T Cell Receptor-induced Activation of Phospholipase C-γ1 Depends on a Sequence-independent Function of the P-I Region of SLP-76

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SLP-76 forms part of a hematopoietic-specific adaptor protein complex, and is absolutely required for T cell development and activation. T cell receptor (TCR)-induced activation of phospholipase C-γ1 (PLC-γ1) depends on three features of SLP-76: the N-terminal tyrosine phosphorylation sites, the Gads-binding site, and an intervening sequence, denoted the P-I region, which binds to the SH3 domain of PLC-γ1 (SH3PLC) via a low affinity interaction. Despite extensive research, the mechanism whereby SLP-76 regulates PLC-γ1 remains uncertain. In this study, we uncover and explore an apparent paradox: whereas the P-I region as a whole is essential for TCR-induced activation of PLC-γ1 and nuclear factor of activated T cells (NFAT), no particular part of this region is absolutely required. To better understand the contribution of the P-I region to PLC-γ1 activation, we mapped the PLC-γ1-binding site within the region, and created a SLP-76 mutant that fails to bind SH3PLC, but is fully functional, mediating TCR-induced phosphorylation of PLC-γ1 at tyrosine 783, calcium flux, and nuclear factor of activated T cells activation. Unexpectedly, full functionality of this mutant was maintained even under less than optimal stimulation conditions, such as a low concentration of the anti-TCR antibody. Another SLP-76 mutant, in which the P-I region was scrambled to abolish any sequence-dependent protein-binding motifs, also retained significant functionality. Our results demonstrate that SLP-76 need not interact with SH3PLC to activate PLC-γ1, and further suggest that the P-I region of SLP-76 serves a structural role that is sequence-independent and is not directly related to protein-protein interactions.

A central early event of the T cell antigen receptor (TCR) signaling pathway is the activation of PLC-γ1. PLC-γ1 catalyzes the formation of 2 second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, which, respectively, trigger the formation of 2 second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, which, respectively, trigger the activation of calcium- and Ras-dependent signaling pathways leading to the activation of nuclear factor of activated T cells (NFAT), a regulator of interleukin-2 transcription (3, 4).

Activation of PLC-γ1 is tightly regulated by at least three hematopoietic-specific adaptor proteins: LAT, Gads, and SLP-76 (5, 6). Upon TCR stimulation, the membrane-anchored adaptor protein, LAT, is heavily tyrosine phosphorylated, and associates with the N-terminal SH2 domain of PLC-γ1 (7–9). This interaction recruits PLC-γ1 to the membrane where it interacts with upstream activators and with the inositol phospholipids that are its substrate (10, 11). Recruitment of PLC-γ1 to the membrane is accompanied by its phosphorylation at tyrosines 771, 783, and 1254 (12, 13). Whereas PLC-γ1 binds directly to LAT, SLP-76 is recruited to LAT by the Grb-2-family adaptor protein, Gads (14, 15). In the absence of SLP-76, PLC-γ1 is still recruited to LAT, but is not efficiently tyrosine phosphorylated or detectably activated, indicating that SLP-76-dependent regulatory events are required to activate PLC-γ1, within the LAT-nucleated complex (16). Such SLP-76-dependent activation of PLC-γ1 is common to a number of signaling pathways initiated by immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptors, including the TCR, Fce receptor, glycoprotein VI collagen receptor, and the Fcγ receptor (16–20).

SLP-76 consists of three domains capable of mediating intermolecular interactions: an N-terminal acidic domain containing three tyrosine phosphorylation sites, a central proline-rich region, and a C-terminal SH2 domain (21, 22). Through these domains, SLP-76 can interact with a large number of signaling proteins (5). The functional importance of each of the domains has been explored by reconstituting SLP-76-deficient mice, bone marrow-derived mast cells, or the SLP-76-deficient T cell line, J14, with various mutant alleles of SLP-76 (23–26). Individual mutation of each domain results in a somewhat different phenotype, suggesting that SLP-76 may exert more than one distinct signaling function. In all cases where a strictly PLC-γ-dependent outcome, such as calcium flux, was measured, the studies support the conclusion that the N-terminal tyrosine phosphorylation sites and Gads-binding site of SLP-76 make a significant contribution to the activation of PLC-γ1 (23–26). By contrast, the SH2 domain is largely dispensable for PLC-γ activation (23, 25, 26), but nonetheless, contributes to important biological responses, such as antigen-induced T cell proliferation (23, 26).

