Requirements for distinct steps of phospholipase Cγ2 regulation, membrane-raft-dependent targeting and subsequent enzyme activation in B-cell signalling

Rosie RODRIGUEZ, Miho MATSUDA, Amy STOREY and Matilda KATAN[1]

Cancer Research UK Centre for Cell and Molecular Biology, Chester Beatty Laboratories, The Institute of Cancer Research, Fulham Road, London SW3 6JB, U.K.

Studies of PLCγ (phospholipase Cγ) have identified a number of regulatory components required for signalling; however, molecular mechanisms and the relationship between events leading to translocation and an increase of substrate hydrolysis have not been well defined. The addition of a membrane-targeting tag to many signal transducers results in constitutive activation, suggesting that these processes could be closely linked and difficult to dissect. The present study of PLCγ2 regulation by cross-linking of the BCR (B-cell antigen receptor) or H_2O_2 stress in DT40 B-cells, demonstrated that the membrane targeting is a separate step from further changes that result in enzyme activation and substrate hydrolysis. Furthermore, we have defined the roles of different domains of PLCγ2 and, using a panel of cell lines deficient in components linked to PLCγ2 regulation, the involvement of signalling molecules with respect to each of the steps. We have found that only the lipid-raft-targeted Lyn–PLCγ2 construct, unlike non-specific membrane targeting, overcame the requirement for the adapter protein BLNK (B-cell linker). The stable expression of Lyn–PLCγ2 was not accompanied by an increase in substrate hydrolysis in resting cells, which followed stimulation and specifically required the presence and/or activation of Syk, Btk, phosphoinositide 3-kinase but not BLNK, as established using deficient cell lines or specific inhibitors. Based on mutational analysis of the specific tyrosine residues [$Tyr^{753} \rightarrow$ Phe (Y753F)/Y759F] and SH2 (Src homology 2) domains (R564A/R672A) in the context of Lyn–PLCγ2, we found that Tyr^{753}/Tyr^{759} were essential, whereas the PLCγ2 SH2 domains did not have an important role in the transient activation of Lyn–PLCγ2 but may serve to stabilize an activated form in sustained activation.

Key words: B-cell signalling, membrane raft, phospholipase activation, phospholipase Cγ2 (PLCγ2), Src homology 2 domain (SH2 domain), tyrosine phosphorylation.

INTRODUCTION

The hydrolysis of PtdIns(4,5)P_2 by PI-PLC (phosphoinositide-specific phospholipase C) occurs in response to a large number of extracellular signals (reviewed in [1–3]). Four families of mammalian PI-PLC, PLCβ (β1–β4), PLCγ (γ1 and γ2), PLCδ (δ1–δ4) and PLCε, have been described. Each family is characterized by the distinct domain organization and type of signalling 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 BCR and TCR (B- and T-cell antigen receptors respectively)] that are linked to the activation of non-receptor tyrosine kinases through a complex signalling network [1–4]. Regulation of PLCγ1 by growth factor receptors, where the receptor provides the main membrane-binding partner and the tyrosine kinase acting on PLCγ, has been studied extensively. The extent to which production of PtdIns(3,4,5)P_3 also contributes to stimulation of PLCγ1 by the tyrosine kinase receptors varies depending on the cell system and the type of receptor [5–7]. Other signalling components may also be involved in regulation of growth-factor-triggered PLCγ1 activity; for example, recent data have implied that c-Cbl has a direct interaction with PLCγ1 [8]. Studies of PLCγ isoforms in haematopoietic cells, in particular PLCγ1 signalling in response to the cross-linking of TCR and PLCγ2 signalling following stimulation of BCR, revealed some general similarities in the type of components required for regulation of PLC activity in these more complex systems [9–11]. The involvement of non-receptor protein tyrosine kinases, such as Src, Syk/Zap70 and Tec families, cell-type specific adapter proteins, PI3K (phosphoinositide 3-kinase) and members of Cbl family, is either critical or plays a role in regulation of intensity and duration of PtdIns(4,5)P_2 hydrolysis. Based on studies in DT40 cells and several other B-cell lines, the signalling components in B-cells involved in regulation of PLCγ2 include the tyrosine kinases Lyn, Syk and Btk, PI3K and an adapter protein BLNK (B-cell linker); it has been suggested that BLNK provides a direct interacting partner and that Btk could be directly involved in phosphorylation of critical tyrosine residues in PLCγ2 [10,11].

A further level of complexity in regulation of PLCγ isoforms may be provided by specific membrane targeting to the lipid rafts, also known as glycolipid-enriched microdomains. There is evidence suggesting the importance of the integrity of these specific microdomains in EGF (epidermal growth factor) receptor stimulation of PLCγ1 [12]. However, the function of lipid rafts have been more extensively studied in B- and T-cells [9,13], with the data generally supporting formation of BCR- and TCR-signalling complexes in this structure, which may also regulate cross-talk between different receptors (e.g. BCR and CD19 in B-cells). Although several reports suggest that the presence of raft structures in the plasma membrane seem to be required for

Abbreviations used: BCR, B-cell antigen receptor; BLNK, B-cell linker; EGF, epidermal growth factor; Fluo 3/AM, Fluo-3 acetoxymethyl ester; PH, pleckstrin homology; PHδ1PLCγ2, chimera of PLCγ2 containing PLCδ1 PH domain; PI3K, phosphoinositide 3-kinase; PI-PLC, phosphoinositide-specific phospholipase C; SH2 and SH3, Src homology 2 and 3 respectively; TCR, T-cell antigen receptor; Y753F etc., $Tyr^{753} \rightarrow$ Phe substitution etc.

[1] To whom correspondence should be addressed (matilda@icr.ac.uk).

PLCγ-mediated signalling [14,15], this signalling may not be confined to the rafts and, in the case of signalling by BCR cross-linking, the raft targeting could be dependent on the stage in B-cell development [16,17].

In contrast with extensive information regarding signalling networks involved in the regulation of PLCγ isoforms by receptor and non-receptor tyrosine kinases, the precise mechanism of transition from an inactive to active form of PLCγ remains unclear. Nevertheless, one requirement that has been defined in both systems is translocation of PLCγ isoforms to the plasma membrane. Studies in growth-factor-stimulated cells demonstrated the role of the SH2 (Src homology 2) domains of PLCγ as a driving force for the translocation to the plasma membrane, which is mediated by direct interaction with the receptor [12,18]. Similarly, the requirements for the PLCγ2 SH2 domains and interaction with BLNK have been shown in B-cells following BCR cross-linking [19–21]. Interestingly, the recruitment of PLCγ1 to signalling complex in T-cells involves interactions with two adapter proteins [Slp-76 (SH2 domain containing leucocyte protein of 65 kDa) and Lat (linker for activation of T-cell)] and requires the SH2 and SH3 domains of PLCγ1 [4]. There is also evidence that in some systems PI3K can indirectly or directly support translocation [5,22]. While the translocation is clearly important, other changes, most notably phosphorylation of specific tyrosine residues, are involved in stimulation of PLCγ isoforms [23–25]. Other ways of stimulation of PLCγ that do not involve tyrosine phosphorylation have also been suggested [26]. There are several lines of experimental evidence (including limited proteolysis and peptide inhibition) suggesting that an intramolecular inhibitory constraint is imposed by regions within the PLCγ-specific domain array [γSA, comprising the 'split' PH (pleckstrin homology) domain, two SH2 domains and one SH3 domain] [27–29]. Therefore, it could be speculated that phosphorylation of essential tyrosine residues in this region (e.g. Tyr^{783} in PLCγ1 and Tyr^{753} and Tyr^{759} in PLCγ2) and/or other interactions with the SH2 or SH3 domains could result in conformational changes that overcome this intramolecular inhibition. Another area where experimental evidence is lacking concerns the relationship between translocation and changes that could result in an increase of substrate hydrolysis, in particular, whether these could, at least in part, be mediated by the same interactions. Specifically, in B-cells signalling components, such as BLNK, could be involved in both translocation and activation of PLCγ2. Similarly, some domains of PLCγ2 (e.g. SH2 domains) could be involved in both processes.

In the present study we have described further insights into PLCγ2 signalling in DT40 B-cell lines following either BCR cross-linking or exposure to H_2O_2, which are known to trigger similar signalling pathways [30–32]. Since it has been reported that PLCγ2 signalling in B-cells could be either stimulated [14] or inhibited [33] by the formation of lipid-raft structures and that BCR signalling could also take place outside lipid rafts in immature B-cells [17], it was important to directly examine the ability of different regions of the plasma membrane to sustain PLCγ2 signalling. We have found that the raft targeting is essential for the BCR and also for stress-induced Ins(1,4,5)P_3 production in DT40 B-cell line.

When the localization to rafts was achieved using the targeting sequence from Src family kinase Lyn, the activation of Lyn–PLCγ2 almost completely overcame the requirement for BLNK. This targeting, not accompanied by an increase in substrate hydrolysis, suggested that it represents a separate step from the stimulation of PLC activity, and therefore enabled further studies of the interactions specifically required for the activation of targeted PLCγ2, which have been difficult to address in previous studies of PLCγ isoforms. While BLNK was clearly not required for the activation, we have found that this step, when triggered by the cross-linking of BCR, involves stimulation of Syk, Btk and PI3K; the requirement for these components was less stringent in H_2O_2-induced stress responses. We have also found that the PLCγ2 SH2 domains did not have an important role in transient activation of the raft-targeted Lyn–PLCγ2, which is in contrast with specific tyrosine residues (Tyr^{753} and Tyr^{759}) that were essential for both BCR cross-linking and H_2O_2-induced responses.

