Effect of GM2 activator protein on the enzymatic hydrolysis of phospholipids and sphingomyelin

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Abstract **GM2 activator protein (GM2AP) is a specific protein cofactor that stimulates the enzymatic hydrolysis of the GalNAc from GM2, a sialic acid containing glycosphingolipid, both in vitro and in lysosomes. While phospholipids together with glycosphingolipids are important membrane constituents, little is known about the possible effect of GM2AP on the hydrolysis of phospholipids. Several recent reports suggest that GM2AP might have functions other than stimulating the conversion of GM2 into GM3 by β-hexosaminidase A, such as inhibiting the activity of platelet activating factor and enhancing the degradation of phosphatidylcholine by phospholipase D (PLD). We therefore examined the effect of GM2AP on the in vitro hydrolyses of a number of phospholipids and sphingomyelin by microbial (*Streptomyces chromofuscus*) and plant (cabbage) PLD. GM2AP, at the concentration as low as 1.08 μM (1 μg/50 μl) was found to inhibit about 70% of the hydrolyses of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol by PLD, whereas the same concentration of GM2AP only inhibited about 20–25% of the hydrolysis of sphingomyelin by sphingomyelinase and had no effect on the hydrolysis of sphingosylphosphorylcholine by PLD. Thus, GM2AP exerts strong and broad inhibitory effects on the hydrolysis of phospholipids carried out by plant and microbial PLDs. High ammonium sulfate concentration (1.6 M or 21.1%) masks this inhibitory effect, possibly due to the alteration of the ionic property of GM2AP.**—Shimada, Y., Y-T. Li, and S-C. Li. **Effect of GM2 activator protein on the enzymatic hydrolysis of phospholipids and sphingomyelin.** ***J. Lipid Res.*** **2003.** 44: **342–348.**

Supplementary key words phospholipase D • phosphatidylcholine • sphingomyelin • phospholipase activity • PC hydrolysis

A number of steps in the degradation of glycosphingolipids (GSLs) by glycosidases require the assistance of a low molecular weight protein cofactor. Five such protein cofactors have been described: GM2 activator protein (GM2AP) (1) and saposins A, B, C, and D that are derived from a single precursor protein, prosaposin (2). The defect or deficiency of any of these protein cofactors can lead to specific pathological manifestation. For example, the deficiency or defect of GM2AP leads to type AB GM2 gangliosidosis characterized by neuronal accumulation of GM2 (1). Among these protein cofactors, only GM2AP is specific in stimulating the hydrolysis of the GalNAc from GM2 by β-hexosamindase A (Hex A). Since its discovery in 1973 (3), GM2AP has been purified from various human tissues, such as kidney (4), liver (5), and brain (6). The genes encoding GM2AP have also been cloned from human (7–9) and from mouse (10). The recombinant human GM2AP has been overexpressed in *Escherichia coli* (11, 12) and transfected Chinese hampster ovary cells (13). The nonglycosylated GM2AP obtained from *E. coli* has full activity as the native protein in stimulating the hydrolysis of GM2 (12); however, the glycosylated native GM2AP was shown to have a slightly lower surface activity than the nonglycosylated GM2AP to bind the lipid monolayers at the air-water interface (14).

The specificity of GM2AP has been extensively studied. Human GM2AP has been found to efficiently stimulate the hydrolysis of the GalNAc from GM2 by Hex A, but not effective in stimulating the hydrolysis of the GalNAc from asialo-GM2 (GA2) by the same enzyme (15). In contrast, mouse GM2AP is not only effective in stimulating the hydrolysis of GM2 by Hex A, but is also more efficient than human GM2AP in stimulating the hydrolysis of the GalNAc from GA2 by Hex A (15). GM2AP was also shown to stimulate the hydrolysis of the NeuAc from GM1 and GM2 by clostridial sialidase (12) and by mammalian membrane-bound sialidases (16–18).

