Phospholipase C-γ1 Association with CD3 Structure in T Cells

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Summary

Recently, we and others have reported tyrosine phosphorylation of phospholipase C-γ1 (PLCγ1) enzyme after CD3 activation of T cells, and have proposed that PLCγ1 mediates signal transduction through the T cell receptor (TCR/CD3). Here, using immunoblotting and immune complex PLC assays, we show that CD3 stimulation of Jurkat cells induces the association of PLCγ1 enzyme with CD3 complex. PLC activity is also found to co-precipitate with the CD3γ chain from activated cells. In addition, in vitro PLC assays show that CD3 activation leads to about 10-fold stimulation of PLCγ1 activity. These results, along with the observation that Jurkat cells preferentially express PLCγ1, indicate that PLCγ1 participates in CD3 signaling.

Materials and Methods

Cells and Reagents. Human leukemic T cell line, Jurkat, was maintained in media (RPMI 1640, 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine) at a density of 1–2 × 10^6 cells/ml, and the media were changed after every 2 d.

Immunoblotting. Proteins in immunoprecipitates or cell lysates were subjected to SDS-PAGE and then transferred to nitrocellulose membranes to blot with appropriate Abs as described (6). These blots were developed with either 11I-protein A or 11I-goat anti-mouse IgG Ab (0.4–0.5 μCi/ml; ICN Chemicals, Irvine, CA) for 2 h at room temperature. After washing, these blots were exposed to Kodak x-ray films for autoradiography.

Immunecomplex Phospholipase C Assay. These assays were performed as described by us previously (6).

Results and Discussion

In vitro measurements of PLC activity show that antiphosphotyrosine (APTyr) Ab isolates ~10-fold or more of the activity from stimulated T cells as compared with that from nonstimulated cells (6–8). Maximal activity is obtained within 2 min of CD3 activation, followed by a subsequent reduction after 10 min (6). Since changes in APTyr Ab–bound PLC activity have been found to correlate with alterations in the level of PLCγ1 tyrosine phosphorylation and the degree of CD3-induced Ca^{2+} mobilization (6), this enzyme has been proposed to mediate the signal transduction via the TCR/CD3 structure.
To demonstrate the direct participation of PLCγ1 in T cell activation, it was decided first of all to examine if the APTyr Ab–bound PLC activity was due to PLCγ1. To achieve this, CD3-activated Jurkat cells were lysed and then subjected to immunoprecipitation with APTyr Ab. The presence of PLCγ1 in these immune complexes was detected by immunoblotting with an anti-PLCγ1 Ab. From parallel samples PLCγ1 was also immunoprecipitated and subjected to similar analysis. These experiments demonstrate that APTyr Ab used in PLC assays binds to PLCγ1 enzyme (Fig. 1, lane B). Since these experiments were performed with excess amounts of APTyr and anti-PLCγ1 Abs, comparison of counts present in PLCγ1 bands (lanes B vs. D) indicates that <5% of the cellular PLCγ1 enzyme is precipitated with APTyr Ab. However, under similar conditions of precipitation, PLCγ1 present in APTyr Ab immunoprecipitate accounts for 60–70% of the total PLCγ1 activity from stimulated T cells. For example, a representative experiment shows that PLCγ1 isolated from 3 min-activated Jurkat cells (5 × 10⁶ cells/sample) using anti-PLCγ1 and APTyr Abs produces 4,162 and 3,050 pmols of IP3 in 30 min, respectively. This indicates that CD3-induced tyrosine phosphorylation of PLCγ1 enhances its enzymatic activity.

To further examine the stimulation of PLCγ1 activity after CD3 activation, the enzyme was precipitated from activated and nonactivated cells, and PLC activities were measured. As described in the case of EGF receptor stimulation (14), we detected CD3-mediated stimulation of PLCγ1 activity. The enzyme isolated from activated T cells has 1.3–1.7 times higher activity than the one from nonactivated cells (Table 1) (n = 4). Maximal stimulation of enzyme activity was detected within 2 min of CD3 activation, which gradually decreases to the control level. Therefore, considering that ∼5% of the cellular PLCγ1 is tyrosine phosphorylated, the present data demonstrate 7–15%-fold stimulation of PLCγ1 activity after CD3 activation. Taken together, these results indicate an involvement of PLCγ1 in TCR signaling.

### Table 1. Activation of PLCγ1 after CD3 Stimulation

| Treatment of cells | pmol of IP3 formed/20 min |
|--------------------|---------------------------|
| Nonactivated       | 601.8                     |
| Activated (1 min)  | 1029.3 (1.71 ×)           |
| Activated (5 min)  | 862.5 (1.43 ×)            |
| Activated (10 min) | 751.3 (1.25 ×)            |

Jurkat cells (5 × 10⁶/0.5 ml/sample) were stimulated with OKT3 (3 μg/ml) for different time periods and then lysed in 200 μl of lysis buffer. From precleared lysates, PLCγ1 was precipitated, and the PLC activities present in immune complexes were determined in the presence of 0.05% Triton X-100 (14) and 0.125% of octyl-glucoside. For details, see Materials and Methods. The specific activity of [3H]PIP2 was 1,200 cpm/nmol.

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**Figure 1.** CD3-induced tyrosine phosphorylation of PLCγ1. Jurkat cells (2 × 10⁷/2 ml/sample) in RPMI 1640 were treated with (A, B, and E) or without (C, D, and F) anti-CD3 Ab OKT3 (3 μg/ml) for 3 min at 37°C, lysed, and then precipitated with (A) control Ab ST2-59 against HLA-DR molecules, (B and C) antiphosphotyrosine Ab 4G10 (reference 13), (D) anti-PLCγ1 Ab, and (E and F) protein A-Sepharose beads alone for background. These precipitates were subjected to SDS-PAGE and processed for immunoblotting with anti-PLCγ1 Ab. Membranes were exposed to X-OMAT Kodak films for 3 d at −70°C for autoradiography.

