Sphingolipids such as ceramide and sphingosine have been regarded as novel signal mediators in cells. However, the mechanisms of generation of these lipids upon various stimulation remain to be elucidated. Neutral sphingomyelinase (N-SMase) is one of the key enzymes in the generation of ceramide, and recently the cloning of a putative N-SMase was reported. Because the function of the protein was unclear in the previous report, we investigated the role it plays in cells. N-SMase activity in cells overexpressing the protein with hexa-histidine tag was immunoprecipitated with anti-hexa-histidine antibody. The metabolism of ceramide and SM was not apparently affected in overexpressing cells. Radiolabeling experiments using [3H]palmitic acid or [3H]hexadecanol demonstrated an accumulation of 1-acyl-2-lyso-glycerol-3-phosphocholine in overexpressing cells. In vitro studies showed that both 1-acyl-2-lyso-snglycerol-3-phosphocholine (lyso-PC) and 1-acyl-2-lyso-sn-glycerol-3-phosphocholine (lyso-platelet activating factor lyso-PAF) are good substrates of the protein. In further radiolabeling experiments, 1-acyl-lyso-PC was predominantly and equally metabolized into diacyl-PC in both vector and overexpressing cells. On the other hand, 1-O-alkyl-lyso-PC (lyso-PAF) was metabolized into both diradyl-PC and 1-O-alkyl-glycerol in overexpressing cells but only into diradyl-PC in vector cells. These results suggest that the protein acts as lyso-PAF-PLC rather than lyso-PC-PLC or N-SMase in cells.

Sphingolipids are now recognized as important signal mediators in cells (1, 2). It has been proposed that ceramide, the backbone of various sphingolipids, may play a crucial role in stress responses. Increases in the level of ceramide are associated with apoptosis induced by various stimuli including tumor necrosis factor-a (3), cross-linking of Fas (4, 5), anticancer drugs (6, 7), irradiation (8), heat shock (9) and serum deprivation (10). In most cases, a concomitant decrease in the level of 1-alkyl-2-acyl-PC were detected in overexpressing cells. Furthermore, radiolabeling experiments using [3H]palmitic acid or [3H]hexadecanol demonstrated an accumulation of 1-acyl-2-lyso-glycerol and a corresponding decrease of 1-alkyl-2-acyl-sn-glycerol-3-phosphocholine in overexpressing cells. In vitro studies showed that both 1-acyl-2-lyso-sn-glycerol-3-phosphocholine (lyso-PC) and 1-acyl-2-lyso-sn-glycerol-3-phosphocholine (lyso-platelet activating factor lyso-PAF) are good substrates of the protein.

Furthermore we show that both 1-acyl-2-lyso-sn-glycerol-3-phosphocholine (lyso-PC) and 1-acyl-2-lyso-sn-glycerol-3-phosphocholine (i.e. lyso-platelet activating factor lyso-PAF) are good substrates for the overexpressed protein in vitro. An accumulation of 1-acyl-sn-glycerol (1-alkyl-glycerol) with corresponding decrease of 1-alkyl-2-acyl-PC were detected in overexpressing cells. Furthermore, radiolabeling experiments showed that although 1-acyl-lyso-PC was predominantly converted to diradyl-PC, lyso-PAF was almost equally metabolized into both diradyl-PC and 1-alkyl-glycerol in overexpressing cells. These results suggest that the putative N-SMase protein acts as lyso-PAF-phospholipase C (PLC) in cells.

* This work was supported in part by National Institutes of Health Grant GM43825 (to Y. A. H.) and CHP-Seed Money for Education and Research Reform Grant (to N. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29425. Tel.: 843-792-4921; Fax: 843-792-4322; E-mail: hannun@musc.edu.

‡ The abbreviations used are: SM, sphingomyelin; A-SMase, acid sphingomyelinase; N-SMase, neutral sphingomyelinase; PC, phosphatidylcholine; lyso-PC, 1-acyl-2-lyso-sn-glycerol-3-phosphocholine; PAF, platelet activating factor; lyso-PAF, 1-O-alkyl-2-lyso-sn-glycerol-3-phosphocholine; 1-alkyl-glycerol, 1-O-alkyl-sn-glycerol; PLC, phospholipase C, lyso-PLD, lysophospholipase D; PCR, polymerase chain reaction; CHAPS, 3-(3-cholamidopropyl)dimethylammoniomethyl-1-propanesulfonic acid.