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Abbreviations: ARF, adenosine ribosylation factor; AS, ammonium sulfate; GTP-γ-S, guanosine-5′-*O*-(3-thiotriphosphate); Hex A, β-hexosaminidase A; GM2, GalNAcβ4(Neu5Acα3)Galβ4GlcβCeramide; GM2AP, GM2 activator protein; PAF, platelet activating factor; GM1, Galβ3-GalNAcβ4(Neu5Acα3)Galβ4GlcβCeramide; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLD, phospholipase D; PI, phosphatidylinositol; PLC, phospholipase C; SM, sphingomyelin; SMase, sphingomyelinase; SPCh, sphingosine-1-phosphatecholine; C-1-P, ceramide-1-phosphate; S-1-P, sphingosine-1-phosphate.

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The crystal structure of GM2AP showed that this protein had a novel β-cup topology, forming a hydrophobic pocket (19). Negatively charged residues (D^{125} and E^{127}) were present at the rim of this cavity. A protruded hydrophobic loop ($V^{59}AGLW^{63}$) at one side of the molecule was proposed to control the diameter of the entrance cavity and to guide the lipid moiety to the hydrophobic pocket. In accordance with this hypothesis, GM2AP might be a lipid carrier protein, and hydrophobic interaction could be the major binding force between GM2AP and the lipids. Giehl et al. (14) suggested a similar model that GM2AP might insert into a lipid bilayer through hydrophobic interaction. In vitro, GM2AP has been found to bind a variety of acidic and neutral GSLs (4, 20–24). This binding activity, however, has not been correlated with the biological functions of individual GSLs.

The most intriguing fact is that GM2AP is widely distributed in tissues (4–7), but GM2 ganglioside is largely absent in tissues other than the CNS. The role of GM2AP in visceral organs remains unclear. Recently, additional activities for GM2AP have been described. *a*) GM2AP enhanced the ADP-ribosylation factor-dependent phospholipase D (PLD) activity in vitro (25–27). *b*) GM2AP inhibited the stimulatory activity of platelet activating factor (PAF) to release intracellular Ca^{2+} pools (28). *c*) The immunoreactivity of rat kidney against a human autoimmune GM2AP antibody (29) suggested that GM2AP has a cytoplasmic localization in kidney intercallated (IC) cells where GM2 is practically not present, and that GM2AP might be involved in the vesicular transport of proton pumps in the IC cells. For exploration of additional functions of GM2AP, we have examined the effect of GM2AP on the in vitro hydrolyses of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), sphingomyelin (SM), and sphingosylphosphorylcholine (SPCh) by PLDs from cabbage and from *Streptomyces chromofuscus*. We found that GM2AP strongly inhibited the hydrolyses of PC, PE, PI, and SM by plant and microbial PLDs, but had little or no effect on the hydrolyses of SM by *Streptomyces* sphingomyelinase (SMase) and SPCh by PLD from *S. chromofuscus*.

MATERIALS AND METHODS

Materials

Human and mouse GM2APs were expressed in *E. coli* and purified as previously reported (12, 15). Recombinant saposin B was also prepared using the same procedure as that for the recombinant GM2AP (12). The following enzymes and reagents were purchased from Sigma-Aldrich: PLD from *S. chromofuscus*, PLD type IV from cabbage, phospholipase C (PLC) type IV from *Bacillus cereus*, SMase from *Streptomyces sp.*, PC type III-E from egg yolk, phosphatidic acid (PA) and PE from egg yolk, PI from soybean, 1,2-dioleoyl-*sn*-glycerol (C18:1, [*cis*]-9), SPCh, ceramide-1-phosphate (C-1-P) from bovine brain, sphingosine-1-phosphate (S-1-P), SM, ceramide, 4-methylumbelliferyl-phosphate, BSA, and triethylamine. Precoated silica gel-60 TLC plates were purchased from EM Science (Gibbstown, NJ), and P-11 phosphocellulose, from Whatman (Clifton, NJ).