In addition to the above, a 67-amino acid stretch of SLP-76, known as the P-I region, is required for TCR-induced activation of PLC-γ1 and NFAT (25). Located between the N-terminal tyrosine phosphorylation sites and the Gads-binding site of SLP-76, the proline-rich, P-I region can bind to the SH3 domain of PLC-γ1 (SH3PLC) (25). These findings are consistent with the notion that an interaction between SH3PLC and the P-I region

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‡ The abbreviations used are: TCR, T cell antigen receptor; Gads, Grb2-related adaptor downstream of Shc; GST, glutathione S-transferase; LAT, linker for activation of T cells; NFAT, nuclear factor of activated T cells; PLC-γ1, phospholipase C-γ1; SH3PLC, SH3 domain of PLC-γ1; SLP-76, SH2 domain-containing leukocyte protein of 76 kDa.
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of SLP-76 may contribute to the activation of PLC-γ1. However, other interpretations are possible. For example, the P-I region may mediate an essential interaction with another signaling protein that participates in the regulation of PLC-γ1 (27, 28); alternatively, the P-I region may serve an essential structural role required to support the proper function of other regions of SLP-76.

In the present study, we have taken a closer look at the P-I region of SLP-76, with the goal of distinguishing between the above hypotheses. Although we have reproduced the previously reported results concerning the P-I deletion, a detailed structure-function analysis of this region suggests that there are no essential protein-protein interactions mediated by the P-I region of SLP-76. Rather, this region appears to serve a sequence-independent function, and may act as a spacer, or hinge region that facilitates the active conformation of other functional regions of SLP-76.

EXPERIMENTAL PROCEDURES

Antibodies—The monoclonal antibody C305 was used for anti-TCR stimulation (29). M2 (anti-FLAG epitope) was from Sigma. Polyclonal anti-PLC-γ1 was from Santa Cruz Biotechnology. Anti-PLC-γ1 mixed monoclonal antibodies, anti-Gads polyclonal antibody, and anti-phosphotyrosine monoclonal antibody (4G10) were from Upstate Cell Signaling Solutions. Phospho-specific anti-PLC-γ1 (pY687) and phospho-specific anti-PLC-γ1 (pY685) were from BIOSOURCE. A rabbit polyclonal antiserum was raised against full-length, recombiant human SLP-76, using the Sigma custom antisera service.

Plasmids—All human SLP-76 alleles were FLAG-tagged at the N terminus and were subcloned into pEFBos (30) for transient transfections, or into pAWneo3 for stable transfections. The SLP-76 PI mutant (Δ157–223) was described previously (25). SLP-76Δ-P-I (Δ158–180), SLP-76Δ-P-Il (Δ181–203), SLP-76Δ-P-I (Δ203–223), and SLP-76Δ-P-I (Δ177–212) were created using a standard overlapping PCR technique. Construction of the overlapping PCR technique was used to create SLP-76-scrambled. Briefly, amino acids 177–212 were deleted, and the remaining residues of the P-I region (amino acids 157–176 and 213–223) were scrambled in a random order. Table I compares the amino acid sequence of the scrambled region to the corresponding region of SLP-76Δ-P-Ia, ... . All PCR-generated constructs were verified by sequencing. pRSu-FLAG-PTEN (31) was a gift from R. Wange (National Institute of Health). The SLP-76 luciferase reporter construct was a gift from G. Crabtree (Stanford University).

