Immunology 1992 **75** 441–447

Regulation of CD3-induced phospholipase C-gamma1 (PLCγ1) tyrosine phosphorylation by CD4 and CD45 receptors

S. B. KANNER,* J. P. DEANS† & J. A. LEDBETTER*† *Bristol-Myers Squibb Pharmaceutical Research Institute and †Department of Microbiology, University of Washington, Seattle, Washington, U.S.A.

Accepted for publication 31 October 1991

SUMMARY

Stimulation of the signal transduction cascade in T cells through the T-cell receptor (CD3) coincides with activation of the phosphatidylinositol–phospholipase C (PI-PLC) pathway. Enzymatic activation of phospholipase C-gamma1 (PLCγ1) occurs through tyrosine phosphorylation in T cells following surface ligation of CD3 receptors with CD3-specific monoclonal antibodies (mAb). Here we show that cross-linking of CD4 molecules with CD3 augments the tyrosine phosphorylation of PLCγ1, while co-ligation of CD3 with CD45 (a receptor tyrosine phosphatase) results in reduced PLCγ1 tyrosine phosphorylation. Mobilization of intracellular calcium correlated with the extent of PLCγ1 tyrosine phosphorylation, indicating that PLCγ1 enzymatic activity in T cells may be regulated by its phosphorylation state. The time-course of PLCγ1 tyrosine phosphorylation in cells stimulated by soluble anti-CD3 was transient and closely paralleled that of calcium mobilization, while the kinetics in cells stimulated by immobilized anti-CD3 were prolonged. The PI-PLC pathway in T cells was not stimulated by tyrosine phosphorylation of PLCγ2, a homologue of PLCγ1, demonstrating the strict regulation of PLCγ isoform usage in CD3-stimulated T cells. A 35,000/36,000 MW tyrosine phosphorylated protein in T cells formed stable complexes with PLCγ1, and its tyrosine phosphorylation was co-regulated with that of PLCγ1 by CD4 and CD45 receptors. Enzymatic activation and tyrosine phosphorylation of PLCγ1 occurs during growth factor stimulation of fibroblasts, where PLCγ1 exists in multi-component complexes. The observation that PLCγ1 exists in complexes with unique tyrosine phosphorylated proteins in T cells suggests that haematopoietic lineage-specific proteins associated with PLCγ1 may play roles in cellular signalling.

INTRODUCTION

Activation of the phosphatidylinositol–phospholipase C (PI-PLC) pathway in T lymphocytes occurs through stimulation of the T-cell receptor (CD3), either by specific antigen presentation or by cross-linking of CD3 receptors with antibody.[1,2] However, the CD3-induced signal is modified by co-receptors such as CD2 and CD4 that can directly initiate PI-PLC activation when separately cross-linked,[2] suggesting that T-cell co-receptors provide a complex regulatory system for CD3-induced activation. The production of the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) from the breakdown of PIP_2 by PLC results in the activation and translocation of protein kinase C and an increase in cytoplasmic calcium.[1,2] These early events, if sustained, can trigger downstream cascades that ultimately lead to interleukin-2 (IL-2) receptor α-chain (CD25) expression, IL-2 synthesis, T-cell effector functions and clonal expansion.[2]

Previous studies have shown that activation of the PI-PLC signal transduction pathway in fibroblasts by epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) results in tyrosine phosphorylation of PLCγ1, the 150,000 MW isoform of PLC.[3] The tyrosine phosphorylated form of PLCγ1 has increased enzymatic activity[4] and can bind to activated PDGF receptors through the *src* homology 2 (SH2) domains.[5] The formation of an activated enzyme complex appears necessary for progression through the signalling cascade.

In T cells stimulated through CD3, mobilization of intracellular calcium follows an early but transient increase in tyrosine phosphorylation of multiple cellular proteins.[6] The tyrosine kinase inhibitor herbimycin A reduces tyrosine phosphorylation of protein substrates and inhibits the coincident calcium release.[7] Clearly, tyrosine phosphorylation of critical proteins regulates downstream effects mediated by surface cross-linking of CD3. In this report, we have investigated the role of tyrosine phosphorylation of PLCγ1 in T-cell receptor-mediated PI-PLC signalling through antibody stimulation. CD3-mediated tyrosine phosphorylation of PLCγ1 was augmented by co-aggregation of CD3 with CD4 (the receptor for class II molecules), whereas cross-linking of the receptor tyrosine phosphatase CD45 with CD3 diminished PLCγ1 phosphorylation. In addition, tyrosine phosphorylated PLCγ1 exists in stable complexes with novel tyrosine-phosphorylated haematopoietic cell pro-

Correspondence: Dr S. B. Kanner, Bristol-Myers Squibb Pharmaceutical Research Institute, 3005 First Avenue, Seattle, WA 98121, U.S.A.

teins that may function co-ordinately with PLCγ1 as early components of the T-cell signal transduction pathway. These results indicate that PI-PLC signalling in T cells appears to involve complex regulatory activities by both accessory surface molecules and multiple intracellular phosphoproteins.

