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Disruption of Phospholipase Cδ4 Gene Modulates the Liver Regeneration in Cooperation with Nuclear Protein Kinase C

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Phospholipase Cδ4 (PLC δ4) gene has been cloned from the cDNA library of regenerating rat liver. Using PLC δ4 gene–disrupted mice (PLC $\delta4^{-/-}$), we studied a role of PLC δ4 during liver regeneration after partial hepatectomy (PH). In PLC $\delta4^{-/-}$, liver regeneration occurred in an apparently normal way. However, BrdU-indices indicated that PLC δ4 gene disruption delayed the onset of DNA synthesis by 2 h. Noticeably, the BrdU-indices in PLC $\delta4^{+/+}$ remained rather constant throughout S phase, 25–35%, whereas in PLC $\delta4^{-/-}$, it fluctuated drastically from 25% at 34 h to 65% at late S, 42 h after PH. This fact showed that PLC δ4 gene disruption caused a higher synchronization of cell proliferation. The mRNA for PLC δ4 in PLC $\delta4^{+/+}$ appeared at late G1, and the expression continued throughout S phase. PLC activity increased transiently in chromatin at the late G1 and S phases in only PLC $\delta4^{+/+}$, but not in PLC $\delta4^{-/-}$. The specific increases in PLC activity well correlated with the transient increases of protein kinase C (PKC) α in chromatin of PLC $\delta4^{+/+}$. PKC ε also increased transiently in chromatin from PLC $\delta4^{+/+}$ at late S. It is concluded that PLC δ4 regulates the liver regeneration in cooperation with nuclear PKC α and ε.

Key words: knockout mouse, liver regeneration, partial hepatectomy, phospholipase Cδ4, protein kinase C.

Abbreviations: BrdU, 5-bromo-2′-deoxy-uridine; chromatin, chromatin-rich fraction; DG, diacylglycerol; DMSO, dimethylsulphoxide; PH, partial hepatectomy; PLC, phosphoinositide-specific phospholipase C; PLC $\delta4^{-/-}$, PLC δ4 gene knockout mouse; PLC $\delta4^{+/+}$, wild mouse; PKC, protein kinase C; PI, phosphatidylinositol; RT-PCR, reverse-transcription polymerase chain reaction.

Phosphoinositides and their metabolites play important roles in intracellular signaling for various cell functions such as cell growth and differentiation (*1*, *2*). Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in cellular signaling that hydrolyses phosphoinositides to generate diacylglycerol (DG) which activates protein kinase C (PKC) (*3*). Now, PLC isoforms are classified into 6 classes, β, γ, δ, ε, ζ and η (*4*, *5*).

We have previously reported that PLC activity in the isolated nucleus from regenerating rat liver increased transiently at S phase (*6*). Then we purified a new type of PLC, tentatively named N4 which was unique to nucleus of regenerating rat liver or rat asceites hepatoma, AH7974 cells (*7*). Liu *et al.* cloned a gene for a new type of PLC δ family from the DNA library of regenerating rat liver and designated as PLC δ4 (*8*). Evidences suggest that N4 corresponds PLC δ4 (*7*, *8*). The mRNA of PLC δ4 expresses more predominantly in regenerating liver than in resting liver (*8*). In rat, PLC δ4 is distributed in brain, testis and in tumor cells such as hepatoma and *src*-transformed cells (*8*, *9*), and is localized in nuclei (*8*). It increases at a phase transition from G1 to S and the high level continues to M phase of cells in synchronized culture (*8*). These results strongly suggest that PLC δ4 relates to cell proliferation. Three splicing variants of PLC δ4, Alt I, II and III have been cloned (*9*, *10*), and Alt III is identified as a negative regulator of PLC δ4 (*10*).

Liver regulates precisely its own growth and total mass. After surgical removal of two-thirds of the liver, hepatocytes exit a mitotically resting state (G0 phase) and enter to cell cycle, proceeding to G1, S, G2 and M phases. The hepatic mass is restored within 2-weeks after partial hepatectomy (PH) of rat or mouse. The molecular mechanism of such a strictly controlled liver regeneration has been extensively studied so far, and many growth factors and cytokines were found to be involved in the regulation of priming from G0 to G1 and the subsequent progression of G1 in liver regeneration (*11*, *12*). Much attention has

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been focused on the mechanism of G1/S transition during liver regeneration, because hepatocytes are committed to the cell division process at this point.

In the present study, we investigated the role of PLC δ4 in liver regeneration using PLC δ4 gene–knockout mouse (PLC $\delta4^{-/-}$) in comparison with the wild type (PLC $\delta4^{+/+}$). The onset of proliferation delayed around 2 h in PLC $\delta4^{-/-}$, measured by DNA replication. Furthermore, in PLC $\delta4^{-/-}$, the BrdU-labeled cells reached 65% at 42 h after PH, much higher than those in PLC $\delta4^{+/+}$, keeping 25–35% throughout. Interestingly, changes in nuclear PLC activity are well associated with those in the amount of nuclear PKC during liver regeneration in PLC $\delta4^{+/+}$. However, this relationship was destroyed by the disruption of PLC δ4 gene.

