, pY—magenta. No image enhancement procedures have been performed.

FRET was measured by the donor-sensitized acceptor fluorescence technique as described previously (Barda-Saad *et al*, 2005). Briefly, three images were acquired for each set of measurements: YFP excitation/YFP emission image (YFP channel); CFP excitation/CFP emission image (CFP channel) and CFP excitation/YFP emission image (FRET channel). A set of reference images was acquired from single-labeled CFP or YFP-expressing cells for each set of acquisition parameters and a calibration curve was derived to allow elimination of the non-FRET components from the FRET channel. The FRET efficiency was calculated on a pixel-by-pixel basis using the following equation:

$$\text{FRET}_{\text{eff}} = \text{FRET}_{\text{corr}} / (\text{FRET}_{\text{corr}} + \text{CFP}) 100\%$$

where FRET_{corr} is the pixel intensity in the corrected FRET image and CFP is the intensity of the corresponding pixel in the CFP channel image. To estimate the significance of the obtained FRET efficiency values and to exclude the possibility of getting false-positive FRET, three negative control systems were prepared: cells expressing LAT-CFP chimera and free YFP; cells expressing SLP-76 YFP chimera and free CFP; and cells expressing free CFP and free YFP. FRET efficiency values obtained from the negative control samples were subtracted from the ones obtained in the main experiments and the resulted efficiency values were reported.

Measurement of intracellular calcium concentration

Cells were incubated with 5 μ M Indo-1-AM (Molecular Probes) and 0.5 mM probenecid (Sigma) in RPMI 1640 medium at 37°C for 45 min. The cells were washed with RPMI 1640, resuspended in imaging buffer containing 0.5 mM probenecid, and kept at room temperature for 30–45 min. The cells were incubated at 37°C for 5 min before measurements and analyzed using the LSR II (BD Biosciences). The data were processed using Tree Star FlowJo software.

Immunoblotting and immunoprecipitation

Jurkat cells were either stimulated with anti-CD3 C305 or OKT3 for 1 min or left untreated. The optimal concentration of the stimulatory antibody was determined by titration. Murine cells were stimulated by incubating with biotinylated anti-CD3 ϵ and anti-CD28 (10 μ g each) for 15 min on ice, crosslinking with streptavidin (25 μ g) and incubating at 37°C for 4 min. Cells were lysed in ice-cold lysis buffer containing 1% Brij, 1% *n*-Octyl- β -D-glucoside, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1 mM Na₃VO₄ and complete protease inhibitor tablets (Roche). Protein A/G Plus-Agarose beads (Santa Cruz Biotechnology) were

used for immunoprecipitation. Protein samples were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and immunoblotted with appropriate primary antibodies. Immunoreactive proteins were detected with either anti-mouse or anti-rabbit horseradish peroxidase-coupled secondary antibody (Upstate Biotechnology) followed by detection with enhanced chemiluminescence (Amersham Biosciences).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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