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On the mechanism coupling phospholipase Cγ1 to the B- and T-cell antigen receptors

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Introduction

An early consequence of ligand-induced activation of numerous different receptors is the activation of phosphoinositide (PtdIns)-specific phospholipase C-γ (PLCγ). PLCγ hydrolyzes phosphatidylinositol (4,5)-bisphosphate ($PtdInsP_2$) to inositol (1,4,5)-trisphosphate and diacylglycerol, metabolites that control calcium mobilization and protein kinase C activation, respectively (reviewed in Rhee and Bae, 1997). Together, these second messengers coordinate the activation of downstream signaling pathways that ultimately control the metabolic and functional

Abbreviations: Ag: antigen; BCR; B-cell receptor; GST; glutathione *S*-transferase; ITAM; immume receptor tyrosine-based activation motif; EFGR; epidermal growth-factor receptor; FGFR; fibroblast growth factor receptor; GST; glutathione *S*-transferase; HA; hemagglutinin epitope tag; NF-AT; nuclear factor of activated T cells; PDGFR platelet-derived growth factor receptor; PLC: phospholipase C (PtdIns-specific phosphodiesterase; EC 3.1.4.11); TCR; T-cell receptor; PtdIns; phosphoinositide; $PtdInsP_2$; phosphatidylinositol (4; 5)-bisphosphate; RTK; receptor tyrosine kinase; SDS-PAGE; sodium dodecylsulfate-polyacrylamide gel electrophoresis; TKB domain; tyrosine kinase-binding domain.

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response of cells of different biological systems. PLCγ-coupled receptors include growth factor receptors, Fc receptors, IgE receptors and the antigen (Ag) receptors of B (BCR) or T (TCR) lymphocytes.

Most of the initial knowledge on PLCγ receptor coupling and activation stemmed from work on receptor tyrosine kinases (RTK's), such as the epidermal growth factor receptor (EGFR) or the platelet-derived growth factor receptor (PDGFR). Until recently, less information was available on PLCγ regulation by multichain receptors, such as the Ag receptors, but significant new insights into the regulation of PLCγ by this class of receptors has been recently emerging. This report attempts to summarize current knowledge and presents some recent findings from our laboratory that further our understanding of the mechanism coupling PLCγ to the BCR and TCR.

Structural features of PLCγ1: the Src-homology domains

Two structurally related PLCγ isozymes have been identified, PLCγ1 and PLCγ2 (reviewed in Rhee and Bae, 1997). PLCγ1 is widely expressed, while PLCγ2 expression is restricted to cells of the hematopoietic lineage. All known PtdIns-specific PLC enzymes contain two conserved regions, termed X and Y, which are required for catalysis. Expression of the X and Y regions from PLCγ1 as two independent polypeptides resulted in self-assembly into a product that was catalytically active in vitro (Horstman et al., 1996). Interestingly, the in vitro catalytic activity of the self-assembled X and Y regions was greater than that of the holoenzyme, suggesting that other regions of PLCγ1 may exert regulatory functions. In PLCγ, the X and Y regions are separated by an insertion that includes a tandem of Src-homology 2 (SH2) domains followed by a single Src-homology 3 (SH3) domain (Fig. 1).

SH2 domains are conserved modular regions of ~100 residues that bind tyrosine-phosphorylated proteins (reviewed in Pawson, 1995). The binding selectivity of SH2 domains is primarily conferred by the amino acid in position βD5, which makes contact with residues at the +1 and +3 positions immediately carboxyl-terminal to the phosphotyrosine of the bound peptide (Songyang et al., 1993). Both SH2 domains of PLCγ1 belong to a group that recognizes target sequences with the motif pY-hydrophobic-X-hydrophobic. Amino acid differences within this consensus

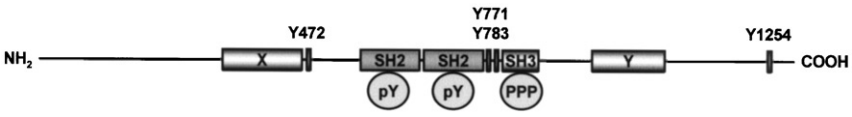

Fig. 1. Structure of phospholipase Cγ. Two domains (X and Y), required for the catalytic activity, are separated by a region that includes two SH2 domains and a single SH3 domain. The SH2 domains bind target molecules via recognition of phosphorylated tyrosine residues. The SH3 domain interacts with proline-rich polypeptides. Tyrosine residues phosphorylated upon PLCγ1 activation by RTKs are indicated (Y).

further select between the N-terminal (SH2N) and C-terminal (SH2C) SH2 domains (Songyang et al., 1993). The SH2N domain prefers an acidic residue in position +2, whereas the SH2C domain prefers a hydrophobic residue in the same position (Songyang et al., 1993, 1994). The arrangement of SH2 domains in tandem, as in PLCγ1, is found in other proteins, such as Zap-70, a T lymphocyte-specific kinase of the Syk family. Zap-70 SH2 domains cooperate by simultaneously engaging a single target protein (Hatada et al., 1995). The significance of the arrangement of the PLCγ1 SH2 domains is unknown.

Several observations suggested that the SH2 domains play a fundamental role in PLCγ1 regulation. Stimulation of the EGFR or PDGFR induces the translocation of PLCγ1 from the cytosol to the membrane via recruitment to phosphorylated tyrosine residues on the intracellular tails of the receptor (Todderud et al., 1990; Valius and Kazlauskas, 1993). This was confirmed by the failure of PLCγ1 to be recruited by forms of the PDGFR or EGFR bearing mutations in certain critical tyrosine residues (Valius et al., 1993; Vega et al., 1992). Fusion proteins encompassing the SH2 domains of PLCγ1 bound tyrosine phosphorylated EGFR (Margolis et al., 1990; Posner and Levitzki, 1994), PDGFR (Valius et al., 1993), and the fibroblast growth factor receptor (FGFR) (Mohammadi et al., 1991). An analysis of the polypeptide sequences preferred by the SH2 domains of PLCγ1 suggested that, of the two, the SH2C domain is best suited to couple PLCγ1 to the EGFR or PDGFR (Margolis et al., 1990; Songyang et al., 1993; Valius et al., 1993; Vega et al., 1992). This conclusion was based upon the observation that sequences found in either the EGFR or PDGFR tails (Y^{992}LIP and Y^{1021}IIP, respectively) match the motif preferred by the isolated SH2C domain. This domain favors Pro at the +3 position, whereas the SH2N prefers Leu (Songyang et al., 1993).

A study employing recombinant fragments of PLCγ1 suggested that the SH2N domain could also bind the intracellular tail of the phosphorylated EGFR (Rotin et al., 1992). Furthermore, a consensus sequence for this domain was identified in the tail of the FGFR (Y^{766}LDL) (Songyang et al., 1993). These findings, however, were contradicted by the observation that only fusion proteins encompassing the SH2C but not the SH2N domain of PLCγ1 bound the relevant phosphorylated tyrosine ($pTyr^{766}$) in a recombinant fragment of the FGFR (Mohammadi et al., 1991). Therefore, stronger evidence supported a role for the SH2C domain in coupling PLCγ1 to the receptors. The NMR resolution of the structure of PLCγ1 SH2C domain bound to a PDGFR peptide lent further credit to the role of this domain in PLCγ1 activation by documenting the physical nature of the interaction (Pascal et al., 1994).

SH3 domains bind proteins that contain proline-rich regions (Pawson, 1995). The PLCγ1 SH3 domain prefers proteins containing a PPVPP motif (Gout et al., 1993; Seedorf et al., 1994; Sparks et al., 1996), with sequences surrounding the SH3 domain contributing to the stabilization of the core interaction (Graham et al., 1998). Fusion proteins encompassing PLCγ1 SH3 domain co-distributed with cytoskeletal structures (Bar-Sagi et al., 1993; Yang et al., 1994), suggesting that this domain targets PLCγ1 to the cell cytoskeleton. The role of the SH3 domain in PLCγ1 regulation, however, remains poorly understood.

