Presence of different phospholipase C isoforms in the nucleus and their activation during compensatory liver growth

Vladiana Crljen, Dora Višnjić, Hrvoje Banfić*

Department of Physiology and Croatian Institute for Brain Research, School of Medicine, University of Zagreb, Šalata 3, 10 000 Zagreb, Croatia

Received 26 May 2004; revised 4 June 2004; accepted 7 June 2004

Available online 1 July 2004

Edited by Richard Marais

Abstract Phospholipase C (PLC) was purified from the membrane-depleted rat liver nuclei. About 60% of the total PLC-activity corresponded to β_{1b} isoform, 30% to PLC-γ_1 and less than 10% to PLC-δ_1. PLC-β_{1b} and -γ_1 were found in the nuclear matrix, while PLC-δ_1 was detected in the chromatin. Two peaks of an increase in the total PLC-activity were detected occurring at 6 and 20 h after partial hepatectomy. An early increase in PLC-β_{1b} activity in the nuclear matrix was associated with serine phosphorylation of the enzyme, while the later increase paralleled the increase in the amount of protein. The increase in the PLC-γ_1 activity measured at 6 and 20 h after partial hepatectomy was associated with tyrosine phosphorylation of the enzyme. The activity of PLC-δ_1 and the amount of the protein found in the chromatin was increased only at 20 h after partial hepatectomy.

Keywords: Phospholipase C; Rat liver nucleus; Partial hepatectomy

1. Introduction

Phosphatidylinositol-specific phospholipases C (PLC) are responsible for converting phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$), which promote the activation of protein kinase C (PKC) and the release of Ca^{2+} from intracellular stores, respectively [1,2]. PLC family comprises twelve isoforms that can be subdivided into five types (β, γ, δ, ε and ζ) based on their structural differences. The activation of PLCs located in the plasma membrane, in response to hormone binding, has been extensively investigated and many aspects of their regulation are now understood [1,2]. However, evidence has accumulated suggesting the presence of PLCs in the cell nucleus, but little is known about regulation, function and possible subnuclear localization of different PLC isoforms [3,4]. It is known that PLC-β_1 and -γ_1 are localized in the nuclei [5,6]. On the other hand, there has been some inconsistency in showing nuclear PLC-δ localization [7,8], but Yamaga et al. [9] observed that δ_1 isoform might actually be cycling in and out of the nucleus. In the last couple of years, evidence has been accumulated that there is compartmentalization of inositol lipid metabolism within the cell nucleus [10–12].

In the present study, PLC purification was achieved from membrane-depleted nuclei and different subnuclear fractions obtained from rat liver nuclei to investigate possible subnuclear localization of different PLC isoforms, since there has been much inconsistency in showing their presence and subnuclear localization by immunocytochemical studies [6,13,14]. Moreover, using a well described model of the liver regeneration in which we have previously shown an increase in the nuclear DAG concentration due to PLC activation [15], the present investigation was undertaken to find out which nuclear PLC isoforms are activated during the process of liver hypertrophy.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: EGTA, EDTA, HEPES, Tris, Pipes, actinomycin D, leupeptin, aprotinin, DTT, phenylmethylsulfonyl fluoride (PMSF), PtdIns(4,5)P_2, Triton X-100, SDS, normal sheep serum (NSS), paraformaldehyde, DNase I and RNase A from Sigma, St. Louis, MO; calpain inhibitors I and II from Calbiochem, Nottingham, UK; [*methyl*-^{3}H]thymidine and [^{3}H]-PtdIns(4,5)P_2 from Perkin–Elmer Life Sciences, Boston, MA; heparin–Sepharose CL-6B and enhanced chemiluminescence kit from Amersham–Pharmacia Biotech., Uppsala, Sweden; YM 30 membrane and Centricon 30 from Amicon, Millipore, Bedford, MA; TSKgel heparin-5PW HPLC column (7.5 × 75 mm) from TosoHaas, Montgomeryville, PA; Microscope slides coated with poly-L-lysine from Fisher Scientific, Pittsburgh, PA; Fluorescein-conjugated affinity pure sheep anti-mouse IgG from Jackson ImmunoResearch Laboratories, West Grove, PA; anti-P-Tyr antibody, anti-phospho MAPK and monoclonal anti-PLC β_1, anti-PLC γ_1, anti-PLC δ_1 antibodies from Upstate Biotechnology, Lake Placid, NY; anti-PLC β_2, anti-PLC β_3, anti-PLC β_4, anti-PLC γ_1, anti-PLC γ_2, anti-PLC δ_1, anti-PLC δ_2, anti-lamin B, anti-histone H2B and anti-tubulin antibodies from Santa Cruz Biotechnology, Santa Cruz, CA; anti-P-Ser antibody from Alexis Biochemicals, Laufelfingen, Switzerland. All other chemicals were of analytical grade.

2.2. Partial hepatectomy, isolation of liver nuclei, assessment of their purity and [*methyl*-^{3}*H*]thymidine incorporation

Male Wistar rats (150–250 g body wt.) were used in all experiments. When partial hepatectomy was performed, two-thirds of the liver was surgically removed [15]. In the experiments where protein synthesis was blocked by actinomycin D, the antibiotic was dissolved in 0.9% NaCl and injected intraperitoneally at a dose of 0.1 mg/kg body mass 30 min before performing partial hepatectomy and 12 h after the operation.

* Corresponding author. Fax: +385-1-4590-207/4596-942.
E-mail address: hrvoje.banfic@zg.tel.hr (H. Banfić).

Abbreviations: PLC, phospholipase C; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; Ins(1,4,5)P_3, inositol(1,4,5)P_3; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; PMSF, phenylmethylsulfonyl fluoride; NSS, normal sheep serum

0014-5793/$22.00
doi:10.1016/j.febslet.2004.06.051

Livers were collected on ice and nuclei were isolated as described previously [12]. The purity of nuclei was estimated by the determination of marker enzymes (leucine arylamidase, Na^+–K^+-ATPase, succinate:cytochrome *c* oxidoreductase, KCN-resistant NADH oxidoreductase and 5-nucleotidase) and by electron microscopy [12].

[*Methyl*-^{3}H]thymidine was injected (0.5 μCi/g of body weight) 2 h before sacrifice. Liver was homogenized in the phosphate-buffered saline (PBS) and [*methyl*-^{3}H]thymidine incorporation was measured after DNA precipitation with 15% trichloroacetic acid and two successive washes with 10% and 5% trichloroacetic acid.

2.3. Preparation of membrane-depleted nuclei, nuclear matrix and chromatin

The membrane-depleted nuclei were prepared as described previously [12] and resuspended and incubated for 20 min at 37 °C in a buffer containing 100 mM NaCl, 0.3 M sucrose, 3 mM $MgCl_2$, 0.04% Triton X-100 (w/v), 0.5 mM $CaCl_2$, 10 mM Pipes (pH 6.8), 1.2 mM PMSF, 100 μg/ml DNase I and 50 μg/ml RNase A in a concentration of 10^7 nuclei/ml. Subsequently, the chromatin-associated proteins were released by adding 2 M ammonium sulfate dropwise to a final concentration of 0.25 M. After 15 min of incubation on ice, the nuclear matrices were pelleted at $2000 \times g$ for 10 min on a cushion containing 43% glycerol and 2 M sucrose in PBS. The matrices were collected with a pipette and diluted with the above mentioned buffer without DNase I and RNase A to a final concentration of approximately 2 mg/ml [10].