Hydrolysis of phospholipids by PLD

The reaction at pH 8.0 was carried out in a final volume of 50 μl that contained 20 μg of a phospholipid substrate, 1 μl (1 unit) of PLD, 0–4.0 μg of GM2AP, and 10 mM of Tris-HCI buffer (pH 8.0) with 10 mM $CaC1_2$. When the reaction was carried out at pH 5.6, the 10 mM Tris buffer was replaced by 10 mM of sodium acetate buffer (pH 5.6) containing 10 mM $CaC1_2$. After incubation at 30°C for a preset time, the incubation mixture was heated in a boiling water bath for 3 min to stop the reaction. The lipid substrate and the product were extracted according to the method of Bligh and Dyer (30) and dried in a SpeedVac concentrator (Savant, Farmingdale, NY). When SPCh was used as substrate, the reaction mixture was directly dried without extraction. The lipids were then dissolved in 20 μl of chloroform-methanol (2:1, v/v) and applied onto a TLC plate. To ensure the complete transfer of the lipids to the TLC plate, the tubes were washed twice with another 20 μl of the same solvent and applied onto the plate. The plate was developed with one of the following solvent systems: Solvent A, chloroform-ethanol-triethylamine-water (30:34:30:8, v/v/v/v) for resolving PA from PC, or C-1-P from SM (31); or solvent B, *n*-butanol-acetic acid-water (2:1:1, v/v/v) for resolving PA from PE and PI or S-1-P from SPCh. Lipids were visualized by spraying the plate with the copper sulfate reagent (32) and heated at 110°C for 20 min. For the detection of S-1-P produced from SPCh, the plate was sprayed with 1% ninhydrin in acetone and air dried. The PLD activity was estimated from the amount of the lipid product detected on the TLC plate. The plates were scanned using a HP Scan Jet IIcx (Hewlett Packard) and the color intensities of the bands were quantified with NIH Image 1.61.

Previously, GM2AP was reported to stimulate the rat kidney PLD in vitro (26) in an incubation mixture that contained 1.6 M ammonium sulfate (AS). Since 1.6 M AS (21.1% solution) is an unusually high concentration to be present in an enzymatic incubation mixture, we compared the hydrolysis of PC by *S. chromofuscus* PLD under our normal assay condition and the condition in the presence of 1.6 M AS. We also performed the experiment in the presence of 1.6 M NaCl instead of 1.6 M AS to verify whether the effect of GM2AP on PLD activity in the presence of 1.6 M AS was due to the presence of a high concentration of salt. After incubation, each mixture was heated in a boiling water bath for 3 min. Then 200 μl of water and 1.0 ml of chloroform-methanol (2:1, v/v) were added. Reaction mixtures devoid of AS were adjusted to 1.6 M AS upon the termination of the reaction and before the addition of chloroform-methanol. After mixing, the two liquid phases were separated by centrifugation at 2,000 rpm for 5 min. An aliquot (660 μl) of the lower phase was transferred to a fresh tube, dried, and analyzed by TLC as described early in this section.

Removal of phosphatase from PLD

The *S. chromofuscus* PLD obtained from Sigma-Aldrich was found to be heavily contaminated with a phosphatase activity (about 6 units of phosphatase activity/1 unit of PLD activity) that splits S-1-P to sphingosine and phosphate and that interfered with the determination of S-1-P produced from SPCh. A P-11 phosphocellulose column was used to remove the phosphatase activity from the purchased PLD. PLD (1,500 units in 150 μl) was applied onto a phosphocellulose column (5.5 mm × 15 mm) equilibrated with 10 mM Tris-HCI buffer, pH 8.0. The column was washed with the same buffer and 0.5 ml-fractions were collected. For checking the phosphatase activity, an aliquot (50 μl) of each fraction was incubated with 10 μl of 4-methylumbelliferylphosphate (1 μg/μl) and 40 μl of 10 mM phosphate buffer, pH 8.0. After incubation for 16 h at 37°C, the reaction was stopped

by adding 1.5 ml of 0.2 M borate buffer, pH 9.8. The resulted fluorescence was measured at λ_{ex} = 360 nm and λ_{em} = 415 nm using a Sequoia-Turner fluorometer. One unit of phosphatase activity was defined as the amount of the enzyme that liberated 1 nmol of 4-methylumbelliferone per min. Fractions containing less than 0.2 unit of phosphatase activity per unit of PLD were used for SPCh hydrolysis.

