Increased Activity of Small GTP-binding Protein-dependent Phospholipase D during Differentiation in Human Promyelocytic Leukemic HL60 Cells

Kenji Ohguchi‡, Shigeru Nakashima‡, Zhiming Tan§, Yoshiko Banno‡, Shuji Dohi§, and Yoshinori Nozawa¶

From the Departments of ‡Biochemistry and §Anesthesiology and Critical Care Medicine, Gifu University School of Medicine, Tsukasamachi-40, Gifu 500, Japan

In response to dibutyryl cyclic AMP (dbcAMP) and all-trans retinoic acid, human promyelocytic leukemic HL60 cells differentiate into granulocyte-like cells. In cell lysate and in vitro reconstitution system, phospholipase D (PLD) activity in response to guanosine 5′-O-(3-thiotriphosphate) (GTPγS) was up-regulated by dbcAMP or all-trans retinoic acid treatment. In the present study, the mechanism(s) for increased PLD activity during differentiation was examined. Western blot analysis revealed that the contents of ADP-ribosylation factor, Rac2, and Cdc42Hs but not RhoA and Rac1 in the cytosolic fraction were elevated during differentiation. However, the cytosolic fraction from undifferentiated cells was almost equally potent as the cytosolic fraction from differentiated cells in the ability to stimulate membrane PLD activity. It was shown that the GTPγS-dependent PLD activity in membranes from differentiated cells was much higher than that in membranes from undifferentiated cells, suggesting that the increased PLD activity during differentiation was due to alterations in some membrane component(s). Clostridium botulinum ADP-ribosyltransferase C3 and C. difficile toxin B, which are known as inhibitors of RhoA and Rho family proteins, respectively, effectively suppressed PLD activity in membranes from differentiated cells. In fact, the amount of membrane-associated RhoA was increased during differentiation. Furthermore, the extent of GTPγS-dependent PLD activity partially purified from membranes from differentiated cells was greater than that from membranes from undifferentiated cells in the presence of recombinant ADP-ribosylation factor 1. The PLD (hPLD1) mRNA level was observed to be up-regulated during differentiation, as inferred by reverse transcription-polymerase chain reaction. Our results suggest the possibility that the increased Rho proteins in membranes and the changed level of PLD itself may be, at least in part, responsible for the increase in GTPγS-dependent PLD activity during granulocytic differentiation of HL60 cells.

Increasing evidence has indicated that phospholipase D (PLD)1 plays an important role in signal transduction in many types of cells (1). PLD hydrolyzes membrane phospholipids, especially phosphatidylcholine (PC), and produces phosphatic acid, which can be further metabolized to diacylglycerol by phosphatidic acid phosphohydrolase (2). PLD is activated by many extracellular signal molecules, and several factors are involved in its activation. In reconstitution experiments, activation of membrane-associated PLD by nonhydrolyzable guanine nucleotide, guanosine 5′-O-(3-thiotriphosphate) (GTPγS), was observed only when cytosol was present in the reaction mixture (3). Similar findings were obtained in permeabilized cells in which the loss of cytosolic components resulted in the reduction of GTPγS-dependent PLD activity (4). These results imply that cytosolic factors for PLD activation are presumed to be GTP-binding proteins. Indeed, two small GTP-binding proteins have been identified as regulatory factors for PLD activity (1). ADP-ribosylation factor (Arf) from the brain cytosol acts as a cytosolic factor to activate PLD in human promyelocytic leukemia HL60 membranes (5, 6). In addition to Arf, evidence for the involvement of Rho family proteins in PLD activation comes from the demonstration that Rho-specific GDP dissociation inhibitor inhibits activation of PLD by GTPγS in neutrophil membranes (7), rat liver plasma membranes (8), and HL60 membranes (9). Our recent study (10, 11) has demonstrated that RhoA and protein kinase C exerted a synergistic stimulation of membrane-associated PLD activity in HL60 cells.

