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Expression of Enzymatically-active Phospholipase Cγ2 in *E. coli*

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Phospholipase C-gamma-2 (PLCγ2) activation is a key signaling event for many cell functions. In order to delineate the pathways that lead to PLCγ2 activation, we devised a quick method for obtaining sufficient PLCγ2. We obtained the full-length cDNA for human PLCγ2 and expressed it in *E. coli* using the expression vector pT5T. To enhance the protein expression, tandem AGG-AGG arginine codons at the amino acid positions 1204-1205 were replaced by CGG-CGG arginine codons. The protein expression was detected in a Western blot analysis by both anti-PLCγ2 antibodies and the antibodies that are raised against the tripeptide epitope (Glu-Glu-Phe) tag that are genetically-engineered to its carboxyl terminal. Crude lysates that were prepared from bacteria that express PLCγ2 were found to catalyze the hydrolysis of phosphatidylinositol 4,5 bisphosphate. Similar to previous reports on PLCγ2 that is isolated from mammalian tissue, the recombinant enzyme was Ca^{2+} dependent with optimal activity at 1-10 uM Ca^{2+}.

Keywords: Bacterial expression, Enzymatic activity, Phospholipase Cγ2, Subcloning

Introduction

Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C is an important signaling event in the regulation of cell functions by different stimuli (Berridge, 1993). This hydrolysis, which results in the production of two intracellular messengers, diacylglycerol and inositol 1,4,5-trisphosphate, is catalyzed by any one of the three types of phospholipase C that is expressed in mammalians; β, γ, and δ. Beta isoforms are secondarily activated to the G protein coupled receptors, these activators are Gαq and Gβγ. Gamma isoforms are secondarily activated to the activation of the poorly defined tyrosine kinase-signaling pathways, and the activation mechanisms of δ isoforms are yet to be investigated.

PLCs are located in cytoplasm in a resting state; upon activation they translocate to cell membranes and stay anchored to catalyze PIP_2 hydrolysis. Therefore, PLCs have evolved as large molecules with multidomain structures; each domain has a unique function (Noh *et al*., 1995). A Ca^{2+} ion, which is located at the active site, directly participates in catalysis; therefore, all of the eukaryotic PLC isozymes require Ca^{2+} for activity. Calcium is also required for the function of the C2 domain. In addition to the multidomain structure that is observed with all of the PLC isozymes, PLCβ and PLCγ isozymes have additional COOH-terminal and SH regulatory domains, respectively. These regulatory domains are responsible for the fact that different PLC isozymes are linked to receptors through distinct mechanisms (Rhee and Bae, 1997).

Partly because of its multidomain complex structure, the purification of PLCs by conventional biochemical methods from natural resources has drawbacks. These include low yields, low activities, and the high cost of labor. Koblan and colleagues published the PLCg1 expression in *E. coli* using the expression vector pT5T (Koblan *et al*., 1995). Therefore, we reasoned that since the bacterial expression had produced enzymatically-active PLCγ1, then similar methodology should produce a fully active PLCγ2. Therefore, we decided to express PLCγ2 in *E. coli*. Here, we report the expression of enzymatically-active PLCγ2, and show its regulation by calcium.

Materials and Methods

Materials Human PLCγ2 cDNA that contains the pMT2 plasmid was a gift from Dr. Joseph Baldassare (St. Louis University). The pT5T vector was a gift from Dr. Kenneth S. Koblan (Merck Research Labs, West Point, USA). Competent DH5α cells

