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Molecular Cloning and Expression of a Phosphoinositide-specific Phospholipase C of *Dictyostelium discoideum**

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A number of phosphoinositide-specific phospholipases C (PLC) of different species have recently been cloned. The predicted amino acid sequences of these isoforms contain two highly conserved domains. Here we report the identification of a PLC gene of *Dictyostelium* by using the polymerase chain reaction. Primers were designed coding for highly conserved amino acid regions located within one of the conserved domains of PLCs. Cloning and sequencing of the polymerase chain reaction product revealed one unique PLC-like sequence. This sequence was used to screen a library and isolate several overlapping cDNA clones. The complete cDNA was expressed in *Dictyostelium* cells resulting in increased basal levels of inositol 1,4,5-trisphosphate and enhanced PLC activity. The identified *Dictyostelium* PLC, DdPLC, encodes a protein with a calculated molecular mass of 91 kDa. The deduced amino acid sequence contains the two conserved domains found in other PLC isoforms, separated by a short variable region. The C-terminal part of the protein shows strong homology with the mammalian PLC- δ isoform. DdPLC is expressed at all stages of development, with an increase in transcription during starvation and in the culminating fruiting body.

Phosphoinositide-specific phospholipase C (PLC)¹ is a major enzyme in the transmembrane signal transduction pathway. It activates the inositol cycle by hydrolyzing phosphatidylinositol 4,5-bisphosphate to form the intracellular second messenger molecules inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (1). Ins(1,4,5)P₃ releases Ca²⁺ from internal stores, whereas diacylglycerol activates protein kinase C. Calcium mobilization and protein kinase C activation are essential for many cellular activities as they mediate processes such as secretion, cell growth, motility, and proliferation (2).

The transmembrane signal transduction pathway in the cellular slime mold *Dictyostelium discoideum* has been studied

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M95783.

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¹ The abbreviations used are: PLC, phospholipase C; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; kb, kilobase(s); bp, base pair(s); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenedis(oxyethylenenitrilo)]tetraacetic acid.

extensively (3). Both chemotaxis and cell differentiation are a result of signal transduction events. Extracellular cAMP is the signal molecule that binds to surface receptors (4) and thus activates the effector enzymes adenylate cyclase, guanylate cyclase, and PLC. During starvation cAMP acts as a chemoattractant and causes the single amoebas to aggregate. In the aggregates extracellular cAMP regulates the expression of cell type-specific genes to induce differentiation (5). Stimulation of Ins(1,4,5)P₃ formation by GTP γ S in permeabilized cells indicates the involvement of G proteins in the activation of PLC (6, 7). The signal transduction pathway in higher eukaryotes appears to be similar to that of *D. discoideum* (8). In mammalian cells a hormone is the extracellular activating signal comparable with cAMP in *Dictyostelium*.

A number of mammalian PLC cDNAs and two PLC genes from *Drosophila melanogaster*, *norpA* and *plc-21*, have been isolated (9-17). They encode proteins varying in size from 85 to 154 kDa. The PLC isoforms are divided into three structural groups, β , γ , and δ , based on the location of two conserved domains in the primary structures of the enzymes (18). There are indications for the presence of a fourth group, α , not containing the conserved domains and with a lower molecular weight than enzymes in the other groups (19-22). The *norpA* gene from *Drosophila* is transcribed specifically in the head and is involved in the phototransduction pathway. The *plc-21* gene encodes two transcripts that are expressed in the central nervous system. The function of the multiple isoforms in mammalian cells has still to be established. They are expressed in a number of tissues, and one cell type can express more than one isoform. In *in vitro* experiments the isoforms show different activities and substrate specificities (18).

We have used PCR (23) to identify a PLC gene from *D. discoideum*, DdPLC,² using degenerate oligonucleotide primers coding for highly conserved amino acid sequence regions within one of the conserved domains. DdPLC is a single copy gene with a 2.8-kb transcript encoding a 91-kDa protein. Transcription appears to be enhanced during cell aggregation, it decreases during slug formation, and increases in the culminating fruiting body. The deduced amino acid sequence of DdPLC predicts the A and B domains that are found in other PLC isoforms and a C-terminal domain that is homologous with the terminus of bovine PLC- δ . To examine whether DdPLC codes for an active phospholipase C enzyme we overexpressed DdPLC in *D. discoideum* and observed an increase in both basal Ins(1,4,5)P₃ levels and PLC activity.