HL60 cells can be differentiated into a mature granulocyte-like phenotype by compounds such as dibutyryl cyclic AMP (dbcAMP) (12), dimethyl sulf oxide (13), and retinoic acid (14). Differentiated HL60 cells possess many of the functional characteristics of granulocytes (15). Several studies have indicated the possibility that receptor-mediated PLD activation may play essential roles in the secretory response and O2 or H2O2 generation in neutrophils and differentiated HL60 cells (16–25). Differentiated (but not undifferentiated) HL60 cells exhibit formylmethionylleucylphenylalanine (fMLP)- and ATP-induced PLD activation (17). Moreover, it was shown that PLD activities stimulated by 4β-phorbol 12-myristate 13-acetate in intact cells and by GTPγS in electroporpermabilized cells increased during differentiation induced by dbcAMP (17).

To gain further insight into the mechanism underlying the enhancement of PLD activity during differentiation, we have studied GTPγS-dependent PLD activation in the lysate and the reconstitution system (the membrane and cytosolic fractions) prepared from undifferentiated and differentiated HL60 cells.
GTP$^7$S-dependent membrane PLD activity is higher in differentiated cells than in undifferentiated cells, although comparable amounts of Arf and Rho family proteins were present in the cytosolic fractions from both undifferentiated and differentiated HL60 cells. The results obtained in the present study suggest the possibility that the translocation of RhoA from the cytosol to membrane and the changed level of PLD protein may be, at least in part, responsible for the increase in GTP$^7$S-dependent PLD activity during differentiation.

EXPERIMENTAL PROCEDURES

Materials—RPMI 1640 medium, penicillin, streptomycin, and recombinant inverse transcartse were obtained from Life Technologies, Inc. [H]Olive acid and palmitoyl-2-Hidipalmitoyl phosphatidylcholine (DPPC) were from Du Pont NEN. dbcAMP, all-trans retinoic acid (ATRA), nitroblue tetrazolium (NBT), NAD, UDP-glucose, brefeldin A, phosphatidylinositol 4,5-bisphosphate, egg PC, and phosphatidylethanolamine were from Sigma. GTP$^7$S was from Boehringer Mannheim. Silica Gel 60 (LKD6) plates were from Whatman. Reparin-Sepharose was from Pharmacia Biotech Inc. Protein concentrator equipment (Centricon 10) was from Amicon. Protein determination reagents were from Bio-Rad. Antibodies against Rho family small GTP-binding proteins (RhoA, Rac1, Rac2, and Cdc42Hs) were from Santa Cruz Biotechnology. E. coli bearing Clostridium botulinum ADF-ribosyltransferase C3 (C3 toxin) plasmid (27) was generously provided by Dr. Alan Hall (University College London, London, England). DNA polymerase and random hexamer were provided by Dr. David M. Lyerly (TechLab, Inc., VPI Corporate Research Center, VA). Other reagents were of the highest quality available.

Cell Culture and Differentiation of HL60 Cells—The human promyelocytic leukemia HL60 cell line was kindly supplied by Dr. T. Okazaki (Osaka Dental University, Japan). HL60 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 mg/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO$_2$ at 37°C. For differentiation, cells were cultured in serum-free RPMI 1640 medium supplemented with 5 mg/ml insulin and 5 mg/ml transferrin for 24 h. Differentiation was initiated by the addition of 0.5 mM dbcAMP or 1 mM ATRA. For the differentiation marker, NBT reduction activity was measured (12). For assay of PLD activity utilizing the endogenous substrate, cells were labeled with [H]Olive acid (0.5 mg/ml) and monolonal antibody against Arf were generous gifts from Dr. Joel Moss (National Institutes of Health). E. coli bearing Clostridium botulinum ADF-ribosyltransferase C3 (C3 toxin) plasmid (27) was generously provided by Dr. Alan Hall (University College London, London, United Kingdom). C. difficile toxin B (Toxin B) was kindly provided by Dr. David M. Lyerly (TechLab, Inc., VPI Corporate Research Center, VA). Other reagents were of the highest quality available.

