Tyrosine Residues in Phospholipase Cγ2 Essential for the Enzyme Function in B-cell Signaling*

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Phospholipase Cγ (PLCγ) isoforms are regulated through activation of tyrosine kinase-linked receptors. The importance of growth factor-stimulated phosphorylation of specific tyrosine residues has been documented for PLCγ1; however, despite the critical importance of PLCγ2 in B-cell signal transduction, neither the tyrosine kinase(s) that directly phosphorylate PLCγ2 nor the sites in PLCγ2 that become phosphorylated after stimulation are known. By measuring the ability of human PLCγ2 to restore calcium responses to the B-cell receptor stimulation or oxidative stress in a B-cell line (DT40) deficient in PLCγ2, we have demonstrated that two tyrosine residues, Tyr[753] and Tyr[759], were important for the PLCγ2 signaling function. Furthermore, the double mutation Y753F/Y759F in PLCγ2 resulted in a loss of tyrosine phosphorylation in stimulated DT40 cells. Of the two kinases that previously have been proposed to phosphorylate PLCγ2, Btk, and Syk, purified Btk had much greater ability to phosphorylate recombinant PLCγ2 *in vitro*, whereas Syk efficiently phosphorylated adapter protein BLNK. Using purified proteins to analyze the formation of complexes, we suggest that function of Syk is to phosphorylate BLNK, providing binding sites for PLCγ2. Further analysis of PLCγ2 tyrosine residues phosphorylated by Btk and several kinases from the Src family has suggested multiple sites of phosphorylation and, in the context of a peptide incorporating residues Tyr[753] and Tyr[759], shown preferential phosphorylation of Tyr[753].

The hydrolysis of phosphatidylinositol 4,5-bisphosphate by phosphoinositide-specific phospholipase C occurs in response to a large number of extracellular signals (reviewed in Refs. 1–4). Four families of mammalian phosphoinositide-specific phospholipase C (PLC),[1] PLCβ (β1–β4), PLCγ (γ1, γ2), PLCδ (δ1–δ4), and PLCε, have been described. Each family is characterized by the distinct domain organization and type of signaling pathways that regulate enzyme activity.

PLCγ isoforms are mainly regulated through receptors with intrinsic tyrosine kinase activity (*e.g.* growth factor receptors) or receptors (such as B- and T-cell antigen receptors) that are linked to the activation of nonreceptor tyrosine kinases through a complex signaling network (3–5). The two isoforms of PLCγ have distinct tissue distributions; whereas PLCγ1 is expressed ubiquitously, the pattern of expression of PLCγ2 is characterized by high levels in cells of hematopoietic origin. Transgenic studies suggested that the biological function of these isoforms is reflected in their cellular distribution. Thus, a deficiency in PLCγ1 is embryonic lethal in mice (6), whereas homozygous disruption of PLCγ2 allowed normal development but resulted in functional and signaling disorders in a subset of cell types including B-cells, platelets, and mast cells (7).

The importance of PLCγ2 in signaling in B-cells has not only been documented in experiments using transgenic animals deficient in PLCγ2 (7) but also by studies of a chicken B-cell lymphoma cell line (DT40) (reviewed in Refs. 8 and 9) with the property of extraordinarily high frequency of homologous recombination when DNA constructs are introduced into the cells. Generation of a number of targeted mutations in specific genes in DT40 cells provided valuable information about signaling components linking the activation of the B-cell receptor (BCR) to an increase in intracellular calcium concentrations. Using this system, it has been found that protein-tyrosine kinases from Src, Tec (*e.g.* Btk), and Syk/ZAP70 families are essential signaling components of the BCR pathway (10, 11). In addition, an adapter BLNK (B-cell linker protein), inositol 1,4,5-trisphosphate receptors, and PLCγ2 itself were required for calcium responses triggered by the BCR (12–15). Although each of these components may have more than one function and could be integrated in different pathways in B-cells, the current model (8, 9) suggests that the Src family kinase Lyn interacts with BCR and becomes activated upon the receptor aggregation. Activation of Syk kinase results in phosphorylation of BLNK that could provide binding sites for PLCγ2 and a number of other proteins. Syk, together with Btk, has also been implicated in phosphorylation of PLCγ2, which, through inositol 1,4,5-trisphosphate production, results in calcium mobilization. A similar pathway seems to be involved in calcium responses to oxidative stress after exposure of B-cells to hydrogen peroxide (16–18). It has been reported that the BCR complex and tyrosine kinases Syk, Lyn, and Btk, are components required for calcium responses. In addition, phosphorylation of several protein components, including BLNK and PLCγ2, has been described.

Despite extensive genetic dissection of B-cell signal transduction, it has not been shown which tyrosine kinase(s) directly

[1] The abbreviations used are: PLC, phospholipase C; BCR, B-cell receptor; PBS, phosphate-buffered saline; GFP, green fluorescent protein; EGF, epidermal growth factor; GST, glutathione *S*-transferase; SH2 and SH3, Src homology 2 and 3, respectively; PH, pleckstrin homology.

phosphorylate PLCγ2 or which sites in PLCγ2 become phosphorylated in response to BCR activation or oxidative stress. Similarly, the relative importance of specific tyrosine residues for signaling function of PLCγ2 has not been clarified. More generally, the molecular mechanism of activation of PLCγ and the role of phosphorylation in this process is not well understood. Previous studies of PLCγ phosphorylation have been mainly restricted to PLCγ1 in signaling through growth factor receptors (19–21). These studies revealed multiple phosphorylation sites, not all of which appear to be functionally critical at least in the context of a specific signaling pathway.

To analyze phosphorylation and importance of specific tyrosine residues in PLCγ2, we used DT40 cell lines stimulated by BCR cross-linking or by oxidative stress. In experiments where the human wild-type and mutated PLCγ2 constructs were tested for reconstitution of calcium responses in DT40 PLCγ2⁻ cells, two tyrosine residues have been identified as important for PLCγ2 phosphorylation and activation in B-cells. Further experiments, using purified protein components, implicated tyrosine kinase Btk and possibly some kinases from the Src family in direct phosphorylation of PLCγ2 and suggested that the requirement for Syk kinase in PLCγ2 activation mainly involves phosphorylation of the adapter protein BLNK.

EXPERIMENTAL PROCEDURES

Generation of DT40 Cell Lines Expressing Human PLCγ2—For the expression of human PLCγ2 in DT40 cells, the full-length cDNA (22) was subcloned into the pApuro vector as described previously (23). The original sequencing data contained a sequencing error close to the C terminus; the corrected frame of the amino acid sequence shows good alignment with the rat PLCγ2 sequence in this region (Fig. 1*B*).

The mutations of tyrosine residues, Y753F, Y759F, and Y753F/Y759F, were generated using a two-stage PCR-based overlap extension method and introduced into pApuro/PLCγ2 construct. The plasmids were used for stable transfection of DT40/PLCγ2⁻ cells (15) as described previously for the wild type PLCγ2 (23). Briefly, the linearized constructs of PLCγ2 were introduced into the cells by electroporation (950 V, 25 microfarads, ∞ Ω), and puromycin (0.35 µg/ml) was added to the medium. 10–12 days after the selection, colonies were picked, and the puromycin selection was repeated for 5–8 days. Subsequently, the puromycin-resistant colonies were grown in normal medium (RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.) and 1% (v/v) chicken serum (Life Technologies)), and the expression of PLCγ2 was confirmed by Western blotting.

Analysis of PLCγ2 Phosphorylation and Calcium Responses in DT40 Cell Lines after Stimulation—DT40 cell lines (DT40 PLCγ2-deficient cells and these cells stably transfected with either the wild type or Y753F, Y759F, and Y753F/Y759F mutants of human PLCγ2) were stimulated by the addition of either anti-chicken IgM or H_2O_2. Typically, a 6×10^6-cell aliquot of these cell lines was stimulated with 10 µg/ml goat anti-chicken IgM (M4) (Universal Biologicals) or 2 mM H_2O_2 at 37 °C for up to 5 min in 200 µl of PBS. The cell pellet was resuspended in 200 µl of lysis buffer (1% Triton, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.5% Nonidet P-40, protease inhibitor mixture (Roche), and phosphatase inhibitor mixture (Sigma)), and the cells were lysed by incubation for 30 min at 4 °C. The supernatant was removed and added to anti-PLCγ2 antibody-protein G complexes (prepared by mixing 2 µg of anti-PLCγ2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with 9 µl of protein G (Roche)) and incubated at 4 °C for 1.5 h. After the incubation, immunocomplexes were washed, resuspended in SDS gel loading buffer, and subjected to SDS-PAGE (7.5% polyacrylamide gels) and subsequent Western blotting. For detection of PLCγ2 in immunocomplexes or in cell extracts and membrane fractions (prepared as described in Ref. 14), the anti-PLCγ2 antibody (1:4000; Santa Cruz Biotechnology) was used, while the detection of PLCγ2 phosphorylation present in immunocomplexes was performed using anti-phosphotyrosine antibody (1:1000; Transduction Laboratories). After incubation with the secondary antibody (anti-rabbit or anti-mouse Ig horseradish peroxidase-linked whole antibody from Amersham Biosciences, Inc., diluted 1:3000), the visualization was performed using the enhanced chemiluminescence (ECL) system (Amersham Biosciences). Total protein transferred to polyvinylidene difluoride membrane was stained using Amido Black solution. For detection of BLNK in PLCγ2 immunoprecipitates, mouse anti-

chicken antibody (described in Ref. 14) was used for Western blotting; the same protein was detected using anti-phosphotyrosine antibody.

