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A New Tyrosine Phosphorylation Site in PLCγ1: The Role of Tyrosine 775 in Immune Receptor Signaling

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Phospholipase Cγ (PLCγ) is a ubiquitous gatekeeper of calcium mobilization and diacylglycerol-mediated events induced by the activation of Ag and growth factor receptors. The activity of PLCγ is regulated through its controlled membrane translocation and tyrosine (Y) phosphorylation. Four activation-induced tyrosine phosphorylation sites have been previously described (Y472, Y771, Y783, and Y1254), but their specific roles in Ag receptor-induced PLCγ1 activation are not fully elucidated. Unexpectedly, we found that the phosphorylation of a PLCγ1 construct with all four sites mutated to phenylalanine was comparable with that observed with wild-type PLCγ1, suggesting the existence of an unidentified site(s). Sequence alignment with known phosphorylation sites in PLCγ2 indicated homology of PLCγ1 tyrosine residue 775 (Y775) with PLCγ2 Y753, a characterized phosphorylation site. Tyrosine 775 was characterized as a phosphorylation site using phospho-specific anti-Y775 antiserum, and by mutational analysis. Phosphorylation of Y775 did not depend on the other tyrosines, and point mutation of PLCγ1 Y775, or the previously described Y783, substantially reduced AgR-induced calcium, NF-AT, and API-1 activation. Mutation of Y472, Y771, and Y1254 had no effect on overall PLCγ1 phosphorylation or activation. Although the concomitant mutation of Y775 and Y783 abolished downstream PLCγ1 signaling, these two tyrosines were sufficient to reconstitute the wild-type response in the absence of functional Y472, Y771, and Y1254. These data establish Y775 as a critical phosphorylation site for PLCγ1 activation and confirm the functional importance of Y775.


A n early consequence of ligand-induced activation of a variety of cell surface receptors is the activation of phosphoinositide-specific phospholipase Cγ (PLCγ). The PLCγ isoforms γ1 and γ2 are downstream of receptors with intrinsic tyrosine kinase activity, such as the growth factor receptors for epidermal growth factor and plasma-derived growth factor (PDGF), and receptors without intrinsic kinase activity, such as the B and T cell Ag receptors (BCR and TCR, respectively). The latter depend on the recruitment of non-receptor tyrosine kinases for their activation (1–5). PLCγ is a cytoplasmic enzyme that requires membrane translocation and tyrosine phosphorylation for its activation. T cells predominantly express the PLCγ1 isoform, PLCγ2 is expressed at high levels in B cells (2, 6, 7). The activated enzyme hydrolyzes phosphatidylinositol 4,5-bisphosphate to form inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). DAG participates in the activation of the RasGRP/PKC effector pathways leading to activation of the transcription factor API-1 (reviewed in Ref. 8). IP3 mediates intracellular Ca2+ mobilization, which is required for downstream events such as activation of the transcription factor NF-AT (8, 9). Both the Ca2+ and DAG signaling pathways are required for T cell cytokine production and proliferation.

Four phosphorylated tyrosines (pY) have been characterized in PLCγ1 Y472, Y771, Y783, and Y1254 (amino acid numbering is based on the sequence of the bovine protein), after epidermal growth factor and PDGF receptor stimulation (10, 11) and in response to TCR activation (12). Studies in both receptor systems demonstrated a requirement for PLCγ1 Y783 phosphorylation in phosphoinositide breakdown (12) and gene induction (13). In addition, early studies indicated Y1254 was needed for maximal growth factor-induced stimulation, while Y771 was proposed as a negative regulator. However, in later studies, the role of these two tyrosines has been questioned (14).

In an effort to resolve these issues, we initiated a study that investigated what effect mutation of each site individually or in combination had on PLCγ1 phosphorylation and activation after Ag receptor (AgR) stimulation. Because previous work from our laboratory had indicated that activation requirements for PLCγ1 were similar for TCR or BCR stimulation (15), models using both receptor systems were analyzed. These studies resulted in the identification of a new phosphorylation site (Y775) that is required for AgR-induced PLCγ1 activation. Our data indicate that phosphorylation of Y775 and Y783 play equally important roles in the activation of PLCγ1 and further suggest that Y472, Y771, and Y1254 are not required for PLCγ1-mediated Ca2+ mobilization or activation of the DAG signaling pathway.

