Phospholipase C-γ1 (PLC-γ1) activation depends on a heterotrimeric complex of adaptor proteins composed of LAT, Gads, and SLP-76. Upon T cell receptor stimulation, a portion of PLC-γ1 is recruited to a detergent-resistant membrane fraction known as the glycosphingolipid-enriched membrane microdomains (GEMs), or lipid rafts, to which LAT is constitutively localized. In addition to LAT, PLC-γ1 GEM recruitment depended on SLP-76 and, in particular, required the Gads-binding domain of SLP-76. The N-terminal tyrosine phosphorylation sites and P-I region of SLP-76 were not required for PLC-γ1 GEM recruitment, but were required for PLC-γ1 phosphorylation at Tyr783. Thus, GEM recruitment can be insufficient for full activation of PLC-γ1 in the absence of a second SLP-76-mediated event. Indeed, a GEM-targeted derivative of PLC-γ1 depended on SLP-76 for T cell receptor-induced phosphorylation at Tyr783 and subsequent NFAT activation. On a biochemical level, SLP-76 inductively associated with both Vav and catalytically active ITK, which efficiently phosphorylated a PLC-γ1 fragment at Tyr783 \textit{in vitro}. Both associations were disrupted upon mutation of the N-terminal tyrosine phosphorylation sites of SLP-76. The P-I region deletion disrupted Vav association and reduced SLP-76-associated kinase activity. A smaller deletion within the P-I region, which does not impair PLC-γ1 activation, did not impair the association with Vav, but reduced SLP-76-associated kinase activity. These results provide new insight into the multiple roles of SLP-76 and the functional importance of its interactions with other signaling proteins.

A trio of hematopoiesis-specific adaptors, including LAT, Gads, and SLP-76, serve as pathway-specific regulators of phospholipase C-γ1 (PLC-γ1) (1). PLC-γ1 is a ubiquitous enzyme, regulated by phosphorylation at Tyr772 and Tyr783 (2-4) and involved in many different signaling pathways (5, 6). When activated, PLC-γ1 hydrolyzes phosphatidylinositol 4,5-bisphosphate, producing the second messengers phosphatidic acid and diacylglycerol, which trigger calcium flux and contribute to protein kinase C and Ras activation, respectively. In T cells, the coordinated activation of calcium- and Ras-dependent signaling pathways leads to the activation of NFAT, a regulator of interleukin-2 transcription (7, 8). Given the importance of PLC-γ1-dependent signaling events, it is not surprising that the activation of PLC-γ1 is tightly regulated.

The LAT, Gads, and SLP-76 adaptor proteins appear to constitute an integrated signaling unit that couples immunoreceptor-mediated activation of cytoplasmic tyrosine kinases to the activation of PLC-γ1 (1, 9). The use of three adaptor proteins to regulate one enzyme is a recurrent theme in complex signaling pathways. In a similar fashion, three adaptors (FRS2, Grb2, and Gab1) cooperate to activate phosphatidylinositol-3-kinase in the fibroblast growth factor receptor signaling pathway (10, 11). Evidence that LAT, Gads, and SLP-76 work in concert includes their similar expression patterns and localization to the same signaling complex; similar signaling defects observed in LAT- and SLP-76-deficient T cell lines; and similar T cell and mast cell phenotypes observed in LAT-, SLP-76-, and Gads-deficient mice (1, 9, 12). Most convincingly, SLP-76 alone cannot reconstitute B cell receptor signaling in a B cell line lacking the SLP-76 analog BLNK (B cell linker); rather, cotransfection of SLP-76, LAT, and Gads is required (13, 14). Over the past years, the mechanisms whereby these adaptors mediate the activation of PLC-γ1 are beginning to be understood, as summarized briefly below.

LAT, a transmembrane adaptor protein, is constitutively localized to specialized membrane microdomains known as glycosphingolipid-enriched membrane domains (GEMs), lipid rafts, or detergent-insoluble glycolipid-enriched membrane domains (15, 16). Upon T cell receptor (TCR) stimulation, LAT is heavily tyrosine-phosphorylated (17, 18), and a motif encompassing LAT phospho-Tyr132 (19–22) binds with nanomolar affinity (23) to the N-terminal SH2 domain of PLC-γ1 (24, 25).

Mutation of either the N-terminal SH2 domain of PLC-γ1 or its
ef{1} This work was supported by the Rappaport Family Institute for Research in the Medical Sciences and by the Technion V.P.R. Research Fund-Charles Krown Research Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡1 Present address: AGES PharmMed, A-1030 Vienna, Austria.

‡2 To whom correspondence should be addressed: Rappaport Faculty of Medicine, Technion-Israel Inst. of Technology, P. O. Box 9649 Bat Galim, Haifa 31096, Israel. Tel.: 972-4-829-5393; Fax: 972-4-829-5255; E-mail: debyag@tx.technion.ac.il.

