A New Phospholipase C δ4 Is Induced at S-phase of the Cell Cycle and Appears in the Nucleus*

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To discover a new phospholipase C (PLC) related to cell growth, we screened a cDNA library prepared from regenerating rat liver. A novel PLC (PLC δ4) encoding a polypeptide of 770 amino acids with structural similarity to PLC δ-type isozymes was isolated. PLC δ4 mRNA is expressed more remarkably in regenerating liver than in normal resting liver. It is also distributed abundantly in tumor cells such as hepatoma and src-transformed cells. Furthermore, its expression can be induced markedly by serum treatment and reaches a maximum at 8 h. Western blot analysis and immunocytochemical staining showed that PLC δ4 is dominantly present in nucleus. Nuclear PLC δ4 dramatically increases at the transition from G1- to S-phase, and the high content continues to the end of M-phase. PLC δ4 almost disappears when cells re-enter the next G1-phase. On the other hand, the contents of PLC β1, PLC γ1, and PLC δ1 do not change significantly during the cell cycle. These results suggest that PLC δ4 is expressed in nucleus in response to mitogenic stimulation and plays important roles in cell growth as one of the early genes expressed during the transition from G1- to S-phase in the cell cycle.

Phospholipase C (PLC) catalyzes a critical step in the signaling systems of a variety of physiological stimuli through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate and diacylglycerol, both of which act in cells as second messengers. Inositol 1,4,5-trisphosphate activates protein kinase C, leading to various cellular responses (Berridge and Irvine, 1984; Majerus, 1992; Nishizuka, 1984). These events seem to regulate not only short-term cellular responses but also longer-term responses such as cell growth and differentiation (Berridge and Irvine, 1989). So far, nine PLC CDNs have been cloned from mammalian cells and can be divided into three types, β, γ, and δ, on the basis of their structures (Rhee et al., 1989; Kritz et al., 1991; Lee et al., 1993; Jhon et al., 1993). The existence of multiple forms of PLC suggests that each isozyme may differ in tissue distribution, intracellular location, regulatory mechanism, and further downstream function. In fact, it has been shown that each PLC distributes differently and couples to different signaling systems. The β-type isozymes are activated by GTP binding proteins such as Gxi and the βγ-subunits of G proteins (Taylor et al., 1991; Smrcka et al., 1991; Camp et al., 1992; Katz et al., 1992). On the other hand, γ-type isozymes have been shown to be activated through receptor activation encoding tyrosine kinases (Wahl et al., 1989; Meisenhelder et al., 1989; Margolis et al., 1989; Morrison et al., 1990; Kim et al., 1991). However, the regulatory mechanisms of PLC δ isozymes remain unclear.

Recently, polyphosphoinositide turnover has been demonstrated to occur in the nucleus as well as in the plasma membrane during cell growth and differentiation (Cocco et al., 1987, 1989; York and Majerus, 1994; Divecha et al., 1989). Moreover, much evidence has shown that a PIP2-hydrolyzing activity exists in the internal nuclear matrix and that PIP2 synthesis activity is present in the envelope-depleted nucleus (Divecha et al., 1991; Payrastre et al., 1992; Capitani et al., 1990). These results suggest the involvement of PLC in nuclear function, probably playing a key role in gene expression and DNA synthesis. The signaling mechanisms of the receptor-mediated activation of PLC, which takes place around plasma membranes, are well established, at least for β-type and γ-type isozymes. Concerning PLCs in the nucleus, PLC β1 has been shown to be present in the nuclei of Swiss 3T3 cells, in which polyphosphoinositides are hydrolyzed quickly in response to insulin growth factor-1 (IGF-1), although there is no direct evidence that the breakdown is induced by nuclear PLC β1 (Martelli et al., 1992). More recently, Asano et al. (1994) have presented evidence that there is a new PLC in the nucleus. They purified a nuclear PLC from rat ascites hepatoma cells (AH 7974). The PLC isozyme was only detected in the nuclei of regenerating liver, not in adult resting liver cells.

Independently, we have been trying to isolate a new PLC cDNA clone expressed dominantly in growing cells and present in the nucleus using a cDNA library prepared from regenerating liver.

Here, we report the molecular cloning of a novel PLC, PLC δ4, which localizes in nuclei, and present evidence that the expression of PLC δ4 is very high in growing cells and induced in S-phase during the cell cycle.

