Association of the Tyrosine Kinase LCK with Phospholipase C-γ1 after Stimulation of the T Cell Antigen Receptor

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Summary

Stimulation of the T cell antigen receptor (TCR) activates a protein tyrosine kinase and leads to the tyrosine phosphorylation of phosphoinositide-specific phospholipase C-γ1 (PLCγ1). The molecular interactions involved in this phosphorylation are not known. After stimulation of the TCR on Jurkat T cells, tyrosine-phosphorylated proteins of 36, 38, 58, and 63 kD coprecipitate with PLCγ1. An identical pattern of proteins precipitate with TrpE fusion proteins that contain the Src homology (SH) 2 domains of PLCγ1, indicating that these regions of PLCγ1 are responsible for binding. TCR stimulation leads to an association between the SH2 domains of PLCγ1 and a protein tyrosine kinase, which, by peptide mapping, is identical to $p56^{lck}$. These studies establish that $p56^{lck}$ associates with PLCγ1 as a result of TCR stimulation of Jurkat cells, suggesting that $p56^{lck}$ plays a central role in coupling the TCR to the activation of PLCγ1.

Activation of a protein tyrosine kinase (PTK)[1] plays a central role in signal transduction by the TCR (1, 2). Lacking intrinsic kinase activity, the TCR likely couples to a Src-like PTK, probably $p59^{fyn}$, which coprecipitates with the TCR (3, 4). A second early TCR-mediated signaling event is the activation of a phosphoinositide (PI)-specific phospholipase C (PLC) (5). The resulting PI hydrolysis generates second messengers that mobilize intracellular calcium and that activate protein kinase C. TCR-mediated PI turnover is sensitive to PTK inhibitors, suggesting that tyrosine phosphorylation regulates PLC activity (6, 7). Consistent with this possibility, stimulation of the TCR on Jurkat T cells leads to the tyrosine phosphorylation of PLCγ1 and, subsequently, to an association between PLCγ1 and the CD3 components of the TCR (8–12).

The molecular interactions that are required for TCR-mediated activation of PLCγ1 in Jurkat cells, and the PTK involved, are not known. Critical to the activation of PLCγ1 by growth factor receptor protein tyrosine kinases, such as the receptors for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), is the direct binding of PLCγ1 to the receptor (13–17). This binding requires autophosphorylation of the receptor and appears to be mediated by the Src homology 2 (SH2) domains of PLCγ1 (18–20). SH2 domains are conserved sequences of ~100 amino acids that are found in a number of signaling molecules (20). By recognizing specific peptide sequences that contain phosphotyrosine, SH2 domains likely play a key role in directing protein–protein interactions during signal transduction (20). Herein, we have examined the possibilities that PLCγ1 associates with tyrosine phosphorylated molecules as a result of TCR stimulation of Jurkat cells, and that this association involves the SH2 domains of PLCγ1.

Materials and Methods

Reagents. TrpE fusion proteins containing the SH2 domains of PLCγ1 and ras GTPase activating protein (GAP) were isolated from *Escherichia coli* RR1 possessing the appropriate pATH expression plasmid as described (18, 19). Rabbit antisera to $p56^{lck}$ and to PLCγ1 were supplied by Drs. J. Bolen (Bristol-Meyers Squibb, Princeton, NJ) and R. Schatzman (Syntex Corp., Palo Alto, CA), respectively. Jurkat cells and the mAb C305 (reactive with the Jurkat TCR) were gifts of Dr. A. Weiss (University of California, San Francisco, CA).

[1] *Abbreviations used in this paper:* EGF, epidermal growth factor; GAP, GTPase activating protein; PDGF, platelet-derived growth factor; PI, phosphoinositide; PLCγ1, phospholipase C-γ1; PTK, protein tyrosine kinase; PVDF, polyvinylidene difluoride.