PLCγ1 activation by receptor tyrosine kinases

A common feature of PLCγ1-coupled receptors is that they are themselves tyrosine kinases or are coupled to tyrosine kinase effectors. PLCγ1 was first studied in fibroblasts and epithelial cells where it was characterized as a cytoplasmic enzyme activated by growth factor RTKs. Tyrosine phosphorylation of the receptor-associated PLCγ1 by the RTK ensues, leading to an increase in catalytic activity by predominantly increasing the V_{max} (Carpenter et al., 1992). Another proposed mechanism whereby tyrosine phosphorylation regulates PLCγ1 activation is that it may allow the enzyme to overcome the substrate sequestration and inhibitory effect of the actin- and $PtdInsP_2$-binding protein, profilin (Goldschmidt-Clermont et al., 1991).

PLCγ1 regulation by the antigen receptors of B and T cells

Multichain receptors, such as the BCR or TCR, have no intrinsic enzymatic activity. These receptors are coupled to several different protein tyrosine kinases that, in turn, lead to the activation of various signaling pathways, including the PLCγ1/Ca^{2+} mobilization pathway (reviewed in Kane et al., 2000; Weiss and Littman, 1994). Members of the Src family of protein tyrosine kinases are activated by these receptors and mediate the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the intracellular tails of invariant subunits associated with the clonotypic chains of the receptors (Fig. 2). A second class of cell-specific kinases of the Syk family (Syk–68 in B lymphocytes and certain T cells, Zap-70 in T lymphocytes) is then recruited. Induction of tyrosine kinase activity preceded PLCγ1 activation in T cells (June et al., 1990a) and tyrosine kinase inhibitors blocked Ag receptor-induced PtdIns hydrolysis and Ca^{2+} mobilization (June et al., 1990b).

Early studies into the mechanism of PLCγ coupling to the lymphocyte Ag receptors showed that receptor engagement induced tyrosine phosphorylation of both PLCγ1 and PLCγ2 isoforms in B lymphocytes (Coggeshall et al., 1992; Hempel et al., 1992; Roifman and Wang, 1992) and of PLCγ1 in T lymphocytes (Augustine et al., 1991; Park et al., 1991; Secrist et al., 1991; Weiss et al., 1991). Additional studies reported the association of PLCγ with the CD3 invariant chains associated with the TCR complex (Dasgupta et al., 1992) or with kinases of the Src and Syk families (Law et al., 1996; Peri et al., 1993; Pleiman et al., 1993; Sillman and Monroe, 1995; Weber et al., 1992). PLCγ1 was also shown to interact with adapter molecules, proteins devoid of enzymatic activity that participate in the assembly of multi-molecular complexes. A complex of PLCγ1, a 36–38 kDa phosphoprotein (pp 36–38), and Grb2 was well documented in activated T cells (Buday et al., 1994; Fukazawa et al., 1995; Nel et al., 1995; Richard et al., 1995; Sieh et al., 1994). The pp 36–38 was subsequently identified as the T cell-specific adapter, Lat (Weber et al., 1998; Zhang et al., 1998a). Additionally, PLCγ was shown to interact with the T cell-specific adapter, Slp-76 (Jackman et al., 1995) or its B cell homologue, Blnk/Slp-65 (Fu and Chan, 1997; Fu et al., 1998; Wienands et al., 1998).

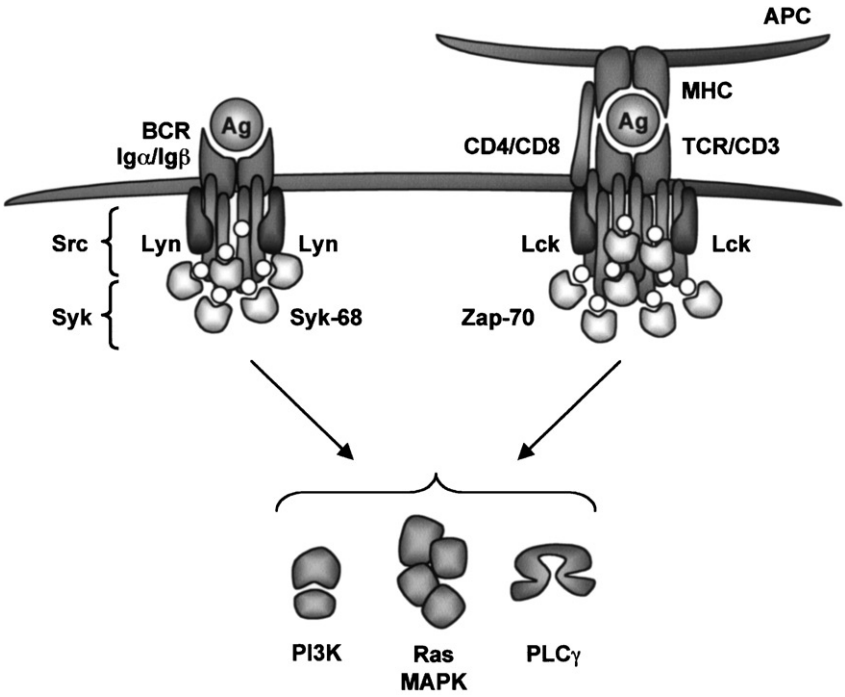

Fig. 2. Signal transduction by the B- and T-cell antigen receptors. Engagement of the receptor by the ligand, soluble Ag for B lymphocytes or processed Ag in the context of the major histocompatibility complex of Ag-presenting cells (APC) for T lymphocytes, induces activation of specific Src-family members. Co-receptor molecules (CD4 for CD8), that associate with Lck, participate in the interaction between T cells and Ag-presenting cells. Phosphorylation of conserved ITAM's recruits kinases of the Syk family to the tails of invariant chains (Igα/Igβ or CD3) of the receptor complex. Downstream events include the activation of phosphoinositide 3-kinase (PI3 K), the Ras/mitogen-activated protein kinase (MAPK) pathway, and PLCγ.

Studies that used cells defective for the expression of different signaling molecules also helped shed light onto the mechanism of PLCγ1 activation. These studies demonstrated that Lck (Straus and Weiss, 1992), Zap-70 (Williams et al., 1998), Lat (Finco et al., 1998; Zhang et al., 1998a) and Slp–76 (Yablonski et al., 1998) played a role in TCR-induced PLCγ1 tyrosine phosphorylation and/or activation in T lymphocytes. Expression of Syk in B lymphocytes was also found to be necessary for PLCγ2 phosphorylation and activation (Takata et al., 1994). Although Syk was capable of phosphorylating PLCγ1 in vitro (Law et al., 1996), co-expression of a functional BCR together with Fyn and Syk in non-lymphoid cells did not induce PtdIns hydrolysis or Ca^{2+} mobilization (Richards et al., 1996), suggesting that additional molecules had to be involved in coupling PLCγ to Syk.

Despite the large number of molecules shown to co-precipitate with PLCγ in lymphocytes, an understanding of the mechanisms governing these interactions

with PLCγ and its coupling to the Ag receptors remained poorly defined. Our laboratory has focused its efforts on elucidating the mechanisms by which PLCγ1 is coupled to and regulated by the BCR and TCR by analyzing the role of the structural elements of PLCγ1. The involvement of multiple molecules in PLCγ1 activation via the Ag receptors suggested an elaborate molecular network in which interactions with regulatory proteins mediated by the SH domains of PLCγ1 were likely to play an important role. Here we review the results of a structure–function analysis of these domains in PLCγ1 activation by the Ag receptors. These data, while contradicting early predictions, have revealed distinct roles for each of the SH2 and SH3 domains. In addition, by using a strategy of forced membrane compartmentalization, we have shown a role for specialized membrane microdomains in PLCγ1 coupling to and regulation by the B- and T-cell Ag receptors.