Preparation of Lysates, the Membrane, and Cytosolic Fractions from HL60 Cells—HL60 cells were washed twice with buffer A (25 mm Na-HEPES, pH 7.4, 100 mm KCl, 3 mm NaCl, 5 mm MgCl$_2$, 1 mm EGTA, 5 mm Na-HEPES, pH 7.5, 80 mm KCl, 1 mm dithiothreitol, and 10 mM MgCl$_2$, 3 mm EGTA, and 2 mM CaCl$_2$ to give a final free Ca$^{2+}$ concentration of 300 mM (total, 0.1 mL) and stimulated with 10 mM GTP$^7$S at 37°C for 30 min in the presence of butanol (0.3%, v/v). To measure oleate-dependent PLD activity using exogenous substrate, the assays were carried out with egg PC and [H]PBut was identified by comigration with PBut standard. The spots scraped off the plate were mixed with scintillation mixture, and the radioactivity was counted in a liquid scintillation counter (Beckman LS-6500).

Western Blot Analysis—The membrane and cytosolic fractions were isolated from HL60 cells as described above. Membranes were washed once in buffer D (20 mm Tris-HCl, pH 7.4, 10 mM EGTA, 2 mM EDTA, 5 mm dithiothreitol, 1 mM phenylmethyisulfon fluoride, and 10 mM MgCl$_2$) and resuspended in buffer D containing 1% Triton X-100. After incubation at 4°C for 60 min, the suspension was centrifuged at 100,000 g for 60 min to obtain the membrane extract. Proteins of the membrane extracts and cytosolic fractions were separated by SDS-polyacrylamide gel electrophoresis on a 13% polyacrylamide gel (31) and then electrophoretically transferred onto nitrocellulose membrane (32). Blocking was performed in Tris-buffered saline containing 5% skimmed-milk powder and 0.05% Tween 20. Western blot analysis using specific antibodies was performed as described previously (33). The intensity of the bands was quantized by a densitometer (Densitograph, ATTO, Japan).

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA was isolated from HL60 cells by acid guanidine thiocyanate method (34). 2 g of RNA was reverse-transcribed by using random hexamer mixed primers. Temperatures used for PCR were: denaturation, 94°C for 30 s; annealing, 58°C for 1 min; and extension, 72°C for 1 min. Primers for hPLD1 were 5'-2549-TGTCGG-GATAACCTTCTGCGCA-3' (sense) and 5'-3080)-AGCATTCGCGACT-GCTGGTGA-3' (antisense) (35). For normalization, glyceraldehyde-3-phosphate dehydrogenase was amplified simultaneously using the sense primer 5'-2575-AGCAGTGTGCTTATGG-3' and the antisense primer 5'-2575-TGACCTTGGAGGAGGTC-3' (36). Amplification cycles were determined for individual primer sets to maintain an exponential rate of product amplification. Amplified DNA fragments were subjected to electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. The intensity of the bands was quantified by a densitometer (Densitograph, ATTO, Japan).

RESULTS

Changes in GTP$^7$S-dependent PLD Activity during Cell Differentiation—The temporal correlation between cell differentiation and PLD activity was investigated by examining changes in GTP$^7$S-dependent PLD activity in HL60 cell lysates at various stages during differentiation. Differentiation was initiated by the addition of 0.5 mM dbcAMP and assessed by NBT reduction test. Less than 5% of cells expressed NBT reduction activity in the undifferentiated state. With dbcAMP treatment, NBT-positive cells increased in a time-dependent manner and reached the maximal level at 72 h (Fig. 1A). As shown in Fig. 1B, PLD activation induced by 10 mg GTP$^7$S in the cell lysates was up-regulated after dbcAMP treatment and reached an almost maximal level at 72 h (an approximately 4-fold increase compared with that of undifferentiated cells). The temporal profile of NBT reduction activity showed a good correlation with that of GTP$^7$S-dependent PLD activation. In addition, ATRA, which is also known to induce granulocytic differentiation of HL60 cells, caused an almost identical increase in NBT-positive cells (Fig. 1C). GTP$^7$S-induced PLD activity in HL60 cells was also increased by ATRA treatment (an approximately 1.8-fold increase compared with that of un-
differentiated cells) but the magnitude was lower than that observed in dbcAMP-treated cells (Fig. 1D).