For measurements of intracellular calcium concentrations in DT40 cells, a cell suspension containing 5×10^6 cells was loaded with 2 µM Fluo-3 AM (Molecular Probes, Inc., Eugene, OR) in RPMI medium for 1 h at room temperature. The cells were washed with PBS, resuspended in RPMI medium, and stimulated with 10 µg/ml M4 antibody or 2 mM H_2O_2, and their calcium mobilization was simultaneously measured at 40 °C, with constant stirring in a LS-50B fluorimeter (PerkinElmer Life Sciences). The excitation wavelength was 490 nm, and emission was monitored at a wavelength of 535 nm.
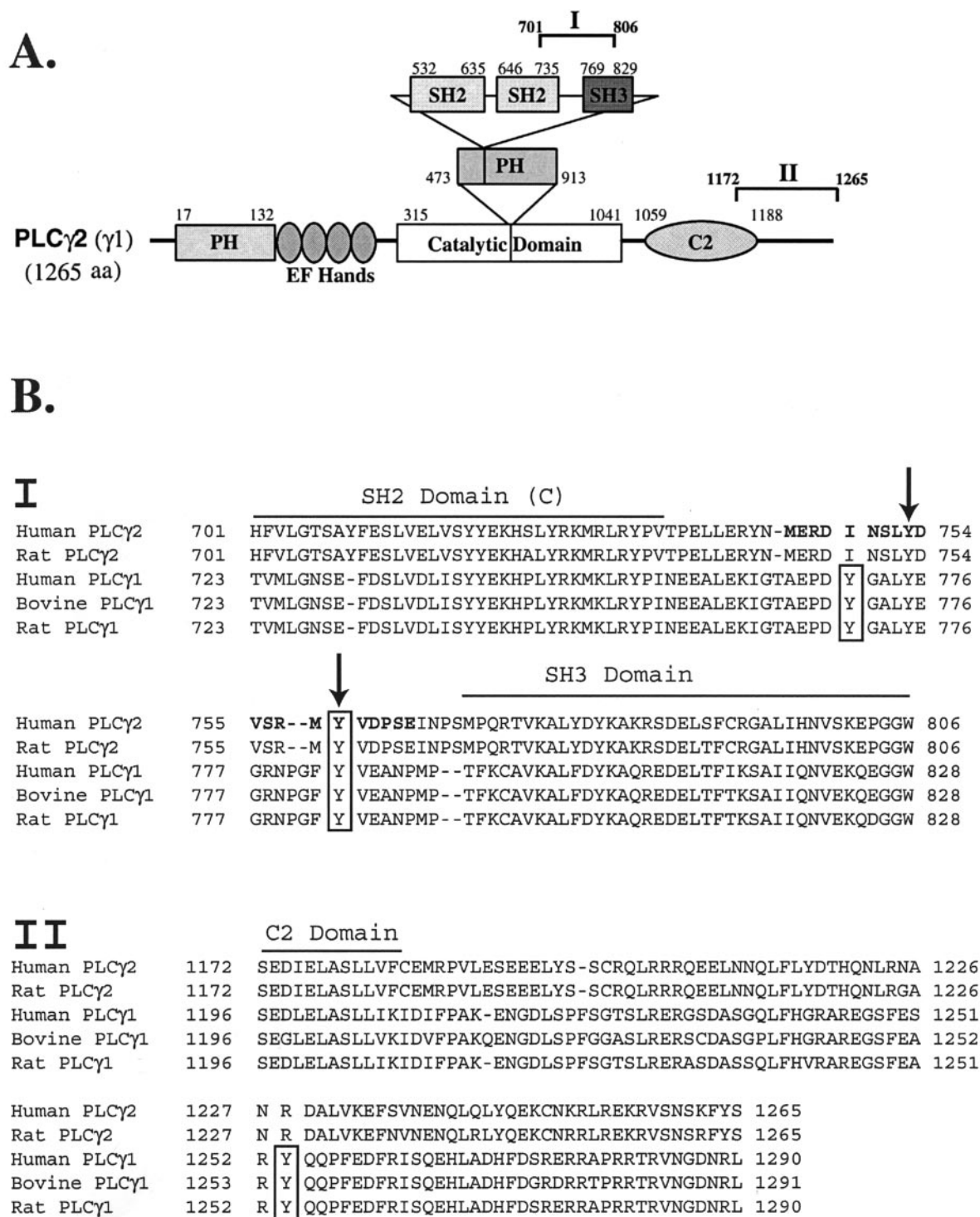
Immunofluorescence Confocal Microscopy—Localization of PLCγ2 constructs containing a GFP tag was analyzed after transfection of A431 cells, exactly as described previously (23), using EGF for stimulation. Similar experiments were performed with A20 B-cells, microinjected with the PLCγ2 plasmids. The images were recorded before and after stimulation of A20 cells with anti-IgG2a (30 µg/ml).

Constructs for Expression of Recombinant Proteins—For expression of the full-length PLCγ2 protein containing the His₆ tag at the C terminus, the DNA fragment encoding the PLCγ2 sequence (with the PCR-generated tag) was subcloned into baculovirus vector pVL1393 (Pharmingen) using the *Xba*I site. The fragment of PLCγ2 with the Y753/Y759F mutations was also subcloned into pVL1393 using *Eco*RI and *Xba*I sites. The PLCγ2 construct for baculovirus expression, encoding a deletion of 1187–1265 amino acid residues, was also made as a His₆ tag protein at the C terminus. A region encoding a PLCγ-specific array of domains of PLCγ2 (γ2SA) (amino acids 468–919) was subcloned from the pEGFP/γ2SA construct described previously (23) into bacterial expression vector pGEX-2T (Amersham Biosciences) using the *Bgl*II and *Eco*RI site. After generation of Y753F/Y759F mutations by PCR in the pEGFP/γ2SA construct, the same strategy was used to subclone the fragment into pGEX-2T vector. Both γ2SA constructs contained a GST tag at the N terminus. Expression of pEGFP/γ2SA constructs incorporating either the wild-type sequence or Y753F/Y759F mutations was analyzed in A431 cells, and translocation of encoded GFP fusion proteins was monitored as described previously (23).

The constructs for expression of different protein-tyrosine kinases (Btk, Syk, Lck, Fyn, and Src) and the adapter protein BLNK, using baculovirus expression, incorporated PCR-generated His₆ tag in frame with the N terminus of the protein. The full-length cDNA encoding human Btk was subcloned into pVL1393 using the *Bgl*II and *Bam*HI sites. To generate a truncated Btk (Δ213-Btk), a fragment encoding residues 214–659 was produced by PCR and cloned as a *Bam*HI/*Not*I fragment into pVL1393 cut with the same enzymes. The construct encoding GST-Syk fusion protein described previously (13) was used to make a truncated catalytically active version of Syk (Δ318-Syk). The region encoding amino acid residues 319–635 was amplified using PCR with a forward primer encoding a *Bam*HI site upstream of the initiation codon and a reverse primer encoding a *Not*I site downstream from the stop codon. Following digestion with *Bam*HI and *Not*I, the PCR product was cloned into the pVL1393 baculovirus transfer vector cut with the same enzymes. The His₆-BLNK construct, containing an N-terminal His₆ tag (Met-Asp-His₆) attached to residue 2 of human BLNK, was cloned in pVL1393 using the previously described BLNK plasmid (13). Constructs for baculovirus expression of Src family kinases were made by subcloning the cDNAs (24) encoding activated Src (Y527F Src), activated Fyn (Y531F Fyn), and activated Lck (Y525F Lck) into the baculovirus transfer vector pVLplink.2.² All viruses were constructed using Baculogold genomic DNA (Pharmingen), according to the manufacturer's instructions.

Expression and Purification of Recombinant Proteins—Insect (Sf9) cells, grown at 27 °C in shaker flasks in TNM-FH medium (supplemented with 10% (v/v) fetal calf serum, 1× lipid mixture (Life Technologies), 5 mM glutamine, penicillin (50 IU/ml), streptomycin (50 µg/ml), and fungizone (0.25 µg/ml)), were infected with baculoviruses for 48–56 h. For purification of His₆-tagged PLCγ2, BLNK, Δ318-Syk, and the full-length Btk and Δ213-Btk, cell pellets were sonicated in PBS supplemented with 5% glycerol and 2 mM imidazole (40 ml of buffer/liter of original Sf9 culture) and centrifuged at $100,000 \times g$. The supernatant was loaded onto a 5-ml nickel column equilibrated in 20 mM Tris-HCl, pH 8.0, 50 mM KH_2PO_4, pH 8.0, 0.4 M NaCl, 5% glycerol, and 15 mM imidazole. The column was washed first with the same buffer and then with a buffer containing 50 mM KH_2PO_4, pH 8.0, 0.1 M $(NH_4)_2SO_4$, 5% glycerol, and 20 mM imidazole, followed by elution in a buffer containing 10 mM HEPES, pH 7.5, 5% glycerol, 100 mM $(NH_4)_2SO_4$, 100 mM EDTA,

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² R. Marais, unpublished results.

A.

B.

I

SH2 Domain (C)

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Human PLCγ2   701  HFVLGTSAYFESLVELVSYYEKHSLYRKMRLRYPVTPELLERYN-MERD I NSLYD  754
Rat PLCγ2     701  HFVLGTSAYFESLVELVSYYEKHALYRKMRLRYPVTPELLERYN-MERD I NSLYD  754
Human PLCγ1   723  TVMLGNSE-FDSLVDLISYYEKHPLYRKMKLRYPINEEALEKIGTAEPD Y GALYE  776
Bovine PLCγ1  723  TVMLGNSE-FDSLVDLISYYEKHPLYRKMKLRYPINEEALEKIGTAEPD Y GALYE  776
Rat PLCγ1     723  TVMLGNSE-FDSLVDLISYYEKHPLYRKMKLRYPINEEALEKIGTAEPD Y GALYE  776
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SH3 Domain

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Human PLCγ2   755  VSR--M Y VDPSEINPSMPQRTVKALYDYKAKRSDELSFCRGALIHNVSKEPGGW  806
Rat PLCγ2     755  VSR--M Y VDPSEINPSMPQRTVKALYDYKAKRSDELTFCRGALIHNVSKEPGGW  806
Human PLCγ1   777  GRNPGF Y VEANPMP--TFKCAVKALFDYKAQREDELTFIKSAIIQNVEKQEGGW  828
Bovine PLCγ1  777  GRNPGF Y VEANPMP--TFKCAVKALFDYKAQREDELTFTKSAIIQNVEKQEGGW  828
Rat PLCγ1     777  GRNPGF Y VEANPMP--TFKCAVKALFDYKAQREDELTFTKSAIIQNVEKQDGGW  828
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II