MATERIALS AND METHODS

Materials—5-Bromo-2′-deoxy-uridine (BrdU) and dimethylsulphoxide (DMSO) HYBR-MAX® were purchased from Sigma-Aldrich Chemical Co. (St.Louis, MO). Polyclonal antibodies against rabbit PKCα and PKCε were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ECL Western blotting detection system was from PerkinElmer Japan Co., Ltd. (Yokohama, Japan). [^{3}H]-phosphatidylinositol ([^{3}H]-PI) was purchased from PerkinElmer Japan Co., Ltd.

PLC $\delta4^{-/-}$ was produced as described previously (*13*). C57BL6/J mice used as PLC $\delta4^{+/+}$ were purchased from Japan SLC Corporation (Hamamatsu, Japan). Mice used here were 7- to 9-week-old male. They were bred and maintained under specific pathogen free conditions and kept in a temperature-controlled animal room with a 12 h dark/light cycle. All experiments were conducted in accordance with the guidelines on animal care of Nagoya University, Nagoya, Japan.

Partial Hepatectomy (PH)—Under ether anesthesia, mice were subjected to 70% PH according to the method of Higgins and Anderson (*14*) which has been first established for rat. The remnant liver was harvested at the regular time intervals indicated. Some was fixed in 70% ethanol for evaluation of DNA synthesis, and another was pickled in RNAlater® (Ambion Inc., Austin, TX) for reverse-transcription polymerase chain reaction (RT-PCR). The remnant was kept on ice for sub-cellular fractionation.

Mouse differed from rat in liver regeneration in some ways. As reported by Weglarz and Sandgren (*15*), mouse hepatocytes started to replicate DNA at approximately 32 h after PH (Fig. 4), *i.e.*, 10 h later than that of rat liver (*6*).

Immunohistochemical Detection of DNA Synthesis in Hepatocyte—DNA synthesis in hepatocyte was monitored by immunohistochemical staining for BrdU using ABC method (BrdU Labeling and Detection kit II, Roche Diagnostics Inc., Basel, Switzerland) (*16*). Mouse was given an intraperitoneal injection of 100 mg/g body weight of BrdU at 2 h prior to sacrifice. Ethanol-fixed liver tissues were embedded in paraffin and sectioned. After deparaffinization, the sections were treated with microwave (500 W, 15 min) in 0.01 M citrate buffer, and were further processed according to manufacturer's instruction. The number of BrdU-labeled hepatocytes was counted among more than 1,000 hepatocytes per sample.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)—The total RNA was isolated using ISOGEN® (Nippon Gene, Tokyo, Japan) from samples pickled in RNAlater® according to the manufacturer's protocol. High-salt precipitation solution (1.2 M NaCl and 0.8 M sodium citrate) was used only for liver tissues. First-strand cDNAs were synthesized with 5 mg of total RNA by using SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) for RT-PCR. For mouse PLC δ4, two sets of mouse PLC δ4–specific primers were used: first, nucleotides 1152–1171 and 1639–1658, and second, nucleotides 1180–1199 and 1639–1658. The amplification of the first amplification of mouse PLC δ4 was performed for 35 cycles of 1 min at 94°C, 45 s at 62°C and 1 min at 72°C with 1 ml of the cDNA products and the first set of primers, and the final extension step was increased to 8 min. The second-round amplification of mouse PLC δ4 was achieved by 30 cycles with the second set of primers and 1 ml of the first-round PCR products as template. The final products were separated on a 2% agarose gel (Agarose S, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The PCR product was electro-eluted from each band, purified and subjected to DNA sequencing using DNA sequencer (3100 Genetic Analyzer, Applied Biosystem, Foster City, CA). It was confirmed that the expected 383-bp and 479-bp oligonucleotides in mouse tissues were amplified specifically from PLC δ4 and Alt I cDNA, respectively. Efficiency of the cDNA synthesis and ability of the resulting cDNA to serve as a template for amplification were evaluated for each RNA sample by amplifying the first-strand products with mouse GAPDH primer; 35 cycles of PCR were performed under the same conditions as described above, and a 503-bp fragment in mouse tissues amplified.

