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Phospholipase Cγ2 Plays a Role in TCR Signal Transduction and T Cell Selection

Guoping Fu,* Yuhong Chen,* James Schuman,†§ Demin Wang,*¶∥ and Renren Wen*

One of the important signaling events following TCR engagement is activation of phospholipase Cγ (PLCγ). PLCγ has two isoforms, PLCγ1 and PLCγ2. It is known that PLCγ1 is important for TCR signaling and TCR-mediated T cell selection and functions, whereas PLCγ2 is critical for BCR signal transduction and BCR-mediated B cell maturation and functions. In this study, we report that PLCγ2 was expressed in primary T cells, and became associated with linker for activated T cells and Src homology 2-domain containing leukocyte protein of 76 kDa and activated upon TCR stimulation. PLCγ1/PLCγ2 double-deficient T cells displayed further block from CD4 and CD8 double-positive to single-positive transition compared with PLCγ1 single-deficient T cells. TCR-mediated proliferation was further impaired in PLCγ1/PLCγ2 double-deficient T cells compared with PLCγ1 single-deficient T cells. TCR-mediated signal transduction, including Ca2+ mobilization and Erk activation, was further impaired in PLCγ1/PLCγ2 double-deficient relative to PLCγ1 single-deficient T cells. In addition, in HY TCR transgenic mouse model, thymic positive and negative selections were reduced in PLCγ1 heterozygous- and PLCγ2 homozygous-deficient (PLCγ1+/−/PLCγ2−/−) relative to wild-type, PLCγ2 single-deficient (PLCγ2−/−), or PLCγ1 heterozygous-deficient (PLCγ1+/-) mice. Taken together, these data demonstrate that PLCγ2 participates in TCR signal transduction and plays a role in T cell selection. The Journal of Immunology, 2012, 189: 2326–2332.

T cell progenitors in the thymus lack CD4 and CD8 expression and are called double-negative (DN) thymocytes. The DN cells are divided, based on their developmental stages, into DN1 (CD44+CD25−), DN2 (CD44+CD25+), and DN3 (CD44−CD25+) cells (1, 2). DN thymocytes develop into CD4 and CD8 double-positive (DP) thymocytes, which then differentiate into CD4 or CD8 single-positive (SP) cells. For αβ T cells, TCR β-chain is rearranged at DN3 stage, followed by α-chain rearrangement at DP stage (3, 4). DP thymocytes that have successfully rearranged both the TCR β- and α-chain genes express the α and β heterodimers that are in association with the CD3 molecules on their cell surface to form the TCR complex and are subjected to thymic selection (5). The random rearrangement of the TCR β- and α-chain genes results in a broad spectrum of affinity between the TCR and its ligand: the peptide/MHC (pep/MHC). Selection of DP thymocytes is based on the interaction affinity between the TCR and the pep/MHC complex presented by APCs in the thymic microenvironments (6). Thymocytes with low affinity between the TCR and the pep/MHC complex will die of neglect. Thymocytes with high affinity between the TCR and the pep/MHC complex are considered autoreactive and will be deleted (negative selection). Thymocytes with intermediate affinity between the TCR and the pep/MHC complex will further develop into CD4 and CD8 SP thymocytes (positive selection) (5, 7, 8). The signal emanating from the TCR signaling complex is the single most important driving force for the thymic selection.

The TCR transduces its signal through the CD3 molecules. Engagement of the TCR by the pep/MHC complex initiates the activation cascade of protein tyrosine kinases (PTKs), which in turn mediates the activation of a series of signaling molecules (9). Inositol phospholipid-specific PLCγ1 is a key signaling molecule that is activated at the early stages of TCR signal transduction. Following TCR engagement, PLCγ1 is recruited to the TCR signaling complex through two adaptor molecules, linker for activated T cells (LAT) and Src homology 2-domain containing leukocyte protein of 76 kDa (SLP-76) (10–13), and is phosphorylated on tyrosine residues (14–17), an indicator of PLCγ activation (18).

Activated PLCγ hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (19–21). DAG activates protein kinase C (PKC), and IP3 mediates calcium (Ca2+) mobilization. In T cells, DAG has also been shown to activate the Ras/ERK pathway through RasGRP (22–24). Whereas Ca2+ mobilization activates NFAT through calcineurin phosphate, PKC activates NF-κB and AP-1 through IκB kinase and JNK, respectively (25, 26). Among the pathways downstream of PLCγ, the Ras/Erk signaling cascade has been shown to be important for thymic positive selection (27–30).