Chromatin was extracted from membrane-depleted nuclei using a modification of the method by Shaw and Huang [16] in which the membrane-depleted nuclei were gently resuspended for 5 min in a buffer containing 0.25 M sucrose, 10 mM Tris (pH 7.4), 0.04% Triton X-100 (w/v) and 1 mM PMSF in a concentration of 10^7 nuclei/ml and centrifuged at $7700 \times g$ for 5 min. The nuclear pellet was resuspended with three strokes of a Potter–Elvejhem homogenizer with teflon pestle at low velocity (600 rpm) followed by magnetic stirring for 1 h at 4 °C and the procedure was repeated four times [17]. The extraction was completed by washing the material in a progressively less concentrated Tris solution (50, 10, 2 and 0.4 mM) (pH 8) containing 1 mM PMSF. The material was recovered at $8000 \times g$ for 10 min and resuspended each time with three strokes of a Potter–Elvejhem homogenizer with a Teflon pestle at low velocity (600 rpm). Two washings for each solution were made. Finally, the material was resuspended in 100 ml of 1 mM PMSF (pH 8) and stirred overnight at 4 °C. The solution was then centrifuged at $90\,000 \times g$ for 30 min and the clear chromatin sediment was used for PLC purification (please see below). At each step, the material was controlled with the light microscope after methylene blue staining in order to ascertain that no nuclei were present during preparation [17].

2.4. Preparation of homogenate, cytosolic fraction and postnuclear membranes

Homogenate of liver tissue was prepared using a power-driven pestle in a buffer containing 10 mM HEPES (pH 7.5), 5 mM $MgCl_2$ and 25 mM KCl (solution B) as described above. Cytosolic fraction was prepared by homogenization in solution B and afterwards samples were spun at $106\,000 \times g$ for 90 min at 4 °C in a Beckman SW 28.1 rotor, and the clear supernatant was considered to be cytosolic fraction. Preparation of postnuclear membranes was achieved by centrifugation of the supernatant that remained above the cushion after the nuclear fraction had been obtained. The supernatant was diluted in solution B to give final 162 mM sucrose and spun at $106\,000 \times g$ for 90 min at 4 °C in a Beckman SW 28.1 rotor and the obtained pellet was considered to contain postnuclear membranes [12].

2.5. PLC assay

100 μg of nuclear protein was incubated in 2 mM $MgCl_2$, 140 mM NaCl, 250 mM sucrose and 10 mM HEPES (pH 7.0) and the reaction was started by the addition of 5 nmol PtdIns(4,5)P_2 containing 60 000 dpm [^{3}H] PtdIns(4,5)P_2 in a total volume of 200 μl. After 5 min at 37 °C, the reaction was stopped by adding 1 ml chloroform/methanol (1:1, v/v) followed by 250 μl of 2.4 N HCl. After a brief centrifugation, the top phase was removed and counted in a liquid scintillation counter [5].

2.6. Purification of nuclear PLC

The membrane-depleted liver nuclei (100 mg of protein) were homogenized in 50 ml of homogenization buffer (10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, leupeptin (10 μg/ml), aprotinin (10 μg/ml), and calpain inhibitors I and II (each at 4 μg/ml) using a glass homogenizer with a motor-driven Teflon pestle (10 strokes). The homogenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was adjusted to 2 M KCl by adding solid KCl, stirred for 2 h at 4 °C, and then centrifuged at $35\,000 \times g$ for 30 min. The resulting supernatant was dialyzed overnight against 4 L of homogenization buffer and centrifuged again. The supernatant was applied to a heparin–Sepharose CL-6B column that had been equilibrated with 20 mM HEPES (pH 7.0) containing 1 mM EGTA and 0.1 mM DTT. Bound proteins were eluted at a flow rate of 4 ml/min with the equilibration buffer containing 1.2 M NaCl. The fractions (16 ml) were collected and assayed for PLC-activity. Essentially, all detectable PLC-activity was eluted in six fractions as described by Lee and Rhee [8], which were pooled and concentrated in a stirred ultrafiltration cell with YM 30 membrane. After the final NaCl concentration was adjusted to 50 mM, the concentrate was centrifuged at $100\,000 \times g$ for 10 min and the proteins from the supernatant were injected into TSKgel heparin-5PW HPLC column that had been equilibrated with 20 mM HEPES (pH 7.0) and 1 mM EGTA. Proteins were eluted at a flow rate of 1.0 ml/min by the application of equilibrium buffer for 15 min, a linear gradient from 0 to 0.64 M NaCl for 40 min, and a second linear NaCl gradient from 0.64 to 1.0 M for 10 min. Finally, the column was washed with equilibrium buffer containing 1.0 M NaCl for 5 min [18]. The fractions (1 ml) were collected and PLC-activity was assayed by adding 2 μl directly into PLC assay buffer as described above. For immunoblot analysis, the fractions were concentrated with Centricon-30, and 5 μl was added into 25 μl of a sample loading buffer [19] and subjected to electrophoresis as described below.

2.7. Preparation of antibodies to PLC-β_{1a}, -β_{1b}, -δ_4, immunoprecipitation of PLC isoforms and Western blot analysis

Peptide (ENPGKEFDTPL) corresponding to residues 1206–1216 in PLC-β_{1a} [20], peptide (TPPNPQALKW) corresponding to residues 1164–1173 in PLC-β_{1b} [20] and peptide (KDEGSDLDPAS) corresponding to residues 454–464 in PLC-δ_4 [8] were synthesized and conjugated to keyhole limpet hemocyanin and the antisera to the peptides were generated separately in the female New Zealand white rabbits which were immunized with 5 μg of peptides injected intradermally. The rabbits were boosted with 5 μg of peptides in Freund's incomplete adjuvant at 4 and 7 weeks after the primary immunization and the sera were collected 9 weeks after the primary immunization and the immunoglobulin was purified on protein A–agarose [8,20].

Chromatin or active fractions obtained from HPLC column were resuspended in 0.5 ml of buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100 (w/v), 0.5% Na^+-deoxycholate (w/v), 0.1% SDS (w/v), 2 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin and spun at $100\,000 \times g$ for 90 min at 4 °C. Different isoforms of PLC were immunoprecipitated overnight at 4 °C with 5 μg of isoform specific antibody and protein G–agarose. Immunoprecipitates were washed once with the above mentioned buffer, then three times with 5 mM HEPES/2 mM EDTA (pH 7.5) and then subjected to Western blot analysis.

