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CD3 stimulation causes phosphorylation of phospholipase C-γ1 on serine and tyrosine residues in a human T-cell line

(inositol phosphate/tyrosine kinase)

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ABSTRACT **The human T-cell line Jurkat was found to contain at least two immunologically distinct isoforms of inositol phospholipid-specific phospholipase C (PLC), PLC-β1 and PLC-γ1. Treatment of Jurkat cells with antibody to CD3 led to phosphorylation of PLC-γ1 but not of PLC-β1. The phosphorylation of PLC-γ1 occurred rapidly and transiently on both serine and tyrosine residues; tyrosine phosphorylation reached a maximum level less than 1 min after stimulation and decreased rapidly, both in the presence and in the absence of orthovanadate. Two-dimensional phosphopeptide map analysis revealed that the major sites of tyrosine and serine phosphorylation in PLC-γ1 from activated Jurkat cells are the same as those in PLC-γ1 from cells treated with peptide growth factors such as epidermal growth factor and platelet-derived growth factor. Previously, it has been shown that multiple phosphorylation of PLC-γ1 by the growth factor receptor tyrosine kinases leads to activation of PLC-γ1. Thus, the current data suggest that inositol phospholipid hydrolysis triggered by the T-cell antigen receptor–CD3 complex is due, at least in part, to activation of PLC-γ1 and that the mechanism by which this activation is achieved involves phosphorylation of multiple tyrosine residues on PLC-γ1 by a nonreceptor tyrosine kinase coupled to the T-cell antigen receptor–CD3 complex.**

The occupancy of the multicomponent T-cell antigen receptor (TCR)–CD3 complex by antigen, lectins, or antibodies to CD3 activates multiple signal transduction pathways that include the rapid phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate to two second messengers, inositol-1,4,5-trisphosphate and diacylglycerol (1–3). Thus, TCR–CD3 belongs to a large number of receptors that have been shown to elicit mobilization of intracellular Ca^{2+} and the activation of protein kinase C through inositol-1,4,5-trisphosphate and diacylglycerol, respectively (4). Another rapid event that follows TCR–CD3 activation is the phosphorylation on tyrosine residues (5, 6) of at least 12 proteins, including the TCR ζ chain (7) and proteins of 135 and 100 kDa (8).

Analysis of the time course of protein-tyrosine phosphorylation after stimulation of resting T cells or Jurkat T-leukemia cells revealed distinct patterns (8). The earliest substrates to exhibit tyrosine phosphorylation after stimulation by antibody to CD3 were the 135- and 100-kDa proteins. The phosphorylation of these substrates was very rapid (half-maximal at 14–30 sec) and preceded the increase in inositol-1,4,5-trisphosphate and cytosolic Ca^{2+} concentrations (8). Stimulation of the CD45 protein-tyrosine phosphatase inhibited the CD3-mediated Ca^{2+} mobilization in T cells (9, 10). In addition, inhibitors of protein-tyrosine kinases prevented TCR–CD3-mediated substrate-tyrosine phosphorylation, PLC activation, and increase in cytosolic Ca^{2+} (11, 12). These results suggest that tyrosine phosphorylation is required for PLC activation.

It has been shown by several investigators that stimulation of appropriate cells with epidermal growth factor or platelet-derived growth factor (PDGF) elicits phosphorylation of the PLC-γ1 isozyme on both serine and tyrosine residues (13–15) and that the tyrosine phosphorylation is responsible for the activation seen in response to the growth factors (16, 17). The phosphorylation of PLC-γ1 on tyrosine residues was found to be mediated directly by intrinsic tyrosine kinase activity of the growth factor receptors (18, 19). Although none of the TCR–CD3 components is a protein kinase, T cells contain nonreceptor protein-tyrosine kinases such as fyn (20) and lck (21). Furthermore, the TCR was shown to become associated with fyn (20).

We therefore investigated whether any PLC isozymes become phosphorylated in Jurkat cells in response to TCR–CD3 stimulation. Our data show that CD3 stimulation causes an increase in the phosphorylation of PLC-γ1 on both serine and tyrosine residues.