Sub-Cellular and Sub-Nuclear Fractionations—The liver tissues were fractionated as reported previously (*17*). Briefly, each liver was homogenized in a hypotonic buffer with Kontes homogenizer (loose fitting, 10 strokes). Sucrose was added to the homogenate and adjusted to 8.5% sucrose concentration. The homogenate was centrifuged and the resultant supernatant was used as a post-nuclear fraction. The pellet was re-homogenized in 70% sucrose and centrifuged. The pellet as highly purified nuclei was re-suspended in the solution containing 8.5% sucrose, 10 mM $MgCl_2$, 0.1 mM PMSF, 15U DNase I (TAKARA BIO INC., Otsu, Japan) and 50 mM Tris/HCl (pH 7.5) and incubated at 4°C overnight. Then, the solution was centrifuged, and the pellet was washed with low salt and high salt buffers, respectively, as described previously (*17*). The soluble fractions thus obtained from DNase I–digested nuclei were put together and used as a chromatin-rich fraction (chromatin).

Immunoblot Analysis—For immunoblot analysis of PKC, aliquots of chromatin and post-nuclear fraction were subjected for electrophoresis on sodium dodecylsulfate–10% polyacrylamide gels. Protein bands were transferred to a polyvinylidene difluoride membranes. After blocking in PBS containing 5% non-fat dry milk, the membranes were incubated first with antibodies against PKCα, PKCβII, PKCδ or PKCε, followed by the second incubation with a horseradish peroxidase–linked anti–rabbit IgG antibody. Membranes were processed with ECL Western blotting detection system (PerkinElmer Japan Co., Ltd., Yokohama, Japan) according to the method recommended by the manufacturer. The bands were quantified using densitometer (Atto Co., Tokyo, Japan).

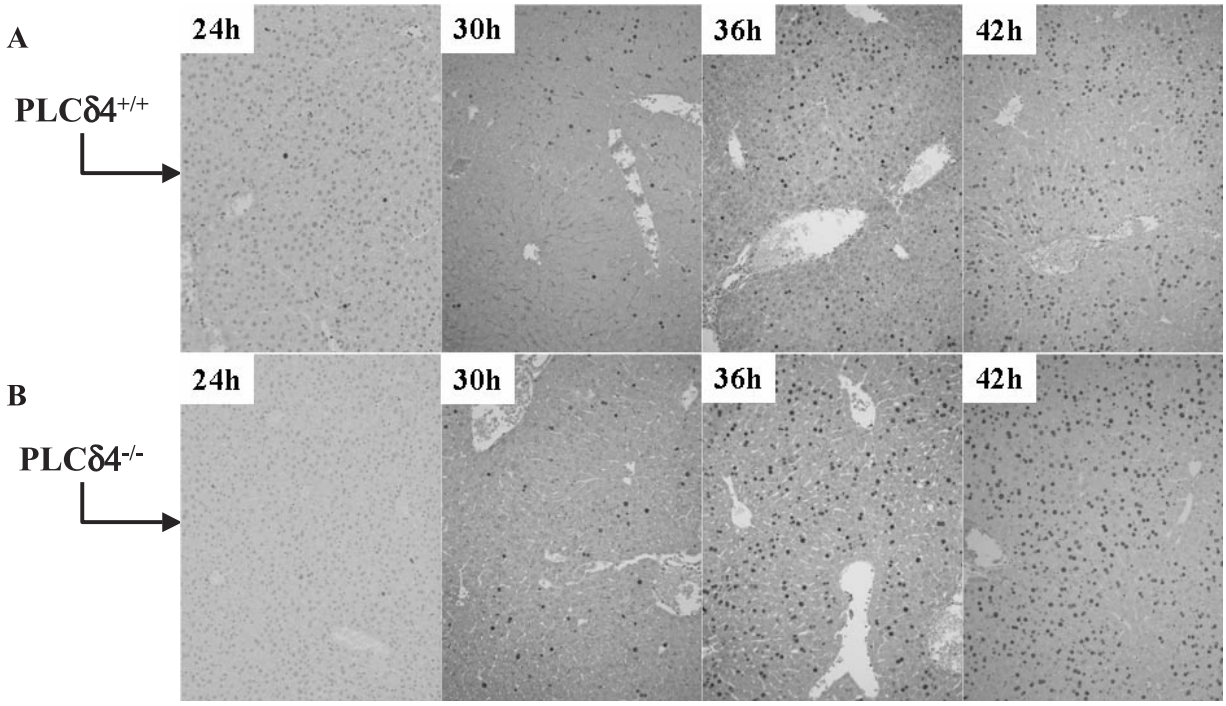

Fig. 1. **Immunohistochemical detection of BrdU-labeled hepatocyte in regenerating mouse liver after PH.** The level of DNA synthesis in hepatocytes was monitored by BrdU incorporation into nuclei. Mice were given an intraperitoneal injection of 100 mg/g body weight of BrdU at 2 h prior to sacrifice at the indicated times. The fixed and sectioned livers were incubated with anti-BrdU antibody and then anti–mouse IgG antibody. After treatment with streptavidine, peroxidase was detected by 3,3′-diaminobenzidine, and livers were counterstained with hematoxylin. A: PLC $\delta4^{+/+}$; B: PLC $\delta4^{-/-}$.