MATERIALS AND METHODS

Monoclonal antibodies (mAb) and reagents

CD3 mAb (G19-4, IgG1), CD4 mAb (G17-2, IgG1), CD45 mAb (9·4, IgG2a) and PLCγ1 mAb were previously described.[8-10] Rabbit antiserum to PLCγ2 was kindly provided by G. Carpenter (Vanderbilt University, Nashville, TN). Purification of mAb was by protein A chromatography or by DEAE chromatography and conjugated to biotin with biotin-succinimide (Sigma Chemical Co., St Louis, MO) as described previously.[11] Avidin (Sigma) was used to cross-link biotin conjugated mAb at a 5:1 (wt/wt) ratio.

Cell culture and receptor cross-linking

Human cell line CEM ($CD3^+$ T-cell clone CEM.6) and RAMOS (B-cell line) were maintained in RPMI-1640 supplemented with 10% foetal calf serum (FCS) at $0{\cdot}5$–1×10^6 cells/ml. CD3, CD4 and/or CD45 receptors were cross-linked by incubating 10^7 CEM cells with 5 μg/ml biotinylated mAb for 5 min at 37°, followed by the addition of 5 μg avidin/μg mAb. Surface IgM was cross-linked by incubating 10^7 RAMOS cells with 10 μg/ml affinity-purified $F(ab')_2$ goat anti-human μ-chain (Jackson ImmunoResearch, Bar Harbor, ME).

Immunoprecipitation and Western immunoblotting

Immunoprecipitates of PLCγ1 and PLCγ2 were prepared by lysis of 10^7 cells (~1 mg cellular protein) in 0·5 ml modified RIPA buffer (1% NP-40, 0·25% Na-deoxycholate, 150 mM NaCl, 50 mM Tris, pH 7·5) containing proteinase and phosphatase inhibitors as previously described,[12,13] followed by incubation of cellular lysates with 2 μg protein A-purified mAb to PLCγ1 or 5 μl rabbit antiserum to PLCγ2 for 1–2 hr at 4°. Immunocomplexes were recovered by the addition of 50 μl of protein A-Sepharose beads (Pharmacia, Piscataway, NJ) for PLCγ2, or beads that had been preincubated with 4 μg of affinity-purified rabbit anti-mouse IgG (Jackson ImmunoResearch) for PLCγ1. The beads were washed three times in modified RIPA buffer, followed by one wash in TN buffer [50 mM Tris-hydrochloride (pH 7·5), 150 mM NaCl]. The proteins were eluted in SDS–PAGE sample buffer, subjected to SDS/8%-PAGE, transferred to nitrocellulose filters, and the filters were incubated with 1 μg/ml affinity-purified rabbit anti-phosphotyrosine (anti-pTyr) or mAb to PLCγ1 as previously described.[3,12,13] Proteins were detected by incubation of filters with [^{125}I]protein A or [^{125}I]goat anti-mouse IgG, followed by autoradiography with X-RP film (Eastman Kodak Co., Rochester, NY) at −70° with intensifying screens. Densitometric analyses and data integration of autoradiographs were performed on a Visage 2000 analyser (Millipore Corp., Ann Arbor, MI).

Cytoplasmic calcium measurements

Intracellular calcium ($[Ca^{2+}]_i$) responses were measured using indo-1 (Molecular Probes, Eugene, OR) and a model 50 HH/2150 flow cytometer (Ortho, Westwood, MA) as described.[14] The histograms were analysed by programs that calculated the mean indo-1 violet/blue fluorescence ratio versus time. In addition, the percentage of responding cells was analysed by programs that first determined the value of the indo-1 ratio that was two standard deviations above the ratio for control cells above the threshold value versus time. There are 100 data points on the *x*-axis (time) on all flow cytometric analyses.

RESULTS

Tyrosine phosphorylation and expression of PLCγ1 in T cells

The tyrosine phosphorylation of PLCγ1 in fibroblasts stimulated with soluble growth factors has been reported previously.[3] To determine whether PLCγ1 in the T-cell line CEM was phosphorylated after surface ligation of the T-cell receptor (CD3) with an anti-CD3 mAb, PLCγ1 was immunoprecipitated from cell lysates and immunoblotted with antibodies to phosphotyrosine (anti-pTyr). In Fig. 1a, tyrosine phosphorylation of PLCγ1 in immune complexes was readily detected in CD3-stimulated cells in contrast to unstimulated cells. In addition, a tyrosine phosphorylated protein of ~35,000 MW (pp35/36) was co-immunoprecipitated with tyrosine phosphorylated PLCγ1. Separate ligation of CD4 also induced tyrosine phosphorylation of PLCγ1, while co-ligation of CD4 with CD3 augmented the tyrosine phosphorylation of PLCγ1. Down-modulation of the tyrosine phosphorylation signals was induced by co-ligation of the CD45 tyrosine phosphatase with either or both CD3 and CD4, although the signal induced by all three receptors appeared somewhat resistant to CD45 inhibition.