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(subcloning efficiency) and competent BL21 (DE3) cells were purchased from GIBCO/BRL (Gaithersburg, USA). The cloning vectors Bluescript KS +/- and Ready-To-Go T4 DNA ligase tube were from Amersham Pharmacia Biotech (Piscataway, USA). PtdIns(4,5)P_2 ammonium salt was either from Boehringer Mannheim (Indianapolis, USA) or Sigma (St. Louis, USA), and $[^3H]$PtdIns(4,5)P_2 was obtained from DuPont-NEN (Boston, USA). Monoclonal antibody YL 1/2 was from Harlan Bioproducts For Science (Indianapolis, USA). The polyclonal anti-PLCγ2 antibody was a gift from Dr. Graham Carpenter (Vanderbilt University). The phosphatase-labeled secondary antibodies and BCIP/NBT membrane phosphatase substrate were from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, USA). Synthetic oligonucleotides were obtained from Genosys (Woodlands, USA). PCR products and plasmids were purified using a QIAquick gel extraction kit and QIAGEN plasmid kit from Qiagen Inc. (Chatsworth, USA). Restriction enzymes and WizardTM Plus Minipreps DNA Purification System were obtained from Promega (Madison, WI). All of the other reagents were purchased from Sigma (St. Louis, USA), unless otherwise indicated.

Subcloning of the Human PLCγ2 cDNA into the bacterial expression vector pT5T The polyclonal site in the pT5T vector has few cloning sites, and the human PLCγ2 cDNA is relatively long (3795 bp) with many internal restriction sites; therefore, the insertion of the full-length cDNA of PLCγ2 into pT5T was achieved in two steps that involved intermediate subcloning steps.

The subcloning of the human PLCγ2 into the bacterial expression vector pT5T included PCR reactions at both ends of the cDNA molecule. These PCR reactions provided genetically-engineered proper restriction sites for subcloning purposes. For the 5' end PCR reaction, primer #1, primer #2, and template DNA in pMT2 were used. The resulting product was digested with *Xba* I and *Eco* RV to generate a 222-bp fragment. Primer #1 (sense: 5' GCTCTAGATCT ATGTCCACCACGGTCAAT 3') contained *Xba* I and *Bgl* II sites (newly engineered into the noncoding region). Primer #2 (antisense: 5' CGTTTTCGTCAAGCGGTC 3') spanned a stretch beyond the internal *Eco* RV site that is found at position 291. This fragment, as well as the 2716 bp *Eco* RV, *Sal* I fragment that was obtained from cDNA, were subcloned into *Xba* I, *Sal* I that digested Bluescript II KS +/-. This generated a 5898 bp construct that was designated PLCγ2pBS1. This construct was propagated in DH5a cells. For the 3 end PCR reaction, the primers #3 and #4, as well as the template DNA in pMT2, were used. The resulting product was digested with *Pvu* II and *Hind* III to generate a 196 bp fragment. Primer #3 (sense: 5' GTCGCCAGCTGCGGCGGCGGCAA 3') mutated AGGAGG arginine codons to CGGCGG arginine codons at positions 1204 and 1205. Primer #4 (antisense: 5' CCCCAAGCTT CTAAAATTCTTCTGAGTAAAACTTGCTGACTCTCTTCTCT CTTAACCTCTTGTTGACTTTCTCCTGGTACAACTGGA 3') contained a DNA sequence that encodes the Glu-Glu-Phe epitop tag that is attached to the end of the coding sequence, as well as a *Hind* III restriction site, after the stop codon and C to T point mutation to abolish the *Pvu* II site at position 3699. This 196 bp fragment, as well as the 692-bp *Sal* I, *Pvu* II fragment that was obtained from the template DNA in pMT2, were subcloned in to *Sal* I, *Hind* III that digested Bluescript II KS +/-, which generated a 3848-bp construct (designated PLCγ2pBS2). Digestion of PLCγ2pBS1 with *Bgl* II and *Sal* I, and digestion of PLCγ2pBS2 with *Sal* I and *Hind* III, yielded 3935 bp and 888 bp fragments, respectively. These two fragments were then ligated into *Bam* HI and *Hind* III sites in the polyclonal region of the pT5T vector, which generated 8708 bp PLCγ2pT5T. The presence of inserts were verified by *Sal* I and *Hind* III digestions. The pT5T vector has ampicillin and tetracycline genes, and the recombinant enzyme is produced under the control of the T7 promoter. DH5α competent cells were used for transformation by PLCγ2pBS1, PLCγ2pBS2, and PLCγ2pBS3 constructs. For the bacterial expression of PLCγ2, the PLCγ2pT5T-transformed *E.coli* BL21 (DE3) strain was grown in a Luria-Bertani medium that was supplemented with 100 μg/ml ampicillin at RT. Upon reaching an optical density at 550 nm (OD_{550}) of 0.8-1.0, the cells were induced with 0.01 mM IPTG, then harvested by centrifugation at indicated periods of induction time.