The proteins for electrophoresis were prepared so that the concentration of each sample was 50 μg/25 μl of the sample loading buffer [19] and electrophoresis was carried out using a Bio-Rad Minigel apparatus at an acrylamide concentration of 7% (w/v) or 10% (w/v) when the presence of PLC-δ_1, tubulin, lamin B, phospho-MAPK and histone H2B was determined. After electrophoresis, the proteins were transferred to nitrocellulose using a Bio-Rad wet-blotting system. The blot was blocked with a buffer containing 4% (w/v) dried milk, 20 mM Tris, 140 mM NaCl, and 0.05% (v/v) Tween 20. It was then probed for 2 h with primary antibody (1:1000), then washed with a blocking buffer and incubated with the secondary antibody conjugated to horseradish peroxidase. Visualization was carried out using enhanced chemiluminescence kit. The molecular mass markers used were as follows: fumarase (48.5 kDa); albumin, bovine serum (66 kDa); α-lactalbumin, bovine milk (14.2 kDa); α_2-macroglobulin (180 kDa); β-galactosidase *E. coli* (116 kDa); and ovalbumin (45 kDa).

2.8. Immunoflorescence and confocal microscopy analysis

Per sample, 1×10^6 isolated membrane-depleted nuclei were dropped on glass microscope slides coated with poly-L-lysine, allowed to stick for 60 min at room temperature and fixed in 4% paraformaldehyde/PBS. After mild permeabilization with 0.2% Triton X-100 for 8 min,

slides were incubated to block non-specific binding with 2% BSA, and 3% NSS in PBS (immunoreaction buffer) for 60 min at room temperature and then reacted with monoclonal primary antibodies to PLC isoforms diluted 1:50 in immunoreaction buffer overnight at 4 °C. After three washing steps, the primary antibody binding was visualized by incubating the specimens with fluorescein-conjugated affinity pure sheep anti-mouse IgG diluted at 1:500 in 1% BSA/PBS for 1 h at room temperature [6]. The samples were analyzed by a confocal laser scanning LSM 510 Meta microscope (Carl Zeiss, Germany).

2.9. Statistical evaluation

The data are shown as means ± S.E.M. For statistical analyses, the Student's *t* test for unpaired samples at the level of significance of 0.05 was used.

3. Results

3.1. Assessment of nuclear purity and purity of nuclear fractions

The purity of isolated nuclei was estimated by the determination of marker enzymes and by electron microscopy. Most marker enzymes were below measurable levels in membrane-depleted nuclei, and electron microscopy showed no other obvious components present (results not shown). The exception in this respect was for endoplasmic reticulum KCN-resistant NADH oxidoreductase, whose specific activity in the membrane-depleted nuclei was reduced to 11% of the specific activity found in the homogenate, while the total activity in the membrane-depleted nuclei was reduced to only 0.04% of the total activity found in the homogenate (Table 1). The presence of KCN-resistant NADH oxidoreductase in nuclear preparations may be taken as evidence for residual endoplasmic reticulum contamination that is finally removed by a detergent, but it is equally likely that it is present in the nuclear membrane, which is also removed by detergent [11,12,15]. It is important to note that similar residual contamination of nuclear preparations by endoplasmic reticulum was observed when another microsomal marker enzyme (glucose-6-phosphatase) was measured [21,22]. As shown in Fig. 1A, immunoblot analysis of the nuclei or nuclear fractions detected the tubulin in the native but not in the membrane-depleted nuclei, confirming that the residual cytoskeleton contamination of the nuclei was effectively removed by a detergent [10]. The lamin B was detected in all preparations which includes the nuclear matrix, but has not been detected in the chromatin, while the histone H2B was not detected in the isolated nuclear matrix, showing that the nuclear matrix and the chromatin could be isolated from the membrane-depleted nuclei without noticeable cross-contamination. These data demonstrate that the membrane-depleted nuclei and/or subnuclear fractions were purified to a satisfactory degree.

3.2. Presence of different PLC isoforms in the nuclei

Total PLC-activity in the native nuclei was 3.4 ± 0.4 (nmol of produced Ins(1,4,5)P_3/min/mg of nuclear protein) and the activity dropped to 2.2 ± 0.3 (nmol of produced Ins(1,4,5)P_3/min/mg of nuclear protein) in the nuclei that were effectively depleted of the membranes by a detergent, showing that the PLC-activity associated with the nuclear membrane and cytoskeleton accounts for about 30% of the total PLC-activity in the native nuclei ([10,11] and present study). Western blot analysis was performed to test for the presence of particular PLC isoforms in the rat liver nucleus. As shown in Fig. 1B, immunoblotting data with antibodies specific for two splicing variants of PLC-β isoform revealed the presence of PLC-β_{1b} and, a smaller amount, of PLC-β_{1a} in the native nuclei, as showed previously by Bahk et al. [23]. However, the membrane-depleted nuclei contained only PLC-β_{1b}. The presence of PLC-γ_1 was detected in the native liver nuclei, but the amount of the enzyme decreased when the nuclear membrane and residual cytoskeleton were removed by a detergent, supporting previous evidence made by immunocytochemical studies that nuclear PLC-γ_1 is localized in the nuclear matrix and lamina [6,13,14]. No other immunoreactive bands were detected in the native or the membrane-depleted nuclei by other PLC isoform-specific antibodies (β_2, β_3, β_4, γ_2, δ_1 and δ_4) under the same experimental conditions as those in Fig. 1B (results not shown).

Having in mind inconsistency in showing nuclear PLC localization, in particular of δ isoforms [7,8], and knowing that a trace amount of protein in the immunoreactive band obtained from the crude or the membrane-depleted nuclei may be too low to be recognized by isoform-specific antibody, a more extensive study was performed in which nuclear PLCs were purified from the membrane-depleted nuclei (100 mg of total protein) on a conventional heparin column (mainly to remove turbid material) and then on a heparin HPLC column [8,18]. Each fraction was assayed for PLC-activity with PtdIns(4,5)P_2 as substrate and the fractions containing PLC-activity were immunoblotted with isoform-specific antibodies. As shown in Fig. 1C and D, three different PLC isoforms were eluted from HPLC column, γ_1 at 37 min, δ_1 at 42 min and β_{1b} at 51–52 min and their retention times were identical to those observed by Lee and Rhee [8]. Moreover, based on PLC-activity in each peak, about 60% of the total PLC-activity in the membrane-depleted nuclei corresponds to β_{1b} isoform, about 30% corresponds to γ_1 isoform and less than 10% corresponds to δ_1 isoform. No PLC-activity was eluted from HPLC column at 62 min, when under the same experimental conditions, Lee and Rhee [8] observed PLC-activity due to PLC-δ_4 elution. No immunoreactive bands were recognized by other PLC isoform-specific antibodies (β_2, β_3, β_4, γ_2 and δ_4) in the active fractions eluted from heparin HPLC column (results not shown).

Table 1
The activity of KCN-resistant NADH oxidoreductase in nuclear preparations

	Total activity (μmol/total liver protein)	Specific activity (μmol/mg of liver protein)
Homogenate	413.62 ± 51.37 (100)	0.55 ± 0.07 (100)
Nuclei	0.72 ± 0.12 (0.17)	0.17 ± 0.03 (30.9)
Membrane-depleted nuclei	0.18 ± 0.03 (0.04)	0.06 ± 0.01 (10.9)

The activity of the enzyme was measured as described in Section 2. The results are means ± S.E.M. for three different experiments, each performed in duplicate and relative percent values are given in brackets.