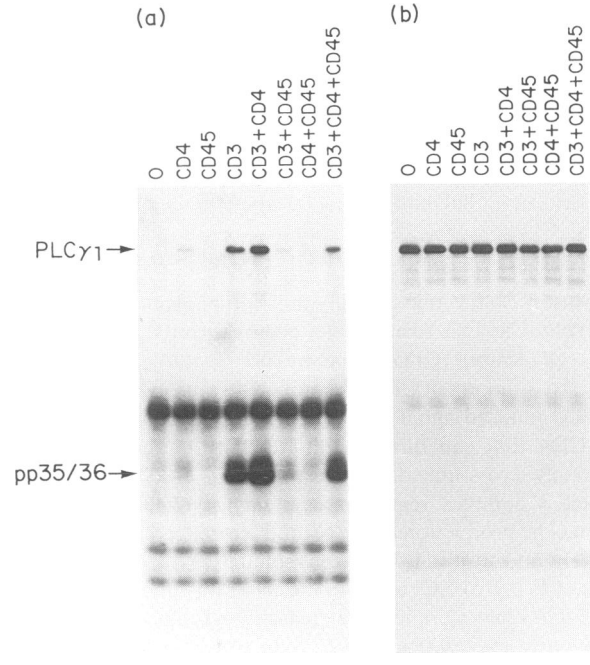

Figure 1. Tyrosine phosphorylation and expression of PLCγ1 in CEM cells. Cells were stimulated with biotinylated mAb to CD3, CD4 and/or CD45 for 5 min, followed by avidin cross-linking for 30 seconds. Immunoprecipitates of PLCγ1 were prepared from cell lysates of 2×10^7 cells, and half of each immune complex was immunoblotted in parallel with either anti-pTyr (a) or anti-PLCγ1 (b). 0, unstimulated cells. PLCγ1 and tyrosine-phosphorylated proteins pp35/36 co-immunoprecipitating with PLCγ1 are indicated on the left. Bands at ~50,000 and 25,000 MW are the IgH and IgL chains, respectively.

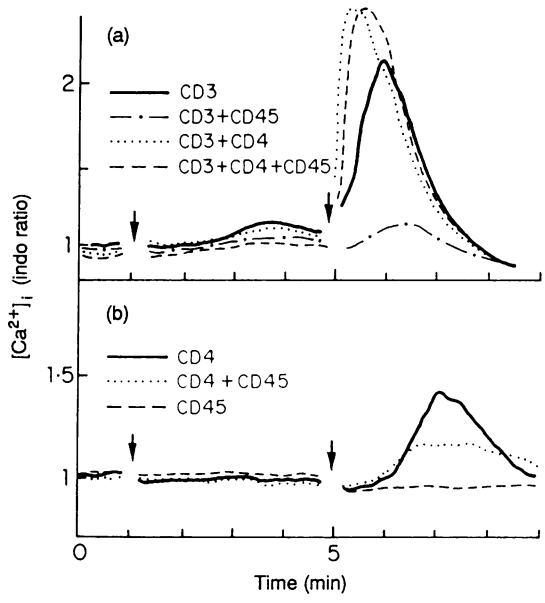

Figure 2. Intracellular calcium ($[Ca^{2+}]_i$) mobilization in CEM cells. Cells incubated in EDTA were stimulated with biotinylated mAb for 4 min, followed by avidin cross-linking, indicated by arrow at 5 min. (a) Stimulation with mAb to CD3, CD4 and/or CD45. (b) Stimulation with mAb to CD4, CD45 or both. Indo-1 ratio was determined as described in Materials and Methods.

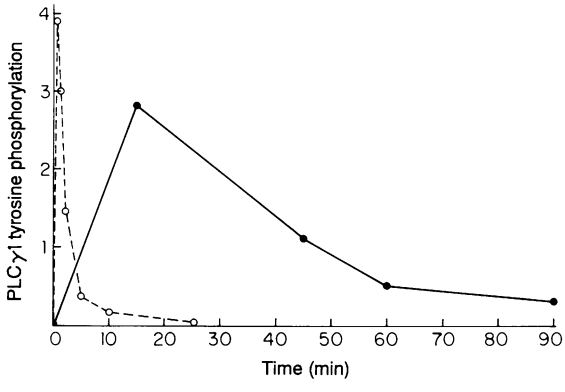

Figure 3. Kinetics of CD3-mediated PLCγ1 tyrosine phosphorylation. CEM cells were stimulated with either soluble anti-CD3 mAb at 50 μg/ml (○) or immobilized anti-CD3 mAb (●) for the indicated time-points. Anti-CD3 was immobilized on plastic dishes at 10 μg/ml overnight prior to blocking with media containing 10% FCS, followed by the addition of cells. Immunoprecipitates of PLCγ1 from 10^7 cells were immunoblotted in parallel with anti-pTyr or anti-PLCγ1. Autoradiographs were analysed by densitometry, phosphotyrosine-containing PLCγ1 bands were corrected for protein content and PLCγ1 tyrosine phosphorylation was expressed in linear units.

Virtually identical results were obtained using purified $CD4^+$ cells from peripheral blood (data not shown). Analysis of the expression level of PLCγ1 in these cells either before or after cell surface stimulation revealed a constitutive steady-state level (Fig. 1b). Thus, CD3-mediated tyrosine phosphorylation of PLCγ1 was increased by CD4 cross-linking while CD45 ligation prevented both CD3- and CD4-induced phosphorylation.