Western blotting Bacterial pellets were solubilized in a Laemmli sample buffer and subjected to 8% SDS- PAGE according to Laemmli (Laemmli, 1970). Freshly-electrophoresed SDS-polyacrylamide gels were dipped into a transfer buffer (192 mM glycine and 25 mM Tris-HCI, pH 8.3, 10% methanol), then laid flat on pre-wetted nitrocellulose paper that was supported on a layer of transfer buffer-wetted filter paper. The gel was overlaid with a wetted-filter paper. The assembly was placed in the transfer tank. The proteins were then electrophoretically-transferred to Immobilon-P using the transfer buffer for 2 h at 200 mA. The Immobilon was briefly rinsed in a transfer buffer and treated overnight at 4ºC with a blocking buffer (5% bovine serum albumin, 150 mM NaCI, 50 mM Tris-HCI, pH 8.0, 0.1% NaN_3). The attachment of specific antibodies to proteins was achieved by the incubation of the blots at 25ºC for 1 h with antiglu-glu-phe mAb (Monoclonal antibody YL 1/2, 1 μg/ml), anti-PLCγ2 antibody (1/10,000 dilution). The blots were washed by shaking in a 100 ml washing buffer (25 mM Tris-HCl, pH 7.4, containing 0.05% Tween-20 and 0.1% albumin, 0.05% NaN_3), 3 times for 5 min, at RT. Probing of the antibody binding was by incubation for 1 h with suitably-diluted alkaline phosphatase-conjugated secondary antibodies. The secondary antibodies were goat anti-rat IgG antibodies (0.2 μg/ml) for antiglu-glu-phe mAb and goat anti-mouse IgG antibodies (0.1 μg/ml) for anti-PLCγ2 antibodies. The blots were pre-rinsed in a little substrate buffer (100 mM Tris-Cl, pH 9.5). The antibody that bound to the blots was visualized by the reaction of BCIP/NBT membrane phosphatase substrate.

Assay of PLCγ2 activity PIP_2 hydrolyzing activity was measured in a reaction mixture that contained lipid micelles, according to previously published protocol (Wahl *et al.*, 1992). Ptd$[^3H]$ Ins 4,5-P_2 (2 μM) was added to lyophilized Ptd Ins 4,5-P_2 ammonium salt (1 mg), and dried by evaporation in a Speed-Vac to ~10 μl. The buffer (180 μl) that contained 50 mM NaH_2PO_4 (pH 6.8) plus 100 mM KCl was added, then the mixture was vortexed gently to suspend the Ptd$[^3H]$ Ins 4,5-P_2. While chilled in ice water, the phospholipid suspension (5 mM) was sonicated at the lowest setting for 2 min. Next, 18 μl of 10 mM of n-octylglucoside was added to yield a 360-μl stock substrate solution that contained Ptd$[^3H]$ Ins 4,5-P_2 (2.5 mM) and 5 mM of n-octylglucoside. Prior to use, this stock solution was mixed with a buffer to give final assay concentrations of 35 mM NaH_2PO_4 (pH 6.8), 70 mM KCl, 1 mM EDTA, 2 mM