Materials and Methods

Cells and reagents

Jurkat E6.1 (T cell line), Jurkat TAg (16), Jurkat Jy1 (PLCγ-deficient Jurkat) (13), P10–14 (17) (DT40-derivative B cell line deficient in PLCγ1 and y2) and stable PLCγ1 P10–14 transfectants were maintained in RPMI 1640 supplemented with 10% FBS, 25 mM HEPES, 1 mM sodium pyruvate, 1× non-essential amino acids (Biosource), and 2 mM L-glutamine.
The anti-TCR Ab C305 (IgM) was a gift from A. Weiss (University of California, San Francisco, CA), the anti-hemagglutinin (HA) Ab 12CA5 from A. Weissman (National Cancer Institute, Bethesda, MD) and the NF-AT and AP-1 luciferase reporter gene constructs from G. Crabtree (Stanford University, Stanford, CA). Purified goat-anti-chicken IgM was purchased from Bethyl Laboratories, anti-py Ab 4G10 from Upstate Biotechnology, anti-pY783 from Cell Signaling Technology, and anti-HA 3F10 from Roche. The anti-pY775 rabbit serum was generated through a commercial contract with Biosource International by immunizing rabbits with the peptide Ac-YGAL(pY)EGRNPGF(Ahx)C-amide. The serum was purified on an agarose gel, eluted with low pH glycine, and quantitated spectrophotometrically at 280 nm.

**DNA plasmds, transfections, and generation of stable transfectants**

The construction of vectors expressing bovine PLCγ1 in pBluescript II-SK (pBluSK) and HA-tagged PLCγ1 (PLCγ1-HA) in pCIneo (Promega) has been previously described (15). Tyrosine (Y) to phenylalanine (F) mutations on PLCγ1 were created using the QuickChange site-directed mutagenesis kit (Stratagene) or by subcloning fragments with the point mutations created with this kit. Transfections were performed by electroporation (15). Tyrosine to phenylalanine mutation constructions were named by a 5-letter code, according to the numerical sequence of tyrosines Y472, Y771, Y775, Y783, and Y1254, where “Y” indicates retained tyrosines and “F” indicates tyrosines that have been mutated to phenylalanine (e.g., WT corresponds to “YYYYY”, the single mutation of 775 or 783 as YYYFY and YYYYY, respectively). Stable P10–14 lines were generated by transfection with 20 μg of the indicated pCIneo PLCγ1-HA construct and 2 μg of pBABEpuro (18) followed by selection with puromycin (Sigma-Aldrich).

**Cell activation, immunoprecipitation, and immunoblotting**

Transfected Jurkat cells were cultured at 0.5 × 10⁶/ml for 18 h and subjected to Ficoll separation. For stimulation, 10⁵ Jurkat cells were treated with medium or C305 Ab for 2 min at 37°C. Peripheral blood lymphocytes were obtained from the National Institute of Health blood bank. RBC were removed by Ficoll-Hypaque centrifugation, and the cells were stimulated overnight with 2 μg/ml PHA. PHA was then removed, and the cells were cultured for 6 days in 20 U/ml IL-2. PHA blasts (3 × 10⁶ cells/point) were incubated with the anti-TCR antibody, OKT3 (150 μg/ml; eBioscience) on ice for 20 min, washed, and then cross-linked with a goat anti-mouse IgG Ab for 3 min at 37°C. Cells were then lysed, immunoprecipitated, and resolved by SDS-PAGE followed by immunoblot analysis and chemiluminescent detection as previously described (15).

**Reporter gene assays**

Jurkat cells were transfected with 5 μg of the respective PLCγ1-HA construct and 5 μg of an NF-AT or AP-1 plasmid containing a luciferase reporter. After 18 h, the cells were distributed in duplicate wells of a 24-well plate containing either medium alone, C305 Ab, or C305 Ab for 3 min at 37°C. Luciferase activity was measured as previously described (15).

**Calcium mobilization**

Cells were suspended in HBSS supplemented with 1% FBS and 10 mM HEPES. They were loaded with Indo-1/AM (Molecular Probes) at 1°C for 30 min, washed and adjusted to 1 × 10⁷/ml. Calcium flux was measured on a BLS flow cytometer equipped with a helium-cadmium laser (325 UV). Cell loading was assessed by stimulation with ionomycin; equal ionomycin responses were a prerequisite for the continuation of the experiments. P10–14 cells and P10–14 cells stably reconstituted with wild-type (WT) PLCγ1 served as negative and positive controls, respectively, in each experiment. Appropriate band-pass filters were used, and the data were analyzed using FlowJo software (Tree Star).