C2 Domain

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Human PLCγ2   1172  SEDIELASLLVFCEMRPVLESEEELYS-SCRQLRRRQEELNNQLFLYDTHQNLRNA  1226
Rat PLCγ2     1172  SEDIELASLLVFCEMRPVLESEEELYS-SCRQLRRRQEELNNQLFLYDTHQNLRGA  1226
Human PLCγ1   1196  SEDLELASLLIKIDIFPAK-ENGDLSPFSGTSLRERGSDASGQLFHGRAREGSFES  1251
Bovine PLCγ1  1196  SEGLELASLLVKIDVFPAKQENGDLSPFGGASLRERSCDASGPLFHGRAREGSFEA  1252
Rat PLCγ1     1196  SEDLELASLLIKIDIFPAK-ENGDLSPFSGTSLRERASDASSQLFHVRAREGSFEA  1251
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Human PLCγ2   1227  N R DALVKEFSVNENQLQLYQEKCNKRLREKRVSNSKFYS  1265
Rat PLCγ2     1227  N R DALVKEFNVNENQLRLYQEKCNRRLREKRVSNSRFYS  1265
Human PLCγ1   1252  R Y QQPFEDFRISQEHLADHFDSRERRAPRRTRVNGDNRL  1290
Bovine PLCγ1  1253  R Y QQPFEDFRISQEHLADHFDGRDRRTPRRTRVNGDNRL  1291
Rat PLCγ1     1252  R Y QQPFEDFRISQEHLADHFDSRERRAPRRTRVNGDNRL  1290
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FIG. 1. **Domain organization and sequence similarity between PLCγ1 and PLCγ2.** *A*, PLCγ1 and PLCγ2 share the same domain organization including the N-terminal PH domain, EF-hand domain, catalytic domain, and the C2 domain. In addition, and specific to the PLCγ family, they have a specific array of domains (γSA) inserted through a loop in the catalytic domain comprising the "split PH domain," two SH2 domains and one SH3 domain. Residues at the boundaries for all domains are indicated for PLCγ2, for two regions located between the second SH2 (C-SH2) domain and the SH3 domain (region I), and for the C terminus (region II). *B*, alignment of mammalian PLCγ1 and PLCγ2 sequences (ClustalW 1.81) is shown for regions I and II, where the main tyrosine phosphorylation sites in PLCγ1 have been mapped. These tyrosine residues in PLCγ1 (771, 783, and 1254) are *boxed*. Conserved tyrosine residues between PLCγ1 and PLCγ2 within the C-SH2/SH3 linker are indicated by the *arrows*. Accession numbers are as follows: human PLCγ1, P19174; rat PLCγ1, P10686; bovine PLCγ1, P08487; human PLCγ2, P16885; rat PLCγ2, P24135.

pH 8.0, and increasing concentrations of imidazole. For purification of Src family kinases Fyn, Src, and Lck, a buffer containing 25 mM Tris, pH 7.5, 0.1% (v/v) Triton X-100, 1 mM dithiothreitol, and complete protease inhibitors (Roche Molecular Biochemicals) was used. The cells were lysed by sonication and subjected to centrifugation at $100,000 \times g$ for 1.5 h at 4 °C. The supernatant was added to Probond nickel resin

(Invitrogen) equilibrated in 20 mM HEPES, pH 8.0, 400 mM NaCl, 5% (v/v) glycerol, 1 mM 2-mercaptoethanol, and 5 mM imidazole, and incubation was carried out for 1.5 h at 4 °C. The resin was washed with the equilibration buffer, and bound proteins were eluted with the same buffer containing increasing concentrations of imidazole. The His$_6$-Src family kinases were eluted with 100 mM (Fyn), 60 mM (Src), or 30 mM
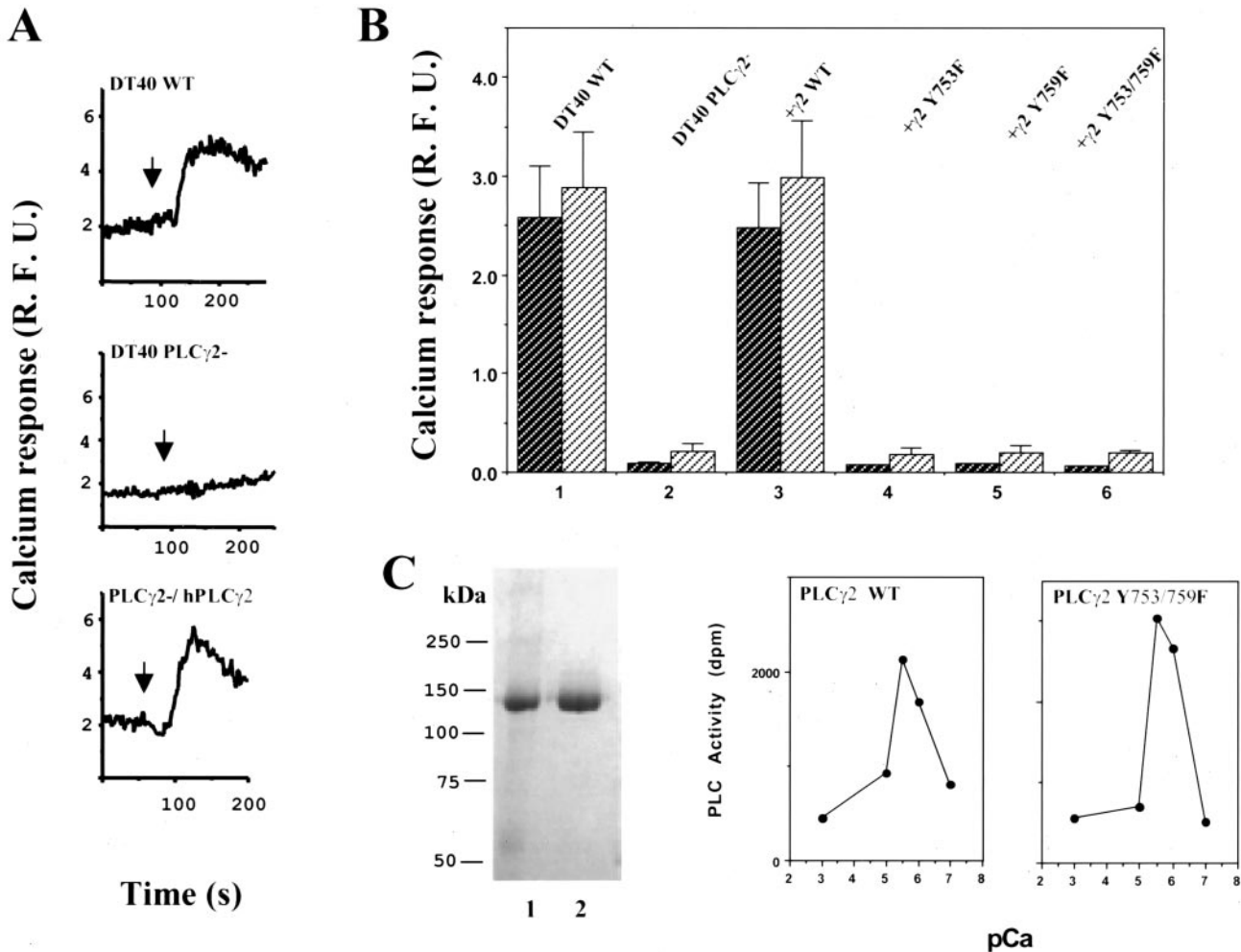
FIG. 2. **Restoration of calcium responses in PLCγ2-deficient DT40 cells by human PLCγ2.** *A*, calcium responses in Fluo-3 AM-loaded cells were analyzed in the wild-type DT40 cells (*wt, top*), PLCγ2-deficient DT40 cells (*PLCγ2⁻, middle*), and a cell line generated by stable transfection of human PLCγ2 into PLCγ2-deficient DT40 cells (*PLCγ2⁻/h PLCγ2, bottom*) after stimulation with the M4 antibody. *B*, stimulation of different DT40 cell lines was carried out using the M4 antibody (*dark shaded bars*) or hydrogen peroxide (*light shaded bars*) as described for *A*, and the region under the peak of calcium responses was quantitated. The measurements were performed in duplicates. The DT40 cell lines were as follows: the wild-type DT40 (*lane 1*); PLCγ2-deficient DT40 cells (*lane 2*); and PLCγ2-deficient DT40 cells transfected with either the wild-type human PLCγ2 (*lane 3*), PLCγ2 mutant Y753F (*lane 4*), PLCγ2 mutant Y759F (*lane 5*), or the double mutant PLCγ2 Y753F/Y759F (*lane 6*). *C*, the wild type (*lane 1*) or Y753F/Y759F double mutant of PLCγ2 (*lane 2*) was expressed as His₆-tagged constructs using a baculovirus system (*left panel*). The activity of the wild-type (*middle panel*) and PLCγ2 Y753F/Y759F mutant (*right panel*) proteins was measured over the range of calcium concentrations using an *in vitro* assay for phosphatidylinositol 4,5-bisphosphate hydrolysis.

(Lck) imidazole in 20 mM Hepes, pH 8.0. Proteins were either buffer-exchanged into the appropriate assay buffer using a NAP-5 (Sephadex G-25) column (Amersham Biosciences) or subjected to further purification. PLCγ2 was further purified on a heparin-Sepharose column (Amersham Biosciences) followed by gel filtration on a Superdex 200 16/60 column (Amersham Biosciences). His₆-BLNK and Δ213-Btk were further purified on a 5-ml HiTrapQ column (Amersham Biosciences) followed by gel filtration on a Superdex 200 16/60 column, while Δ318-Syk was purified by gel filtration on a Superdex 75 16/60. For purification of GST-Syk, the supernatant was incubated with glutathione-Sepharose (Amersham Biosciences) equilibrated in PBS, and bound protein was isolated by centrifugation at 4,000 × *g* for 5 min.

The γ2SA domains were expressed as GST fusion proteins in *Escherichia coli*. After induction with 0.2 mM isopropyl-1-thio-β-D-galacto-pyranoside (Calbiochem), cells were grown for 18 h at 20 °C. Cell pellets were resuspended in (8 ml/liter of original culture) PBS supplemented with 2 mM dithiothreitol, 1 mM EDTA, 1% (v/v) Triton X-100 with complete protease (Roche) and phosphatase inhibitors (Sigma), lysed by sonication, and subjected to centrifugation (10,000 × *g* for 10 min). The supernatant was added to glutathione-Sepharose and incubated at 4 °C for 45 min. After extensive washing with PBS, the fusion protein was eluted with 50 mM Tris, pH 8.0, supplemented with 10 mM reduced glutathione (Sigma) and 0.1% (v/v) Triton X-100.