Determination of PLC Activity—PLC activity was determined according to the method of Kuriki *et al.* (*6*) in a 50 μl assay mixture containing 2.5–3.5 μg protein of chromatin or 1.2–1.5 μg of post-nuclear fraction as enzyme source, 100 mM Tris/HCl (pH 6.9), 0.06% taurodeoxycholate, 10 mM $CaCl_2$ and 5 nmol of [^{3}H] PI (8,000 dpm/nmol, adjusted by cold PI). The assay mixture was incubated at 37°C for 30 min. Under these conditions, the reaction proceeded linearly for 60 min. The reaction was terminated by adding 200 μl of chloroform/methanol (2:1, v/v). The radioactivity in the upper phase was determined by scintillation counter (Beckman LS-1701, Beckman Coulter Inc., Fullerton, CA).

RESULTS

Disruption of PLC δ4 Gene Affects the Pattern of DNA Synthesis in Liver Regeneration—Proliferating hepatocytes were detected as DNA replicating cells incorporating BrdU, which have dark stained nuclei as shown in Fig. 1. It is clearly seen that hepatocytes can proliferate in either PLC $\delta4^{+/+}$ (Fig. 1A) or PLC $\delta4^{-/-}$ (Fig. 1B), indicating that PLC δ4 is dispensable for the liver regeneration. Actually, after 70% PH, liver mass was restored within 2 weeks even in PLC $\delta4^{-/-}$, as in PLC $\delta4^{+/+}$, without obvious histological changes (Fig. 2 and data not shown). However, we found that the hepatic response to PH was not the same between PLC $\delta4^{-/-}$ and PLC $\delta4^{+/+}$. The onset of proliferation slightly delayed in PLC $\delta4^{-/-}$, measured by DNA replication (Fig. 3). PLC $\delta4^{+/+}$ liver displayed a small peak of BrdU-labeling at around 32 h, and the labeled cells increased subsequently as a large broad peak until 42 h post-hepatectomy. In contrast, BrdU-labeling in PLC $\delta4^{-/-}$ started to increase at 34 h, 2 h later than that in PLC $\delta4^{+/+}$ (Fig. 3). Noticeably, in PLC $\delta4^{+/+}$, the index was relatively stable from 25 to 35% through S phase, whereas in PLC $\delta4^{-/-}$, it increased up to 65% at the last stage of S phase, 42 h after PH.

Expression of the mRNA for PLC δ4 Is Associated with BrdU-Labeling Index in Regenerating Liver of PLC$^{+/+}$—To know the possible mechanism of such a difference described above in liver regeneration between PLC $\delta4^{+/+}$ and PLC $\delta4^{-/-}$ at first, we analyzed the expression of PLC δ4 isoforms during liver regeneration of PLC $\delta4^{+/+}$ (Fig. 4). The mRNA for PLC δ4 appeared at around 24 h after PH and its expression continued throughout S phase, then decreased. On the other hand, Alt I was expressed in a constitutive manner throughout liver regeneration, and in sham-operated livers as well as in resting liver (Fig. 4). Alt II and Alt III were not expressed in livers at all stage examined (Fig. 4). The sequences of each band corresponding to 479 bp and 383 bp on the gel were consistent with a part of Alt 1 (NM148937) and genuine PLC δ4 (BC066156) genes, respectively. Neither genuine PLC δ4 nor its variant was expressed in resting liver as well as regenerating livers of PLC $\delta4^{-/-}$ (Fig. 4).

These results are quite consistent with the concept that PLC δ4 is expressed in association with S-phase in the cell cycle of hepatocyte that has been deduced from experiments with the synchronized rat Swiss 3T3 cells (*8*). In contrast, a splicing variant, Alt I showed a constitutive expression in hepatocyte and did not have any correlation with the cell cycle.

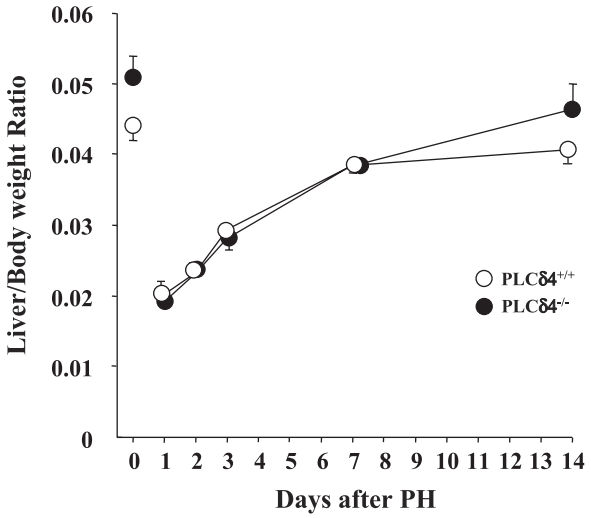

Fig. 2. **Rate of the increase of remnant liver weight after PH.** The level of recovery of remnant liver after PH was evaluated by the ratio of liver weight versus body weight. The remnant livers were cut from both PLC $\delta4^{-/-}$ and PLC $\delta4^{+/+}$ at the indicated days after PH and their weights were compared. Closed and open circles denote the ratios in PLC $\delta4^{-/-}$ and PLC $\delta4^{+/+}$, respectively. The results shown are the mean ± SE from more than 3 mice at the indicated each time.