**Results and Discussion**

**Tyrosine 775 of PLCγ1 is phosphorylated upon TCR and BCR activation**

To address the contribution of the previously described PLCγ1 tyrosine (Y) phosphorylation sites in AgR-induced PLCγ1 phosphorylation, PLCγ1 mutants were generated in which tyrosines 472, 771, 783, or 1254 were replaced by phenylalanine (F) individually or in combination, and the constructs were ectopically expressed in the Jurkat TAg cell line. The degree of AgR-induced phosphorylation of each mutant was then compared with that observed with WT PLCγ1 by anti-HA immunoprecipitation, and immunoblotting with the anti-pY Abs 4G10 (Fig. 1A) or PY20 (unpublished data) (16). None of the individual Y to F point mutations impaired the overall phosphorylation of PLCγ1 (Fig. 1A). Surprisingly, a PLCγ1 construct in which all four tyrosines had been mutated to phenylalanine was still found to be heavily phosphorylated upon TCR stimulation (67% of WT, Fig. 1B). The same results were observed after anti-IgM induced activation of the PLCγ-deficient P10–14 cell line stably reconstituted with WT PLCγ1 or the PLCγ1 Y to F mutant constructs (unpublished data). These data suggest the existence of one or more unidentified AgR-induced tyrosine phosphorylation sites within PLCγ1.

Multiple BCR-induced phosphorylation sites have been identified for PLCγ2, including Y753 and Y759, which reside in the region between the carboxy Src homology 2 (SH2C) domain and the Src homology 3 (SH3) domain of PLCγ2 (19–22). The tyrosine phosphorylation sites on PLCγ1 were characterized by phosphopeptide mapping and sequencing (10, 11) and PLCγ1 Y771 and Y783 were suggested as the homologues to PLCγ2 Y753 and Y759 (14, 19, 22, 23). Upon reassessment of the sequences, we proposed that PLCγ1 Y775 rather than PLCγ1 Y771 was the more likely equivalent for PLCγ2 Y753 (Fig. 2A).

To investigate whether PLCγ1 Y775 was indeed an AgR-induced phosphorylation site, we engineered a PLCγ1 mutant in which Y775 was mutated to phenylalanine in combination with Y472, Y771, Y783, and Y1254. We adopted a 5-letter code for the various constructs, listing the respective tyrosines/phenylalanines in ascending amino acid order. As an example, the construct cited above with all the previously characterized phosphorylation sites exchanged for phenylalanine is named FFFFFF and the construct with the additional Y775F mutation is referred to as FFFFFF. The AgR-induced phosphorylation of the FFFFFF construct (Y775 intact) was measured at 67% of WT as detected by 4G10, whereas the PLCγ1 FFFFF construct with the additional mutation of Y775 showed marked reduction to 23% of WT in transiently transfected Jurkat cells (Fig. 1B) or in a stable PLCγ1 FFFFFF expressing P10–14 cell line (unpublished data). These data...
and YYFYY PLCγ1 constructs as controls in experiments that used the anti-pY775 antibody. Phosphorylation of Y775 did not require the presence of Y783 (Fig. 2B), nor did the phosphorylation of Y783 require Y775 (unpublished data). These data indicate that PLCγ1 Y775 is phosphorylated upon TCR and BCR stimulation and that its phosphorylation contributes to the signal intensity observed with anti-phosphotyrosine antibodies, such as 4G10. Furthermore, Y775 phosphorylation is independent of the presence of the other four tyrosines, as there was no alteration in its level of phosphorylation in the FFFYF construct compared with WT PLCγ1 (Fig. 2B).