A Strategy for an Analysis of PLCγ1 in Antigen Receptor Signaling

The murine B cell line, WEHI 231.4, and the chicken B cell line, P10–14 (a DT-40 cell derivative), were utilized as B lymphocyte models. In P10-14 cells, the PLCγ2 gene had been disrupted by gene targeting (Takata et al., 1995). These cells were used for reconstitution experiments since they do not express PLCγ1 and therefore lack any endogenous PLCγ. Jurkat human T cells were used as a T lymphocyte model. These cells have been extensively used in the biochemical characterization of TCR signal transduction. These cells, however, are defective in PTEN phosphatase (Shan et al., 2000), a molecule involved in D3-phosphorylated PtdIns metabolism that controls certain signaling events. Prudence in interpreting and generalizing data obtained from Jurkat cells is therefore necessary. Nonetheless, they remain an invaluable source of information because of ease of handling, efficient ectopic protein expression, and the convenient availability of mutant lines (Abraham, 2001). Antibody-mediated perturbation of the receptor complex (anti-BCR/IgM, anti-CD3, or anti-TCR) was used as a surrogate for Ag-induced activation.

A triple influenza hemagglutinin (HA) epitope-tagged PLCγ1 (PLCγ1–HA) was constructed for transfection studies and used in all our experiments (Stoica et al., 1998). Mutagenesis of the SH2 domains of PLCγ1 was carried out by substituting the Arg residue in the conserved Phe–Leu–Val–Arg motif of either domain (position 586 of the SH2N domain and 694 of the SH2C domain) with Lys (Stoica et al., 1998). This conserved Arg forms an ion pair through hydrogen bonding with the phosphate of the phosphorylated tyrosine (Waksman et al., 1992). A peculiarity of the PLCγ1 SH2C domain is the presence of a second Arg in position 696 which can interact with both the phosphate and the aromatic ring of the phosphotyrosine (Pascal et al., 1994). Because of this structural feature, Arg^{694} and Arg^{696} could potentially compensate for each other in binding phosphorylated proteins. Therefore, a double mutant of Arg^{694} and Arg^{696} was also engineered (Stoica et al., 1998).

The Role of the Src-Homology Domains in Coupling PLCγ1 to the T- and B-Cell Antigen Receptors

Recombinant glutathione *S*-transferase (GST)-fusion proteins encompassing the individual SH2 domains or the corresponding mutants were used in pull-down experiments to assess the individual function of the SH2 domains of PLCγ1 and confirm the loss of binding by the mutations introduced. The GST–SH2C domain showed a wide spectrum of tyrosine-phosphorylated polypeptides from murine WEHI-231.4 B cells, whereas a single protein bound the GST–SH2N domain (Fig. 3). These data confirm the observation in activated P10–14 chicken B cells (DeBell et al., 1999) or Jurkat human T cells (Stoica et al., 1998). The 65-kDa protein observed in B cells (an 81-kDa molecule in chicken B cells) was identified by us and others as Blnk (DeBell et al., 1999; Ishiai et al., 1999). The SH2N domain-bound phosphoprotein in T cells was identified as Lat (Zhang et al., 1998a). These phosphorylated molecules were also recognized by the GST–SH2C domain of PLCγ1, by GST–Grb2, and co-precipitated with PLCγ2 (Fig. 3) or PLCγ1 (data not shown). The mutated SH2 domains failed to bind phosphorylated proteins from activated cells, confirming that the amino acid substitutions abrogated the phosphotyrosine-binding ability of either PLCγ1 SH2 domain. Thus, the in vitro binding selectivity of the SH2N domain of PLCγ1 is much greater than that of the SH2C domain.

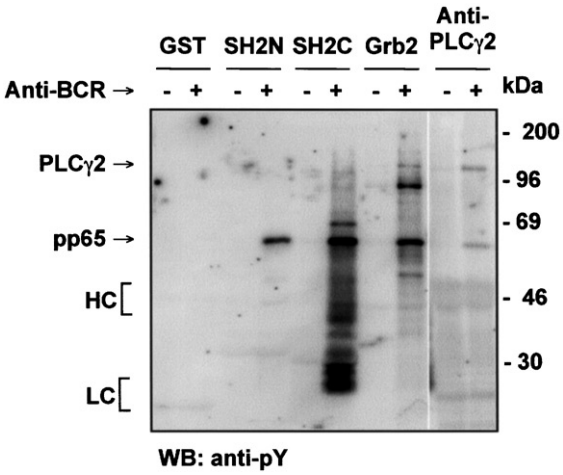

Fig. 3. Tyrosine-phosphorylated proteins recognized by the SH2 domains of PLCγ1 in B cells. WEHI-231.4 B cells were activated with an anti-BCR antibody (anti-murine IgM) for 2 min at 37°C or treated with medium alone. Clarified lysates were pulled down with the indicated GST-fusion proteins (200 pmol/sample) or an anti-PLCγ2 antibody. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes and blotted with an anti-phosphotyrosine (pY) antibody (4G10, Upstate Biotechnology, Lake Placid, NY). HC and LC indicate the position of the heavy and light chains of the antibody used in immunoprecipitation.

Membrane translocation and tyrosine phosphorylation are critical initial steps in the activation of PLCγ1. Immunofluorescence data from Jurkat T cells stably transfected with HA-tagged PLCγ1 document the critical role of PLCγ1 SH2N domain in Ag receptor-induced membrane translocation. Whereas wild type PLCγ1 was present as a cytoplasmic fluorescence in unstimulated cells, within minutes of Ag receptor stimulation, a portion of PLCγ1 localized to the cell membrane and the juxtamembrane areas (Fig. 4). This translocation was abrogated upon mutation of the SH2N domain. These data confirm our studies of B cells transfected with various PLCγ1 mutants that also showed that translocation was independent of the SH2C domain and partially reduced by mutation of the SH3 domain (DeBell et al., 1999). These results were unexpected and not predicted by the GST fusion protein data or previous evidence of PLCγ1 coupling to RTKs. We concluded that, contrary to previous suggestions, the SH2N domain was absolutely required for PLCγ1 membrane translocation, the SH3 domain contributed additional anchoring functions, whereas the SH2C domain played no apparent role in Ag receptor-induced translocation of PLCγ1.

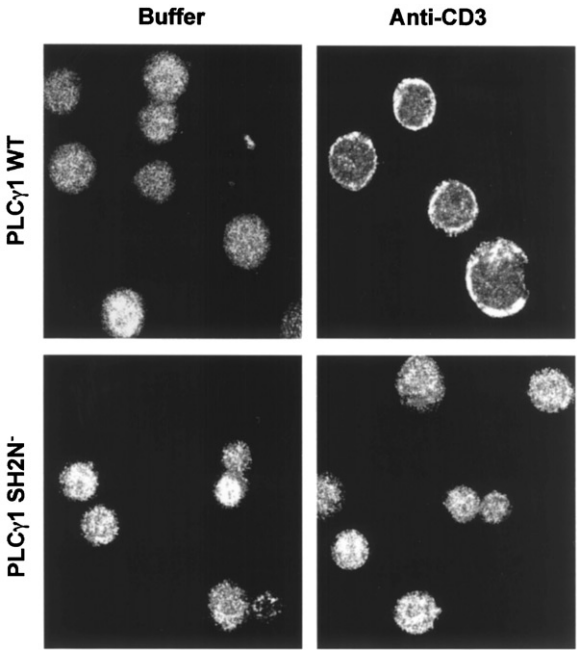

Fig. 4. The SH2N domain is required for TCR-induced redistribution of PLCγ1-HA in Jurkat T cells. Jurkat T cells were transfected with a mammalian expression vector (pCI-Neo, Stratagene, La Jolla, CA) encoding wild type PLCγ1-HA (PLCγ1 WT) or the SH2N-domain mutant of PLCγ1-HA (PLCγ1 SH2N$^-$). Stable transfectants, expressing similar levels of PLCγ1-HA by immunoblot analysis (data not shown), were activated with an anti-receptor antibody (right panels, Anti-CD3) for 1 min at 37°C or treated with medium alone (left panels, Buffer). Cells were fixed with formaldehyde, permeabilized, reacted with anti-HA, and stained with a secondary fluorescein-conjugated antiserum. .