EXPERIMENTAL PROCEDURES

Standard DNA techniques were performed as described by Sambrook *et al.* (24) unless otherwise stated.

² The *D. discoideum* PLC sequence isolated is named DdPLC in this paper. It will be renamed DdPLC-A if more *Dictyostelium* PLC genes are identified.

TABLE I
PLC activity and *Ins(1,4,5)P₃* levels

Phospholipase C activity (pmol of *Ins(1,4,5)P₃* produced $\text{min}^{-1}/10^6$ cells) and basal *Ins(1,4,5)P₃* levels (pmol of *Ins(1,4,5)P₃*/10⁶ cells) were measured as described under "Experimental Procedures." The data are presented as the mean \pm S.D. of three independent experiments in triplicate. The difference between the control cells and the DdPLC_{sense} overexpressing cells is significant, with $p < 0.02$.

	Phospholipase C activity			<i>Ins(1,4,5)P₃</i> levels		
	DdBS18	DdPLC _{sense} bulk	DdPLC _{sense} 12	DdBS18	DdPLC _{sense} bulk	DdPLC _{sense} 12
		pmol/min $\times 10^6$ cells			pmol/10 ⁶ cells	
Exp. I	7.67 \pm 2.70	32.08 \pm 13.08	188.42 \pm 56.99	1.28 \pm 0.26	10.83 \pm 2.00	10.44 \pm 4.31
II	7.42 \pm 2.44	35.00 \pm 7.43	101.08 \pm 15.55	1.28 \pm 0.35	2.42 \pm 0.20	3.44 \pm 0.31
III	18.17 \pm 4.48	47.00 \pm 20.73	86.25 \pm 23.35	0.92 \pm 0.54	3.25 \pm 0.53	5.69 \pm 0.55

to the 5' end of each oligonucleotide.

Genomic DNA from *D. discoideum* strain NC-4 was isolated as described by Nellen *et al.* (25). A 30-cycle PCR was used for DNA amplification with a 1-min denaturing step at 94 °C and a 3-min annealing and extension step at 40 or 50 °C. Reactions were performed in 50 mM KCl, 10 mM Tris-HCl, pH 8.0, 4 mM MgCl₂, 60 $\mu\text{g}/\text{ml}$ bovine serum albumin, 0.2 mM dNTPs, 100 μM concentrations of 5' and 3' PCR primer, 10 ng of genomic DNA, and 2.5 units of Taq DNA polymerase (Promega Corp.) in a total volume of 100 μl . The amplified DNA was precipitated with ethanol and digested with the appropriate restriction enzymes. PCR products of the expected length were excised from low melting point agarose gel and purified. The fragments were ligated into compatible pGEM-7ZF(+) vector (Promega Corp.) and sequenced according to the dideoxy chain termination method (26). One of these clones, named PCR240bp, was used to screen a cDNA library.

cDNA Screen and Sequencing—A $\lambda\text{gt}11$ library prepared from 6-h developed *D. discoideum* cells was provided by P. Devreotes, Baltimore. The PLC-like clone PCR240bp was used as a probe to screen the library. The cDNA insert isolated in the first screen was used to screen the same library a second time to obtain the complete sequence.

Filters were prehybridized at 65 °C in 1% bovine serum albumin, 0.5 M NaH₂PO₄, 5 mM EDTA, 7% SDS and hybridized in the same solution containing the probe. Probes were ³²P labeled using the random priming method. Filters were washed for 30 min in 5% SDS, 1% bovine serum albumin, 5 mM EDTA at 65 °C and subsequently twice in 1% SDS, 1% bovine serum albumin, 5 mM EDTA. Phage DNA was isolated by elution over Qiagen columns (Qiagen Inc). The inserts were excised with *EcoRI* or *KpnI/SacI* double digestions and ligated into pGEM-7 vector for sequencing. Both strands of the cDNA were sequenced using overlapping clones and subcloning of restriction fragments.