Hirofumi Sawai, Naouchika Domae, Narasimhan Nagan, and Yusuf A. Hannun

From the 3Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425, the 4Department of Medicine, Osaka Dental University, Osaka, Japan, and the 5Department of Medical Laboratory Sciences, Medical University of South Carolina, Charleston, South Carolina 29425

This paper is available on line at http://www.jbc.org
EXPERIMENTAL PROCEDURES

Materials—[Choline-methyl-1-3H]SM and [acetate-1-4C]C2- ceramide were provided by Dr. Alicja Bielaswa (Medical University of South Carolina, Charleston, SC). [Choline-methyl-1-3H]Choline chloride (75 Ci/mmol), [9,10-3H]palmitic acid (43 Ci/mmol), [1-0-octadecyl-9,10-3H]PAF (160 Ci/mmol), [choline-methyl-1-3H]digalactosylcytolipotycol PC (159 mCi/mmol), [1-palmitolyl-3H]lyso-PC (56.7 mCi/mmol), and [1-32P]ATP (3000 Ci/mmol) were from NEN Life Science Products. [1-0-octadecyl-3H]lyso-PAF (161 Ci/mmol) was from Amersham Pharmacia Biotech. SM, PC, lyso-PC, lyso-PAF, and phosphatidylserine were from Avanti Polar Lipids. Frozen stripped rat brains were purchased from Pel-Freeze Biologicals. Other chemicals were from Sigma.

Cell Culture—Human embryonic kidney 293 cells and human leukemia Molt-4 cells were purchased from ATCC. The cells were cultured in minimum essential medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum at 37 °C in a humidified 5% CO2 incubator.

Preparation of SMase cDNA—A 1514 bp fragment containing the translation initiation codon of human N-SMase was obtained by PCR amplification of cDNA from human breast carcinoma cells (MCF-7) using the following primers: 5'-ATGAAGCTCAACTTCTCCCTGCGACTG and 3'-TTATTGTTCTTTAGTTCTGTCCCCCTCCTGCTG) were synthesized by using Exponent (26) and separated by TLC in solvent system A (chlorform/methanol/10% acetic acid, 1:1:0.2). The DNA fragment was subcloned into a mammalian expression vector (p3.1NSM), respectively. The inserts were cut out between EcoRI and XhoI sites and ligated into the same sites in pcDNA3.1/HisC and p3.1NSM, respectively.

Preparation of Enzyme—N-SMase was partially purified from the membrane fraction of rat brain Molt-4 cells (28) as described in SM synthase assay. Rat brains were homogenized in the same buffer by using a Teflon pestle glass homogenizer. Lysate was centrifuged at 800 mg for 5 min, and the supernatant was centrifuged at 100,000 g for 1 h. The pellet was used as the membrane fraction.

Partial Purification of N-SMase from Rat Brain or Molt-4 Cells—N-SMase was partially purified from the membrane fraction of rat brain or Molt-4 cells as described (28) except that Hitrap Q column (1 ml, Amersham Pharmacia Biotech) was used instead of DEAE-Sepharose column.

Western Blot—Western blot was performed as described (29) except that 10% running gel and nitrocellulose membrane (Bio-Rad) were used. Anti-His6 monoclonal antibody (CLONTECH), rabbit polyclonal antibody against the cloned human N-SMase (MEMOREC Stoffel GmbH, Germany), goat anti-mouse antibody, and anti-rabbit antibody (Santa Cruz) were used at 1:1000 dilution.

Mass Measurement of SM and PC—Cellular Lipids were extracted by the method of Bligh and Dyer. For the measurement of SM, the lipids in chloroform mixture were mixed with the same volume of 0.2 N NaOH in methanol and incubated at 37 °C for 1 h. The phase was separated by the addition of 0.45 volume of 0.2 N HCl. Lipids were separated by TLC in solvent system A and visualized with iodine vapor, and the bands corresponding to SM and PC were scraped. Lipids were extracted from the silica gel by the method of Bligh and Dyer, and the level of lipid phosphate was determined as described before (30).

Ceramide Measurement—The level of ceramide was determined by using diacylglycerol kinase assay as described (29).

Preparation of [9,10-3H]Hexadecanol—[9,10-3H]Hexadecanol was made by a modification of the procedure described (31). Briefly, the methyl ester of [9,10-3H]hexadecanoic acid was reduced by treatment with Vitride [sodium bis(2-methoxyethoxy)aluminum hydride] (Red-AlTM, Aldrich). Following reduction, the excess vitride was destroyed by addition of ice-cold 20% ethanol, and the radioactive alcohol was extracted with chloroform. The radioactive alcohol was localized by autoradiography following a preparative TLC in a silica gel-G plate (Analtech) (hexane:diethyl ether-acetic acid 70:30:1). The corresponding spot was eluted from the TLC plate by extraction of the silica gel scrapings with chloroform/methanol (2:1). The yield (99%) and purity (>98%) of the product was assessed by TLC in the same solvent system described above. The specific activity of the product was 0.2–0.3 Ci/mmol.