Immunoprecipitation, Precipitations Using TrpE Fusion Proteins, and Immunoblotting. Cells were lysed in an ice-cold buffer containing 1% Triton X-100 as described (18). After a 30-min incubation on ice, the lysates were subjected to a 15-min centrifugation at 14,000 *g*. To immunoprecipitate PLCγ1 or $p56^{lck}$, the clarified lysate was incubated with the appropriate rabbit antiserum complexed to Pansorbin (Calbiochem Corp., La Jolla, CA). For precipitations with the TrpE fusion proteins, lysates were incubated with the indicated TrpE fusion protein immobilized by a TrpE mAb (Oncogene Science, Inc., Manhasset, NY) complexed to Pansorbin with a rabbit antiserum to mouse Ig (Cappel Laboratories, Durham, NC). After incubation with the Jurkat lysate for 90 min at 4°C, the precipitation complexes were recovered by centrifugation, washed three times, and then eluted in Laemmli sample buffer. Samples were subjected to electrophoresis under reducing conditions in 5–15% polyacrylamide gradient gels, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA), and analyzed by immunoblotting with a mouse IgG2b mAb (4G10) to phosphotyrosine (Upstate Biologicals, Inc., Lake Placid, NY), revealed by an alkaline phosphatase-conjugated mAb specific for mouse IgG2b (PharMingen, San Diego, CA).

In Vitro Kinase Assays. Precipitation complexes were washed in a kinase wash buffer (1% Triton X-100; 150 mM NaCl; 50 mM Hepes, pH 7.5; 1 mM Na_3VO_4), incubated for 15 min on ice with 27 μCi/sample γ-[^{32}P]ATP (Amersham International, Amersham, Bucks, UK, 6,000 Ci/mmole) in a kinase reaction buffer (100 mM NaCl; 20 mM Hepes, pH 7.4; 5 mM $MnCl_2$; 5 mM $MgCl_2$) (final vol, 25 μl), and then washed twice in the above wash buffer containing 1 mM EDTA. The reaction products were solubilized in Laemmli sample buffer and resolved by electrophoresis under reducing conditions in 5–15% polyacrylamide gels. After fixation for 1 h in 10% glacial acetic acid/10% isopropanol, the gels were treated with 1 M KOH for 2 h at 55°C followed by incubation in 10% glacial acetic acid/10% isopropanol.

Peptide Mapping and Phosphoamino Acid Analysis. In vitro kinase reactions using precipitations from lysates of TCR-stimulated Jurkat cells were analyzed as described above except that the gels were not treated with KOH. For peptide mapping, the bands of interest were identified by autoradiography, precisely excised from the dried gel, rehydrated, and digested with staphylococcal V8 protease (Calbiochem Corp.) as described by Cleveland et al. (21). For phosphoamino acid analysis, the ^{32}P-labeled proteins were transferred to PVDF membranes, eluted, and then hydrolyzed in 6N HCl for 1 h at 110°C as described (22). Phosphoamino acids were resolved on thin-layer cellulose plates by two-dimensional electrophoresis using a Hunter thin-layer electrophoresis system (HTLE-7000; CBS Scientific, Del Mar, CA) (22).

Results

The addition of a TCR mAb to Jurkat cells induces the tyrosine phosphorylation of PLCγ1 (8–12). When PLCγ1 is immunoprecipitated from lysates of TCR-stimulated Jurkat cells, we observe that four prominent tyrosine-phosphorylated proteins of 36, 38, 58, and 63 kD (pp36, pp38, pp58, and pp63) consistently coprecipitate with PLCγ1 (Fig. 1). To determine whether the coprecipitation of these molecules is a result of their binding to the SH2 domains of PLCγ1, we used fusion proteins of TrpE and PLCγ1 SH2 domains as affinity ligands. Fusion proteins that have both the NH_2- and COOH-terminal PLCγ1 SH2 domains precipitate tyrosine-phosphorylated proteins that exactly comigrate with

