Nuclear ADP-ribosylation Factor (ARF)- and Oleate-dependent Phospholipase D (PLD) in Rat Liver Cells

INCREASES OF ARF-DEPENDENT PLD ACTIVITY IN REGENERATING LIVER CELLS

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Two forms of phospholipase D (PLD) have been found to be present in nuclei isolated from rat hepatocytes by measuring phosphatidylbutanol produced from exogenous radiolabeled phosphatidylcholine in the presence of butanol. In nuclear lysates from either rat liver or ascites hepatoma AH 7974 cells, the PLD activity was markedly stimulated by a recombinant ARF-ribosylation factor (rARF) in the presence of the guanosine 5'-O-(3-thiotriphosphate) (GTPγS) and phosphatidylinositol 4,5-bisphosphate. ATP and phorbol-12-myristate 13-acetate had no synergistic effect on this PLD activity. On the other hand, the nuclear PLD was stimulated by unsaturated fatty acids, especially by oleic acid. The ARF-dependent nuclear PLD activity was increased in the S-phase of the regenerating rat liver after partial hepatectomy and also was much higher in AH 7974 cells than in the resting rat liver. In contrast, the levels of the oleate-dependent PLD activity remained constant throughout the cell cycle in liver regeneration. The intranuclear levels of the stimulating proteins of the nuclear PLD activity, e.g. ARF, RhoA, and protein kinase Cδ increased in the S-phase of the regenerating liver. These results suggested that the nuclear ARF-dependent PLD activity may be associated with cell proliferation.

It has been known that cell nuclei contain a variety of enzymes generating lipid second messengers, such as sphingomyelinase (1), phospholipase A2 (2), PI1-specific phospholipase C (3–6) and lipid kinases (7). Growth factors seem to be able to affect the phosphoinositide metabolism in nuclei, suggesting a role of PLD in nuclei (8, 9). In addition, accumulation of diacylglycerol (DG) in nuclei and translocation of protein kinase C to them have been demonstrated in a variety of cell types (10, 11). A large rise in mass of DG, with only small changes in mass of phosphoinositides, suggested a source of DG other than polyphosphoinositides, for example, phosphatidylcholine (PC), in nuclei (12). It was also shown that phosphatidylethanol formation from phosphatidylcholine, which was specifically catalyzed by phospholipase D (PLD) in the presence of ethanol, was induced by PMA in nuclei isolated from kidney cells and that nuclei possess the ability to generate DG and phosphatidic acid through the PLD-phosphatidic acid phosphohydrolase pathway. Thus, upon cell stimulation with agonists, the enzymes involving PC metabolism are considered to be activated in the nucleus as well as in the plasma membrane.

It was demonstrated that PLD activity can be regulated by several factors (14, 15); oleate, small G proteins (ARF and Rho family), phosphatidylinositol 4,5-bisphosphate (PIP2), phosphatidylethanolamine (PE), Ca2+, protein kinase C, protein tyrosine kinase. Recently, two different types of PLD, oleate-dependent and ARF-dependent, were isolated from rat brain membranes (16). The oleate-dependent PLD was purified from pig liver microsomes (17), and ARF-dependent PLD activity was found to be abundant in Golgi-enriched membranes from several cell lines (18). More recently, a gene of human ARF-dependent PLD (hPLD1) has been cloned (19). It has been shown that the membrane-associated PLD activity was modulated by protein kinase C and Rho family (20–24). These observations suggest the presence of different isoforms of PLD.

The nuclear PLD activity from Madin-Darby canin kidney (MDCK)-D1 cells was observed to be regulated by protein kinase C and RhoA (23). It was also shown that an oleate-dependent form of PLD was present in rat brain neuronal nuclei and inhibited by acidic phospholipids, such as PIP2 (25). However, the physiological roles of PLD in nuclei remain unclear.

In this study, we have demonstrated that two forms, ARF-dependent and oleate-dependent PLD activities, were present in the isolated nuclei from rat resting liver, regenerating liver, and rat ascites hepatoma cells AH7974 and characterized their PLD activities using exogenous radiolabeled PC substrates. Furthermore, we have shown a transient elevation of the nuclear ARF-dependent PLD activity, but not the oleate-dependent PLD activity during S-phase of liver regeneration.