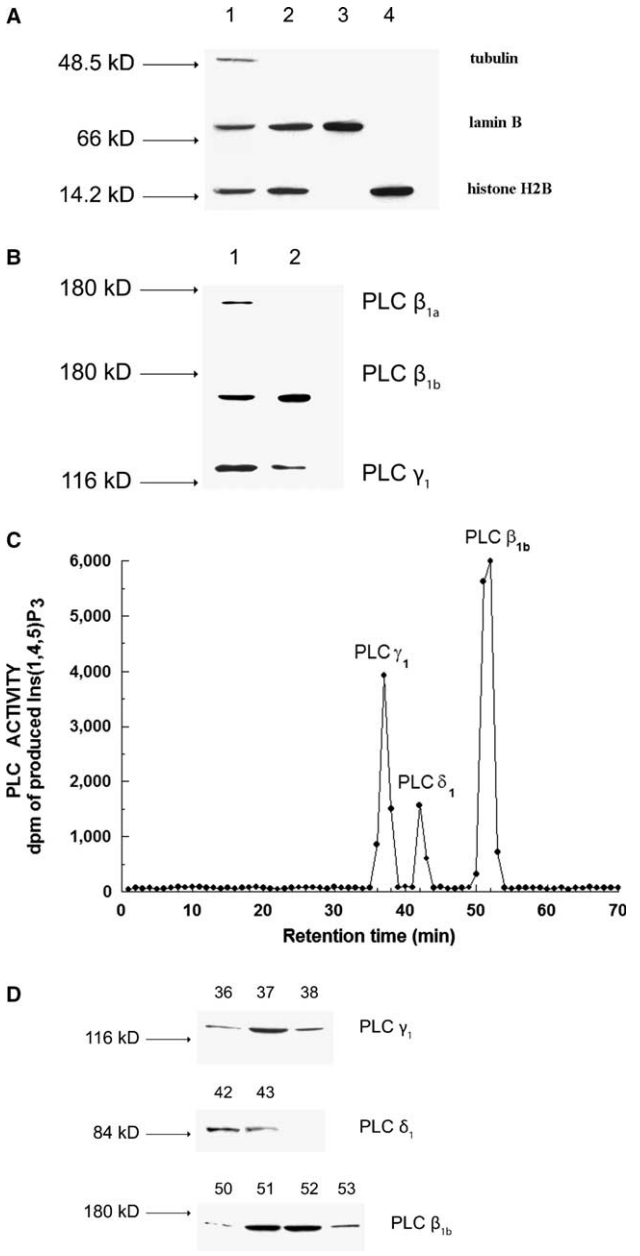

Fig. 1. (A) Western blot analysis of native nuclei (lane 1), membrane-depleted nuclei (lane 2), nuclear matrix (lane 3) and chromatin (lane 4) for the presence of tubulin, lamin B and histone H2B. Protein (50 μg) was subjected to SDS–PAGE, transferred to nitrocellulose, and probed separately with anti-tubulin, or anti-lamin B or anti-histone H2B antibody. (B) Western blot analysis of PLC-β_1 and -γ_1 in the native (1) and membrane-depleted nuclei (2). Protein (50 μg) was subjected to SDS–PAGE, transferred to nitrocellulose, and probed separately with anti-PLC β_{1a}, anti-PLC β_{1b} or anti-PLC γ_1 antibodies. (C) Purification of PLC from the membrane-depleted nuclei on TSKgel heparin-5PW HPLC column. (D) Western blot analysis of PLC isoforms in active fractions. The number above each lane corresponds to the retention time for each PLC-active fraction. The position of the molecular mass marker is indicated on the left side by the arrow.

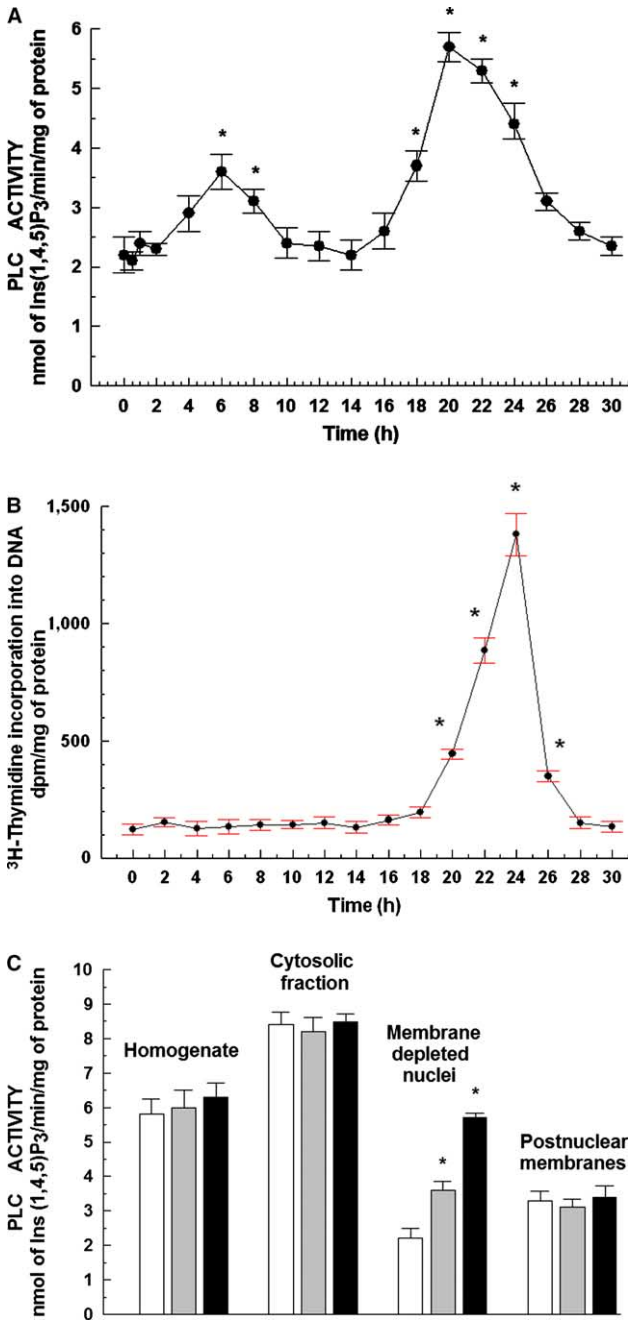

Fig. 2. (A) Time course of changes in the total PLC-activity in the membrane-depleted nuclei, (B) [*methyl*-^{3}H]thymidine incorporation into DNA and (C) PLC-activity in the homogenate, cytosolic fraction, membrane-depleted nuclei and postnuclear membranes following partial hepatectomy. The control samples (open bars), and the samples obtained at 6 h (gray bars) or 20 h (black bars) after partial hepatectomy. The results are means ± S.E.M. for three different experiments, each performed in duplicate. *$P < 0.05$ (Student's t test) with respect to the control.

