Association of the Tyrosine Kinase LCK with Phospholipase C-\(\gamma\)1 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-\(\gamma\)1 (PLC\(\gamma\)1). 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 PLC\(\gamma\)1. An identical pattern of proteins precipitate with TrpE fusion proteins that contain the Src homology (SH) 2 domains of PLC\(\gamma\)1, indicating that these regions of PLC\(\gamma\)1 are responsible for binding. TCR stimulation leads to an association between the SH2 domains of PLC\(\gamma\)1 and a protein tyrosine kinase, which, by peptide mapping, is identical to p56\(\kappa\kappa\). These studies establish that p56\(\kappa\kappa\) associates with PLC\(\gamma\)1 as a result of TCR stimulation of Jurkat cells, suggesting that p56\(\kappa\kappa\) plays a central role in coupling the TCR to the activation of PLC\(\gamma\)1.

A ctivation 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\(\kappa\kappa\), 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 PLC\(\gamma\)1 and, subsequently, to an association between PLC\(\gamma\)1 and the CD3 components of the TCR (8–12).

The molecular interactions that are required for TCR-mediated activation of PLC\(\gamma\)1 in Jurkat cells, and the PTK involved, are not known. Critical to the activation of PLC\(\gamma\)1 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 PLC\(\gamma\)1 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 PLC\(\gamma\)1 (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 PLC\(\gamma\)1 associates with tyrosine phosphorylated molecules as a result of TCR stimulation of Jurkat cells, and that this association involves the SH2 domains of PLC\(\gamma\)1.

Materials and Methods

Reagents. TrpE fusion proteins containing the SH2 domains of PLC\(\gamma\)1 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\(\kappa\kappa\) and to PLC\(\gamma\)1 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).

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\(^1\) Abbreviations used in this paper: EGF, epidermal growth factor; GAP, GTPase activating protein; PDGF, platelet-derived growth factor; PI, phosphoinositide; PLC\(\gamma\)1, phospholipase C-\(\gamma\)1; PTK, protein tyrosine kinase; PVDF, polyvinylidene difluoride.
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 NH2- and COOH-terminal PLCγ1 SH2 domains precipitate tyrosine-phosphorylated proteins that exactly comigrate with PLCγ1-associated molecules. Comparable levels of all four tyrosine-phosphorylated proteins also bind to a fusion protein that has only the NH2-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 NH2-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 p21GAP (Fig. 1) or with TrpE alone (Fig. 2). The TrpE/p21GAP 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 1. Tyrosine-phosphorylated proteins from TCR-stimulated Jurkat cells coprecipitate with PLCγ1 and bind to TrpE fusion proteins containing the NH2-SH2 domain of PLCγ1. Jurkat cells (6 x 10⁶ 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 NH2- and COOH-terminal SH2 domains of PLCγ1 (PLCγ-SH2), the NH2-terminal SH2 domain alone (PLCγ-SH2[N]), the COOH-terminal domain alone (PLCγ-SH2[ψ]), or the NH2- and COOH-terminal SH2 domains of p21GAP (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). (Arrow) Tyrosine-phosphorylated proteins of 36, 38, 58, and 63 kD that coprecipitate with endogenous PLCγ1 and with TrpE fusion proteins containing the NH2-terminal SH2 domain of PLCγ1.
Figure 2. Time course of the binding of tyrosine-phosphorylated proteins to a TrpE fusion protein containing the NH₂- and COOH-terminal SH2 domains of PLCγ1. Jurkat cells (2 × 10⁷ 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).

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 kDa at the earliest time points, a well as a 55-kDa 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 kDa. 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 kDa and 63 kDa tyrosine-phosphorylated proteins that associate with PLCγ1 have apparent Mr similar to p59f11 and to p56lck, a Src-like PTK which is noncovalently associated with the CD4 and CD8 coreceptors and which, because of differential serine phosphorylation, has two elec-
trophoretic mobilities in activated T cells (23, 24). On phosphotyrosine immunoblots, the two PLCγ1-associated molecules comigrate with p56kk immunoprecipitated from TCR-stimulated Jurkat cells (data not shown). Furthermore, p56kk, 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 p56kk, 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 p56kk.

Because TCR ligation induced an association between PLCγ1 and tyrosine-phosphorylated p56kk, we determined whether TCR stimulation altered the tyrosine phosphorylation of the latter. Consistent with previous reports that some p56kk is tyrosine-phosphorylated in unstimulated T cell lines (25, 26), we observed that p56kk immunoprecipitated from unstimulated Jurkat cells contained phosphotyrosine (Fig. 5). There was a clear increase in the tyrosine phosphorylation of p56kk within 60 s of TCR stimulation (Fig. 5, second lane). In contrast to the immunoprecipitates from resting cells, several tyrosine-phosphorylated molecules coprecipitated with p56kk 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 p56kk, 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 p56kk 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 p56kk from the lysates of unstimulated cells (Fig. 5, compare the first and third lanes).

Discussion

We observe that stimulation of the TCR on Jurkat cells leads to the binding of several tyrosine-phosphorylated molecules, including p56kk, 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 p56kk 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 p56kk is transient and peaks several minutes before the reported association of PLCγ1 with CD3 (12). The time course of the association between

Figure 4. Comparison of the V8 protease peptide maps of the comigrating phosphoproteins generated by in vitro kinase reactions using p56kk 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.

Figure 5. Effect of TCR stimulation on the tyrosine phosphorylation of p56kk. Jurkat cells (2 × 10^6 cells/sample) were either lysed directly (−) or stimulated with a TCR mAb for 60 s before lysis (+). p56kk 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 p56kk.
Our results lend additional support to earlier indications that p56 \( \kappa \) may participate in TCR-mediated signaling. Recently Abraham et al. (27) observed augmented responses to antigen after expression of an activated form of p56 \( \kappa \) 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, \( \sim 30-60\% \) of p56 \( \kappa \) is complexed to these coreceptors (23–26). Interactions with p56 \( \kappa \) 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 \( \kappa \), we could not detect p59 \( \mu \) in our PLC\( \gamma \)1 precipitates (data not shown). p59 \( \mu \) precipitates with the TCR, and there are compelling data that p59 \( \mu \) has a role in TCR-mediated signaling (3, 4). For example, \( \kappa \) cells from transgenic mice that overexpress p59 \( \mu \) have enhanced TCR-mediated Ca\( ^{2+} \) mobilization, suggesting that p59 \( \mu \) regulates the coupling of the TCR to PI turnover (4). Our inability to detect an association between p59 \( \mu \) and PLC\( \gamma \)1 does not argue against a role for this, or any other PTK in TCR-mediated phosphorylation of PLC\( \gamma \)1. Activation of an “upstream” PTK, such as p59 \( \mu \), might induce PLC\( \gamma \)1 phosphorylation indirectly by triggering the interaction between p56 \( \kappa \) and PLC\( \gamma \)1. Alternatively, an association between PLC\( \gamma \)1 and p59 \( \mu \) (or other PTKs) might not be stable under the conditions used here for precipitation. We also cannot be certain that p56 \( \kappa \) is the only PTK present in our PLC\( \gamma \)1 precipitates, particularly in view of the precipitation of tyrosine-phosphorylated molecules (pp36, pp38, pp72, pp105) whose identity is uncertain.

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