PLCγ has two isoforms, PLCγ1 and PLCγ2, which share the same domain structure (21, 31). PLCγ1 is ubiquitously expressed, whereas PLCγ2 expression is restricted to the hematopoietic system (32). Studies showed that PLCγ1 is the predominant isoform expressed in both primary T cells and T cell lines (33), and it is activated following TCR stimulation (14–17). Immunodepletion of PLCγ1 following TCR stimulation essentially depletes PLCγ1 activity from Jurkat T cell protein extracts (16). Moreover, T cell-
specific deletion of PLCγ1 at DP stage impairs TCR signaling transduction, affects both positive and negative selection, causes severe reduction of SP thymocytes, and results in peripheral T cell lymphopenia (34), whereas PLCγ2 deficiency has no effect on T cell development and function (35). Based on these findings, it is generally thought that PLCγ1 is responsible for PLCγ activity downstream of TCR in T cells.

However, the observation that PLCγ1-deficient T cells exhibit residual Ca2+ influx in response to TCR stimulation implicates the involvement of other PLCγ isoform in TCR signal transduction (34). In this study, we found that PLCγ2, the other PLCγ isoform, was expressed in T cells and activated upon TCR stimulation. Importantly, PLCγ1/PLCγ2 double deficiency further blocked the transition of DP thymocytes to the SP stage compared with PLCγ1 single deficiency. TCR-mediated Ca2+ mobilization and Erk activation were completely abolished in PLCγ1/PLCγ2 double-deficient relative to PLCγ1 single-deficient T cells. Moreover, in the HY TCR transgenic mice, thymic positive and negative selections were reduced in PLCγ1 heterozygous- and PLCγ2 homozygous-deficient relative to wild-type, PLCγ2 single-deficient, or PLCγ1 heterozygous-deficient T cells. Taken together, these results demonstrate an unappreciated role of PLCγ2 in TCR signal transduction and TCR-mediated thymic selection.

Materials and Methods

Mice

HY TCR and CD4Cre transgenic mice were purchased from Taconic Laboratory. PLCγ1−/− and Rosa26-YFP mice were provided by G. Carpenter (Vanderbilt University, Nashville, TN) (36) and F. Costantini (Columbia University, New York, NY) (37), respectively. CD4Cre/PLCγ1−/− and PLCγ2−/− mice have been described previously (34, 35). Due to increased lethality of CD4Cre/PLCγ1−/− and PLCγ2−/− mice when they were backcrossed to B6 background, the mice in the study were backcrossed to B6 mice for only three to four generations before intercrossing. Mice were bred and maintained in the Biological Research Center of the Medical College of Wisconsin. All animal protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Western blotting analysis

For detecting PLCγ1 and PLCγ2, DN, DP, CD4SP, and CD8SP thymocytes, and CD4+ and CD8+ splenic T cells were sorted using FACSAria (BD Biosciences). Cells were lysed, and 20 μg of each sample was subjected to direct Western blotting analysis with Abs against PLCγ1 (sc-81; Santa Cruz Biotechnology) or PLCγ2 (sc-407; Santa Cruz Biotechnology). For detecting tyrosine phosphorylation of PLCγ1, splenic T cells were purified by negative selection using B220-conjugated magnetic beads and MACS columns (Miltenyi Biotec). Purified T cells were stimulated for 2 min on tissue culture plate coated with 10 μg/ml anti-CD3 Abs, and cells were lysed on the plate. PLCγ1 or PLCγ2 proteins in the cell lysates were immunoprecipitated with anti-PLCγ1 or anti-PLCγ2 Abs, respectively, and then subjected to Western blotting analysis using anti-phosphorylated tyrosine Ab (4G10; Millipore). For detecting PLCγ1 phosphorylation, 1 × 106 purified splenic T cells were used. For detecting PLCγ2 phosphorylation, 5 × 106 purified splenic T cells were used. For detecting phosphorylation of PLCγ2 at Y759 and interaction of PLCγ2 with SLP-76 and LAT, purified splenic T cells (3 × 106 cells at 5 × 106/ml) were coated with biotin-anti-CD3 (10 μg/ml), followed by cross-linking with streptavidin (8 μg/ml; Pierce) for the indicated time. A total of 50 μg of each cell lysate was subjected to Western blotting analysis for detecting phosphorylation at Y759 with a pY759 Ab (3874; Cell Signaling). The rest of the cell lysates were immunoprecipitated with anti-PLCγ2 Abs, followed by Western blotting analysis with an Ab that specifically recognizes SLP-76 (66-548; Millipore) or LAT (66-807; Millipore). For detecting Erk phosphorylation, FACS-sorted YFP+DP thymocytes or total thymocytes were coated with biotin anti-CD3, followed by cross-linking with streptavidin for the indicated time. The cells were lysed, and 50 μg of each cell lysate was subjected to direct Western blot analysis using anti-p-Erk (sc-7383; Santa Cruz Biotechnology) and anti-Erk (sc-93, sc-154; Santa Cruz Biotechnology) Abs.