Labeling Experiments of the Cells—For [3H]Choline labeling, cells were seeded to 100-mm culture dishes in 6 ml of medium (21) and incubated for 3 days. The cells were then washed twice with 2 μl of [3H]Choline. For chase experiments, the cells were labeled for 4 days, and then medium was replaced by new medium, and the cells were further incubated for the indicated hours. Lipids were extracted by the method of Bligh and Dyer. For the experiments with bacterial SMase treatment, the medium was replaced by new medium, the cells were rested for 2 h, 100 million/mL of bacterial SMase from Staphylococcus aureus (Sigma) was added to the medium, and after 25 min of

PAP-FLC Assay—The assay was performed as described in lyso-PAF-PLC assay except that the reaction mixture contained 10 nmol [1-0-octadecyl-3H]PAF (200,000 dpm) instead of lyso-PAF.

SM Synthase Assay—Cells were suspended in the buffer containing 25 mA Tris (pH 7.4), 5 mA EDTA, 1 mA phenylmethylsulfonyl fluoride, 5 mA of each of chymostatin, leupeptin, antipain, and pepstatin A, centrifuged by using the method of Bligh and Dyer (27) with modifications. Briefly, 10 μl of cell lysate was added to 100 μl of reaction mixture containing 50 mA Tris (pH 7.4), 25 mA KCl, 0.5 mA EDTA, and 10 nmol [choline-methyl-14C]PC (200,000 cpm). After 30 or 60 min of incubation at 37 °C, the lipid was extracted by the method of Bligh and Dyer (27).

Ceramide Measurement—The level of ceramide was determined by using diacylglycerol kinase assay as described (29).

Preparation of [9,10-3H]Hexadecanol—[9,10-3H]Hexadecanol was made by a modification of the procedure described (31). Briefly, the methyl ester of [9,10-3H]hexadecanoic acid was reduced by treatment with Vitride [sodium bis(2-methoxyethoxy)aluminum hydride] (Red-AlTM, Aldrich). Following reduction, the excess vitride was destroyed by addition of ice-cold 20% ethanol, and the radioactive alcohol was extracted with chloroform. The radioactive alcohol was localized by autoradiography following a preparative TLC in a silica gel-G plate (Analtech) (hexane:diethyl ether-acetic acid 70:30:1). The corresponding spot was eluted from the TLC plate by extraction of the silica gel scrapings with chloroform/methanol (2:1). The yield (99%) and purity (>98%) of the product was assessed by TLC in the same solvent system described above. The specific activity of the product was 0.2–0.3 Ci/mmol.
incubation the medium was replaced by new medium, and the cells were further incubated for the indicated times. Cells were lysed in 0.6 ml of water, and a portion was used for determination of protein concentration as described (27). For labeling with $^3$H|palmitic acid or $^3$H|hexadecanol, cells were incubated with 5 $\mu$Ci of $^3$H|palmitic acid or 2 $\mu$Ci of $^3$H|hexadecanol for the indicated times. For labeling with [1-O-octadecyl-2$^3$H|lyso-PAF, [1-palmitoyl-1$^4$C|lyso-PC, or [1-O-octadecyl-2$^3$H|PAF, medium was replaced by serum-free medium, and then cells were incubated with either 1 $\mu$Ci of $^3$H|lyso-PAF, $^1$4C|lyso-PC, or $^3$H|PAF for the indicated times. Lipids were extracted by the method of Bligh and Dyer and separated by TLC in solvent system A or B or C (chloroform:methanol:acetic acid 98:2:1).

RESULTS

Overexpression of Putative N-SMase in HEK293 Cells—Two primers corresponding to the amino- and carboxyl-terminal of the open reading frame were synthesized from the cDNA sequence of the putative human N-SMase, and PCR was performed using a human cDNA library generated from neuroblastoma cells as a template. The PCR product of the expected size (approximately 1.3 kilobase pairs) was subcloned into pT7Blue-3, and it was confirmed that its sequence was identical to that previously reported. The insert was cut out and ligated in BamHI-XhoI sites of pcDNA3.1/HisC for hexa-histidine-tagged expression of the protein (pHisNSM). When the pHisNSM was stably transfected into human embryonic kidney 293 cells, N-SMase activity in the cell lysate was increased by approximately 20-fold compared with vector transfected cells (Table I). The N-SMase activity was Mg$^{2+}$-dependent and stimulated by reducing reagents such as dithiothreitol and $\beta$-mercaptoethanol when lysis buffer did not contain reducing reagents (data not shown). However, PC-PLC activity was not increased in pHisNSM transfected cells (Table I) in contrast to the previous report, which described an increase of PC-PLC activity equivalent to about 30% of N-SMase activity. Also, SM synthase activity was not increased in pHisNSM transfected cells (Table I). A-SMase activity was decreased in pHisNSM transfected cells compared with wild type or vector transfected cells (Table I).