Cell Culture and Transfections—The SLP-76-deficient cell line, J14, its SLP-76-reconstituted derivative, J14-76-11, and the SLP-76Δ-P-I-reconstituted derivative have been described previously (16, 25). J14 cells were stably transfected by electroporation with SLP-76Δ-P-Ia, ..., following transfection of J14 cells by limiting dilutions in RPMI medium supplemented with 10% fetal calf serum and 2 mg/ml G418 (Calbiochem). Three clones expressing different levels of SLP-76Δ-P-Ia, ..., and similar levels of surface TCR were chosen for further analysis. Transient transfections were performed by electroporation using the Gene Pulser (Bio-Rad Laboratories), at a setting of 234 V and 1000 microfarads, using a 0.4-cm cuvette (Bio-Rad).

GST Fusion Protein—All glutathione S-transferase (GST) fusion proteins were expressed in Escherichia coli BL21 bacteria, using the pGEX-2TK bacterial expression vector (Amersham Biosciences), purified on glutathione-agarose beads (Sigma) by standard procedures (32), and used to “pull-down” associating proteins from precleared cell lysates as described previously (25). PCR was used to amplify different fragments of SLP-76 prior to subcloning into pGEX-2TK (see Fig. 4A for details). Constructs encoding GST-SH3b ( GST-SH3b (ΔN-terminus)) were generously provided by Ottmar Janssens (University Hospital Kiel, Kiel, Germany). The construct encoding GST-SH3b (SH3b was constructed previously (25).

Cell Lysis and Immunoprecipitation—Jurkat cells were washed in phosphate-buffered saline containing CaCl2 and MgCl2 (Dulbecco’s phosphate-buffered saline), preheated to 37 °C for 10 min, stimulated or mock stimulated as indicated, collected, and lysed at 106 cells/ml in cold lysis buffer containing 1% n-dodecyl-β-D-maltoside (Calbiochem), 50 mM Tris, pH 7.5, and 150 mM NaCl, supplemented with 50 mM NaF, 2 mM Na3VO4, 10 μg/ml aprotinin, 0.5 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 2 mM phenylmethylsulfonyl fluoride. After 20 min at 4 °C, lysates were centrifuged at 4 °C in a microcentrifuge at 13,000 rpm for 10 min. Supernatants were used directly for immunoprecipitation, followed by SDS-PAGE electrophoresis and Western blotting, performed using standard procedures.

Luciferase Assay—Cells were transiently cotransfected with 15 μg of anti-TCR reporter plasmid along with the plasmids indicated in the figure legends. 16–20 h later cells were aliquoted into a 96-well cell culture dish at 2 × 105 cells/well and stimulated for 6 h at 37 °C, with the stimuli indicated in the figure legends. Cells were lysed and assayed for luciferase activity as described previously (33). To correct for variations in transfection efficiency, the NFAT luciferase activity obtained upon receptor stimulation was normalized to the activity obtained upon treatment with phorbol 12-myristate 13-acetate (50 ng/ml) plus ionomycin (1 μM).

Intracellular Calcium—Cells were loaded with the fluorescent calcium indicator dye Indo-1 (Molecular Probes), washed, and resuspended in calcium buffer, consisting of 25 mM Hepes (pH 7.4), 1 mM CaCl2, 125 mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 0.5 mM MgCl2, 1 glifter glucose, and 100 μg/ml bovine serum albumin. A Victor 2 fluorimeter (PerkinElmer Life Sciences) was used for kinetic measurement of fluorescence at 37 °C, with excitation at 355 nm and emission measured at 405 and 486 nm. An automatic injector was used to stimulate cells with anti-TCR (C305) followed by stimulation with 1 mM thapsigargin (Sigma), at the time points indicated in the figure. Calcium concentration was calculated based upon the ratio of F405/F486 (as described (34)).