MATERIALS AND METHODS

Materials. Monoclonal antibodies to the PLC isozymes were prepared as described (22). The mixture of monoclonal antibodies used for immunoprecipitation and immunoblotting contained K-32-3, K-82-3, and K-92-3 for PLC-β1; F-7-2, B-20-3, B-6-4, D-7-4, E-8-4, and E-9-4 for PLC-γ1; and S-11-2, R-29-1, R-39-2, and Z-78-5 for PLC-δ1. For the immunoblot analysis of PLC-β1, polyclonal antibodies were used. Antibodies to phosphotyrosine were kindly provided by David Kaplan (National Cancer Institute). The monoclonal antibody to CD3, OKT3 (ascitic fluid), was a gift from Carl June (Naval Medical Research Institute).

Cells. Jurkat leukemia cells were maintained in RPMI 1640 medium (GIBCO/BRL) with 10% (vol/vol) fetal bovine serum (HyClone) under a humidified atmosphere of 95% air/5% CO_2 at 37°C. Cells were harvested at 5×10^5 cells per ml for experiments.

Phosphorylation of PLC. Cells were harvested by centrifugation, washed twice with RPMI 1640 medium containing 0.5% dialyzed fetal bovine serum, and resuspended at 1×10^7 cells per ml in the same medium containing 0.5% fetal bovine serum and 50 mM Hepes (pH 7.3). Cells (1×10^7 cells per sample) were preincubated with 1 mM sodium orthovanadate for 10 min and then stimulated with OKT3 (1:500 dilution of ascitic fluid) at 37°C. Stimulations were terminated by aspirating the medium after brief centrifugation, and the cells were washed twice with an ice-cold phosphate-buffered saline. Each sample was treated with 600 μl of ice-cold lysis buffer [20 mM Hepes, pH 7.2/1% Triton X-100/10% (vol/

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Abbreviations: PLC, phospholipase C; TCR, T-cell antigen receptor; PDGF, platelet-derived growth factor.

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vol) glycerol/50 mM NaF/1 mM phenylmethylsulfonyl fluoride/1 mM Na_3VO_4/leupeptin (10 μg/ml)] for 30 min on ice.

For phosphoamino acid analysis and measurement of total phosphate incorporated into serine, threonine, and tyrosine, cell proteins were metabolically labeled by incubating the cells with indicated amounts of [^{32}P]orthophosphate (ICN) in phosphate-free RPMI medium plus 0.5% dialyzed fetal bovine serum and 50 mM Hepes (pH 7.3) for 3 hr at 37°C. The cells were then stimulated, washed, and lysed as described above.

Immunoprecipitation and Immunoblot Analysis. Immunoprecipitation was performed with mixtures of monoclonal antibodies to PLC-γ1 and PLC-β1 as described (23). The immunoprecipitates were washed with a buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 50 mM Tris·HCl (pH 8.5). Immunoprecipitated proteins were released by adding 50 μl of Laemmli buffer and heating for 5 min at 95°C. SDS/polyacrylamide gel electrophoresis was performed on 6% polyacrylamide gels by the method of Laemmli, and the separated proteins were then transferred to nitrocellulose filters for 1 hr at 50 V. After blocking with 2% (wt/vol) bovine serum albumin, filters were probed with monoclonal antibodies to phosphotyrosine. The immune complex was detected with phosphatase-labeled antibody to mouse IgG and a phosphatase substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD) or with ^{125}I-labeled protein A (Amersham) and autoradiography.

Phosphoamino Acid Analysis. [^{32}P]Phosphoproteins were subjected to immunoprecipitation with antibodies to PLC-γ1, separated on a 6% polyacrylamide gel containing SDS, and then transferred to an Immobilon [poly(vinylidene difluoride)] membrane (Millipore) as described (24). After autoradiography, the [^{32}P]PLC-γ1 band was cut from the membrane and treated with 5.7 M HCl at 110°C for 60 min, and phosphoamino acid analysis was performed as described (24).

Tryptic Phosphopeptide Mapping. [^{32}P]Phosphoproteins were separated on a 6% polyacrylamide gel containing SDS after immunoprecipitation with anti-PLC-γ1 antibodies. The gel was dried and exposed to Kodak XAR film for autoradiography. The PLC-γ1 band visualized by autoradiography was cut from the gel, and PLC-γ1 was extracted and subjected to tryptic digestion as described (14). The resulting phosphopeptides were separated in two dimensions on a 100-μm thin-layer cellulose plate (EM Science) by electrophoresis for 30 min at 1 kV with buffer [acetic acid/88% (vol/vol) formic acid/water, 156:50:1794 (vol/vol), pH 1.9], followed by chromatography [1-butanol/pyridine/acetic acid/water, 75:50:15:60 (vol/vol)].