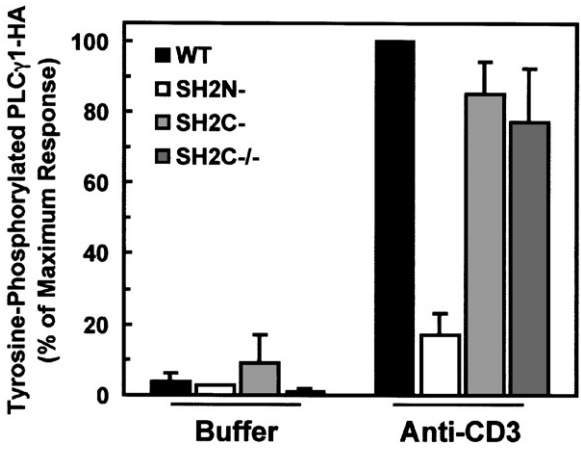

Fig. 5. TCR-induced tyrosine phosphorylation of SH2-domain PLCγ1 mutants in Jurkat T cells. Jurkat T cells were transiently transfected with a mammalian expression vector (pCI-Neo) encoding wild type PLCγ1 (WT) or the indicated SH2-domain mutants of PLCγ1. After 24 h, viable cells were collected by Ficoll gradient centrifugation and 5×10^6 cells/sample were activated with an anti-receptor antibody (anti-CD3) for 2 min at 37°C or treated with medium alone. Clarified lysates were immunoprecipitated with anti-HA, resolved by SDS-PAGE, transferred to nitrocellulose membranes and blotted with an anti-phosphotyrosine antibody (4G10). Immunoblots were scanned and the data expressed as percentage of tyrosine-phosphorylated PLCγ1-HA. Shown is the mean ± s.e.m. of five independent determinations.

Consistent with the translocation data, the SH2N PLCγ1 mutant demonstrated reduced levels of tyrosine phosphorylation in T cells (Fig. 5) compared to the wild type protein. In contrast, either mutation of the SH2C domain had virtually no effect on Ag receptor-induced tyrosine phosphorylation of PLCγ1. Furthermore, mutation of the SH3 domain did not decrease the stimulated level of Ag receptor-induced tyrosine phosphorylation of PLCγ1 (data not shown and DeBell et al., 1999).

In Jurkat cells, phosphorylated Lat did not co-precipitate with the PLCγ1 SH2N-domain mutant, whereas normal levels were present with the PLCγ1 SH2C-domain mutants (data not shown). In P10–14 B cells, wild type PLCγ1 co-precipitated with Blnk, which was absent from immunoprecipitates from stimulated cells transfected with the SH2N-domain mutant (DeBell et al., 1999). Mutation of the SH2C or the SH3 domains resulted in no detectable difference in co-precipitating Blnk (DeBell et al., 1999), indicating that the function of these domains was not important for binding this molecule.

The role of the SH domains in PLCγ1 activation and signal transduction was established in PLCγ1-reconstituted P10-14 B cells. BCR-induced generation of inositol phosphates was restored by expressing wild type PLCγ1, but remained deficient in cells expressing either the SH2N-domain mutant or the SH2C-domain mutant and was partially reduced in cells expressing the SH3-domain mutant (DeBell et al., 1999). A consequence of inositol phosphate-induced increase in intracellular Ca^{2+} is the activation of calcineurin, a phosphatase that dephosphorylates the cytoplasmic transcription factor, nuclear factor of activated T cells

(NF–ATp), thereby enabling it to enter the nucleus (Crabtree and Clipstone, 1994). NF–AT elements are present in numerous promoters, including that of the interleukin 2 gene (Crabtree and Clipstone, 1994). The activation of an NF–AT reporter is therefore a marker for the activation of Ca^{2+}-dependent events. Reporter gene studies showed a lack of activation of the PLCγ1 SH2N- or SH2C-domain mutants, consistent with the role of these domains in PtdIns hydrolysis (DeBell et al., 1999). Similarly, an intermediate result was obtained with the PLCγ1 SH3-domain mutant, concordant with the contribution of this domain to membrane translocation. Therefore, all SH domains must be functional for ligand-dependent PLCγ1 activation and each domain plays an independent role in coupling PLCγ1 and the Ag receptors. The requirement for a functional SH2C domain, however, suggests that other steps, in addition to tyrosine phosphorylation and membrane translocation, control PLCγ1 activation.

The SH2C Domain of PLCγ1: A Domain with an Enigmatic Function

The precise function of the SH2C domain in TCR- or BCR-induced PLCγ1 activation remains elusive. To further explore the role of PLCγ1 SH2C domain in mediating protein–protein interactions, we performed pull-down experiments by using tyrosine-phosphorylated peptides immobilized on a solid matrix and cells transfected with the HA-tagged PLCγ1 constructs. We selected a peptide sequence, KDND(p)YIIPLPDPK, which was derived from the PDGFR and closely matched the consensus recognized by the isolated SH2C domain of PLCγ1 (Songyang et al., 1993). Although there was a complete loss of binding to the SH2N-domain PLCγ1 mutant, no loss of binding was seen with either of the SH2C-domain mutants (Fig. 6). These in vitro data are consistent with our in vivo studies that suggest the SH2C domain plays a negligible role in mediating the initial association of PLCγ1 with other proteins.

In vitro assays of enzyme activity of PLCγ1 SH2-domain mutants offered another intriguing result (DeBell et al., 1999). As expected, the in vitro enzyme activity of wild type PLCγ1 from P10–14 B cells was increased upon BCR aggregation, while the SH2N-domain mutant demonstrated only basal levels, irrespective of BCR aggregation. In contrast, the SH2C-domain mutant of PLCγ1 showed in vitro activity levels comparable to those of activated wild type PLCγ1, independently of BCR ligation. Hence, the SH2C domain may play a role as an intrinsic negative regulator of the protein's enzyme activity. This potential inhibitory function revealed by the in vitro activity assay, however, contrasts with the domain's requirement for PtdIns hydrolysis in vivo. Arguably, the SH2C domain plays a more complex role in vivo than that captured by the in vitro assay.

Our studies exclude a function for the SH2C domain in the initial binding to a target molecule and membrane translocation. They also exclude the possibility that the SH2-domain tandem of PLCγ1 functions by locking onto the same protein in a coordinated, synergistic fashion, as in the case of Zap-70 (Hatada et al., 1995). Whereas gross defects in tyrosine phosphorylation of the PLCγ1 SH2C-domain mutant have been ruled out, we cannot eliminate the possibility that this domain is