DNA and RNA Analysis—Total RNA isolated at 2-h intervals from a 28-h developmental program of *D. discoideum* NC-4 cells was a gift from P. Schaap, Leiden (27). The Northern blots were probed with the clone PCR240bp at 42 °C in 50% formamide, 5 \times Denhardt's solution, 1% SDS, 5 \times SSPE (75 mM NaCl, 50 mM NaH₂PO₄, 5 mM EDTA, pH 7.4). Washing was performed at the same temperature for 30 min in 5 \times SSPE, 1% SDS. DdPLC transcript was quantitated using cross-hybridization with ribosomal RNA as an internal standard.

Genomic DNA from NC-4 cells was digested with a number of restriction enzymes. One μg of digested DNA was added per gel lane for electrophoresis on 1% agarose gels. Hybridizations were performed at 42 °C in 50% formamide, 6 \times SSC (0.9 M NaCl, 0.09 M sodium citrate), 10% dextran sulfate, 1 \times Denhardt's, 1% SDS, 20 mM Tris-HCl, pH 7.6. The blots were washed at 50 °C in 6 \times SSC, 0.5% SDS. Two of the isolated cDNA clones were used as probes.

Transformation of Dictyostelium with DdPLC—The cDNA clones L9 and L15 in the pGEM-7 vector were combined to obtain a construct containing the full-length DdPLC sequence. Both clones were digested with restriction enzyme *NcoI*. The 3' PLC fragment after the *NcoI* restriction site of clone L9 was replaced by the 3' PLC sequence of clone L15. To facilitate further cloning the *EcoRI/SacI* fragment containing full-length DdPLC was ligated with the *EcoRI/HindIII*-digested Bluescript SK(-) vector (Stratagene), so that the DdPLC sequence was flanked by two *BamHI* restriction enzyme sites. As this clone does not contain the internal *EcoRI* site because of a 2-base pair deletion in clone L15, the mutated sequence was converted by oligonucleotide-directed mutagenesis (28). The restored full-length

DdPLC cDNA construct was digested with *BamHI* and ligated with the *BglII*-restricted BS18 *Dictyostelium* expression vector (29, provided by R. Firtel, San Diego). The sense orientation of DdPLC relative to the actin 15 promoter was determined by partial dideoxy sequence analysis and used for further expression studies. A calcium phosphate precipitate of this construct was used to transform *Dictyostelium* strain AX3 cells (30). Control cells were transformed with the BS18 vector lacking the DdPLC insert. The cells were selected in modified HL5 medium (31, with 10 g of D-glucose/liter of medium) containing G418 at 10 $\mu\text{g}/\text{ml}$.

Measurements of Basal *Ins(1,4,5)P₃* Levels and PLC Activity—Vegetative *Dictyostelium* cells were washed and resuspended in 40 mM Hepes/NaOH, pH 6.6, 5.9 mM EGTA. The cells were rapidly lysed through a Nucleopore polycarbonate filter (pore size 3 μm). Basal *Ins(1,4,5)P₃* levels were determined in samples treated with an equal volume of perchloric acid (3.5%, v/v) directly after lysis. Phospholipase C activity was determined as the amount of *Ins(1,4,5)P₃* produced during a 20-s incubation after the addition of 6.9 mM CaCl₂, as described (32). *Ins(1,4,5)P₃* levels were measured in the neutralized lysate by isotope dilution assay (33).

RESULTS

Amplification of a Genomic *D. discoideum* PLC Fragment—The highly conserved amino acid sequence regions within the different PLC isoenzyme forms from rat, bovine, and *Drosophila* were used to design primers to isolate a new PLC gene from *D. discoideum*. The sense primers PLC5'A and PLC5'B correspond to amino acid regions ELDCW and HNTYL, whereas antisense primers PLC3'A, PLC3'B, and PLC3'C correspond to amino acid regions PYPVIL, ELDCW, and YPVIL, respectively.

PCR using primer combinations PLC5'A + PLC3'A, PLC5'B + PLC3'C, PLC5'B + PLC3'B, or PLC5'A + PLC3'C with annealing and extension reactions at 40 °C resulted in products of no distinct size but smears between 100 and 400 bp. PCR with annealing and extension reactions at 50 °C gave smears when using primers PLC5'B + PLC3'B, or PLC5'A + PLC3'C. However, a fragment of the expected size of about 240 bp was obtained with primers PLC5'B + PLC3'C.