Intracellular calcium $[Ca^{2+}]_i$ mobilization correlates with PLCγ1 tyrosine phosphorylation

Activation of the PI-PLC pathway increases the intracellular levels of IP_3, the second messenger that regulates the release of Ca^{2+} from intracellular stores.[2] In order to determine whether $[Ca^{2+}]_i$ increased co-ordinately with tyrosine phosphorylation of PLCγ1, CD3 was cross-linked on the surface of CEM cells and $[Ca^{2+}]_i$ was measured by the indo-1 dye binding assay.[14] In Fig. 2a, flow cytometry analysis of CD3-mediated $[Ca^{2+}]_i$ mobilization in the absence of extracellular calcium is shown. Co-ligation of CD3 with CD4 augmented the release of $[Ca^{2+}]_i$ compared to CD3 cross-linking alone, while CD45 ligation with CD3 clearly reduced calcium mobilization. Analysis of CD3/CD4-mediated $[Ca^{2+}]_i$ release demonstrated a resistance to the effect of CD45 cross-linking with CD3, correlating with the increased tyrosine phosphorylation and complex formation observed by this stimulation. In addition, the CD4-induced $[Ca^{2+}]_i$ mobilization was partially inhibited by CD45 co-ligation (Fig. 2b) which paralleled the loss of signal observed in the PLCγ1 tyrosine phosphorylation analysis. Hence, the activation of PLCγ1 enzymatic activity as a function of $[Ca^{2+}]_i$ coincided with the tyrosine phosphorylation state of PLCγ1.

Kinetics of PLCγ1 tyrosine phosphorylation

Stimulation of T cells with soluble anti-CD3 results in a transient calcium response and desensitization of the cells to further activation.[15,16] In contrast, use of immobilized anti-CD3 to activate the PI-PLC pathway, which more closely mimics an antigen-presenting cell interaction, induces a sustained response that leads to the production of IL-2 receptor α-chain (CD25), cytokines and ultimately cell proliferation.[17] Examination of the time-course of PLCγ1 tyrosine phosphorylation showed that soluble anti-CD3 induced a transient peak at 30 seconds, followed by complete dephosphorylation of PLCγ1 by 25 min (Fig. 3). Immobilized anti-CD3 increased the duration of tyrosine phosphorylation and appeared to slow the rate of dephosphorylation, correlating with previous reports that immobilized mAb to CD3 prolonged tyrosine phosphorylation of multiple protein substrates.[18] Earlier time-points in the immobilized anti-CD3 assay showed that PLCγ1 tyrosine phosphorylation increased steadily to a peak at 15 min before decreasing (data not shown).

PLCγ isoform usage and protein complex formation in T-cell signalling

An additional isoform of PLCγ expressed in haematopoietic lineage cells (PLCγ2) has approximately 50% homology with PLCγ1.[19] Although it has been demonstrated that PLCγ2 is phosphorylated on tyrosine in the RAMOS B-cell line (S. B. Kanner and J. A. Ledbetter, unpublished results), we addressed the question whether PLCγ2 was phosphorylated on tyrosine in CEM cells in response to activation through CD3 and CD4. For comparative analysis, PLCγ1 was immunoprecipitated from CEM activated by ligation of both CD3 and CD4 (Fig. 4a) versus RAMOS activated with anti-IgM. Clearly, PLCγ1 tyrosine phosphorylation was induced by CD3/CD4 in T cells, and PLCγ1 was tyrosine phosphorylated after IgM cross-linking in B cells. Interestingly, the associated phosphoprotein in B cells was an 80,000 MW species in comparison to the 35,000