Calcium flux analysis

Thymocytes (2 × 106) were resuspended in 1 ml PBS plus 2% FBS in the presence of 10 μg/ml indo-1AM (Invitrogen) at room temperature in the dark for 30 min. The cells were then incubated in 200 μl PBS plus 2% FBS in the presence of FITC-anti-CD4, PE-anti-CD8 Abs at room temperature in the dark for 15 min. After being washed, the cells were resuspended in 1 ml primary T cell culture medium at room temperature. The cells were run on a LSRII (BD Biosciences), and data were collected for 2 min. Biotin anti-CD3 Ab was then added to a final concentration of 20 μg/ml, and the cells were run and data were collected for 30 s. Following addition of streptavidin (Pierce) to a final concentration of 8 μg/ml to cross-link the TCR, the cells were run and data were collected for another 9 min.

In vitro negative selection

DP thymocytes were purified from 6-wk-old wild-type and PLCγ1−/−, PLCγ2−/− mice by sorting. A total of 1 × 106 DP thymocytes was cultured in 500 μl primary lymphocyte culture medium (RPMI 1640 supplemented with 10% FBS, 2 mM l-glutamine, 10 mM nonessential amino acid, 1 mM sodium pyruvate, 50 μM 2-ME) in a 24-well plate coated overnight with anti-CD3 (10 μg/ml) and anti-CD28 (50 μg/ml) for 20 h. The cells were then harvested and stained with propidium iodide (PI; 20 μg/ml) and analyzed by FACS.

Cell proliferation assay

Total splenocytes were enumerated and resuspended in PBS (2 × 106/ml). The cells were then labeled with 1 μM PKH Fluorescent Cell Linker Dye (PKH26, Sigma-Aldrich), according to the manufacturer’s recommendation. The cells were then washed twice and resuspended at 2 × 106/ml. The cells (0.5 ml) were cultured in a 48-well plate in medium alone, or stimulated with plate-bound anti-CD3 (2 μg/ml), plate-bound anti-CD2 (2 μg/ml) plus anti-CD28 (2 μg/ml), plate-bound anti-CD3 (2 μg/ml) plus IL-2 (10 U/ml), and PMA (10 ng/ml) plus ionomycin (500 ng/ml). Seventy-two hours later, the cells were harvested and stained with PE-anti-CD4 and allophycocyanin anti-CD8, followed by FACS analysis for cell proliferation.

Results

PLCγ2 is expressed in primary T cells and activated following TCR stimulation

PLCγ1-deficient T cells exhibit residual Ca2+ influx following TCR ligation, implicating that the other PLCγ isoform, PLCγ2, may also be involved in TCR signaling (34) (data not shown). To explore a potential role of PLCγ2 in TCR signaling, we first examined the expression levels of PLCγ2 in T cells at different developmental stages. PLCγ2 was expressed in DN, DP, CD4SP, CD8SP thymocytes, and CD4, CD8 splenic T cells, and the expression was highest in DN thymocytes (Fig. 1A). Of note, PLCγ1 expression level was consistent throughout T cell development (Fig. 1A). To determine whether PLCγ2 expressed in T cells is functional during TCR signaling, we assessed TCR-induced activation of PLCγ2. Upon anti-CD3 stimulation of purified splenic T cells, PLCγ2 was markedly phosphorylated on tyrosine residues (Fig. 1B), which is an indicator of PLCγ activation (18, 21). As expected, PLCγ1 was also phosphorylated on tyrosine residue following anti-CD3 stimulation (Fig. 1B). Phosphorylation of PLCγ2 at Y759, which is an activation-dependent phosphorylation site (38, 39), was also observed following TCR stimulation (Fig. 1C). In addition, TCR engagement induced the association of PLCγ2 with LAT and SLP-76 (Fig. 1D), suggesting that PLCγ2 is recruited to the TCR signaling complex using the same adaptor molecules employed by PLCγ1. Thus, PLCγ2 is expressed in T cells, is associated with LAT and SLP-76, and is activated upon TCR engagement.