After restriction enzyme digestion the fragment of 240 bp was ligated into the sequencing vector. Eight clones containing the 240-bp PCR product were sequenced. Of these, three clones were different from each other and did not contain a PLC-like structure. The other five clones were nearly identical and contained a PLC-like sequence as deduced from their putative amino acid sequence. One clone showed a 1-base substitution, probably caused by an error during PCR. The clone used in further studies was named PCR240bp.

Cloning and Sequence Analysis of Phospholipase C cDNA—The PLC-like insert from clone PCR240bp was used as a ³²P-labeled probe to screen a $\lambda\text{gt}11$ cDNA library. Two hybridizing clones of about 1.6 kb were isolated from 100,000 plaque-forming units and shown to be identical by restriction enzyme

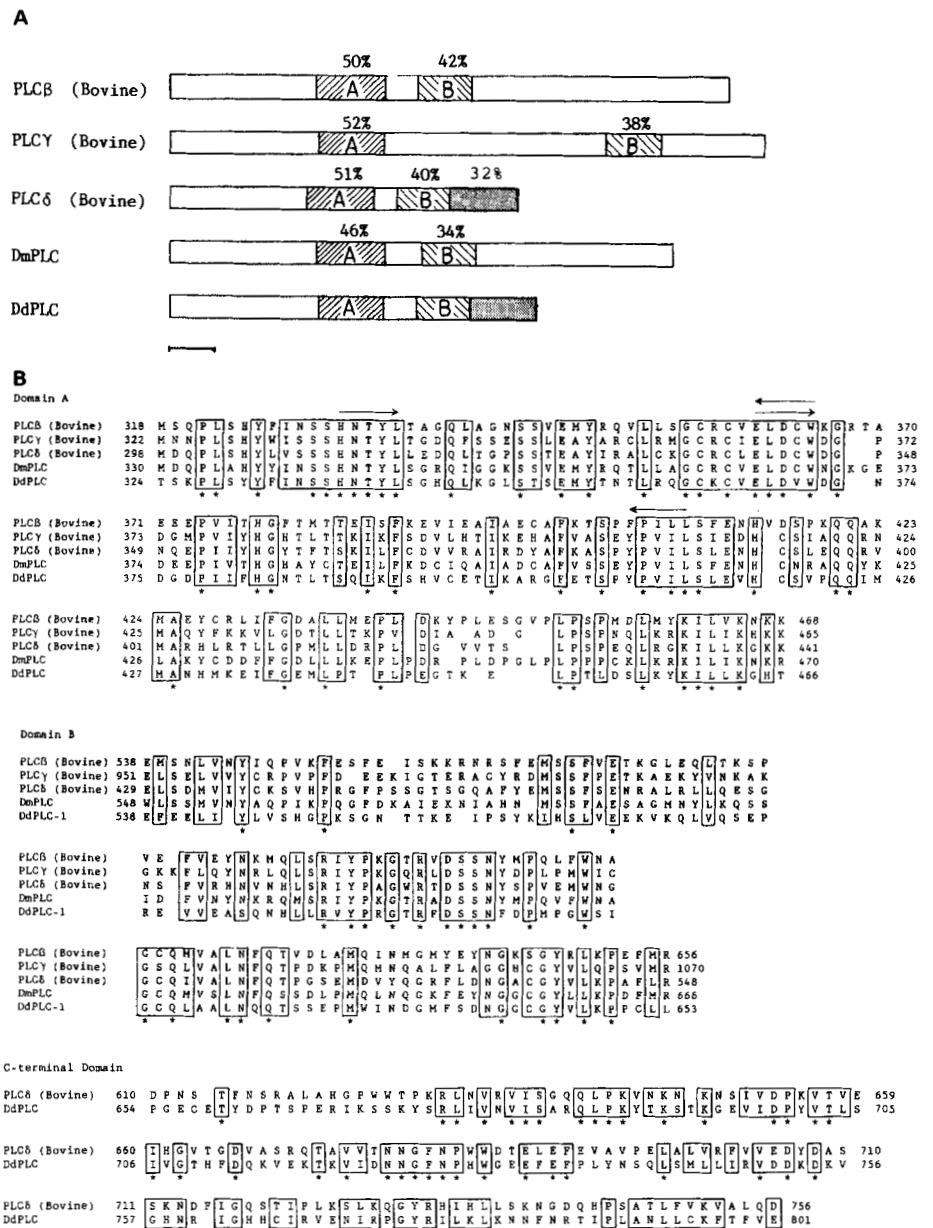


FIG. 5. Comparison of the primary structures of phospholipase C isoenzymes from bovine, *Drosophila norPA* (*Dm*), and *Dictyostelium* (*Dd*). *A*, the locations of the two conserved regions found in phosphoinositide-specific PLCs are denoted by boxes *A* and *B*. The numbers above each box refer to the percentage of identity in comparison with *Dictyostelium* PLC. The length of the bar represents 100 amino acids. *B*, amino acid sequence comparison of DdPLC with the conserved domains *A* and *B* of bovine, *Drosophila*, and *Dictyostelium* PLC, and with the C-terminal domain of bovine PLC- δ . Highly conserved regions in all of the sequences are boxed, and identical residues are indicated by an asterisk. Arrows indicate the sequences used for designing PCR primers. Sequence data were obtained from the Swiss-Prot Sequence Database. Note: the N-terminal sequence of bovine PLC- δ has not been described; amino acid numbers refer to those of the nearly identical rat PLC- δ .

analysis and end sequencing. One clone, named L1.5, was sequenced entirely. It contained a 1,570-bp-long open reading frame encoding the conserved A domain and part of the B domain. As probe PCR240bp hybridized to a \approx 3-kb mRNA in Northern analysis, L1.5 was used as a probe to screen the library a second time to obtain the complete sequence. In total one million plaque-forming units were screened, and 21 hybridizing clones were identified.