EXPERIMENTAL

Constructs for expression in DT40 B-cells

For the expression of wild-type human PLCγ2 in DT40 (chicken B-lymphoma) cell line, the full-length cDNA of PLCγ2 was subcloned into the pApuro vector to generate the previously described pApuroPLCγ2 construct [18]. The chimera PHδ1PLCγ2, encoding amino acids 1–134 from rat PLCδ1 and residues 134–1265 from human PLCγ2, was generated using the two-stage PCR-based extension method. The PCR fragment, encoding the N-terminal portion of the chimera, was subcloned into a vector generated by EcoRI cleavage of pAPuroPLCγ2 to produce pAPuroPHδ1PLCγ2 plasmid. The two-stage PCR approach was also used to generate construct incorporating 9 amino acids from the N-terminus of the Src kinase Lyn (G^2CIKSKGKD^{10}) between the first and second amino acids residues of PLCγ2. The PCR fragment incorporating this Lyn-tag was subcloned into pAPuroPLCγ2, using unique *Eco*RI sites, generating pAPuroLyn-PLCγ2. This PCR fragment was also subcloned into a plasmid encoding PLCγ2 with mutations of two critical tyrosine residues, pAPuroPLC$\gamma 2^{Y753F/Y759F}$ (where Y753F is the substitution $Tyr^{753} \rightarrow$ Phe etc.) [24], creating a Lyn-tag at the N-terminus of the construct (pAPuroLyn-PLC$\gamma 2^{Y753F/Y759F}$). To generate pAPuroLyn-PLC$\gamma 2^{R564A/R672A}$, where mutations are introduced into the N- and C-terminal SH2 domains in the context of Lyn–PLCγ2, the following strategy was used. First, pAPuroPLC$\gamma 2^{R564A/R672A}$ was made by subcloning the entire coding region from pVL1393PLC$\gamma 2^{R564A/R672A}$ (the mutations were generated as described in [18]) into pAPuro, using *Kpn*I and blunt-ended *Eco*RI restriction sites. The PCR fragment incorporating the Lyn-tag was subsequently subcloned into pAPuroPLC$\gamma 2^{R564A/R672A}$ using the same strategy as described for pAPuroLyn-PLCγ2. A fragment incorporating sequence of human BLNK was subcloned into pApuro from a previously described plasmid [24], using *Kpn*I and blunt-ended *Bam*HI restriction sites.

Generation of stable D40 cell lines

DT40 cells (the wild-type and PLCγ2-, BLNK-, Btk- and Syk-deficient) were maintained in RPMI 1640 medium, supplemented with 10 % (v/v) foetal bovine serum (Gibco), 1 % (v/v) chicken serum (Gibco) and 3 mM glutamine, in the presence of 5 % CO_2 at 40 °C. Stable transfectants of DT40 cells were generated as described previously [18,24]. Briefly, various PLCγ2 and BLNK constructs in pApuro were linearized and introduced into DT40 cell lines (PLCγ2-, BLNK-, Syk- or Btk- deficient) by electroporation (950 V, 25 μF, ∞ Ω; Gene Pulser, Bio-Rad Laboratories). After 24 h, puromycin (0.35 μg/ml for PLCγ2-deficient and 0.29 μg/ml for BLNK-, Syk- and Btk-deficient cells) was added to the medium. After the selection (10–12 days), colonies were isolated and puromycin (0.3 μg/ml) selection repeated for 5–8 days. Subsequently, the puromycin-resistant colonies were grown in normal medium for a further 6 days. Generated stable cell lines are listed in Table 1.

Table 1 DT40 cell lines

Background cell line	Construct introduced	Stable cell line generated
PLCγ2$^-$	None	
	Human wild-type PLCγ2	PLCγ2$^-$/PLCγ2
	PHδ1PLCγ2	PLCγ2$^-$/PHδ1PLCγ2
	Lyn–PLCγ2	PLCγ2$^-$/Lyn–PLCγ2
	Lyn–PLCγ2$^{Y753F/Y759F}$	PLCγ2$^-$/Lyn–PLCγ2$^{Y753F/Y759F}$
	Lyn–PLCγ2$^{R564A/R672A}$	PLCγ2$^-$/Lyn–PLCγ2$^{R564A/R672A}$
BLNK$^-$	None	
	Human BLNK	BLNK$^-$/BLNK
	Human wild-type PLCγ2	BLNK$^-$/PLCγ2
	PHδ1PLCγ2	BLNK$^-$/PHδ1PLCγ2
	Lyn–PLCγ2	BLNK$^-$/Lyn–PLCγ2
	Lyn–PLCγ2$^{Y753F/Y759F}$	BLNK$^-$/Lyn–PLCγ2$^{Y753F/Y759F}$
	Lyn–PLCγ2$^{R564A/R672A}$	BLNK$^-$/Lyn–PLCγ2$^{R564A/R672A}$
Btk$^-$	None	
	Lyn–PLCγ2	Btk$^-$/Lyn–PLCγ2
Syk$^-$	None	
	Lyn–PLCγ2	Syk$^-$/Lyn–PLCγ2

Measurements of Ca^{2+} responses and Ins(1,4,5)P_3 production

Intracellular Ca^{2+} concentrations in DT40 cells were measured using fluorescence spectroscopy, essentially as described previously [18,24]. Briefly, a cell suspension of 5×10^6 cells was loaded with 2 μM Fluo 3/AM (Fluo-3 acetoxymethyl ester) (Molecular Probes) in RPMI 1640 medium for 1 h at 20 °C. The cells were washed with PBS, resuspended in RPMI 1640 medium and stimulated with 10 μg/ml M4 antibody or 2 mM H_2O_2 and their Ca^{2+} mobilization simultaneously measured at 40 °C, with constant stirring in a LPS-220B fluorimeter (Photon Technology International). Excitation wavelength was at 490 nm and emission was monitored at a wavelength of 535 nm. The responses were quantitated as described previously in [18,24], taking into account the area of the peak corresponding to immediate rise in Ca^{2+} concentrations.

Production of Ins(1,4,5)P_3 was measured using BIOTRAK IP_3 assay system (Amersham Pharmacia Biotech) or Inositol-1,4,5-Triphosphate [^{3}H] Radioreceptor Assay Kit (NEN), according to the manufacturers' instructions. For these measurements, aliquots of DT40 cells (2×10^6) were used, resuspended in 50 μl of culture medium and stimulation carried out with 10 μg/ml M4 or 2 mM H_2O_2 at 37 °C for the time indicated in the Figure legends. The incubation was stopped with 50 μl of ice-cold 15 % (w/v) trichloroacetic acid. After centrifugation at 5000 rev./min for 15 min, the supernatants were washed four times with 1 ml of water-saturated diethyl-ether and used for Ins(1,4,5)P_3 mass measurements with the reagents supplied by the manufacturers.

For experiments using the PI3K inhibitors LY294002 (Calbiochem) and wortmannin (Calbiochem), DT40 cells were loaded with 2 μM Fluo 3/AM for 30 min at 40 °C (for Ca^{2+} measurements only) and incubated with 50 μM LY294002 or 100 nM wortmannin for a further 30 min. Cells were then stimulated with 2 mM H_2O_2 or 10 μg/ml M4. Intracellular Ca^{2+} mobilization and Ins(1,4,5)P_3 production was measured as described above.

Analysis of cellular localization and phosphorylation of different PLCγ2 constructs

Localization of PLCγ2 protein in various DT40 cell lines was performed by immunostaining. The cells were fixed in 4 % (v/v) paraformaldehyde for 10 min, washed with PBS for 2 min and permeabilized with 0.1 % (v/v) Triton X-100, 100 mM glycine in PBS for 15 min. Permeabilized cells were incubated with 10 % (v/v) foetal calf serum for 30 min. Localization of PLCγ2 in cells was visualized using a polyclonal anti-PLCγ2 antibody (Santa Cruz Biotechnology, dilution 1:2000). After 1 h, cells were washed with PBS and incubated with FITC-labelled goat anti-rabbit antibody (Pharmingen; dilution 1:200) for 30 min.

Cell fractionation and preparation of the lipids rafts (glycolipid-enriched membranes) was based on previously published methods [34,35]. DT40 cells (3×10^7) were resuspended in 200 μl of lysis buffer (60 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, protease and phosphatase inhibitors) without detergent, and sonicated three times with 5-s pulses. Samples were adjusted to a total volume of 1 ml in lysis buffer containing 1 % (w/v) Brij 58 (Sigma) and maintained on ice for 1 h. The lysates were then mixed with 85 % (w/v) sucrose in 60 mM Tris/HCl (pH 8.0), containing 150 mM NaCl and 5 mM EDTA (pH 8.0) and transferred to polyallomer centrifuge tubes (Beckman). A 2-ml volume of 30 % (w/v) sucrose was layered on top, followed by 1 ml of 5 % (w/v) sucrose. Samples were subjected to centrifugation in a SW55Ti rotor at 200000 ***g*** for 16 h at 4 °C. Fractions (400 μl) were collected from the top of the gradient and 40 μl aliquots were analysed by Western blotting. Alternatively, for comparison of non-stimulated and stimulated cells, starting from 1×10^8 cells, the fractions containing the raft-marker Lyn were pooled and concentrated prior to analysis by Western blotting. For detection of PLCγ2, the anti-PLCγ2 antibody (1:4000; Santa Cruz Biotechnology) was used, whereas chicken Lyn present in DT40 cells was visualized using anti-chicken Lyn antibodies (1:2000; a gift from T. Kurosaki, Department of Molecular Genetics, Institute for Liver Research, Kansai Medical University, Japan). After incubation with the secondary antibody (donkey anti-rabbit antibody, dilution 1:4000; Amersham Pharmacia Biotech), the visualization was performed using the enhanced chemiluminescence (ECL®) system (Amersham Pharmacia Biotech).