PLCγ2 plays a role in T cell development and activation

PLCγ2 single deficiency has no detectable effect on T cell development or TCR-dependent activation, whereas PLCγ1 single deficiency markedly impairs TCR-mediated T cell development and function (34, 35). However, it is possible that PLCγ2 plays a role in TCR-mediated T cell development and activation, and the
role of PLCγ2 might be revealed when the expression of PLCγ1 is absent or reduced. Thus, we compared T cell development in mice deficient for both PLCγ1 and PLCγ2 with that in mice lacking only PLCγ1. To this end, we generated CD4Cre/YFP/PLCγ1fl/fl PLCγ2−/−, CD4Cre/YFP/PLCγ1fl/fl, and CD4Cre/YFP mice. In these mice, the CD4 enhancer/promoter/silencer initiates Cre expression at the thymic DP stage (40), and Cre-mediated YFP expression can track floxed PLCγ1 deletion (34, 37). Consistent with previous report (34), thymocytes from CD4Cre/YFP/PLCγ1fl/fl mice displayed a severe block at DP to SP transition, as shown in the increase of the percentage of YFP+DP cells and the reduction in the percentage and number of YFP+SP cells compared with those in wild-type CD4Cre/YFP mice (Fig. 2A, Table I). Importantly, the percentage of YFP*DP thymocytes was further increased, and the percentages and numbers of YFP* CD4SP and YFP*CD8SP thymocytes were further reduced in CD4Cre/YFP/PLCγ1fl/fl PLCγ2−/− mice compared with CD4Cre/YFP/PLCγ1fl/fl mice (Fig. 2A, Table I). Consistent with the defect in thymus, splenic YFP+CD4+ and YFP+CD8+ T cell numbers were significantly reduced in CD4Cre/YFP/PLCγ1fl/fl mice compared with wild-type CD4Cre/YFP mice (Table I), and were further reduced in CD4Cre/YFP/PLCγ1fl/fl PLCγ2−/− mice compared with CD4Cre/YFP/PLCγ1fl/fl mice (Table I). Percentage of YFP* CD8+ cells was also substantially reduced in CD4Cre/YFP/
Thymic DP to SP transition is driven by signals emanating from the MHC class I Db molecule (44). T cells with the HY transgenic TCR (34). HY TCR transgenic mice express a transgenic TCR that is positive and negative selection using the HY TCR transgenic model. TCR-mediated Erk activation in DP thymocytes is important for thymic negative selection in female wild-type mice, resulting in deletion of HY transgenic T cells. Because both PLCγ2 deficiency (35) and T cell-specific PLCγ1 deficiency (data not shown) caused certain level of lethality perinatally or several weeks after birth, the frequency of obtaining CD4Cre/PLCY1/fl/YFP/PLCY2−/− mice that also carry an HY TCR transgene was extremely low. However, we observed that T cells in PLCγ2−/− mice displayed reduced expression of PLCγ1 proteins (data not shown). Importantly, we observed that PLCγ1−/−PLCγ2−/− thymocytes showed reduced TCR-mediated Erk activation (Fig. 4A). Therefore, we examined thymic selection in PLCγ1−/−PLCγ2−/− HY mice, in which PLCγ2 was absent and PLCγ1 expression is reduced. The breeding was set up in a way to generate wild-type HY, PLCγ2−/−/HY, PLCγ1−/−/HY, and PLCγ1−/−PLCγ2−/−/HY mice and ensure each experimental mouse to carry one copy of HY transgene. The total thymocyte cell numbers were comparable in the four types of female HY transgenic mice (Table II). The percentages of CD8SP thymocytes and positively selected T3-70high SP cells were not significantly different in female wild-type HY, PLCγ2−/−/HY, and PLCγ1−/−/HY mice (Fig. 4B, Table II), indicating that absence of PLCγ2 alone or reduction of PLCγ1 PLCγ1−/−PLCγ2−/− mice compared with CD4Cre/YPFP/PLCY1/fl mice (Fig. 2B). T cells from CD4Cre/YPFP/PLCY1/fl mice also exhibited dramatic reduction of proliferation in response to stimulation with anti-CD3, anti-CD3/anti-CD28, or anti-CD3/IL-2 compared with those from CD4Cre/YPFP mice, and this proliferation was further reduced in T cells derived from CD4Cre/YPFP/PLCY1/fl/PLCY2−/− mice (Fig. 2C). These data demonstrate that PLCγ2 plays a role in TCR-mediated thymic DP to SP transition and TCR-mediated proliferation.