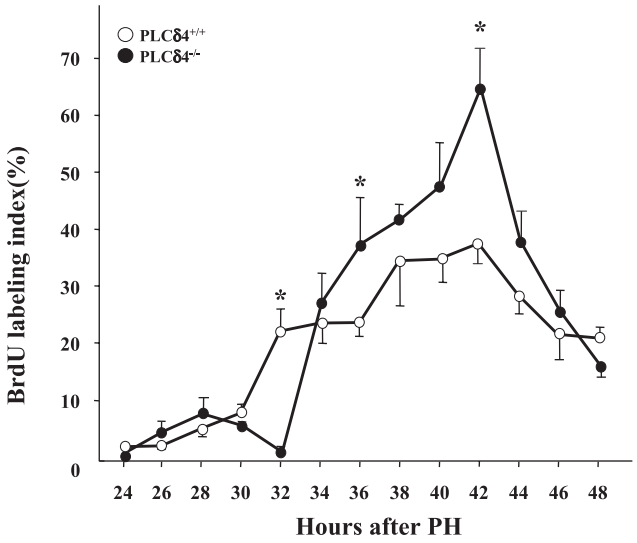

Fig. 3. **Kinetics of BrdU-labeling in hepatocytes of regenerating mouse liver after PH.** The average number of BrdU-positive hepatocytes was determined for each liver by counting more than 1,000 hepatocytes and expressed as the percentage to all hepatocytes counted. The results shown are the mean ± SE from more than 4 mice at the indicated each time. Statistical analyses were performed using Mann-Whitney U-test and the differences with a less *P*-value than 0.05 were judged to be statistically significant (*$P < 0.05$).

PLC δ4 Gene Disruption Changes PLC Activity and Content of PKC in Chromatin from Regenerating Liver Nucleus—PLC δ4 is localized in nucleus (*8*). Large part of nuclear phosphoinositides associates with chromatin (*18*, *19*) and may modulate DNA synthesis (*20*). Action of nuclear PLC may result in the increased DG content, which induces translocation of PKC to nucleus from cytosol. The nuclear PKC is implicated in transition at G1/S as well as G2/M boundary (*21–24*). In this context, we measured changes of the activity of nuclear PLC in both PLC $\delta4^{+/+}$ and PLC $\delta4^{-/-}$ livers, in relation to changes in each amount of various PKC isoforms (Fig. 5).

In PLC $\delta4^{+/+}$ liver regeneration, PLC activity in chromatin exhibited twin peak: the first peak was seen at 26 h after PH (late G1), and the second one was at 40–44 h after PH (late S) (Fig. 5A). In PLC $\delta4^{-/-}$, PLC activity was also detected in chromatin. This may be due to other PLC isoforms that compensate PLC δ4, however, the activity pattern was not the same as that in PLC $\delta4^{+/+}$. Namely, twin peak of PLC activity disappeared and the activity was lower during G1 and S (Fig. 5A). In the post-nuclear fraction, there was no difference between PLC $\delta4^{+/+}$ and PLC $\delta4^{-/-}$ with respect to PLC activity during liver regeneration (Fig. 5A).

Using immunoblotting method, we measured the contents of PKCα, βII, δ and ε in chromatin from regenerating liver nucleus, and patterns were compared between PLC $\delta4^{+/+}$ and PLC $\delta4^{-/-}$. In chromatin of PLC $\delta4^{+/+}$, PKCα exhibited two peaks, at early and late S phases (Fig. 5B). This correlated well with twin peak of chromatin PLC in PLC $\delta4^{+/+}$ (Fig. 5A). In PLC $\delta4^{-/-}$ liver regeneration, PKCα in chromatin gradually increased from early to late S phases, in a pattern which was entirely different from that of PLC $\delta4^{+/+}$ (Fig. 5B). Besides PKCα, PKCε also increased markedly and transiently at late S phase in chromatin of PLC $\delta4^{+/+}$ regenerating hepatocytes, whereas it showed only a small increase at late S in chromatin of PLC $\delta4^{-/-}$ (Fig. 5C). Neither of PKCβII nor δ in chromatin showed PLC $\delta4^{+/+}$–specific changes (data not shown). In the post-nuclear fraction of PLC $\delta4^{+/+}$ regenerating hepatocytes, the amounts of PKCα and ε did not show any noticeable changes (Fig. 5, B and C).