Analysis of PLCγ2 tyrosine phosphorylation was performed essentially as previously described [18,24]. Typically, a sample of 6×10^6 cells from various DT40 cell lines was stimulated with 2 mM H_2O_2 at 37 °C for 4–5 min or with 10 μg/ml M4 antibody for 1–3 min. The cell pellet was resuspended in 200 μl of lysis buffer [1 % (v/v) Triton, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.5 % (v/v) Nonidet P40, protease inhibitor cocktail (Roche), and phosphatase inhibitor cocktail (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 and incubated at 4 °C for 1.5 h. After the incubation, immunocomplexes were washed with lysis buffer, resuspended in SDS gel-loading buffer and subjected to SDS/PAGE (7.5 % polyacrylamide gels) and then Western blotting. For the detection of PLCγ2, the anti-PLCγ2 antibody was used as described above. The detection of PLCγ2 phosphorylation was performed using anti-phosphotyrosine antibody (1:1000; Transduction Laboratories) and the secondary antibody (goat anti-mouse Ig-horseradish-peroxidase-linked whole antibody from Amersham Pharmacia Biotech; diluted 1:3000). The visualization was performed by enhanced chemiluminescence and the intensity of selected areas assessed using NIH Image 1.62 program.

RESULTS

Constitutive membrane targeting of PLCγ2 in DT40 cells

Following stimulation in a number of cellular systems, PLCγ1 and PLCγ2 translocate to the plasma membrane. In analysis

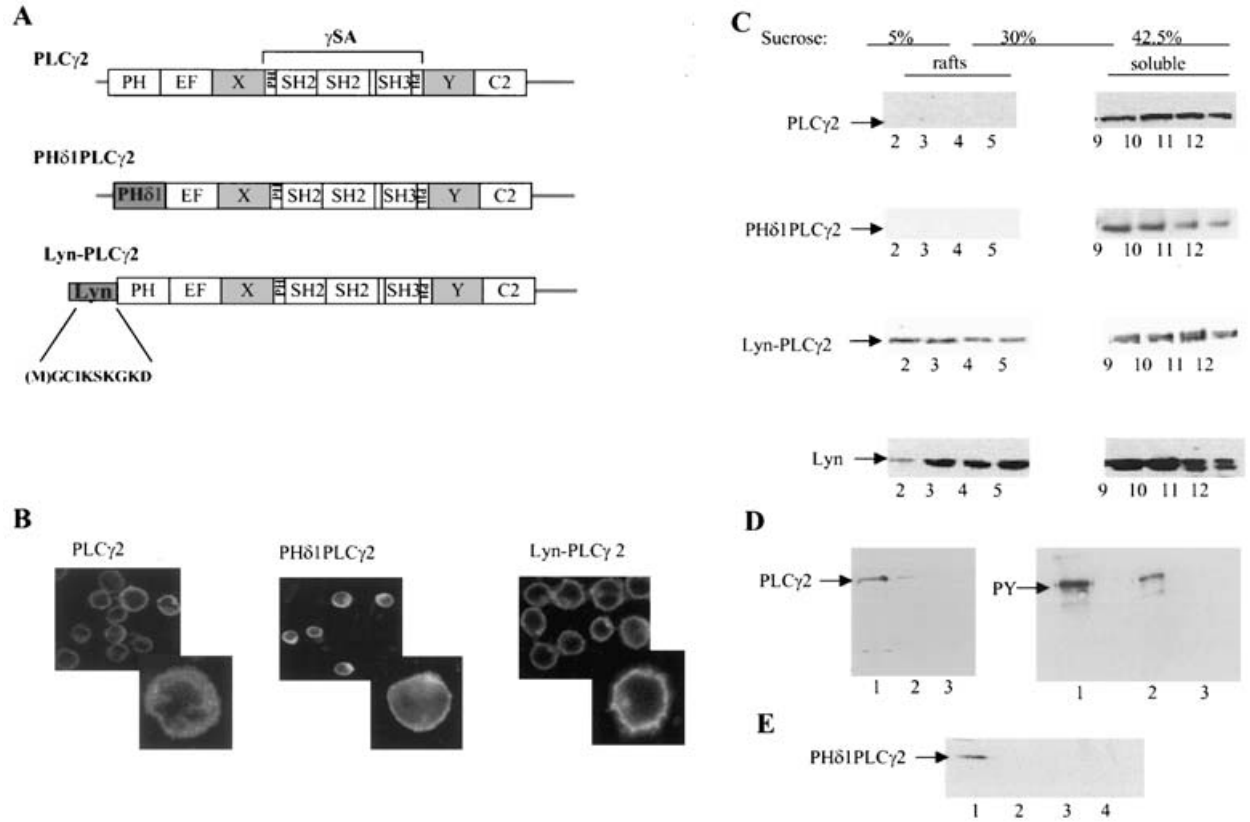

Figure 1 Localization of the wild-type, PLCδ1 PH domain chimera and Lyn-tagged PLCγ2 in DT40 cells

(**A**) Representation of the constructs encoding the wild-type PLCγ2 (top), the PLCδ1PH domain chimera of PLCγ2 (middle) and the Lyn-tagged PLCγ2 (bottom). The domains within the PI-PLC core structure (the N-terminal PH domain, EF-hands, catalytic domain incorporating X and Y regions and C2 domain) and the domains specific for PLCγ isoforms indicated as γSA (the 'split' PH domain, two SH2 domains and SH3 domain) are shown. (**B**) PLCγ2 localization in DT40 stable cell lines. Stably transfected DT40 cell lines PLCγ2$^-$/PLCγ2 (left), PLCγ2$^-$/PHδ1PLCγ2 (middle) and PLCγ2$^-$/Lyn–PLCγ2 (right) were analysed by immunofluorescence confocal microscopy using anti-PLCγ2 antibody. Fields of cells and enlarged single cell images are shown. (**C**) Lipid raft compartmentalization of the wild-type, PH domain PLCδ1 chimera and Lyn-tagged PLCγ2 in DT40 cells. Extracts from the stably transfected cells, PLCγ2$^-$/PLCγ2, PLCγ2$^-$/PHδ1PLCγ2, and PLCγ2$^-$/Lyn–PLCγ2, were fractionated on discontinuous sucrose gradients as described in the Experimental section. The fractions (2–5 enriched in detergent insoluble rafts and 9–12 enriched in the detergent-soluble proteins), analysed by Western blotting using antibody against PLCγ2, are shown for all three cell lines (top panels). Fractions containing endogenous chicken Lyn are shown in the bottom panel. (**D**) The DT40 PLCγ2$^-$/PLCγ2 cell line was analysed for the presence of the wild-type PLCγ2 in the pool of fractions 2–5 obtained after fractionation on discontinuous sucrose gradients, subjected to Western blotting using anti-PLCγ2 antibody (left panel). The analysis of tyrosine phosphorylation was performed using anti-phosphotyrosine (PY) antibodies in samples containing comparable amounts of PLCγ2 (right panel). Cells were stimulated with 2 mM H_2O_2 for 4 min (lane 1), 10 μg/ml of M4 antibody for 2 min (lane 2) or not stimulated (lane 3). It is estimated that the phosphorylation of the wild-type PLCγ2 triggered by M4 represents 25–30 % of phosphorylation in the presence of H_2O_2. (**E**) The cell lines PLCγ2$^-$/PHδ1PLCγ2 (lanes 1 and 2) and BLNK$^-$/PHδ1PLCγ2 (lanes 3 and 4) were analysed for the presence of PLCγ2 in the pool of fractions 2–5 obtained after fractionation on discontinuous sucrose gradients as described in (**D**). Analysis was performed with H_2O_2 stimulated (lanes 1 and 3) and non-stimulated cells (lanes 2 and 4).

of function and regulation of proteins that interact with the plasma membrane, specific targeting signals, such as the C-terminal sequence of H- and K-Ras (including the CAAX box) and the N-terminal sequences of Src kinases, have been used to achieve constitutive targeting [36,37]. Among PI-PLCs, only the PLCδ1 PH domain provides a membrane anchor in the absence of stimulation by binding to PtdIns(4,5)P_2 [38,39]. To constitutively target PLCγ2 to the plasma membrane of DT40 cells, we have chosen to replace the PH domain of PLCγ2 with the PLCδ1 PH domain that would localize PLCγ2 to the plasma membrane sites where PtdIns(4,5)P_2 substrate is present. We have also chosen the N-terminus of the Src family kinases, known to determine targeting to raft microdomains, as a targeting signal for PLCγ2. In DT40 B-cells, Lyn kinase has been described as constitutively present in the membrane rafts. Within the sequence M^{1}GCIKSKGKD^{10} of Lyn, two residues (Gly^2 and Cys^3) are likely to undergo lipid modifications, myristoylation and palmitoylation [36]. The constructs of PLCγ2 containing either PLCδ1 PH domain (PHδ1PLCγ2) or the N-terminal tag of Lyn (Lyn–PLCγ2) were made (Figure 1A).