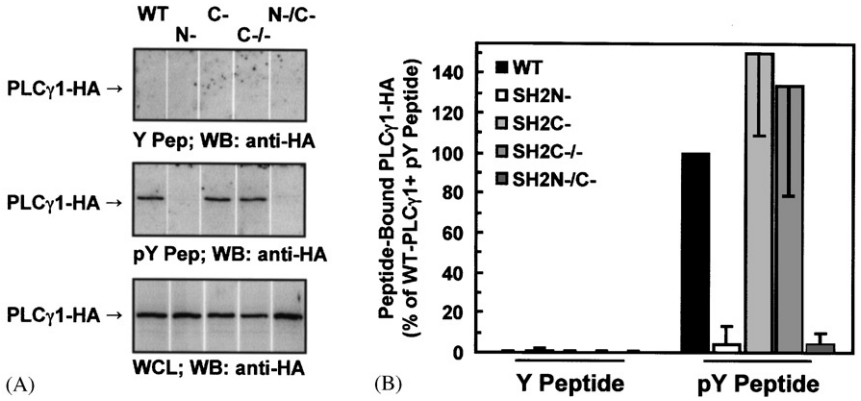

Fig. 6. PLCγ1 SH2C-domain mutant binds to immobilized tyrosine-phosphorylated peptides. Jurkat T cells were transiently transfected with a mammalian expression vector (pCI-Neo) encoding wild type PLCγ1 (WT) or the indicated SH2-domain mutants of PLCγ1. After 24 h, viable cells were collected by Ficoll gradient centrifugation and lysates from 5×10^6 cells/sample were pulled down with the indicated peptides immobilized on Affi-Gel 15 beads (Bio-Rad), resolved by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-HA. Whole-cell lysates (WCL) from the same cells were also blotted to assess transfection efficiencies. Immunoblots were scanned and the data expressed as percent binding relative to wild-type PLCγ1-HA bound to the tyrosine-phosphorylated peptide. Shown is the mean ± s.e.m. of four independent determinations. Y Pep, non-phosphorylated peptides; pY Pep, tyrosine-phosphorylated peptides.

required for the phosphorylation of specific tyrosine residues undetected by anti-phosphotyrosine immunoblotting but critical for activation. Nevertheless, an interaction of the SH2C domain with a potential target appears necessary for PLCγ1 activation in vivo. Alternate possibilities include binding of this domain to regulatory phospholipids, such as D3-phosphorylated PtdIns (Rameh et al., 1998).

Lipid Rafts Regulate PLCγ1 Activation in T Cells

The plasma membrane bilayer contains microdomains, termed lipid rafts, which are enriched in sphingolipids and cholesterol. These microdomains form liquid-ordered assemblies with distinct functions, including a role in signal transduction (Brown and London, 2000; Janes et al., 2000; Simons and Ikonen, 1997). Several effectors and regulatory molecules implicated in signal transduction are segregated in the membrane of resting cells based in part on their differential compartmentalization in lipid rafts. In Ag receptor signaling, molecules, such as the Src kinases (van't Hof and Resh, 1997) and Lat (Lin et al., 1999; Zhang et al., 1998b), associate constitutively with lipid rafts. Proper compartmentalization of Lck to the lipid rafts is required for TCR signaling (Kabouridis et al., 1997). Furthermore, a Lat mutant which failed to associate with lipid rafts was not phosphorylated and resulted in defective TCR signaling (Zhang et al., 1999, 1998b).

After TCR activation, Lat acts as a transmembrane raft scaffold that mediates the inducible re-compartmentalization of proteins via binding its tyrosine-phosphorylated residues. As discussed earlier, Lat recruits PLCγ1 directly via the SH2N domain (Stoica et al., 1998; Zhang et al., 1998a). The tyrosine-phosphorylated form of PLCγ1 is enriched in lipid rafts (Xavier et al., 1998; Zhang et al., 1998b). Furthermore, disruption of raft organization impairs T cell activation (Xavier et al., 1998).

Receptor-induced re-compartmentalization of molecules to these microdomains may promote their interaction by increasing their local concentration or via the formation of localized complexes with molecules constitutively present in this compartment. A critical question, therefore, is what role re-compartmentalization of PLCγ1 to these microdomains plays in controlling PLCγ1 phosphorylation and activation status. To investigate this, we have engineered forms of PLCγ1 that constitutively associate with the lipid rafts.

The addition of palmitic acid to certain Cys residues can target proteins to the cytoplasmic leaflet of these membrane microdomains (Wolven et al., 1998). We used this strategy to force the compartmentalization of PLCγ1 to the lipid rafts. In one such approach, we added an N-terminal myristoylation and palmitoylation signal from the human Fyn sequence (Fyn–PLCγ1). By expressing this fusion protein in Jurkat T cells, we have demonstrated that forced raft compartmentalization bypassed the requirement for Ag receptor engagement and was sufficient to constitutively phosphorylate and activate PLCγ1 (Veri et al., 2001). Interestingly, phosphorylation of Fyn–PLCγ1 still required Lck (Veri et al., 2001). Lck is necessary for Ca^{2+} mobilization in T cells (Straus and Weiss, 1992) and functions by phosphorylating and activating Zap-70 (Chan et al., 1995), whose expression is also required for TCR-induced PLCγ1 activation (Williams et al., 1998). Fyn–PLCγ1, however, was constitutively tyrosine phosphorylated in Zap-70-deficient Jurkat cells (Veri et al., 2001). Accordingly, we also observed that Lat and Slp-76 were also dispensable (Veri et al., 2001). Therefore, forced PLCγ1 compartmentalization to the lipid rafts leads to its constitutive tyrosine phosphorylation without requiring Zap-70 and Zap-70-dependent events.

The choice of a Fyn sequence as a PLCγ1 fusion partner was dictated by the extensive characterization of the function of this signal (Wolven et al., 1998). To further confirm the role of rafts in PLCγ1 signaling, we have engineered a second form of raft-targeted PLCγ1 by fusing it with the transmembrane and juxtamembrane region of Lat (Lat–PLCγ1), another raft-associated protein (Zhang et al., 1998b). Microdomain localization of the Lat–PLCγ1 was compared to that of Fyn–PLCγ1 by using sucrose gradient ultracentrifugation and Lck, a protein in large part associated with this microdomain fraction, as a marker (Fig. 7A). The results showed that Lat–PLCγ1 is constitutively present in lipid rafts. Consistent with our previous data, Lat–PLCγ1 was constitutively phosphorylated (Fig. 7B). Furthermore, expression of Lat–PLCγ1 also induced the constitutive activation of gene transcription (Fig. 7C), consistent with the notion that tyrosine phosphorylation controls PLCγ1 activation. Thus, a raft-associated PLCγ1 acts as dominant positive variant independently of the nature of the signal targeting it to the raft compartment.