SMase assay

SM (20 μg) and 0–2.0 μg of GM2AP were dispersed in 49 μl of 20 mM sodium phosphate buffer, pH 7.5. The reaction was initiated by adding 0.125 units (1 μl) of SMase at 37°C. After incubation for 30 min, the reaction mixture was heated in a boiling water bath for 3 min to stop the reaction. The intact SM and the product, ceramide, were resolved by TLC using solvent C, chloroform-methanol-14.8 N ammonium hydroxide (80:20:2, v/v/v). The lipids were visualized by spraying the plate with the copper sulfate reagent (32) and heated at 110°C for 20 min. The amount of ceramide produced was quantified by densitometry as described in the section "Hydrolysis of phospholipids by PLD."

Hydrolysis of PC by PLC

PC (50 μg) was suspended in 48 μl of 10 mM Tris-HCI buffer (pH 7.3) containing 10 mM $CaC1_2$ and 0–2.0 μg of GM2AP. The reaction was started by the addition of 2 μl (0.02 units) of PLC. After incubation at 37°C for 30 min, the mixture was placed in a boiling water bath for 3 min to stop the reaction. The lipids were subsequently extracted by the method of Bligh and Dyer (30), dried, and analyzed by TLC using the following two solvents. The plate was first developed with solvent D, chloroform-methanol-acetic acid (98:2:1, v/v/v), until the solvent reached the top of the plate. Under this solvent system, the diacylglycerol migrated with a R_f of 0.85, while PC remained near the origin. To have a well-resolved band of PC, the air-dried plate was again developed with solvent A until the solvent reached the half-height of the plate. Diacylglycerol produced from PC was visualized and quantified by the method described in the section "Hydrolysis of phospholipids by PLD."

RESULTS

Effect of GM2AP on the PLD-catalyzed hydrolysis of phospholipids

Under our assay condition, we initially observed that a 1.08-μM (1 μg/50 μl) concentration of GM2AP exerted over 50% inhibition of the hydrolysis of PC by the *S. chromofuscus* PLD at pH 8.0. We subsequently examined the dose dependency of the inhibition by GM2AP (0–4.0 μg) for this reaction. As shown in **Fig. 1A**, the production of PA from PC was inhibited about 70% by 1.08 μM (1 μg/50 μl) of GM2AP, and this inhibition was dose-dependent (Fig. 1A, line a). When PE (Fig. 1A, line b) or PI (Fig. 1A, line c) was used as the substrate, a similar extent of inhibition by GM2AP was observed. We further examined the effect of GM2AP on the cabbage PLD activity under the same condition. Since PI was refractory to the cabbage PLD (33), only the hydrolyses of PC and PE were examined (Fig. 1B). Both the hydrolyses of PC (Fig. 1B, line a) and PE (Fig. 1B, line b) by the cabbage PLD were inhibited over 70% by 1.08 μM (1 μg/50 μl) concentration of GM2AP. These results show that GM2AP broadly inhibits the hydrolysis of various phospholipids carried out by PLDs.

