REVIEW ARTICLE

PROKARYOTIC EXPRESSION AND PURIFICATION OF PLC-γ2

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Phospholipase C-gamma 2 (PLC-γ2) is an enzyme that plays an important role in intracellular signal transduction pathways. It has been shown that PLCγ2 is the predominant isoform of phospholipase C in platelets and B-lymphocytes, being implicated in collagen-induced signal transduction in platelets and antigen-dependent signaling in B-lymphocytes. The activation of PLCγ2 in these cells is induced by phosphorylation of two specific tyrosine residues (Y753 and Y759) situated in its SH2-SH3 linker region. So far, *in vitro* and *in vivo* studies have revealed several protein tyrosine kinases that are able to directly interact and phosphorylate PLCγ2: Btk, Syk, Lyn, Fyn and Lck. Although PLCγ2 phosphorylation has been intensively approached, there are no reports about its dephosphorylation.

In order to identify the tyrosine phosphatase involved in PLCγ2 dephosphorylation and further investigate its *in vitro* dephosphorylation, we applied a quick and cheap method for obtaining sufficiently pure PLCγ2. First, human PLCγ2 full-length was expressed in bacteria. In order to obtain a higher quantity of soluble protein, different cultivation temperatures and IPTG concentrations were tested. An increased proportion of the soluble protein was observed when the cultivation temperature and IPTG concentration were lowered. Further, the expressed protein was purified by affinity chromatography using calmodulin-Ni-Sepharose resin. Finally, we set up an efficient method to obtain recombinant PLCγ2 with a relatively high degree of purity. The recombinant protein obtained can be used for further *in vitro* studies.

Key words: phospholipase C-γ2, prokaryotic expression, protein purification.

INTRODUCTION

Phospholipase C enzymes are essential signaling mediators involved in agonist-stimulated phosphoinositide metabolism and calcium signaling. There are six major families of PLC enzymes (β, γ, δ, ε, ζ and η) that share a common core of domains related to catalysis and are distinguished by some regulatory regions (1, 2). Under the control of cell surface receptors, these enzymes cleave

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phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] generating two intracellular molecules: inositol 1,4,5-triphosphate ($InsP_3$), a universal calcium-mobilizing messenger, and diacylglycerol (DAG), an activator of protein kinase C (1).

PLCγ encompasses two isoforms, PLCγ1 and PLCγ2, which are regulated through receptors with intrinsic tyrosine kinase activity (e.g. growth factor receptors or receptors that are linked to the activation of non-receptor tyrosine kinases [e.g. BCR (B-cell antigen receptor) and TCR (T-cell receptor)] (3, 4). They constitute large molecules with multidomain structures that contain: an N-terminal pleckstrin homology domain (PH), four EF-hand motifs, two catalytic domains (PLCγc X and Y), a "split" PH domain (spPH), two Src homology 2 domains (SH2), one Src homology 3 domain (SH3) and a C-terminal calcium binding domain (C2) (Fig. 1). The two isoforms have distinct tissue distribution: PLCγ1 is expressed ubiquitously while PLCγ2 is mainly expressed in the hematopoietic lineage. Also, studies using knockout animals suggested different biological functions for the two isoforms. Thus, deficiency in PLCγ1 proved to be lethal in embryonic mice (5), whereas homozygous disruption of PLCγ2 allowed normal mice development but generated functional and signaling disorders in B-cells, platelets, and mast cells (6).

PLCγ2 is the predominant isoform in B-lymphocytes where it has been shown to constitute an essential component in signalization through BCR (6–9). After its activation through phosphorylation, PLCγ2 plays a dominant role in triggering the BCR-induced calcium signaling that further generates a series of biochemical events such as gene transcription, cellular proliferation, and differentiation. Recently, it has been demonstrated that PLCγ2 is required for efficient generation and maintenance of memory B-lymphocytes in a B lymphocytes-intrinsic manner (10). Also, PLCγ2 is abundantly expressed in platelets where it is involved in signal transduction following collagen stimulation and in adhesion of platelets to the sites of vascular injury, thus promoting thrombus growth (11, 12). In these cells PLCγ2 was found to mediate Ca^{2+} release from intracellular stores, leading to cytoskeleton reorganization and filopodia extension (12).