In Vitro Assays for Analysis of Protein Phosphorylation, Formation of Protein Complexes, and PLC Activity—For phosphorylation reaction *in*

vitro, purified preparations of PLCγ2 (5 μg), γ2SA proteins (1–5 μg), or synthetic peptides (10–30 μg) (Genosphere Biotechnologies) were used as a substrate for the purified protein kinases (0.1–0.5 μg). The reaction mixture contained 50 mM Tris, pH 8.0, 2 mM MnCl₂, 2 mM MgCl₂, 1 mM Na₃VO₄, 50 μM ATP, 2 mM dithiothreitol and, when specified, also included 1–5 μCi of [γ-³²P]ATP. Reactions were carried at 30 °C for 20–30 min (or longer, when indicated) and terminated by the addition of 4× SDS loading buffer, and protein was subjected to SDS-PAGE (7.5 and 10% polyacrylamide for PLCγ2 and γ2SA proteins, respectively) or a 10–20% gradient of polyacrylamide (Invitrogen) for the peptides). Further analysis was by Western blotting using anti-phosphophotyrosine antibody as described above or, when [γ-³²P]ATP was included in reaction mixtures, using a PhosphorImager (Storm 860; Molecular Dynamics, Inc., Sunnyvale, CA) or scintillation counting of extracted peptides. Kinetic analysis of PLCγ2 and BLNK phosphorylation by Syk and Btk was performed in the presence of 5 μCi of [γ-³²P]ATP and quantitated using a PhosphorImager. For measurements of initial velocity (V_0), it was verified that reaction rates were linear with respect to time for all concentrations of substrates. Values for V_0 were expressed as PhosphorImager units/min of incubation time/mg of kinase (units/min/mg). Apparent K_m and V_{max} values were determined by plotting results as the double reciprocal Lineweaver-Burk plot.

For analysis of interactions between PLCγ2 and phosphorylated or nonphosphorylated BLNK, 8 μg of each protein was incubated for 40 min at 4 °C in a reaction mixture containing 20 mM Tris, pH 7.5, 150 mM

Important Tyrosine Residues in PLCγ2
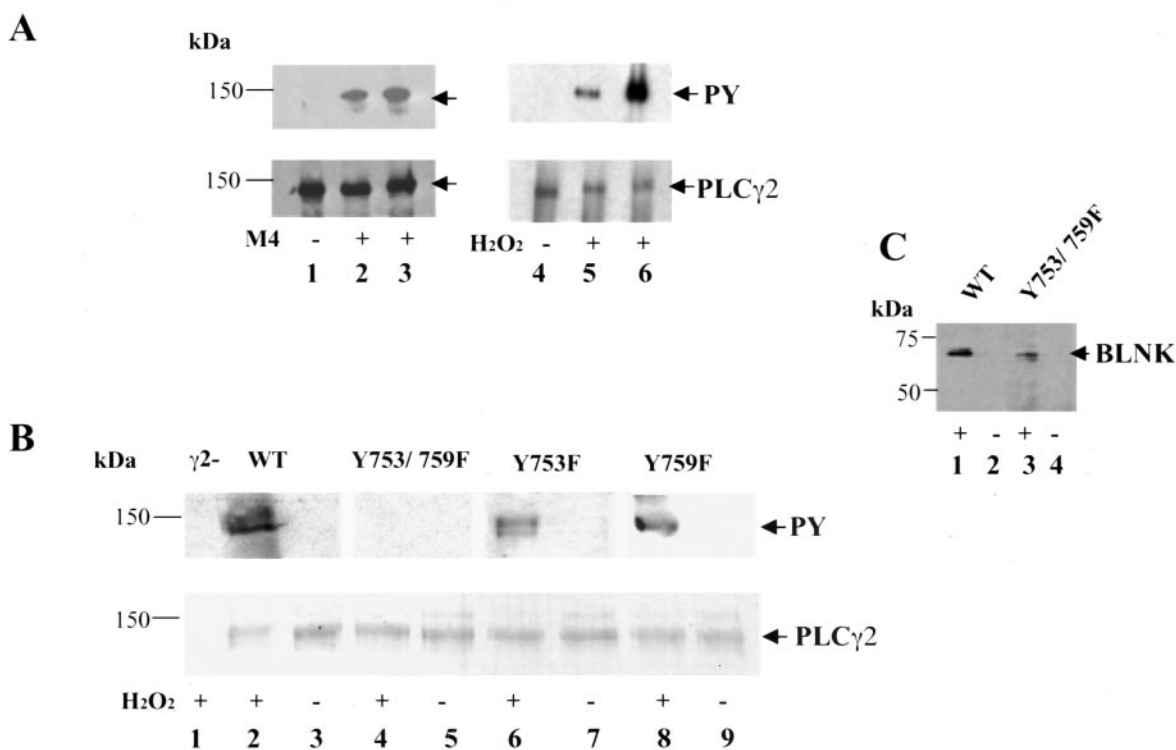
F‍IG. 3. **Tyrosine phosphorylation of PLCγ2 in different DT40 cell lines.** *A*, the phosphorylation of PLCγ2 was analyzed in the PLCγ2-deficient DT40 cells stably transfected with human PLCγ2 (PLCγ2⁻/h PLCγ2). The analysis was performed before (*lane 1*) and 2 and 5 min after stimulation with M4 (*lanes 2* and *3*). The phosphorylation was also analyzed before (*lane 4*) and 2 and 5 min after stimulation by hydrogen peroxide (*lanes 5* and *6*). PLCγ2 from various cell extracts was isolated by immunoprecipitation and analyzed by Western blotting using either anti-phosphotyrosine antibody (*PY, top panels*) or antibody to PLCγ2 (PLCγ2, *bottom panels*). *B*, the PLCγ2-deficient DT40 cells (γ2⁻) (*lane 1*) and these cells stably transfected either with the wild-type human PLCγ2 (*wt*) (*lanes 2* and *3*) or PLCγ2 mutants Y753F/Y759F (*lanes 4* and *5*), Y753F (*lanes 6* and *7*), and Y759F (*lanes 8* and *9*) were analyzed for PLCγ2 phosphorylation. After immunoprecipitation using anti-PLCγ2 antibody, Western blotting was performed using anti-phosphotyrosine antibody (*PY, top panel*). The PLCγ2 protein was visualized on the same nitrocellulose membrane by Amido Black staining (PLCγ2, *bottom panel*). *C*, immunoprecipitation of the wild-type PLCγ2 (*lanes 1* and *2*) and PLCγ2 Y753F/Y759F mutant (*lanes 3* and *4*) from the stable DT40 cell lines was performed as described for *A* and *B*. The presence of BLNK in the immunoprecipitates from stimulated (*lanes 1* and *3*) and unstimulated cells (*lanes 2* and *4*) was analyzed by Western blotting.

NaCl, 1 mM EDTA, and 1 mM dithiothreitol and subjected to gel filtration on a Superose 12 PC 3.2/3.0 column (Amersham Biosciences) using the same buffer. The conditions used to phosphorylate BLNK were as described for PLCγ2, except that GST-Syk bound to glutathione-Sepharose was used; after incubation, BLNK was separated from the enzyme by centrifugation.

Analysis of interaction between PLCγ2 and nonphosphorylated or phosphorylated BLNK was also performed by band shift on 12.5% polyacrylamide native PHAST gels (Amersham Biosciences) after incubation of protein components at 25 °C for 15 min. To ensure full phosphorylation of BLNK for this analysis, BLNK was prepared from Sf9 cells co-infected with Syk, further phosphorylated by Syk *in vitro*, and purified using chromatography steps described above.

Phospholipase C activity was measured using detergent-mixed micelles containing sodium cholate and [³H]phosphatidylinositol 4,5-bisphosphate at different concentrations of free calcium, as previously described (25).

Separation of Peptides and Mass Spectrometry—Separation of peptides was carried out using μRPC C2/C18 C2.1/10 (Amersham Biosciences) or 5 μC18 300A (Phenomenex) reverse phase columns, and elution was performed by increasing concentrations of MeCN in 0.1% trifluoroacetic acid.

All mass spectra were acquired in reflector mode using a Voyager-DE™ STR BioSpectrometry™ work station fitted with a 337-nm nitrogen laser. All samples were prepared using the dried droplet method with freshly prepared α-cyano-4-hydroxycinamic acid at 10 mg/ml in 50% MeCN, 0.1% trifluoroacetic acid.

RESULTS

Calcium Responses in DT40 Cell Lines after B-cell Receptor and Hydrogen Peroxide Stimulation—PLCγ2 is an essential component in calcium signaling triggered either by the stimulation of BCR (8, 9) or, as described below (Fig. 2*B*), by stress

responses to hydrogen peroxide in DT40 cells. The stimulation of the PLCγ2 activity in these cells by both agonists is accompanied by phosphorylation of the enzyme at tyrosine residues (15, 17). To analyze which tyrosine residues may be involved in activation of PLCγ2 in these systems, the sequences of PLCγ1 and PLCγ2 from two regions were compared. The first region corresponds to linker between the C-terminal SH2 (C-SH2) domain and the SH3 domain (within the "specific array of domains" unique to the PLCγ family (γSA)), and the second region is located at the C terminus of PLCγ (Fig. 1). In PLCγ1, two phosphorylated residues have been mapped to region I (Tyr⁷⁷¹ and Tyr⁷⁸³) and one residue (Tyr¹²⁵⁴) within region II. However, only one of these residues, Tyr⁷⁸³, appears to be critical for the enzyme signaling function after platelet-derived growth factor stimulation (21). The amino acid sequence alignment of mammalian PLCγ1 and PLCγ2 enzymes shows conservation of Tyr⁷⁸³, which corresponds to Tyr⁷⁵⁹ in PLCγ2 (Fig. 1*B*). The sequence around this residue, however, is not strictly conserved. Residues Tyr⁷⁷¹ and Tyr¹²⁵⁴ seem to be unique for PLCγ1. Analysis of sequence similarity between PLCγ1 and PLCγ2 has also revealed that another tyrosine residue within the C-SH2/SH3 linker, Tyr⁷⁷⁵ in PLCγ1 and Tyr⁷⁵³ in PLCγ2, is conserved.