§1 The abbreviations used are: LAT, linker for activation of T cells; Gads, Grb2-related adaptor downstream of Src; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa; PLC-γ1, phospholipase C-γ1; NFAT, nuclear factor of activated T cells; GEMs, glycosphingolipid-enriched membrane domains; TCR, T cell receptor; SH, Src homology; ITK, interleukin-2-inducible T cell tyrosine kinase; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; MES, 4-morpholineethanesulfonic acid; MBS, MES-buffered saline; GST, glutathione S-transferase; PMA, phorbol 12-myristate 13-acetate.
binding site on LAT greatly reduces TCR-induced tyrosine phosphorylation of PLC-γ1 (20–22, 24, 25), suggesting that recruitment to LAT is essential for PLC-γ1 activation. The PLC-γ1/LAT interaction may serve to recruit PLC-γ1 to the GEMs. Indeed, mutation of the GEM-targeting motif within LAT prevents both tyrosine phosphorylation of LAT and activation of PLC-γ1 (15, 16), whereas fusion of a GEM-targeting motif to PLC-γ1 confers a degree of constitutive activity to the enzyme (26). Nevertheless, LAT is insufficient to activate PLC-γ1 in the absence of SLP-76 (27), which is recruited to LAT indirectly via Gads (9).

SLP-76 consists of three domains that mediate interactions with many different signaling proteins: an N-terminal acidic domain containing three tyrosine phosphorylation sites, a large central proline-rich region, and a C-terminal SH2 domain (1). PLC-γ1 activation depends on the N-terminal tyrosine phosphorylation sites and the Gads-binding site, found within the proline-rich domain of SLP-76 (28–31), whereas the SH2 domain of SLP-76 is largely dispensable for PLC-γ activation (28–30), but contributes to antigen-induced T cell proliferation (29, 30).

Located between the tyrosine phosphorylation sites and the Gads-binding site, a portion of the proline-rich domain known as the P-I region is also required for PLC-γ1 activation (23, 28, 32). Whereas deletion of the P-I region abrogates PLC-γ1 activation, a smaller deletion within this region, denoted ΔP-Ib+++, does not impair PLC-γ1 activation (33). Both deletions equally impair the binding of SLP-76 to the SH3 domain of PLC-γ1 (33), and to date, the biochemical basis for the strikingly different functional consequences of these two similar deletions has not been established.

Among the proteins that bind to SLP-76, Vav and ITK appear to be involved, directly or indirectly, in the activation of PLC-γ1. Mice lacking either Vav or ITK exhibit reduced TCR-induced phosphorylation and activation of PLC-γ1 (34, 35). Furthermore, both Vav and Rlk (a kinase related to ITK) can synergize with SLP-76 to activate the NFAT transcription factor (36, 37). It is currently not clear whether Vav and ITK act independently or as part of an SLP-76-nucleated complex. Their observed co-immunoprecipitation with SLP-76 is consistent with the second possibility; however, both proteins bind SLP-76 with very low stoichiometry. Indeed, some studies have suggested that SLP-76 may function independently of its binding to Vav (38, 39).

Given the presence of SLP-76 in the LAT-nucleated, GEM-localized signaling complex, we wondered whether SLP-76 might contribute to the TCR-induced GEM localization of PLC-γ1 and what the significance of this contribution might be. Here, we show that SLP-76 is required for efficient GEM recruitment of PLC-γ1. Nonetheless, GEM recruitment is insufficient to activate PLC-γ1 in the absence of a second SLP-76-mediated function required for PLC-γ1 phosphorylation at Tyr783. Consistent with the second function, we detected an SLP-76-associated kinase activity (identified as ITK) that efficiently phosphorylated a recombinant PLC-γ1 fragment at Tyr783. This activity also appeared to be insufficient for full activation of PLC-γ1, and we present evidence suggesting that the full activity of SLP-76 depends on its assuming a conformation capable of associating with Vav.

**Experimental Procedures**

**Antibodies and Reagents**—Monoclonal antibody C305 (specific for the Jurkat T β-chain) (40) was used for anti-TCR stimulations. Monoclonal antibodies 3F10 (Roche Applied Science) and 12CA5 were used to detect hemagglutinin (HA)-tagged PLC-γ1. Rabbit anti-LAT antibody was the generous gift of Larry Samelson or was purchased from Upstate. Anti-FLAG antibody M2 was from Sigma. Anti-Akt phospho-Ser473 and anti-ERK were from Cell Signaling Technology. Rabbit anti-PLC-γ1 and anti-Vav antibodies, mouse anti-PTEN and anti-ITK (2F12) monoclonal antibodies, and protein A/G-Sepharose beads were from Santa Cruz Biotechnology, Inc. Rabbit anti-ITK antibody BL12 was provided by Michael G. Tomlinson and Joseph Bolen. Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate. Anti-PLC-γ1 phospho-Tyr783, anti-LAT phospho-Tyr132, and anti-SLP-76 phospho-Tyr145 antibodies were from BIOSOURCE. Horseradish peroxidase-conjugated chola toxin subunit B was from Calbiochem. Anti-Lck monoclonal antibody 1F6 was generously provided by Arthur Weiss.

**Plasmids**—C-terminally HA-tagged forms of PLC-γ1 were generously provided by Ezio Bonvini and Barbara L. Reallah, including pCIneo-PLCγ1-HA (wild-type), pCIneo-Palm-PLCγ1-HA (palmitoylated), and pCIneo-(GC→AS)Palm-PLCγ1-HA (bearing a mutationally inactivated palmitoylation signal) (26). The NFAT-luciferase reporter construct, in which the expression of luciferase is driven by multiple copies of the NFAT DNA-binding element, was a gift from G. Crabtree (Stanford University).