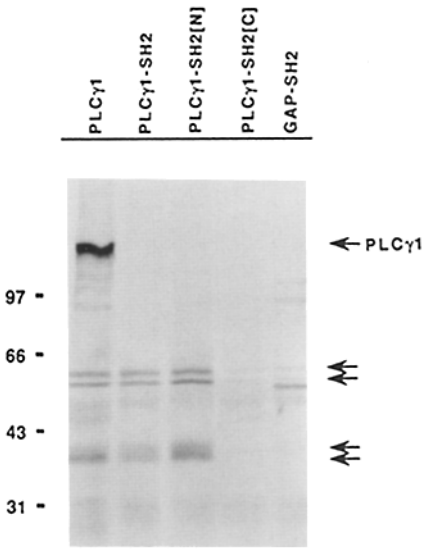

Figure 1. Tyrosine-phosphorylated proteins from TCR-stimulated Jurkat cells coprecipitate with PLCγ1 and bind to TrpE fusion proteins containing the NH_2 SH2 domain of PLCγ1. Jurkat cells (8 × 10^6 cells/sample) were stimulated for 2 min at 37°C with the TCR mAb C305 and then lysed with an ice-cold buffer containing 1% Triton X-100 and phosphatase inhibitors as described (13). Endogenous PLCγ1 was immunoprecipitated using a specific antiserum as described in Materials and Methods. Alternatively, lysates were subjected to precipitations using TrpE fusion proteins that contained both the NH_2- and COOH-terminal SH2 domains of PLCγ1 (*PLCγ-SH2*), the NH_2-terminal SH2 domain alone (*PLCγ1-SH2[N]*), the COOH-terminal domain alone (*PLCγ1-SH2[C]*), or the NH_2- and COOH-terminal SH2 domains of $p21^{ras}$ GAP (*GAP-SH2*). After resolution under reducing conditions on SDS-PAGE and transfer to PVDF membranes, tyrosine-phosphorylated proteins were detected by immunoblotting. The 150-kD tyrosine-phosphorylated band in the PLCγ1 immunoprecipitate comigrates with PLCγ1 as detected by immunoblotting with anti-PLCγ1 (data not shown). (*Arrows*) Tyrosine-phosphorylated proteins of 36, 38, 58, and 63 kD that coprecipitate with endogenous PLCγ1 and with TrpE fusion proteins containing the NH_2-terminal SH2 domain of PLCγ1.

PLCγ1-associated molecules. Comparable levels of all four tyrosine-phosphorylated proteins also bind to a fusion protein that has only the NH_2-terminal SH2 domain. In contrast, a fusion protein containing the COOH-terminal PLCγ1 SH2 domain precipitates only barely detectable levels of pp58 and pp63 molecules and does not precipitate pp36 and pp38 (Fig. 1). The NH_2-terminal SH2 domain, therefore, appears to be primarily responsible for the binding of these tyrosine-phosphorylated molecules to PLCγ1. The binding is specific for PLCγ1 SH2 domains in that little or no binding is observed with a TrpE fusion protein that contains the SH2 domains of $p21^{ras}$ GAP (Fig. 1) or with TrpE alone (Fig. 2). The TrpE/$p21^{ras}$ GAP fusion protein effectively precipitates tyrosine-phosphorylated PDGF and EGF receptors (18, 19).

Only low level binding of the tyrosine-phosphorylated proteins to the TrpE/PLCγ1 SH2 fusion protein is detected in lysates from unstimulated Jurkat cells (Fig. 2). Similarly, these molecules are either absent or only barely detectable when the endogenous PLCγ1 is immunoprecipitated from unstimu-

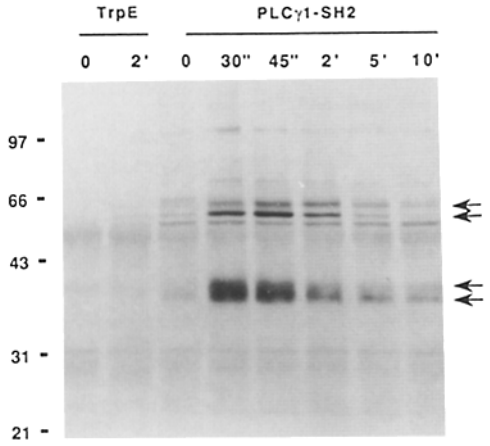

Figure 2. Time course of the binding of tyrosine-phosphorylated proteins to a TrpE fusion protein containing the NH_2- and COOH-terminal SH2 domains of PLCγ1. Jurkat cells (2×10^7 cells/sample) were either lysed directly (0) or stimulated with the TCR mAb C305. At the indicated time interval, the reactions were terminated by the addition of ice-cold lysis buffer. Precipitation experiments using either TrpE alone or the TrpE/PLCγ1 fusion protein were performed and, after electrophoresis under reducing conditions on SDS-PAGE and transfer to PVDF, were analyzed by antiphosphotyrosine immunoblotting as described in Materials and Methods. The H and L chains of the precipitating antibodies are visible in each lane and are seen in control immunoblots developed in the absence of the antiphosphotyrosine mAb (data not shown).

lated cells (data not shown). Within 30 s of TCR stimulation a substantial increase in the binding of all four molecules occurs (Fig. 2). Binding is maximal by 45 s and declines after 2 min (Fig. 2). In most, but not all experiments (compare Figs. 1 and 2), we observed induced bands of 72 and 105 kD at the earliest time points, a well as a 55-kD band whose binding was unaffected by receptor stimulation.