GTPγS-induced PLD Activation in Reconstitution System from Undifferentiated and Differentiated HL60 Cells—The reconstitution systems from both undifferentiated and differentiated HL60 cells were designed to further investigate the mechanisms for the increase in GTPγS-dependent PLD activity during differentiation. Recent studies with the cell-free system (3) and cytosol-depleted permeabilized cells (4) have indicated that some cytosolic factors are required for activation of PLD by GTPγS. We have examined PLD activity by GTPγS in the membranes from undifferentiated and differentiated HL60 cells labeled with [3H]oleic acid. In the absence of the cytosolic fraction, GTPγS caused a marginal PLD activation in undifferentiated membranes (Fig. 2A). However, when the cytosolic fraction from undifferentiated HL60 cells was present in the reaction mixture, the PLD activity was enhanced nearly 4-fold by the addition of 10 μM GTPγS (Fig. 2A). In contrast, as shown in Fig. 2B, GTPγS and the cytosolic fraction exerted much greater effects on the membrane PLD activation in differentiated HL60 cells (an approximately 3-fold increase compared with that of undifferentiated cells). When the GTPγS-dependent PLD activity in the cytosolic as well as membrane fractions was determined using the exogenous substrate (phosphatidylethanolamine/phosphatidylinositol 4,5-bisphosphate/egg PC, 10:1:5:1), the differentiation induced by dbcAMP caused a small but significant enhancement in cytosolic PLD activity (Fig. 3). The GTPγS-induced PLD activation in both undifferentiated and differentiated membranes was dependent on the cytosolic fraction from undifferentiated and differentiated cells, respectively. Furthermore, the maximal PLD activation in membranes from differentiated cells was approximately 3-fold higher than that in undifferentiated membranes (Fig. 3B). Thus, similar results were obtained in both endogenous and exogenous substrate systems.

Because recent studies have demonstrated the implication of Arf and Rho family proteins as cytosolic factors in the regulation of PLD activity in HL60 cells (5, 6, 9), changes in the contents of Arf and Rho family proteins in the cytosolic fractions were examined by Western blot analysis. As shown in Fig. 4, the contents of Arf, Rac2, and Cdc42Hs in the cytosolic fractions from differentiated cells (Cyt-D) were approximately 3.5-fold higher than those in undifferentiated cells (Cyt-UD). In contrast, there were no significant increases in the levels of RhoA and Rac1 (Fig. 4).

In the next experiments, the effects of the cytosolic fraction from undifferentiated or differentiated cells were examined for GTPγS-dependent PLD activity in undifferentiated membranes (Mem-UD). Two cytosolic fractions (Cyt-D and Cyt-UD) enhanced PLD activity in a concentration-dependent manner (Fig. 5). However, relatively modest differences were observed between the undifferentiated and differentiated cytosolic fractions. The PLD activity by GTPγS in membranes isolated from undifferentiated or differentiated cells was compared in the presence of undifferentiated cytosol (Cyt-UD). The activation of GTPγS-dependent PLD in membranes (Mem-D) from differentiated cells was markedly distinct from that in membranes (Mem-UD) from undifferentiated cells (Fig. 6). These results suggested that the increased PLD activity during differentiation was caused primarily by changes of membrane component(s), e.g., activating factor(s) and/or PLD itself, but not of cytosolic factors.