**Cell Culture and Transfections**—All cell lines used derive from the Jurkat T cell line and have been described previously. These include J14, an SLP-76-deficient cell line (27); J.CaM2.5, a LAT-deficient cell line (41); stable transfectants of J14 expressing FLAG-tagged wild-type or mutant forms of SLP-76 (27, 28, 33); and a stable transfectant of J.CaM2.5 expressing wild-type LAT (41). Stable transfectants of Jurkat cells that inducibly express either wild-type PTEN or phosphatase-dead forms (42) were generously provided by Arthur Weiss. All cells were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum, penicillin, streptomycin, and glucose. Stable transfectants were maintained in the same medium but with 10% fetal calf serum and 2 mg/ml G418, which was washed out 2–7 days prior to the use of cells for experiments. Transient transfections were performed by electroporation using the Gene Pulser (Bio-Rad) at a setting of 234 V and 1000 microfarads using a 0.4-cm cuvette (Bio-Rad).

**Isolation of GEMs**—Previously described protocols for GEM isolation (16, 43, 44) were slightly modified; we used the detergent Brij-58 (Pierce), which preserves GEM integrity better than Triton X-100 (45). Briefly, 100 × 10^6 cells were washed, preheated to 37 °C for 10 min, and stimulated for 1 min with anti-TCR antibody C305 or mock-stimulated in 0.5 ml of Dulbecco’s phosphate-buffered saline. Cells were lysed by the addition of an equal volume of ice-cold 2× MES-buffered saline (MBS) with Brij-58 and protease inhibitors to achieve a final concentration of 1 × MBS (25 mM MES (pH 7.0), 150 mM NaCl, 50 mM NaF, and 2 mM orthovanadate) supplemented with 1%.
Brij-58, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 2 μg/ml pepstatin, and 4 mM phenylmethylsulfonyl fluoride. Following 20 min on ice, lysates were homogenized with 10 strokes of a Teflon homogenizer and centrifuged at 2200 rpm for 7 min at 4 °C in a microcentrifuge. One ml of supernatant was transferred to an ultracentrifuge tube, mixed with an equal volume of 80% (w/v) sucrose dissolved in 1 X MBS, overlaid with 2 ml of 30% sucrose in 1 X MBS followed by 1 ml of 5% sucrose in 1 X MBS, and centrifuged in an SW 55Ti rotor at 260,000 x g for 18 h at 4 °C. Twelve 400-μl fractions were collected from top to bottom and analyzed by Western blotting.

**Cell Lysis and Immunoprecipitation**—Cells were stimulated for 1 min and lysed at a concentration of 10^6 cells/ml of lysis buffer essentially as described (33). For co-immunoprecipitation experiments, lysis buffer contained 1% n-dodecyl β-d-maltoside (Calbiochem), 150 mM NaCl, 50 mM Tris (pH 7.5), 50 mM NaF, 2 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mM EDTA, 1 μg/ml pepstatin, and 2 mM phenylmethylsulfonyl fluoride. For in vitro kinase experiments, lysis buffer contained 20 mM HEPES (pH 7.3), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM Na3VO4, 10 μg/ml aprotinin, 2 mM EDTA, 10 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride. After 20 min at 4 °C, lysates were centrifuged 13,000 rpm for 10 min at 4 °C in a microcentrifuge, and the supernatants were cleared by centrifugation at 137,000 x g for 11 min at 4 °C prior to immunoprecipitation.

**In Vitro Kinase Assay**—Anti-FLAG immune complexes (prepared from cleared lysates of 15 × 10^6 cells) were washed twice with lysis buffer and once with kinase reaction buffer (25 mM HEPES (pH 7.3), 7.5 mM MgCl2, and 1 mM Na3VO4). Beads were resuspended in 30 μl of kinase reaction buffer containing 100 ng of recombinant GST-PLC-γ1SH2-SH3 fusion protein (28) and preheated for 2 min at 30 °C. Reactions were initiated by the addition of ATP to a final concentration of 1 μM and terminated after 15 min by the addition of EDTA to 12.5 mM. Supernatants were collected, separated by SDS-PAGE, and probed by Western blotting with anti-PLC-γ1 and probed by Western blotting with anti-PLC-γ1.

**Scanning and Densitometry**—ECL films were scanned under conditions of transillumination. In all cases in which a figure subsection contains multiple panels probed with the same antibody, all panels were scanned from the same exposure of the same Western blot and were subsequently cropped into separate sections for clarity. Exposures used for densitometry were chosen such that there was no saturation of the bands to be quantified.

**RESULTS**

**LAT-mediated Recruitment of PLC-γ1 to the GEMs**—Upon TCR stimulation, there is a measurable increase in the amount of PLC-γ1 found in a detergent-resistant buoyant membrane fraction known as the GEMs (16, 43). GEM recruitment of PLC-γ1 peaked at ~1 min following TCR stimulation (data not shown), approximately coinciding with peak tyrosine phosphorylation of LAT. These kinetics suggested that GEM recruitment of PLC-γ1 may reflect its SH2 domain-mediated binding to tyrosine-phosphorylated LAT (19–25). However, PLC-γ1 also contains a pleckstrin homology domain, which can contribute to the membrane recruitment and activation of PLC-γ1 (11, 46) by binding to phosphatidylinositol 3,4,5-trisphosphate (11), present at high levels in Jurkat-derived cell lines due to their lack of expression of the phosphatidylinositol 3-phosphatase PTEN (47). It was therefore not apparent which mechanism is primarily responsible for the TCR-induced GEM recruitment of PLC-γ1.