Activation of PLCγ2 has been shown to correlate with its tyrosine phosphorylation. Several studies on B-lymphocytes indicated that two protein tyrosine kinases, Btk (Bruton's tyrosine kinase) and Syk, are the most likely enzymes that directly phosphorylate and activate PLCγ2 (13, 14). Also, it was demonstrated that Lyn, Fyn and Lck tyrosine kinases are able to *in vitro* dephosphorylate PLCγ2 (15). There are four tyrosine residues in PLCγ2 identified as Btk-dependent phosphorylation sites required for BCR-coupled Ca^{2+} signaling, but two of them (Y753 and Y759) were found to be essential for activation of PLCγ2 (16, 17).

Although the tyrosine phosphorylation of PLCγ2 was thoroughly studied, no information about its dephosphorylation pathway has been reported so far. *In vitro* studies involving pure PLCγ2 preparations would be important to this purpose. So

far, two articles regarding preparation of recombinant PLCγ2 have been reported (15, 18). However, the yield of final product was relatively low and the suggested preparation protocol requested expensive commercial consumables. Therefore, we expressed human PLCγ2 full-length in *E. coli* and set up an efficient purification procedure yielding to a slightly higher amount of protein and relatively high purity of the final preparation.

MATERIAL AND METHODS

EXPRESSION AND PURIFICATION OF HUMAN PLCγ2

For the bacterial expression of PLCγ2, *E. coli* BL21(DE3) strain was transformed with pCAL-n-PLCγ2 plasmid (kindly provided by Dr. J. Daniel, Temple University, Philadelphia, USA). The bacterial cells were grown on Luria-Bertani medium supplemented with 100 μg/mL ampicillin at 25°C until it reached an optical density at 600 nm of 0.7–0.8. Then, protein expression was induced at different temperatures (30, 28, 25 and 20°C) in order to increase the level of expressed soluble protein. Also, two concentrations of IPTG were tested (0.1, respectively 0.01 mM). The cells were harvested 6 h after induction for cultures induced at 30° and 28°C, respectively 18 h after induction for cultures induced at 25° and 20°C. Further, the pellet was resuspended in lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 4 mM DTT, 1 mM $MgCl_2$, 1 mM imidazole, 2 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitors cocktail (Roche), 1% Nonidet P-40]. The resuspended cells were lysed by sonication and the cellular debris were removed by centrifugation 20 min at 14000xg, 4°C. The resulted supernatant was incubated with calmodulin-His-Ni-Sepharose (previously prepared as indicated below) overnight, at 4°C. The fusion protein was eluted with elution buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM DTT, 1 mM $MgCl_2$, 1 mM imidazole, and 2 mM EGTA). Analysis of protein expression and purification was done by 10 % SDS-PAGE and by Western blotting.

CALMODULIN-Ni-SEPHAROSE RESIN PREPARATION

First, calmodulin N-terminally fused with six histidine residues was expressed in a prokaryotic system. Thus, *E. coli* BL21(DE3) strain was transformed with pET28-(6xHis)-calmodulin plasmid (kindly provided by Dr. Nadia Bucurenci, Cantacuzino Institute, Bucharest, Romania). Protein expression was induced at 37°C, for 4 h, by the addition of 1 mM IPTG. The cells were harvested by centrifugation 10 min at 5000 rpm, 4ºC, resuspended in lysis buffer (50 mM Tris buffer pH 7.4, 500 mM NaCl, 20 mM imidazole, 4 mM DTT, 1mM

PMSF, 1% Triton X-100) and sonicated 5×10 s. The lysate was cleared by centrifugation 20 min, 14000xg, 4°C and the supernatant was incubated 1 h 30 min at 4°C with Ni-Sepharose High Performance resin (GE Healthcare). Analysis of (6xHis)-tagged calmodulin expression and binding to the resin was performed by 15% SDS-PAGE.

HUMAN PLATELETS ISOLATION AND LYSIS

Fresh human blood was collected in acid/citrate/dextrose (2.5 g sodium citrate, 1.5 g citric acid, 2 g glucose in 100 ml ddH_2O). Platelet rich plasma was obtained by centrifugation at 180×g for 15 min at ambient temperature and incubated with aspirin (1 mM) at 37°C for another 45 min. Platelets were isolated from the incubation medium by centrifugation (800×g for 15 min, room temperature) and small aliquots of cell pellets were stored at -70°C. For PLCγ2 detection, the isolated platelets were lysed in Laemmli sample buffer and separated by 10% SDS-PAGE.