To determine whether a PTK associates with PLCγ1, we performed in vitro kinase reactions using precipitates with the TrpE/PLCγ1 SH2 fusion protein and immunoprecipitates of the endogenous PLCγ1 (Fig. 3, *A* and *B*). There is little or no kinase activity in precipitates from unstimulated cells or in precipitates using TrpE alone. After TCR stimulation, a protein kinase precipitates with the TrpE/PLCγ1 SH2 domain fusion protein and with the endogenous PLCγ1. In both cases, the in vitro kinase reaction yields a prominent doublet of phosphoproteins of 58 and 63 kD. Phosphoamino acid analysis of each of these phosphoproteins demonstrated that the phosphorylation occurred in tyrosine residues (Fig. 3, *C*, and data not shown).

The 58 kD and 63 kD tyrosine-phosphorylated proteins that associate with PLCγ1 have apparent Mr similar to $p59^{fyn}$ and to $p56^{lck}$, a Src-like PTK which is noncovalently associated with the CD4 and CD8 coreceptors and which, because of differential serine phosphorylation, has two elec-

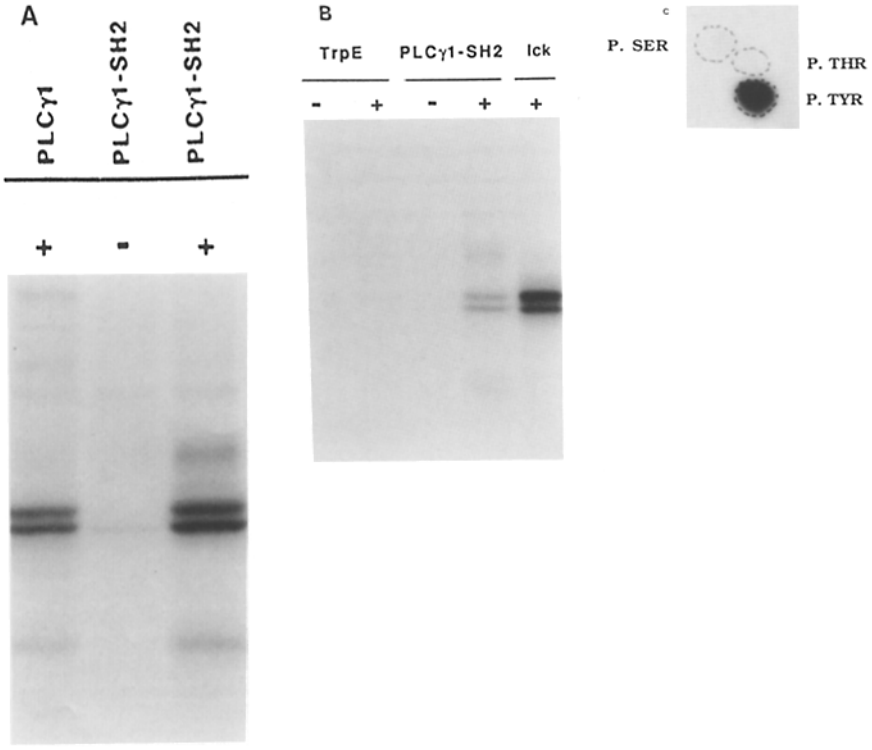

Figure 3. Detection of a PTK activity in precipitates of the endogenous PLCγ1 and in TrpE fusion protein containing the NH_2- and COOH-terminal SH2 domains of PLCγ1. (*A*) Jurkat cells were either lysed directly (−) or after stimulation (+) for 2 min with the TCR mAb C305. The dried gel was exposed to Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY) for 8 h. Each lane represents the reaction products generated by precipitations from 20×10^6 cells. (*B*) Jurkat cells were either lysed directly (−) or after stimulation (+) with the TCR mAb C305. Precipitations were performed using TrpE alone, the TrpE fusion protein containing the NH_2- and COOH-terminal SH2 domains of PLCγ1, or a rabbit antiserum to $p56^{lck}$. Kinase reactions were performed, and the reaction products analyzed as described in Materials and Methods. The autoradiograph was exposed for 3 h. The reaction products of the $p56^{lck}$ immunoprecipitates are from 10^6 Jurkat cells, whereas the other lanes represent precipitations from 10×10^6 cells. (*C*) Phosphoamino acid analysis of the 62-kD phosphoprotein generated from an in vitro kinase assay using the TrpE/PLCγ1-SH2 fusion protein. The positions of the phosphoamino acid standards are shown.