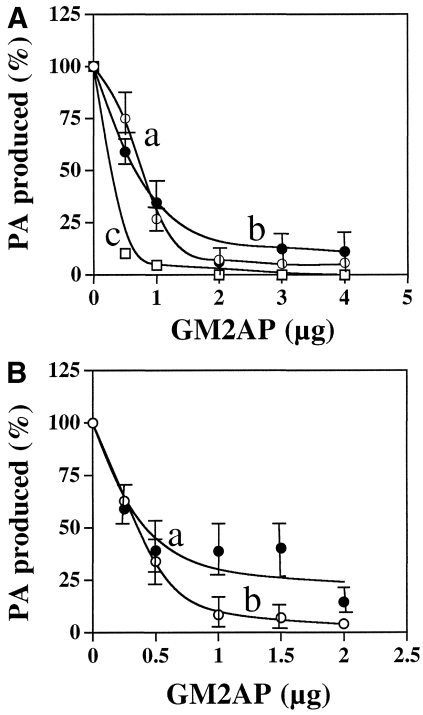

Fig. 1. Effect of GM2AP on phospholipase D (PLD)-catalyzed hydrolysis of phospholipids. Hydrolyses of phospholipids by PLDs were determined at pH 8.0 in the presence of GM2AP as indicated. The concentration of the phospholipid substrate was 20 μg/50 μl of the incubation mixture. A: The hydrolyses of phosphatidylcholine (PC) (line a), phosphatidylethanolamine (PE) (line b), and phosphatidylinositol (PI) (line c) by 2.5 units of the *Streptomyces chromofuscus* PLD. The incubation time for PC and PE were 30 min and that for PI, 60 min. B: The hydrolyses of PC (line a) and PE (line b) by 5 units and 1 unit of the cabbage PLD, respectively. The incubation time for PC was 2 h and for PE, 17.5 h. GM2AP, GM2 activator protein; PA, phosphatidic acid. The vertical line on each point indicates the error range of the duplicate determinations, except for B, line a, the range of the triplicate determinations. For additional incubation conditions, see Materials and Methods.

Effect of GM2AP on the hydrolyses of SM by SMase, SM and SPCh by PLD, and PC by PLC

We subsequently examined the effects of GM2AP on the hydrolyses of SM by SMase, SM and SPCh by PLD, and PC by PLC. SM is known to be a good substrate for the *S. chromofuscus* PLD (34). As in the case for the hydrolysis of PC by PLD, the hydrolyses of SM by PLD (**Fig. 2**, line a) and PC by PLC (Fig. 2, line b) were also inhibited over 75% by 1.08 μM (1 μg/50 μl) of GM2AP. This inhibition was also dose dependent. However, under the same condition, the hydrolysis of SM by SMase was only inhibited about 20–25% (Fig. 2, line c) and the hydrolysis of SPCh by PLD was not affected by the presence of GM2AP (Fig. 2, line d). It is of interest to note the striking difference GM2AP exerted on the hydrolyses of SPCh and SM using the same PLD (Fig. 2, lines a and d). As SPCh is the deacylated SM, the hydrophobicity of the substrate must play an important role in the inhibitory activity of GM2AP. It had been previously reported that GM2AP could bind many GSLs (4, 20–24) and the hydrophobicity of GSLs was important for GM2AP to elicit the stimulatory activity (12).

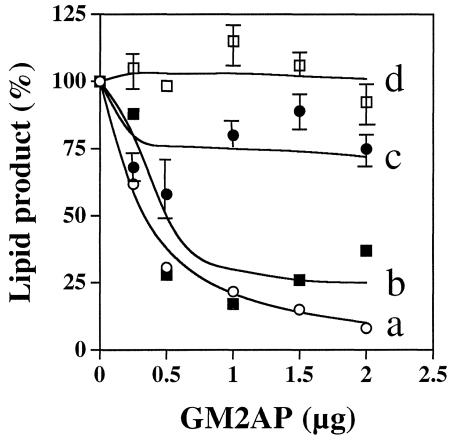

Fig. 2. Effect of GM2AP on the hydrolyses of SM by SMase, SM and SPCh by PLD, and PC by PLC. The hydrolyses of SM, SPCh, and PC were examined in the presence of different amounts of GM2AP as indicated. Line a, production of C-1-P from SM (20 μg) by 2.5 units of the *S. chromofuscus* PLD in 30 min; line b, production of diacylglycerol from PC (50 μg) by 20 mU of PLC in 30 min; line c, production of ceramide from SM (20 μg) by 0.125 units of SMase in 30 min; line d, production of S-1-P from SPCh (20 μg) by 0.5 units of the purified *S. chromofuscus* PLD in 2 h. GM2AP, GM2 activator protein. The vertical line on each point indicates the error range of the duplicate determinations. For additional incubation conditions, see Materials and Methods.

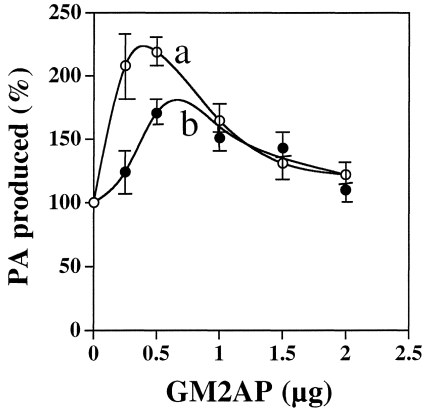

Fig. 3. Effect of GM2AP on the hydrolysis of PC at pH 5.6. In each experiment, the substrate concentration was 20 μg/50 μl of the incubation mixture. Line a, hydrolysis of PC by 1 unit of the *S. chromofuscus* PLD in 30 min; line b, hydrolysis of PC by 2.5 units of the cabbage PLD in 30 min. GM2AP, GM2 activator protein; PA, phosphatidic acid. The vertical line on each point indicates the error range of the duplicate determinations. For additional incubation conditions, see Materials and Methods.