To analyze the role of conserved tyrosine residues within the region I for PLCγ2 signaling in B-cells, stable cell lines were generated by transfection of human PLCγ2 into PLCγ2-deficient DT40 cells. As shown in Fig. 2*A*, human PLCγ2 containing the wild-type sequences restored calcium responses to BCR
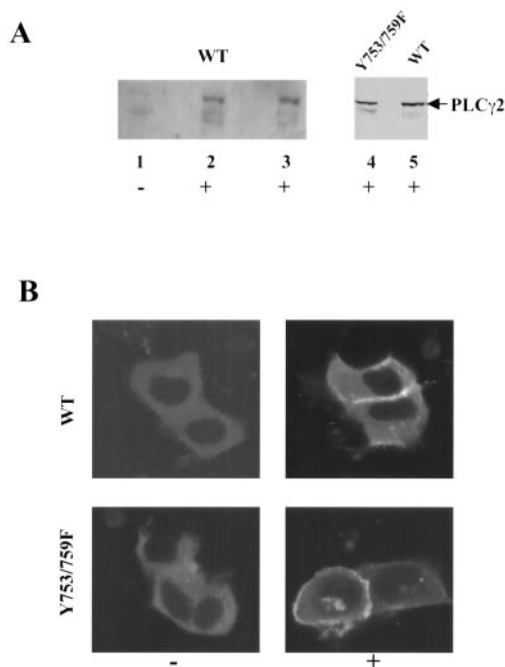
FIG. 4. **Analysis of the association of PLCγ2 with isolated membrane fractions and membrane localization in cells.** *A*, the presence of PLCγ2 in a membrane fraction isolated from unstimulated DT40 cells stably transfected with the wild-type PLCγ2 (*WT*) (*lane 1*) or from these cells stimulated with M4 for 2 or 5 min (*lanes 2* and *3*) was analyzed by Western blotting. The same analysis was performed 5 min after stimulation to compare DT40 cell lines expressing the wild-type (*WT*) (*lane 4*) and Y753F/Y759F mutant (Y753F/Y759F) (*lane 5*) PLCγ2. *B*, constructs encoding GFP fusion proteins of PLCγ2 (*GFP-γ2SA*) incorporating either the wild-type sequences (*WT*, *top panel*) or Y753F/Y759F mutation (*Y753F/Y759F*, *bottom panel*) were expressed in A431 cells and analyzed before (−, *left panels*) or after (+, *right panels*) stimulation. A similar experiment in A20 B-cells resulted in translocation of both constructs (the wild type and Y753F/Y759F) but with more uneven plasma membrane appearance.

stimulation (*bottom panel*) to levels similar as measured in the wild-type DT40 cells (*top panel*) but lacking in PLCγ2-deficient cells (*middle panel*). In addition to the wild-type PLCγ2, the constructs incorporating mutations Y753F, Y759F, and Y753F/Y759F were also used to generate stable DT40 cell lines (PLCγ2⁻/wtPLCγ2, PLCγ2⁻/PLCγ2 Y753F, PLCγ2⁻/PLCγ2 Y759F, and PLCγ2⁻/PLCγ2 Y753F/Y759F). Expression of PLCγ2 in all cell lines was initially analyzed by Western blotting, and clones expressing similar amounts of PLCγ2 were selected for further study. Immunoprecipitation confirmed that these cell lines expressed similar amounts of human wild-type or mutant PLCγ2 (see Fig. 3*B*, *bottom panel*). The selected cell lines were analyzed for calcium responses to stimulation by either the M4 antibody, which binds to BCR, or to hydrogen peroxide (Fig. 2*B*). In all DT40 cell lines, the M4 antibody and hydrogen peroxide had a similar effect on calcium responses. Both agonists stimulated calcium responses in the wild-type DT40 cells and PLCγ2⁻/wtPLCγ2. In contrast, DT40 cell lines PLCγ2⁻/PLCγ2 Y753F, PLCγ2⁻/PLCγ2 Y759F, and PLCγ2⁻/PLCγ2 Y753F/Y759F showed no calcium responses (in addition to those seen in PLCγ2-deficient cells). These results indicate that Tyr^{753} and Tyr^{759} are essential for PLCγ2 function in B-cells.

To confirm the possibility that the mutation of tyrosine residues had an effect on PLCγ2 signaling function, rather than by causing more general changes in the catalytic properties of this PLC, the wild-type and PLCγ2 Y753F/Y759F mutant were expressed using a baculovirus system, and the purification based on the presence of His$_6$ tag was carried out. Preparations of pure

proteins (Fig. 2*C*, *left panel*) were analyzed for PLC activity *in vitro* using conditions measuring basal catalytic activity. Under similar conditions, measurements of PLCγ1 activity gave the same values for the enzyme isolated from nonstimulated and stimulated cells (26). In this assay, the wild-type and PLCγ2 Y753F/Y759F mutant had similar specific activities (in the range of 120–180 μmol/mg). Measurements of PLC activity over the range of calcium concentrations (Fig. 2*C*, *middle* and *right panels*) also demonstrated similar calcium dependence with the highest activity at 5–10 μM. These data demonstrated an intact function of the PLCγ2 Y753F/Y759F catalytic domain and suggested the importance of Tyr^{753} and Tyr^{759} residues in the context of the BCR signal transduction and stress responses. Further evidence ruling out gross changes in protein structure and correct folding is presented in Figs. 3*C* and 4, demonstrating that Y753F/Y759F mutation did not affect interaction with BLNK or the ability of PLCγ2 to translocate to the plasma membrane, previously shown to require functional SH2 domains (23).

Phosphorylation of PLCγ2 in DT40 Cell Lines—The tyrosine phosphorylation of PLCγ2, endogenously present in the wild-type DT40 cell, has previously been observed after stimulation by the M4 antibody or hydrogen peroxide (15, 17). In the experiments shown in Fig. 3*A*, the phosphorylation of human PLCγ2 in the DT40 PLCγ2⁻/wtPLCγ2 cell line could also be detected 2 and 5 min following stimulation with either M4 (*right panel*) or hydrogen peroxide (*left panel*). The stimulation in the presence of hydrogen peroxide appeared to be more potent and particularly prominent after 5 min of stimulation.

Phosphorylation of PLCγ2 was also analyzed in different DT40 cell lines: PLCγ2⁻, PLCγ2⁻/wtPLCγ2, PLCγ2⁻/PLCγ2 Y753F, PLCγ2⁻/PLCγ2 Y759F, and PLCγ2⁻/PLCγ2 Y753F/Y759F. Essentially the same data were obtained using either M4 or hydrogen peroxide to stimulate the DT40 cells. As illustrated in Fig. 3*B* for hydrogen peroxide stimulation, the tyrosine phosphorylation of PLCγ2 present in immunoprecipitates obtained using anti-PLCγ2 antibody was clearly seen for the wild-type PLCγ2 (*lane 2*), PLCγ2 Y753F (*lane 6*), and PLCγ2 Y759F (*lane 8*) proteins. The PLCγ2 with the double mutation of tyrosine residues, Y753F/Y759F (*lane 5*), did not contain any detectable phosphotyrosine residues.

An attempt was made to map tyrosine residues in PLCγ2 that become phosphorylated after stimulation of DT40 cells. Analysis of tryptic peptides from tyrosine-phosphorylated PLCγ2 demonstrated that several peaks (resolved by reverse-phase chromatography) contained tyrosine-phosphorylated peptides. Further analysis of the peak fractions by mass spectroscopy revealed that masses corresponding to phosphorylated peptides containing Tyr^{753} and Tyr^{759} were present under two of these peaks. However, the fractions contained a mixture of peptides, and phosphorylation of Tyr^{753} and Tyr^{759} was not confirmed by sequencing due to limiting amounts (data not shown).

Further analysis of PLCγ2 Y753F/Y759F mutant in DT40 cell has suggested that, as previously shown for the wild type PLCγ2 (13–15), it could also interact with BLNK (Fig. 3*C*) and the plasma membrane (Fig. 4). Whether or not levels of the mutant in glycolipid-enriched microdomains were comparable with that of the wild-type was not demonstrated conclusively due to the background presence of PLCγ2 in the absence of stimulation (data not shown). Nonetheless, the translocation of a PLCγ2 construct incorporating the Y753F/Y759F mutation in a system previously used to demonstrate a requirement for functional SH2 domains (23) was clearly demonstrated (Fig. 4*B*). Using the same approach, translocation of the Y753F/Y759F mutant was also confirmed in A20 B-cells (data not shown).
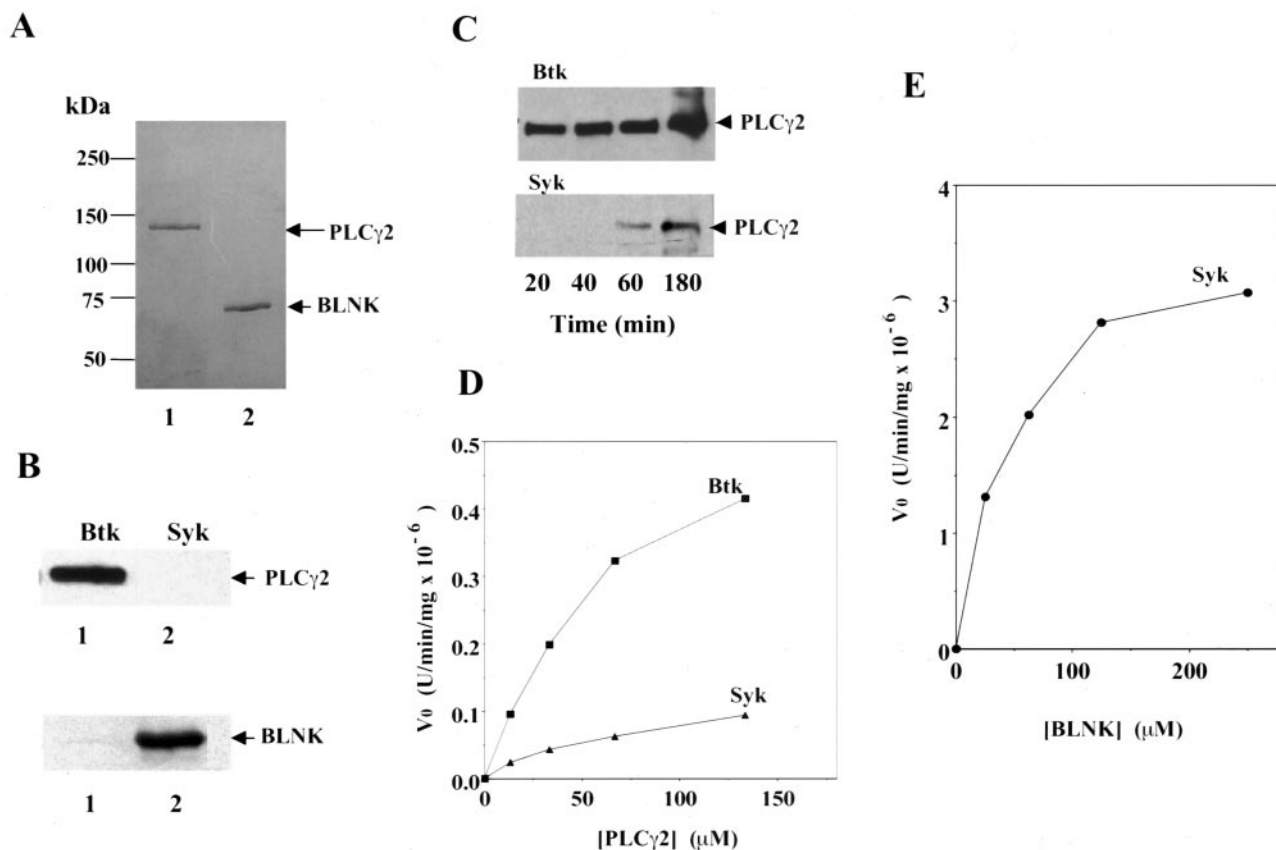
FIG. 5. ***In vitro* phosphorylation of PLCγ2 and BLNK recombinant proteins by purified tyrosine kinases Btk and Syk.** *A*, His$_6$-PLCγ2 (*lane 1*) and His$_6$-BLNK (*lane 2*) were expressed using a baculovirus system, and the purified proteins were analyzed by SDS-PAGE. *B*, purified PLCγ2 (*top panel*) and BLNK (*bottom panel*) proteins were used as a substrate, in an *in vitro* phosphorylation assay, in the presence of purified Btk (*lane 1*) or Syk (*lane 2*). Tyrosine phosphorylation was analyzed by Western blotting using anti-phosphotyrosine antibody. *C*, time course of PLCγ2 phosphorylation by Btk (*top*) and Syk (*bottom*), visualized as in *B*. *D*, kinetic analysis of PLCγ2 phosphorylation by Syk and Btk, performed in the presence of [^{32}P]ATP (see "Experimental Procedures"). *E*, kinetic analysis of BLNK phosphorylation by Syk, performed as in *D*.