To test the involvement of LAT, we isolated GEM fractions from LAT-deficient or LAT-reconstituted J.CaM2 cells prior to or following TCR stimulation. TCR-induced recruitment of PLC-γ1 to the GEMs was abrogated in the LAT-deficient cell line, but restored in LAT-reconstituted cells (Fig. 1A). To test whether PTEN deficiency is a contributing factor in PLC-γ1 GEM recruitment, we utilized a Jurkat-derived cell line stably transfected with wild-type PTEN under the control of a tetra-
cycloheximide-inducible promoter (42). TCR-induced recruitment of PLC-γ1 to the GEMs was unaffected by expression of PTEN (Fig. 1B), despite a marked reduction in the basal level of phospho-Akt (Fig. 1C), indicative of reduced basal levels of phosphatidylinositol 3,4,5-trisphosphate. There was no appreciable difference in GEM recruitment of PLC-γ1 between cells expressing wild-type or catalytically inactive PTEN (Fig. 1B). These results suggest that TCR-induced GEM recruitment of PLC-γ1 primarily reflects the inducible binding of PLC-γ1 to LAT and is not an artifact of PTEN deficiency.

**GEM Recruitment of PLC-γ1 Depends on SLP-76**—SLP-76 forms part of the LAT-nucleated signaling complex and is required to mediate TCR-induced activation of PLC-γ1 by an incompletely understood mechanism. TCR-induced GEM recruitment of PLC-γ1 was markedly impaired in the SLP-76-deficient cell line, J14, compared with a paired J14-derived cell line stably reconstituted with wild-type SLP-76 (Fig. 2A, upper two panels). In contrast to their reduced PLC-γ1 content, GEMs isolated from SLP-76-deficient cells consistently contained at least as much GM1, Lck, and LAT as did GEM fractions from SLP-76-expressing cells, whereas ERK was excluded from the GEMs in both cell types (Fig. 2A). Furthermore, SLP-76 was not required for efficient TCR-induced phosphorylation of LAT at Tyr132 (Fig. 2B), the tyrosine that binds to the SH2 domain of PLC-γ1. Together, these data suggest that the GEM fraction of J14 cells is intact; nonetheless, GEM-localized, tyrosine-phosphorylated LAT is insufficient to mediate the efficient GEM recruitment of PLC-γ1 in the absence of SLP-76.

**SLP-76 Acts within the LAT-nucleated Signaling Complex to Promote PLC-γ1 GEM Recruitment**—To identify the domains of SLP-76 that are required for PLC-γ1 GEM recruitment, we used previously described J14-derived cell lines, each stably reconstituted with a different mutant allele of SLP-76 (28, 33). Previous studies showed that TCR-induced activation of PLC-γ1 is profoundly impaired upon mutation of the N-terminal tyrosine phosphorylation sites of SLP-76 (SLP-76(Y3F)), deletion of the Gads-binding site (SLP-76ΔGad), or deletion of a 67-amino acid proline-rich region designated the P-I region (SLP-76ΔP-I). We therefore focused our studies on these three mutants, including, for comparison, a fourth mutant (SLP-76ΔP-ΔI) that bears a smaller deletion within the P-I region, but retains the ability to activate PLC-γ1. As technical considerations precluded the preparation of GEMs from more than three cell lines at a time, we assayed each mutant-bearing cell line separately, comparing the TCR-induced change in PLC-γ1 GEM recruitment. SLP-76 and SLP-76-reconstituted J14 wild-type SLP-76-reconstituted cells assayed side by side. Twelve μl of each fraction was used for detection of Lck, LAT, and ERK in the GEM (fractions 2 and 3) and non-GEM (fraction 10) fractions were detected by Western blotting (WB), and the indicated cell lines (J14 (SLP-76ΔP-I) and SLP-76-reconstituted J14 (SLP-76ΔP-I′)) were mock-stimulated or stimulated for 1 min with anti-TCR antibody prior to fractionation of GEMs as depicted in the legend to Fig. 1A. The presence of PLC-γ1, Lck, LAT, and ERK in the GEMs (fractions 2 and 3) and non-GEM (fraction 10) fractions was detected by Western blotting (WB). Fractions 2–3 (110 μl) and fraction 10 (12 μl) were used for detection of PLC-γ1. Twelve μl of each fraction was used for detection of Lck, LAT, and ERK. GM1 was detected by spotting 1 μl of each fraction onto nitrocellulose, followed by detection with horseradish peroxidase-conjugated cholera toxin subunit B. This experiment was repeated at least 10 times with similar results. B, GEM fractions from the experiment shown in A were resolved by SDS-PAGE and probed with anti-LAT phospho-Tyr132 antibody (α-pLAT-Y132, upper panels), followed by stripping and reprobing with anti-LAT antibody (lower panels). This experiment was repeated four times with similar results.

