In response to α-melanocyte-stimulating hormone (α-MSH) or cAMP-elevating agents (forskolin and isobutylmethylxanthine), mouse B16 melanoma cells underwent differentiation characterized by increased melanin biosynthesis. However, the mechanism(s) underlying the regulation of melanogenesis during differentiation has not yet been clearly understood. Phospholipase D (PLD) has been reported to be involved in differentiation. This enzyme cleaves phosphatidylcholine upon stimulation with stimuli to generate phosphatidic acid. In the current study, the involvement of PLD in the regulation of melanogenesis characteristic of differentiation was examined using mouse B16 melanoma cells. Treatment of B16 cells with α-MSH was found to cause marked decreases in the PLD1 activity concurrent with its reduced protein level. Moreover, treatment of exogenous bacterial PLD also inhibited α-MSH-induced melanogenesis. To further investigate the role of PLD1 in the regulation of melanogenesis, we examined the effects of overexpression of PLD1 on melanogenesis in B16 melanoma cells. The B16 cells overexpressing PLD were prepared by transfection with the vector containing the cDNA encoding PLD1. The melanin contents in PLD1-overexpressing cells (B16/PLD1) were observed to be lower compared with those in the vector control cells (B16/Vec), concomitant with the decreases in both activity and protein level of tyrosinase, a key regulatory enzyme in melanogenesis. Moreover, overexpression of PLD1 resulted in a marked inhibition of melanogenesis induced by α-MSH. The inhibition of melanogenesis was well correlated with the decrease in the tyrosinase activity associated with its expression. These results indicated that PLD1 negatively regulated the melanogenic signaling by modulating the expression of tyrosinase in mouse B16 melanoma cells.

Phospholipase D (PLD) cleaves phosphatidylcholine (PC) in response to a variety of cell stimuli to generate phosphatidic acid (PA) (1–3), which acts as a second messenger and can be further converted to other messenger molecules, 1,2-diacylglycerol, and lysoPA (1–3). Two mammalian PLD genes, PLD1 and PLD2, have been cloned (4). PLD1 is regulated by small GTP-binding proteins such as ADP-ribosylation factor and Rho family proteins and also by protein kinase C (2, 3). On the other hand, PLD2 is constitutively active and requires phosphatidylinositol 4,5-bisphosphate (PIP2) in the intracellular system (2, 3), but the precise mechanisms that regulate PLD2 activity are still undefined. PLD is considered to be implicated in a variety of cellular responses including cell differentiation (5, 6). Several lines of evidences support a role for PLD in differentiation (7). Our previous studies have demonstrated changes in the mRNA expression and activity of PLD during differentiation of HL-60 cells (8–10) and C6 glioma cells (11). In addition, similar findings were obtained in differentiation of epidermal keratinocytes (12), PC12 pheochromocytoma cells (13, 14), HT-29 colon cells (15), and LA-N-1 neuroblastoma cells (16). However, little information is available regarding the precise role and regulatory mechanism of PLD in cell differentiation.

Melanogenesis is a principal parameter of differentiation in melanocytes and melanoma cells (17). Synthesis of melanin starts from the conversion of l-tyrosine to 3,4-dihydroxyphenylalanine (l-dopa), and then the oxidation of l-dopa yields dopaquinone by tyrosinase, the rate-limiting step enzyme in the melanin biosynthesis (18, 19). In melanocytes or melanoma cells, melanogenesis can be induced by α-melanocyte-stimulating hormone (α-MSH) and cAMP-elevating agents (forskolin and isobutylmethylxanthine) (20, 21). α-MSH, which induces differentiation in melanocytes or melanoma cells, binds to its specific receptor (MC1R), resulting in the activation of stimulatory GTP-binding protein (Gα), which in turn stimulates adenylate cyclase to produce cAMP (20, 21). cAMP increases melanogenesis mainly via activation of microphthalmia-associated transcription factor, a melanocyte-specific transcription factor, thereby leading to induction of tyrosinase expression (21–26). However, the mechanism(s) for the regulation of melanogenesis has not fully been understood. Thus, it is of interest to know whether PLD is implicated in the melanogenic differentiation of pigment cells. In this context, the current was designed to examine the involvement of PLD in melanogenesis of B16 melanoma cells and we demonstrate for the first time that PLD1 exerts a negative regulatory role in the melanogenic signaling by modulating expression of tyrosinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—l-dopa, [Nle4,D-Phe7]-α-MSH, synthetic melanin, Strep-toomyces chromofuscus PLD, Clostridium perfringens PLC, the antibody to β-actin, and 45-phorbol 12-myristate 13-acetate (PMA) were from Sigma. Guanosine 5′-O-(3-thiotriphosphate) (GTPγS) was from Roche Applied Science. [palmitoyl-1-14C]Dipalmitoylphosphatidylcholine was from PerkinElmer Life Science, LipofectAMINE and G418 were from Invitrogen. WST-1 cell proliferation assay system was from TaKaRa. The antibody to tyrosinase was from Santa Cruz Biotechnology. Poly-
cloned anti-PLD1 and anti-PLD2 antibodies were prepared as described previously (27). Anti-rabbit antibody conjugated with horseradish peroxidase and the chemiluminescence (ECL) kit was obtained from Amersham Biosciences. Expression plasmid of wild-type human PLD1 was kindly supplied by Dr. Michael A. Frohman (Institute for Cell and Developmental Biology, State University of New York, New York, NY). Other reagents were of the highest quality available.