The data presented in Fig. 3 have demonstrated that the residues Tyr^{753} and Tyr^{759} are not only important for calcium signaling function in B-cells (Fig. 2*B*) but also for phosphorylation of the entire PLCγ2 protein. Although the mapping of tyrosines phosphorylated *in vivo* has not shown this conclusively, the loss of phosphorylation observed for the Y753F/Y759F mutant suggests that these are the tyrosine residues that become phosphorylated in response to stimulation. Furthermore, this phosphorylation could be a requirement for phosphorylation of other tyrosine residues, which may be present in other regions of PLCγ2.

Role of Btk and Syk in Phosphorylation and Complex Formation in Vitro—Genetic studies of B-cell signal transduction have demonstrated that nonreceptor tyrosine kinases from at least three families, Src, ZAP-70/Syk, and Tec, were important for an increase in intracellular calcium (8, 9). The tyrosine kinases Syk and Btk, present in DT40 and other B-cells, have been considered to directly phosphorylate PLCγ2. Previous analysis of PLCγ2 phosphorylation in cells after overexpression of a particular tyrosine kinase (27, 28), however, has not been conclusive, since the possibility that this tyrosine kinase could contribute to PLCγ2 phosphorylation through activation of another tyrosine kinase(s) endogenously present in the cell could not be ruled out. Furthermore, concentrations of tyrosine kinases or the PLCγ2 substrate could not be controlled in those experiments. To circumvent these problems, purified proteins (prepared either as His$_6$ or GST fusion proteins) were used in a phosphorylation assay *in vitro* (Fig. 5). While Btk was able to use purified PLCγ2 protein as a substrate, phosphorylation of this PLC by Syk kinase was much lower (Fig. 5, *B* and *C*).

Using the same preparation of Syk kinase, autophosphorylation (Fig. 8*A*) and phosphorylation of purified BLNK protein (Fig. 4*B*, *bottom panel*) could be demonstrated clearly. Essentially the same results were obtained using a purified GST fusion protein of the full-length Syk as with a truncated, catalytically active His$_6$-tagged protein. Syk kinase could also phosphorylate γSA of PLCγ1 at tyrosine residue 783, as demonstrated using a specific antibody to this phosphorylation site (data not shown). When longer incubation times and increased concentrations of PLCγ2 were used (Fig. 5*C*, *bottom panel*) or when greater amounts of purified kinases were included in the reaction (data not shown), phosphorylation of PLCγ2 by Syk could also be measured. Detailed kinetic analysis, directly comparing phosphorylation of PLCγ2 by Syk and Btk, is illustrated in Fig. 5*D* and has allowed calculation of apparent K_m values and relative values for V_{max}. The difference between K_m values was about 2-fold (50.0 μM for Btk and 83.3 μM for Syk), and the difference between values for V_{max} (expressed as units/min/mg) was about 7-fold (6.6 for Btk and 1.1 for Syk). The kinetic analysis was extended to phosphorylation of BLNK by Syk (Fig. 5*E*), demonstrating even greater differences between phosphorylation of BLNK and PLCγ2 by Syk than when the two kinases were compared for PLCγ2 phosphorylation. An apparent K_m value for phosphorylation of BLNK by Syk was 43.2 μM (compared with 83.3 μM with PLCγ2 as a substrate), and V_{max} was 37.1 units/min/mg, about 40 times greater than PLCγ2 phosphorylation (1.1 units/min/mg).

Analysis of phosphorylation by Btk was also performed using a synthetic peptide incorporating Tyr^{753} and Tyr^{759} residues, ^{745}MERDINSLYDVSRMYVDPSE^{764}, designated as peptide 1

A

B

Peptide 1

MERDINSLYDVSRMYVDPSE

Peptide 2

ERDINSLFDVSRMYVDPSE
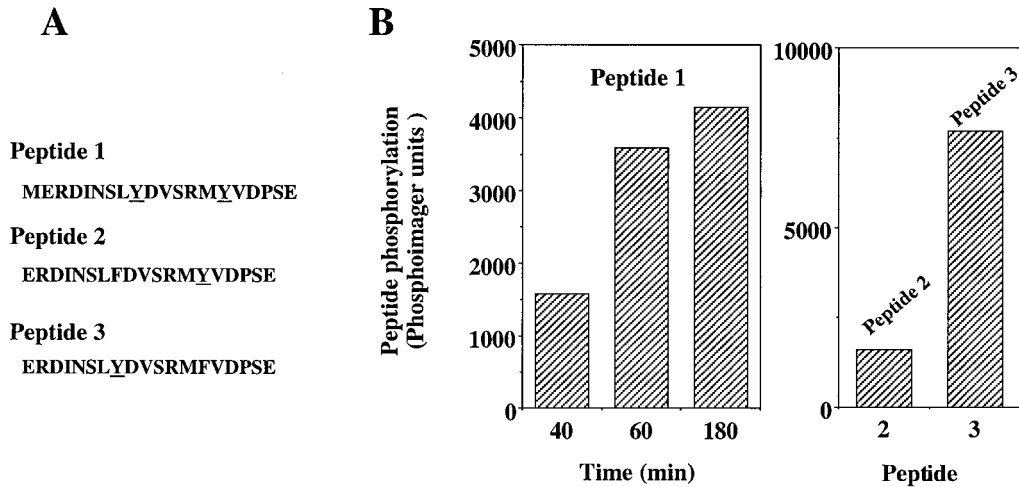
Peptide 3

ERDINSLYDVSRMFVDPSE

FIG. 6. **Phosphorylation of peptides incorporating Tyr^{753} and Tyr^{759} of PLCγ2 by Btk.** *A*, sequences of the peptides designated as peptides 1, 2, and 3. *B*, time course of phosphorylation of peptide 1, linear over 60 min (*left panel*) and phosphorylation of peptides 2 and 3, analyzed after 60 min of incubation and a prolonged exposure (*right panel*). The phosphorylation reaction was carried out in the presence of [^{32}P]ATP. After separation by SDS-PAGE, the gel was subjected to analysis using a PhosphorImager. Relative intensity of the area containing the peptide was analyzed and expressed as PhosphorImager units.

(Figs. 1*B* and 6*A*). This peptide was phosphorylated, separated from nonphosphorylated peptide, and analyzed by mass spectrometry, demonstrating an increase in mass (by 80, from 2420.00 to 2499.98) corresponding to the phosphorylation of one tyrosine residue. When two additional peptides incorporating either the Y753F (peptide 2) or Y759F (peptide 3) mutation were used, it was shown that Tyr^{753} was phosphorylated in preference to Tyr^{759} (Fig. 6*B*). Further phosphorylation studies *in vitro* using the wild type and Y753F/Y759F mutant in the context of the full-length PLCγ2 demonstrated phosphorylation of both proteins (data not shown). Thus, additional Btk phosphorylation sites, outside the region represented by the peptide, are present in PLCγ2. Some of the additional sites could be within the γ2SA protein (containing 24 tyrosine residues), as suggested in Fig. 8*D*.

Interaction of purified PLCγ2 and BLNK has been analyzed by gel filtration (Fig. 7*A*) and band shift on native gels (Fig. 7*B*) and demonstrated that phosphorylation of BLNK by Syk resulted in incorporation of PLCγ2 in high molecular weight complexes. These data are consistent with previous observations of co-immunoprecipitation of these proteins after B-cell stimulation (13, 14). When purified preparation of PLCγ2 Y753F/Y759F mutant protein was tested, it was also incorporated into a complex with phosphorylated BLNK in this *in vitro* assay (Fig. 7*B*, *right panel*).

In Vitro Phosphorylation of PLCγ2 by Various Tyrosine Kinases—In addition to Syk and Btk, several other nonreceptor tyrosine kinases from the Src family were tested for their ability to phosphorylate PLCγ2 (Fig. 8, *A* and *B*). It has been previously reported that partially purified preparations of several of these kinases could phosphorylate PLCγ2 *in vitro* (29). The Src family kinases used in our study included Src, Lck, and Fyn, and all contained a mutation (corresponding to Y527F in Src) known to prevent phosphorylation and inhibition by other tyrosine kinases in cells (30). The proteins were expressed using a baculovirus system and contained a His$_6$ tag for purification. Like Syk and Btk, all Src kinases were autophosphorylated *in vitro* (Fig. 8*A*). Also, all Src kinases, like Btk, phosphorylated PLCγ2 (Fig. 8*B*). Thus, among the tyrosine kinases tested at the specific conditions, only Syk kinase was unable to efficiently phosphorylate the full-length PLCγ2.