EXPERIMENTAL PROCEDURES

Materials and Cells—Phosphatidyl[2-3H]inositol 4,5-bisphosphate (173.9 MBq/mmol) was purchased from DuPont NEN. Phosphatidylinositol 4,5-bisphosphate was prepared by the method of Schacht (1978), [γ-32P]Deoxyctidine triphosphate (dCTP) was from Amer sham Corp. Anti-PLC γ1 and δ1 antibodies were produced by the methods described before (Homma et al., 1990). Anti-PLC β1 antibody was from UBI.

Reverse transcriptases were from Life Technologies, Inc. Rat 3-Y1, Src/
356 Nuclear Phospholipase C 64

3Y1, RLH 84, RH 34, and Swiss 3T3 cells were from the Japanese Cancer Research Resources Bank. They were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Isolation of mRNA from Regenerating Rat Liver—Regenerating livers were prepared from partially hepatectomized rats (Donryu strain) 24 h after the removal of approximately two-thirds of liver mass according to the method of Higgins and Anderson (1931). The poly(A) RNA was isolated by the guanidine method (Han et al., 1987) and oligopexid (dT) 30 (Roche, Japan) apan.

Reverse Transcriptase PCR—A total of 6 µg of RNA from regenerating livers was used in 120 µl of the olig(dT) 3 primed reverse transcription reaction mixture. The cDNA products were used as a source of PCR amplification. Three sense primers responding to the highly conserved X domain sequences (R/K/I)(L/I/V/I)(V/K/N/H/G)KK of t-ψ, and t-type PLCs, respectively, and one antisense primer responding to the Y domain DSSNY(M/D/S/N)P were synthesized.

The underlined sequences of the primers were as follows: 5'—GACAAGCTTATTG/C/CTTTTG/A(T/C)GA/AA(A/G)TA/G/GAA-3' (P-Xb), 5'—GACAAGCTTAAGATC/T(G/A)CT/GAT/CTA/AA(G/A)GA/AA-3' (P-Xa), 5'—GACAAGCTTAAAGATC/T(T/G)CT/GAT/CTA/AA(A/G)GA/AA-3' (P-Xc), and 5'—ATGGGATCCIGG(C/G)(A/T)(C/T)(A/G)TC-3' (P-Xd).

Three nucleotide sites determined by the autocycle sequencing method using an A.L.F. DNA sequencer (Pharmacia Biotech Inc.). The 420-bp PCR product was further subcloned into pBlue vector (Bethesda Research Laboratories). The overexpressed GST-PLC 4 cDNA fragment was sequenced by the dideoxy chain-termination method with [32P]dCTP labeling.

Construction of a Regenerating Rat Liver cDNA Library—Oligo(dT) 3 primed cDNA from regenerating rat liver was synthesized using a λ-ZAP cDNA synthesis kit (Stratagene), and the different sizes were fractionated on 10% agarose gel. Purification was done with Geneclean II (Bio 101, Inc.). The cDNA >1.2 kb was cloned into Uni-ZAP vector. The original library contained over 3.6 x 10 6 independent recombinant clones.

Library Screening—[32P]dCTP-labeled probes were prepared from a 420-bp PCR product by a random priming procedure and used to screen the regenerating rat liver cDNA library. Among 8 x 10 6 clones screened, a positive bacteriophage containing a 2.7-kb insert was isolated. The insert was cloned into pBlueScript II vector (Pharmacia Biotech Inc.). The 420-bp clone coding a new PLC DNA was expressed in JM109 (Pharmacia Biotech Inc.) as a fusion protein with a glutathione-Sepharose column.

Production and Purification of Polyclonal Anti-PLC 4 Antibody—The 420-bp cDNA fragment (Bgl II-Xho II) was constructed into pGEX-3X expression vector. The overexpressed GST-PLC 4 fusion proteins were used as the source of antigen. The fused proteins were lysed with a lysis buffer composed of 40 mM Tris/HCl (pH 7.6), 5 mM EDTA, 0.1 mM PMSF, 0.1 mM diisopropyl fluorophosphate, 1% Triton, 0.5% deoxycholate, and 0.1% SDS. The lysates were applied to a glutathione-Sepharose column and eluted with 50 mM Tris/HCl (pH 8.0) and 50 mM glutathione. To obtain the pure antigen, disk electrophoresis (Nihon Eido NA-1800) was further carried out. The fractions at 44 kDa were collected and lyophilized. The antigen (200 µg) was mixed with complete Freund's adjuvant (Difco) for the first and second immunizations and with incomplete Freund's adjuvant (Difco) for the third and fourth immunizations.

The mixture was agitated by gentle shaking at 4 °C for 30 min. The band was washed three times with PBS, and the antibody was eluted from the membranes with 0.1 M glycine-HCl (pH 2.7) and neutralized immediately with 1.0 M Tris/HCl (pH 9.0).