![Figure 2](image)

FIGURE 2. SLP-76 modulates TCR-induced recruitment of PLC-γ1 to the GEMs. A, the indicated cell lines (J14 (SLP-76ΔP-I) and SLP-76-reconstituted J14 (SLP-76ΔP-I′)) were mock-stimulated or stimulated for 1 min with anti-TCR antibody prior to fractionation of GEMs as described in the legend to Fig. 1A. The presence of PLC-γ1, Lck, LAT, and ERK in the GEMs (fractions 2 and 3) and non-GEM (fraction 10) fractions was detected by Western blotting (WB). Fractions 2–3 (110 μl) and fraction 10 (12 μl) were used for detection of PLC-γ1. Twelve μl of each fraction was used for detection of Lck, LAT, and ERK. GM1 was detected by spotting 1 μl of each fraction onto nitrocellulose, followed by detection with horseradish peroxidase-conjugated cholera toxin subunit B. This experiment was repeated at least 10 times with similar results. B, GEM fractions from the experiment shown in A were resolved by SDS-PAGE and probed with anti-LAT phospho-Tyr132 antibody (α-pLAT-Y132, upper panels), followed by stripping and reprobing with anti-LAT antibody (lower panels). This experiment was repeated four times with similar results.

contrast, SLP-76ΔGads failed to support efficient TCR-induced GEM recruitment of PLC-γ1 (Fig. 3D). Densitometric analysis of 2–10 experiments for each cell line revealed that the amount of GEM-localized PLC-γ1 found in TCR-stimulated cells expressing SLP-76ΔGads did not exceed that observed in SLP-76-deficient cells; in contrast, the Y3F, ΔP-I, and ΔP-I′ mutants mediated PLC-γ1 GEM recruitment that was not significantly different from that mediated by wild-type SLP-76 (Fig. 3E).

Notably, SLP-76ΔGads-expressing cells exhibited intact LAT-containing GEMs and TCR-inducible phosphorylation of LAT at Tyr132 (Fig. 3F). Thus, recruitment of PLC-γ1 to the GEMs depends on the SLP-76/Gads interaction, which mediates recruitment of SLP-76 to LAT. Within the LAT-nucleated complex, SLP-76 promotes GEM localization of PLC-γ1 via
PLC-γ1 is recruited to GEMs upon TCR stimulation, whereas nearly 90% of total PLC-γ1 was found in the non-GEM fractions.

A similar analysis of cells expressing the Y3F or ΔP-1 allele of SLP-76 revealed that both alleles are severely impaired in their ability to mediate the phosphorylation of both GEM-localized and non-GEM-localized PLC-γ1 at Tyr783 (Fig. 4C, upper panels). These results provide evidence that GEM recruitment of PLC-γ1 can be insufficient to trigger its phosphorylation at Tyr783 because even GEM-recruited PLC-γ1 was not efficiently phosphorylated in cells expressing the Y3F or ΔP-1 mutant of SLP-76.

To further test this conclusion, we investigated whether SLP-76 is required to mediate the phosphorylation of a PLC-γ1 construct (referred to as Palm-PLC) that is constitutively localized to the GEMs by virtue of an N-terminal palmitoylation signal (26). Palm-PLC bound inducibly to LAT upon TCR stimulation (Fig. 5A), suggesting that it should be fully accessible to any TCR-induced tyrosine kinases found within the GEMs or within the LAT-nucleated signaling complex. Nonetheless, TCR-induced phosphorylation of Palm-PLC at Tyr783 was significantly reduced in J14 cells compared with SLP-76-reconstituted cells (Fig. 5B, left panels). Indeed, Palm-PLC resembled endogenous wild-type PLC-γ1 in its TCR-inducible, SLP-76-dependent phosphorylation at Tyr783 (Fig. 5B, compare left and right panels). These results strongly support the conclusion that SLP-76 is required, following the GEM recruitment of PLC-γ1, to mediate its phosphorylation at Tyr783.

One of the downstream consequences of PLC-γ1 phosphorylation and activation is activation of the NFAT transcription factor. To probe the significance of PLC-γ1 phosphorylation as opposed to PLC-γ1 GEM recruitment, we assessed the ability of Palm-PLC to mediate NFAT activation in the presence or absence of SLP-76. As described previously (26), Palm-PLC exhibited a degree of constitutive activity and could mediate SLP-76-independent activation of the NFAT transcription factor in unstimulated cells (Fig. 6A, left) or phorbol 12-myristate 13-acetate (PMA)-treated cells (Fig. 6A, middle). Nonetheless, the level of constitutive NFAT activation achieved by Palm-PLC was severalfold lower than that observed upon TCR stimulation of wild-type cells, suggesting that GEM localization may be insufficient to fully activate PLC-γ1. Despite equal expression in the two cell lines (Fig. 6B), Palm-PLC did not support TCR-induced activation of NFAT in the absence of SLP-76 (Fig. 6C).
6A, right). Thus, Palm-PLC exhibits a low level of SLP-76-independent constitutive activity, which is apparent in unstimulated and PMA-stimulated cells, but depends on SLP-76 to achieve the full level of activity triggered by TCR stimulation. These results are consistent with the idea that GEM recruitment is insufficient to couple PLC-γ1 to TCR-induced tyrosine kinases; rather, SLP-76 is required to facilitate the phosphorylation of GEM-recruited PLC-γ1, leading to its full activation.