Changes in GTPγS-dependent PLD Activity and Contents of Rho Family Proteins in Undifferentiated and Differentiated HL60 Membranes—PLD activity in differentiated membranes was stimulated by GTPγS even in the absence of cytosolic...
C3 toxin, which inhibits RhoA (27), on GTP
S-dependent PLD activation in differentiated HL60 membranes was almost abolished by Toxin B treatment. These results suggest that RhoA may play a major role in GTPγS-induced PLD activation in differentiated HL60 membranes. Thus, to further examine the roles of Cdc42Hs and Rac in PLD activation of differentiated HL60 membranes, we used Toxin B, which was recently shown to glucosylate Rho family proteins and inhibit their interaction with effectors (28). As shown in Fig. 7B, GTPγS-induced PLD activation in differentiated HL60 membranes was almost abolished by Toxin B treatment. These results suggest that RhoA may play a major role in GTPγS-induced PLD activation in differentiated HL60 membranes. Then, the contents of Rho family proteins in membranes of both undifferentiated and differentiated cells were examined by Western blot analysis. As shown in Fig. 8, A and B, the amounts of RhoA greatly increased in membranes during differentiation. There was a smaller but appreciable increase in Cdc42Hs. The levels of Rac1 and Rac2 in membranes
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were unchanged during differentiation. Furthermore, the RhoA contents of HL60 membranes also increased during ATRA-induced differentiation (Fig. 8, C and D). In contrast, brefeldin A, which is often used as an inhibitor of Arf protein (37), did not affect PLD activation by GTPγS (data not shown). In addition, the level of Arf1 in membranes as assessed by Western blot analysis was not altered during differentiation induced by dbcAMP and ATRA (data not shown).

Effects of Arf1 and Oleate on Membrane-associated PLD Activity from Undifferentiated and Differentiated HL60 Cells—At least two types of PLD have been described in several tissues: the small GTP-binding proteins-dependent type and the oleate-dependent type (29, 38–40). The effects of Arf1 and sodium oleate were examined for PLD activity in undifferentiated and differentiated membranes. The Arf1-stimulated PLD activity in differentiated membranes (Mem-D) was found to be 3.5-fold higher than that in undifferentiated membranes (Mem-UD, Fig. 9A). On the other hand, sodium oleate (4 mM) failed to activate PLD in membranes from either undifferentiated or differentiated cells (Fig. 9B). The oleate-dependent PLD activity in rat brain membranes was observed under the same assay condition (data not shown).

To separate PLD from RhoA, membranes were treated with 1% cholate and subjected to heparin-Sepharose column chromatography. RhoA was recovered in the flow-through fraction (Fig. 10A), in which Arf1-dependent PLD activity was not detected (Fig. 10B). In contrast, Arf1-dependent PLD activity was obtained in 1 M NaCl extract, in which RhoA protein was undetectable by Western blot analysis (Fig. 10, A and B). The effects of recombinant Arf1 on this partially purified PLD from undifferentiated and differentiated membranes were examined. As shown in Fig. 10C, GTPγS in the presence of recombinant Arf1, but not alone, enhanced PLD activity. The Arf1-mediated PLD activity from differentiated membranes (PLD-D) was found to be nearly 3-fold higher than that from undifferentiated membranes (PLD-UD, Fig. 10C).

Changes in hPLD1 mRNA Level during HL60 Cell Differentiation—Recently, a gene encoding Arf-dependent PLD (hPLD1) has been cloned from the HeLa cell cDNA library (35). To investigate the possible changes responsible for the increase in Arf-induced PLD activation during differentiation, hPLD1 mRNA levels were examined by RT-PCR. As shown in Fig. 11, A and B, treatment of HL60 cells with 0.5 mM dbcAMP resulted in the elevated expression of hPLD1 mRNA. The hPLD1 messages nearly reached maximal levels at 48 h. Increases in the levels of hPLD1 mRNA were also observed in ATRA-treated HL60 cells (Fig. 11, C and D). In contrast with dbcAMP treatment, a relatively modest elevation was observed in hPLD1 by RT-PCR. Because the antibody for hPLD1 is not yet available, we are unable to determine the level of hPLD1 protein. However, the observed increase in hPLD1 mRNA levels leads us to presume that its protein level would be increased in differentiated HL60 cells induced by dbcAMP or ATRA.