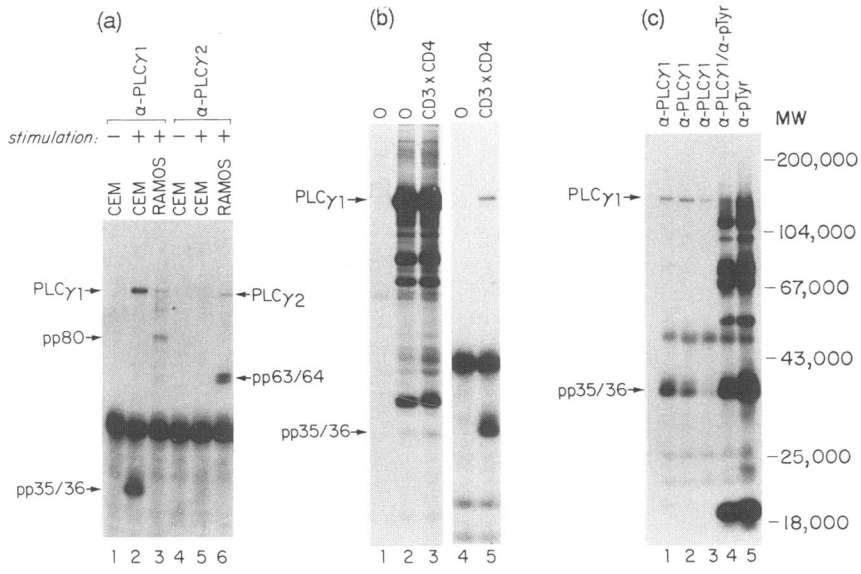

Figure 4. Association of phosphoproteins with PLCγ1. (a) CEM cells were unstimulated (−) or stimulated with both anti-CD3 and anti-CD4 (+). RAMOS cells (B-cell line) were stimulated with anti-IgM (+). Lysates were prepared, PLCγ1 and PLCγ2 were immunoprecipitated as indicated from 10^7 cells each and immune complexes were immunoblotted with anti-pTyr. Tyrosine phosphorylated proteins co-immunoprecipitating with PLCγ1 and PLCγ2 are indicated as pp35/36, pp80 and pp63/64. The band at ~50,000 MW is the IgH chain. (b) Immunoprecipitation of PLCγ1 from [^{35}S]methionine/cysteine-labelled CEM cells. A control lane without mAb to PLCγ1 was included (lane 1). 0, unstimulated; CD3 × CD4-stimulated with biotinylated mAb and avidin. For comparison, PLCγ1 was immunoprecipitated in parallel and immunoblotted with anti-pTyr (lanes 4 and 5). (c) CEM cells were stimulated with biotinylated mAb to both CD3 and CD4, followed by avidin cross-linking. Lysates from 10^7 cells were immunoprecipitated with anti-PLCγ1, followed by immunoblotting with anti-pTyr (lane 1). Consecutive immunoprecipitations of PLCγ1 from the remaining lysate (lanes 2 and 3) were performed to deplete PLCγ1 from the same lysate. After three rounds of anti-PLCγ1 immunoprecipitation, the remaining lysate was immunoprecipitated with anti-pTyr (lane 4). For comparison, non-depleted lysates from 10^7 cells were immunoprecipitated with anti-pTyr (lane 5). PLCγ1 and associated phosphoproteins pp35/36 are indicated on the left. Molecular weight markers are indicated on the right.

MW protein seen in the T-cell line. In contrast, immunoprecipitation of PLCγ2 from the same cell lysates did not reveal tyrosine phosphorylation in T cells [although the protein was expressed (data not shown)], while the control B-cell line showed tyrosine phosphorylation of PLCγ2, co-immunoprecipitating with a 63,000 MW phosphoprotein. PLCγ2 is thus not a tyrosine kinase substrate in T cells stimulated through CD3/CD4 and may not play a central role in the PI-PLC activation pathway.

Activation of PLCγ1 by CD3 and/or CD4 ligation in T cells results in the tyrosine phosphorylation of pp35/36, a protein in a stable complex with PLCγ1. Since pp35/36 is a major substrate of tyrosine kinases in activated T cells,[20,21] we investigated (1) whether pp35/36 was associated with PLCγ1 prior to T-cell stimulation, and (2) whether the entire pool or a fraction of phosphorylated pp35/36 was associated with PLCγ1. PLCγ1 was immunoprecipitated from [^{35}S]methionine/cyteine-labelled cells both before and after CD3 × CD4 cross-linking (Fig. 4b). A protein of identical M_r to pp35/36 was observed in complex with PLCγ1 both before and after stimulation, in addition to other proteins, suggesting that the proteins interact in resting cells. Further, PLCγ1 was depleted from lysates of cells (stimulated through surface ligation of CD3 and CD4) by consecutive rounds of immunoprecipitation, followed by immunoblotting with anti-pTyr (Fig. 4c). Following removal of PLCγ1, total phosphotyrosine-containing proteins were immunoprecipitated with anti-pTyr (lane 4). For comparison, lysates not depleted for PLCγ1 were used for anti-pTyr immunoprecipitation (lane 5), demonstrating that proteins of identical M_r to pp35/36 remained in the PLCγ1-depleted lysate, suggesting that only a fraction of pp35/36 is associated with PLCγ1.

DISCUSSION

Evidence has accumulated recently that tyrosine phosphorylation of cellular protein tyrosine kinase substrates is critical for PI-PLC signalling in T cells.[2] Several specific substrates have been identified, including PLCγ1, the tyrosine kinase pp56^{lck} [22,23] and the ζ-chain of CD3;[24] however, the majority of the phosphoproteins detected by immunoblotting with anti-pTyr remain unidentified. The functional contribution of most tyrosine-phosphorylated proteins in activated lymphocytes is largely unclear, but both the presence and duration of phosphorylation of individual substrates correlates with either desensitization of T cells or cell proliferation.[2]

Ligation of surface receptors CD3 and CD4 together augments the tyrosine phosphorylation signals that each receptor may induce individually.[6,18] Specifically, the substrates phosphorylated on tyrosine in response to ligation of each receptor are both unique and partially overlapping, wherein co-aggregation of CD3 with CD4 influences the entire repertoire of substrate phosphorylations.[18,20] Here we show that intracellular calcium responses and the extent of phosphorylation of PLCγ1 are both affected by co-clustering of CD3 and CD4 molecules. Separate but simultaneous ligation of CD3 and CD4 receptors using homoconjugated mAb (CD3 × CD3 and CD4 × CD4) did not result in the increased tyrosine phosphorylation of PLCγ1