The stable cell lines expressing the membrane-targeted constructs of PLCγ2 were first made in PLCγ2-deficient cells (Table 1) to confirm that the constructs were localized to the plasma membrane and that the changes have not resulted in chimeras/tagged proteins that are no longer functional in B-cell signalling. In B-cells, translocation of PLCγ2 requires BLNK, and BLNK-deficient DT40 cells do not support translocation of PLCγ2 [20]. Therefore, the PLCγ2 constructs were also introduced into BLNK-deficient DT40 cells (Table 1) to test their ability to bypass the function of BLNK. As shown in Figure 1(B), the stable cell lines of the targeted constructs (PHδ1PLCγ2 and Lyn–PLCγ2, middle and right panels), unlike the cells containing the wild-type construct of PLCγ2 (PLCγ2, left panel), demonstrated the presence of PLCγ2 in the plasma membrane. Essentially the same results were obtained when the constructs were introduced into PLCγ2-deficient and BLNK-deficient cells, since

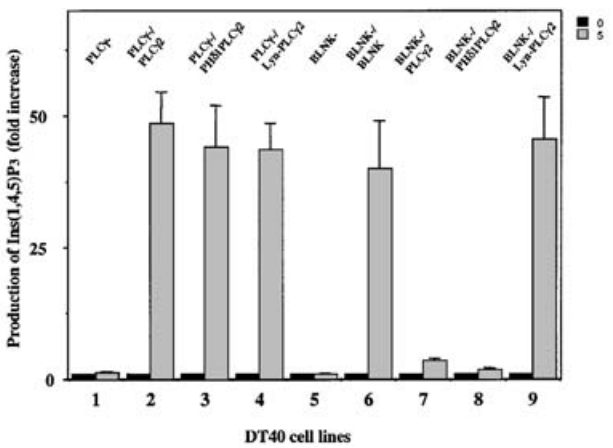

Figure 2 Production of Ins(1,4,5)P_3 in various DT40 cell lines

Samples of 2×10^6 cells from non-stimulated DT40 cells and DT40 cells stimulated with 2 mM H_2O_2 for 5 min were analysed for Ins(1,4,5)P_3 production as described in the Experimental section. The DT40 cell lines were as follows: PLCγ2-deficient (PLCγ2-) (1), PLCγ2-deficient cells stably transfected with the wild-type human PLCγ2 (PLC$\gamma2^-$/PLCγ2) (2), PLCγ2-deficient cells stably transfected with the PLCδ1PH domain chimera of PLCγ2 (PLC$\gamma2^-$/PHδ1PLCγ2) (3), PLCγ2-deficient cells stably transfected with the Lyn-tagged PLCγ2 (PLC$\gamma2^-$/Lyn–PLCγ2) (4), BLNK-deficient (BLNK-) (5), BLNK-deficient cells stably transfected with the human BLNK (BLNK$^-$/BLNK) (6), BLNK-deficient cells stably transfected with the wild-type PLCγ2 (BLNK$^-$/PLCγ2) (7), BLNK-deficient cells stably transfected with the PLCδ1PH domain chimera of PLCγ2 (BLNK$^-$/PHδ1PLCγ2) (8) and BLNK-deficient cells stably transfected with the Lyn-tagged PLCγ2 (BLNK$^-$/Lyn–PLCγ2) (9). Ins(1,4,5)P_3 production in stimulated cells is shown by grey bars. The results are expressed as the means $\pm$ S.D. for three independent experiments performed in duplicate.

endogenous PLCγ2 in BLNK-deficient cells only contributed to the staining in the cytoplasm.

Since the stimulation of B- and T-cells results in enrichment of the wild-type PLCγ in specialized plasma membrane microdomains ([9,13] and Figure 1), a further analysis of different DT40 cell lines was performed. To define membrane microdomains where PLCγ2-targeted constructs are present, a separation method designed to isolate membrane rafts was performed (Figure 1C). Comparison was made between stable cell lines (made using PLCγ2-deficient background) expressing the wild-type PLCγ2, the PHδ1PLCγ2 chimera or Lyn–PLCγ2. As shown in Figure 1(C), the wild-type PLCγ2 and PHδ1PLCγ2 chimera (upper panels) were not present in fractions containing lipid rafts (fractions 2–5), whereas Lyn–PLCγ2 and endogenously present Lyn kinase (lower panels) were clearly present in the membrane raft fractions. Thus, although two targeted constructs of PLCγ2 were present in the plasma membrane, only the Lyn-tagged PLCγ2 construct resulted in the enrichment of PLCγ2 in the raft microdomains in the absence of stimulation. Interestingly, the PHδ1PLCγ2 chimera was found in lipid rafts only after stimulation in PLCγ2-deficient cells, but not in BLNK-deficient cells (Figure 1E).

Effects of membrane targeting of PLCγ2 on DT40 responses to BCR cross-linking and H_2O_2 stress

Following the BCR stimulation or H_2O_2 stress, phosphorylation of the wild-type PLCγ2 can be readily detected (Figure 1D, right panel) and is accompanied by an increase in PLC activity (Figure 2). The constitutive membrane targeting of PLCγ2, particularly to the rafts where many tyrosine kinases are present, could lead to constitutive phosphorylation and activation PLCγ2. Analysis of Lyn-targeted PLCγ2, however, demonstrated that it did not contain phosphotyrosine in unstimulated cells (Figure 3B). Stimulation of the stable cell line expressing Lyn–PLCγ2 resulted in phosphorylation of its tyrosine residues, as has been observed for the wild-type PLCγ2 (Figure 3B). Similarly, the appearance of membrane patches, visualized by anti-PLCγ2 antibody, could be detected only after stimulation (Figure 3A). Essentially the same results were obtained when Lyn–PLCγ2 was introduced to BLNK-deficient (Figure 3A) and PLCγ2-deficient cells (results not shown). As previously reported [19–21], in the control cell line where the wild-type PLCγ2 was introduced into BLNK-deficient cells, translocation could not be detected (Figure 3A, bottom panel), whereas the phosphorylation was greatly reduced (in response to H_2O_2, Figure 3B, right panel) or absent (in response to BCR stimulation, results not shown).

To assess activation of PLCγ2, the formation of Ins(1,4,5)P_3 was measured in different stable DT40 cell lines after stimulation by BCR cross-linking (using M4 antibody) or stress responses to H_2O_2. Under the conditions used in the present study, the maximum Ins(1,4,5)P_3 production was at about 1 min after addition of M4 and about 5–10 min after addition of H_2O_2. These time points were used to compare different stable cell lines and demonstrated that the relative responses to M4 and H_2O_2 for a given cell line were similar. The Ins(1,4,5)P_3 responses of different cell lines are shown in Figure 2, using H_2O_2 as a stimulus. Analysis of Ins(1,4,5)P_3 production by PHδ1PLCγ2 chimera and Lyn–PLCγ2 in a PLCγ2-deficient background demonstrated that both constructs were functional and could restore the Ins(1,4,5)P_3 generation lacking in PLCγ2-deficient DT40 cells. However, only Lyn–PLCγ2 was able to restore Ins(1,4,5)P_3 production in DT40 cells deficient in BLNK; the levels of Ins(1,4,5)P_3 produced were similar to those in the stable cell line where human BLNK have been introduced into a BLNK-deficient background. These experiments suggested that the PHδ1PLCγ2 chimera had properties of the wild-type protein both in raft-localization after stimulation and the ability to restore Ins(1,4,5)P_3 production only in PLCγ2-deficient background (Figure 1 and 2). In contrast, Lyn-tagged PLCγ2 can bypass requirements for BLNK by providing more specific membrane-targeting signal resulting in localization of PLCγ2 to membrane rafts.

Since the analysis of Lyn–PLCγ2 have not revealed clear differences in BLNK$^-$ and PLCγ2-deficient cells, further characterization is shown for the BLNK$^-$/Lyn–PLCγ2 cell line in comparison with a control cell line, the wild-type PLCγ2 in PLCγ2-deficient cells (Figure 3). The time course for production of Ins(1,4,5)P_3 is shown in Figure 3(C). The responses to H_2O_2 were similar with some reduction in Ins(1,4,5)P_3 generation (about 20–25 %) observed for BLNK$^-$/Lyn–PLCγ2 cell line at earlier time points (Figure 3C, left panel). Transient responses to M4 for the two cell lines were also similar, and again only a small reduction at earlier time points was observed (Figure 3C, right panel). The differences in Ins(1,4,5)P_3 production between the cell lines were reflected in Ca^{2+} responses (Figure 3D).