$MgCl_2$, 0.6 mM $CaCl_2$ (1 μM final Ca^{2+} concentration), 5 μM BSA, 5 mM DTT, 200 μM Ptd[^{3}H] Ins 4,5-P_2 (25,000 dpm), and 5 mM n-octyl glycoside. After thermally pre-equilibrating the assay mixture in the absence of enzyme, an aliquot of the PLCγ2 suspension (5 μl) was added to the substrate solution (45 μl). The reaction mixture was incubated at 25°C for 15 min. The reaction was stopped by transferring to an ice bath with the addition of 0.5 ml of chloroform/methanol/HCl (100 : 100 : 0.6), followed by 0.15 ml of 1 N HCl that contained 5 mM EDTA. The aqueous and organic phases were separated by centrifugation, and a 200 μl portion of the upper aqueous phase was removed for liquid scintillation counting. Ca^{2+} dependence was measured in a reaction mixture in which the free Ca^{2+}concentration was adjusted by varying the ratio of Ca^{2+} to EDTA.

Results

Bacterial expression of Human PLCγ2 The pT5T vector is derived from the phage T7 promoter-polymerase expression system that was originally developed by Studier and co-workers (Studier and Moffatt, 1986), and modified by Squires et al. (Squires *et al*., 1988). Since the polyclonal site in the pT5T vector has few cloning sites, and the human PLCγ2 cDNA is relatively long (3795 bp) with many internal restriction sites, our first task was to plan a strategy that would allow the insertion of the full length cDNA of PLCγ2 into pT5T. Two possible human PLCγ2 cloning sites in pT5T are the *Bam* HI and *Hind* III sites. To avoid the interference of the internal *Bam* HI and *Hind* III sites in PLCγ2 during the subcloning procedure, we needed to genetically-engineer these sites to the two ends of the cDNA. Therefore, we performed two different PCR reactions at each end of the cDNA molecule. The presence of inserts were verified by *Sal* I and *Hind* III digestions. The pT5T vector has ampicillin and tetracycline resistance genes. The recombinant enzyme is produced under the control of the T7 promoter.

For the human PLCγ2 expression, the *E. coli* BL21 cells were transformed with the PLCγ2pT5Tconstruct. This bacteria strain is employed for the high level expression of genes that are cloned into expression vectors that contain the T7 promoter. The bacteria were grown in the presence of ampicillin and tetracycline at room temperature, and induced with isopropyl thio-galactoside (IPTG). The recombinant enzyme was produced under the control of the T7 promoter by translationally-coupling its expression to that of the phage-ø10 coat protein in the pT5T vector. On the assumption that CGG-CGG codons would enhance the protein expression (Bonekamp and Jensen, 1988), tandem AGG-AGG arginine codons at the amino acid positions 1204-1205 were replaced by CGG-CGG arginine codons. In Western blots, a protein band with an electrophoretic mobility that is compatible with PLCγ2 (145 kDa) was detectable with anti-PLCγ2 antibodies (donated by Dr. Graham Carpenter, Vanderbilt University) at 6 and 12 hour induction times in the PLCγ2pT5T-transformed E.coli (Fig. 1, panel A). This band was undetectable in the