**PLCγ2 plays a role in TCR signal transduction in thymocytes**

Thymic DP to SP transition is driven by signals emanating from the TCR. Our observation that PLCγ2 played a role in thymic DP to SP transition suggested contribution of PLCγ2 to TCR signal transduction in thymocytes. To determine the role of PLCγ2 in TCR-mediated signaling, we examined TCR-regulated signaling events in DP thymocytes from CD4Cre/YPFP, CD4Cre/YPFP/PLCY1/fl, and CD4Cre/YPFP/PLCY1/fl/PLCY2−/− mice. YFP+DP thymocytes from CD4Cre/YPFP/PLCY1/fl mice displayed markedly reduced, but not abolished, TCR-induced Ca2+ flux compared with those derived from wild-type CD4Cre/YPFP mice (Fig. 3A). In contrast, YFP+DP thymocytes from CD4Cre/YPFP/PLCY1/fl mice were unable to elicit TCR-induced Ca2+ flux (Fig. 3A). Similarly, TCR-mediated Erk activation was substantially reduced in CD4Cre/YPFP/PLCY1/fl YFP+DP thymocytes, and was abolished in CD4Cre/YPFP/PLCY1/fl/PLCY2−/− YFP+DP thymocytes (Fig. 3B). Collectively, these data demonstrate that, in addition to PLCγ1, PLCγ2 plays a role in TCR-mediated signaling, and their functions are additive in TCR signal transduction in DP thymocytes.

**PLCγ2 plays a role in T cell selection**

TCR-mediated Erk activation in DP thymocytes is important for thymic positive selection (27–29, 41–43). Our observation that PLCγ2 played a role in thymic DP to SP transition (Fig. 2A, Table I) and TCR-mediated Erk activation in DP thymocytes (Fig. 3B) prompted us to examine the role of PLCγ2 in thymic positive selection. Previously, we reported that PLCγ1 plays a role in T cell positive and negative selection using the HY TCR transgenic model (34). HY TCR transgenic mice express a transgenic TCR that is specific for a peptide derived from the HY male Ag in the context of MHC class Iα molecule (44). T cells with the HY transgenic TCR are negatively selected in H-2b male mice, resulting in deletion of CD8+ T cells expressing the transgenic TCR. However, HY transgenic T cells are positively selected in female H-2k mice, leading to skewed development of thymocytes toward the CD8+ lineage. Selection of T cells bearing the transgenic TCR can be followed by the clonotypic Ab, T3-70 (45, 46).

**Table I. Analysis of YFP+ T cell populations in CD4Cre/YFP, CD4Cre/YFP/PLCY1/fl, and CD4Cre/YFP/PLCY1/fl/PLCY2−/− mice**