Preparation of Cell Fractions and Western Blotting—Nuclear and cytoplasmic fractions of Swiss 3T3 cells were prepared as described by Divecha et al. (1991). Swiss 3T3 cells were harvested with 0.2% trypsin and washed twice with cold PBS. The cells were suspended in buffer A (20 mM Tris/HCl (pH 7.8), 1% Nonidet P-40, 10 mM (β-mercaptoethanol, 0.5 mM PMSF, 1 µM aprotinin and leupeptin) for 2 min on ice. An equal volume of distilled H 2 O was added, and the cells were allowed to swell for 2 min. The cells were sheared by ten passages through a 22-gauge needle. The nuclei were recovered by centrifugation and washed once with buffer B (10 mM Tris/HCl (pH 7.4), 2 mM MgCl 2, 0.5 mM PMSF, 1 µM aprotinin and leupeptin) for 20 min, percollashed with 0.2% Triton X-100 in PBS for 5 min, and washed with PBS. The cells were incubated with 1:250 diluted anti-PLC 4 antibody and stained with alkaline phosphatase-conjugated anti-rabbit IgG.

Immunofluorescent Staining—Swiss 3T3 cells were cultured on glass coverslips. The cells were serum-starved for 3 h in Dulbecco's modified Eagle's medium containing 1 mg/ml bovine serum albumin, 5 µM insulin, 5 µM transferrin, and sodium selenite (5 ng/ml) and then stimulated by adding 10% fetal calf serum for the indicated times. The cells were fixed with 3% PFA for 20 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and washed with PBS. The cells were incubated with 1:250 diluted anti-PLC 4 antibody for 90 min and washed three times with PBS. Then, they were incubated for 40 min with 1:40 diluted secondary antibody conjugated to fluorescein isothiocyanate and washed three times. The stained cells were observed by Axioskop (Zeiss, Germany).

RESULTS

Isolation of PLC 4 cDNA Clones—It is very important to select a good gene pool containing many high copies of the gene to be searched. Regenerating rat liver was chosen as the gene source for this research because it was thought to provide a good model for elucidating the relationship between cell proliferation and polyphosphoinositide turnover in the nucleus. We...
isolated mRNA from regenerating rat liver 24 h after partial hepatectomy and carried out RT-PCR. The primers were designed according to the highly conserved amino acid sequence in the X and Y domains with consideration given to the codon usage and notable amino acid similarity among PLC isozymes. We obtained several products. Among them, a 420-bp PCR product contained a PLC-like sequence distinct from those of known PLC isozymes. Therefore, we screened the regenerating rat liver cDNA library with the 420-bp PCR product and obtained one positive clone, named 7A. Clone 7A had a 2.7-kb insert. Complete sequencing of clone 7A revealed an open reading frame of 2310 bp surrounded by 135 bp of 5'-noncoding sequence and 102 bp of 3'-noncoding sequence (Fig. 1). The initiation methionine was designated at position 136 because there was an in-frame stop codon upstream of this methionine; comparing the sequence with known PLCs further supports this designation. The 3'-noncoding region contained a poly(A) tail, but no polyadenylation addition signal was found. The deduced amino acid sequence of clone 7A revealed an overall structure similar to the PLCδ type. Thus, the protein encoded by the 7A clone was named PLCδ4. PLCδ4 consists of 770 amino acids with a calculated molecular size of 85 kDa. The amino acid sequence of PLCδ4 is more similar to that of PLCδ2 than to PLCδ1 or PLCδ3, especially in the region extending from the amino-terminal to the beginning of the X domain (Fig. 2). Both PLCδ4 and PLCδ2 have possible nuclear translocation signal peptide sequences near the amino-terminal. This probably indicates a functional similarity between PLCδ4 and PLCδ2. A PH domain and an EF-hand-like domain are also included in PLCδ4.

To confirm that clone 7A encodes an inositol phospholipid-specific PLC, the whole coding region of clone 7A was subcloned into pGEX-3X expression vector and overexpressed in E. coli. The soluble GST-PLCδ4 fusion proteins were purified on a glutathione-Sepharose column. GST-PLCδ4 showed PIP2-hydrolyzing activity. The specific activity of GST-PLCδ4 was found to be 1.6 μmol/min/mg protein, indicating that it was a PLC isozyme.