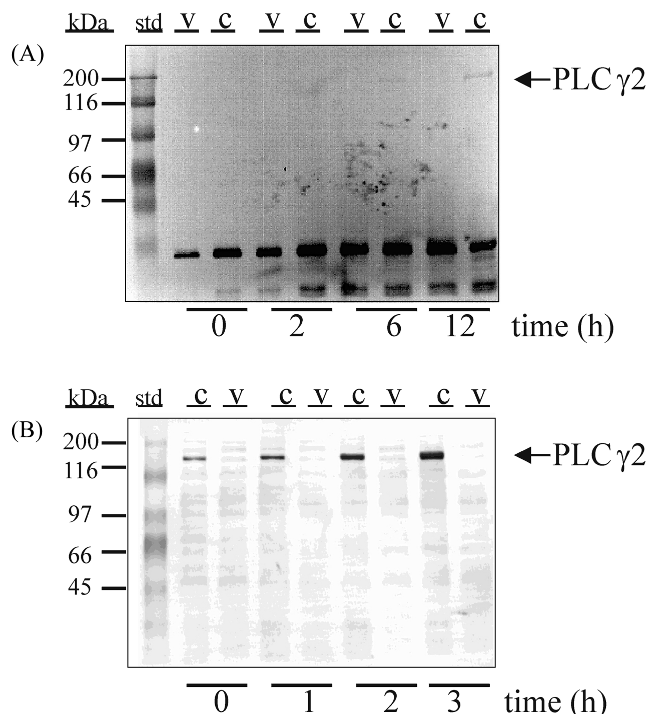

Fig. 1. Western blot of PLCγ2-expressed in *E. coli*. *E.coli*, transformed with either pT5T (v) or PLCγ2pT5T (c), were grown as described, induced for indicated periods of time with 0.01 mM IPTG, and lysed with a SDS sample buffer. Whole lysates were subjected to 8% SDS/PAGE, and the resolved proteins that were transferred to Immobilon-P. Immunoblots were performed either with (A) anti-PLCγ2 antibodies (1/10000 dilution) or (B)antibodies against Glu-Glu-Phe epitope tag (mAb YL 1/2, 1 μg/ml). The first lanes in the SDS/PAGE are molecular weight standards.

vector-only transformed and induced controls. Genetically-engineered carboxyl-terminal glu-glu-phe epitope tag facilitated the detection of the expressed PLCγ2 with the monoclonal antibody YL 1/2 (Stammers *et al*., 1991). PLCγ2 was detectable much earlier with the monoclonal antibody, and the time-dependent increase in expression was apparent (Fig. 1, panel B).

Assay of PLCγ2 activity We further analyzed the bacterial extracts for their ability to hydrolyze PtdIns(4,5)P_2, when the substrate was presented in micelles. Extracts from bacteria were transformed by the plasmid that contained the enzyme gene (PLCγ2pT5T), but not from the E. coli extracts that were transformed by the plasmid vector (pT5T) showed catalytic activity (not shown). This result confirmed the expression of catalytically-active human PLCγ2 in *E.coli*. We also demonstrated that the calcium dependence of the recombinant enzyme was similar to that of the native enzyme that was reported by others (Wahl *et al*., 1992), the maximum activity was between 1 and 10 micromolar calcium concentration (Fig. 2). Therefore, we were able to express the

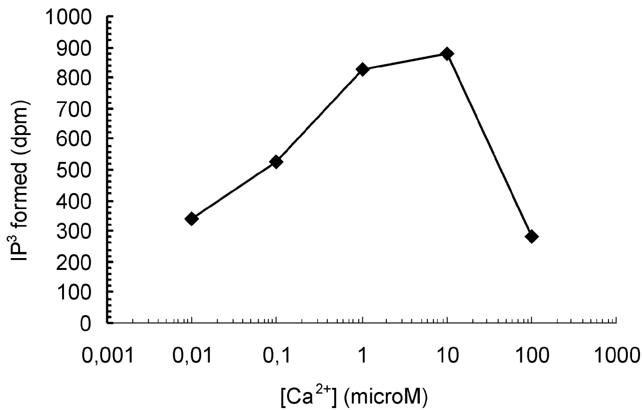

Fig. 2. Ca^{2+} dependence of PIP_2 hydrolysis by PLCγ2. PIP_2-hydrolyzing activity of crude lysates that were prepared from *E.coli* that was transformed with the construct (PLCγ2pT5T) was tested for PIP_2-hydrolyzing activity. Free Ca^{2+} was adjusted by varying the ratio of Ca^{2+} to EDTA.

enzymatically-active enzyme and showed its regulation by calcium. Assuming that the recombinant enzyme is as active as the native enzyme that is reported by others (Homma *et al*, 1990), the estimated yield of the soluble enzyme is ~10 µg/liter of bacterial culture. However, it should be kept in mind that the assay system that is employed by Homma and colleagues is different than ours. This could lead to an underestimation of the active soluble enzyme yield.