RESULTS

The Functional Importance of the P-I Region of SLP-76 Is Not Attributable to Any Particular Part of the Region—Previous studies have identified a functionally important segment, denoted the P-I region and encompassing residues 157–223 of SLP-76, that binds to the SH3 domain of PLC-γ1 and is required for TCR-mediated activation of PLC-γ1 (25). The P-I region consists of 67 amino acids, 19 of which are proline. To identify functional motifs within this region, three new SLP-76 deletion mutants (ΔP-Ib, ..., ΔP-Ic, each lacking one-third of the P-I region (Fig. 1A), were transiently transfected into the SLP-76-deficient cell line, J14, and TCR-induced activation of the NFAT transcription factor was measured (Fig. 1B). All SLP-76 alleles were expressed at levels similar to or higher than the wild-type protein with the exception of the ΔP-Ic mutant, which was consistently expressed at lower levels (data not shown). Unexpectedly, each of the mutants bearing these smaller deletions retained significant activity, reflected in 10–40-fold activation of the NFAT-luciferase reporter, upon TCR stimulation. Whereas none of the mutants was as active as wild-type SLP-76, none showed the striking lack of activity seen in the P-I deletion mutant.

SLP-76 Can Mediate Activation of NFAT, in the Absence of a Binding Site for SH3P-PTC—The above experiments were quite puzzling. Although deletion of the entire P-I region abrogates SLP-76 function, no particular part of the P-I region is absolutely required for SLP-76 function. We therefore created a fourth mutant, denoted ΔP-Ib, ..., in which a deletion of residues 177–212 of SLP-76 results in the removal of 15 of the 19 proline residues of the P-I region (Fig. 1A). When transiently transfected into J14 cells, ΔP-Ib, ..., resembled ΔP-Ia, ..., and ΔP-Ic, in that it retained a significant ability to mediate TCR-induced NFAT activation (Fig. 1B). The ΔP-Ib, ..., mutant was stably transfected into J14 cells, and three clones that expressed different levels of SLP-76Δ-P-Ib, ..., (Fig. 2A, lower panel) but approximately equivalent levels of surface TCR (data not shown) were tested for TCR-induced activation of NFAT. Strikingly, SLP-76Δ-P-Ib, ...,-reconstituted J14 cells exhibited NFAT activation to the same extent as wild-type SLP-76-reconstituted J14 cells (Fig. 2A). This result was observed in all three clones, including two that expressed a lower level of SLP-76 than the wild-type-reconstituted cells. Clone 4, which most closely matched the wild-type-reconstituted cells in the level of SLP-76 expression, was used in all subsequent experiments. Despite clear evidence that the ΔP-Ib, ...,-reconstituted cells have not regained expression of wild-
type SLP-76 (Fig. 2B), the response of SLP-76ΔP-Ib++-reconstituted J14 was similar to wild-type SLP-76-reconstituted J14 cells over a wide range of doses of the stimulatory anti-TCR antibody (Fig. 2C).

Whereas the ΔP-Ib++ mutant appeared fully functional, the slightly larger deletion found in the ΔP-I mutant renders SLP-76 completely non-functional. To explore the biochemical basis for this difference, we tested the major known biochemical attributes of SLP-76. Both mutants were inducibly tyrosine phosphorylated upon TCR stimulation. No differences were noted in the kinetics of tyrosine phosphorylation of these mutants, relative to wild-type SLP-76, whether we looked at their overall tyrosine phosphorylation (using 4G10) or site-specific phosphorylation (using an antiserum specific to phospho-Tyr145 of SLP-76) (Fig. 3C). Furthermore, both mutants bound constitutively to Gads, as does wild-type SLP-76 (Fig. 3A). Consistent with the ability of Gads to recruit SLP-76 into the LAT-nucleated signaling complex (25), both the ΔP-I and the ΔP-Ib++ mutants were observed in anti-PLC-γ1 immune complexes, when isolated from TCR-stimulated cells (data not shown). By contrast, the sensitivity of the co-immunoprecipitation assay did not permit detection of basal interactions between PLC-γ1 and SLP-76 in unstimulated cells. To test the potential for direct, SH3-mediated interactions, we therefore turned to GST pull-down experiments, which revealed that ΔP-Ib++ closely resembled the ΔP-I mutant in its binding characteristics to various SH3 domains. Like SLP-76ΔP-I, SLP-76ΔP-Ib++ bound to the C-terminal SH3 domain of Gads, and to the Lck SH3 domain, but did not bind to the PLC-γ1 SH3 domain (Fig. 3B). Thus, we have generated two SLP-76 mutants, which are similar in their biochemical characteristics, but exhibit a remarkable difference in functionality.