**Y775 and Y783 phosphorylation is required for PLCγ1-dependent calcium mobilization**

PLCγ1 Y783, and its counterpart Y759 in PLCγ2, has been shown by several groups to be important for receptor-mediated activation of PLCγ1 (14, 19, 20, 22, 23). The role of other tyrosines is less clear. Data from Kim et al. (14) suggested that after PDGF receptor (PDGFR) stimulation, mutation of Y1125 inhibited, and mutation of Y771 enhanced PLCγ1 activity. Recent work from this group, however, raises questions about the significance of Y771 and Y1254 phosphorysion in terms of PLCγ1 activity after both AgR and PDGFR stimulation (23). For PLCγ2 activation there is also contradictory data on the role of Y1197 and Y1217 (20, 22) which may be analogous to Y1254 in PLCγ1. To delineate the functional importance of the tyrosine sites for in vivo AgR-induced PLCγ1 activation, we first investigated the requirement for each tyrosine in BCR-induced Ca2+ mobilization in stable transfectants of the P10–14 cell line. This B cell line was chosen because it is devoid of PLCγ1 and PLCγ2 and thus has no Ca2+ mobilization, while the PLCγ1-deficient Jurkat T cell line, Jy1, has residual detectable calcium responses due to endogenous PLCγ2 (13). Clones with matching levels of HA-PLCγ1 and BCR expression were used. All clones responded equally well to stimulation with ionomycin (unpublished data). The positive control cell line expressing WT PLCγ1 showed a characteristic pattern of Ca2+ signaling upon BCR activation (Fig. 3A). As expected, neither the parental PLCγ1-deficient P10–14 cell line (Null), nor the PLCγ1 FFYYF expressing cell line mobilized Ca2+ after BCR cross-linking (Fig. 3A). Significantly, a single mutation in either Y775 or Y783 resulted in a dramatic decrease in BCR-induced Ca2+ mobilization, whereas the single mutation of Y771 had no effect on the Ca2+ response (Fig. 3B). Mutation of both Y775 and Y783 to phenylalanine in the context of intact Y472, Y771 and Y1254 (PLCγ1 YYFYF) produced a PLCγ1 construct that was incapable of mediating a BCR-induced Ca2+ flux (Fig. 3C). Conversely, a PLCγ1 construct that had Y472, Y771 and Y1254 mutated to phenylalanine, but had intact Y775 and Y783 residues (PLCγ1 FFYYF) mediated a BCR-induced Ca2+ response that was comparable to WT PLCγ1 (Fig. 3D). These data confirm the importance of Y783 in PLCγ1 activation and indicate that Y775 plays an equally important role in AgR-induced PLCγ1 activation. Data from the PLCγ1 FFYYF and YYFYF constructs suggest that, of the known tyrosine phosphorylation sites in PLCγ1, Y775 and Y783 are both required and sufficient for full AgR-induced PLCγ1 activation.

**Both Y775 and Y783 are required for PLCγ1-dependent gene transcription events**

To further delineate the impact of PLCγ1 phosphorylation on downstream signaling events, we measured the transcriptional activation of NF-AT and AP-1 using luciferase reporter gene assays. For this purpose, the PLCγ1-deficient Jurkat cell line, Jy1 (13), strongly suggested that Y775 is an AgR-induced PLCγ1 phosphorylation site.

To confirm Y775 as a true phosphorylation site, we generated a rabbit polyclonal phospho-specific Y775 Ab (anti-pY775) and tested the antibody’s reactivity on anti-HA immunoprecipitates from the stable PLCγ1-expressing P10–14 cell lines. Anti-pY775 showed no reactivity on PLCγ1 immunoprecipitated from resting cells, but yielded a single immunoreactive band at the m.w. of PLCγ1 after immunoprecipitation of HA-PLCγ1 from activated cells (Fig. 2B). No phosphorylation of the FFFFY construct was observed with anti-pY775 and little or no phosphorylation of the YYFYF construct (Y775F). Binding of anti-pY775 to PLCγ1 from activated cells was blocked by the phosphorylated peptide used as the immunogen for Ab production but not by its non-phosphorylated counterpart (Fig. 2B). Similar results were seen in Jurkat cells transiently transfected with the respective PLCγ1 constructs (unpublished data). In addition, Y775 was phosphorylated on endogenous PLCγ1 after TCR stimulation of Jurkat cells, PHA blasts generated from human peripheral blood lymphocytes (Fig. 2C), or mouse CD4+ lymphocytes (unpublished data). Although the reactivity of anti-pY775 was substantially higher in PLCγ1 constructs with intact Y775, the Ab also exhibited a low level of reactivity with the YYFYF construct in stimulated P10–14 cells (Fig. 2B). This low level of cross-reactivity was not observed in T cells, nevertheless, we routinely included the WT

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**FIGURE 2.** AgR-induced phosphorylation of PLCγ1 Y775. A. Sequence alignment of human PLCγ2 (P16885), human PLCγ1 (P19174), and bovine PLCγ1 (P08487). B. The indicated HA-tagged PLCγ constructs were immunoprecipitated from stably transfected P10–14 cells, which were either left unstimulated or were stimulated with anti-IgM Ab for 2 min at 37°C. Anti-HA precipitates were split into three equal parts, resolved on SDS-PAGE gels, transferred and blotted with either anti-PLCγ or reprobed with anti-HA. Each panel originates from one film, black bars represent lanes that were rearranged for reasons of presentation but stem from the same experiment and film.
FIGURE 3. PLCγ1 Y775 and Y783 are required for BCR-induced calcium mobilization. P10–14 stable cell lines expressing the indicated PLCγ1 phosphorylation mutant were loaded with Indo-1/AM and stimulated with anti-IgM Ab. The ratio of fluorescence emission at 405 and 495 nm was plotted as a function of time. In total, three stable clones per construct were tested at least three times. The representative kinetics in A, B, and D are from the same experiment. WT PLCγ1 and parental P10–14 cells (null) served as positive and negative controls, respectively, in each experiment.