Importance of the SH2 domains and tyrosine residues implicated in phosphorylation for the activation of raft-targeted PLCγ2

Since the Lyn-targeted PLCγ2 is not constitutively active and bypasses targeting to the membrane alone, it provides a system to test the requirement of the SH2 domains and tyrosine residues (implicated in phosphorylation by Btk) that are specifically related to changes leading to a high rate of PtdIns(4,5)P_2 hydrolysis. Therefore, for this analysis, mutations in both SH2 domains or mutations of Tyr^{753} and Tyr^{759} have been made in the context of Lyn-tagged PLCγ2 (Figure 4A) and the stable cell lines

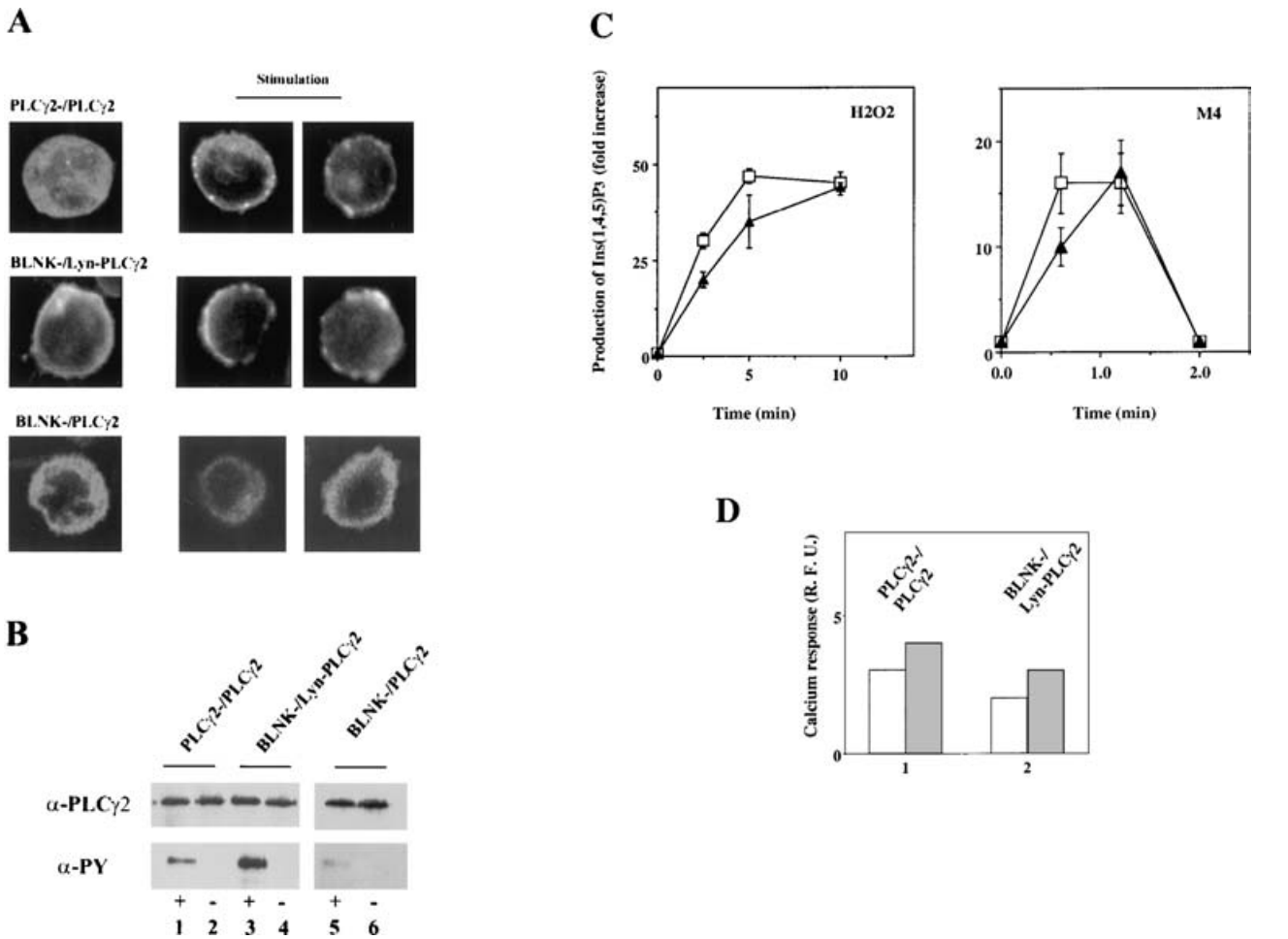

Figure 3 Comparison of the BLNK$^-$/Lyn–PLCγ2, BLNK$^-$/PLCγ and the control PLCγ2$^-$/PLCγ2 DT40 cell line following stimulation by H_2O_2 or M4 antibody

(**A**) Immunofluorescence confocal analysis of PLCγ2 localization in PLCγ2$^-$/PLCγ2 (top panels), BLNK$^-$/Lyn–PLCγ2 (middle panels) and BLNK$^-$/PLCγ2 (bottom panels) was performed, as described in Figure 1, before (left panels) and after (right panels) stimulation. (**B**) PLCγ2 phosphorylation in PLCγ2-deficient cells stably transfected with the wild-type PLCγ2 (PLCγ2$^-$/PLCγ2, lanes 1 and 2) and BLNK-deficient cells stably transfected with the Lyn-tagged PLCγ2 (BLNK$^-$/Lyn–PLCγ2, lanes 3 and 4) was analysed before (−) and after (+) stimulation with 2 mM H_2O_2. PLCγ2 was immunoprecipitated from cell extracts using anti-PLCγ2 antibody and the Western blotting performed with either anti-PLCγ2 antibody (top panels) or anti-phosphotyrosine antibody (α-PY; bottom panels). After the stimulation with M4 antibody, reduction of PLCγ2 phosphorylation in PLCγ2$^-$/PLCγ2 DT40 cells was similar to that shown in Figure 1(D); a similar 3–4-fold reduction was observed in BLNK$^-$/Lyn–PLCγ2, while the phosphorylation in BLNK$^-$/PLCγ2 could not be detected (results not shown). (**C**) Time-course analysis of Ins(1,4,5)P_3 production. Ins(1,4,5)P_3 was measured in extracts prepared from 2×10^6 cells of BLNK$^-$/Lyn–PLCγ2 (▲) and PLCγ2$^-$/PLCγ2 (□) DT40 cell lines. Stimulation was either with 2 mM H_2O_2 (left panel) or 10 μg/ml M4 (right panel) for the indicated times. The results are expressed as the means ± S.D. for experiments performed in duplicate. (**D**) Ca^{2+} mobilization was measured in Fluo 3/AM-loaded PLCγ2$^-$/PLCγ2 (1) and BLNK$^-$/Lyn–PLCγ2 (2) DT40 cells after stimulation with 2 mM H_2O_2 (dark grey) or 10 μg/ml M4 (light grey), as described in the Experimental section. R.F.U., relative fluorescence units.

generated (Table 1). Both constructs (Lyn–PLCγ2$^{R564A/R672A}$ and Lyn–PLCγ2$^{Y753F/Y759F}$) were expressed at similar levels as Lyn–PLCγ2 and were targeted to the membrane (Figure 4B). As shown in Figure 4(C), phosphorylation of both constructs could be detected; the phosphorylation levels were reduced for Lyn–PLCγ2$^{Y753/759F}$, but not as severely as in the context of non-targeted PLCγ2 as shown previously [24]. It is possible that by introducing the Lyn-tag, the amount of PLCγ2 and/or time that PLCγ2 is exposed to raft-associated kinases may be increased, resulting in further phosphorylation of tyrosine residues other than Tyr^{753} and Tyr^{759}, known to be accessible to phosphorylation, at least *in vitro* [24,25].

Several previous studies of non-targeted PLCγ2 in DT40 cells have demonstrated that both the SH2 domain mutation (R564A/R672A) and Y753F/Y759F mutation have diminished or greatly reduced the ability of PLCγ2 to restore responses in PLCγ2-deficient cells without non-specifically affecting catalytic properties *in vitro* [19,24,25]. Analysis of Ins(1,4,5)P_3 production at the fixed time-points in cell lines expressing Lyn-tagged PLCγ2 in PLCγ2- or BLNK-deficient background have shown that Lyn–PLCγ2 and also Lyn–PLCγ2$^{R564A/R672A}$ can restore responses to M4 antibody or H_2O_2, whereas Lyn–PLCγ2$^{Y753F/Y759F}$ could only restore about 20–25 % of the Ins(1,4,5)P_3 production observed for other constructs (Figure 5A). The differences between the cell lines have also been confirmed by analysis of Ca^{2+} responses to M4 and H_2O_2 (results not shown).

The Ins(1,4,5)P_3 production by Lyn–PLCγ2$^{R564A/R672A}$ and Lyn–PLCγ2$^{Y753F/Y759F}$ mutants was further analysed in a time-course experiment following stimulation by H_2O_2 (Figure 5B, left panel) or M4 (Figure 5B, right panel). The amount of Ins(1,4,5)P_3 produced in the stable cell line expressing Lyn–PLCγ2$^{Y753F/Y759F}$ were greatly reduced. Nevertheless, similar to the control cells and Lyn–PLCγ2 (see Figures 3 and 6), the responses to H_2O_2 were sustained and those following addition of M4 antibodies were only transient. In the stable cell line expressing Lyn–PLCγ2$^{R564A/R672A}$, the amounts of Ins(1,4,5)P_3 produced at the late time points

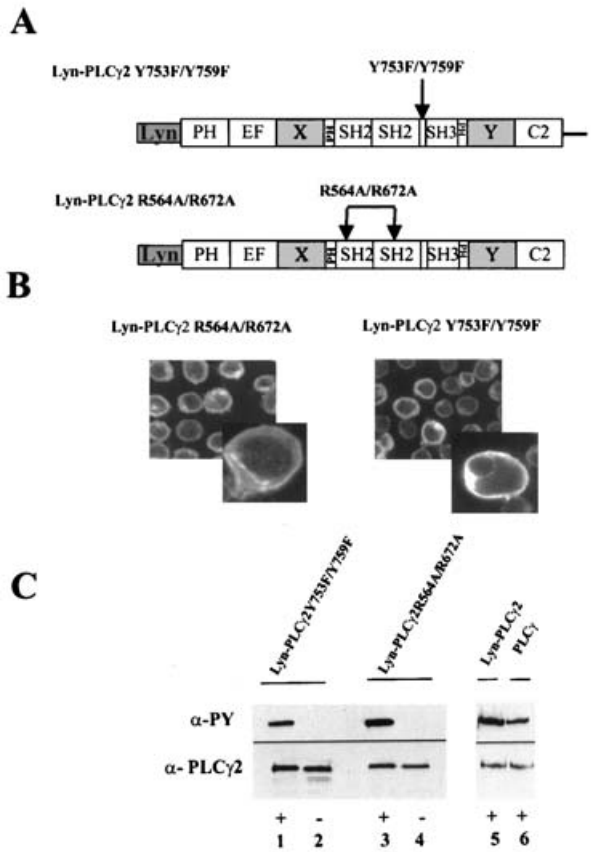

Figure 4 Mutations in the SH2 domains and critical tyrosine residues in the context of Lyn-tagged PLCγ2