seen by mixed cross-linking of receptors (CD3 × CD4) (data not shown). We conclude that the tyrosine kinase(s) responsible for phosphorylation of PLCγ1 do not simultaneously interact with a single PLCγ1 molecule unless the CD3 and CD4 receptors co-aggregate. Perhaps CD4-associated pp56^{lck} [22,23] phosphorylates a fraction of the pool of PLCγ1 when CD4 is ligated separately, whereas upon engagement with CD3 receptors (potentially associated with pp59^{fyn}) [25] PLCγ1 is multiply phosphorylated. Alternatively, pp56^{lck} and pp59^{fyn} (or another unidentified tyrosine kinase) may interact with one another, thereby regulating enzymatic activity or phosphorylation of specific tyrosine residues on substrates. Furthermore, perhaps ligation of the CD45 tyrosine phosphatase with CD3 and CD4 results in effects on specific phosphotyrosine residues that may or may not affect enzymatic activity. For example, we have shown that cross-linking of CD3 with CD4 and CD45 together results in a diminished level of PLCγ1 (and pp35/36) tyrosine phosphorylation, with no apparent change in $[Ca^{2+}]_i$ fluxing. Recently, it was shown by mutagenesis of phosphorylated PLCγ1 tyrosine residues that although two out of three residues affected the activity of PLCγ1, only one was critical for PLCγ1 enzymatic activation.[26] It thus remains possible that CD3 × CD4 ligation results in phosphorylation of PLCγ1 tyrosine residues that are affected by CD45 co-aggregation but do not result in altered enzymatic activity.

The effects of the CD45 receptor tyrosine phosphatase on the responsiveness of T cells to CD3 stimulation include down-modulation of: (1) IP_3 synthesis, (2) Ca^{2+} mobilization, (3) tyrosine phosphorylation of several protein substrates, and (4) IL-2 production.[2,9,20,21] By inference, CD45 may dephosphorylate a critical component of the PI-PLC pathway, such as PLCγ1, or pp56^{lck} as shown previously.[27,28] PLCγ1 may not be a direct substrate of CD45 *in vivo* since dephosphorylation was not demonstrated *in vitro*;[4] however, additional regulatory molecules such as profilin[29] generally lacking in *in vitro* systems may be necessary to demonstrate specificity. The activity of CD45 appears to be dependent upon its co-aggregation with CD3 and other co-stimulatory molecules. For example, addition of CD45 antibody to T cells may partially inhibit CD3-mediated Ca^{2+} mobilization,[20] but cross-linking of CD45 with CD3 appears to cluster several intracellular components that has profound effects on signalling.[20] In fact, it has been demonstrated that under gentle lysis conditions, CD45 and CD3 associate in immune complexes.[30]

Cross-linking of CD4 molecules alone on T cells causes Ca^{2+} mobilization and weak, but significant tyrosine phosphorylation of PLCγ1. CD45 has been shown previously to both enhance[9] and inhibit[31] CD4-induced Ca^{2+} mobilization. Additional experiments have shown that the stoichiometry of receptor cross-linking affects activation of the PI-PLC pathway. For example, addition of excess anti-CD45 to resting T lymphocytes results in enhancement of CD4-induced Ca^{2+} mobilization, while co-ligation with equal amounts of anti-CD45 and anti-CD4 leads to a diminished response (data not shown). Perhaps the localization of a high density of CD45 receptors favours deregulation of tyrosine kinase activity, thereby increasing phosphorylation of PLCγ1 and leading to an increase in IP_3 production. Here we show that simultaneous cross-linking of CD3, CD4 and CD45 resulted in only a partial reduction in tyrosine phosphorylation of PLCγ1 without a concomitant loss of Ca^{2+} mobilization. This suggests that CD45 regulation of the T-cell receptor is modified by the presence of CD4 in the complex. A sufficient level of IP_3 production by activated PLCγ1 under these conditions may stimulate cytoplasmic Ca^{2+} release and override the partial dephosphorylation of PLCγ1 detected in our assays. Hence, the contribution of CD45 to CD3 × CD4-induced PI-PLC activation appears to include effects on the tyrosine phosphorylation state of PLCγ1 and its associated proteins without detectable inhibition of PLCγ1 enzymatic activity.

Analysis of the tyrosine phosphorylation of PLCγ1 in purified CD4$^+$ T cells from fresh PBL in response to activation through CD3, CD4 and/or CD45 receptors was virtually identical to that observed in CEM cells (data not shown). In addition, a correlation also was observed between mobilization of $[Ca^{2+}]_i$ in CD4$^+$ PBL and the state of tyrosine phosphorylation of PLCγ1. Thus, CEM cells display both phenotypic and physiological responses similar to those of normal T cells. We have also noted that most cell lines derived from haematopoietic lineage, including T cells, B cells, myeloid cells and their progenitors, express PLCγ1 at levels comparable to or greater than that observed in fibroblasts (data not shown). In addition, it was recently reported that CD3 ligation induces tyrosine phosphorylation of PLCγ1 in the Jurkat T-cell line.[32–34] Perhaps tyrosine phosphorylation of PLCγ1 regulates early signalling events in most haematopoietic cells. Indeed, it has been demonstrated recently that PLCγ1 is tyrosine phosphorylated in B cells stimulated through ligation of surface IgM receptors.[35]