Immunoprecipitation of N-SMase Activity—Because the overexpressed protein was not purified in the previous report, it was not clear whether the overexpressed protein itself has N-SMase activity or not. Therefore, the overexpressed protein was immunoprecipitated by anti-His$_6$ antibody (Fig. 1A), and N-SMase activity of the immunoprecipitant was measured. As shown in Fig. 1B, approximately 40% of the activity were recovered in the immunoprecipitant, and only 20% remained in the supernatant. However, the same amount of mouse IgG did not immunoprecipitate either the overexpressed protein (Fig. 1A) or N-SMase activity (Fig. 1B). We also purified the overexpressed protein with heza-histidine tag by using TALON spin metal affinity column (CLONTECH). The molecular mass of the purified protein was about 48 kDa, consistent with the expected molecular size of the hexa-histidine-tagged protein. The 48-kDa protein was also detected by Western blot using anti-His$_6$ antibody. However, the purified protein had little N-SMase activity, possibly because of the inhibition by Co$^{2+}$ used for the affinity column (data not shown).

Comparison of N-SMase Activity of the Overexpressed Protein with That of Partially Purified Rat Brain N-SMase—In earlier experiments we found that a high concentration (1%) of Triton X-100 in the lysis buffer inhibited N-SMase activity of the overexpressed protein, whereas activity was rather stable in the presence of 0.1% Triton X-100 in the lysis buffer. Because it was previously reported that 1% Triton X-100 in the extraction buffer did not inhibit N-SMase activity in rat brain membrane fraction (14), the effect of Triton X-100 in the extraction buffer was examined on N-SMase activity in the membrane fraction of p3.1NSM transfected cells. The final concentration of Triton X-100 in the N-SMase assay was adjusted to 0.1% in these experiments. As shown in Fig. 2, 1% Triton X-100 in the extraction buffer inhibited N-SMase activity of the transfected cells by approximately 80%. However, rat brain N-SMase activity was not inhibited by 1% Triton X-100 in the extraction buffer (Fig. 2) as previously reported. The effects of other detergents on N-SMase activity were also examined. As shown in Fig. 2, both 1% Nonidet P-40 and 1.5% $\beta$-octylglucoside in the extraction buffer inhibited N-SMase activity of transfected cells by approximately 90%, whereas 10 mM deoxycholic acid inhibited N-SMase activity of rat brain much more than that of transfected cells. These results suggested that the overexpressed N-SMase is distinguishable from rat brain N-SMase.

Lack of Recognition of N-SMase Purified from Molt-4 Cells by an Antibody against the Cloned N-SMase—In this experiment, p3.1NSM, which does not contain the hexa-histidine tag, was used for the overexpression of the cloned protein in the native form. A band of approximately 45 kDa was detected in p3.1NSM transfected cells by Western blot using an antibody against the cloned human N-SMase protein (Fig. 3). However, the antibody detected little, if any, 45-kDa protein in a partially purified N-SMase preparation from Molt-4 cells. In these experiments, equal N-SMase activities were loaded. These results
suggest that the cloned N-SMase is immunologically distinct from the endogenous N-SMase in Molt-4 cells.

_Growth Rates of Transfectant Cells—_Because of the above results showing N-SMase activity distinct from purified N-SMases, we next investigated the function of the cloned N-SMase in cells. To this end, 293 cells were transfected with either vector alone or pHisNSM expressing the putative N-SMase. There was no apparent phenotypic difference between vector and pHisNSM transfectant cells. As shown in Fig. 4, growth rates of both vector and pHisNSM transfectant cells were similar.

_The Levels of SM and Ceramide in Transfectant Cells—_To examine whether the overexpressed protein functions as N-SMase in cells, the levels of SM and ceramide in wild type, vector, or pHisNSM transfectants of 293 cells were measured. The level of SM and PC were slightly higher in vector transfectant, and the level of SM was slightly lower in pHisNSM transfectant cells compared with wild type cells (Fig. 5, A and B). The level of ceramide was increased in pHisNSM transfectant cells by approximately 20% compared with vector transfectant cells and by only 10% compared with wild type cells (Fig. 5C). These changes in the levels of SM and ceramide in overexpressing cells seemed very small compared with the 20-fold increase in __in vitro__ N-SMase activity.

_Metabolism of SM and PC in Transfectant Cells—_When vector or pHisNSM transfectant cells were continuously labeled with [3H]choline, the time course of the increase in labeled PC or SM was similar in both transfectant cells (Fig. 6A). There was a slight decrease in the level of labeled SM at 3 days in pHisNSM transfectant cells compared with that in vector transfectant cells. When vector or pHisNSM transfectant cells were labeled with [3H]choline in equilibrium and then chased after replacement of medium, labeled SM and PC in pHisNSM transfectant cells decreased in a similar time course with those in vector transfectant cells (Fig. 6B). These results argue against any significant effect of the enzyme on the catabolism of either SM or PC.