Another interesting observation is the drastic difference between the hydrolysis of SM by PLD and by SMase in the presence of GM2AP (Fig. 2, lines a and c). The hydrolysis of SM by PLD was inhibited more than 75% by 1.08 μM (1 μg/50 μl) of GM2AP; however, under the same condition, GM2AP only inhibited about 25% of the hydrolysis of SM by SMase. PLD cleaves SM at the phosphoester linkage next to the choline residue, and SMase cleaves SM at the phosphoester linkage next to the ceramide residue. These results suggest that GM2AP may compete with ceramide moiety for interacting PLD, which is important for the action of PLD, whereas the action of SMase does not depend on the interaction with the ceramide residue.

Effect of GM2AP on PLD activity at pH 5.6

As shown in Fig. 1, the cabbage and the *S. chromofuscus* PLD activities were inhibited similarly by GM2AP at pH 8.0. Since the optimum pH of the cabbage PLD is 5.6 and that of the *S. chromofuscus* PLD is pH 8.0, we examined the hydrolysis of PC by these two PLDs at pH 5.6. Interestingly, the hydrolysis of PC by the *S. chromofuscus* PLD at pH 5.6 was found to be about 2-fold stimulated in the presence of a 0.54-μM (0.5 μg/50 μl) concentration of GM2AP (**Fig. 3**, line a). When the concentration of GM2AP was greater than 1.08 μM, the stimulation became less significant. Similar results were obtained for PC-hydrolysis by the cabbage PLD (Fig. 3, line b). These results indicated that the behaviors of the two kinds of PLD toward PC hydrolysis were similar, despite the fact that they were from different sources. Also noteworthy is the observation that GM2AP at 0.54 μM (0.5 μg/50 μl) concentration inhibited about 25–30% of the hydrolysis of PC by both PLDs at pH 8.0 (Fig. 1), yet at pH 5.6 (Fig. 3) the same concentration of GM2AP showed 2-fold stimulation of the same reaction.

Effect of 1.6 M AS on the hydrolysis of PC by PLD

Nakamura et al. (26) reported that GM2AP stimulated the in vitro hydrolysis of PC carried out by rat kidney PLD in the presence of 1.6 M AS. Our results showed that in the absence of AS, 1.08 μM of GM2AP inhibited over 70% of the hydrolysis of PC by both microbial and plant PLDs (Fig. 1). Therefore, the effect of 1.6 M AS on the hydrolysis of PC by *S. chromofuscus* PLD was examined under our assay condition. **Figure 4A** shows that, in the absence of AS, the hydrolysis of PC by *S. chromofuscus* PLD was inhibited more than 75% by the presence of 78 pmol (1.5 μg) of GM2AP. Interestingly, 1.6 M AS alone also inhibited about 80% of the PLD activity. However, in the presence of both GM2AP (1.5 μg) and 1.6 M AS, the activity of PLD, although lower than what was observed in the absence of both GM2AP and AS, was about 2 times higher than the activity in the presence of only GM2AP or AS. For comparison, a similar experiment was performed in the presence of 78 pmol of saposin B, a nonspecific activator protein for the hydrolysis of GSLs (35). As shown in Fig. 4B, saposin B did not have any effect on the PLD activity, even in the presence of 1.6 M AS. To further compare the effects of GM2AP and saposin B on the hydrolysis of PC by *S. chromofuscus* PLD in the presence of 1.6 M AS, we have carried out the dose-response experiment of these two activator proteins. As can be seen in Fig. 4C, the stimulatory effect of GM2AP was dose dependent, reaching a maximum of about 2-fold at 100 pmol (2 μM concentration). Saposin B, on the other hand, did not show any effect throughout the concentrations tested. BSA has been often used to stabilize a highly purified enzyme. When 0–150 pmol of BSA was used in the place of GM2AP, no effect on