These results suggest that PKCα and ε in PLC $\delta4^{+/+}$ regenerating hepatocytes translocate to chromatin in response to the production of DG in chromatin by transiently expressed PLC δ4 in liver regeneration.

DISCUSSION

Though PLC δ4 gene was first cloned from a regenerating rat liver cDNA library (*8*), its role in liver regeneration has remained unclear. In the present study, we investigated the role of PLC δ4 in liver regeneration using PLC $\delta4^{-/-}$ that lacks this enzyme.

The macroscopic feature of liver regeneration in PLC $\delta4^{-/-}$ was almost the same as that in PLC $\delta4^{+/+}$. Therefore, the enzyme PLC δ4 seems to be dispensable for liver regeneration. However, the comparison of the patterns of BrdU-labeling revealed that the disruption of PLC δ4 gene delayed the entry into S phase by two hours (Fig. 3). Furthermore, the pattern of BrdU-labeling in PLC $\delta4^{-/-}$ differed from that in PLC $\delta4^{+/+}$. While 25 to 35% of hepatocytes were labeled in PLC $\delta4^{+/+}$ throughout S-phase, as high as 65% were labeled in PLC $\delta4^{-/-}$ at late S phase (Fig. 3). Thus, the proliferation was synchronized more intensely in PLC $\delta4^{-/-}$ than in PLC $\delta4^{+/+}$. It is known that PLC and its substrates, phosphoinositides, exist in chromatin (*18*, *19*). Furthermore, PLC δ4 is localized in nuclei (*8*). During liver regeneration, twin peak of PLC activity was detected in chromatin of PLC $\delta4^{+/+}$, at late

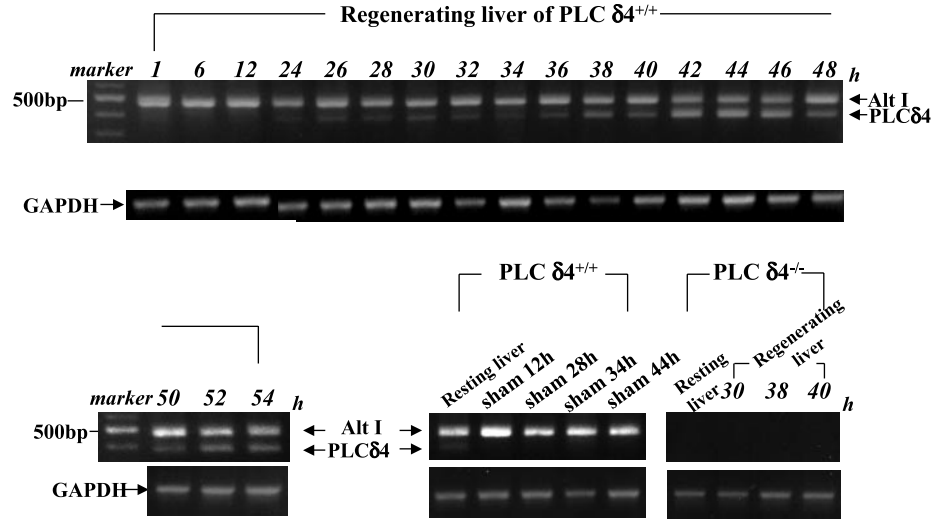

Fig. 4. **Expression of the mRNA for PLC δ4 isoforms during mouse liver regeneration.** The expression of the mRNA for PLC δ4 isoforms during liver regeneration was analyzed by RT-PCR as described in "MATERIALS AND METHODS". First-strand cDNAs were generated with 5 μg of total RNA from each liver and an oligo(dT) primer. PCR was performed with PLC δ4 specific primers and the final products were separated on a 2% agarose gel. Gel markers with a 100-bp ladder (New England BioLabs®, Inc.) were loaded in the side lane. The indicated times show hours after PH, and sham means sham-operated liver.