<table>
<thead>
<tr>
<th></th>
<th>Thymocytes</th>
<th>Spleenocytes</th>
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<tbody>
<tr>
<td></td>
<td>YFP*</td>
<td>YFP*CD4SP</td>
</tr>
<tr>
<td>CD4Cre/YFP</td>
<td>% 89 ± 3.5</td>
<td>12 ± 2.2</td>
</tr>
<tr>
<td>(n = 10)</td>
<td># (×10⁶)</td>
<td>77 ± 2.6</td>
</tr>
<tr>
<td>CD4Cre/YFP/PLCY1/fl</td>
<td>% 79 ± 3.9**</td>
<td>96 ± 0.6**</td>
</tr>
<tr>
<td>(n = 8)</td>
<td># (×10⁶)</td>
<td>105 ± 26</td>
</tr>
<tr>
<td>CD4Cre/YFP/PLCY1/fl/PLCY2−/−</td>
<td>% 84 ± 3.9</td>
<td>98 ± 0.49*</td>
</tr>
<tr>
<td>(n = 3)</td>
<td># (×10⁶)</td>
<td>57 ± 50</td>
</tr>
</tbody>
</table>

The age of the mice analyzed was between 6 wk and 4 mo. Data presented are average percentage (%) or absolute number (#) of each T cell subset. Percentage of YFP+ cells is gated in total lymphocytes, and other populations are gated in YFP+ lymphocytes. The p value (two tailed) was calculated by comparing the percentage or absolute number of each T cell subset from CD4Cre/YPFP mice with those from CD4Cre/YPFP/PLCY1/fl mice (annotations added to the data derived from CD4Cre/YPFP/PLCY1/fl mice), or by comparing the percentage or absolute number of each T cell subset from CD4Cre/YPFP mice with those from CD4Cre/YPFP/PLCY1/fl mice (annotations added to the data derived from CD4Cre/YPFP/PLCY1/fl mice). In this table, CD4Cre/YPFP/PLCY1/fl included CD4Cre/YPFP/PLCY1/fl and CD4Cre/YPFP/PLCY1/fl genotypes.

*p < 0.05, **p < 0.001.

FIGURE 3. Role of PLCγ2 in TCR-mediated signal transduction. Thymocytes were purified from CD4Cre/YPFP, CD4Cre/YPFP/PLCY1/fl, and CD4Cre/YPFP/PLCY1/fl/PLCY2−/− mice. (A) TCR-induced Ca2+ flux in PLCγ1/PLCγ2 double-deficient DP thymocytes. Thymocytes were loaded with indo-1 and were stained with Abs to CD4 and CD8. Biotin-conjugated anti-CD3 (first arrow) and subsequently streptavidin (second arrow) were added to the cells to cross-link TCR. Induction of Ca2+ flux was determined by LSR II. Ca2+ analysis was gated on YFP+DP thymocytes. (B) TCR-induced Erk activation in PLCγ1/PLCγ2 double-deficient DP thymocytes. FACS-sorted YFP+DP thymocytes were stimulated with biotin-conjugated anti-CD3 plus streptavidin. Cell lysates were subjected to SDS-PAGE, followed by Western blotting analysis with the indicated Ab. Data shown in (A) and (B) are a representative of two independent experiments.
FIGURE 4. Thymic selection in PLCγ1 heterozygous- and PLCγ2 homozygous-deficient mice. (A) TCR-induced Erk activation in PLCγ1WT/−/PLCγ2−/− thymocytes. Total thymocytes from wild-type and PLCγ1WT/−/PLCγ2−/− mice were stimulated with biotin-conjugated anti-CD3 plus streptavidin. Cell lysates were subjected to direct Western blotting analysis with the indicated Abs. Data are a representative of two independent experiments. (B) Positive selection in PLCγ1WT/−/PLCγ2−/− HY mice is impaired. CD4 and CD8 expression profile of thymocytes from 4- to 10-wk-old wild-type HY, PLCγ2−/−/HY, PLCγ1−/−/HY, and PLCγ1−/−/PLCγ2−/−/HY female mice. (C) Negative selection is reduced in PLCγ1−/−/PLCγ2−/− HY mice. CD4 and CD8 expression profile of thymocytes from 4- to 10-wk-old wild-type HY, PLCγ2−/−/HY, PLCγ1−/−/HY, and PLCγ1−/−/PLCγ2−/−/HY male mice. (D) Impaired negative selection of PLCγ1WT/−/PLCγ2−/− thymocytes in an in vitro negative selection assay. DP thymocytes from individual wild-type (n = 3) and PLCγ1−/−/PLCγ2−/− (n = 2) thymocytes were cultured in a 24-well plate with medium alone (med) or coated overnight with anti-CD3 and anti-CD28. The cells were harvested 20 h later, stained with PI, and analyzed by FACS. *p < 0.05.