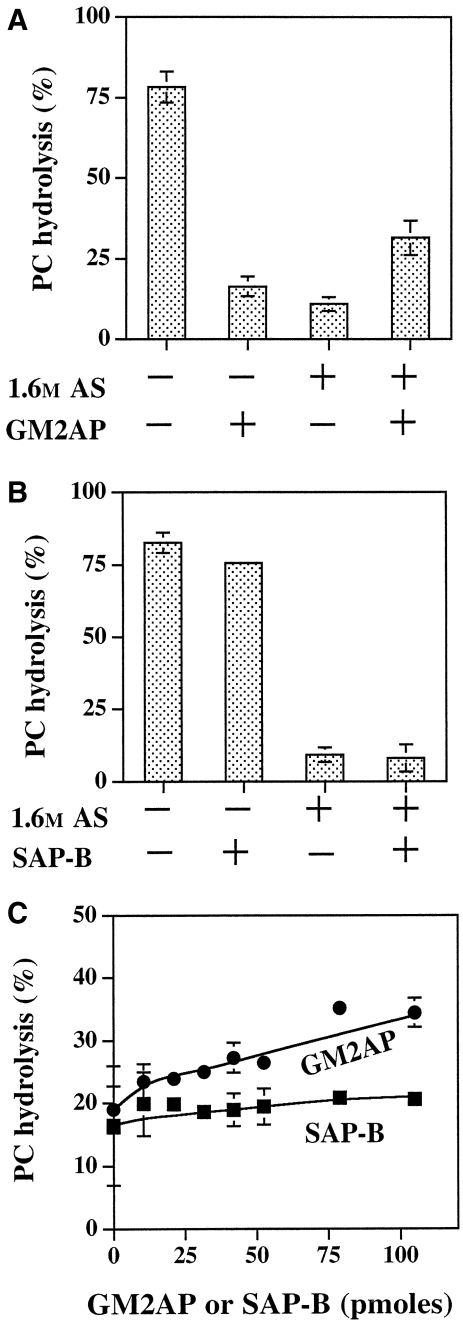

Fig. 4. Effect of 1.6 M AS on the hydrolysis of PC by *S. chromofuscus* PLD. Hydrolysis of PC by the *S. chromofuscus* PLD was examined in the presence or absence of GM2AP, saposin B, and 1.6 M AS. A: Hydrolysis of PC in the presence or absence of 78 pmol of GM2AP and 1.6 M AS. B: Hydrolysis of PC in the presence or absence of 78 pmol of saposin B and 1.6 M AS. C: The dose-response curves for the effect of GM2AP and saposin B on the hydrolysis of PC. Each line shows the average of two separate experiments. AS, ammonium sulfate; GM2AP, GM2 activator protein; SAP-B, saposin B. For additional incubation conditions, see Materials and Methods.

the PLD activity was observed with or without 1.6 M AS (data not shown). These results suggest that the stimulation of PLD activity by GM2AP in the presence of AS was not simply due to the stabilization of PLD by GM2AP. When 1.6 M NaCl was used to replace 1.6 M AS in the reaction mixture, the PLD activity was completely inhibited even in the presence of GM2AP.

DISCUSSION

Each member of the PLD superfamily has two active-site motifs of $HXK(X)_4D(X)_6GSXN$ (HKD motifs), and histidine residues in these motifs are considered to be responsible for electron exchanges between the enzyme and the substrate (36–38). The crystal structure of bacterial endonucrease (39), a member of the phospholipase D family, revealed that the active sites of PLDs consisted of hydrophobic interface and a variable loop region that might be important determinants for substrate specificity. The crystal structure of the PLD from *Streptomyces sp.* (40) further supports the proposed PLD superfamily relationship. The variable loop regions of PLDs in the superfamily differ in both length and amino acid composition, which may account for the observed differences in substrate specificity of the PLDs from Streptomyces and cabbage. The former hydrolyzes PI as well as PC or PE and the latter hydrolyzes PC and PE, but not PI. The hydrophobic interface of PLD was suggested to bind lipid substrate (36–38). When GM2AP is present together with a lipid substrate and PLD, GM2AP may be positioned at the active site of PLD either by direct hydrophobic interaction or by indirect attachment as the lipid-bound GM2AP. In either case, the presence of GM2AP at the active site of PLD may prevent the enzyme from having functional contacts with the lipid substrate and hinder the hydrolytic process.