Mapping the PLC-γ1 Binding Site within the P-I Region of SLP-76—To localize the PLC-γ1 binding site within the P-I region of SLP-76, the GST fusion constructs shown in Fig. 4A were tested for their ability to bind PLC-γ1 (Fig. 4B). Whereas GST fused to the entire P-I region (residues 157–223) interacted with PLC-γ1, preliminary mapping showed that fragments including the N-terminal (residues 150–176) or C-terminal (residues 194–223) portion of the P-I domain, did not bind PLC-γ1, despite an equivalent amount of the GST fusion protein (Fig. 4B). All fragments including residues 170–196 of SLP-76 bound PLC-γ1; however, a fragment containing residues 170–191 did not. We conclude that SH3PLC binds to the P-I domain of SLP-76, via a single binding site, located at the C-terminal end of fragment 170–196 of SLP-76. Indeed, residues 186–197 of SLP-76, PPVPQPRMAAL, closely resemble the canonical PLC-γ1 SH3 binding site, described by Sparks et al. (PPVPPRPZXNL) (35).

It is important to note that the PLC-γ1-binding site identified in these experiments is deleted in the ΔP-Ib++, mutant (Fig. 4A). Consistent with this finding, the ΔP-Ib++ mutant fails to bind to the SH3 domain of PLC-γ1 (Fig. 3B), nonetheless, this mutant remains functional (Fig. 2). Taken together the above results demonstrate that interaction of the PLC-γ1 SH3 domain with SLP-76 is not required to mediate TCR-induced NFAT activation.

Activation of PLC-γ1 by the ΔP-Ib++, Mutant of SLP-76—Robust activation of NFAT by a SLP-76 mutant that fails to bind PLC-γ1 was an unexpected finding. To verify that SLP-76ΔP-Ib++-mediated NFAT activation is indeed associated with activation of PLC-γ1; we assessed the ability of the mutant to mediate TCR-induced tyrosine phosphorylation of PLC-γ1. Because overall tyrosine phosphorylation of PLC-γ1 does not change between PLC-γ1 and PLC-γ1ΔP-Ib++, we considered the possibility that SLP-76ΔP-Ib++ may have an effect on PLC-γ1 activity in the absence of NFAT activation.
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not always correlate well with activation (24, 25, 36, 37), we examined phosphorylation of tyrosine 783 of PLC-γ1, a phosphorylation site required for activation (12), using phosphospecific antibodies. Over a range of stimulation times, TCR-induced activity of these and other SLP-76 mutants (data not shown).

Consistent with this result, TCR-induced calcium flux was of similar magnitude in SLP-76ΔP-Ib++-reconstituted J14 cells as compared with wild-type-reconstituted cells (Fig. 5B). By contrast, J14 cells or SLP-76ΔP-I-reconstituted J14 cells exhibited very low TCR-induced calcium flux. Thus, the ΔP-Ib++ mutant of SLP-76 mediates a level of activation of PLC-γ1 that is sufficient for normal, TCR-induced calcium flux. To test whether the lack of PTEN in Jurkat cells (31) is responsible for the unexpected activity of the ΔP-Ib++ mutant, we tested the functionality of the ΔP-Ib++-reconstituted cells following transient transfection with PTEN. Even upon expression of PTEN, wild-type and ΔP-Ib++-reconstituted cells exhibited similar TCR-induced NFAT activation (data not shown). Taken together, the above findings are inconsistent with the notion that SLP-76 must bind to PLC-γ1 to activate it. We conclude that the ability of the P-I region of SLP-76 to bind SH3PLC-γ1 does not underlie the defect in function seen upon deletion of this region.