The formation of stable complexes of PLCγ1 with the tyrosine kinase substrate pp35/36 in T cells parallels that observed in fibroblasts stimulated with soluble growth factors. For example, PLCγ1 in fibroblasts associates with activated PDGF receptors[5] in addition to other proteins.[3,36] Although the identity of pp35/36 remains unknown, its tyrosine phosphorylation is co-regulated with that of PLCγ1 by both CD4 and CD45 receptors, suggesting that it may function as a component of the active enzyme complex. Examination of total tryosine-phosphorylated cellular proteins after CD3-mediated stimulation demonstrates that a protein with identical M_r as pp35/36 is not only a major tyrosine kinase substrate, but that its phosphorylation is affected by cross-linking of CD4 or CD45,[18,20,21] similar to that observed with PLCγ1-associated pp35/36. Although it appears that only a fraction of the pool of tyrosine-phosphorylated pp35/36 is associated with PLCγ1, it is equally possible that a large proportion of PLCγ1 associated with pp35/36 is unphosphorylated. The definitive experiments thus await the generation of antibodies to pp35/36. The observation that PLCγ1 exists in complexes with different haematopoietic lineage-specific phosphoproteins (i.e. pp80 associated with PLCγ1 and pp63/64 associated with PLCγ2 in B cells) suggests that these components may play a role in the activity and regulation of PLCγ1 in receptor-mediated activation pathways. Finally, the tyrosine phorphorylation state of PLCγ1 and the associated complex phosphoproteins appear to influence its enzymatic activity, and its status is ultimately affected by surface receptor-associated kinase/phosphatase (CD4/CD45) regulation.

ACKNOWLEDGMENTS

We thank S. G. Rhee for monoclonal antibody to PLCγ1, G. Carpenter for antiserum to PLCγ2, G. Schieven for affinity-purified anti-phosphotyrosine, T. Tsu and D. Hewgill for technical assistance, M. West for

editorial assistance, T. Purchio and P. Linsley for critical reviews of the manuscript, and S-L. Hu for encouragement. Supported in part by NIH grant GM42508.