PLCγ2 DETECTION BY WESTERN BLOTTING

After separation by SDS-PAGE, the proteins were transferred to Immobilon P membrane (Millipore) for 2 h at 200 mA, using a semidry transfer system. The membrane was further treated for 1h at room temperature or overnight at 4°C with blocking buffer (5% skimmed low-fat milk (Fluka) in a 50 mM Tris, pH 8.0, 150 mM NaCl buffer). Then, the membrane was incubated with mouse monoclonal antibody anti-PLCγ2 (Santa Cruz Biotechnology) for 1h at room temperature, followed by incubation with the secondary antibody, anti-IgG mouse HRP-conjugated (Sigma) for 1h at room temperature. The blots were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and exposed to Amersham Hyperfilm ECL (GE Healthcare).

RESULTS AND DISCUSSION

Full-length PLCγ2 is a large (Mw 148 kDa), multimodular protein composed of ten domains (Fig. 1). Preparation of a reasonable quantity of pure PLCγ2 from natural sources by conventional biochemical methods, proved to have some drawbacks including low yield and high cost of labor (15). Therefore, we have chosen to express PLCγ2 in a prokaryotic system. For this reason, we used pCAL-n-PLCγ2 prokaryotic vector containing the gene that encodes for the full length PLCγ2 (fl PLCγ2), N-terminally tagged with a calmodulin binding peptide (CBP).

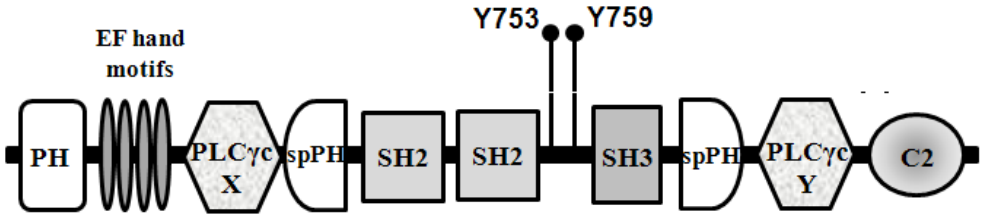


Fig. 1. – Schematic representation of the multimodular structure of full length PLCγ2. The domains within the structure of PLCγ2 are: the N-terminal pleckstrin homology domain (PH), EF-hand motifs, catalytic domain incorporating X and Y regions (PLCγc X and Y), the "split" PH domain (spPH), two Src homology 2 domains (SH2), one Src homology 3 domain (SH3) and the C-terminal calcium binding domain (C2). Y753 and Y759 represent the two tyrosine residues that, when phosphorylated, they are essential for activation of PLCγ2.

Being a protein with a multidomain complex structure, PLCγ2 was difficult to be expressed into a prokaryotic system: it has been expressed with a low yield and solubility. In order to increase the efficiency of its bacterial expression, different conditions of expression were tested. Thus, bacterial cells have been induced at different temperatures (30, 28, 25 and 20 °C) with two different IPTG concentrations (0.1 and 0.01 mM) in order to increase the level of expressed soluble protein (Fig. 2).

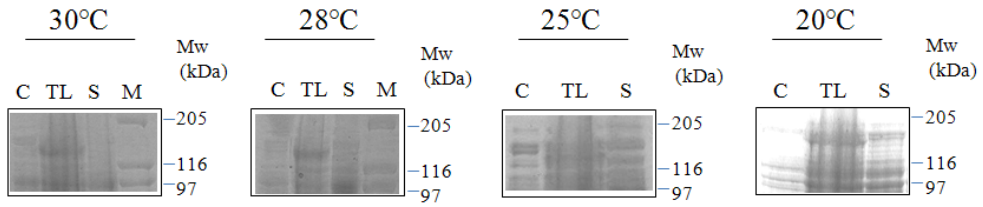


Fig. 2. – Prokaryotic expression of PLCγ2 at different temperatures. C (Control) – non-induced culture; TL – total lysate after induction with IPTG; S – soluble proteins; M – Molecular marker. The temperatures shown above represent the expression induction temperatures tested in order to increase the yield and solubility of the recombinant protein. Samples were run in a 10% SDS-PAGE.

At higher temperatures (28 and 30°C), PLCγ2 was expressed with a slightly higher yield but it was less soluble than in the case of the expression performed at 20°C. Finally, the expression was performed at 20°C for 16 h, using 0.1 mM IPTG. PLCγ2 was expressed as a recombinant protein N-terminally fused with a CBP-tag that allows its purification on calmodulin affinity resin. Because the yield of the purified protein was low, the relatively high price of the affinity resin did not justify its usage. Therefore, we adopted another strategy to obtain the calmodulin binding resin in the lab. This strategy consisted in the prokaryotic expression of calmodulin fused with 6XHis affinity tag and further binding of the recombinant

calmodulin to an affinity support (Ni-Sepharose 4B). Calmodulin binding to the resin followed by purification of recombinant PLCγ2 is schematically represented in Fig. 3.