The P-I Region of SLP-76 Does Not Have an Essential Binding Partner—Once we demonstrated that the P-I region need not bind PLC-γ1, we considered the possibility that this region may not mediate any essential protein-protein interactions, but may serve a structural role, which is dependent on a minimal length of the region and not on any specific sequence motif. To test this hypothesis, we created a new SLP-76 mutant, in which residues 177–212 were deleted (as in the ΔP-Ib++ mutation) and the remainder of the P-I region was randomly scrambled, to destroy any putative sequence-dependent protein-protein interaction motifs, while retaining any sequence-independent functions. The sequence of the scrambled region is shown in Table I. The scrambled construct was transiently transfected into J14 cells, and its ability to mediate NFAT activation was measured. SLP-76-scrambled was expressed as well or better than the other constructs, and was able to mediate TCR-induced activation of NFAT (Fig. 6). Whereas this construct...
phospho-PLC-Immunoprecipitates were resolved on SDS-PAGE and probed with anti-deletion mutants, /H9004 the scrambled mutant appears to be as active as the smaller was at least 7-fold more active than the /H11003 from the lysates of 8 8368 same type of NFAT assay following transient expression (com- pare Fig. 1B). Lysates from each time point were probed with anti-TCR (bottom), B, the indicated GST fusion proteins were used to affinity purify associating proteins from the lysates of 25 × 10⁶ Jurkat cells. Top panel, associating proteins or total cell lysates (from 0.3 × 10⁶ Jurkat cells) were separated by SDS-PAGE and probed with a mixture of anti-PLC-γ1 monoclonal antibodies. Bottom panel, I/15 of each reaction was separated on SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and stained with Amido Black, to visualize the relative amount of each fusion protein.

**FIG. 4.** Mapping the PLC-γ1-binding site within the P-I region of SLP-76. A, sequence of the P-I region of human SLP-76 (top). A sequence resembling the consenus PLC-γ1 SH3 domain-binding site (35) is shaded in gray. The residues deleted in SLP-76ΔP-Ib, are underlined. The indicated fragments (bottom) were expressed as GST fusion proteins, and purified on glutathione-agarose. Fragments that interacted with PLC-γ1 are hatched, and those that did not are shaded (see B). Fragments: a, residues 150–223; b, 157–223; c, 150–196; d, 150–176; e, 170–191; f, 170–196; g, 194–223. B, the indicated GST fusion proteins were used to affinity purify associating proteins from the lysates of 25 × 10⁶ Jurkat cells. Top panel, associating proteins or total cell lysates (from 0.3 × 10⁶ Jurkat cells) were separated by SDS-PAGE and probed with a mixture of anti-PLC-γ1 monoclonal antibodies. Bottom panel, I/15 of each reaction was separated on SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and stained with Amido Black, to visualize the relative amount of each fusion protein.

**FIG. 5.** SLP-76ΔP-Ib- mediate TCR-induced activation of PLC-γ1. A, the indicated cell lines were mock stimulated or stimulated for the indicated times with anti-TCR. PLC-γ1 was immunoprecipitated from the lysates of 8 × 10⁶ cells using anti-PLC-γ1 (Santa Cruz). Immunoprecipitates were resolved on SDS-PAGE and probed with anti-phospho-PLC-γ1-pY783 (top), then stripped and reprobed with anti-PLC-γ1 (middle). Lysates from each time point were probed with anti-FLAG to detect SLP-76 (bottom). B, the indicated cell lines were loaded with indo-1 and calcium concentration was measured in resting cells and following stimulation with anti-TCR (blue arrow) and thapsigargin (black arrow), which was used to control for cell viability and intact calcium stores.