Since the mutagenesis of tyrosine residues identified Tyr^{753} and Tyr^{759} as important for PLCγ2 signaling function and tyrosine phosphorylation in stimulated DT40 cells, γ2SA pro-

tein (which includes these tyrosine residues) was also used as a substrate. γ2SA encoding the wild-type sequences and the protein incorporating Y753F/Y759F mutations were expressed as GST fusion proteins (Fig. 8*C*). When the panel of protein-tyrosine kinases (Syk, Btk, Src, Lck, and Fyn) was used with the wild-type γ2SA as a substrate, Btk and Lck phosphorylated this protein better than other kinases. Further comparison of these kinases using both the wild-type and Y753F/Y759F γ2SA demonstrated that the mutation abolished phosphorylation by Lck but not with Btk (Fig. 8*D*). This demonstrates that Lck can phosphorylate one or both of these tyrosine residues in PLCγ2.

The studies of phosphorylation of Tyr^{753} and Tyr^{759} were also performed in the context of a synthetic peptide corresponding to residues 745–764 in PLCγ2 (peptide 1) (Fig. 8*E*). Phosphorylation of the peptide by Syk, Btk, Lck, Fyn, and Src was analyzed in a reaction mixture containing [γ-^{32}P]ATP, and the peptide was separated from other components by SDS-PAGE. When low concentrations of the enzymes (0.1 μg) and short incubation times (20 min) were used, Lck was clearly the most efficient tyrosine kinase from the panel (Fig. 8*E*). Purified preparations of Lyn, prepared as a GST fusion protein, could phosphorylate the peptide to levels comparable with Btk and Fyn but not Lck (data not shown). Analyses of the peptide phosphorylated by Lck by mass spectrometry revealed phosphorylation of only one tyrosine residue in the peptide (an increase of the peptide mass by 80, from 2420.00 to 2500.14). Further analysis using peptides with either Tyr^{753} or Tyr^{759} replaced by phenylalanine identified Tyr^{753} as the main site phosphorylated by Lck (data not shown).

DISCUSSION

Phosphorylation of both PLCγ1 and PLCγ2 has been well documented for the majority of cellular systems where the activation of PLCγ isoforms takes place (3–5). However, phosphorylation sites and the importance of specific tyrosine residues that become phosphorylated have been analyzed only for PLCγ1 in cells stimulated through growth factor receptors. Within a complex profile of PLCγ1-phosphorylated peptides, obtained after EGF stimulation, two main tyrosine-phosphorylated residues have been mapped as Tyr^{771} and Tyr^{1254}, and one minor site has been found to correspond to Tyr^{783} (19). More recently, the use of phosphospecific antibodies to Tyr(P)^{783} confirmed phosphorylation of this site in stimulated cells (23, 31). Similar patterns of phosphorylation have been seen
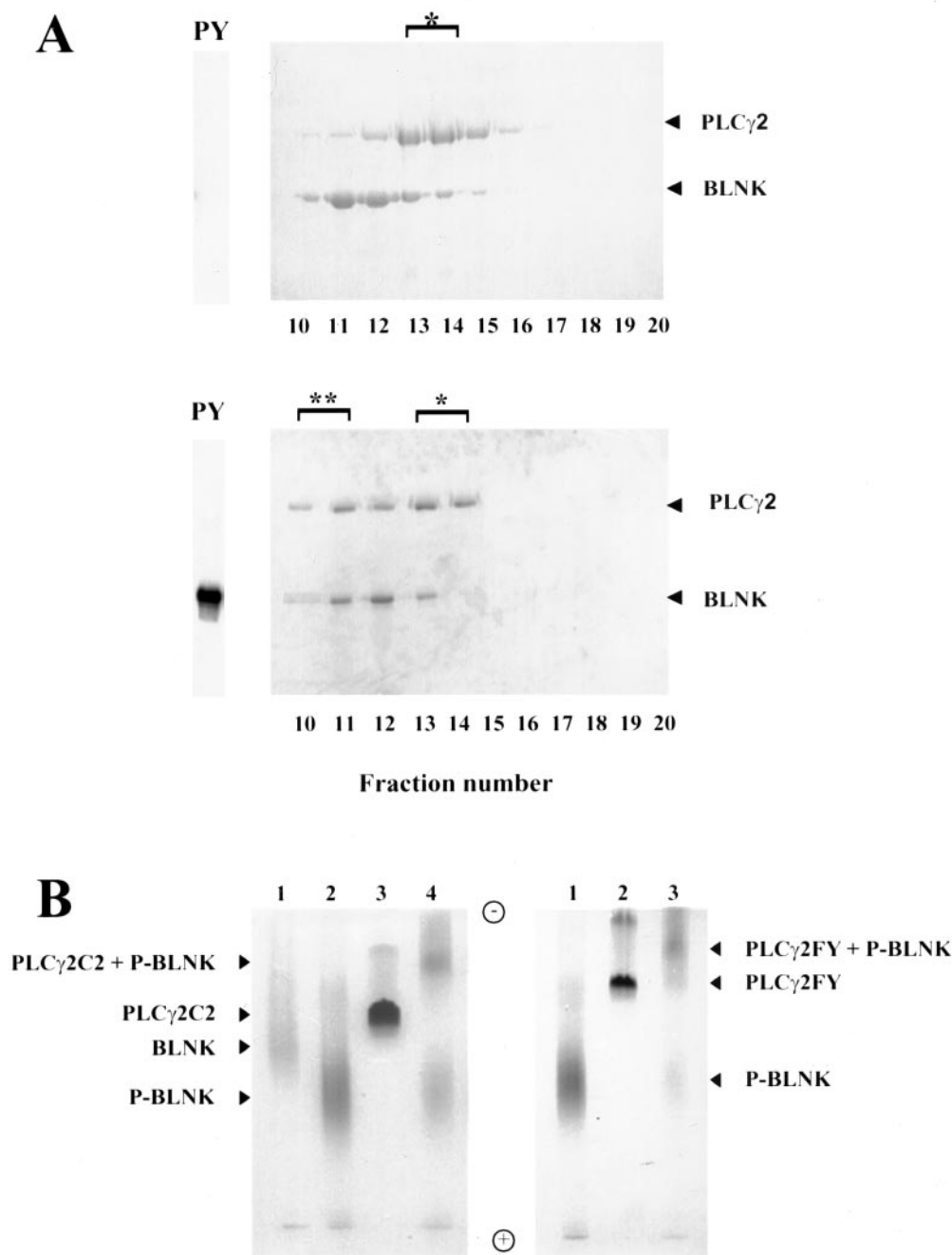
FIG. 7. **Formation of complexes containing purified PLCγ2 and BLNK.** *A*, formation of protein complexes was analyzed by gel filtration chromatography using preparations of PLCγ2 and either nonphosphorylated BLNK (*top panel*) or BLNK isolated after phosphorylation by Syk *in vitro* (*bottom panel*). The phosphorylation was analyzed by Western blotting with anti-phosphotyrosine antibodies (*lanes* indicated as *PY*). When nonphosphorylated BLNK was used in the incubation reaction with PLCγ2, the elution of BLNK (homo-oligomers, about 200,000 kDa) and PLCγ2 (a monomer, about 150,000 kDa) was as observed when each component was analyzed individually. Formation of complexes with phosphorylated BLNK resulted in the presence of PLCγ2 protein not only in fractions corresponding to a monomer (*) but also in fractions corresponding to high molecular weight proteins (**). *B*, phosphorylation of BLNK and complex formation with PLCγ2 were analyzed by band shift on 12.5% polyacrylamide native gels. In the *left panel*, nonphosphorylated BLNK (*lane 1*) shows a lower mobility than BLNK phosphorylated by Syk (*lane 2*). *Lane 3* shows migration of PLCγ2 C2 (protein lacking the C-terminal sequences after the C2 domain of PLCγ2). After incubation with an excess of purified phosphorylated BLNK, PLCγ2 is shifted completely. In the *right panel*, a complex formation between PLCγ2 mutant Y753F/Y759F (PLCγ2 FY) (*lane 2*) and phosphorylated BLNK (*lane 1*) was analyzed. PLCγ2 Y753F/Y759F is completely shifted into a complex (*lane 3*).

after stimulation of fibroblasts with platelet-derived growth factor and in several other systems (3–5, 21). These (Tyr[771], Tyr[783], and Tyr[1254]) and some additional sites have been identified after *in vitro* phosphorylation of purified PLCγ1 by EGF receptor kinase (20). Interestingly, mutational studies have revealed that only Tyr[783] was critical, while other residues had less impact on PLCγ1 function when tested in platelet-derived growth factor signaling (21), demonstrating that not all phos-