(**A**) Representation of the constructs encoding the Lyn-tagged PLCγ2 incorporating Y753F/Y759F (upper panel) or R564A/R672A (lower panel) mutations. (**B**) Localization of PLCγ2 constructs in stably transfected PLCγ2^{-}/Lyn–PLCγ2$^{R564A/R672A}$ and PLCγ2^{-}/Lyn–PLCγ2$^{Y753F/Y759F}$ DT40 cells was analysed by immunofluorescence confocal microscopy using anti-PLCγ2 antibody. The fields of cells and enlarged single cell images (insets) are shown. (**C**) The cell lines described in (**B**), were also analysed for tyrosine phosphorylation of PLCγ2 before (−) and after (+) stimulation with H_2O_2 (lanes 1–4). Stimulated PLCγ2^{-}/Lyn–PLCγ2 and PLCγ2^{-}/PLCγ2 cells are shown for comparison (lanes 5 and 6). After immunoprecipitation using anti-PLCγ2 antibody, Western blotting was performed with either anti-phosphotyrosine antibody (α-PY; top panel) or anti-PLCγ2 antibody (bottom panel). The comparison of the ratios of intensities obtained with the two antibodies resulted in an estimate that Lyn–PLCγ2 was phosphorylated about two-fold more than PLCγ2. Phosphorylation of Lyn–PLCγ2$^{R564A/R674A}$ was similar to Lyn–PLCγ2, whereas Lyn–PLCγ2$^{Y753F/Y759F}$ was reduced to about 30 % of Lyn–PLCγ2$^{R564A/R674A}$. The fold reduction after M4 stimulation for Lyn–PLCγ2$^{R564A/R672A}$ and Lyn–PLCγ2$^{Y753F/Y759F}$ constructs was as described for the wild-type PLCγ2 in Figure 1(D).

following H_2O_2 stimulation were reduced; instead of sustained production of Ins(1,4,5)P_3, the response was transient and completely diminished after 10 min (Figure 5B, left panel).

The results described above suggest that within the membrane-raft-targeted construct of PLCγ2, the stimulation of PtdIns(4,5)P_2 hydrolysis requires the presence and, presumably, phosphorylation of Tyr^{753} and Tyr^{759}. In contrast, the SH2 domains appear to have a less critical role, but could be important for sustained activation of PLCγ2.

Involvement of Btk, Syk and PI3K in activation of the raft-targeted PLCγ2

In DT40 cells deficient in Btk or Syk tyrosine kinase, the BCR stimulation or H_2O_2 treatment resulted in reduced or diminished production of Ins(1,4,5)P_3 and Ca^{2+} responses [31,32,40,41]. It is possible that these kinases could contribute to both translocation and activation of PLCγ2. To test the involvement of Syk and Btk in the activation of PLCγ2 further, Lyn–PLCγ2 construct was introduced in Btk- or Syk-deficient cells (Table 1). As expected, the Lyn–PLCγ2 was present in the plasma membrane of generated stable cell lines (Figure 6A).

Analysis of Ins(1,4,5)P_3 production in Syk^{-}/Lyn–PLCγ2 and Btk^{-}/Lyn–PLCγ2 cell lines demonstrated some response to H_2O_2 and M4 stimulation (Figure 6C), which, in agreement with previous observations [31,32,40,41], could not be detected in Syk- or Btk-deficient cells (results not shown) and were mediated by endogeous non-tagged PLCγ2. However, as shown in Figure 6(C), these responses were different from those in the control cell line (PLCγ2^{-}/PLCγ2). Stimulation by H_2O_2 resulted in delayed and somewhat reduced Ins(1,4,5)P_3 production (Figure 6C, left panel). Under these conditions Lyn–PLCγ2 was phosphorylated (Figure 6B), most likely by tyrosine kinases present in the rafts and due to the inhibition of protein phosphotyrosine phosphatases by H_2O_2. However, when stimulation through BCR cross-linking was analysed, the phosphorylation of Lyn–PLCγ2 was hardly detectable and this construct was very poor in restoring cellular responses (Figures 6B and 6C). The production of Ins(1,4,5)P_3 in both cell lines, Syk^{-}/Lyn–PLCγ2 and Btk^{-}/Lyn–PLCγ2, was greatly reduced and transient (Figure 6C, right panel). These results demonstrated that both Syk and Btk tyrosine kinases are required for activation of PLCγ2 under these more physiological conditions, and further supported previous findings that their activation in BCR signalling directly or indirectly results in PLCγ2 phosphorylation.

Analysis of DT40 cells deficient in PI3K suggested an involvement of this enzyme in stimulation of PLCγ2 activity [42]. Previous experiments have also suggested that the PI3K product, PtdIns(3,4,5) P_3, could have several links with PLCγ2, the most important being the involvement in translocation/stimulation of Btk [43]. Therefore, it would be expected that the inhibition of PI3K by LY294002 or wortmannin have a similar effect as Btk deficiency. The roles of Btk and PI3K, however, seem to be somewhat different in signalling through the BCR compared with H_2O_2-induced stress. It has been reported that inhibition of PI3K by wortmannin completely abolished Ca^{2+} mobilization in the wild-type DT40 cells stimulated by the BCR cross-linking and only reduced (up to 50 %) the Ca^{2+} responses to H_2O_2 stress; also, only in response to H_2O_2 could the inhibition be overcome by Btk over-expression [30]. As shown in Figure 7(B), the analysis of PLCγ2^{-}/PLCγ2 control cells is in agreement with the previous observation using the wild-type DT40 cells [30], and demonstrated the reduction (20–25 %) of Ins(1,4,5)P_3 production by H_2O_2 (Figure 7B, left panel) and complete inhibition of responses to M4 (Figure 7B, right panel) in the presence of either LY294002 or wortmannin. The same differences were observed using BLNK^{-}/Lyn–PLCγ2 cells (Figure 7C) demonstrating that effects of PI3K inhibitors do not require the presence of BLNK and that involvement of PI3K in BCR signalling may be related to activation rather than translocation of PLCγ2, since it cannot be bypassed by Lyn targeting.

DISCUSSION

In the present study, we have described the analysis of regulation of PLCγ2 in B-cell signalling by generating a number of DT40 B-cell lines which expressed various PLCγ2 constructs, membrane-targeted and lacking specific functional domains or residues, in a DT40 cell background deficient in different signalling

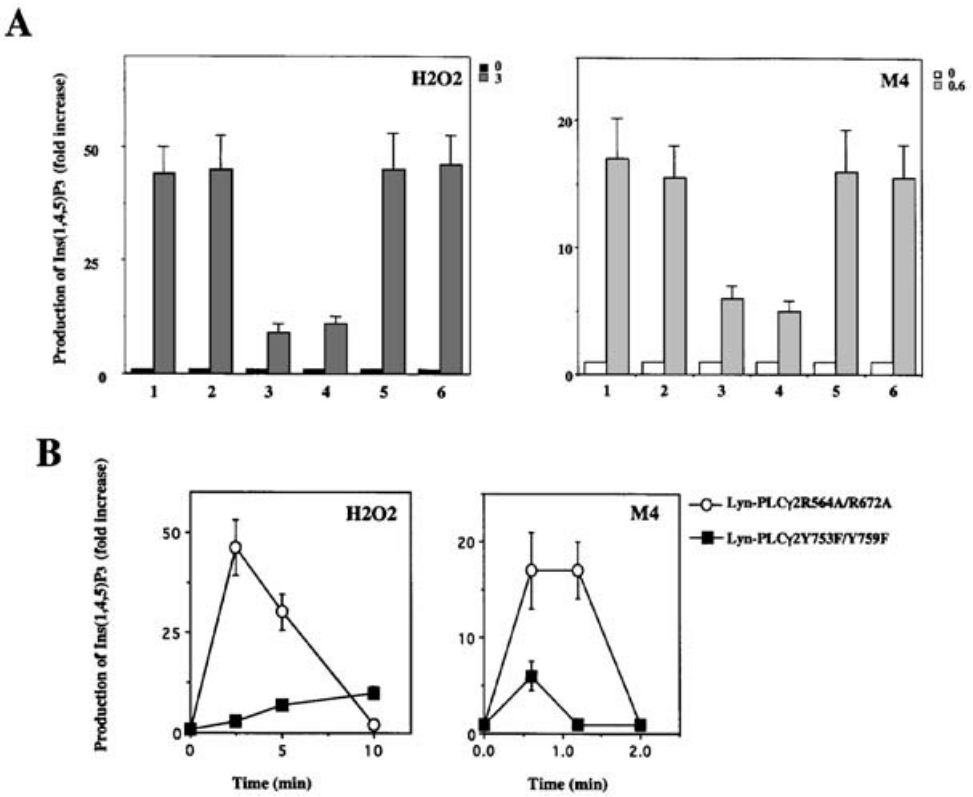

Figure 5 Analysis of Ins(1,4,5)P_3 production by Lyn–PLCγ2, Lyn–PLC$\gamma 2^{Y753F/Y759F}$ and Lyn–PLC$\gamma 2^{R564A/R672A}$ in various DT40 cell lines