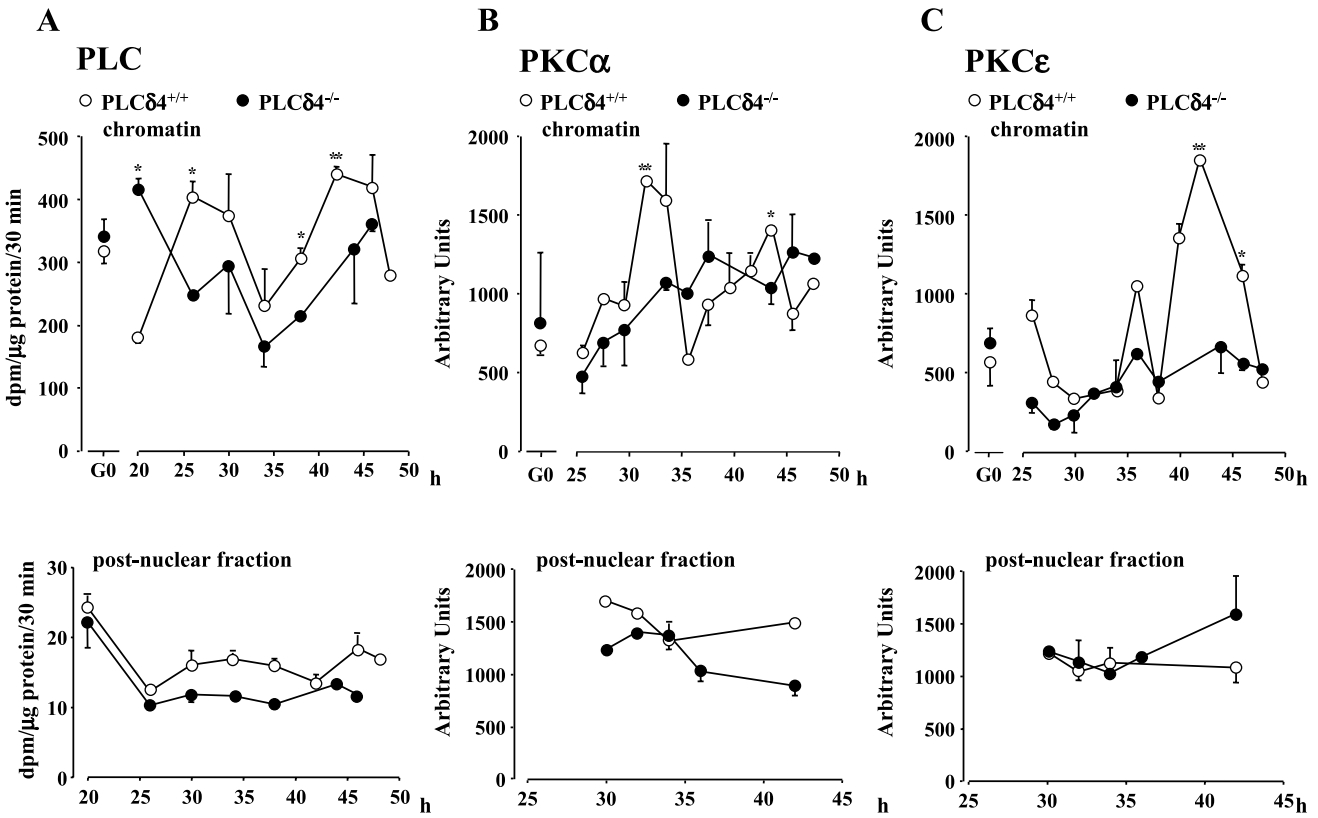

Fig. 5. **Changes in the PLC activity and the amounts of PKCα and ε in chromatin and post-nuclear fraction during mouse liver regeneration.** Highly purified nuclei and post-nuclear fraction were prepared from the resting (G0) and regenerating livers of PLC $\delta4^{+/+}$ and PLC $\delta4^{-/-}$ as described in "MATERIALS AND METHODS". The nuclei were further sub-fractionated to obtain chromatin. (A) Using chromatin and post-nuclear fraction as an enzyme source, the activity of PLC was measured. The indicated times show hours after PH. Open and closed circles denote PLC $\delta4^{+/+}$ and PLC $\delta4^{-/-}$, respectively. PLC activities in chromatin and post-nuclear fraction are shown in upper and lower panels of (A), respectively. (B) The amounts of PKCα in chromatin and post-nuclear fraction of resting (G0) and regenerating livers were determined by immunoblotting using anti-PKCα antibody. The indicated times show hours after PH. Open and closed circles denote PLC $\delta4^{+/+}$ and PLC $\delta4^{-/-}$, respectively. The amount of PKCα in chromatin and post-nuclear fraction is shown in upper and lower panels of (B), respectively. (C) Respective amounts of PKCε in chromatin and post-nuclear fraction of resting (G0) and regenerating livers were determined using anti-PKCε antibody. The indicated times show hours after PH. Open and closed circles denote PLC $\delta4^{+/+}$ and PLC $\delta4^{-/-}$, respectively. Amounts of PKCε in chromatin and post-nuclear fraction are shown in upper and lower panels of (C), respectively. The results shown are the mean ± SE from more than 3 mice at the indicated each time. Statistical analyses were performed using student *t*-Test and the differences with a less *P*-value than 0.05 were judged to be statistically significant (* or **). *Comparison is made at exactly the same time at: 26 h, 38 h in PLC; 44 h in PKC α; 46 h in PKC ε. **Comparison is made at times that differed slightly each other by a technical reason: 42 h (PLC $\delta4^{+/+}$) and 44 h (PLC $\delta4^{-/-}$) in PLC; 32 h (PLC $\delta4^{+/+}$) and 34 h (PLC $\delta4^{-/-}$) in PKC α; 42 h (PLC $\delta4^{+/+}$) and 44 h (PLC $\delta4^{-/-}$) in PKC ε.