**Role of ITK and Vav in SLP-76-mediated Phosphorylation of PLC-γ1**—ITK and Vav are two signaling proteins that inducibly associate with SLP-76 (36, 49) and have been implicated in the phosphorylation and activation of PLC-γ1 (34, 35). We immunoprecipitated FLAG-tagged SLP-76 from the lysates of TCR-stimulated or unstimulated cells and confirmed that ITK inducibly associates with SLP-76 (Fig. 7A, second panel, lanes 1 and 2).

For more sensitive detection of SLP-76-associated ITK, we used an immune complex in vitro kinase assay. Upon immunoprecipitation of FLAG-tagged SLP-76, we observed a TCR-inducible, SLP-76-associated kinase activity that phosphorylated a recombinant PLC-γ1 fragment at Tyr783 in vitro (Fig. 7A, upper panel, lanes 1 and 2). An activity with identical catalytic specificity was observed upon immunoprecipitation of ITK (Fig. 7A, lanes 5 and 6). The SLP-76-associated activity was definitively identified as ITK because removal of ITK by immunoprecipitating abolished the SLP-76-associated kinase activity (Fig. 7A).

We then tested a number of SLP-76 mutants to determine whether the observed SLP-76-associated kinase activity could account for the ability of SLP-76 to mediate PLC-γ1 phosphorylation in intact cells (Fig. 7B, upper panel). No activity co-immunoprecipitated with the Y3F mutant, consistent with the inability of this mutant to mediate TCR-induced phosphorylation of PLC-γ1. A reduced but clearly detectable kinase activity associated with the ΔP-I and ΔP-Ib++ mutants. Similarly, a reduced but clearly detectable association of the ΔP-I and ΔP-Ib++ mutants with ITK protein was observed (Fig. 7B, middle panel). This result is consistent with the inducible phosphorylation of both SLP-76ΔP-I and SLP-76ΔP-Ib++ at Tyr783 (Fig. 7C, third panel from top), which has been suggested to bind to ITK (50). The similar association of the ΔP-I and ΔP-Ib++ mutants with ITK protein and kinase activity was unexpected in light of their dramatically differing ability to mediate PLC-γ1 phosphorylation in intact cells (compare Figs. 4A and 7B).

To obtain further insight, we examined other protein interactions mediated by SLP-76. SLP-76ΔP-I exhibited a moderately reduced TCR-inducible association with PLC-γ1, whereas SLP-76ΔP-Ib++ associated with PLC-γ1 as efficiently as did wild-type SLP-76 (Fig. 7C, first panel). More strikingly, both the...
ΔP-I and Y3F mutations abrogated the interaction of SLP-76 with Vav, whereas the ΔP-Ib++ mutation did not reduce this association (Fig. 7C, second panel). Of all the interactions we tested, the interaction of SLP-76 with Vav is the parameter that best correlates with the ability of SLP-76 to mediate PLC-γ1 phosphorylation in intact cells. Further research will be required to determine whether a quaternary complex containing SLP-76, PLC-γ1, Vav, and catalytically active ITK may be required to mediate TCR-induced phosphorylation of PLC-γ1 at Tyr783.

**DISCUSSION**

This study furthers our understanding of the ways in which SLP-76, Gads, and LAT cooperate to bring about the GEM localization and activation of PLC-γ1. We have shown that SLP-76 has two genetically separable roles that depend on different domains of the adaptor. SLP-76 facilitates the LAT-mediated recruitment of PLC-γ1 to the GEMs and also promotes the phosphorylation of PLC-γ1 at Tyr783. Tyr783 is one of two tyrosines that are necessary for PLC-γ1 activation (4); indeed, evidence suggests that its phosphorylation triggers a conformational change that is important for PLC-γ1 activation (2). We suggest that Tyr783 of PLC-γ1 is phosphorylated by SLP-76-associated catalytically active ITK; however, the full biological activity of SLP-76 may also depend on its ability to associate with Vav.

Cooperative Interactions between SLP-76 and LAT in PLC-γ1 GEM Recruitment—LAT is a GEM-localized adaptor (15, 16) that binds directly to PLC-γ1 via a monovalent moderate affinity interaction involving the N-terminal SH2 domain of PLC-γ1 and Tyr132 of LAT (19–25). As expected, LAT was required for TCR-induced recruitment of PLC-γ1 to the GEMs. Notwithstanding the centrality of LAT, GEM-localized, Tyr132-phosphorylated LAT was insufficient for PLC-γ1 GEM recruitment in the absence of SLP-76. This function of SLP-76 depended on its binding site for Gads, which bridges the binding of SLP-76 to LAT, thereby recruiting SLP-76 to the GEMs (9, 32, 44). We therefore suggest that SLP-76 acts within the LAT complex to facilitate the stable binding of PLC-γ1 to the LAT-nucleated complex.