**Figure 2.** Precipitation of PLC activity with CD3 complex. (A) Precooled Jurkat cells (10⁷/ml/sample) in RPMI 1640 were treated with OKT3 (3 μg/ml) on ice for 7 min and then transferred to 37°C. At indicated time points, cells were quickly washed and lysed in 250 μl of lysis buffer as described Materials and Methods. From these lysates CD3 complexes were immunoprecipitated on protein A-Sepharose beads and PLC activities were measured as described in Materials and Methods. (B) Cell lysates prepared as above were subjected to immunoprecipitation with APTyr-Ab, and PLC activities were measured in these complexes. The specific activity of [3H]PIP2 was 2,700 cpm/nmol.
Figure 3. PLCγ1 co-precipitates with CD3 complex. Jurkat cells (10⁷/ml) were activated with OKT3 for 5 min at 37°C and CD3 complexes were immunoprecipitated as described in the legend to Fig. 2 A. Control immunoprecipitates using mouse IgG (A) and CD3-immune complexes (C) from 3 x 10⁷ activated cells, and 6 x 10⁷ cell lysate (B) were processed for immunoblotting with anti-PLCγ1. For autoradiography, membranes were exposed to X-OMAT Kodak films for 3 d at -70°C.

The ligand binding to epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors have been shown to promote PLCγ association with the receptors (15-17). In our experiments, association of PLC activity with the CD3 structure was first indicated by the observation that, within 2-3 min of activation, a low but significant amount of PLC activity (180-340 pmol IP3 formed/30 min) was precipitated by anti-CD3 Ab (n = 4). CD3 protein forms a complex with TCR/CD3 and apparently plays a crucial role in signal transduction (18). However, variable results were obtained when CD3-anti-CD3 complexes, isolated from the activated cell lysates, were tested for the presence of PLC activity. This variation could be due to relatively weak binding and a rapid dissociation of OKT3 Ab from CD3 at 37°C (19). Therefore, instead of adding anti-CD3 to T cells at 37°C precooled T cells were coated with OKT3 Ab at 4°C for 7-8 min, where the binding is largely bivalent, complete, and stronger (19). The precoated cells were then transferred to 37°C to induce CD3 stimulation. Using this procedure, PLC activity was found to co-precipitate with the CD3 structure in a reproducible manner (n = 6). The level of activity associated with the CD3 complex after 4-6 min of activation ranges from 478 to 805 pmol of IP3 formed/30 min (n = 4). The data from a representative experiment are shown in Fig. 2 A. In parallel experiments, APTyr Ab precipitated a high level of PLC activity from cell lysates (Fig. 2 B) (n = 4), consistent with the increased PLCγ tyrosine phosphorylation (data not shown). Similar experiments performed with anti-CD45RO Ab, UCHL1, do not show precipitation of PLC activity with CD45RO Ag, indicating a specific association of PLC activity with the CD3 structure.

To determine if this activity is due to PLCγ1, CD3 complexes immunoprecipitated from the lysates of activated T cells were subjected to SDS-PAGE, and the presence of PLCγ1 was detected by immunoblotting with anti-PLCγ1 Ab. Fig. 3 shows that CD3 complex from activated cells contains PLCγ1 (lane C). Radioactive counts present in the PLCγ1 protein band precipitated with CD3 complex from 3 x 10⁷ Jurkat cells (Fig. 3, lane C) are about one half of that in PLCγ1 from 6 x 10⁷ Jurkat cell lysate (Fig. 3, lane B), indicating ~1% of the cellular PLCγ1 in association with the CD3 structure.

The present work demonstrates that CD3 activation of T cells leads to the association of PLCγ1 enzyme with the CD3 complex and also stimulates its enzymatic activity. These results, along with the observation that Jurkat cells preferentially express PLCγ1 and not PLCγ2 (results not shown), indicate that the former isozyme of PLC mediates TCR/CD3 signaling. Although in comparison with EGF and PDGF receptor stimulation, the extent of CD3-induced PLCγ1 tyrosine phosphorylation and its association with the CD3 complex is small, these values appear consistent when the density of CD3 on T cells (2 x 10⁴/cell; reference 19) is compared with that of EGF or PDGF receptors (1.5-5 x 10⁴/cell; reference 20). The CD3/PLCγ1 association observed in activated cells apparently indicates that CD3-linked protein tyrosine kinase p59 (1) may be responsible for PLCγ1 tyrosine phosphorylation. However, a fairly high level of PLCγ1 tyrosine phosphorylation occurs before a significant CD3/PLCγ1 association (Fig. 2), suggesting that in T cells increased tyrosine phosphorylation of PLCγ1 does not correlate with its association with the CD3 structure. Similar observations were made in NIH3T3 cells where, despite low levels of tyrosine phosphorylation, mutant PLCγ1 associates with the PDGF receptor (21). On the contrary, in HER14 cells PDGF-mediated PLCγ1 tyrosine phosphorylation does not induce EGF receptor/PLCγ1 association (20). These studies suggest that the association of PLCγ1 with receptor molecules depends upon the biochemical state of the latter, perhaps determined by their phosphorylation. Therefore, the question to be addressed is whether the cytosolic or the TCR/CD3-bound protein tyrosine kinase phosphorylates PLCγ1 after CD3 activation of T cells.

We thank Dr. George Wermers for his helpful discussions and critical reading of the manuscript. This work was supported by a grant from the American Cancer Society to J. D. Dasgupta.

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Received for publication 9 August 1991.

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