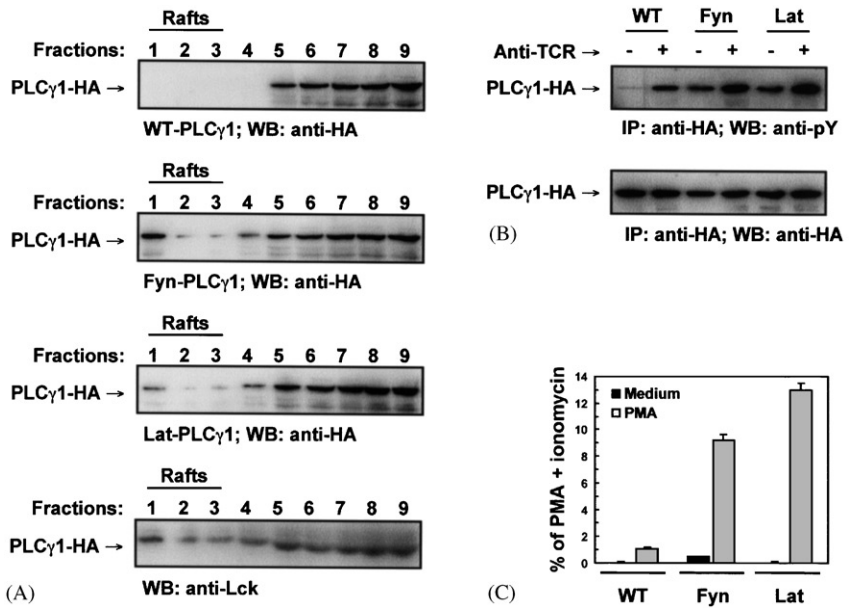

Fig. 7. Constitutive tyrosine phosphorylation and activation of raft-targeted Lat–PLCγ1 in Jurkat T cells. (A). Lysates from transiently transfected Jurkat T cells were separated on discontinuous sucrose gradients. Fractions were analyzed by SDS–PAGE and immunoblotted with anti-HA or for endogenous Lck (SantaCruz Biotechnology, Santa Cruz, CA). (B). Transiently transfected Jurkat T cells were activated with an anti-receptor antibody (anti-TCR C305) for 2 minutes at 37°C or treated with medium alone as indicated. Lysates were immunoprecipitated with anti-HA, resolved by SDS–PAGE, immunoblotted with an anti-phosphotyrosine (anti-pY) antibody (4G10), stripped and re-probed with anti-HA. (C). Jurkat T cells were transiently transfected with the indicated constructs along with a NF–AT luciferase reporter and an adenovirus major-late promoter β-galactosidase-encoding vector to control for transfection efficiency. Transfected cells were activated for 6 h at 37°C with an anti-receptor antibody (anti-TCR, C305) with or without 4β-phorbol 12-myristate 13-acetate (PMA), or PMA plus ionomycin. By activating Ras, PMA induces the AP-1 component of NF–AT-dependent transcription (Crabtree and Clipstone, 1994). The combination of PMA and ionomycin provides receptor-independent Ras activation and Ca^{2+} influx for maximal NF–AT transcriptional activation. Data were normalized for β-galactosidase activity and expressed as percentage of maximum activation with PMA plus ionomycin. The data shown are the means ± s.e.m. of triplicate values of a representative experiment out of three performed.

These data indicate that tyrosine kinases that phosphorylate PLCγ1 are associated with the raft microdomains where they can interact with raft-targeted PLCγ1.

c-Cbl Negatively Regulates Antigen Receptor-Induced PLCγ1 Activation

During the analysis of proteins that interact with the SH domains of PLCγ1, our laboratory identified c-Cbl as the predominant tyrosine-phosphorylated protein pulled down by the recombinant SH3 domain of PLCγ1 (Graham et al., 1998). Early work from our laboratory indicated that over-expression of c-Cbl in T lymphocytes inhibits TCR-induced activation of the Ras pathway (Rellahan et al., 1997). Subsequent studies assessed whether c-Cbl regulates PLCγ1. We found that c-Cbl

Table 1
Effect of c-Cbl over-expression on TCR-induced phosphoinositide hydrolysis and NF-AT activation

	% of Control	
	pCI-Neo	pCI-c-Cbl
PtdIns Hydrolysis[a]	94.0% ± 6.0	56.3% ± 4.0
NF-AT Activation[b]	43.6% ± 5.9	15.3% ± 3.0

[a] Jurkat cells were transfected with a green-fluorescence protein (GFP) expression plasmid together with the indicated control or c-Cbl vectors, FACS-sorted based on GFP fluorescence, labeled with *myo*-[2-3H]-inositol (15 μCi/ml), and treated with medium alone or an anti-receptor antibody (OKT3) for 45 min at 37°C in the presence of 300 μM LiCl. Total inositol phosphates were separated by ionic exchange chromatography. Data are shown as the percentage of TCR-induced PtdIns hydrolysis observed in negatively sorted, GFP-negative, matched control cells (mean ± SD of three separate experiments).

[b] Jurkat cells were transfected with an NF-AT-luciferase reporter plasmid together with the indicated control or c-Cbl vectors. Cells were treated with medium, an anti-receptor antibody (OKT3), or the combination of the phorbol ester, 4β-phorbol 12-myristate 13-acetate (PMA) and thapsigargin, to induce receptor-independent, maximal activation. Cell extracts were assayed for luciferase activity as described (DeBell et al., 1999). Data are expressed as the percent of NF-AT-induced activation by PMA plus thapsigargin (mean ± SD of five separate experiments).

over-expression in Jurkat T cells inhibited TCR-induced PtdIns hydrolysis and NF-AT activation (Table 1) (Graham et al., 2000).

c-Cbl is an adapter protein with ubiquitin-ligase activity that has been identified as a negative regulator of RTKs (reviewed in Thien and Langdon, 2001). c-Cbl's structural complexity, together with the numerous protein interactions that have been documented for this molecule, suggest that c-Cbl is involved in the regulation of a variety of different cellular substrates. The tyrosine kinase-binding (TKB) domain and the RING/Ub-ligase domains are essential for c-Cbl's ability to regulate tyrosine kinases (Thien and Langdon, 2001). The TKB has been shown to mediate the association of c-Cbl with the tyrosine kinase, with the RING/Ub ligase domain mediating the ubiquitination of the kinase itself (Levkowitz et al., 1999; Thien et al., 2001). c-Cbl is also phosphorylated on multiple carboxyl-terminal tyrosines and has been shown to interact with the SH2 domains of numerous proteins through these residues (Tsygankov et al., 2001).

c-Cbl constitutively interacts with PLCγ1 in Jurkat T cells (Fig. 8A). The region of c-Cbl that extends from residue 494 to residue 826 contains multiple SH3 domain-binding sites and c-Cbl has been shown to interact with the SH3 domains of several proteins, including Grb2, Fyn, Src, Itk, and PLCγ1 (Bunnell et al., 1996; Graham et al., 1998; McCormick, 1993; Rellahan et al., 1997; Touhara et al., 1995). Three of these potential SH3 domain-binding sites contain a minimal consensus sequence for the SH3 domain of PLCγ1 (PPVPP) (Gout et al., 1993; Seedorf et al., 1994; Sparks et al., 1996). The SH3 domain of PLCγ1 interacts with both canonical and non-canonical sites in the proline-rich region of c-Cbl and mediates an interaction required for its regulation by c-Cbl (Rellahan et al., submitted). Although c-Cbl interacts in a constitutive manner with SH3 domain of PLCγ1, the inhibitory ability of c-Cbl depends on its activation-induced phosphorylation (Rellahan et al.,