None of the interactions mediated by the N-terminal tyrosine phosphorylation sites or P-I region of SLP-76 were required to promote PLC-γ1 GEM recruitment. This observation may represent a functional redundancy in these SLP-76 domains such that either one of the domains is sufficient to promote PLC-γ1 GEM recruitment. Alternatively, PLC-γ1 GEM recruitment may be mediated by other regions of SLP-76 that were not perturbed by these mutations. In particular, SLP-76 contains a very large proline-rich region encompassing 250 amino acids, only a small part of which is removed by the ΔP-I mutation. This region has the potential to mediate multiple, perhaps low affinity interactions with the SH3 domain of PLC-γ1 (51) or with other regions of PLC-γ1. In principle, such low affinity interactions could promote the retention of
Dual Role of SLP-76 in PLC-γ1 Activation

FIGURE 7. An SLP-76-associated kinase activity phosphorylates PLC-γ1 at Tyr783. A, J14 cells stably reconstituted with FLAG-tagged SLP-76 were mock-stimulated or stimulated for 1 min with anti-TCR antibody and lysed. Lysates were precleared by three rounds of immunoprecipitation (IP) with anti-FLAG antibody and were left alone as indicated. Anti-FLAG or anti-ITK immune complexes were then prepared from the lysates of 20 × 10^6 cells (for anti-FLAG) or 5 × 10^6 cells (for anti-ITK) and assayed for kinase activity using the GST-PLC-γ1 phospho-Tyr783 antibody (α-pY783, upper panel). Immune complex beads were probed with anti-FLAG antibody (middle panel). Lysates were blotted for FLAG to confirm that the preclearing did not remove substantial amounts of SLP-76 (lower panel). This experiment is representative of three. B and C, J14-derived cell lines stably expressing the indicated FLAG-tagged alleles of SLP-76 were mock-stimulated or stimulated for 1 min with anti-TCR antibody and lysed. A, anti-FLAG immune complexes prepared from lysates of 15 × 10^6 cells were assayed for associated kinase activity (upper panel) as described for A. Immune complex beads were probed with anti-ITK antibody (middle panel) and then stripped and probed with anti-FLAG antibody (lower panel). A representative experiment of five is shown. In all five experiments, the phosphorylation of GST-PLC-γ1 was quantified by densitometry and normalized to the result obtained using wild-type SLP-76 (WT) in the same experiment. The graph depicts the average normalized activity; error bars indicate the S.D. The Y3F mutant was not included in this quantitative analysis because it did not display any detectable associated kinase activity in any of the experiments. C, anti-FLAG immune complexes prepared from lysates of 25 × 10^6 cells were probed for co-immunoprecipitating PLC-γ1 and Vav and then stripped and reprobed with anti-FLAG phospho-Tyr145 antibody (α-pY145). Total cell lysates were probed with anti-FLAG antibody. This experiment was repeated at least six times with similar results.

PLC-γ1 in the vicinity of LAT, where it would be available for rebinding following transient detachment from LAT. Alternatively, by virtue of its presence within the LAT-nucleated signaling complex, SLP-76 may provide steric interference with the diffusion of PLC-γ1 out of the complex. Because the binding of Gads to SLP-76 can induce a conformational change in the proline-rich region of SLP-76 (23), it is possible that SLP-76 wraps around PLC-γ1, thereby locking it into the complex. Additionally, the binding of SLP-76 to the LAT-nucleated complex may induce a conformational change in LAT that could increase its binding affinity for PLC-γ1. All of the above mechanisms are consistent with the dependence of the GEM-recruiting function of SLP-76 on its Gads-binding site and its independence of other known functional sites within SLP-76.

This study expands on previous observations that together support the notion of cooperative interactions between LAT, recruitment of PLC-γ1. In our hands, we found no difference in Cbl expression between SLP-76-deficient and SLP-76-reconstituted J14 cells, and SLP-76 expression did not affect the TCR-induced association of Cbl with PLC-γ1 (data not shown).

Biological Significance of PLC-γ1 GEM Recruitment—The functional significance of the GEMs has been a topic of much controversy (54). GEMs are operationally defined as a buoyant fraction of the plasma membrane that is relatively resistant to non-ionic detergent extraction. In essence, the GEM flotation procedure yields a fraction of the cell membrane that contains very little protein, but is highly enriched in LAT; thus, it essentially reflects the TCR-induced binding of PLC-γ1 to LAT. This assay may more accurately reflect LAT-mediated interactions compared with our previously published co-immunoprecipitation assays (27) because the
highly concentrated immobilized antibodies used for immuno-precipitation can potentially nucleate de novo formation of complexes post-lysis.

It remains controversial whether the GEMs exist as an identifiable submembrane compartment in live cells or are an artifact of the detergent extraction process, which nonetheless reflects the biophysical properties of the membrane components and interactions between proteins that partition into the GEM fractions (55). Relevant to this controversy, a recent study by Zhang and co-workers (56) demonstrated that a membrane targeted but non-GEM-localized chimera including the cytoplasmic domain of LAT supports TCR signaling in LAT-deficient cells. Together with recent imaging studies (57), the study of Zhang et al. supports the notion that the protein/protein interactions mediated by LAT at the plasma membrane are more important than its GEM localization.

Our study does not relate directly to the current controversy over the importance of GEMs. Rather, we utilized GEM localization as a way of visualizing the protein/protein interactions between PLC-γ1 and LAT and demonstrating the contribution of SLP-76 to this process. Although SLP-76 was required for GEM recruitment of PLC-γ1, this function of SLP-76 was not sufficient to mediate activation of PLC-γ1 because two inactive alleles of SLP-76 nonetheless mediated efficient PLC-γ1 GEM recruitment. Strikingly, even a constitutively GEM-targeted allele of PLC-γ1 that retained the ability to interact inducibly with LAT depended on SLP-76 for its TCR-induced phosphorylation at Tyr783 and activation. Thus, although potentially important, GEM recruitment of PLC-γ1 is clearly not sufficient for its activation.