In another set of experiments, we employed bacterial SMase to evaluate the topology of SM and its resynthesis. [3H]choline-labeled cells were treated with exogenous bacterial SMase for 25 min and then chased (Fig. 6C). Treatment with bacterial SMase diminished the level of SM by approximately 80% in both vector and pHisNSM transfectant cells. These results show that the distribution of SM between bacterial SMase-sensitive (outer leaflet) and resistant (internal) pools in pHisNSM transfectant cells is similar to that in vector transfectant cells. Moreover, the level of SM did not recover at least during 24 h in both transfectants, showing that the gene under investigation did not stimulate this pool of SM synthesis. This is consistent with the result of __in vitro__ SM synthase assay, which showed no increase of SM synthase activity (Table I), and with the result of the pulse choline labeling experiment, which showed no stimulation of SM synthesis in pHisNSM transfectant cells compared with vector transfectant cells (Fig. 6A).

_Strain-induced Ceramide Generation in Transfectant Cells—_Because it has been reported that N-SMase may be involved in stress-induced ceramide generation, we investigated whether overexpression of the protein might stimulate ceramide generation induced by hydrogen peroxide, a known inducer of ceramide (9, 32). The level of ceramide was increased by treatment with 1 mM hydrogen peroxide in a similar time course in both vector and pHisNSM transfectant cells (Fig. 7). These results showed that the overexpressed protein is not involved in stress-induced ceramide generation.
To investigate whether the levels of lipids other than SM, PC, or ceramide might be changed in pHisNSM transfec-
tant compared with vector transfectant cells, both transfec-
tants were labeled with \(^{3}\text{H}\)palmitic acid, and the levels of
labeled lipids were compared between vector and pHisNSM
transfectant cells. The majority of labeled lipids showed no
apparent difference in various TLC solvent systems. However,
a band was increased in pHisNSM cells compared with vector
transfectant cells (Fig. 8A). This band was scraped from the
TLC plate, and the lipid was extracted by the method of Bligh
and Dyer and subjected to mild base hydrolysis. The extracted
lipid was resistant to mild base hydrolysis, indicating that the
lipid does not contain ester linkages (data not shown). This
band was not labeled with \(^{3}\text{H}\)dihydrosphingosine or
\(^{3}\text{H}\)sphingosine (data not shown). These results suggested that
1-alkyl-2-acyl-PC in overexpressing cells. A lane labeled with [3H]hexadecanol than with [3H]palmitic acid in glycerol. It was found that the band was more prominently in pHisNSM transfectant cells (Fig. 8C), suggesting that 1-alkyl-2-acyl-PC was used as the source of 1-alkyl-glycerol through deacylation at sn-2 position followed by the action of the overexpressed protein (discussed below).

Lyso-PAF and Lyso-PC-PLC Activity of the Overexpressed Protein in Vitro—Assuming that the overexpressed enzyme has PLC activity, we suspected that lyso-PAF might be another substrate of the enzyme. Therefore, we conducted in vitro experiments using [1-alkyl-3H]lyso-PAF as a substrate. As expected, lyso-PAF was a good substrate for this activity, and we observed an increased generation of 1-alkyl-glycerol (Table II). Lysate of pHisNSM transfectant cells had 20 times as high PLC activity on lyso-PAF as that of vector transfectant cells. Lyso-PC-PLC activity was dependent on Mg2+ and was stimulated by dithiothreitol (data not shown). In contrast to N-SMase activity, which requires detergent such as Triton X-100, lyso-PC-PLC activity was detected in the absence of Triton X-100. In fact, addition of Triton X-100 in the assay inhibited lyso-PC-PLC activity (data not shown). The protein immunoprecipitated with anti-His antibody also demonstrated high lyso-PC-PLC activity (Fig. 9). Approximately 30% of the lyso-PC-PLC activity was recovered in the immunoprecipitant, whereas about 20% of the activity remained in the supernatant. Lyso-PC was also a good substrate of the enzyme in vitro (Table II). The Km and Vmax values of the immunoprecipitated protein for lyso-PAF and lyso-PC were similar (Table III). The Km and Vmax for SM could not be determined because N-SMase activity in the absence of Triton X-100 in the assay was very low (less than 2% compared with the activity in the presence of 0.05% Triton X-100 in the assay). The Km for SM in the presence of 0.05% Triton X-100 was similar to and Vmax was higher than that for lyso-PAF or lyso-PC. No PAF-PLC activity was detected in either vector or pHisNSM transfectant cells (Table II), suggesting the requirement of 2-lyso structure of the glycerolipid substrates.