**Cell Culture and Induction of Melanogenesis—**Mouse B16 melanoma cells were purchased from Riken Cell Bank (Tsukuba, Japan). B16 melanoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) that was supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified CO2-controlled (5%) incubator. Induction of melanogenesis was initiated by the addition of 1 μM [Nle4,D-Phe7]-MSH. For treatment with the bacterial phospholipases (S. chromofuscus PLD or C. perfringens PLC), cells incubated with 100 units/ml PLD or PLC.

**Transfections—**For establishment of transfectants overexpressing PLD1, we used a pCGN eukaryotic expression vector. B16 melanoma cells were transfected with pCGN-PLD1 by using LipofectAMINE according to the manufacturer’s lipofection protocol. The transfectants then were selected by using medium containing 0.7 mg/ml G418 for 7 days. The stable clones (B16/PLD1) were established, and their expression level of PLD1 was confirmed by Western blot analysis. The clone (B16/Vec) was transfected with a vector alone.

**Measurement of PLD Activity—**Cells were washed twice with phosphate-buffered saline (PBS) and resuspended in buffer A (25 mM HEPES, pH 7.4, 3 mM NaCl, 10 mM KCl, 5 mM MgCl2, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and 10 μg/ml leupeptin). Cells were then sonicated three times at 15 s each. After unbroken cells and nuclei were removed by centrifugation at 900 × g for 5 min, the resulting supernatant was used as cell homogenate for additional experiments. PLD activity in cell homogenate was measured by production of [3H]phosphatidylbutanol (PBut), a specific product via transphosphatidylation activity. PLD activity in cell homogenate was measured using the exogenous substrate of phospholipid vesicles. Mixed lipid vesicles (phosphatidylethanolamine/PIP2/egg PC, 10:1.5:1 molar ratio) were added to cell homogenate (50 μg protein) in the reaction mixture containing 50 mM HEPES, pH 7.5, 3 mM EGTA, 80 mM KCl, 2.5 mM MgCl2, 2 mM CaCl2, and 1 mM dithiothreitol and were stimulated with 50 μM GTPγS and 100 nM PMA at 37°C for 1 h in the presence of 0.3% butanol. After extraction of lipids, [3H]PBut was separated by TLC and measured by a densitometer. The intensity of the bands was quantified by a densitometer.

**Cell Growth Assay—**Cells were seeded on a 96-well plate at a concentration of 1 × 104 cells/well in a volume of 100 μl and grown for 48 h. Cell numbers were measured colorimetrically using a WST-1 cell proliferator assay system.

**Electrophoresis and Western Blot Analysis—**Proteins were separated by SDS-PAGE on 7.5% polyacrylamide gel for PLD and tyrosinase. Proteins were transferred electrophoretically onto a polyvinylidene fluoride membrane. Blocking was performed in Tri-buffered saline containing 5% skim milk powder and 0.05% Tween 20. Western blot analysis using specific antibodies was performed as described previously (29). The intensity of the bands was quantified by a densitometer.