DISCUSSION

The possible involvement of PLD in secretory response and O2− or H2O2 generation has been indicated in neutrophils and differentiated HL60 cells (16–24). Because Arf, an activator of PLD, is known to play an important role in vesicular trafficking (44), Arf-mediated PLD activation can be considered to take part in the vesicular transport process (42, 45). A recent report (46) showed evidence that PLD plays important roles in cytoskeletal organization. These findings also suggest that Arf-

FIG. 7. Changes of GTPγS-dependent PLD activity in undifferentiated and differentiated HL60 membranes. A, membranes (20 μg of protein/assay) from undifferentiated HL60 cells (UD) or differentiated cells treated with 0.5 mM dbcAMP for 72 h (D) were incubated with 10 μM GTPγS and the substrate phospholipid vesicles containing [palmitoyl-3H]DPPC at 37°C for 30 min in the presence of 0.3% butanol (total, 0.1 ml). B, membranes (20 μg of protein/assay) from differentiated cells treated with 0.5 mM dbcAMP for 72 h were preincubated with 1 μg/ml C3 toxin in the presence of 10 μM GTPγS or 1 μg/ml Toxin B in the presence of 50 μM UDP-glucose at 37°C for 15 min and then incubated with 10 μM GTPγS and the substrate phospholipid vesicles containing [palmitoyl-3H]DPPC at 37°C for 30 min in the presence of 0.3% butanol (total, 0.1 ml). PLD activity was determined by measuring the formation of [3H]Phos as described under “Experimental Procedures.” Data represent the means ± S.D. from two different experiments, each carried out in duplicate.

FIG. 8. Changes in the contents of Rho family proteins in HL60 membranes during differentiation. A, membrane extracts (Triton X-100-soluble fraction) from HL60 cells treated with 0.5 mM dbcAMP for the indicated times were electrophoresed. The amount of membrane proteins used in these experiments was 20 μg for RhoA and 50 μg for Cdc42Hs, Rac1, and Rac2. Western blot analyses with specific antibodies were performed as described under “Experimental Procedures.” B, quantification of relative amounts of proteins was performed by scanning the spots on the film with a densitometer. Data represent the means from two different experiments. C, membrane extracts (Triton X-100-soluble fraction) from HL60 cells treated with 0.5 mM dbcAMP (72 h) or 1 μM ATRA (72 h) were electrophoresed. Western blot analysis with anti-RhoA antibody was performed as described under “Experimental Procedures.” D, quantification of the relative amounts of proteins was performed by scanning the spots on the film with a densitometer. Data represent the means from two different experiments.
and Rho-mediated PLD activity may be involved in the regulation of bacterial function and motile responses in neutrophils. HL60 cells could be a useful model to verify these hypotheses because the cells are endowed with neutrophil-like functions during differentiation. It is well documented that HL60 cells are differentiated into granulocyte-like cells by treatment with dbcAMP (12, 17, 19, 47, 48). Xie et al. (17) have demonstrated that dbcAMP-differentiated but not undifferentiated HL60 cells exhibited receptor-mediated PLD activation. HL60 cell differentiation is accompanied by the expression of cell surface receptors for fMLP (15). Moreover, PLD activities stimulated by 4β-phorbol 12-myristate 13-acetate in intact cells and by GTPyS in electropermeabilized cells were increased during HL60 differentiation (17). We have also observed that PLD activity in response to fMLP or 4β-phorbol 12-myristate 13-acetate was enhanced in dbcAMP-treated HL60 cells (data not shown). The increase in PLD activity during differentiation correlated well with that of NBT reduction activity (Fig. 1). These findings have prompted us to investigate the mechanism underlying increased PLD activity during differentiation. GTPyS-dependent PLD activity increased in a time-dependent manner during differentiation induced by dbcAMP in HL60 cells (Fig. 1), suggesting a lack of either activating factor(s) for PLD or of PLD itself in undifferentiated cells. In fact, the protein levels of Arf, Rac2, and Cdc42Hs in the HL60 cytosolic fraction were elevated during differentiation (Fig. 4). However, the levels of cytosolic small GTP-binding proteins for the membrane-associated PLD activation were thought to be sufficient in undifferentiated cells because the membrane-associated PLD activity was equally activated by the cytosolic fractions from either undifferentiated or differentiated cells (Fig. 5).