3.3. *Increased nuclear PLC-activity during compensatory liver growth*

It is known that the concentration of nuclear DAG increases due to the activation of nuclear PLC during liver regeneration following partial hepatectomy [6,15]. As shown in Fig. 2A, two peaks of an increase in the total PLC-activity were detected in the membrane-depleted nuclei following partial hepatectomy. The first increase in the total PLC activity was measured at 6 h after partial hepatectomy and the second, more pronounced increase, was detected at 20 h. Time course analysis of [*methyl*-^{3}H]thymidine incorporation into DNA of the regenerating rat liver tissues revealed that the maximum increase in the rate of

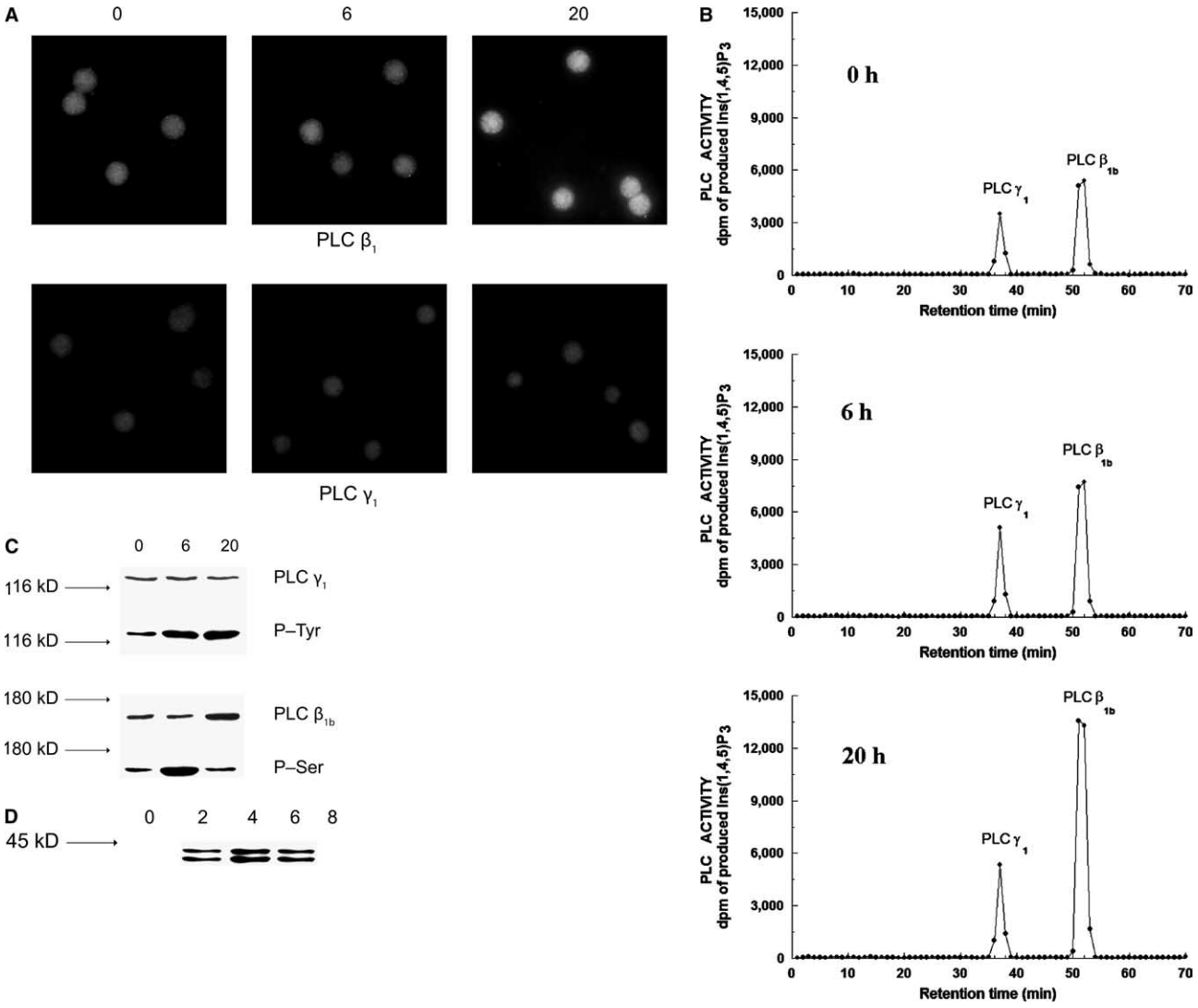

Fig. 3. (A) Immunohistochemical localization of PLC-β_1 and -γ_1 in the membrane-depleted nuclei obtained from intact liver (0 h) or 6 and 20 h after partial hepatectomy. The nuclei were fixed, stained with anti-PLC β_1 or anti-PLC γ_1 antibodies and analyzed by confocal laser microscopy as described in Section 2. (B) Purification of PLC on TSKgel heparin-5PW HPLC column from the nuclear matrix isolated from the intact nuclei (0 h) and the nuclei harvested at 6 and 20 h after partial hepatectomy. (C) Western blot analysis of active fractions purified from the control nuclear matrix (0 h) and nuclear matrices isolated at 6 and 20 h after partial hepatectomy. Active fractions which correspond to each isoform were pooled, concentrated and immunoblotted with isoform-specific anti-PLC antibody. When Western blot analysis of PLC phosphorylation was performed, the enzymes were first immunoprecipitated using isoform-specific antibodies and immunoprecipitates were then probed with anti-P-Tyr or anti-P-Ser antibodies. (D) Western blot analysis of phosphorylated forms of MAPK in cell lysates isolated at different times after partial hepatectomy. Protein (50 μg) was subjected to SDS–PAGE, transferred to nitrocellulose, and probed with anti-phospho MAPK antibody. The position of the molecular mass marker is indicated on the left side by the arrow.

DNA synthesis followed the second increase in the nuclear PLC-activity (Fig. 2B). No increase in the total nuclear PLC-activity was observed in the nuclei isolated at later time points, up to 168 h following partial hepatectomy (results not shown). Moreover, the increase in the total PLC-activity was specific for the membrane-depleted nuclei only, since no increase in the total PLC-activity could be observed in the homogenate, cytosolic fraction and postnuclear membranes isolated at 6 and 20 h (Fig. 2C). Immunocytochemical analysis of PLC presence in the membrane-depleted nuclei isolated at 0, 6 and 20 h after the partial hepatectomy was performed using commercially available monoclonal antibodies against PLC-β_1, -γ_1 and -δ_1 isoforms, since polyclonal antibodies which were produced to PLC-β_1 splice variants were not shown to be efficient in immunohistochemical studies. As shown in Fig. 3A, the presence of both PLC-β_1 and -γ_1 was detected in the control membrane-depleted nuclei. The increase in the level of PLC-β_1 was observed at 20 h, while no change in the intensity of PLC-γ_1 signal was detected after partial hepatectomy.

3.4. Compartmentalization of PLC isoforms in the nuclei and their activation following partial hepatectomy

Knowing that PLC-β_{1b}, -γ_1 and -δ_1 isoforms could be found in the membrane-depleted nuclei, and that there are two peaks of increase in the total PLC-activity following partial hepatectomy, further studies were performed to reveal the subnuclear localization of different PLC isoforms and the mechanism of their activation during the process of liver hypertrophy.