Metabolism of Lyso-PAF, Lyso-PC, and PAF in Transfectant Cells—To investigate the metabolism of lyso-PAF or lyso-PC in cells, both transfectants were labeled with [1-alkyl-3H]lyso-PAF (Fig. 10A) or [1-acyl-3H]lyso-PC (Fig. 10B). In vector transfectant cells both lyso-PAF and lyso-PC were predominantly metabolized into diradyl-PC. (The radioactivities in diradyl-PC and 1-alkyl- or 1-acyl-glycerol compared with the total radioactivities in vector transfectant cells were 21.8 ± 0.6% and 0.7 ± 0.1% for lyso-PAF labeling and 39.8 ± 2.3% and 0.3 ± 0.1% for lyso-PC labeling, respectively.) In pHisNSM transfectant cells lyso-PAF was metabolized not only into diradyl-PC but also into 1-alkyl-glycerol, whereas lyso-PC was almost exclusively metabolized into diradyl-PC. (The radioactivities in diradyl-PC and 1-alkyl- or 1-acyl-glycerol compared with the total radioactivities in pHisNSM transfectant cells were 14.0 ± 0.1% and 11.1 ± 0.4% for lyso-PAF labeling and 34.8 ± 2.3% and 0.5 ± 0.1% for lyso-PC labeling, respectively.) These results suggested that the overexpressed protein in cells

![Diagram](image-url)

**Fig. 7.** No effects of the overexpressed protein on stress-induced ceramide generation. Vector and pHisNSM transfectant cells were treated with 1 mM hydrogen peroxide for the indicated hours, and the levels of ceramide were determined by diacylglycerol kinase assay as described under “Experimental Procedures.” The results are averages of three different experiments. Error bars indicate S.D. Closed circles, vector transfectant cells; open circles, pHisNSM transfectant cells.

**Table II**

|                 | Wild type | Vector | pHisNSM |
|-----------------|-----------|--------|---------|
| Lyso-PAF-PLC    | 5.0 ± 2.7 | 6.1 ± 2.9 | 135.7 ± 30.7 |
| Lyso-PC-PLC     | 11.4 ± 3.2 | 16.7 ± 3.4 | 124.1 ± 10.4 |
| PAF-PLC         | 0         | 0       | 0       |

supported that the band was 1-alkyl-glycerol. When lipids from cells labeled with [3H]hexadecanol were subjected to mild base hydrolysis, a decrease of 1-alkyl-lyso-PC (lyso-PAF) was detected in pHisNSM transfectant cells compared with vector transfectant cells (Fig. 8C), suggesting that 1-alkyl-2-acyl-PC was used as the source of 1-alkyl-glycerol through deacylation at sn-2 position followed by the action of the overexpressed protein (discussed below).
functions much more specifically as lyso-PAF-PLC rather than lyso-PC-PLC. Furthermore, generation of 1-alkyl-glycerol was also detected when pHisNSM transfectant cells were labeled with [1-alkyl-3H]PAF (Fig. 10C). These results strongly suggest that the overexpressed protein is involved in the metabolic pathways of PAF.

Comparison of Lyso-PAF-PLC Activity in the Overexpressed Protein and Rat Brain N-SMase—Lyso-PAF-PLC activity in partially purified rat brain N-SMase was also examined. Lyso-PAF-PLC activity was only 1% of N-SMase activity in partially purified rat brain protein (Table IV), whereas lyso-PAF-PLC activity was almost the same as N-SMase activity in the overexpressed protein (Tables I and II). These results further suggest that rat brain N-SMase is unrelated to the overexpressed protein.

**DISCUSSION**

In this paper we demonstrated that the cloned putative N-SMase protein does not function as N-SMase in cells and that the protein exerted lyso-PAF-PLC activity both in vitro and in cells. Because the overexpressed protein has high N-SMase activity in vitro, there seems to be two possibilities to explain why it does not act as N-SMase in cells. One possibility is the localization of the protein in cells. Approximately 80% of SM is localized in the outer leaflet of plasma membrane judging from the results using exogenous bacterial SMase, and SM involved in stress-induced responses is supposed to be located in the inner leaflet of plasma membrane or caveolae (33). It is possible that this protein is not localized in the plasma membrane so that it cannot gain access to most of SM in cells. In fact, overexpression of a green fluorescent protein fusion protein showed that this protein mainly exists in the ER. Another possibility is the requirement of specific detergents for N-SMase activity. The protein has little N-SMase activity in the absence of detergents in the assay (Table III). Among the detergents tested, Triton X-100 and Nonidet P-40 could stimulate N-SMase activity. The protein has little N-SMase activity under prevailing conditions in cells. In contrast, lyso-PAF-(or lyso-PC-)PLC activity did not require detergents in vitro, and this protein also exerted lyso-PAF-PLC activity in cells.