**TABLE I**

| Sequence of the SLP-76-scrambled mutant |
|----------------------------------------|
| SLP-76 mutant | Sequence of region between SLP-76 residues Leu<sup>156</sup> and Asn<sup>24</sup> |
| SLP-76ΔP-Ib<sub>b</sub> | QNSILPAKPPNSMSTDQRTNHEEPRSR |
| (177–212) | |
| SLP-76-scrambled | ASDPISNPQERSHMQSSTPNLKERRFIR |

appeared to be slightly less potent than the ΔP-Ib- mutant, it was at least 7-fold more active than the ΔP-I mutant. Notably, the scrambled mutant appears to be as active as the smaller deletion mutants, ΔP-Ia, ΔP-Ic, and ΔP-Ib, when tested in the same type of NFAT assay following transient expression (compare Fig. 1B and 6). Based on these results we suggest that the function of the P-I region is at least partly sequence-indepen-

dent; that is, that the function of the P-I domain depends on a minimal length of this domain, but not on its exact sequence.

**DISCUSSION**

Many immunoreceptor tyrosine-based activation motif (ITAM)-containing receptor-initiated signaling pathways exhibit SLP-76-dependent activation of PLC-γ1 (16–20). In SLP-76-deficient T cells, PLC-γ1 is recruited to LAT, but is not activated (16). Previous studies demonstrated that PLC-γ1 activation depends on three domains of SLP-76: the N-terminal tyrosine phosphorylation sites, the Gads-binding site, and the P-I region (23–26). The striking functional defect produced by deletion of the P-I region has been attributed to its ability to bind PLC-γ1 (25). However, recently published results cast doubt on this interpretation, by showing that the interaction between the P-I domain of SLP-76 and the SH3 domain of PLC-γ1 is of very low affinity, and virtually undetectable at physiologic temperatures (38). In this study, we demonstrate that SLP-76 mediates TCR-induced phosphorylation of PLC-γ1...
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at tyrosine 783, by a mechanism that depends on a sequence-independent role played by the P-I region of SLP-76. Strikingly, this role of the P-I region does not depend on its ability to bind the SH3 domain of PLC-γ1.

To better understand the mechanistic basis for the importance of the P-I region, we undertook a functional analysis of smaller deletion mutations within this region. Each of the SLP-76 mutants bearing smaller deletions retains significant functionality, demonstrating that no particular part of the P-I region is functionally essential. This result could be interpreted to mean that PLC-γ1 can bind to multiple, redundant sites within the P-I region. To rule out this interpretation, we mapped the PLC-γ1 binding site within the P-I region of SLP-76 and found that it includes only one binding site for PLC-γ1, which resembles the previously reported SH3PLC binding site (35). This consensus site is completely removed in the SLP-76ΔP-Ia construct; nonetheless, this construct retains significant functionality. Furthermore, an enlarged form of this deletion (ΔP-Ib+) in which amino acids 177–212 were removed, results in the removal of 15 of the 19 prolines in the P-I domain, including all consensus SH3 domain-binding sites. We confirmed that SLP-76ΔP-Ib+ fails to bind to SH3PLC; nonetheless, this mutant mediates TCR-induced phosphorylation of PLC-γ1 at tyrosine 783, and calcium flux, to the same extent as wild-type SLP-76. Furthermore, this mutant mediates TCR-induced NFAT activation about as well as wild-type SLP-76, even under less than optimal conditions, such as upon expression of SLP-76ΔP-Ib+ at a lower level than wild-type SLP-76, or when low doses of anti-TCR antibody were used for stimulation, or in the presence of the lipid phosphatase PTEN. Together, our unexpected findings demonstrate that a direct interaction between SLP-76 and SH3PLC is not necessary to mediate TCR-induced activation of PLC-γ1. This conclusion is consistent with several reports, which demonstrate, in different experimental systems, that the SH3 domain of PLC-γ1 does not play an essential role in its activation (39–41).

It is important to note the overall biochemical similarity between SLP-76ΔP-I and SLP-76ΔP-Ib+. Both SLP-76 mutants are inductively tyrosine phosphorylated upon TCR stimulation, including phosphorylation at Tyr783, the tyrosine closest to the site of the deletion. Both mutants bind constitutively to Gads, and both fail to bind to the SH3 domain of PLC-γ1. Indeed, we did not identify any biochemical difference between these mutants; nonetheless, the ΔP-Ib+ mutant is completely inactive, whereas the ΔP-Ia+ mutant is active, suggesting that the known biochemical parameters do not fully reflect the physiologic function of SLP-76. Future studies will be required to identify the additional parameter, which is abrogated by a deletion of residues 157–223, but not by deletion of residues 177–212.