Previous study has shown an increase in the PLC-activity associated with chromatin after partial hepatectomy [24]. Therefore, the nuclear matrix and chromatin were isolated from membrane-depleted nuclei obtained from sham-operated animals (time 0) or nuclei harvested 6 or 20 h after partial hepatectomy, proteins were fractionated using heparin columns, and the fractions centered about each peak of PLC-activity were pooled together and subjected to immunoblot analysis. As shown in Fig. 3B, two peaks of activities corresponding to PLC-β_{1b} and -γ_1 isoforms were detected in the nuclear matrix, and their activities were increased at 6 and 20 h after partial hepatectomy. Western blot analysis of the active fractions that correspond to γ_1 isoform (Fig. 3C) revealed no changes in the amount of a PLC-γ_1 immunoreactive band, which corroborates finding by immunohistochemical studies shown in Fig. 3A. However, when enzyme activity was immunoprecipitated using isoform-specific antibody and immunoprecipitates subjected to Western blot analysis using anti-P-Tyr antibody, an increase in the intensity of an immunoreactive band was detected at 6 and 20 h after partial hepatectomy. Immunoblot analysis of the active fractions that correspond to PLC-β_{1b} isoform (Fig. 3C) revealed an increase in the amount of protein recognized by anti-PLC β_{1b} antibody at 20 h after partial hepatectomy, similar to results obtained by immunohistochemical analysis (Fig. 3A). When enzyme activity was immunoprecipitated using isoform-specific antibody and immunoprecipitates probed with an anti-P-Ser antibody, an increase in the amount of immunoreactive band was detected only at 6 h after partial hepatectomy. As previous studies suggested that the activation of nuclear PLC-β_{1b} is mediated by MAPK-induced phosphorylation, the kinetics of MAPK activation was assessed by using antibody against phosphorylated forms of MAPK. As shown in Fig. 3D, maximal phosphorylation was observed slightly earlier than the increase in nuclear PLC-β_{1b} activity.

When proteins were extracted from chromatin and fractionated using heparin columns, only PLC-δ_1 was found and its activity was increased at 20 h after partial hepatectomy (Fig. 4A). Western blot analysis of the active fractions that correspond to δ_1 isoform revealed an increase in the amount of PLC-δ_1 protein at 20 h (Fig. 4B). No immunoreactive band was found when immunoprecipitates were probed with either anti-P-Ser or anti-P-Tyr antibody (results not shown). To test whether the increased amount of PLC-β_{1b} and -δ_1 in the nuclei harvested at 20 h after partial hepatectomy was due to translocation or higher expression of the enzymes, protein synthesis was blocked by actinomycin D [25]. As shown in Fig. 4C, the inhibition of protein synthesis did not affect the increase in the amount of PLC-$\beta_{1\beta}$ in the nuclear matrix, or PLC-δ_1 in the chromatin.

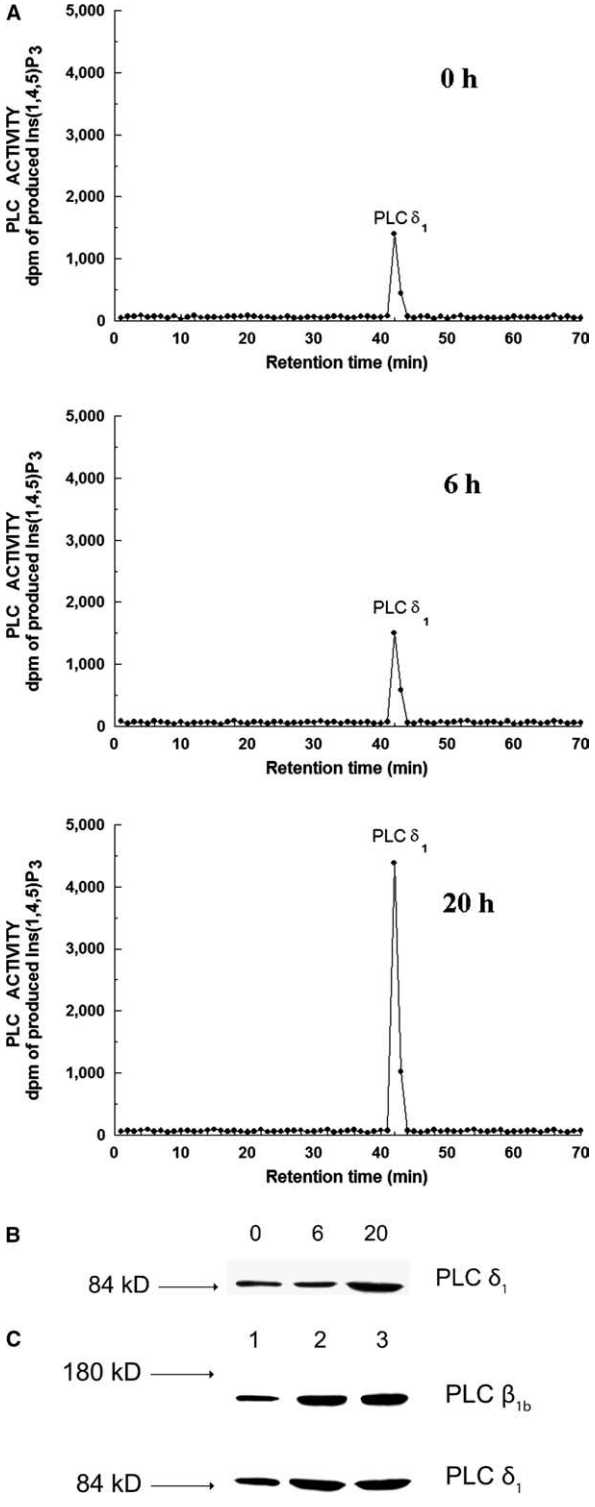

Fig. 4. (A) Purification of PLC on TSKgel heparin-5PW HPLC column from chromatin isolated from the intact nuclei (0 h) and the nuclei harvested at 6 and 20 h after partial hepatectomy. (B) Western blot analysis of active fractions purified from the control chromatin (0 h) and chromatin isolated at 6 and 20 h after partial hepatectomy. Active fractions which correspond to each isoform were pooled, concentrated and immunoblotted with isoform-specific anti-PLC antibody. (C) The effect of actinomycin D on the amount of PLC-β_{1b} in nuclear matrix and PLC-δ_1 in chromatin. Protein (50 μg) was subjected to SDS–PAGE, transferred to nitrocellulose, and probed with anti-PLC β_{1b} antibody. When Western blot analysis of PLC-δ_1 in chromatin was performed, the enzyme was first immunoprecipitated from chromatin using isoform-specific antibody and immunoprecipitates were then probed with anti-PLC δ_1 antibody: lane 1, control nuclear matrix or chromatin; lane 2, nuclear matrix or chromatin harvested from the nuclei at 20 h after partial hepatectomy; lane 3, nuclear matrix or chromatin harvested from the nuclei at 20 h after partial hepatectomy obtained from actinomycin D treated animals. The position of the molecular mass marker is indicated on the left side by the arrow.

4. Discussion

Compensatory hepatic growth is a very informative model for natural cell proliferation [26] in which an increase in nuclear DAG concentration due to PLC activation was demonstrated [6,14,15]. In this study, we have extended previous observations by showing: (a) the presence of three different PLC isoforms in the intact membrane-depleted nuclei, (b) their different subnuclear localization and (c) that following partial hepatectomy increase in their activity may be differently regulated.