In Fig. 1A the cDNA clones are shown which were used to sequence the complete DdPLC cDNA sequence. Unfortunately, no full-length cDNA clone could be isolated. L15 does not contain the internal *EcoRI* restriction enzyme site because of a 2-bp deletion. The overlapping clones form a 2,788-bp cDNA sequence.

The nucleotide and deduced amino acid sequences of DdPLC are presented in Fig. 1B. The sequence contains an open reading frame of 2,403 nucleotides following the first ATG. This putative initiation codon at position 289 is flanked by a sequence that fits Kozak's criteria for a translation initiation codon (34). The next ATG at position 323 does not

comply to Kozak's rule and is followed by an in-frame stop codon at position 337. The long open reading frame encodes a protein of 801 amino acids and has a predicted molecular mass of 91 kDa.

DdPLC RNA Expression—DdPLC gene expression was analyzed to determine whether transcription is developmentally regulated. Northern blots of RNA from a time course of development were hybridized with probe PCR240bp. Fig. 2 demonstrates that the 2.8-kb mRNA is present at all stages of development. After 2 h of starvation DdPLC is expressed at a 2-fold higher level than in vegetative cells. In the aggregating cells and during tip formation the levels are comparable with vegetative levels. Transcription is markedly decreased in the migrating slug. In the culminating fruiting body DdPLC levels are again increased. (The dip in expression at 6 h appears to be an artifact of the RNA isolation at this time point. Expression of a number of developmentally regulated genes using the RNA from this time course of development was also reduced at 6 h, as shown by Peters *et al.* (27).)

Southern Analysis—Genomic *Dictyostelium* DNA was di-

gested with restriction enzymes *EcoRI* and *NcoI* and analyzed by Southern hybridization. cDNA clone L1.5 and a 600-bp fragment 5' from the *EcoRI* site of clone C1 were used as probes (Fig. 3, A and B). As L1.5 contains a *NcoI* site but no *EcoRI* site, hybridization with L1.5 showed two bands for DNA digested with *NcoI* and a single band for *EcoRI*-digested DNA. The lengths of the two bands found in the *EcoRI/NcoI* double digestion together correspond to the length of the band in the *EcoRI* digestion. Hybridization with the 600-bp fragment from clone C1 gave single bands in both the *EcoRI*- and *NcoI*-digested genomic DNA as expected. These results indicate that DdPLC is a single copy gene.