The results of our current study showed that GM2AP is a broad and potent inhibitor for the in vitro hydrolysis of PC, PE, and PI by PLD or PC by PLC. At pH 8.0, 1.08 μM (1 μg/50 μl) of GM2AP inhibited over 60% of the hydrolyses of PC, PE, and PI by both the PLDs from cabbage and *S. chromofuscus* (Fig. 1). At pH 5.6, however, GM2AP enhanced about 2-fold of the PLD activity (Fig. 3). The difference in the effect of GM2AP on PLD activity from pH 8.0 to 5.6 may be due to the diminished net charge of GM2AP for binding the lipid substrate or PLD, as this pH is near the isoelectric point (pH 5.0) of GM2AP. Although the binding of GM2AP with various lipids involves mainly hydrophobic interaction, GM2AP, as shown by its crystal structure (19), may also have ionic binding specificity through the negatively charged residues (D^{125} and E^{127}) at the rim of its lipid binding pocket. Thus, the changes of a pH environment may alter the molecular functionality of GM2AP. This may explain the observation that, under the low pH environment in lysosomes, GM2AP acts as an activator protein for GM2 hydrolysis (1), and in the cytoplasmic environment with pH slightly above pH 7.0, GM2AP acts as a modulator for binding and shuffling proton pumps in kidney IC cells (29).

The hydrolysis of SM by *S. chromofuscus* PLD (Fig. 2, line a) was also inhibited dose dependently by GM2AP in the same manner as the hydrolysis of PC by PLD. However, the hydrolysis of SM by *Streptomyces* SMase was inhibited only about 25% by the presence of 1.08 μM (1 μg/50 μl)

of GM2AP (Fig. 2, line c). This indicates that the two enzymes, SMase and PLD, may have different recognition sites on SM and the site that interacts with PLD can also interact with GM2AP. Alternatively, the active site of PLD may have much stronger affinity to GM2AP than that of SMase. Sphingomyelin has been shown to have little or no binding to GM2AP, and does not inhibit the hydrolysis of GM2 by Hex A at pH 4.0 in the presence of GM2AP (20). Therefore, the inhibitory effect of GM2AP on the hydrolysis of SM by PLD or PC by PLD may be due to the occupation of the active site of PLD by GM2AP. The hydrolysis of SPCh by PLD was not at all affected by the presence of GM2AP (Fig. 2, line d). This is in line with our previous observation that GM2AP was not able to enhance the hydrolysis of the tetrasaccharide derived from GM2 (lipid-free GM2 oligosaccharide) by Hex A (12).

Nakamura et al. found from their experiment in cell free system (26) that 1.6 M AS could unmask the PLD activity from inhibitory agents in the tissue extract. Since then, they have included 1.6 M of AS in the reaction mixture to examine the rat kidney PLD activity in vitro (25–27). They, however, did not examine the effect of GM2AP in the absence of AS except on one occasion in which the PLD activity was assayed in the absence of AS using streptolysin-*O*-permeabilized HL-60 cells (25). In this case, GM2AP enhanced the PLD activity only about 3-fold. High AS concentration may promote hydrophobic interaction and suppress electrostatic interaction among the components. Our results showed that, although AS and GM2AP individually inhibited the hydrolysis of PC by the *S. chromofuscus* PLD, this reaction was enhanced 2- to 3-fold in the presence of both AS and GM2AP (Fig. 4A). This level of stimulation is comparable to the results of Shimooku et al. (41), but lower than that reported by Nakamura et al. (26). This stimulation might be the result of an assay condition containing unusually high AS concentration. Thus, the in vitro assay condition for an enzymatic reaction, especially on the hydrolysis of hydrophobic substrates, should be carefully considered.

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