**RESULTS**

The mouse melanoma cell line B16 has been used for studying the molecular events during melanogenic differentiation. In this study, melanogenesis was initiated by the addition of 1 μM α-MSH and assessed by determination of intracellular melanin content. After stimulation with α-MSH, melanin content increased in a time-dependent manner, reaching the maximal levels at 72 and 96 h (Fig. 1). We then have examined the changes in PLD activity in homogenates of B16 melanoma cells treated with α-MSH for 72 h. It is well accepted that PLD1 is activated by the small GTP-binding proteins and protein kinases in the presence of PIP2. On the other hand, PLD2 exhibits constitutive activity, which requires PIP2 but not other factors. As shown in Fig. 2A, the PLD activity was considerably enhanced by the addition of GTPγS and PMA in the presence of PIP2. However, surprisingly, there was no significant stimulation of the PLD activity by GTPγS and PMA when cells were pretreated with α-MSH for 72 h. GTPγS and PMA-independent PLD activity was unchanged after the same treatment with α-MSH (Fig. 2A). Since Western blot analysis showed the presence of PLD1 but not PLD2 (Fig. 2B), the increased PLD activity by the addition of GTPγS and PMA was attributed to PLD1. Thus, it is thought that PLD1 activity was decreased during melanogenesis induced by α-MSH. To know the mechanism for such marked decrease in the decrease in PLD1 activity associated with melanogenesis, the level of PLD1 protein...
was examined by Western blot analysis. As shown in Fig. 2B, the treatment of B16 melanoma cells with α-MSH for 72 h caused a marked depression PLD1 protein expression with a ~0.2-fold decrease compared with that of untreated cells. These findings indicated that the decreased PLD1 activity in differentiated B16 cells showing active melanogenesis was because of down-regulation of PLD1 and also suggested that PLD1 activity is associated with melanogenesis.

To further investigate the involvement of PLD in the regulation of melanogenesis, the effect of exogenous PLD on α-MSH-induced melanogenesis was examined. In the presence of 100 units/ml S. chromofuscus PLD, when B16 melanoma cells were treated with α-MSH, significant decrease in α-MSH-induced melanogenesis was observed compared with that in the absence of exogenous PLD (Fig. 3). In contrast, C. perfringens PLC was ineffective on α-MSH-induced melanogenesis (Fig. 3). These observations led us to assume that PLD1 exerts a negative regulatory role in melanogenesis in B16 melanoma cells stimulated with α-MSH.

To get more insight into the role of PLD in melanogenesis, we examined the effects of PLD1 overexpression on melanogenesis in the cells. The stable cell line overexpressing PLD1 (B16/PLD1) was established by the transfection with a vector containing the cDNA encoding wild-type PLD1. Cells transfected with an empty vector served as controls (B16/Vec). Western blot analysis using specific antibody as described under “Experimental Procedures.” Data represent the mean ± S.D. of two different experiments each carried out in duplicate. Statistical analysis was performed using Student’s t test.

Fig. 3. Effect of exogenous bacterial phospholipases on α-MSH-induced melanogenesis in B16 melanoma cells. The cells were treated with 1 μM [Nle4, D-Phe7]α-MSH for 72 h with 100 units/ml S. chromofuscus PLD or 100 units/ml C. perfringens PLC, and the melanin content was determined as described under “Experimental Procedures.” Data represent the mean ± S.D. of two different experiments each carried out in duplicate. Statistical analysis was performed using Student’s t test.

Fig. 4. Overexpression of PLD1 in B16 melanoma cells. The cells stably transfected with empty vector or PLD1 cDNA were maintained in DMEM supplemented with 10% fetal bovine serum containing 0.7 mg/ml G418. A, the levels of PLD1 expression in the transfected B16 melanoma cells (B16/Vec and B16/PLD1) were monitored by Western blot analysis using specific antibody as described under “Experimental Procedures.” B, cell growth of B16/Vec and B16/PLD1 melanoma cells were measured by WST-1 assay as described under “Experimental Procedures.” Data represent the mean ± S.D. of two different experiments each carried out in duplicate. C, B16/Vec and B16/PLD1 melanoma cells under phase-contrast microscopy.