trophoretic mobilities in activated T cells (23, 24). On phosphotyrosine immunoblots, the two PLCγ1-associated molecules comigrate with $p56^{lck}$ immunoprecipitated from TCR-stimulated Jurkat cells (data not shown). Furthermore, $p56^{lck}$, which autophosphorylates during in vitro kinase reactions (23–26), comigrated with the 58- and 63-kD phosphoproteins observed with in vitro kinase reactions from either immunoprecipitated PLCγ1 or the fusion protein precipitates (Fig. 3 *B*). To confirm that the PLCγ1-associated molecules are $p56^{lck}$, we compared the peptide maps of the phosphoproteins. As shown in Fig. 4, the maps of the phosphoproteins generated from precipitates of the TrpE/PLCγ1 SH2 fusion protein are identical to those from $p56^{lck}$.

Because TCR ligation induced an association between PLCγ1 and tyrosine-phosphorylated $p56^{lck}$, we determined whether TCR stimulation altered the tyrosine phosphorylation of the latter. Consistent with previous reports that some $p56^{lck}$ is tyrosine-phosphorylated in unstimulated T cell lines (25, 26), we observed that $p56^{lck}$ immunoprecipitated from unstimulated Jurkat cells contained phosphotyrosine (Fig. 5). There was a clear increase in the tyrosine phosphorylation of $p56^{lck}$ within 60 s of TCR stimulation (Fig. 5, *second lane*). In contrast to the immunoprecipitates from resting cells, several tyrosine-phosphorylated molecules coprecipitated with $p56^{lck}$ from TCR-stimulated cells. The apparent Mr of these molecules are identical to those of the 36-, 38-, and 72-kD molecules that associate with PLCγ1 (Fig. 5, *second lane*). We then precleared lysates with the TrpE/PLCγ1 SH2 domain fusion protein to deplete the lysates of the molecules that bind PLCγ1. This preclearing removed the molecules that coprecipitate with $p56^{lck}$, confirming that these were identical to the PLCγ1-associated molecules (Fig. 5, *fourth lane*). Moreover, preclearing the lysates from TCR-stimulated cells reduced the amount of tyrosine-phosphorylated $p56^{lck}$ to the levels of unstimulated cells (Fig. 5, compare the *second* and *fourth lanes*). Consistent with the absence of PTK activity in precipitations from unstimulated cells (Fig. 3), the fusion protein did not remove any tyrosine-phosphorylated $p56^{lck}$ from the lysates of unstimulated cells (Fig. 5, compare the *first* and *third lanes*).