Several criteria also suggest that the overexpressed protein is distinct from N-SMases partially purified from rat brain and Molt-4 cells. First, N-SMase activity in the overexpressed protein was inhibited by Triton X-100, Nonidet P-40, and β-octyl-
glucoside, whereas that in partially purified rat brain was not inhibited by these detergents but by deoxycholic acid. Second, the partially purified protein from Molt-4 cells was not detected by an antibody against the overexpressed protein. Third, lyso-PAF-PLC activity of the partially purified protein from rat brain was only 1% of N-SMase activity, whereas lyso-PAF-PLC activity was almost the same as N-SMase activity in the overexpressed protein. It was also reported that lyso-PC is not a good substrate for bacterial N-SMase (34). This discrepancy is now explained by the observation that the cloned enzyme is not the previously studied N-SMase. Finally, the overexpressed protein was not involved in stress-induced ceramide generation. These results suggest that the overexpressed protein is not the major N-SMase in rat brain and Molt-4 cells. In support of this is the evidence that the mRNA expression of the mouse homologue of this protein was the most abundant in kidney, whereas N-SMase activity was the highest in brain among various mouse tissues (23). It will be of great significance to identify the protein for N-SMase activity involved in stress responses.

Although this protein demonstrated PLC activity toward both lyso-PAF and lyso-PC in vitro, little 1-acyl-glycerol was accumulated when cells were labeled with lyso-PC, whereas accumulation of 1-alkyl-glycerol was detected by labeling with lyso-PAF or PAF (Fig. 10). This can be explained in several ways. One possibility is that lyso-PC is much more preferably converted into diacyl-PC by acyltransferases or transacylases than into 1-acyl-glycerol by this protein. In this case, it should be also considered that acyltransferases or transacylases prefer lyso-PC rather than lyso-PAF because lyso-PAF was almost equally converted into both diacyl-PC and 1-alkyl-glycerol. It is also possible that accumulation of 1-acyl-glycerol could not be detected because 1-acyl-glycerol was rapidly degraded by lipases, whereas 1-alkyl-glycerol accumulated because of the slower metabolism of 1-alkyl-glycerol. Another explanation is the distribution of lyso-PC in cells where this protein cannot access it as a substrate. However, this appears less probable because the structure of lyso-PC is very close to that of lyso-PAF, and one would expect similar physical properties including solubility, uptake, and partitioning.

Several reports have shown the existence of a lyso-PLD and the generation of 1-alkyl-glycerol from lyso-PAF by lyso-PLD followed by the action of phosphohydrolase (35–37). Here we demonstrated that the overexpressed protein is not lyso-PLD but lyso-PAF because not only cell lysates but also the immuno-precipitated protein produced 1-alkyl-glycerol from lyso-PAF in vitro. In fact, this protein did not generate lysophosphatidic acid even in the presence of 20 mM sodium fluoride, an inhibitor of phosphohydrolase, in the assay. 2 Because of these considerations, we propose the name lyso-PAF-PLC for this enzyme.

The significance of lyso-PLC in the metabolism of PAF has not been described before. Although the existence of lyso-PLC involved in the metabolism of PAF was reported in one study, the possibility of the involvement of lyso-PLD instead of lyso-PLC was not considered there (38). Another report described a lyso-PLC activity that hydrolyzed ether-linked lysophosphoglycerides and was involved in the synthesis of choline plasmalogens from ethanolamine plasmalogens (39). It remains to be elucidated whether this lyso-PLC activity is identical to that described here.

Because the overexpressed protein produced 1-alkyl-glycerol from lyso-PAF generated from PAF by acetylhydrolase in cells, it is highly probable that the protein is involved in the metabolic pathway of PAF in cells. PAF is a biologically very potent molecule that induces platelet aggregation and granule secretion at nanomolar concentrations (40). It is generally consid-