EXPERIMENTAL PROCEDURES

Materials—[2-palmitoyl-9,10-3H]Dipalmitoylphosphatidylcholine (DPPC) (37.5 Ci/mmol) was obtained from DuPont NEN. Egg phosphatidylcholine (egg PC), PIP2, PE, and sodium oleate were from Sigma. GTPγS was from Boehringer (Mannheim, Germany). Antibody against ARF1 was a generous gift from Dr. Joel Moss (National Institutes of Health, Bethesda, MD). Antibodies to PKC isozymes (α, βI, βII,
γ, δ, ε), RhOa, Cdc42, Rac1, ERK-2, and P38 MAP kinase were purchased from Santa Cluz Biotechnology (San Francisco, CA). Anti-Gil2α antibody was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Isolation of Nuclei—Nuclei were isolated and purified from rat ascites hepatoma AH7974 cells and rat liver cells as described previously (26). They were highly pure as examined by electron microscopic examination and by determining marker enzymes for microsomes (glucose-6-phosphatase), plasma membrane (5'-nucleotidase), and mitochondria (cytochrome oxidase). DNA polymerase α activity was measured in isolated nuclei as described previously (27). The isolated nuclei were resuspended in 0.25 M sucrose, 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, and 1 mM phenylmethylsulfonyl fluoride. 

Regeneration of Rat Liver—Rats (7-week-old) of inbred Donryu strain were used in all experiments. Partial hepatectomy was performed under light ether narcosis, and two-thirds of the liver was surgically removed according to the method of Higgins and Anderson (28). Livers were obtained as follows: control livers from nontreated and sham-operated rats and regenerating livers from partially hepatomized rats (1, 2, 5, 8, 12, 18, 20, 22, 24, 26, 28, 30, and 32 h post-hepatectomy).

Nuclear Phospholipase D in Rat Liver Cells

TABLE I

| Rat liver cells | AH cells |
|----------------|---------|
| [H]IP3 formation was measured in the nuclear lysates from rat liver (3 μg of protein), or from AH cells (1 μg of protein) by using [3H]IP3PC in PE/PIP/egg PC vesicles (substrate 1) without or with rARF (5 μM/GTP-S; 30 μM) and [3H]IP3PC in egg PC vesicles (substrate 2) with 0.5 mM oleate as described under “Experimental Procedures.” Results are given as mean ± S.E. from duplicate determinations of three different experiments.

| Rat liver cells | AH cells |
|----------------|---------|
| None (substrate 1) | 0.19 ± 0.08 |
| ARF/GTP-S | 0.61 ± 0.12 |
| None (substrate 2) | 0.08 ± 0.01 |
| Oleate | 0.49 ± 0.09 |
| None | 1.52 ± 0.23 |

Specific activities of the ARF-dependent and oleate-dependent PLD of AH7974 nuclei were approximately 10- to 3-fold higher than those of rat resting liver nuclei, respectively. When used PE/PIP/PC vesicles as substrate, the PLD activity without stimulators was high in the AH7974 nuclei. In the AH7974 nuclei, as shown in Fig. 1, the [H]IP3 formation in the absence of GTP-S showed a linear increase in a time-dependent manner, although a much less increase than that with GTP-S, indicating the presence of a GTP-S-independent PLD activity. Addition of GTP-S (30 μM) to AH7974 nuclear lysates increased the PLD activity in a time-dependent manner (Fig. 1A). The rate of [H]IP3 formation was linear for at least 60 min. The PLD activity without GTP-S was not affected by ATP (0.5 mM). The PLD activities with or without GTP-S were increased by PIP2 in a dose-dependent manner (Fig. 1B). The ratio of PIP2:PC was 3:1 for the maximal activation. The PLD activity without GTP-S in the nuclei isolated from rat resting liver was very low, even in the presence of PIP2.

The nuclear PLD activities of both rat resting liver and AH7974 cells were increased by rARF in the presence of 30 μM GTP-S in a dose-dependent manner, reaching a maximal level at 3 μM (Fig. 2A). Linear increases in the ARF-dependent PLD activity were observed up to 2 and 5 μg of proteins of the AH7974 and rat resting liver nuclear lysates, respectively (Fig. 2B). Both nuclear ARF-dependent PLD activities were linearly stimulated by low concentration of Ca²⁺ with maximal effect at 0.3 μM and inhibited by higher concentrations than 1 μM (Fig. 2C). Mg²⁺ stimulated both nuclear ARF-dependent PLD activities with maximal concentration of 3 mM (Fig. 2D).

It has been known that PKC had a synergistic stimulatory effect of the ARF-dependent PLD activity (32). To examine the effect of PKC on the nuclear ARF-dependent PLD activity, the nuclear lysates of the rat resting liver and AH7974 cells were incubated with GTP-S (30 μM), rARF (5 μM), PMA (100 nM), and ATP (0.5 mM) or their combination. As shown in Fig. 3, rARF was most potent activator for both nuclear PLD activities. PMA had no synergistic stimulatory effect on the nuclear ARF-dependent PLD activity in both nuclei.