phorylation sites may be functionally important. Taking into account the complexity of the phosphorylation pattern and possible functional redundancy, the studies of PLCγ2 described here focused on a mutagenesis approach based on information obtained for the PLCγ1 isoform. Comparison of PLCγ1 and PLCγ2 sequences has revealed that of three tyrosine residues in the loop region between the C-SH2 and the SH3 domain, only two are conserved (Tyr[753] and Tyr[759] in PLCγ2, the latter
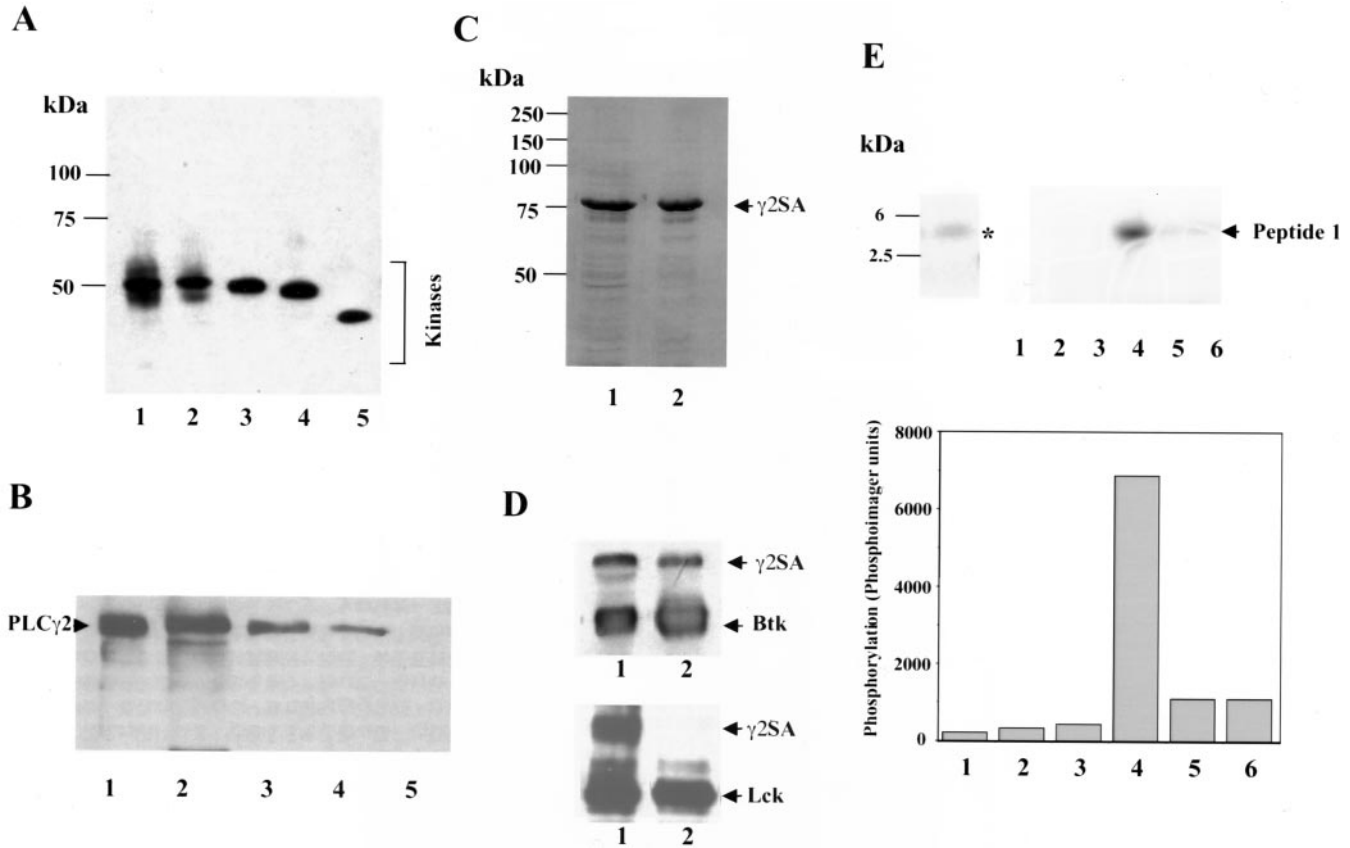
FIG. 8. *In vitro* **phosphorylation of PLCγ2 recombinant proteins by purified tyrosine kinases.** *A*, different tyrosine kinases, containing the His_6 tag, were expressed in a baculovirus system and purified. An aliquot of each protein preparation was incubated in a phosphorylation reaction, and autophosphorylation was analyzed by Western blotting using anti-phosphotyrosine antibody. The protein-tyrosine kinases were Src (*lane 1*), Fyn (*lane 2*), Lck (*lane 3*), Btk (*lane 4*), and Syk (*lane 5*). *B*, purified PLCγ2 protein was used as a substrate for various tyrosine kinases. Phosphorylation of PLCγ2 was analyzed by Western blotting using anti-phosphotyrosine antibody. The protein-tyrosine kinases used to phosphorylate PLCγ2 were Src (*lane 1*), Fyn (*lane 2*), Lck (*lane 3*), Btk (*lane 4*), and Syk (*lane 5*). *C*, GST fusion proteins encoding γ2SA (residues 468–920), containing the wild-type sequences (*lane 1*) or the double mutation Y753F/Y759F (*lane 2*), were isolated from bacterial extracts and analyzed by SDS-PAGE. *D*, GST-γ2SA proteins bound to glutathione-Sepharose were used as a substrate for Btk (*top panel*) or Lck (*bottom panel*). Phosphorylation was analyzed by Western blotting using anti-phosphotyrosine antibody. The wild-type γ2SA (*lane 1*) and the double mutation Y753F/Y759F (*lane 2*) were used. *E*, the phosphorylation reaction was carried out in the presence of [^{32}P]ATP. After separation by SDS-PAGE, the peptide 1 (marked by the *asterisk*) was visualized by GelCode Blue Stain (*left panel*), and the gel was subjected to analysis using a PhosphorImager (*right panel*). The relative intensity of the area containing the peptide was analyzed after incubation in the absence of a tyrosine kinase (*lane 1*) and after phosphorylation in the presence of Syk (*lane 2*), Btk (*lane 3*), Lck (*lane 4*), Fyn (*lane 5*), or Src (*lane 6*). Quantitation of the data is presented in the *bottom panel*. Relative intensities are expressed as PhosphorImager units.

corresponding to phosphorylation site Tyr^{783} in PLCγ1), while there is no conservation of sequences in the C-terminal region, including residue Tyr^{1254} in PLCγ1 (Fig. 1). Our mutagenesis analysis of tyrosines in PLCγ2 within the C-SH2/SH3 loop region demonstrated that both Tyr^{753} and Tyr^{759} are required to restore calcium signaling in DT40 cells deficient in PLCγ (Fig. 2*B*). Thus, the conserved residue corresponding to Tyr^{759} in PLCγ2 and Tyr^{783} in PLCγ1 is important for the function of both isoforms. The other conserved residue (753 in PLCγ2/775 in PLCγ1) has not been mutated in PLCγ1 and has not been identified as one of the major phosphotyrosine sites in response to EGF stimulation. Further studies are required to establish whether or not this site is functionally important in any of a number of different signaling pathways leading to phosphorylation of PLCγ1.

Comparison between properties of a double mutant within the C-SH2/SH3 loop region in PLCγ1 (Y771F/Y783F, where Tyr^{771} is unique for PLCγ1) observed in previous studies (21) with the PLCγ2 Y753F/Y759F double mutant in the same region described here (Figs. 2 and 3) reveals several similarities. For example, both proteins (PLCγ1 Y771F/Y783F and PLCγ2 Y753F/Y759F) retained full *in vitro* catalytic activity. Also, when the function of these proteins has been analyzed in

the context of platelet-derived growth factor signaling for PLCγ1 and in B-cell signaling for PLCγ2, these mutations not only inhibited generation of inositol 1,4,5-trisphosphate and calcium mobilization but also abolished phosphorylation of the PLCγ protein. In the case of PLCγ1, it has been shown that the Y771F/Y783F mutation resulted in a loss of not only phosphorylation in the C-SH2/SH3 loop region but also phosphorylation of Tyr^{1254} at the C terminus. Since the phosphorylation profile of PLCγ2 in stimulated B-cells also appears to be complex, it is possible that the double mutation Y753F/Y759F in PLCγ2 could have a similar effect on other potential phosphorylation sites. It has been speculated that the main impact of tyrosine phosphorylation on the function of PLCγ isoforms could be to, through conformational changes, increase the access of the enzyme to phosphatidylinositol 4,5-bisphosphate present in the plasma membrane and in this way result in a higher rate of substrate hydrolysis (3–5). However, these conformational changes in the C-SH2/SH3 loop region may also be required to expose additional phosphorylation sites.

Genetic analysis of DT40 cells has suggested the importance of several nonreceptor tyrosine kinases for PLCγ2-mediated calcium signaling (8, 9). However, it has not been established which of these enzymes could phosphorylate PLCγ2 directly.

This was examined here (Figs. 5, 6, and 8) using purified preparations of PLCγ2 constructs and various tyrosine kinases with an emphasis on Btk and Syk, both essential for PLCγ2 signaling.

The role of Btk in B-cell signaling has been extensively studied. B-cells deficient in Btk and stable cell lines where the wild-type or different Btk mutants have been transfected into these deficient cells have been assessed for calcium signaling and PLCγ2 phosphorylation (11, 16, 27, 35, 36). While the calcium responses in Btk$^-$ cells were abolished, in most reports only reduction in PLCγ2 phosphorylation has been observed, suggesting the involvement of additional tyrosine kinases in phosphorylation of this PLC. The *in vitro* phosphorylation study using purified components described here demonstrated that Btk could directly phosphorylate PLCγ2, including an important residue, Tyr[753] (Figs. 5, 6, and 8). Our studies have also shown that additional sites are phosphorylated by Btk *in vitro*. However, the identity of all sites remains to be established, together with their physiological relevance. Furthermore, studies of Btk have also suggested that the role of this protein in calcium signaling could be more complex than a requirement for PLCγ2 tyrosine phosphorylation. Mutations in the Btk PH and SH2 domains as well as a mutation affecting the catalytic activity resulted in a loss of signaling function, as measured by restoration of calcium responses in DT40 Btk$^-$ cells (11). While the Btk PH domain could be involved in critical membrane binding interactions, it is possible that the Btk SH2 and/or SH3 domains provide important sites for a formation of a signaling complex. It has been reported recently that a tyrosine kinase-inactivating mutation (in the active site and different from the nonactive site mutation affecting the catalytic activity in a preceding study) did not abolish the function of Btk in calcium signaling (16). This further emphasizes the potential scaffolding role of Btk and the possibility that the important tyrosine residues phosphorylated by Btk, and possibly other critical residues in PLCγ2, could also be phosphorylated by another kinase. Surprisingly, the studies using a panel of different tyrosine kinases (Fig. 8) have identified Lck, an Src family kinase where a link to B-cell signaling was not confirmed in all studies (10, 32, 33, 34), as a tyrosine kinase that can efficiently phosphorylate a peptide incorporating Tyr[753] and Tyr[759] residues of PLCγ2.

Protein-tyrosine kinase Syk has also been implicated in B-cell signaling and shown to be required for both PLCγ2 phosphorylation and calcium responses (10). It has been shown that the essential adapter protein BLNK, forming complexes with a number of signaling components including PLCγ2, needs to be phosphorylated by Syk in order to bind other proteins (13, 14). Therefore, the role of Syk in calcium responses could be to phosphorylate both PLCγ2 and BLNK or to phosphorylate only BLNK, thereby enabling formation of signaling complexes. The data presented here (Fig. 5) show that Syk does not efficiently phosphorylate PLCγ2, but it does phosphorylate BLNK. Furthermore, phosphorylation of BLNK by Syk, in the absence of additional components, could be sufficient to provide docking sites for direct binding of PLCγ2 (Fig. 7).

In summary, we identified tyrosine residues Tyr[753] and Tyr[759] as important for activation and tyrosine phosphorylation of PLCγ2 in B-cells. Based on this observation, the roles of various tyrosine kinases that genetic analysis has implicated in regulation of PLCγ2 were further assessed. Direct phosphorylation of PLCγ2 by Btk is observed; however, the role of Syk may not be to phosphorylate PLCγ2 directly but to provide docking phosphotyrosine sites on the adapter protein BLNK, essential in B-cell signaling.

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