Expression of *Dictyostelium* Phospholipase C—A complete DdPLC cDNA sequence was constructed and inserted into the BS18 *Dictyostelium* expression vector in the sense orientation. AX3 cells transformed with this construct or with a control vector (BS18) were assessed for DdPLC RNA expression, basal $\text{Ins}(1,4,5)\text{P}_3$ levels, and PLC activity. Measurements were done on mass cultured cells (DdPLCsense bulk) and on one clone (DdPLCsense 12) and were compared with control cells (DdBS18). Northern blot analysis revealed a substantially higher expression of the DdPLC messenger in the DdPLCsense-transformed cells (Fig. 4). The PLC activity in the DdPLCsense bulk cells showed a 4-fold increase, whereas in the clonal line the activity was increased up to 20-fold (Table I). Our results show that overexpression of DdPLC leads to a 2–7-fold increase in basal $\text{Ins}(1,4,5)\text{P}_3$ levels (Table I). The difference between the control cells and the DdPLCsense overexpressing cells is significant, with $p < 0.02$. Overexpression of PLC and the resulting increase in basal $\text{Ins}(1,4,5)\text{P}_3$ levels did not influence growth and development in *Dictyostelium*.

DISCUSSION

In this paper we report the cloning of a PLC isoform from *D. discoideum* and overexpression of this PLC in *Dictyostelium* cells. The conserved amino acid sequences of previously identified PLC isoforms from other species were used to design PCR primers. Primers designed from the highly conserved regions HNTYL and YPVIL in domain A were successful in amplifying a PLC-like fragment of the expected size. *Dictyostelium* PCR240bp encodes many of the highly conserved amino acids in the same location as the other phosphoinositide-specific PLC enzymes. However, the cysteine in the sequence ELDCW, which was used to design a primer in other PCR experiments, was substituted for a valine in DdPLC.

As mammalian cells are known to contain more than one PLC isoform, we searched for other possible PLC genes in *Dictyostelium* by PCR and Southern analysis. PCR with primers designed in the regions HNTYL and YPVIL gave one unique PLC-like sequence in addition to a number of unrelated sequences. No other PLC-like sequences were found in the PCR product. Reactions using other primer combinations did not yield products of distinct sizes under the conditions tested. Southern blots hybridized under reduced stringency conditions (30% formamide at 42 °C) with the PLC sequence obtained by PCR did not show cross-reacting bands. In hybridization solutions containing 10% formamide many weakly hybridizing fragments were observed in addition to the strongly hybridizing DdPLC bands. Therefore, we have no indication for other PLC genes in *Dictyostelium*.

The deduced amino acid sequence of the cloned *Dictyostelium* PLC cDNA shows many characteristics of the PLC sequences from vertebrates and *Drosophila* (Fig. 5A). All PLC sequences contain two conserved A and B domains of about 140 and 120 amino acids, respectively. The different PLC isoforms, including DdPLC, show about 50 and 40% sequence

identities in these domains. This suggests that vertebrate PLC β , γ , and δ have about the same evolutionary distance to each other as to *Dictyostelium* with respect to these two conserved domains. The N-terminal part of the proteins up to the A domain shows very little sequence identity but has a nearly identical length of 298–330 amino acids. The region of the PLC proteins between the A and B domain is variable in both sequence and length. DdPLC does not contain regions similar to the nonreceptor tyrosine kinases of the *src* family as found in the PLC γ isoforms. A search for potential biologically significant sites in this region using the PC/Gene program (IntelliGenetics/Genofit) revealed a putative protein kinase C phosphorylation site at position 524, a putative cAMP/cGMP-dependent kinase phosphorylation site at position 531, and an EF-hand calcium binding domain between positions 490 and 502. Finally, the C-terminal part of PLC proteins after the B domain can vary from 147 (PLC- δ) to 560 amino acids (PLC- β). The C-terminal part of DdPLC shows significant homology in length and sequence only with PLC- δ (see Fig. 5B).

In this study we show that overexpressing DdPLC in *Dictyostelium* results in an increase in both basal $\text{Ins}(1,4,5)\text{P}_3$ levels and PLC activity, demonstrating that the cloned sequence encodes a PLC enzyme. The increase in PLC activity does not appear to influence growth or development as compared with control cells. From studies in mammalian systems it is known that overexpression of endogenous PLC- γ results in an increased $\text{Ins}(1,4,5)\text{P}_3$ production in response to platelet-derived growth factor. However, this did not alter intracellular calcium signaling or induce DNA synthesis (35).

The development of efficient gene targeting systems in *Dictyostelium* has made it possible to use the cloned cDNA to study nonlethal mutations (30). By disrupting the DdPLC gene we hope to understand the role of this PLC isoform in the signal transduction pathway and its effect on chemotaxis and cell development.

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