Fig. 5. Effects of PLD1 overexpression on melanin content, tyrosinase activity, and the protein level of tyrosinase in B16 melanoma cells. The B16/Vec and B16/PLD1 melanoma cells were maintained in DMEM supplemented with 10% fetal bovine serum containing 0.7 mg/ml G418. A, the melanin content was determined as described under “Experimental Procedures.” B, tyrosinase activity was determined by measuring the formation of dopachrome as described under “Experimental Procedures.” Data represent the mean ± S.D. of two different experiments each carried out in duplicate. C, the amount of tyrosinase protein in the transfected B16 melanoma cells was performed by Western blot analysis using specific antibody as described under “Experimental Procedures.”
In mammalian cells, PLD is considered to be implicated in a variety of cellular responses, which include regulated secretion, membrane trafficking, cytoskeletal reorganization, proliferation, and meiosis (1–3). In addition, several lines of evidence indicate the involvement for PLD in cell differentiation. Our previous studies have shown that increases in mRNA expression and activity of PLD1/2 are associated with differentiation in HL-60 cells. As shown in Fig. 5B, the tyrosinase activity was decreased in B16/PLD1 cells. Furthermore, the level of the tyrosinase protein was examined by Western blot analysis and was observed to be lower in B16/PLD1 cells than that in control B16(Vec) cells (Fig. 5C). These results suggested that PLD1 participates in melanogenesis probably through regulating the expression of tyrosinase in B16 melanoma cells.

The effects of PLD1 overexpression were examined next for α-MSH-induced melanogenesis. Whereas there was a time-dependent increase in melanin content in control B16/Vec cells stimulated with α-MSH, melanogenesis was almost completely repressed in PLD1-overexpressed cells (Fig. 6). Because melanogenesis is dependent upon the tyrosinase activity, we examined the effect of PLD1 overexpression on α-MSH-mediated increase in tyrosinase activity. Although the tyrosinase activity in B16/Vec cells was enhanced nearly 3-fold by the treatment with α-MSH for 72 h, the activity was not increased in B16/PLD1 cells (Fig. 7A). The expression levels of tyrosinase protein evaluated by Western blot analysis were compared in B16/Vec cells and B16/PLD1 cells with or without stimulation with α-MSH. α-MSH-induced increase in tyrosinase protein levels in B16/Vec cells was no longer observed in B16/PLD1 cells (Fig. 7B). To investigate the relationship between inhibition of melanogenesis and PA production, a primary alcohol 1-butanol was used to inhibit the PA production by PLD. In the presence of 1-butanol but not t-butanol, PBut was efficiently produced at the expense of PA by the transphosphatidylation activity of PLD. 1-Butanol of 0.4% (v/v) or less did not affect cell growth in B16/PLD1 cells (data not shown). As shown in Fig. 8A, the treatment of 1-butanol at 0.4% recovered melanogenesis in B16/PLD1 cells. In contrast, t-butanol was without any effect on melanogenesis in B16/PLD1 cells (Fig. 8B). Under the same condition, treatment of B16/PLD1 cells with 1-butanol (0.4%) induced the expression levels of tyrosinase proteins (Fig. 8B). Taken together, the results presented here have demonstrated evidence to suggest that PLD1 plays a negative regulatory role in melanogenesis through modulating the expression of tyrosinase.

**DISCUSSION**

In mammalian cells, PLD is considered to be implicated in a variety of cellular responses, which include regulated secretion, membrane trafficking, cytoskeletal reorganization, proliferation, and meiosis (1–3). In addition, several lines of evidence indicate the involvement for PLD in cell differentiation. Our previous studies have shown that increases in mRNA expression and activity of PLD1/2 are associated with differentiation in HL-60...
cells (9, 10), C6 glioma cells (11), and PC12 pheochromocytoma cells (13). Other reports (12, 15, 16) have also demonstrated the implication of PLD in cell differentiation of several cell types. However, little information is available regarding the precise role and regulatory mechanism of PLD in cell differentiation. Mouse B16 melanoma cells have been known to undergo differentiation by stimuli such as α-MSH (20, 21). The differentiated B16 melanoma cells are characterized by increased melanin biosynthesis. Thus, the B16 melanoma cells have often been employed as a useful model for studying the molecular basis for melanogenesis during differentiation.

In this study, we have examined the involvement of PLD in the regulation of melanogenesis using B16 melanoma cells. The intracellular melanin content increased in a time-dependent manner during differentiation induced by α-MSH in B16 melanoma cells (Fig. 1). Interestingly, the GTP-yS/PS-dependent PLD activity was found to be decreased after treatment of α-MSH in B16 melanoma cells (Fig. 2A). Since Western blot analysis revealed that PLD1 but not PLD2 was expressed in B16 melanoma cells (Fig. 2B), it can be considered that PLD1 activity was decreased after treatment of α-MSH in B16 melanoma cells (Fig. 2A). Since Western blot analysis revealed that PLD1 but not PLD2 was expressed in B16 melanoma cells, the GTP-yS/PS-dependent PLD activity was considered to reflect PLD1 activity. We then examined the expression level of PLD1 protein in α-MSH-treated B16 melanoma cells. Decreases in PLD1 protein expression determined by Western blot analysis were well correlated with those in GTP-yS/PS-dependent PLD activity (Fig. 2B), suggesting that the α-MSH-induced decrease in PLD1 activity was because of the repressed expression of PLD1 enzyme. These findings led us to indicate that PLD1 is implicated in melanogenesis.