REFERENCES

1. Hannun, Y. A. (1996) Science 274, 1855–1859
2. Hannun, Y. A. (1994) J. Biol. Chem. 269, 3125–3128
3. Kim, M. Y., Linardic, C., Obeid, L., and Hannun, Y. (1991) J. Biol. Chem. 266, 484–489
4. Tepper, C. G., Jayadev, S., Liu, B., Bielawska, A., Wolff, R., Yonehara, S., Hannun, Y. A., and Seldin, M. F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8443–8447
5. Cifone, M. G., De Maria, R., Roncadoi, P., Rippo, M. R., Azuma, M., Lanier, L. L., Santoni, A., and Testi, R. (1994) J. Exp. Med. 177, 1547–1552
6. Strum, J. C., Small, G. W., Pauig, S. B., and Daniel, L. W. (1994) J. Biol. Chem. 269, 15493–15497
7. Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fucks, Z., and Kolesnick, R. (1995) Cell 82, 405–414
8. Haimovitz-Friedman, A., Kan, C. C., Ehleiter, D., Persaud, R. S., McLaughlin, M., Fucks, Z., and Kolesnick, R. N. (1994) J. Exp. Med. 180, 525–535
9. Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Sahoo, E., Zou, L. Y., Kyo, Y., and Haimovitz-Friedman, A., Fucks, Z., and Kolesnick, R. N. (1996) Nature 380, 75–79
10. Jayadev, S., Liu, B., Bielawska, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M. Y., Obeid, L. M., and Hannun, Y. A. (1995) J. Biol. Chem. 270, 2047–2052
11. Quintern, L. E., Weitz, G., Nehrkorn, H., Tager, J. M., Schram, A. W., and Sandhoff, K. (1987) Biochim. Biophys. Acta 923, 325–336
12. Quintern, L. E., Schuchman, E. H., Levrari, O., Suchi, M., Ferlinz, K., Reinke, H., Sandhoff, K., and Desnick, R. J. (1989) EMBO J. 8, 2469–2473
13. Schuchman, E. H., Suchi, M., Takahashi, T., Sandhoff, K., and Desnick, R. J. (1991) J. Biol. Chem. 266, 8551–8559
14. Liu, B., Hassler, D. F., Smith, G. K., Weaver, K., and Hannun, Y. A. (1998) J. Biol. Chem. 273, 34472–34479
15. Gotthardt, T., Bielawska, A., Scotto, K., Bell, R. M., and Hannun, Y. A. (1994) J. Biol. Chem. 269, 4070–4077
16. Schissel, S. L., Schuchman, E. H., Williams, K. J., and Tabas, I. (1996) J. Biol. Chem. 271, 18431–18436
17. Duan, R. D., Nyberg, L., and Nilsson, A. (1995) Biochim. Biophys. Acta 1259, 49–55
18. Duan, R. D., and Nilsson, A. (1997) Hepatology 26, 823–830
19. Ottenbach, B., and Stoffel, W. (1995) Cell 81, 1053–1061
20. Horinouchi, K., Eriich, I., Pap, D., Ferlinz, K., Bisgaier, C. L., Sandhoff, K., Desnick, R. J., Stewart, C. L., and Schuchman, E. H. (1995) J. Biol. Chem. 270, 2560–2565
21. Santana, P., Pena, L. A., Haimovitz-Friedman, A., Martin, S., Green, D., Kolesnick, R. N. (1996) J. Biol. Chem. 271, 823–830
22. Confalonieri, A., Riva, E., Zon, L. I., Kyo, Y., and Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. N. (1996) Nature 380, 75–79
23. Jayadev, S., Liu, B., Bielawska, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M. Y., Obeid, L. M., and Hannun, Y. A. (1995) J. Biol. Chem. 270, 2047–2052
24. Warne, T. R., Buchanan, P. G., and Robinson, M. (1995) J. Biol. Chem. 270, 11147–11154
25. Feng, X., Zhang, J., Barak, L. S., Meyer, T., Caron, M. G., and Hannun, Y. A. (1998) J. Biol. Chem. 273, 10755–10762
Function of AJ 222801 as Lyso-PAF-PLC

26. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–917
27. Luberto, C., and Hannun, Y. A. (1998) J. Biol. Chem. 273, 14550–14559
28. Liu, B., and Hannun, Y. A. (1997) J. Biol. Chem. 272, 16281–16287
29. Sawai, H., Okazaki, T., Takeda, Y., Tashima, M., Sawada, H., Okuma, M., Kishi, S., Umehara, H., and Domae, N. (1997) J. Biol. Chem. 272, 2452–2458
30. Ames, B. N., and Dubin, D. T. (1960) J. Biol. Chem. 235, 769–775
31. Davis, P. A., and Hajra, A. K. (1981) Arch. Biochem. Biophys. 211, 20–29
32. Goldkorn, T., Balaban, N., Shannon, M., Chea, V., Matsukuma, K., Gilchrist, D., Wang, H., and Chan, C. (1998) J. Cell Sci. 111, 3209–3220
33. Linardic, C. M., and Hannun, Y. A. (1994) J. Biol. Chem. 269, 23530–23537
34. Dziewanowska, K., Edwards, V. M., Deringer, J. R., Bohach, G. A., and Guerra, D. J. (1996) Arch. Biochem. Biophys. 335, 102–108
35. Wykle, R. L., and Schremmer, J. M. (1974) J. Biol. Chem. 249, 1742–1746
36. Wykle, R. L., Kraemer, W. F., and Schremmer, J. M. (1977) Arch. Biochem. Biophys. 184, 149–155
37. Wykle, R. L., Kraemer, W. F., and Schremmer, J. M. (1980) Biochim. Biophys. Acta 619, 58–67
38. Okayasu, T., Hoshii, K., Seyama, K., Ishibashi, T., and Imai, Y. (1986) Biochim. Biophys. Acta 876, 58–64
39. Strum, J. C., and Daniel, L. W. (1995) J. Biol. Chem. 268, 25500–25508
40. Snyder, F. (1995) Biochim. Biophys. Acta 1254, 231–249
41. Snyder, F. (1990) Am. J. Physiol. 259, C697–C708
42. Arthur, G., and Bittman, R. (1998) Biochim. Biophys. Acta 1390, 85–102