(**A**) Ins(1,4,5)P_3 generation in BLNK- and PLCγ2-deficient cells stably transfected with the Lyn-tagged constructs, Lyn–PLCγ2, Lyn–PLC$\gamma 2^{Y753F/Y759F}$ and Lyn–PLC$\gamma 2^{R564A/R672A}$, was measured in response to stimulation by H_2O_2 for 3 min (left panel) and M4 antibody for 0.6 min (right panel) as described in the legend for Figure 3. The following cell lines were analysed: PLC$\gamma 2^-$/Lyn–PLCγ2 (1), BLNK$^-$/Lyn–PLCγ2 (2), PLC$\gamma 2^-$/Lyn–PLC$\gamma 2^{Y753F/Y759F}$ (3), BLNK$^-$/Lyn–PLC$\gamma 2^{Y753F/Y759F}$ (4), PLC$\gamma 2^-$/Lyn–PLC$\gamma 2^{R564A/R672A}$ (5) and BLNK$^-$/Lyn–PLC$\gamma 2^{R564A/R672A}$ (6). Ins(1,4,5)P_3 generation in stimulated cells is shown by dark (H_2O_2) and light (M4) grey bars. The results are expressed as the means $\pm$ S.D. for three independent experiments performed in duplicate. (**B**) Time-course analysis of Ins(1,4,5)P_3 production was performed in BLNK$^-$/Lyn–PLC$\gamma 2^{Y753F/Y759F}$ (■) and BLNK$^-$/Lyn–PLC$\gamma 2^{R564A/R672A}$ (○) DT40 cell lines after H_2O_2 (left panel) and M4 (right panel) stimulation. The results are expressed as the means $\pm$ S.D. for experiments performed in duplicate.

components. The results support a two-step model: the membrane targeting, specifically to the lipid rafts, and subsequent activation of the enzyme. These steps require interaction with different signalling components and involve different regions of PLCγ2.

The importance of the lipid rafts in PLCγ signalling has been addressed in this and several previous studies [12–15,35,44]. In response to different extracellular signals (e.g. stimulation of EGF receptor, collagen receptor glycoprotein VI, TCR and BCR), at least a fraction of PLCγ translocates to the lipid rafts [12–15]. In DT40 B-cells, a small but significant portion of PLCγ2 is translocated to this structure; this has also been observed for some other components including BLNK, Btk and more recently, Cbl-b [35,44]. Our studies in DT40 cells provide evidence that the PLCγ2 targeting to the lipid rafts is essential for signalling by this isoform. Unlike targeting mediated through the PtdIns(4,5)P_2 binding by the PLCδ1 PH domain chimera that results in general membrane targeting without enrichment in rafts, the PLCγ2-containing Lyn-targeting sequences was specifically present in these structures and, more importantly, capable of restoring signalling in BLNK-deficient cells (Figures 1 and 2). Although some results suggest that the substrate for PI-PLC, PtdIns(4,5)P_2, is more abundant in rafts [45], at least in B-cells this is not sufficient for the specific targeting. Furthermore, recent studies in cells using the PLCδ1PH domain as a probe, suggest a similar density of PtdIns(4,5)P_2 in the raft (caveolein)-containing and other areas of the plasma membrane [46]. Targeting to the rafts therefore may not be related to the substrate access by PLCγ2, but to proximity to molecules such as Syk and Btk tyrosine kinases, known to be recruited to the rafts [14,47] and required for PLCγ2 signalling in B-cells [31,32,40,41]. Interestingly, the Lyn-tagged PLCγ2 was not constitutively phosphorylated and activated (Figures 2 and 3B).

Consistent with the results from the present study (Figures 1 and 2), previous experiments using a different membrane-targeting PLCγ2-chimera (not fully active, most likely due to intramolecular constrains and folding problems of the construct) have shown a potential of this targeted molecule to partially restore at least some functions of BLNK [19,20]. Although not analysed directly, these studies have also indicated the importance of specific targeting. More recent work in T-cells using targeted construct of PLCγ1 containing the N-terminus of Src kinase Fyn, similar to the Lyn–PLCγ2 construct described in the present study, have demonstrated the presence of this construct in the lipid rafts [34]. Furthermore, Fyn–PLCγ1 could function without interacting with the adapter Lat and also in cells deficient in another adapter protein, Slp-76. However, in contrast to observations in B-cells, Fyn-PLCγ1 was constitutively phosphorylated and implicated in constitutive Ca^{2+}-dependent increase of transcription [34]. This difference could reflect different tyrosine kinases involved in phosphorylation of two

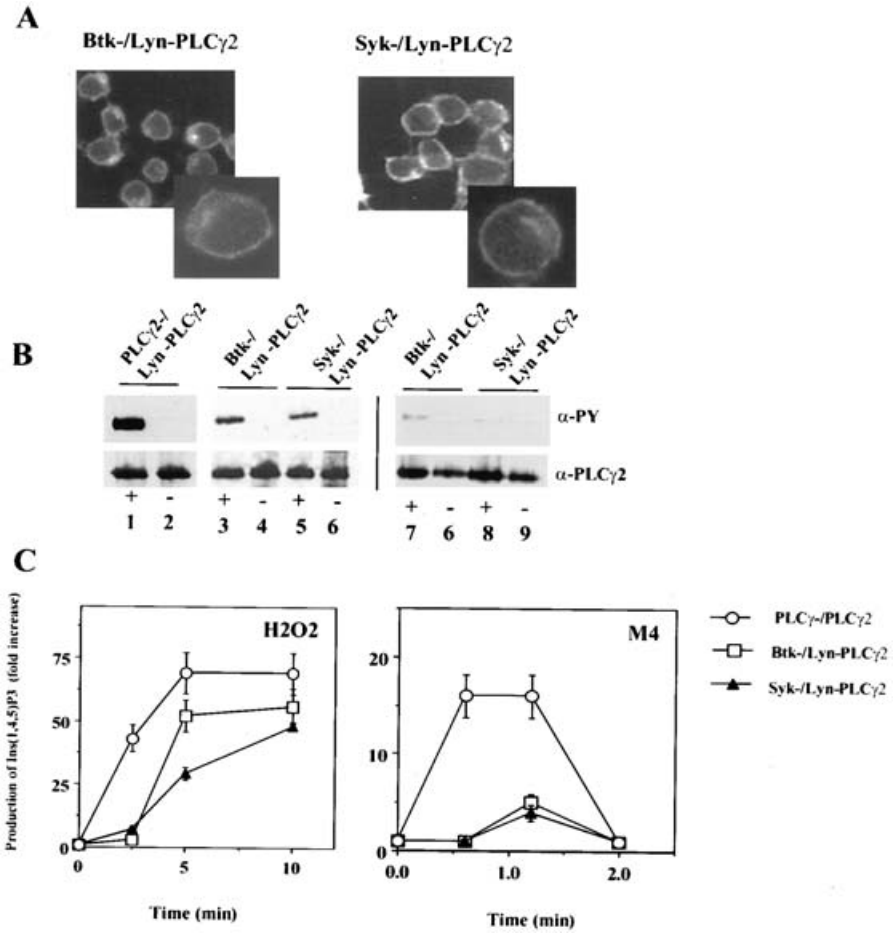

Figure 6 Analysis of the involvement of Btk and Syk in activation of Lyn-targeted PLCγ2

(**A**) Btk- and Syk-deficient cells stably transfected with Lyn-targeted PLCγ2, Btk$^-$/Lyn–PLCγ2 (left panel) and Syk$^-$/Lyn–PLCγ2 (right panel), were analysed for localization of PLCγ2 constructs by immunofluorescence confocal microscopy. (**B**) The detection of PLCγ2 phosphorylation in the PLCγ2$^-$/Lyn–PLCγ2 (lanes 1 and 2), Btk$^-$/Lyn–PLCγ2 (lanes 3 and 4) and Syk$^-$/Lyn–PLCγ2 (lanes 5 and 6) DT40 cell lines was performed before (−) and after (+) H_2O_2 stimulation. After immunoprecipitation of PLCγ2, Western blotting was performed using anti-phosphotyrosine (α-PY; upper panels) and anti-PLCγ2 (lower panels) antibody. It was estimated that the phosphorylation of Lyn–PLCγ2 in Btk- and Syk-deficient cells was reduced 50–60 % compared with PLCγ2-deficient background. The M4 stimulation (lanes 7–9) was performed in a separate experiment with the control cell line having the normal difference in phosphorylation between H_2O_2 and M4, described in Figure 1(D). (**C**) Ins(1,4,5)P_3 generation at different time points after stimulation with H_2O_2 (left panel) and M4 antibody (right panel) was analysed in Btk$^-$/Lyn–PLCγ2 (□) and Syk$^-$/Lyn–PLCγ2 (▲) cells and compared with the control cell line PLCγ2$^-$/PLCγ2 (○).

PLCγ isoforms or a more stringent regulation of PLCγ-directed tyrosine kinase activity in resting DT40 cells compared with a specific T-cell line chosen for studies of T-cell signalling.