Distribution of PLCδ4 and Its Expression in Growing Cells—Northern hybridization was carried out to detect the distribution of PLCδ4 and PLCδ2, and PLCδ3. Open boxes Y and X denote the conserved sequences found in all mammalian PLCs. The numbers above each box refer to the first and last amino acid. The extent of sequence identity to the corresponding region of PLCδ4 is indicated by the percentages.
in normal liver and regenerating liver. The results are shown in Fig. 3a. PLC δ4 was expressed abundantly in regenerating liver, while it was only slightly detected in normal liver. The expression was also very high in intestine and moderately high in thymus. Since the cells in both intestine and regenerating liver have high proliferating activities, it is reasonable to suppose that PLC δ4 is related to cell growth and cell proliferation.

To elucidate whether this is a general phenomenon or just specific to regenerating liver and intestine, we investigated the expression of PLCδ4 in four cell lines, RL-34, RHl-84, 3Y1, and Src3Y1 (Fig. 3b). RL-34 is a rat hepatocyte cell line, RHl-84 is a rat hepatoma cell line, and Src3Y1 is src-transformed 3Y1 cells. When comparisons were made between RL-34 and RHl-84 and between 3Y1 and Src3Y1, we found that PLC δ4 is expressed more vigorously in hepatoma (RHl-84) and src-transformed cells (Src3Y1). These data clearly show that PLC δ4 is closely related to cell proliferation.

Serum Induction of PLC δ4—Swiss 3T3 cells were starved in serum-free medium for 30 h and then stimulated by adding 10% fetal calf serum. After serum stimulation for the indicated times, changes in PLC δ4 mRNA levels were determined by RT-PCR. Fig. 3c shows the effect of serum on the expression of PLC δ4. PLC δ4 was expressed at low levels when the cells were starved. The expression of PLC δ4 mRNA began to increase 4 h after serum stimulation and reached a maximum at 8 h. After that, PLC δ4 mRNA levels decreased gradually until 20 h. We also examined the time course of the changes in PLC δ4 content during the cell cycle by Western blotting. We prepared both nuclear and cytosol fractions from Swiss 3T3 cells according to the methods described by Divecha et al. (1991). The anti-PLC δ4 antibody used for Western blotting was affinity purified, and only a single band appeared at the expected molecular weight when it was used to blot cell lysates. To check whether the nuclear fraction was contaminated by cytosol proteins, we examined for actin contamination as a control. The results are shown in Fig. 4. PLC δ4 appeared mostly in the nuclear fraction, while PLC γ1 and δ1 were in the cytosol. PLC β1 was detected in the nucleus. Because actin was not detected in the nuclear fraction, contamination from the cytosol fraction could be neglected. Among the PLCs examined (PLC β1, γ1, δ1, and δ4), only the content of PLC δ4 changed depending on the phase of the cell cycle. PLC δ4 was only slightly detected at G0-phase and early G1-phase (0 and 6 h). Then, the level was elevated at 12 h and climbed further reaching a maximum at 16 h. The high level continued during S-/M-phase (until 24 h). When the cells entered the next G1-phase (28 h), the PLC δ4 content decreased dramatically. On the other hand, the level of other PLCs remained stable during the cell cycle. These results imply that PLC δ4 is probably one of the early genes expressed during the transition from G1- to S-phase and functions during the S- and M-phases.

Localization of PLC δ4 in Swiss 3T3 Cells—To confirm the localization of PLC δ4 in Swiss 3T3 cells, cells in various stages of the cell cycle were stained for immunofluorescent microscopy using an anti-PLC δ4 antibody (Fig. 5). In resting cells (0 h), immunofluorescent density was very slight. However, at 8 h after serum stimulation, the nuclear area began to be stained, and the density increased up to 16 h while the cytosolic area was not stained. This strong staining in the nucleus continued to the end of M-phase. PLC δ4 in the nuclear areas almost disappeared at 28 h and began to increase again when cells re-entered the next S-phase (36 h, data not shown). All these data show that PLC δ4 is expressed during S-/M-phase and is located in nuclei.

**DISCUSSION**

We isolated PLC δ4 from regenerating rat liver. This PLC δ4 not only shows nuclear localization but also a strong correlation with cell proliferation. It is highly expressed in regenerating liver but was detected at low levels in normal liver. When we
investigated the distribution of PLC\textsubscript{4}, we found that it is not restricted to the regenerating liver but is also expressed strongly in intestine, hepatoma, and src-transformed 3Y1 cells. These data indicate that the distribution of PLC\textsubscript{4} is quite different from that of other known PLCs. It appears to be expressed selectively in cells with high proliferating activity.