Discussion

One of the most important contributions of recombinant DNA technology to our understanding of biology has been the development of DNA vector-based systems for the high level expression of foreign genes in different hosts, particularly *E. coli* cells. With the emergence of efficient expression systems, it is possible to obtain a variety of low abundance proteins. These include hormones, transcription factors, and signaling proteins in quantities that are sufficient for biochemical and biophysical characterization, as well as for the generation of specific antibodies. To date, most biochemical studies have used PLCγ2 that has been purified from mammalian sources (Ryu *et al*., 1987; Banno *et al*., 1990; Homma *et al*., 1990). However, the methods for purifying the enzyme from mammalian tissues are either tedious, or yield low quantities of the enzyme. For enzymological and structure-activity studies, a ready source of the enzyme is essential; therefore, we wanted to devise a method to quickly obtain sufficient amounts of enzyme. Koblan and colleagues (Koblan *et al*., 1995) used the pT5T vector for the expression of PLCγ1 in *E.coli* (BL 21) cells. Vector pT5T is derived from the phage T7 promoter-polymerase expression system that was originally developed by Studier and co-workers (Studier and Moffatt, 1986), and modified by Squires *et al* (Squires *et al*., 1988). Soluble PLCγ1 was produced in *E.coli* BL21 under low induction conditions. After the successful completion of subcloning and transformation procedures, the bacterial expression of PLCγ2 was tested. The expression under standard induction conditions produced insoluble PLCγ2 that accumulated in the cytoplasm of *E. coli* as inclusion bodies as expected (not shown). Even though the fusion proteins may accumulate in cytoplasm at very high levels (up to 25% of the total protein), in the majority of the cases the expressed proteins are in an insoluble form (Marston, 1986). Consistent with the literature (Lilie *et al*., 1998), this difficulty was overcome by lowering the cultivation temperature and decreasing the IPTG concentration to induce bacterial culture. This decreased the synthesis rate. These modifications provided us with soluble recombinant PLCγ2. The yield of recombinant PLCγ2 with the slow synthesis technique is sufficient for our kinetic and structural studies. Refolding the protein from inclusion bodies to increase the yield can be another choice; however, PLCγ2 has a large multidomain structure and we feel that it will be difficult to renature.

When proteins are expressed in heterologous systems, such as bacteria, incorrect folding of the protein during expression is a common problem, and it is detrimental to the function of the protein. One reason for incorrect folding can be the production of a protein with an incorrect location of the disulfide bonds. The presence of disulfide bonds has not been reported for PLCγ2. Since PLCγ2 is an enzyme, the observation of its catalytic activity can be important criteria for correct folding (Lilie *et al*., 1998). Therefore, the fact that recombinant PLCγ2 is active can be taken as evidence of correct folding. We wanted to further test the correct conformation of the enzyme by studying its calcium dependence. Since the free Ca^{2+} concentration is titrated within each assay system in different laboratories, then it can be regarded as independent from the assay system that was used. Ca^{2+} is necessary for the PLCγ2 activity. It interacts with several domains of the enzyme, including the catalytic and C2 domains (Katan, 1998). Low Ca^{2+} concentrations activate the enzyme, whereas high Ca^{2+}concentrations inhibit it, which creates a peak of catalytic activity as a function of free calcium concentration. Therefore, the Ca^{2+} concentrations at which the peak occurs can be taken as strong evidence for the correct conformation of the enzyme. The calcium dependence of the recombinant PLCγ2 was the same as those that were reported for PLCγ2 that was purified from platelets (Banno *et al*., 1990), PLCγ1 that was purified from bovine brain (Wahl *et al*., 1992; Koblan *et al*., 1995), and recombinant PLCγ1 that was expressed in bacteria (Koblan *et al*., 1995); maximal activity was between 1-10 µM free Ca^{2+} concentrations. However, the issue of correct folding can only be definitively addressed when the expressed enzyme is purified, and its specific activity is compared to that of the native enzyme.

In conclusion, we were able to express catalytically-active PLCγ2 in *E. coli* using the pT5T vector. The regulation of the enzyme by calcium is the same as previously reported for the native enzyme. Future structure-activity studies using

recombinant PLCγ2 will help delineate the key residues that are involved in the regulation of the enzyme.

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