RESULTS AND DISCUSSION

PLC Isoforms in Jurkat Cells. Three immunologically distinct PLC isozymes, PLC-β1, PLC-γ1, and PLC-δ1, that exhibit apparent molecular masses of 150, 145, and 85 kDa, respectively, on SDS/polyacrylamide gels, have been purified from bovine brains (25), and their cDNAs have been molecularly cloned and sequenced (26). Subsequently, four additional PLC-related cDNAs were obtained by low-stringency cross-hybridization techniques (27, 28). Sequence alignment of these four additional mammalian PLCs (PLC-β2, PLC-γ2, PLC-δ2, and PLC-δ3) with the three brain PLCs revealed that each of the sequences is similar and structurally related to one of the three brain PLCs. This result suggests that each PLC type (β, γ, and δ) contains several distinct members. Highly specific antibodies to PLC-β1, PLC-γ1, and PLC-δ1 have been prepared (22).

Immunoblot intensities of PLC-β1, PLC-γ1, and PLC-δ1 in detergent extracts of Jurkat cells were compared with those in C_6Bu1 cells (Fig. 1). It has been shown (29) that C_6Bu1 is one of the few established cell lines containing all three isoforms of PLC in measurable quantities: 1 mg of C_6Bu1 cell protein contains 70 ng of PLC-β1, 260 ng of PLC-γ1, and 30 ng of PLC-δ1 (29). The amounts of PLC-β1 and PLC-γ1 in Jurkat cells were approximately 0.5 and 3 times, respectively, the amounts in C_6Bu1 cells; PLC-δ1 was not detectable in Jurkat cells.

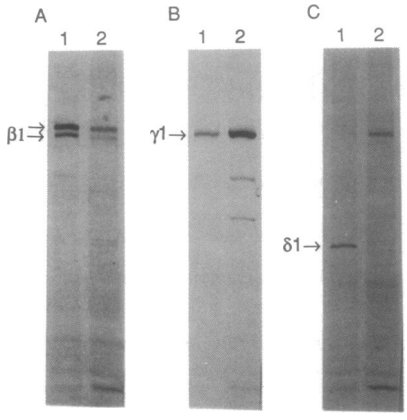

FIG. 1. Immunoblot showing the expression of PLC-β1, PLC-γ1, and PLC-δ1 in C_6Bu1 and Jurkat cells. C_6Bu1 cells (lanes 1) and Jurkat cells (lanes 2) were lysed with a buffer containing 1% Triton X-100 and centrifuged, and 100 μg of supernatant protein was loaded on a 6% polyacrylamide gel containing SDS. Proteins were transferred to nitrocellulose and probed with rabbit antiserum to PLC-β1 (*A*), monoclonal antibodies to PLC-γ1 (*B*), or monoclonal antibodies to PLC-δ1 (*C*). The positions of PLC isozymes are indicated.

Phosphorylation of PLC-γ1 Induced by CD3 Antibody. Under appropriate conditions, antibodies to TCR or CD3 can mimic the effects of antigens and activate T cells (2). To investigate whether either PLC-β1 or PLC-γ1 becomes phosphorylated after treatment with OKT3, a monoclonal antibody to CD3, we metabolically labeled Jurkat cells by incubating them in medium containing [^{32}P]orthophosphate and then treated the cells with OKT3 for 1 min at 37°C. Subsequently, the cells were lysed with detergent buffer, and the soluble extracts were treated separately with monoclonal antibodies to PLC-β1 and PLC-γ1. After SDS/polyacrylamide gel electrophoresis of the immunoprecipitates, we were able to identify bands corresponding to PLC-β1 and PLC-γ1 (Fig. 2 *A* and *B*). The PLC-γ1 band from unstimulated cells contained a significant amount of ^{32}P, and the extent of phosphorylation was increased ≈2-fold after OKT3 treatment. The ^{32}P content of PLC-β1 was very low and appeared to be unchanged after OKT3 treatment. Although the low concentration of PLC-β1 in Jurkat cells (≈5% that of PLC-γ1) hindered detection of changes in ^{32}P content of the PLC-β1 band, appropriate experiments indicated that the PLC-β1 band would have been detected by autoradiography if it had contained ^{32}P radioactivity corresponding to 5% of that of the PLC-γ1 band from activated cells. We next analyzed the phosphoamino acid content of PLC-γ1 precipitated from stimulated and unstimulated cells. PLC-γ1 from control cells contained phosphoserine and small amounts of phosphothreonine, but phosphotyrosine was nearly undetectable (Fig. 2*C*). After stimulation with OKT3, the phosphoserine content increased ≈2-fold and phosphotyrosine became clearly visible (Fig. 2*D*). In Fig. 2*B*, in addition to the 145-kDa PLC-γ1 band, two other phosphoproteins with molecular masses of 80 and 43 kDa were visible. Proteins of similar size have been shown to coimmunoprecipitate with PLC-γ1 from A431 cells (13) and NIH 3T3 cells (14). The time course of PLC-γ1 phosphorylation was followed (Fig. 3). OKT3 stimulated a rapid increase in the ^{32}P content of PLC-γ1 that was apparent within 1 min, and the extent of phosphorylation remained elevated for up to 3 min. After this, in the continued presence of OKT3, there was a rapid fall in the amount of