G1 and S phases (Fig. 5A). Since the twin peak was absent in PLC $\delta4^{-/-}$, it must be derived from either of the genuine PLC δ4 or its splicing variant, Alt I (Fig. 4). We identified PLC β1, β3 and γ1 in nuclei of regenerating livers from either PLC $\delta4^{+/+}$or PLC $\delta4^{-/-}$ by immunoblotting, and found that the amount of each PLC was comparable in both nuclei (data not shown). The total PLC activity in chromatin of PLC $\delta4^{+/+}$ regenerating liver may be a sum of four kinds of PLC. On the other hand, the PLC activity in PLC $\delta4^{-/-}$ chromatin may correspond to the one that lack PLC δ4 and it might has been modified due to compensation by other PLC isoforms.

Albi *et al.* (*25*) reported that PLC β1 existed at chromatin of rat liver nucleus in association with DNA replication site and increased before the peak of DNA replication in liver regeneration, while PLC γ1 presented at the nuclear envelope and might be involved in G2/M phase transition through lamin phosphorylation. Using membrane-depleted rat liver nuclei, Crljen *et al.* (*26*) showed the existence of PLC β1b and γ1 in the nuclear matrix and PLC δ1 in chromatin. They found that total PLC activity increased at 6 and 20 h after partial hepatectomy, presumably due to the activation of PLC β1b and γ1 while PLC δ1 increased only at 20 h. On the other hand, Neri *et al.* (*27*) reported that rat liver regeneration was closely correlated with nuclear inositide cycle *via* modulation of PLC γ1. Concerning with PLC δ4, Liu *et al.* (*8*) suggested its role in rat liver regeneration, whereas others described that no PLC δ4 isoform was found in rat liver (*9*, *26*). This discrepancy may be due to the small amount of PLC δ4 in rat liver. Similarly, the expression of PLC δ4 mRNA was also low in mouse liver (Fig. 4).The nuclear localization of PLC δ4 has been confirmed in PLC δ4-overexpressed Cos7 cells by staining with anti-HA (tag) antibody, though we failed to immuno-stain PLC δ4 in liver section using polyclonal anti–PLC δ4 antibody (data not shown).

The PKC may be recruited into nucleus when DG is accumulated by the action of nuclear PLC, and the nuclear PKC may promote the progression of cell cycle at G1/S and G2/M boundaries (*21*, *24*, *28*). In this context, we measured the amount of four kinds of PKC isoforms, PKC α, βII, δ and ε, in chromatin from PLC $\delta4^{+/+}$ and PLC $\delta4^{-/-}$ during liver regeneration. In PLC $\delta4^{+/+}$, PKCα was detected as two peaks (Fig. 5B), at the times corresponded well to the twin peak of PLC activity in the chromatin (Fig. 5A). Furthermore, a peak of PKCε appeared at S phase chromatin, at exactly the same time as the latter peak of PLC as well as PKCα (Fig. 5C). Such a pattern of increases in PKCα and PKCε in chromatin was detected exclusively in PLC $\delta4^{+/+}$, but not in PLC $\delta4^{-/-}$ (Fig. 5, B and C). Neither PKC βII nor δ showed the different pattern between PLC $\delta4^{+/+}$ and PLC $\delta4^{-/-}$ during liver regeneration. It is suggested, therefore, that PLC δ4 may regulate cell cycle through nuclear PKCα and ε-associated signaling pathways. In the rat liver regeneration, Banfic *et al.* (*29*) detected a peak of nuclear DG at 20 h after PH, accompanied with a translocation of PKC into nucleus, though the type of recruited PKC was unknown. In PLC $\delta4^{-/-}$ chromatin, PKCα increased gradually from the early S phase (Fig. 5B), suggesting the existence of a mechanism that compensates PLC δ4 for recruiting nuclear PKCα in PLC $\delta4^{-/-}$.

It has been well documented that PKC isoforms are translocated into nucleus when nuclear DG is generated from phosphoinositides, but it is still unknown how cytoplasmic PKC recognizes the accumulation of nuclear DG. To solve this issue, it would be important to know where in nucleus DG accumulates. Patterns of expression of PKCα and/or PKCε strongly suggest that generation and accumulation of nuclear DG might be much higher in late S phase compared with that at late G1 phase or G1/S boundary.

In conclusion, it is strongly suggested that PLC δ4 associated with chromatin is involved in the regulation of cell cycle during mouse liver regeneration. PLC δ4 may regulate G1/S boundary through PKCα-associated signaling and also the last step of S phase through PKCα and ε-associated signaling in the nucleus.

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