The presence of PLC-β_1 in the cell nucleus has been demonstrated in numerous cells [4], including liver nuclei [5], where it has been localized by confocal microscopy to be associated with nuclear matrix and lamina [13]. Overexpression of PLC-β_1 and subsequent localization to the nucleus was shown to significantly enhance the mitogenic action of IGF-I in Swiss 3T3 cells [27] and also prevent erythroid differentiation in the mouse erythroleukemia cells [28]. In the serum-starved Friend erythroleukemia cells, overexpression of PLC-β_1 caused an increase in the expression of cyclin D3 and cdk4, hyperphosphorylation of retinoblastoma protein, the activation of E2F-1 transcription factor and a subsequent progression through the cell cycle [29]. Two forms of PLC-β_1 generated by alternative splicing of a single gene were identified [20]; PLC-β_{1a} appeared to be located preferentially in the cytosol, whereas PLC-β_{1b} was found predominantly in the nuclei [23,28,29]. In these studies, no attempts were made to remove the nuclear membrane, which is known to be associated with cytosolic structures. The results of the present study confirmed the presence of PLC-β_{1b} and a smaller amount of PLC-β_{1a} in the native nuclei, and further showed that the PLC-β_{1b} isoform is the only splicing variant that is an integral part of the nuclear matrix, which is responsible for about 60% of the overall PLC-activity found in the membrane-depleted nuclei. Furthermore, two peaks of activities corresponding to PLC-β_{1b} were detected in the nuclear matrices isolated at 6 and 20 h after partial hepatectomy; while the early increase in the activity was associated with serine phosphorylation of PLC-β_{1b}, the later increase is probably due to an increase in the amount of protein in the nuclear matrix. Previous studies have shown that PLC-β_1 is activated by MAPK-mediated phosphorylation on serine in the nuclei of NIH 3T3 cells [30], and the activation of MAPK is considered to be important for the progression through early G_1 phase of hepatocytes from the regenerating liver [31]. In the present study, the similar kinetics of MAPK-activation was detected. Therefore, it is possible that serine phosphorylation of PLC-β_{1b} and an increase in the activity of the enzyme in the nuclear matrix that occurs at 6 h after partial hepatectomy is caused by the activation of MAPK. The later increase in the activity of PLC-β_{1b} in the nuclear matrix that was associated with an increased amount of protein precedes the increase in [*methyl*-^{3}H]thymidine incorporation into DNA, therefore correlating close with DNA synthesis in the hepatic parenchymal cells following partial hepatectomy [32] and suggesting that nuclear PLC-β_{1b} may be involved in the progression of cells through cell cycle during liver regeneration as has been observed in in transfecto experiments on Friend erythroleukemia cells [28]. Moreover, as it is known that an increase in nuclear DAG is maximal at 20 h after partial hepatectomy causing translocation of PKC to the nucleus [15], it is possible that potential targets for activated nuclear PKC such as lamins, DNA polymerase, and topoisomerase II, which are known to be involved in cell cycle regulation (see [4,33–35] for reviews), are also included as a part of complex signaling pathway which is activated upon stimulation of nuclear PLC-β_{1b}.

The present study confirms previous observations made by immunocytochemical studies that nuclear PLC-γ_1 is localized in the nuclear matrix and lamina [6,13,14] and that the enzyme undergoes tyrosine phosphorylation following partial hepatectomy [6]. The results of the present study show that PLC-γ_1 accounts for about 30% of the total PLC-activity found in the membrane-depleted nuclei, and that the activation of the enzyme in nuclear matrices that occurs at 6 and 20 h after partial hepatectomy may be mediated by tyrosine phosphorylation. Activated PLC-γ_1 may contribute to an increased production of nuclear DAG, which can result in nuclear PKC translocation. On the other hand, it is known that PLC-γ_1 may also act as a guanine nucleotide exchange factor for phosphatidylinositol 3-kinase (PI3K) enhancer, which is nuclear GTPase that activates a nuclear PI3K-activity [36] and it is important to note that PI3K-activity was observed in the intact liver nuclei [37].

Regarding the presence of PLC-δ isoforms in the liver nuclei, this study confirms previous observation by Lee and Rhee [8] who were unable to find any PLC-δ_4 isoform in the liver tissue. On the other hand, when PLC was purified from the membrane-depleted nuclei, about 10% of the total PLC-activity was found to correspond to δ_1 isoform. The presence of PLC-δ_1 was detected in the chromatin fraction and the amount of the enzyme increased at 20 h after partial hepatectomy parallel to an increase in the activity of the enzyme, suggesting that an increased activity of PLC-δ_1 in the chromatin may be caused by an increased amount of protein. A study using green fluorescent protein fusion system in Madin–Darby canine kidney cells shows that PLC-δ_1 shuttled between the cytoplasm and the nucleus and accumulated in the nucleus when the nuclear export signal was blocked by Leptomycin B or disrupted [9]. It is possible that an increase in the amount of PLC-δ_1-protein and the activity found in the chromatin harvested at 20 h after partial hepatectomy may be due to the accumulation of the enzyme in the nucleus. It is important to note that we were unable to notice any specific signal by confocal laser microscopy analysis of PLC-δ_1 presence in the membrane-depleted nuclei, which is in accordance with the above mentioned study where PLC-δ_1 nuclear localization could be proven only by trapping the enzyme within the nuclei and corroborates the hypothesis by Irvine [4] that the amount of the enzyme in the native nuclei is too low to be noticeable by immunohistochemical analysis. At the moment one can only speculate about possible function(s) of chromatin-associated PLC-δ_1 isoform, but it is noteworthy that in the yeast, protein product (Plc1p) which is most closely related to mammalian PLC-δ isoforms is involved in kinetochore function [38], messenger RNA export [39] and chromatin remodeling by inositol polyphosphates [40,41] and that the plant homeodomain finger of chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor [42].

In summary, the data presented in this report show that 60% of the total PLC-activity in the membrane-depleted nuclei corresponds to β_{1b} isoform, about 30% to γ_1 isoform and less than 10% to δ_1 isoform. Both PLC-β_{1b} and -γ_1 isoforms were

found to be associated with the nuclear matrix, and their activities increased at 6 and 20 h after partial hepatectomy. An early (6 h) increase in PLC-β_{1b} activity was associated with a serine phosphorylation of the enzyme, while the later increase parallels an increase in the amount of the protein in the nuclear matrix. An increase in the activity of PLC-γ_1 in the nuclear matrices was associated with tyrosine phosphorylation of the enzyme. The activity of PLC-δ_1 isoform was found in chromatin only and the activity that was associated with an increase in the amount of enzyme was detected at 20 h after partial hepatectomy. Finally, the increase in the amount of nuclear PLC-$\beta_{1\beta}$ and -δ_1 was found not to be due to the higher expression of the enzymes.

Acknowledgements: We thank Rene Lui for editing the manuscript before submission, Alan P. Fields for valuable help and advice and Ivan Sabolić for helping us in immunoflorescence studies. This work was supported by the Ministry for Science of the Republic of Croatia (to D. V. and H. B.) and by the Fogarty International Research Collaboration Award (to H. B.).