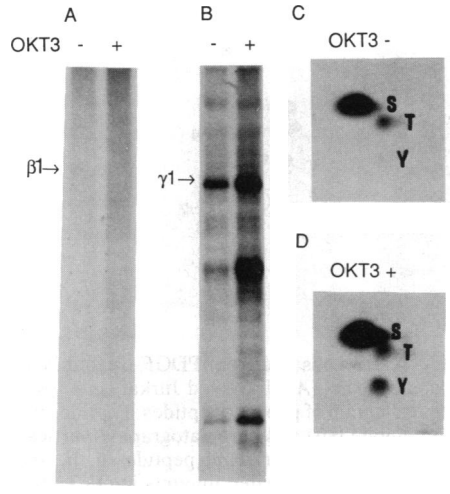

FIG. 2. Effect of OKT3 on phosphorylation of PLC isoforms in Jurkat cells. (*A* and *B*) Jurkat cells were incubated with 1.0 mCi of [^{32}P]orthophosphate for 3 hr prior to treatment with OKT3 at 37°C. The labeled cells were treated with (+) or without (−) OKT3 (1:500 dilution of ascitic fluid) for 1 min. PLC-β1 (*A*) and PLC-γ1 (*B*) were immunoprecipitated individually with monoclonal antibodies specific to each isoform. The immunoprecipitates were separated on 6% polyacrylamide gels containing SDS, and the dried gels were exposed to Kodak XAR film. (*C* and *D*) Jurkat cells were labeled with 3.3 mCi of [^{32}P]orthophosphate for 3 hr and then treated without (*C*) or with (*D*) OKT3 for 1 min at 37°C. PLC-γ1 was selectively immunoprecipitated and separated as described above, and the proteins were then transferred to Immobilon. After autoradiography, the PLC-γ1 bands were cut from the membrane, and phosphoamino acids were analyzed. S, T, and Y denote serine, threonine, and tyrosine, respectively.

^{32}P-labeled PLC-γ1. The time courses of phosphorylation of the two coimmunoprecipitating proteins were significantly slower. We could not measure phosphorylation at times less than 1 min because of the time taken for the experimental procedure.

Time Course of PLC-γ1 Tyrosine Phosphorylation. Treatment of Jurkat cells with OKT3 provoked a rapid and transient phosphorylation of PLC-γ1 on tyrosine residues both in the presence and absence of orthovanadate, a phosphotyrosine phosphatase inhibitor (Fig. 4). Prior treatment of cells with orthovanadate enhanced the tyrosine phosphate content of PLC-γ1 but did not completely prevent dephosphorylation, probably because it only partially blocked the activity of intracellular phosphotyrosine phosphatases such as CD45 (9, 10). Comparison of Figs. 3*B* and 4*C* shows that the time course of tyrosine phosphorylation of PLC-γ1 was significantly different from that of total ^{32}P incorporation, the latter mostly reflecting serine phosphorylation. Total ^{32}P incorporation was maximal between 1 and 3 min and then decreased to control levels after 10 min, whereas tyrosine phosphorylation peaked at 1 min or earlier, rapidly decreased between 1 and 3 min, and then slowly declined.