The unexpected functionality of the ΔP-Ia, ΔP-Ib, ΔP-Ia+, and ΔP-Ib+ mutants suggested to us that the P-I region may not mediate any essential SH3 interactions. To strengthen this conclusion, we removed amino acids 177–212 and scrambled the remaining amino acids in the P-I region, to abolish any remaining sequence-dependent protein–protein interaction motifs within the P-I region. Indeed, this “scrambled” mutant retained significant functionality, supporting the notion that the P-I region does not mediate any essential protein–protein interactions.

How can one explain that deletion of the P-I region results in a profound loss of function while a scrambled P-I region maintains significant functionality? Because the P-I domain is located between two essential functional sites, the N-terminal tyrosine phosphorylation sites and the Gads-binding domain, we suggest that the P-I region serves a structural role, perhaps as a spacer or hinge region. As such, the P-I region may facilitate the adoption of an active conformation by other regions of SLP-76 or it may provide for proper orientation of the different SLP-76 binding partners, relative to each other. Because its role is primarily structural, and appears to be sequence-independent, deletion of any portion of the P-I domain does not significantly impact function, provided the deleted region is not too large.

We have found that SLP-76 can activate PLC-γ1 in the absence of a direct interaction between SLP-76 and SH3PLC. Indeed, under all of the assay conditions we have tested, including upon expression of PTEN or upon stimulation with suboptimal concentrations of anti-TCR, we find that this interaction is not required. Nonetheless, it remains possible that an interaction between the P-I region of SLP-76 and SH3PLC may occur under some circumstances, and may represent a redundant mechanism for stabilizing the recruitment of PLC-γ1 and SLP-76 to the LAT complex. Support for this view can be gleaned from an experiment by Boerth et al. (42), who demonstrated that lipid raft-targeted SLP-76 can substitute for LAT in mediating TCR-induced NFAT activation. Although the mechanism whereby this construct activates PLC-γ1 has not been addressed, it seems reasonable to assume that PLC-γ1 must somehow be recruited to the lipid rafts, possibly via a direct interaction with SLP-76. Indeed, the P-I domain is essential for the function of raft-targeted SLP-76 (43), although whether this is because of its binding to PLC-γ1, or to its structural role remains to be determined.

Another circumstance in which the P-I-SH3PLC interaction may play an essential role is upon disruption of Gads-mediated recruitment of SLP-76 to LAT. Many studies, using independent methodologies, have shown that disruption of Gads-mediated recruitment of SLP-76 to LAT produces a milder phenotype than does deletion of SLP-76 itself (23, 25, 26, 36, 44). These results are consistent with the notion that there may be multiple, partially redundant mechanisms for recruiting SLP-76 to the LAT complex. Upon mutational inactivation of Gads-mediated recruitment, SLP-76 may be recruited to the LAT complex by virtue of its interaction with SH3PLC. Consistent with this idea, Zhang et al. (45) showed that mutational inactivation of the major PLC-γ1-binding site in LAT (Tyr1325) not only eliminates binding of PLC-γ1 to LAT, it also very significantly reduces binding of SLP-76 to LAT, suggesting that a web of interactions may, under some circumstances stabilize the recruitment of SLP-76 to LAT.

The P-I region is part of an extensive, extremely proline-rich segment of SLP-76, which spans 265 amino acids, nearly one-fourth of which are prolines. Whereas proline-rich regions often function as ligands for SH3 domains, we suggest that one should distinguish between two very different, and possibly complementary roles of proline-rich regions within adaptor proteins: on the one hand, their role as ligands for SH3 domains and on the other hand, their role as important structural elements.

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T Cell Receptor-induced Activation of Phospholipase C-γ1 Depends on a Sequence-independent Function of the P-I Region of SLP-76
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