REFERENCES

1. Imboden J.B. & Stobo J.D. (1985) Transmembrane signaling by the T cell antigen receptor: perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. *J. exp. Med.* **161,** 446.
2. Altman A., Coggeshall K.M. & Mustelin T. (1990) Molecular events mediating T cell activation. *Adv. Immunol.* **48,** 227.
3. Meisenhelder J., Suh P.-G., Rhee S.G. & Hunter T. (1989) Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases *in vivo* and *in vitro*. *Cell*, **57,** 1109.
4. Nishibe S., Wahl M.I., Hernandez-Sotomayor S.M.T., Tonks N.K., Rhee S.G. & Carpenter G. (1990) Increase of the catalytic activity of phospholipase C-γ1 by tyrosine phosphorylation. *Science*, **250,** 1253.
5. Anderson D., Koch C.A., Grey L., Ellis C., Moran M.F. & Pawson T. (1990) Binding of SH2 domains of phospholipase Cγ1, GAP, and Src to activated growth factor receptors. *Science*, **250,** 979.
6. June C.H., Fletcher M.C., Ledbetter, J.A. & Samelson L.E. (1990) Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T-cell receptor stimulation. *J. Immunol.* **144,** 1591.
7. June C.H., Fletcher M.C., Ledbetter J.A., Schieven G.L., Siegel J.N., Phillips A.F. & Samelson L.E. (1990) Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. *Proc. natl. Acad. Sci. U.S.A.* **87,** 7722.
8. Ledbetter J.A., June C.H., Grosmaire L.S. & Rabinovitch P.S. (1987) Crosslinking of surface antigens causes mobilization of intracellular ionized calcium in T lymphocytes. *Proc. natl. Acad. Sci. U.S.A.* **84,** 1384.
9. Ledbetter J.A., Tonks, N.K., Fischer E.H. & Clark E.A. (1988) CD45 regulates signal transduction and lymphocyte activation by specific association with receptor molecules on T or B cells. *Proc. natl. Acad. Sci. U.S.A.* **85,** 8628.
10. Suh P.-G., Ryu S.H., Choi W.C., Lee K.-Y. & Rhee S.G. (1988) Monoclonal antibodies to three phospholipase C isozymes from bovine brain. *J. biol. Chem.* **263,** 14497.
11. Ledbetter J.A., June C.H., Rabinovitch P.S., Grossmann A., Tsu T.T. & Imboden J.B. (1988) Signal transduction through CD4 receptors. Stimulatory versus inhibitory activity is regulated by CD4 proximity to the CD3/T cell receptor. *Eur. J. Immunol.* **18,** 525.
12. Kanner S.B., Reynolds A.B., Vines R.R. & Parsons J.T. (1990) Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc. natl. Acad. Sci. U.S.A.* **87,** 3328.
13. Kanner S.B., Reynolds A.B. & Parsons J.T. (1991) Tyrosine phosphorylation of a 120-kilodalton pp60^{src} substrate upon epidermal growth factor and platelet-derived growth factor receptor stimulation and in polyomavirus middle-T-antigen-transformed cells. *Mol. cell. Biol.* **11,** 713.
14. Rabinovitch P.S., June C.H., Grossmann A. & Ledbetter J.A. (1986) Heterogeneity among T cells in intracellular free calcium response after mitogen stimulation with PHA and anti-CD3. Simultaneous use of indo-1 and immunofluorescence with flow cytometry. *J. Immunol.* **137,** 952.
15. Davis L.S., Wacholtz M.C. & Lipsky P.E. (1989) The induction of T cell unresponsiveness by rapidly modulating CD3. *J. Immunol.* **142,** 1084.
16. Jenkins M.K., Pardoll D.M., Mizuguchi J., Chused T.M. & Schwartz R.H. (1987) Molecular events in the induction of a non-responsive state in interleukin 2-producing helper T lymphocyte clones. *Proc. natl. Acad. Sci. U.S.A.* **84,** 5409.
17. Geppert T.D. & Lipsky P.E. (1987) Accessory cell independent proliferation of human T4 cells stimulated by immobilized monoclonal antibodies to CD3. *J. Immunol.* **138,** 1660.
18. Ledbetter J.A., Gilliland L.K. & Schieven G.L. (1990) The interaction of CD4 with CD3/Ti regulates tyrosine phosphorylation of substrates during T cell activation. *Seminars Immunol.* **2,** 99.
19. Emori Y., Homma Y., Sorimachi J., Kawasaki H., Nakanishi O., Suzuki K. & Takenawa T. (1989) A second type of rat phosphoinositide-specific phospholipase C containing a *src*-related sequence not essential for phosphoinositide-hydrolyzing activity. *J. biol. Chem.* **264,** 21885.
20. Ledbetter J.A., Schieven G.L., Uckun F.M. & Imboden J.B. (1991) CD45 crosslinking regulates phospholipase C activation and tyrosine phosphorylation of specific substrates in CD3/Ti stimulated T cells. *J. Immunol.* **146,** 1577.
21. Gilliland L.K., Schieven G.L., Grosmaire L.S., Damle N.K. & Ledbetter J.A. (1990) CD45 ligation in T cells regulates signal transduction through both the interleukin-2 receptor and the CD3/Ti T-cell receptor complex. *Tissue Antigens*, **35,** 128.
22. Rudd C.E., Trevillyan J.M., Dasgupta J.D., Wong L.L. & Schlossman S.F. (1988) The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc. natl. Acad. Sci. U.S.A.*, **85,** 5190.
23. Veillette A., Bookman M.A., Horak E.M., Samelson L.E. & Bolen J.B. (1989) Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56^{lck}. *Nature*, **338,** 257.
24. Patel M., Samelson L.E. & Klausner R.D. (1987) Multiple kinases and signal transduction: phosphorylation of the T cell antigen receptor complex. *J. biol. Chem.* **262,** 5831.
25. Samelson L.E., Phillips A.F., Luong E.T. & Klausner R.D. (1990) Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. *Proc. natl. Acad Sci. U.S.A.*, **87,** 4358.
26. Kim H.K., Kim J.W., Zilberstein A., Margolis B., Kim J.G., Schlessinger J. & Rhee S.G. (1991) PDGF stimulation of inositol phospholipid hydrolysis requires PLC-γ1 phosphorylation on tyrosine residues 783 and 1254. *Cell*, **65,** 435.
27. Mustelin T., Coggeshall K.M. & Altman A. (1989) Rapid activation of the T-cell tyrosine protein kinase pp56^{lck} by the CD45 phosphotyrosine phosphatase. *Proc. natl. Acad. Sci. U.S.A.* **86,** 6302.
28. Ostergaard H.L. & Trowbridge I.S. (1990) Coclustering CD45 with CD4 or CD8 alters the phosphorylation and kinase activity of p56^{lck}. *J. exp. Med.* **172,** 347.
29. Goldschmidt-Clermont P.J., Kim J.W., Machesky L.M., Rhee S.G. & Pollard T.D. (1991) Regulation of phospholipase Cγ1 by profilin and tyrosine phosphorylation. *Science*, **251,** 1231.
30. Volarevic S., Burns C.M., Sussman J.J. & Ashwell J.D. (1990) Intimate association of thy-1 and the T-cell antigen receptor with the CD45 tyrosine phosphatase. *Proc. natl. Acad. Sci. U.S.A.* **87,** 7085.
31. Williams A.F. & Beyers A.D. (1989) From marker antigens for T lymphocyte subsets to molecules that regulate cell activation. *Progress in Immunology*, Vol. VII, p. 131.
32. Park D.J., Rho H.W. & Rhee S.G. (1991) CD3 stimulation causes phosphorylation of phospholipase C-γ1 on serine and tyrosine residues in a human T-cell line. *Proc. natl. Acad. Sci. U.S.A.* **88,** 5453.
33. Secrist J.P., Karnitz L. & Abraham R.T. (1991) T-cell antigen receptor ligation induces tyrosine phosphorylation of phospholipase C-γ1. *J. biol. Chem.* **266,** 12135.
34. Weiss A., Koretzky G., Schatzman R.C. & Kadlecek T. (1991) Functional activation of the T-cell antigen receptor induces tyrosine phosphorylation of phospholipase C-γ1. *Proc. natl. Acad. Sci. U.S.A.* **88,** 5484.

35. Carter R.H., Park D.J., Rhee S.G. & Fearon D.T. (1991) Tyrosine phosphorylation of phospolipase C induced by membrane immunoglobulin in B lymphocytes. *Proc. natl. Acad. Sci. U.S.A.* **88,** 2745.

36. Morrison D.K., Kaplan D.R., Rhee S.G. & Williams L.T. (1990) Platelet-derived growth factor (PDGF)-dependent association of phospholipase C-γ with PDGF receptor signaling complex. *Mol. cell. Biol.* **10,** 2359.