protein was not able to significantly affect positive selection. However, compared with female wild-type HY mice, female PLCγ1WT/−/PLCγ2−/−/HY mice displayed a marked reduction in the percentages of both thymocytes and T3-70high CD8SP thymocytes (Fig. 4B, Table II). Compared with female PLCγ2−/−/HY and PLCγ1−/−/HY mice, female PLCγ1WT/−/PLCγ2−/−/HY mice also showed a significant reduction of T3-70high CD8SP thymocyte percentage (Table II). Thus, PLCγ2 deficiency impairs thymic positive selection when PLCγ1 expression is reduced.

Next, we examined the role of PLCγ2 in negative selection using the HY transgenic mice. There was no significant difference in thymic cellularity and percentages of CD8SP and T3-70high CD8SP thymocytes within male wild-type HY, PLCγ2−/−/HY, and PLCγ1−/−/HY mice, suggesting a similar level of negative selection in these mice. Compared with male wild-type HY, PLCγ2−/−/HY, and PLCγ1−/−/HY mice, PLCγ1−/−/PLCγ2−/−/HY mice showed some level of increase in thymocyte cellularity (Table II). Compared with male wild-type HY, male PLCγ1−/−/PLCγ2−/−/HY mice displayed marked increase in the percentages of CD8SP and T3-70high CD8SP thymocytes (Fig. 4C, Table II), indicating impaired negative selection in PLCγ1WT/−/PLCγ2−/−/HY mice. The increase in the percentage of CD8SP thymocytes was also significant in male PLCγ1WT/−/PLCγ2−/−/HY mice, when compared with male PLCγ2−/−/HY and PLCγ1−/−/HY mice. Thus, PLCγ2 deficiency impairs thymic negative selection when PLCγ1 expression is reduced in the HY transgenic model. We also examined the role of PLCγ2 in negative selection using an in vitro negative selection model, in which DP thymocytes were induced to undergo apoptosis with plate-bound anti-CD3 and anti-CD28 (47). Less PLCγ1WT/−/PLCγ2−/−/HY thymocytes underwent apoptosis than wild-type thymocytes (Fig. 4D). The reduced apoptosis was not due to heterozygous PLCγ1 deficiency, because there was no significant difference in the levels of cell apoptosis between wild-type and PLCγ1WT−/− thymocytes (data not shown). Taken together, these data demonstrate that PLCγ2 plays a role in both thymic positive and negative selection.

Table II. PLCγ2 is important for thymic selection in HY TCR transgenic mice

<table>
<thead>
<tr>
<th>HY Females</th>
<th>Genotype</th>
<th>Thymocyte No. (×10⁶)</th>
<th>CD8SP% in Thymocytes</th>
<th>T3-70highCD8SP% in Thymocytes</th>
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<tr>
<td>HY (n = 9)</td>
<td>112 ± 56</td>
<td>21 ± 11</td>
<td>18 ± 10</td>
<td></td>
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<tr>
<td>PLCγ2−/−/HY (n = 7)</td>
<td>100 ± 27</td>
<td>16 ± 5.0</td>
<td>13 ± 3.8</td>
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<tr>
<td>PLCγ1−/−/HY (n = 7)</td>
<td>101 ± 77</td>
<td>17 ± 7.9</td>
<td>14 ± 7.5</td>
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<tr>
<td>PLCγ1−/−/PLCγ2−/−/HY (n = 10)</td>
<td>109 ± 53</td>
<td>11 ± 3.3*</td>
<td>8.5 ± 3.2**</td>
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<table>
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<tr>
<th>HY Males</th>
<th>Genotype</th>
<th>Thymocyte No. (×10⁶)</th>
<th>CD8SP% in Thymocytes</th>
<th>T3-70highCD8SP% in Thymocytes</th>
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<tbody>
<tr>
<td>HY (n = 6)</td>
<td>8.4 ± 6.0</td>
<td>11 ± 6.2</td>
<td>10 ± 6.0</td>
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<tr>
<td>PLCγ2−/−/HY (n = 5)</td>
<td>6.4 ± 1.8</td>
<td>13 ± 4.1</td>
<td>11 ± 4.7</td>
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<tr>
<td>PLCγ1−/−/HY (n = 5)</td>
<td>7.9 ± 3.4</td>
<td>12 ± 3.9</td>
<td>11 ± 3.5</td>
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<tr>
<td>PLCγ1−/−/PLCγ2−/−/HY (n = 9)</td>
<td>14 ± 8.9</td>
<td>19 ± 5.7*</td>
<td>17 ± 5.6*</td>
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</table>