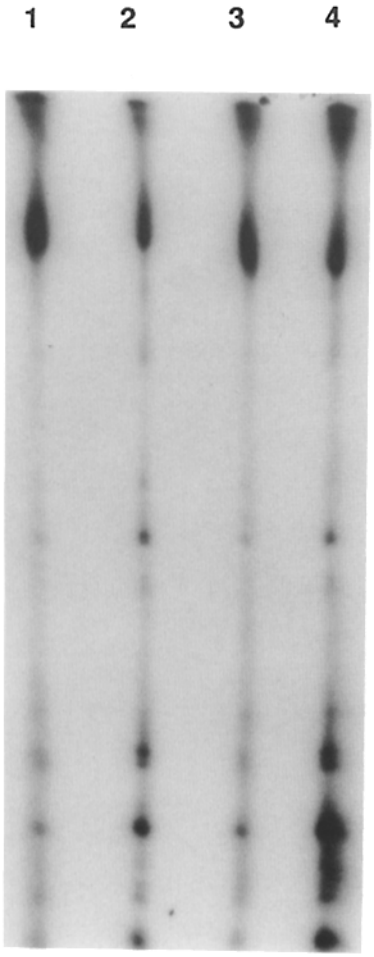

Figure 4. Comparison of the V8 protease peptide maps of the comigrating phosphoproteins generated by in vitro kinase reactions using $p56^{lck}$ immunoprecipitations from 10^6 Jurkat cells and precipitations with the TrpE/PLCγ1 SH2 fusion protein from 10×10^6 Jurkat cells. The digestion products were resolved under reducing conditions on a 15% polyacrylamide gel and visualized by exposing the dried gel to Kodak X-OMAT film (Eastman Kodak Co.) for 24 h. Lanes *1*: (*upper band*) TrpE/PLCγ1 fusion protein reaction, *2*: (*upper band*) anti-LCK reaction; *3*: (*lower band*) TrpE/PLCγ1 fusion protein reaction; *4*: (*lower band*) anti-LCK reaction.

Discussion

We observe that stimulation of the TCR on Jurkat cells leads to the binding of several tyrosine-phosphorylated molecules, including $p56^{lck}$, to PLCγ1. Studies using TrpE/PLCγ1 fusion proteins indicate that these associates are mediated by the SH2 domains of PLCγ1, the same region of PLCγ1 that binds to the tyrosine-phosphorylated EGF and PDGF receptors. The association with $p56^{lck}$ occurs within 30 s of TCR stimulation and thus coincides with the onset of tyrosine phosphorylation of PLCγ1 in Jurkat cells (8–11). The interaction between PLCγ1 and $p56^{lck}$ is transient and peaks several minutes before the reported association of PLCγ1 with CD3 (12). The time course of the association between

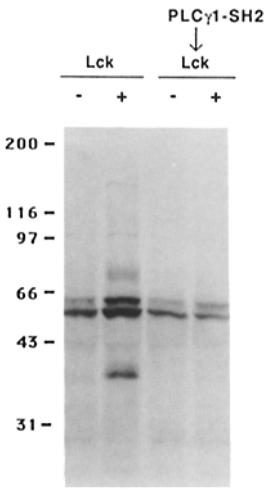

Figure 5. Effect of TCR stimulation on the tyrosine phosphorylation of $p56^{lck}$. Jurkat cells (2×10^7 cells/sample) were either lysed directly (−) or stimulated with a TCR mAb for 60 s before lysis (+). $p56^{lck}$ was immunoprecipitated with a specific antiserum, resolved by electrophoresis under reducing conditions on SDS-PAGE, transferred to PVDF, and then analyzed by immunoblotting with a mAb to phosphotyrosine. In the *third* and *fourth* lanes, the lysates were precleared with the TrpE/PLCγ1 SH2 domain fusion protein before the immunoprecipitation of $p56^{lck}$.

$p56^{lck}$ and PLCγ1, therefore, is consistent with the possibility that $p56^{lck}$ plays a role in TCR-mediated activation of PLCγ1. $p56^{lck}$ may be partly or solely responsible for tyrosine phosphorylation of PLCγ1. Alternatively, $p56^{lck}$ may phosphorylate molecules that associate with PLCγ1, and that are involved in its activation.

Our results lend additional support to earlier indications that $p56^{lck}$ may participate in TCR-mediated signaling. Recently Abraham et al. (27) observed augmented responses to antigen after expression of an activated form of $p56^{lck}$ in an antigen-specific T cell hybridoma. This augmentation occurred in the absence of cell-surface expression of CD4 and CD8 and, therefore, was independent of coreceptor ligation (27). In T cells that express CD4 or CD8, ~30–60% of $p56^{lck}$ is complexed to these coreceptors (23–26). Interactions with $p56^{lck}$ appear to be at least partly responsible for the ability of CD4 to confer enhanced antigen responses upon T cell clones and hybridomas (28). CD4 may be physically associated with the TCR, and there is evidence that maintaining CD4 and TCR in physical proximity enhances TCR-mediated signaling (29, 30). For example, in mAb-induced signaling, coclustering of the TCR and CD4 (by means of heteroconjugates of mAb) substantially augments TCR-mediated PI turnover, whereas crosslinking CD4 and the TCR independently diminishes this response (31).