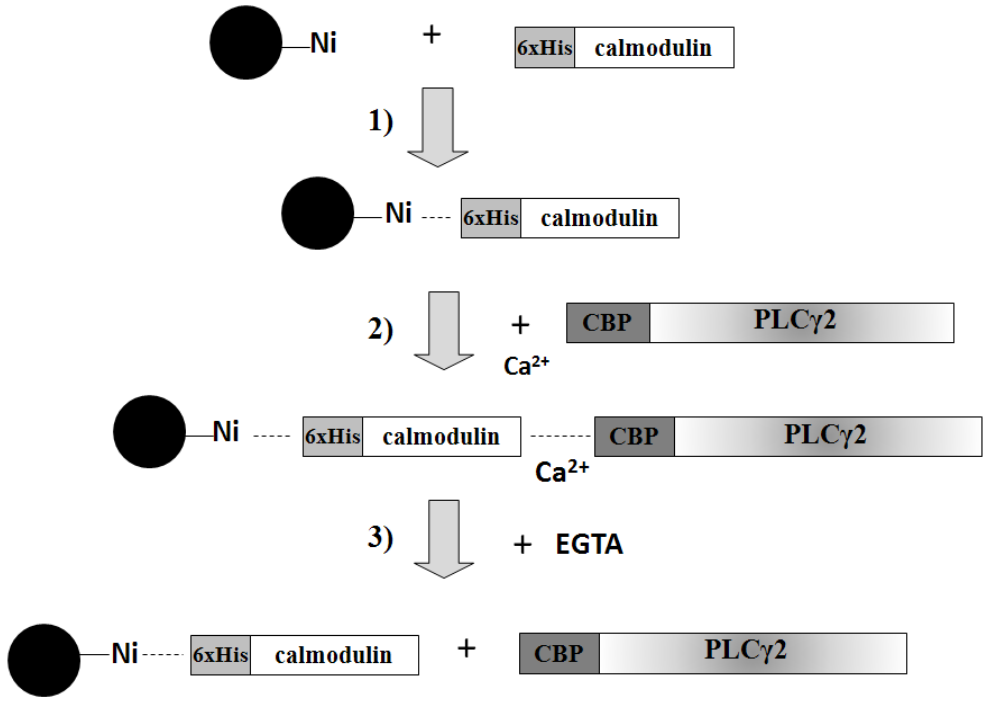


Fig. 3. – Scheme of the strategy used for full length PLCγ2 purification after its expression in *E. coli*. 1) The recombinant calmodulin N-terminally fused with a 6XHis-tag was bound to the Ni-Sepharose resin; 2) The recombinant PLCγ2 N-terminally fused with CBP (calmodulin binding peptide) was bound to Sepharose – Ni – 6XHis-calmodulin in presence of Ca^{+2} ions; 3) Finally, PLCγ2 is eluted from the resin in the presence of EGTA, a chelator of Ca^{+2} ions.

To obtain calmodulin binding resin, initially calmodulin N-terminally tagged with 6xHis residues (Mw 17.6 kDa) was expressed in *E. coli*. The bacterial cells were lysed and the cell lysate was mixed with Ni-Sepharose in a batch system beads and finally the resulting calmodulin-Ni-Sepharose resin was intensively washed to remove the unbound protein and further used for affinity purification of PLCγ2 (Fig. 4). Bacterial pellets obtained after induction of PLCγ2 expression were solubilised in lysis buffer and the clarified cell lysates were incubated with calmodulin-Ni-Sepharose affinity resin and the beads - eluted with an elution buffer containing EGTA (that chelates Ca^{2+}, leading to release of bound PLCγ2 from the resin).