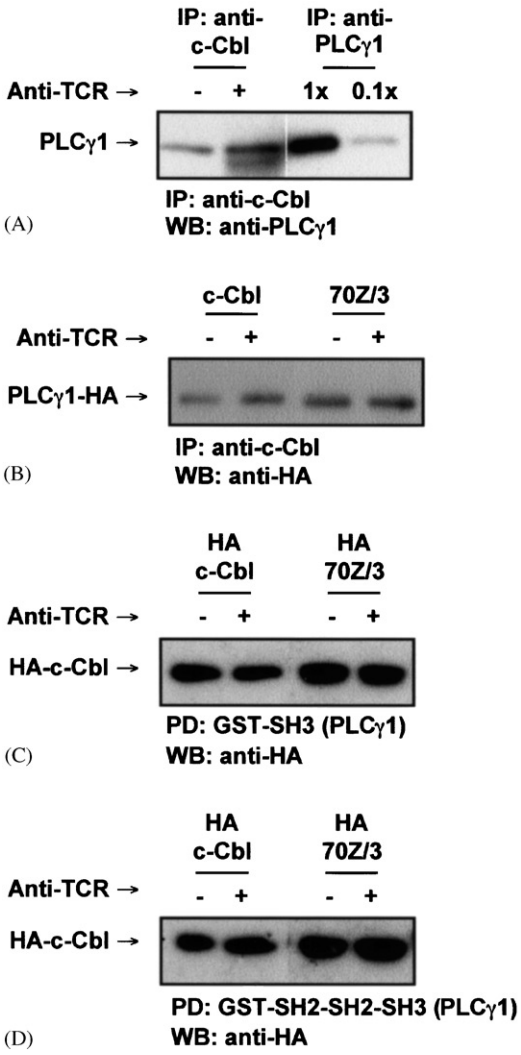

Fig. 8. c-Cbl and 70Z/3 Cbl constitutively interact with PLCγ1 and its SH3 domain. (A). Jurkat T cells were activated with an anti-receptor antibody (anti-CD3) as indicated. Lysates from 5×10^7 cells were immunoprecipitated with an anti-c-Cbl antibody (SantaCruz Biotechnology) or an anti-PLCγ1 antibody (Upstate Biotechnology), resolved by SDS–PAGE, and immunoblotted with an anti-PLCγ1 antibody. 1x, 1×10^6 cell equivalent/lane; 0.1x, 1×10^5 cell equivalent/lane. (B). Jurkat T cells were transiently transfected with a mammalian expression vector (pCI-Neo) encoding wild type c-Cbl or 70Z/3 Cbl together with PLCγ1-HA. After 24 h, viable cells were collected by gradient centrifugation and 5×10^6 cells/sample treated with an anti-receptor antibody (C305) for 2 min at 37°C. Clarified lysates were immunoprecipitated with anti-c-Cbl (SantaCruz Biotechnology), resolved by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-HA. (C and D). Jurkat T cells were transiently transfected with a mammalian expression vector (pCI-Neo) encoding wild type c-Cbl or 70Z/3 Cbl. After 24 h, viable cells were collected by gradient centrifugation and 5×10^6 cells/sample treated with an anti-receptor antibody (C305) for 2 min at 37°C. Clarified lysates were pulled down with the indicated GST-fusion protein of the SH2 and SH3 domains of PLCγ1, resolved by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-HA.

submitted), which likely enables the association between phosphorylated c-Cbl and the SH2-domain(s) of PLCγ1. These data suggest that the constitutive interaction between the SH3 domain of PLCγ1 and c-Cbl is required to tether the proteins together and facilitate a low-avidity interaction between the SH2 domain(s) of PLCγ1 and tyrosine-phosphorylated c-Cbl that further stabilizes the association between the two proteins.

The inhibitory mechanism mediated by this association remains unclear. An oncogenic form of c-Cbl, 70Z/3 Cbl, activates PLCγ1 rather than inhibiting it (Graham et al., 2000). 70Z/3 Cbl, however, associates with PLCγ1 in a manner indistinguishable from that of wild type c-Cbl (Fig. 8B and C and data not shown). 70Z/3 Cbl features a 17-amino acid deletion that disrupts its ubiquitin ligase activity (Thien and Langdon, 2001), which raises the possibility that c-Cbl regulates PLCγ1 by inducing its ubiquitination. Preliminary investigations indicate that PLCγ1 undergoes Ag-receptor induced ubiquitination (Rellahan and Bonvini, unpublished observation). The role of c-Cbl in this event, however, is unclear. No Ag receptor-induced decrease in the protein expression or phosphorylation levels of PLCγ1 has been detected in cells that over-express c-Cbl (Graham et al., 2000). In the absence of an observable decrease in PLCγ1 expression, we favor the hypothesis that c-Cbl regulates other events required for PLCγ1 activation, such as the association of PLCγ1 with other proteins (e.g., Slp-76), its substrate, or its subcellular localization.

A structural model of PLCγ1 coupling to the antigen receptors

While the structural arrangement of the SH2 domains in tandem has initially suggested the possibility of a cooperative interaction, all SH domains appear to function independently of each other and in a sequential manner, by performing autonomous and overlapping functions (Table 2). The role of the SH domains in TCR- and BCR-induced PLCγ1 translocation, phosphorylation, and activation have also been confirmed by Irvin et al. in Jurkat T cell models defective for PLCγ1 (Irvin et al., 2000). Consistent with our findings in B and T cells, recent evidence indicates that the SH2N domain is also a primary player in coupling PLCγ1 to the EGFR and PDGFR (Chattopadhyay et al., 1999; Ji et al., 1999; Poulin et al., 2000). These data have contributed to revise the initial assumptions and suggest a common structural paradigm in PLCγ1 coupling to different receptor families.

In the model, we propose the SH2N domain to be the critical domain, essential for membrane translocation, phosphorylation, and activation (Fig. 9). The engagement

Table 2
The role of the Src-homology domains in antigen receptor-induced PLCγ1 activation

	SH2N	SH2C	SH3
Membrane Translocation	Required and sufficient	Dispensable	Enhancement
Phosphorylation	Required and sufficient	Dispensable	Dispensable
Activation	Required	Required	Enhancement

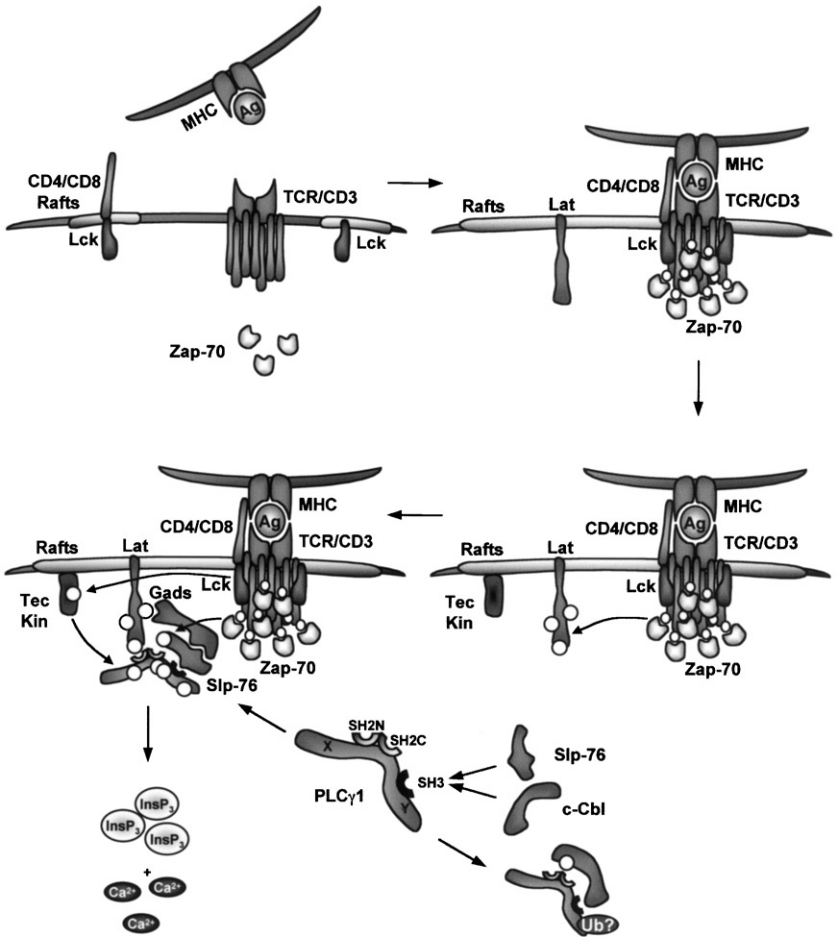

Fig. 9. A hypothetical raft-based model of PLCγ1 coupling to the T-cell receptor. See the discussion for an explanation.

of the SH2N domain with a phosphoprotein initiates the activation sequence by recruiting PLCγ1 to an activation complex within the membrane raft compartment. PLCγ1 phosphorylation ensues as a consequence of the interaction of PLCγ1 with one or more kinases, an event exclusively mediated by the SH2N domain.