To examine the oleate-dependent PLD activity, the rat liver and AH7974 nucleus were incubated with [3H]IP3PC in egg PC vesicles as substrate in the presence of 0.5 mM oleate. The rate of [H]IP3 formation of their nuclear lysates was linear for at least 60 min, and a linear increase of [H]IP3 formation was observed up to 50 μg of protein of their nuclear lysates (data not shown). Characterization of the oleate-dependent PLD activity of the AH7974 nuclei was shown in Fig. 4. An optimal pH was 6.0–7.0 in 50 mM HEPES/NaOH buffer (Fig. 4A). Lower concentration of Ca²⁺ less than 0.1 mM had little effect on the [H]IP3 formation induced by oleate, but its higher concentrations over 0.5 mM were rather inhibitory. On the other hand, Mg²⁺ stimulated the PLD activity with the maximal level at 1
Oleic acid was the most potent in stimulating the AH7974 nuclear PLD activity. The [3H]PBut formation was measured by using [3H]DPPC in PE/PIP2/egg PC vesicles as substrate as described under “Experimental Procedures.” B, [3H]PBut formation was measured by using [3H]DPPC in PE/PIP2/egg PC (indicated various molar ratios of PIP2/egg PC) vesicles as substrate. Results are given as mean ± S.E. from duplicate determinations of three different experiments.

Changes of Nuclear PLD Activity in Regenerating Liver Cells—In order to examine whether the nuclear PLD activity is somehow associated with cell proliferation, PLD activity was measured in nuclei isolated from regenerating liver of partially hepatectomized rats. The ARF-dependent PLD activity began to increase 23 h after hepatectomy, reaching a peak at 26–28 h (Fig. 5B). At 28 h after hepatectomy the nuclear ARF-dependent PLD activity was not affected by GTPγS, PIP2, rARF, or PMA (data not shown), indicating that the nuclear oleate-dependent PLD is distinct from the ARF-dependent one.

Nuclear Phospholipase D in Rat Liver Cells
FIG. 5. Changes in the nuclear PLD and DNA polymerase α activities during liver regeneration. Livers were obtained from nontreated, sham-operated rats, and regenerating livers from partially hepatectomized rats (1, 2, 5, 8, 12, 18, 20, 24, 26, 28, 30, and 32 h post-hepatectomy). Nuclei were prepared from each liver as described under “Experimental Procedures.” [3H]PBut formation was measured in nuclear lysates (3 μg of protein) of regenerating livers from operated rats (●) and of control livers from sham operated rats (○) by using [3H]DPPC in PE/PIP2/egg PC vesicles for the ARF-dependent PLD activity (A, B) and [3H]DPPC in egg PC vesicles for the oleate-dependent PLD activity (C) as described under “Experimental Procedures.” DNA polymerase α activity was determined by the method described previously (27) (D). Results are given as mean ± S.E. from duplicate experiments of three different experiments.

PKCo was not changed, and the PKCβII level rather decreased during liver regeneration. Significant increases in the nuclear levels of p42 and p38 MAP kinases were observed in S-phase. To examine the intranuclear G protein levels, the isolated nuclei from AH7974, the resting rat liver, and regenerating liver (28 h after partial hepatectomy) were blotted with various specific antibodies. Analysis of the Western blotting revealed the presence of low molecular weight GTP-binding proteins, ARF, RhoA, and Cdc42 and heterotrimeric GTP-binding protein, Gi2 (Fig. 7). The nuclear ARF and RhoA levels of AH7974 cell were 9- and 2-fold higher than those of rat resting liver cells, respectively, although the Cdc42 and Gi2 levels were almost similar between both nuclei. ARF protein was scant in the rat resting liver nuclei, but it was 5-fold increased in the nuclei of S-phase. A little increase of nuclear RhoA level was observed in the S-phase (1.3-fold).