The age of the mice analyzed was between 4 and 10 wk. Data presented are average thymocyte number (No.) or percentage (%) of thymocyte subsets. The p values (two tailed) were calculated by comparing the total thymocyte cell number or percentage of each thymocyte subset from PLCγ2−/−/HY, PLCγ1−/−/HY, or PLCγ1−/−/PLCγ2−/−/HY mice with those from HY mice.

* p < 0.05, ** p < 0.01.
Discussion

Immunodepletion of PLCγ1, the predominant isoform in T cells, effectively depletes TCR-induced PLCγ activity from Jurkat T cell extracts (16, 33). In addition, PLCγ1 deficiency in T cells markedly impairs TCR signaling and TCR-mediated T cell development (34). However, PLCγ2 deficiency seems not to affect TCR signaling and TCR-mediated T cell development (35). Thus, PLCγ1 is generally believed to be responsible for TCR-induced PLCγ activity. Nonetheless, the presence of a residual TCR-induced Ca2+ influx in the absence of PLCγ1 suggests a potential involvement of PLCγ2 in TCR signaling. In the current study, we found that PLCγ2 was expressed in T cells and played a role in TCR-mediated Ca2+ mobilization, Erk activation, thymic DP to SP transition, and positive and negative selection. The functions of PLCγ1 and PLCγ2 appear to be redundant to some degree in T cells. The observation that the absence of PLCγ2 alone has no effect on TCR-mediated function implicates that PLCγ2 deficiency can be largely masked by compensatory effects of the full expression of PLCγ1. The impairment of TCR signaling by PLCγ1 deficiency cannot be compensated by mere existence of PLCγ2, but can be further deteriorated by lack of PLCγ2. Thus, an unappreciated role of PLCγ2 in TCR signaling can only be revealed when PLCγ1 expression is reduced or absent.

PLCγ1 and PLCγ2 contribute to TCR signal transduction; however, it is not known whether the contributions made by the two PLCγ isozymes are quantitatively or qualitatively different. Our study suggests that PLCγ2 is recruited to the TCR signaling complex through its interaction with SLP-76 and LAT, which is a mechanism employed by PLCγ1. Similar receptor coupling mechanism is also employed by PLCγ2 in human platelets following collagen receptor activation (48). Both activated PLCγ2 hydrolyze phosphatidylinositol 4,5-bisphosphate to generate DAG and IP3, which lead to PKC and Ras/ERK activation as well as Ca2+ influx (19–24). Both the magnitude and duration of PKC and Ras/ERK activation and Ca2+ flux are therefore dependent upon the amount of PLCγ activity and can differentially affect activation of a number of transcription factors, including AP-1, NFκB, and NFAT (22–26). The degree of both PLCγ1 and PLCγ2 activation, and thus the total amount of PLCγ activity, reflects the strength of TCR signaling and ultimately determines the nature of the T cell response. However, our previous study has shown that overexpression of PLCγ1 in PLCγ2-deficient B cells fails to restore BCR-mediated B cell proliferation or maturation, indicating that PLCγ1 and PLCγ2 have quantitatively different contributions to signal transduction (49). PLCγ1 and PLCγ2 may couple the TCR signaling pathway to different downstream signaling pathways. This possibility assumes that PLCγ1 and PLCγ2 have phospholipase-independent signaling functions. This assumption is supported by the finding that a catalytically inactive mutant of PLCγ1 can initiate a full mitogenic response in fibroblasts (50, 51). Similarly, the Src homology 3 other than the catalytic domain of PLCγ1 is able to promote PC12 and NIH 3T3 cell growth and possesses guanine nucleotide exchange factor activity for the mitogenic GTPase P2KE (50–53). It is possible that either in addition to or instead of the phospholipase activity, other activities of PLCγ1 and/or PLCγ2 may make qualitatively different contributions to TCR-mediated function.

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Disclosures

The authors have no financial conflicts of interest.

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