To further investigate the role of PLD1 in the regulation of melanogenesis, we examined the effect of overexpression of PLD1 on melanogenesis in B16 melanoma cells. It was shown that the melanin synthesis was markedly repressed in PLD1-overexpressed B16 melanoma cells (B16/PLD1) (Figs. 5A and 6C). It can be considered that the PLD1 activity acts as a negative regulatory factor in the melanogenic process. As tyrosinase is known to be the key enzyme that regulates melanogenesis, we examined whether overexpression of PLD1 affects the tyrosinase activity. The PLD1-overexpressed cells (B16/PLD1) clearly showed decreases in its activity compared with the control cells (B16/VeG) (Fig. 5B and 7A). This decreased tyrosinase activity was considered to be attributed to decreases in the expression level of the enzyme as assessed by Western blot analysis using the antibody against tyrosinase (Figs. 5C and 7B). Taken together, we demonstrated that PLD1 down-regulates the expression of tyrosinase protein, although the exact mechanisms remain to be disclosed.

On the other hand, several intracellular signaling pathways have been reported to involve melanogenesis. The activation of extracellular signal-regulated kinase (ERK) cascade leads to an inhibition of melanogenesis (31). ERK phosphorylates microphthalmia-associated transcription factor and promotes its degradation, thereby resulting in inhibition of tyrosinase expression and melanogenesis (32, 33). Furthermore, the specific inhibitors of phosphatidylinositol 3-kinase (PI3K), wortmannin, and LY294002 stimulate melanin synthesis, suggesting that the PI3K pathway is involved in the regulation of melanogenesis (34, 35). One of the effectors of PI3K is p70S6-kinase that the PI3K pathway is involved in the regulation of melanogenesis (32, 33). Furthermore, the specific thalmia-associated transcription factor and promotes its deactivation (31). ERK phosphorylates microphthalmia-associated transcription factor to stimulate melanin synthesis, suggesting that the PI3K pathway is involved in the regulation of melanogenesis (32, 33). Furthermore, the specific thalmia-associated transcription factor and promotes its deactivation (31). ERK phosphorylates microphthalmia-associated transcription factor to stimulate melanin synthesis, suggesting that the PI3K pathway is involved in the regulation of melanogenesis (32, 33). Furthermore, the specific thalmia-associated transcription factor and promotes its deactivation (31). ERK phosphorylates microphthalmia-associated transcription factor to stimulate melanin synthesis, suggesting that the PI3K pathway is involved in the regulation of melanogenesis (32, 33). Furthermore, the specific thalmia-associated transcription factor and promotes its deactivation (31). ERK phosphorylates microphthalmia-associated transcription factor to stimulate melanin synthesis, suggesting that the PI3K pathway is involved in the regulation of melanogenesis (32, 33). Furthermore, the specific thalmia-associated transcription factor and promotes its deactivation (31). ERK phosphorylates microphthalmia-associated transcription factor to stimulate melanin synthesis, suggesting that the PI3K pathway is involved in the regulation of melanogenesis (32, 33). Furthermore, the specific thalmia-associated transcription factor and promotes its deactivation (31). ERK phosphorylates microphthalmia-associated transcription factor to stimulate melanin synthesis, suggesting that the PI3K pathway is involved in the regulation of melanogenesis (32, 33). Furthermore, the specific thalmia-associated transcription factor and promotes its deactivation (31). ERK phosphorylates microphthalmia-associated transcription factor to stimulate melanin synthesis, suggesting that the PI3K pathway is involved in the regulation of melanogenesis (32, 33). Furthermore, the specific thalmia-associated transcription factor and promotes its deactivation (31). ERK phosphorylates microphthalmia-associated transcription factor to stimulate melanin synthesis, suggesting that the PI3K pathway is involved in the regulation of melanogenesis (32, 33). Furthermore, the specific thalmia-associated transcription factor and promotes its deactivation (31). ERK phosphorylates microphthalmia-associated transcription factor to stimul
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