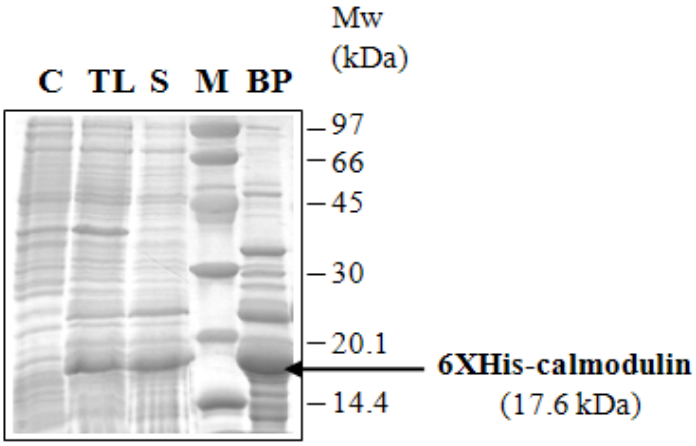


Fig. 4. – Expression in *E. coli* of calmodulin N-terminally fused with a 6XHis-tag and its absorption to Ni-Sepharose. C Control – non-induced culture; TL – total lysate after induction with IPTG; S – soluble proteins; M – Molecular marker; BP – protein bound to Ni-Sepharose. Samples were run in 15% SDS-PAGE.

The expression of PLCγ2 was tested by SDS-PAGE (Fig. 5A) as well as by Western blotting (Fig. 5B) using specific antibodies. In the case of SDS-PAGE detection, the band corresponding to PLCγ2 after purification is weak, but still visible by Coomassie staining (Fig. 5A, lane 4). In addition, no other significant bands of contaminant proteins can be seen after elution. The expression of PLCγ2 was further confirmed by detection with anti-PLCγ2 antibodies (Fig. 5B). Western blotting results suggest that PLCγ2 (Mw 148 kDa) fused with CBP (Mw 4 kDa) was

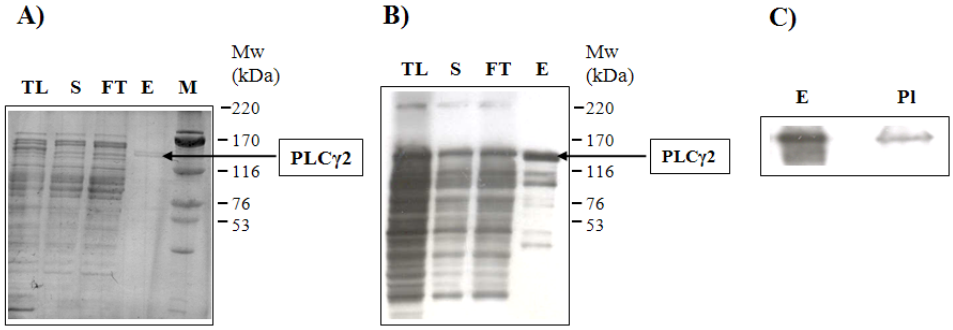


Fig. 5. – Evidentiation of PLCγ2 expression and purification A) SDS-PAGE: C Control – non-induced culture; TL – total lysate after induction with IPTG; S – soluble proteins; FT – flow trough; E – eluted protein; M – Molecular marker. Samples were run in 10% SDS-PAGE. B) Western blotting: TL, S, FT and E samples were immunodetected with monoclonal anti-PLCγ2 antibody (B-10); C) Comparison between PLCγ2 expressed in bacteria and obtained from platelet cell lysates. A sample resulted after PLCγ2 elution from calmodulin-Ni-Sepharose resin (E) and a sample of platelets lysate (Pl) were probed with anti-PLCγ2 antibody (B-10). Samples were run in 10% SDS-PAGE.

expressed in prokaryotic system at its theoretical molecular weight of 152 kDa. As it can be seen the anti-PLCγ2 antibodies reveal the presence of other bands besides those corresponding to the PLCγ2 full-length. Those bands could constitute either shorter forms of PLCγ2 or unspecific bindings of the antibody. To confirm that the protein expressed and detected is indeed the full length form of PLCγ2, a positive control consisting of a lysate of platelets, cells known to express PLCγ2 endogenously were used. The band corresponding to the recombinant PLCγ2 was at the same molecular weight to that obtained in the case of platelets lysates (the 4 kDa difference between the molecular weights of tagged and untagged protein could not be detected in 10% SDS-PAGE gel used for sample running) (Fig. 5C).

In conclusion, we expressed PLCγ2 in a prokaryotic system and set the optimal parameters for an increased expression and solubility. The amount of recombinant PLCγ2 obtained after purification was estimated at 30–40 μg/l soluble protein, being slightly higher than the amount reported before by Ozdener *et al*. (18). PLCγ2 was purified from bacteria cultures to approximately 80% purity as estimated from SDS-PAGE. Thus, we developed a non-expensive and rapid method to obtain recombinant PLCγ2 with a relatively high degree of purity.

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