We have further shown that the basic paradigm for Ag receptor-induced PLCγ1 activation in B and T cells demonstrates similar structural requirements (DeBell et al., 1999; Stoica et al., 1998). There are, however, remarkable mechanistic differences between these two cell types, with an increased regulatory complexity in T cells. In T cells, Lat acts as a trans-membrane, raft-associated scaffold responsible for assembling PLCγ1 with Grb2, Gads and other proteins (Zhang et al., 1998a, 2000). In B cells, Blnk, a cytosolic protein, recruits PLCγ1 to the plasma membrane

via phosphorylation-dependent interactions with other molecules (Fu and Chan, 1997; Fu et al., 1998; Wienands et al., 1998). Interestingly, Blnk is not related to Lat, but, rather, is the homologue of the T cell-specific adapter, Slp-76 (Fu et al., 1998; Jackman et al., 1995; Wienands et al., 1998). Slp-76 is required for TCR-induced PLCγ1 phosphorylation and activation (Yablonski et al., 1998), but binds PLCγ1 constitutively via the SH3 domain (Yablonski et al., 2001) and inducibly via Gads and Lat (Ishiai et al., 2000; Liu et al., 1999). Hence, the interaction of PLCγ1 with Blnk in B cells is a direct one, while in T cells the association with Slp-76 shows added complexity. This complexity in T cells might enable other molecules (e.g., CD2, CD28, adhesion molecules, and other co-stimulatory molecules) to participate in PLCγ1 regulation. Nonetheless, the primary common defect of the PLCγ1 SH2N-domain mutant in both B and T cells is its failure to interact with the phosphorylated adapters.

The function of the SH2C domain is still unclear. The SH2C domain is dispensable for translocation and phosphorylation, but is required for PLCγ1 activation, possibly by binding a yet unidentified phosphoprotein, by interacting with membrane phospholipids (Rameh et al., 1998) or by exerting yet undefined regulatory functions. Interestingly, in T cells the SH2C domain can couple the CD28 co-stimulatory molecule to its downstream pathway in an enzyme-independent fashion (Irvin et al., 2000), suggesting a novel adapter-like function ascribable to the domain that will certainly deserve future attention.

The SH3 domain contributes to Ag receptor-induced PLCγ1 membrane translocation and activity, but has no apparent role in phosphorylation. This is consistent with the observation that, in T cells, the SH3 domain mediates a constitutive interaction with Slp-76 (Yablonski et al., 2001) that is inducibly recruited to the rafts and contributes to stabilizing the raft-based complex (Ishiai et al., 2000). By pivoting between Slp-76 and c-Cbl, the SH3 domain of PLCγ1 can play both positive and negative regulatory roles.

In addition to PLCγ1, several other proteins (Fyn, Lck, Grb2, PI3 K, Lyn) interact with c-Cbl through both their SH3 and SH2 domains (Buday et al., 1996; Dombrosky-Ferlan and Corey, 1997; Jain et al., 1997), suggesting this may be a common interaction strategy between c-Cbl and some of the partners it regulates. We speculate that this interaction through both their SH3 and SH2 domains establishes an orientation between the two proteins that is optimal for c-Cbl to exert its regulatory function. For example, if c-Cbl regulated these proteins via ubiquitination, this orientation may allow ubiquitination of specific Lys on the target protein or it may allow optimal Ub chain growth. Although most evidence suggests that the mechanism of signal down-regulation is dependent on the ability of c-Cbl to ubiquitinate proteins it associates with, more work is needed to fully understand the process by which c-Cbl regulates signal transduction. Recently, Tvorogov and Carpenter also reported an SH3 domain-dependent, EGF-induced association between PLCγ1 and c-Cbl (Tvorogov and Carpenter, 2002), suggesting that this regulatory mechanism may extend to PLCγ1 activation by RTKs.

PLCγ1 membrane raft translocation via SH2N domain-dependent interactions appears to be the primary event which, in itself, is sufficient to induce

phosphorylation by promoting proximity to an active kinase. Compartmentalized segregation of regulatory proteins is a well-known regulatory mechanism, which includes the control of Ras by Sos (Holsinger et al., 1995). Consistent with this paradigm, translocation to the lipid rafts may be a rate-limiting factor in PLCγ1 activation by promoting its interaction with regulatory kinases present in these microdomains. The Tec kinase family has emerged as another tyrosine kinase family involved in PLCγ1 phosphorylation in B and T lymphocytes. One of its members, Btk, was shown to be required for efficient tyrosine phosphorylation of PLCγ in B cells (Takata and Kurosaki, 1996). A more subtle defect in PLCγ1 phosphorylation was observed in T cells lacking the Tek kinase, Itk (Liu et al., 1998). A more pronounced defect, however, was observed in mice bearing a double-knock-out of Itk and a second Tec member, Rlk (Schaeffer et al., 1999). It has been proposed that Tec kinases play a role in controlling the Ag receptor-induced PLCγ activation that lead to the sustained Ca^{2+} influx required for immune cell activation (Fluckiger et al., 1998; Liu et al., 1998; Takata and Kurosaki, 1996). Tec kinases are either constitutively (e.g., Rlk) or inducibly (e.g., Itk) associated with the lipid rafts via an acylation tail or the interaction with $PtsInsP_3$, respectively (Schaeffer and Schwartzberg, 2000). Interestingly, we have found that Lck and Rlk preferentially phosphorylate raft-compartmentalized PLCγ1 (Veri et al., 2001). This can occur either directly or sequentially, since the activation of Tec kinases requires their phosphorylation by a Src kinase such as Lck (Schaeffer and Schwartzberg, 2000). A goal for the future will be to define the specific roles this raft-associated kinase network exerts in the activation of PLCγ1 in B and T cells.

Summary

Mobilization of Ca^{2+} via activation of phospholipase Cγ1 (PLCγ1) is crucial for the activation of lymphocytes by antigens (Ag). Tyrosine phosphorylation of PLCγ1 ensues rapidly after the engagement of the Ag receptors of B (BCR) or T (TCR) lymphocytes and leads to its activation. The molecular mechanism that links PLCγ1 to the Ag receptors is still incompletely understood. The BCR and TCR have no intrinsic enzymatic activity, but are coupled to Src, Syk, and Tec tyrosine kinases as well as to adapters that function by assembling protein complexes that regulate downstream effectors including PLCγ1. Following Ag stimulation, PLCγ1 is inducibly translocated to specialized detergent-insoluble microdomains (lipid rafts) that are thought to facilitate the assembly of these multi-molecular protein complexes.

PLCγ1 has two Src-homology 2 (SH2) domains and a single SH3 domain that mediate protein interactions by binding phosphorylated tyrosine residues or proline-rich sequences, respectively. The PLCγ1 isoform is predominantly expressed and activated in T-lymphocytes, while both PLCγ1 and PLCγ2 isoforms are present and activated in B-lymphocytes. We have found that the SH domains of PLCγ1 play interrelated but distinct roles in coupling this enzyme to the Ag receptors. The amino terminal SH2 (SH2N) domain was absolutely required in Ag receptor-induced

membrane translocation, tyrosine phosphorylation, and enzymatic activation by mediating the ligand-dependent association of PLCγ1 with phosphorylated adapters. The SH3 domain contributed to optimal PLCγ1 membrane association. Additionally, the SH3 domain can interact with c-Cbl, a complex adapter that acted as a negative regulator of Ag receptor-induced PLCγ1 activation. Ag-induced phosphorylation of c-Cbl was required for its ability to inhibit PLCγ1, consistent with c-Cbl functioning as a negative feedback regulator of PLCγ1.

The role of lipid rafts in PLCγ1 activation was explored by forcing the enzyme to these microdomains via amino-terminal acylation. This led to the constitutive, receptor-independent phosphorylation and activation of the enzyme in a manner that evaded the requirement for several up-stream molecules involved in Ag receptor-induced activation of wild-type PLCγ1 in T-lymphocytes. In contrast, a raft-associated Src kinase, Lck, was required. Our findings support a model of Ag receptor-induced positive regulation of PLCγ1 via SH2N domain-mediated re-compartmentalization to lipid rafts, where it associates with regulatory molecules and tyrosine kinases. In addition, they indicate a negative feedback pathway mediated by c-Cbl that is dependent on PLCγ1 SH3 domain. In our studies, the carboxyl-terminal SH2 domain showed no detectable role in PLCγ1 translocation or phosphorylation. This domain, however, was necessary for Ag receptor-induced PLCγ1 activation by an as yet unknown mechanism which is the subject of ongoing studies.

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