A variety of data have shown that polyphosphoinositide turnover also occurs in the nucleus and changes dynamically during cell proliferation and differentiation (Cocco et al., 1987, 1989; York Majorus, 1994; Divecha et al., 1989, 1991). However, no direct evidence has been provided, although many researchers have tried to find a PLC isozyme responsible for polyphosphoinositide turnover in the nucleus. Recently, Martelli et al. (1992) reported that PLC\textsubscript{4} is present in the nuclei of Swiss 3T3 cells and may be responsible for the breakdown of polyphosphoinositide in the nucleus in response to IGF-1. In this case, PLC\textsubscript{4} is activated immediately after IGF-1 stimulation, and the activity returns to normal within 30 min. Therefore, it seems unlikely that PLC\textsubscript{4} plays an important role in events during S-phase and later stages of the cell cycle. PLC\textsubscript{4} behaves differently from PLC\textsubscript{1} since its expression is induced by mitogens and its level reaches a maximum at S-phase. During the cell cycle, the levels of PLC\textsubscript{1} and PLC\textsubscript{4} which are mostly located in the cytosol, did not change. Similarly, PLC\textsubscript{4} levels did not change significantly, although it is present in the nucleus.

PLC\textsubscript{4} is highly homologous to PLC\textsubscript{2} with 69% identity in the amino acid sequence (Meldrum et al., 1989, 1991). However, the functions and distribution of PLC\textsubscript{2} have not been investigated yet. PLC\textsubscript{2} may play a similar role to PLC\textsubscript{4}.

The increased hydrolysis of nuclear polyphosphoinositides during S-phase of the cell cycle has been observed in regenerating liver (Kuriki et al. 1992) and HeLa cells (York and Majorus, 1994), while levels of cytoplasmic polyphosphoinositides remain constant in HeLa cells. There are at least three possible explanations for the increase in nuclear PLC activity. First, PLC in nuclei may be activated by some signaling molecules. This may be the case for PLC\textsubscript{1} activation upon treatment with IGF-1. Second, translocation of PLC from the cytosol to nucleus may occur in response to stimuli. Finally, a certain PLC isozyme may be newly synthesized in response to stimuli. Our results on time course changes of PLC\textsubscript{4} suggest that the last event probably occurs in cells because the expression of PLC\textsubscript{4} increases dramatically at S-phase.

It has been reported that the treatment of nuclear matrix with PLC results in a release of nucleic acid, implying that inositol phospholipids are responsible for the hydrophobic interaction between nuclear matrix and nucleic acids (Cocco et al., 1980). Therefore, the dramatic increase in PLC\textsubscript{4} at S-phase may influence the interaction between nuclear matrix and nucleic acids. It is also likely that PLC\textsubscript{4} may activate a certain protein kinase C through diacylglycerol formation in the nucleus, causing the phosphorylation of lamin and the certain protein kinase C through diacylglycerol formation in the nucleus, causing the phosphorylation of lamin and the activation of DNA polymerase and topoisomerase leading to cell proliferation (Divecha et al., 1989). Since PDGF stimulation of NIH 3T3 cells (Fields et al., 1990) and IGF-1 stimulation of Swiss 3T3 cells (Divecha et al., 1991) induce the translocation of protein kinase C from the cytosol to nucleus, translocated protein kinase C rather than pre-existing protein kinase C may play a crucial role in the nucleus. Furthermore, Sylvia et al. (1988) provided direct evidence that a hydrolyzed product of polyphosphoinositides, inositol 1,4-bisphosphate, in the nucleus could directly regulate DNA synthesis through an in-

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**Fig. 4. Changes in PLC\textsubscript{4} content during the cell cycle.** Swiss 3T3 cells were cultured in serum-free medium for 30 h and then stimulated with serum for various periods. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-PLC\textsubscript{4} antibody (a), anti-PLC\textsubscript{1} antibody (b), anti-PLC\textsubscript{1} antibody (c), anti-PLC\textsubscript{1} antibody (d), or anti-actin antibody (e). Column C refers to the nuclear fraction, and column N refers to the cytosol fraction.

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**Fig. 5. Immunofluorescent staining of serum-stimulated Swiss 3T3 cells with anti-PLC\textsubscript{4} antibody.** Serum-starved Swiss 3T3 cells were stimulated by adding 10% fetal calf serum. The cells were fixed at 0 h (A and a), 8 h (B and b), 16 h (C and c), 24 h (D and d), and 28 h (E and e). A–E are the immunofluorescent micrographs of cells with anti-PLC\textsubscript{4} antibody; a–e are phase-contrast micrographs.
crease in DNA polymerase α activity. PLC δ4 may activate DNA polymerase α by producing inositol 1,4-bisphosphate during S-phase of the cell cycle. It will be very interesting to identify the relationship between the increase in PLC δ4 activities and DNA polymerase activation in S-phase, but it remains to be solved in the future.

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