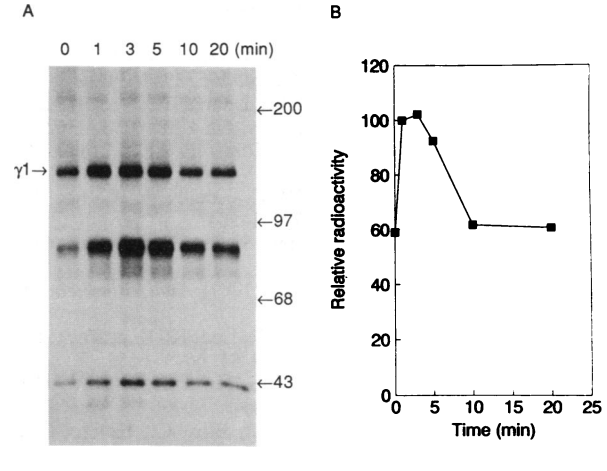

FIG. 3. Time course of phosphorylation of PLC-γ1 in Jurkat cells. Jurkat cells were metabolically labeled with 3.3 mCi of [^{32}P]orthophosphate and stimulated with OKT3 for the designated time intervals. Then PLC-γ1 was immunoprecipitated from the cell extracts and separated on 6% polyacrylamide gels containing SDS. The dried gels were exposed to Kodak XAR film to yield the autoradiogram shown in *A*. The positions of molecular mass standards (in kDa) are indicated. Relative radioactivity in the PLC-γ1 bands shown in *A* was determined with PhosphoImager (Molecular Dynamics, Sunnyvale, CA) and expressed as a percentage of the value at 1 min of OKT3 treatment in *B*.

Phosphopeptide Map of PLC-γ1. Although the primary structures of epidermal growth factor and PDGF are significantly different, the tyrosine kinase activity of each of these receptors phosphorylates PLC-γ1 at identical sites (namely, tyrosine residues 771, 783, and 1254) *in vivo* and *in vitro* (18, 19). The phosphorylation of these three sites is responsible for three PLC-γ phosphopeptides, which are labeled a, b, and c in Fig. 5. Tyrosine phosphorylation was found to enhance the catalytic activity of PLC-γ1 measured under specific assay conditions (16). With site-directed mutagenesis techniques, it was shown that phosphorylation at tyrosine residues 783 and 1254 is essential for the growth-factor-dependent activation of PLC in NIH 3T3 cells (17). Growth factor treatment also enhances phosphorylation of PLC-γ1 on serine residues; Ser-1248 was identified as the major site of phosphorylation (J. W. Kim, N. Ahn, and S.G.R., unpublished work). Tryptic digestion of PLC-γ1 usually generates two Ser-1248-containing phosphopeptides (labeled peptides 1

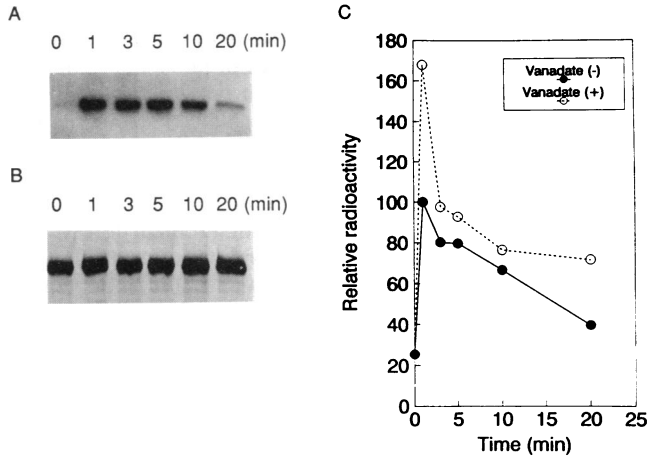

FIG. 4. Time course of tyrosine phosphorylation of PLC-γ1 in Jurkat cells. Jurkat cells were incubated with and without 1 mM sodium orthovanadate for 10 min and then stimulated with OKT3 for timed intervals. PLC-γ1 was immunoprecipitated, electrophoresed on 6% polyacrylamide gels containing SDS, transferred to nitrocellulose, and probed with anti-phosphotyrosine antibodies. Then the immune complexes were detected by ^{125}I-labeled protein A and autoradiography (*A*). Relative radioactivity in the PLC-γ1 bands shown in *A* was determined with PhosphoImager and expressed as a percentage of the value at 1 min of OKT3 treatment in the absence of orthovanadate (*C*). After quantitation, the same nitrocellulose blot was incubated with antibodies to PLC-γ1 to ascertain that each lane received similar amounts of PLC-γ1 (*B*). *A* and *B* show immunoblots obtained with PLC-γ1 phosphorylated in the absence of orthovanadate but not those obtained with PLC-γ1 phosphorylated in the presence of orthovanadate. *C* shows relative ^{125}I radioactivity associated with PLC-γ1 from cells pretreated with (open circle) and without (solid circle) orthovanadate. Each data point represents the mean of three experiments.