Role of SLP-76 and Its Associated Signaling Proteins in PLC-γ1 Phosphorylation at Tyr783—The functional significance of the interactions mediated by SLP-76 may be illuminated by mutational analysis, comparing the biochemical characteristics of each SLP-76 mutant with its ability to mediate PLC-γ1 activation. The Y3F and ΔP-I mutations abrogate TCR-induced phosphorylation of PLC-γ1 at Tyr783 (this study), as well as TCR-induced calcium flux and NFAT activation (28, 32, 33, 48). In contrast, the ΔP-Ib+ mutations, which removes a smaller portion of the P-I region, does not substantially affect TCR-induced phosphorylation of PLC-γ1 at Tyr783, calcium flux, or NFAT activation (this study and Ref. 33). Based on the strong correlation between Tyr783 phosphorylation and PLC-γ1 activation, we suggest that Tyr783 phosphorylation is the primary means by which SLP-76 regulates PLC-γ1 activation. Consistent with this notion, we identified an SLP-76-associated kinase activity that phosphorylates a PLC-γ1 fragment at Tyr783 in vitro. Immunoprecipitation experiments identified this activity as ITK. ITK can bind weakly to SLP-76 (this study and Refs. 49 and 50) and is required for the activation of PLC-γ1 (35). Thus, it is tempting to speculate that SLP-76 may activate PLC-γ1 by regulating the ITK-mediated phosphorylation of PLC-γ1 at Tyr783.

Mutational analysis uncovered the structural requirements for the binding of SLP-76 to ITK. The N-terminal tyrosine phosphorylation sites of SLP-76 were absolutely required for its association with ITK, consistent with the previously demonstrated binding of the SH2 domain of ITK to SLP-76 (49, 50). The ΔP-I and ΔP-Ib+ deletions reduced to a similar extent but did not eliminate the association of SLP-76 with ITK protein and activity. ITK was suggested previously to bind to SLP-76 via a bivalent interaction involving both Tyr145 of SLP-76 and residues 184–208, found within the proline-rich P-I region of SLP-76 (49, 50). This model was based primarily on the in vitro binding of recombinant fragments of ITK to peptides derived from SLP-76 (50). Our results now validate this model using full-length proteins because both the Y3F and ΔP-I mutations decreased the association of SLP-76 with ITK.

Unexpectedly, the above analysis revealed similar binding of the ΔP-I and ΔP-Ib+ mutants to ITK, despite their strikingly different functionality. A previous analysis also did not uncover any significant biochemical differences between the ΔP-I and ΔP-Ib+ mutants of SLP-76, which might explain their strikingly different ability to mediate PLC-γ1 activation (33). Both proteins bind constitutively to Gads; are inducibly phosphorylated upon TCR stimulation, including phosphorylation at Tyr145; and fail to bind the SH3 domain of PLC-γ1 (33). Because both proteins also show similar association with ITK protein and kinase activity, we suggest that the association of SLP-76 with catalytically active ITK may be necessary but is not sufficient for PLC-γ1 phosphorylation and activation.

In addition to binding active ITK, SLP-76 may be required to correctly juxtapose the active site of ITK to Tyr783 within the context of full-length PLC-γ1. The association of SLP-76 with PLC-γ1 is largely indirect and is attributable to the inducible binding of both PLC-γ1 and the Gads-SLP-76 complex to LAT (28), but also depends on Vav (34). The ΔP-I (but not ΔP-Ib+) deletion abrogated the association of SLP-76 with Vav. This result was surprising because Vav binds to the N-terminal tyrosine phosphorylation sites of SLP-76 (39), which are not affected by either deletion. We previously provided evidence that the P-I region may serve as an essential spacer that is required for the proper functioning of other regions of SLP-76 (33). Thus, SLP-76ΔP-I may assume a conformation that is inconsistent with binding to Vav. Together with published evidence of the contribution of Vav to PLC-γ1 activation (34), our study suggests that a quaternary complex consisting of SLP-76, Vav, ITK, and PLC-γ1 may be required to mediate the phosphorylation and activation of PLC-γ1 in intact cells.

Acknowledgments—We thank Arthur Weiss, Zheng Xu, Ezio Bonvini, Barbara L. Relihan, Larry Samelson, Michael G. Tomlinson, and Joseph Bolen for generously providing some of the reagents used in this study and Ron Wange for input concerning the measurement of ITK kinase activity. We also thank Ofer Shenkar of the Interdepartmental Equipment Facility and all members of the Yablonski laboratory for support and constructive input.

REFERENCES

1. Yablonski, D., and Weiss, A. (2001) *Adv. Immunol.* 79, 93–128
2. Poulin, B., Sekiya, F., and Rhee, S. G. (2005) *Proc. Natl. Acad. Sci. U. S. A.* 102, 4276–4281
3. Kim, H. K., Kim, J. W., Zilberstein, A., Margolis, B., Kim, J. G., Schlessinger, J., and Rhee, S. G. (1991) *Cell* 65, 435–441
4. Serrano, C. J., Graham, L., DeBell, K., Rawat, R., Veri, M.-C., Bonvini, E., Relihan, B. L., and Reischl, I. G. (2005) *J. Immunol.* 174, 6233–6237
5. Rhee, S. G. (2001) *Annu. Rev. Biochem.* 70, 281–312