FIGURE 4. PLCγ1 Y775 and Y783 are required for PLCγ1-dependent AgR-induced transciptional events. A. PLCγ1-deficient Jy1 Jurkat cells were transiently transfected with NF-AT luciferase reporter plasmid and the indicated PLCγ1 expression plasmid. Cells were cultured in medium and stimulated with C305 Ab or PMA plus ionomycin. NF-AT activation was normalized to the maximal activity obtained with PMA plus ionomycin in each sample for intra- and interexperimental comparison. B. Jy1 cells were transiently transfected with AP-1-luciferase reporter plasmid and the indicated PLCγ1 expression plasmid. Cells were cultured in medium and stimulated with C305 mAb or PMA. AP-1 activation was normalized to the maximal activity obtained with PMA in each sample. C. PLCγ1-deficient P10–14 cells were transiently transfected with NF-AT luciferase reporter plasmid and the indicated PLCγ1 expression plasmid. Cells were cultured in medium, stimulated with anti-IgM Ab, or PMA plus ionomycin. NF-AT activation was normalized to the maximal activity obtained with PMA plus ionomycin for each construct and normalized to WT for cross-experimental comparison. All panels show the mean and SEM of three experiments, and in all cases, cells were transfected with 5 μg of reporter plasmid and 5 μg of the respective PLCγ1 construct.

was transiently cotransfected with either an NF-AT- or AP-1-driven luciferase reporter construct, and the respective PLCγ1 Y to F mutant. The cells were subsequently activated through the TCR with C305, and the luciferase activity measured (Fig. 4, A and B). Jy1 cells are able to generate small but detectable NF-AT and AP-1 responses, which are probably mediated by low levels of PLCγ2 in these cells (13). PLCγ1 FFFFF was therefore included in all experiments to control for this background activity. Cells transfected with WT PLCγ1 and PLCγ1 FFYYF showed robust NF-AT activation when stimulated with C305 compared with non-stimulated cells (Fig. 4A). No activity was observed in the double Y775 and Y783 mutant (PLCγ1 YYYYY). The single Y775F construct (YYFYY) had reduced TCR-induced NF-AT activation whereas the response of PLCγ1 Y783F (YYYFY) was comparable with that observed with PLCγ1 FFFFF, suggesting that it was incapable of inducing NF-AT activation. Conversely, PLCγ1 FFFYYF retained a marginal level of activity, whereas PLCγ1 FFYFF had baseline NF-AT activation. Similar results were obtained in transiently transfected P10–14 cells (Fig. 4C). These data suggest that phosphorylation of both tyrosines is required for PLCγ1 to mediate full NF-AT activation.

PLCγ1 phosphorylation requirements for TCR-induced AP-1 activation were similar to those for NF-AT activation (Fig. 4B). Both Y775 and Y783 were required for AP-1 activation. Cells transfected with PLCγ1 YYFFY displayed baseline TCR-induced AP-1 responses whereas activation of those transfected with FFFYY retained a marginal level of activity, whereas PLCγ1 FFYYF had baseline NF-AT activation. Similar results were obtained in transiently transfected P10–14 cells (Fig. 4C). These data definitively identify Y775 as a new, functionally important phosphorylation site on PLCγ1. They further indicate that phosphorylation of both Y775 and Y783 is required for PLCγ1 activation as measured by AgR-induced Ca2+ flux, NF-AT and AP-1 activation. Y775 and Y783 are therefore identified as the primary regulatory tyrosines for both the IP3-mediated Ca2+ flux and the DAG-mediated activation of the PKC/RasGRP pathway. No role for AgR-induced Y472, Y771, and Y1254 phosphorylation is apparent from our data, suggesting that phosphorylation of these sites is not required for AgR-induced PLCγ1 activation. In addition, our data suggest that the phosphorylation of Y775 occurs independently of the other tyrosine residues and that it does not affect the phosphorylation of Y783.

These results underscore the importance of tyrosine phosphorylation for the activation of PLCγ1. Additional work is required to distinguish whether phosphorylation alters the intramolecular conformation of PLCγ1 (24), its substrate recognition (12) or protein/protein interactions that are required for activation (25).
Furthermore, although we have shown that Y775 is phosphorylated after TCR engagement in human peripheral T cells and mouse CD4⁺ T cells, the role that Y775 plays in PLCγ1 activation during T and B cell development and lymphocyte activation in vivo remains to be confirmed. Our data emphasize the importance of using site-specific anti-pY Abs for the analysis of proteins with multiple tyrosine phosphorylation sites and delineate the critical roles of Y775 and Y783 for PLCγ1 function.

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Disclosures
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