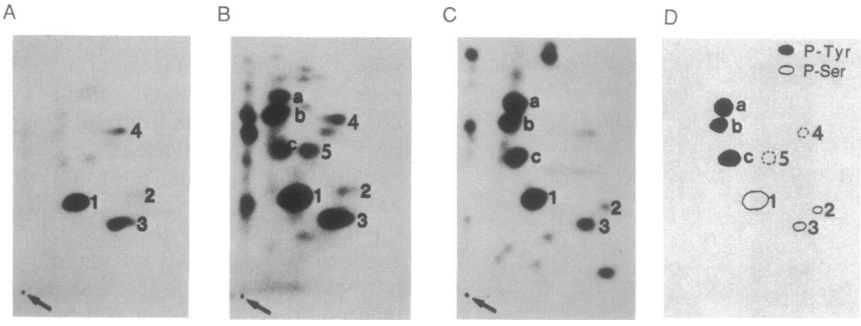

FIG. 5. Comparison of tryptic phosphopeptide maps of PLC-γ1 from OKT3-treated Jurkat cells and from PDGF-treated NIH 3T3 cells. ^{32}P-labeled tryptic digests were prepared from PLC-γ1 immunoprecipitated from ^{32}P-labeled cells. (*A*) Untreated Jurkat cells. (*B*) Jurkat cells treated for 1 min with OKT3. (*C*) NIH 3T3 cells treated for 5 min with PDGF. (*D*) Schematic diagram of phosphopeptides. Peptides were resolved in two dimensions on cellulose thin-layer plates by electrophoresis (horizontal direction; anode left) and chromatography (vertical direction). Origins are indicated by arrows at the lower left. Peptides 1, 2, and 3 contain phosphoserine (P-Ser), and peptides a, b, and c contain phosphotyrosine (P-Tyr). Peptides 4 and 5 contain unidentified phosphoamino acids and are found in greater amounts in OKT3-treated Jurkat cells.

and 2 in Fig. 5), probably due to incomplete digestion. At the present time, neither the function of nor the protein kinase responsible for phosphorylation at Ser-1248 is known. One possible candidate for the serine/threonine kinase is raf-1: Phosphorylation of PLC-γ1 by raf-1 *in vitro* generated peptides 1 and 2 (30). To assess whether the sites of PLC-γ1 that are phosphorylated as a result of OKT3 treatment are identical to those phosphorylated by the transmembrane receptor tyrosine kinases, we performed phosphopeptide mapping studies. OKT3 treatment increased the ^{32}P content of phosphotyrosine-containing peptides a, b, and c and that of phosphoserine-containing peptides 1 and 2. The map of PLC-γ1 from OKT3-treated T cells was very similar to that of PLC-γ1 from NIH 3T3 cells treated with PDGF, except that two additional peptides (peptides 4 and 5) with unidentified phosphoamino acids are visible with T-cell PLC-γ1.

These results and the rapid kinetics of phosphorylation in response to OKT3 are consistent with tyrosine phosphorylation of PLC-γ1 being partly, if not solely, responsible for TCR–CD3-mediated activation of PLC. Thus, this report identifies a nonreceptor tyrosine kinase coupled to a transmembrane receptor phosphorylating and subsequently activating a PLC isozyme. Key questions that remain unresolved are whether the tyrosine kinase phosphorylating PLC-γ1 is the TCR-associated fyn or lck or both and, if it is, how does it communicate with the activated TCR–CD3 complex. In addition, the fact that Jurkat cells contain PLC-β1, albeit at a low concentration, suggests the presence of an additional PLC activation mechanism in T cells that is dependent on a guanine nucleotide-binding protein (G protein); the G protein G_q has been shown to specifically activate PLC-β1 (31).

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