The membrane-targeted Lyn–PLCγ2 was used to further define post-targeting events leading to PLC activation and to analyse which domains of PLCγ2 and which signalling components are required specifically for this step. Our results demonstrated different roles for the SH2 domains and specific tyrosine residues (Tyr^{753} and Tyr^{759}) in the context of Lyn–PLCγ2 (Figures 4 and 5). The functional SH2 domains were only required to support prolonged Ins(1,4,5)P_3 production, whereas the presence of specific tyrosine residues (Tyr^{753} and Tyr^{759}) was essential to fully restore responses by Lyn–PLCγ2 to BCR cross-linking and H_2O_2.

In previous attempts to study requirements for the high rate of PtdIns(4,5)P_2 hydrolysis by PLCγ1 separately from the requirements for translocation, an *in vitro* system using substrate/Triton X-100 mixed micelles has been developed [48,49]. In this system, the difference in activity between phosphorylated and non-phosphorylated PLCγ1 have been observed after isolation of PLCγ1 from cells, supporting the importance of phosphorylation in stimulation of PLC activity [48]. Using a similar system, differences have been shown between non-phosphorylated and PLCγ2 phosphorylated *in vitro*, rather than in cells [50]. Nevertheless, studies of different DT40 stable cell lines have shown that mutations of several tyrosine residues introduced separately and the double mutation Y753F/Y759F of PLCγ2, reduce or abolish Ca^{2+} responses to the BCR cross-linking and H_2O_2 [24,25]. Further experiments have also suggested that Y753F/Y759F mutation did not affect translocation [24]. Consistent with these previous findings, the results presented in the present study (Figures 3 and 4) show that the membrane targeting can not bypass the requirement for these specific tyrosine residues. In addition, several other observations (Figure 6) suggest the importance of tyrosine phosphorylation. One of the effects of Lyn-targeting of PLCγ2 is that this construct becomes more readily phosphorylated than the wild-type PLCγ2, in particular following H_2O_2 stimulation that potently inhibits protein tyrosine phosphatases. This phosphorylation of Lyn–PLCγ2 occurs even in the absence

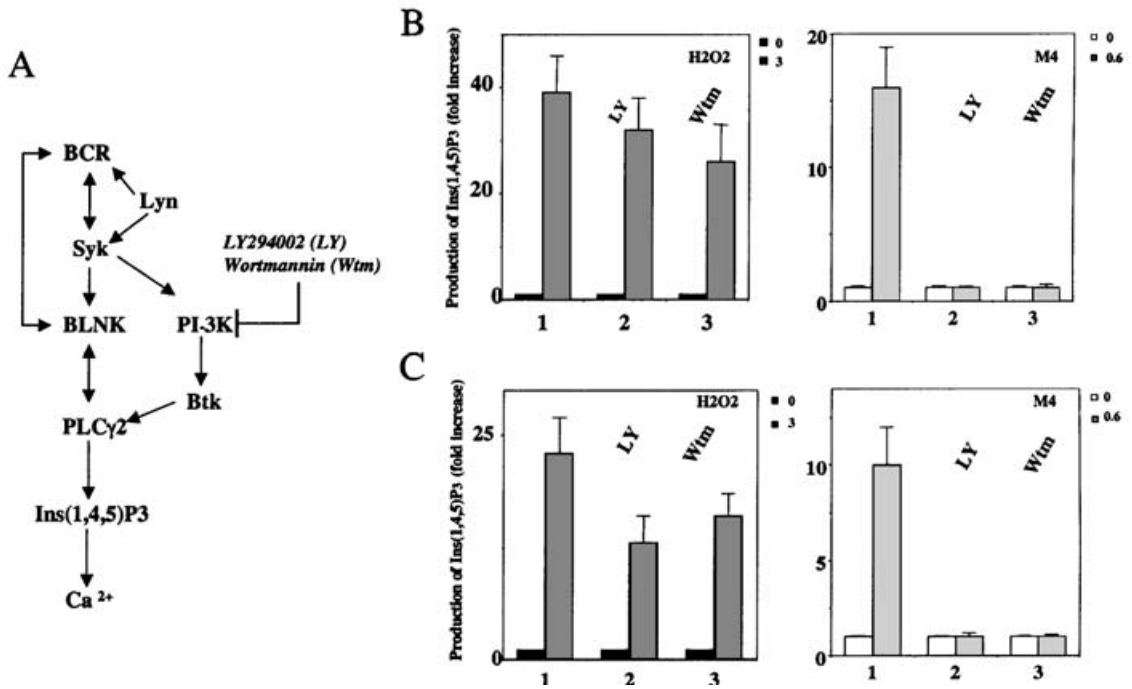

Figure 7 Effects of PI3K inhibitors, LY294002 and wortmannin

(**A**) Diagram showing inhibition of PI3K by LY294002 and wortmannin in the context of BCR-mediated signalling. The diagram is based on recent reviews [10,11]. (**B**) The control DT40 cell line PLCγ2^{-}/PLCγ2 was either untreated (1) or incubated with 50 μM LY294002 (2) or 100 nM wortmannin (3) for 30 min. Ins(1,4,5)P_3 present in the cell extracts was measured before and after stimulation with either H_2O_2 for 3 min (left panel) or M4 antibody for 0.6 min (right panel). Ins(1,4,5)P_3 production in stimulated cells is shown by dark (H_2O_2) and light (M4) grey bars. The results are expressed as the means $\pm$ S.D. for three independent experiments performed in duplicate. (**C**) The same experiment as described in (**B**) was performed using BLNK^{-}/Lyn–PLCγ2 cell line.

of Btk and Syk, presumably by some other kinases present in rafts, and is accompanied by activation of Lyn–PLCγ2.

Previous studies of the PLCγ1 SH2 domains have suggested that these domains could be involved not only in translocation, but also in PLC activation [1–3]. For example, binding of tyrosine-phosphorylated peptides, corresponding to the PLCγ1-binding sites in the EGF receptor, induced conformational changes of recombinant PLCγ1 and resulted in an increase of PLC activity *in vitro* [49]. Since the suggested binding partner for the PLCγ2 SH2 domains is the adapter protein BLNK, the PLCγ2–BLNK interactions could have a similar effect. Our results using Lyn-targeted PLCγ2 (Figures 2, 3 and 5), however, do not support this possibility and show that neither the SH2 domain mutations nor BLNK deficiency have a significant effect on Lyn–PLCγ2 signalling following the BCR cross-linking. Furthermore, in response to H_2O_2 stress, the effects of the SH2 mutations and BLNK deficiency are quite different; only in the case of SH2 domain mutations were the prolonged responses to H_2O_2 affected. Similar observations in Syk-deficient and Btk-deficient cells (Figure 6) do not favour Syk or Btk (the latter previously implicated in sustained substrate hydrolysis) as components responsible for the SH2 domain-mediated effects on prolonged activation. When considering allosteric regulation through the SH2 domain binding, there is also a possibility that the interactions may occur intramolecularly between phosphorylated tyrosines in PLCγ2 (Tyr^{753}, Tyr^{759} or others) and one of its SH2 domains. Regardless of the identity of phosphotyrosine residues that bind the SH2 domains, this interaction could stabilize an activated form of PLCγ2 (which could be caused by phosphorylation of critical tyrosine residues) and/or protect the phosphorylated tyrosines from the action of phosphatases, thus underlying the observed requirement in prolonged activation.

Analysis of DT40 cells revealed a number of components that contribute to PLCγ2 signalling in this system, including Syk, Btk and PI3K; the requirements for Btk and PI3K are less stringent under some conditions [30–32,40–42,51]. Further studies in this and other systems suggested that the requirements for these components in PLCγ2 signalling could reflect their involvement at several steps leading to PLC responses and could also include both indirect effects and direct interactions with PLCγ2. For example, Syk kinase is not only required for phosphorylation of BLNK [20,21,24], but also contributes to activation of Btk and PI3K [52,53]. In the case of Btk, in addition to the suggested role in direct phosphorylation of several tyrosine residues in PLCγ2 [24,25], this protein may be an important structural component of a scaffold required for the assembly of BCR signalling complexes [54]. The requirement for PI3K in PLCγ2 signalling in B-cells could be due to its involvement in the regulation of Btk [43]. However, the link between PI3K and PLCγ isoforms could be more direct and affect either translocation or PLC activation [5–7]. Our results (Figures 6 and 7) show that Syk and Btk proteins and PI3K activity are all required for the activation step of the raft targeted PLCγ2 in response to BCR cross-linking. Interestingly, stimulation by H_2O_2, accompanied by enhanced phosphorylation of the Lyn–PLCγ2, can partially restore responses in Btk- and Syk-deficient cells by this targeted construct, suggesting that their effects at the activation step are at least in part related to phosphorylation of PLCγ2. In contrast with our present study, the analysis of Fyn–PLCγ1 in T-cell signalling have not supported the role of the Syk family kinase Zap-70

(zeta-associated protein 70) or the production of PtdIns(3,4,5)P_3 in the regulation of PLC activity of this construct [34]. However, it is not clear whether these differences reflect selected cell types, where the lipid-raft targeting is coupled or uncoupled with constitutive phosphorylation and activation, or physiological differences between regulation of PLCγ1 activity in T-cells and regulation of PLCγ2 in B-cells.

The results described in the present study, together with previous observations [14,19,20], support the important role of the lipid-raft targeting as a first step in regulation of PLCγ2. They also suggest that post-targeting events, resulting in fully active PLC, involve additional modifications and interactions that in the BCR signalling require specific tyrosine residues that are implicated in the phosphorylation of PLCγ2 and the activation of Syk, Btk and PI3K.

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