References

[1] Rhee, S.G. (2001) Annu. Rev. Biochem. 70, 281–312.
[2] Berridge, M.J., Bootman, M.D. and Roderick, H.L. (2003) Nat. Rev. Mol. Cell Biol. 4, 517–529.
[3] Divecha, N., Banfic, H. and Irvine, R.F. (1993) Cell 74, 405–407.
[4] Irvine, R.F. (2003) Nat. Rev. Mol. Cell Biol. 4, 1–12.
[5] Divecha, N., Rhee, S.G., Letcher, A.J. and Irvine, R.F. (1993) Biochem. J. 289, 617–620.
[6] Neri, L.M., Ricci, D., Carini, C., Marchisio, M., Capitani, S. and Bertagnolo, V. (1997) Cell. Signal. 9, 353–362.
[7] Liu, N., Fukami, K., Yu, H. and Takenawa, T. (1996) J. Biol. Chem. 271, 355–360.
[8] Lee, S.B. and Rhee, S.G. (1996) J. Biol. Chem. 271, 25–31.
[9] Yamaga, M., Fujii, M., Kamata, H., Hirata, H. and Yagisawa, H. (1999) J. Biol. Chem. 274, 28537–28541.
[10] Payrastre, B., Nievers, M., Boonstra, J., Breton, M., Verkleij, A.J. and Van Bergen en Henegouwen, P.M.P. (1992) J. Biol. Chem. 267, 5078–5084.
[11] Vann, L.R., Wooding, F.B.P., Irvine, R.F. and Divecha, N. (1997) Biochem. J. 327, 569–576.
[12] Sindic, A., Aleksandrova, A., Fields, A.P., Volinia, S. and Banfic, H. (2001) J. Biol. Chem. 276, 17754–17761.
[13] Bertagnolo, V., Mazzoni, M., Ricci, D., Carini, C., Neri, L.M., Previati, M. and Capitani, S. (1995) Cell. Signal. 7, 669–678.
[14] Albi, E., Rossi, G., Maraldi, N.M., Magni, M.V., Cataldi, S., Solimando, L. and Zini, N. (2003) J. Cell. Physiol. 197, 181–188.
[15] Banfic, H., Zizak, M., Divecha, N. and Irvine, R.F. (1993) Biochem. J. 290, 633–636.
[16] Shaw, L.M.J. and Huang, R.C.C. (1970) Biochemistry 9, 5430–5441.
[17] Viola-Magni, M.P., Gahan, P.B. and Pacy, J. (1985) Cell Biochem. Funct. 3, 71–78.
[18] Lee, C.W., Park, D.J., Lee, K.H., Kim, C.G. and Rhee, S.G. (1993) J. Biol. Chem. 268, 21318–21327.
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] Bahk, Y.Y., Lee, Y.H., Lee, T.G., Seo, J., Ryu, S.H. and Suh, P.G. (1994) J. Biol. Chem. 269, 8240–8245.
[21] Visnjic, D., Crljen, V., Curic, J., Batinic, D., Volinia, S. and Banfic, H. (2002) FEBS Lett. 529, 268–274.
[22] Visnjic, D., Curic, J., Crljen, V., Batinic, D., Volinia, S. and Banfic, H. (2003) Biochim. Biophys. Acta 1631, 61–71.
[23] Bahk, Y.Y., Song, H., Baek, S.H., Park, B.Y., Kim, H., Ryu, S.H. and Suh, P.G. (1998) Biochim. Biophys. Acta 1389, 76–80.
[24] Albi, E. and Magni, M.V. (1999) Biochem. Biophys. Res. Commun. 265, 640–643.
[25] Salihagic, A., Mackovic, M., Banfic, H. and Sabolic, I. (1988) Pflugers Arch. 413, 190–196.
[26] Alison, M.R. (1986) Physiol. Rev. 66, 499–541.
[27] Xu, A., Suh, P.G., Marmy-Conus, N., Pearson, R.B., Seok, O.Y., Cocco, L. and Gilmour, R.S. (2001) Mol. Cell. Biol. 21, 2981–2990.
[28] Faenza, I., Matteuci, A., Bavelloni, A., Marmiroli, S., Martelli, A.M., Gilmour, R.S., Suh, P.G., Manzoli, L. and Cocco, L. (2002) Biochim. Biophys. Acta 1589, 305–310.
[29] Faenza, I., Matteuci, A., Manzoli, L., Billi, A.M., Aluigi, M., Peruzzi, D., Vitale, M., Castoria, S., Suh, P.G. and Cocco, L. (2000) J. Biol. Chem. 275, 30520–30524.
[30] Martelli, A.M., Billi, A.M., Manzoli, L., Faenza, I., Aluigi, M., Falconi, M., De Pol, A., Glimour, R.S. and Cocco, L. (2000) FEBS Lett. 486, 230–236.
[31] Talarmin, H., Rescan, C., Cariou, S., Glaise, D., Zanninelli, G., Bilodeau, M., Loyer, P., Guguen-Guillouzo, C. and Baffet, G. (1999) Mol. Cell. Biol. 19, 6003–6011.
[32] Fabrikant, J.I. (1968) J. Cell Biol. 36, 551–565.
[33] Irvine, R.F. and Divecha, N. (1992) Semin. Cell Biol. 3, 225–235.
[34] Fields, A.P. and Thompson, L.J. (1995) in: Progress in Cell Cycle Research (Meijer, L., Guidet, S. and Tung, H.Y.L., Eds.), vol. 1, pp. 271–286, Plenum Press, New York.
[35] Murray, N.R., Thompson, L.J. and Fields, A.P. (1997) in: Protein Kinase C (Parker, P.J. and Dekker, L.V., Eds.), pp. 97–120, R.G. Landes Company, Austin.
[36] Ye, K., Aghdasi, B., Luo, H.R., Moriarity, J.L., Wu, F.Y., Hong, J.J., Hurt, K.J., Bae, S.S., Suh, P.G. and Snyder, S. (2002) Nature 415, 541–544.
[37] Lu, P.J., Hsu, A.L., Wang, D.S., Yan, H.Y., Yin, H.L. and Chen, C.S. (1998) Biochemistry 37, 5738–5745.
[38] Lin, H., Choi, J.H., Hasek, J., DeLillo, N., Lou, W. and Vancura, P. (2000) Mol. Cell. Biol. 20, 3597–3607.
[39] York, J.D., Odom, A.R., Murphy, R., Ives, E.B. and Wente, S.R. (1999) Science 285, 96–100.
[40] Shen, X., Xiao, H., Ranallo, R., Wu, W.H. and Wu, C. (2003) Science 299, 112–114.
[41] Steger, D.J., Haswell, E.S., Miller, A.L., Wente, S.R. and O'Shea, E.K. (2003) Science 299, 114–116.
[42] Gozani, R., Karuman, P., Jones, D.R., Ivanov, D., Cha, J., Lugovsky, A.A., Baird, C.L., Zhu, H., Field, S.J., Lessnick, S.L., Villasenor, S., Mehrotra, B., Chen, J., Rao, V.R., Brugge, J.S., Ferguson, C.G., Payrastre, B., Myszka, D.G., Cantley, L.C., Wagner, G., Divecha, N., Prestwich, G.D. and Yuan, J. (2003) Cell 114, 99–111.