DISCUSSION

We have demonstrated here that two forms of PLD activity, GTPγS-dependent and oleate-dependent, were present in the isolated nuclei from rat resting, regenerating liver cells, and rat ascites hepatoma AH7974 cells. Their GTPγS-dependent PLD activities were markedly stimulated by rARF. The nuclear ARF-dependent PLD activity was very high in the AH7974 cells, and its elevation was observed at S-phase of the regenerating liver cells. The nuclei isolated from the regenerating liver and AH cells contained ARF, RhoA, Cdc42, and various
PKC isozymes (PKCa, PKCβII, and PKCb). The ARF-dependent PLD has been known to be activated synergistically by either RhoA, Cdc42, or PKCa (21, 22, 32, 34, 35). Balboa et al. (13, 23) have reported that isolated nuclei from MDCK cells contain an ATP-dependent PLD activity in the presence of GTPγS was inhibited by protein kinase C inhibitors (chelerythrine and calphostin C) and ATP-ribosylation of RhoA by C3 exoenzyme, indicating that the MDCK nuclear PLD activity is synergistically regulated via RhoA and protein kinase C. However, we have not observed synergy in the nuclear ARF-dependent PLD activities of both rat liver cells and AH7974 cells by either ATP or PMA when assayed with an exogenous substrate (PE/PIP2/PC vesicles), although considerable PKCa was present in these nuclei. C3 exoenzyme is known to block the binding to effector enzymes by ADP-ribosylating Rho proteins (RhoA, RhoB, and RhoC) (36). Recently, Clostridium difficile toxin B has been found to be available for analyzing the role of Rho family proteins in PLD activation (37, 38). Toxin B is monoglucosyltransferase catalyzing the incorporation of glucose into threonyl residue at position 37 of Rho family proteins (Rho, Rac, and Cdc42) (39). The ARF-dependent nuclear PLD activities of the hepatic cells were partially inhibited by toxin B, but not by C3 exoenzyme, suggesting that other Rho family protein rather than RhoA (most likely Cdc42) may be involved in the nuclear ARF-dependent PLD activity of rat hepatic cells (data not shown). Thus the rat liver nuclear PLD may be different from the MDCK nuclear enzyme in lower activation by PKC and insensitivity to C3 exoenzyme.

Several studies have shown that ARF plays an essential role in membrane trafficking by controlling the reversible assembly of the coat protein complex on the surface of Golgi membranes (40) and that ARF and ARF-stimulated PLD activity is enriched in Golgi membranes (18, 41). On the other hand, Boman et al. (42) have suggested a role for ARF in nuclear vesicle dynamics during mitosis. Our results indicated that the specific activity of the nuclear ARF-dependent PLD activity, but not oleate-dependent enzyme, was transiently elevated in S-phase of rat regenerating liver cells. Moreover, the ARF-dependent PLD activity was extremely high in the nuclei of rat hepatoma AH7974 cells compared with that of the resting liver cells. The nuclear ARF levels was high in the AH7974 cells, and the increase of nuclear ARF level was observed in S-phase. Thus, one would speculate that the nuclear ARF-dependent PLD may be activated during S-phase to generate nuclear signaling molecules required for DNA synthesis or to enter into G2 phase.

There are some possible explanations for the transient elevation of the nuclear ARF-dependent PLD activity during S-phase of rat liver regeneration. First, the GTPγS-dependent PLD activity in nuclei is stimulated by translocation of cytosolic ARF to nuclei. This possibility could be supported by our finding that level of the nuclear ARF was increased in the S-phase and were higher in the AH7974 nuclei. Second, certain PLD is newly expressed by receptor activation by various stimuli produced after hepatectomy. The level of PLCβ4 in nuclei has been known to increase in liver regeneration (6). Finally, translocation of PLD to nuclei occurs in response to external stimuli induced by hepatectomy. Recent reports have demonstrated that cPLA2 and MAP kinases also are translocated to nuclear envelope by cell stimulation with growth factors and A23187 (43–45). However, the mechanism underlying the transient elevation of the nuclear ARF-dependent PLD activity in the rat-regenerating liver remains to be disclosed.

A number of studies have provided evidence suggesting roles for the activation of nuclear PKC in nuclear functions involving phosphorylation of transcription factors and DNA replication factors (46). Also it has been shown that the activation of nuclear PKC is correlated with an increase in nuclear DG. Divecha et al. (8, 10) have demonstrated that a turnover of nuclear PI is important in modulation of nuclear PKC in NIH3T3 cells. Furthermore, these notions are supported by the presence of nuclear PLC (3–6). On the other hand, Jarpe et al. (12) have shown that the source of the thrombin-induced nuclear DG in fibroblasts is PC, not PI by examining the molecular species profiles. These reports suggest that both PI and PC contribute to the formation of nuclear DG, but the hydrolyzing enzymes are differentially located in the nucleus; PLC is associated with the internal matrix (7), while the PC-hydrolyzing enzyme exists in the nuclear envelope (11). The result was supported by our results; the envelope-depleted nuclei from rat liver cells and AH7974 cells after treatment with Triton X-100 as described by Payrastre (7) had less activity of the nuclear ARF-dependent PLD, suggesting that the ARF-dependent PLD activity was located in the nuclear envelope (data not shown).

Many laboratories have demonstrated the nuclear localization of PKC isozymes in both resting and stimulated cells (11, 47–49). PKCβ is located in the nuclei from unstimulated liver (47). We have demonstrated in this study that various levels of PKC isozymes, high levels of PKCa and PKCβII and low level of PKCb, were present in the isolated nuclei from rat resting liver cells and that the nuclear PKCβ level, but not PKCβII, was increased during S-phase. These results lead us to assume that nuclear translocation of the PKCβ during S-phase may result from increase of nuclear DG levels produced by activation of the nuclear ARF-dependent PLD.

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