It was noted that the PLD activity in differentiated membrane was higher than that in undifferentiated membrane (Fig. 7A), suggesting that activating factor(s) for PLD activity and/or
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PLD itself in membrane were increased during differentiation. Indeed, C3 toxin and Toxin B effectively inhibited GTPγS-induced [3H]IP2But formation (Fig. 7B). These results indicate that RhoA may play a major role in GTPγS-induced PLD activation in differentiated HL60 membranes. This notion was supported by Western blot analysis showing the increases in RhoA and Cdc42Hs in differentiated membranes (Fig. 8). The enhancement of GTPγS-dependent PLD activity during differentiation can be explained, at least in part, by an increase in RhoA content in the membrane. RhoA and Arf exert a synergetic effect on PLD activation in several cell types, including HL60 cells (9, 41, 43). On the other hand, our previous study (10) has shown that Rho-specific GDP dissociation inhibitor, which removes Rho proteins from membranes, suppressed synergistic PLD activation by GTPγS and protein kinase C in HL60 membranes and has also shown that this suppressed PLD activation was restored by the addition of recombinant RhoA. Thus, RhoA plays a key role in Arf- and protein kinase C-induced PLD activity in HL60 cells.

On the other hand, the increase in RhoA content in membranes could be explained by its translocation from cytosol or newly synthesized RhoA to membrane probably after post-translational modification (geranyl geranylation). The pre-treatment of cells with mevastatin to inhibit hydroxymethylglutaryl-CoA reductase involved in the formation of isoprenoids (49) did not prevent an increase in membrane-associated RhoA during differentiation induced by dbcAMP (data not shown). Thus, the former hypothesis is more likely. Cytosolic Rho family proteins form complexes with the Rho-specific GDP dissociation inhibitor in the GDP-bound form (50). Translocation to membrane, which is believed to be an activation process, requires dissociation from the Rho-specific GDP dissociation inhibitor and an exchange of bound GDP for GTP (48). Aepfelbacher et al. (51) have demonstrated that monocyctic differentiation in U-937 cells was associated with an increase in membrane-associated Cdc42Hs. However, its detailed mechanism is not clearly understood. Further investigation of the increases in membrane-associated RhoA during differentiation will provide a clue toward the better understanding of the mechanism underlying the membrane translocation of RhoA.

There is an alternate possibility for the differentiation-dependent increase in the membrane PLD activity. Arf-mediated PLD activity in differentiated membranes was shown to be higher than in undifferentiated membranes (Figs. 9 and 10), suggesting the increased expression of membrane-associated PLD during differentiation. This notion was supported by RT-PCR with primers designed for hPLD1. The hPLD1 mRNA levels were increased by dbcAMP or ATRA treatment of HL60 cells (Fig. 11). However, at present we do not know whether the hPLD1 protein level was increased because its antibody is not yet available.

In summary, GTPγS-dependent membrane PLD activity was elevated during HL60 cell differentiation induced by dbcAMP. Similar results were obtained in experiments with differentiated HL60 cells induced by ATRA, thus supporting the notion that the increase in RhoA content in membranes and changes in PLD expression are associated with granulocytic differentiation. However, additional studies are required to disclose the precise mechanism for the increased GTPγS-induced PLD activation during the differentiation of HL60 cells.

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Addendum—After submission of this manuscript, a paper was published describing the lysophosphatidic acid-induced translocation of RhoA in Rat1 fibroblasts and its possible implication in PLD activation (Malcolm, K. C., Elliott, C. M., and Exton, J. H. (1996) J. Biol. Chem. 271, 13135–13139).

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