In contrast to the findings presented here with $p56^{lck}$, we could not detect $p59^{fyn}$ in our PLCγ1 precipitates (data not shown). $p59^{fyn}$ coprecipitates with the TCR, and there are compelling data that $p59^{fyn}$ has a role in TCR-mediated signaling (3, 4). For example, T cells from transgenic mice that overexpress $p59^{fyn}$ have enhanced TCR-mediated Ca^{2+} mobilization, suggesting that $p59^{fyn}$ regulates the coupling of the TCR to PI turnover (4). Our inability to detect an association between $p59^{fyn}$ and PLCγ1 does not argue against a role for this, or any other PTK in TCR-mediated phosphorylation of PLCγ1. Activation of an "upstream" PTK, such as $p59^{fyn}$, might induce PLCγ1 phosphorylation indirectly by triggering the interaction between $p56^{lck}$ and PLCγ1. Alternatively, an association between PLCγ1 and $p59^{fyn}$ (or other PTKs) might not be stable under the conditions used here for precipitation. We also cannot be certain that $p56^{lck}$ is the only PTK present in our PLCγ1 precipitates, particularly in view of the precipitation of tyrosine-phosphorylated molecules (pp36, pp38, pp72, pp105) whose identity is uncertain.

The $p56^{lck}$ that associates with PLCγ1 represents <5% of the $p56^{lck}$ that can be immunoprecipitated directly with a specific antiserum (note that, in Fig. 3, one-tenth the cell number was used for the $p56^{lck}$ immunoprecipitates). The precise mechanism by which the TCR induces an association between PLCγ1 and this subset of $p56^{lck}$ remains to be determined. We find that TCR stimulation triggers an increase in the tyrosine phosphorylation of $p56^{lck}$ and that preclearing the lysates of PLCγ1-binding proteins reduces the level of tyrosine-phosphorylated $p56^{lck}$ to that of unstimulated cells. This observation, coupled with the inability of PLCγ1 to bind to $p56^{lck}$ from unstimulated cells, suggests that PLCγ1 selectively binds the subset of $p56^{lck}$ that is tyrosine phosphorylated as a result of TCR stimulation. We are currently investigating whether this reflects phosphorylation of different tyrosine residues of $p56^{lck}$ and whether the PLCγ1-associated $p56^{lck}$ derives from the pool that is associated with CD4. Another important issue is whether interaction with a small fraction of cellular $p56^{lck}$ can account for PLCγ1 phosphorylation. It is interesting in this regard that the tyrosine phosphorylation of PLCγ1 after TCR stimulation appears to involve <5% of cellular PLCγ1, with only 1% of PLCγ1 associating with CD3 (12).

In addition to $p56^{lck}$, we observed several other tyrosine-phosphorylated molecules that associate with PLCγ1 as a result of TCR stimulation. The identities of these molecules are not known. Their size distinguishes them from known components of the TCR complex. The time course for the binding of the pp36 and pp38 molecules to the PLCγ1 SH2 fusion protein is very similar to that of $p56^{lck}$. The presence of pp36, pp38, and $p56^{lck}$ in the PLCγ1 precipitates suggests two general models for the association of $p56^{lck}$ with PLCγ1. In the first model, $p56^{lck}$ binds directly to the SH2 domains of PLCγ1, and this binding is independent of pp36 and pp38. In the alternative model, the association of $p56^{lck}$ with PLCγ1 requires the participation of pp36 and pp38. For example, $p56^{lck}$ might bind directly to pp36 and pp38, and the resulting complex would interact with the SH2 domains of PLCγ1. In this model, pp36 and p38 would subserve a docking function that allows the physical approximation of $p56^{lck}$ and PLCγ1, and the tyrosine phosphorylation of pp36 and pp38 could represent a key step in TCR-mediated activation of PLCγ1. Further characterization of pp36 and pp38 should distinguish between these possibilities and shed light on the physiological functions of these molecules.

We thank J. Bolen for the antiserum to $p56^{lck}$, R. Schatzman for the antiserum to PLCγ1, and A. Weiss for C305.

This work was supported by RO1 AI-26644 from the National Institutes of Health (NIH), by the Department of Veterans Affairs, and by grants from the National Cancer Institute of Canada (NCIC) and Medical Research Council of Canada. John R. Weber is supported by training grant DK-07007 from the NIH, and Gregory M. Bell is a recipient of a NIH Physician-Scientist Award. Tony Pawson is a Terry Fox Cancer

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Received for publication 3 March 1992 and in revised form 28 April 1992.

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