Neutral Sphingomyelinase 2 Is Palmitoylated on Multiple Cysteine Residues

ROLE OF PALMITOYLATION IN SUBCELLULAR LOCALIZATION

Motohiro Tani and Yusuf A. Hannun

From the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425

The neutral sphingomyelinas (nSMases) are considered major candidates for mediating the stress-induced production of ceramide. nSMase2, which has two hydrophobic segments near the NH2-terminal region, has been reported to be located at the plasma membrane and play important roles in ceramide-mediated signaling. In this study, we found that nSMase2 is palmitoylated on multiple cysteine residues via thioester bonds. Site-directed mutagenesis of cysteine residues to alanine indicated that two cysteine clusters of the enzyme are multiply palmitoylated; one cluster is located between the two hydrophobic segments, and the second one is located in the middle of the catalytic region of the protein. When overexpressed in the confluent phase of MCF-7 cells, wild-type nSMase2 was strictly localized in the plasma membranes, and the cysteine mutants of each palmitoylated cysteine cluster were seen not only at the plasma membrane but also in some punctate structures. Furthermore, mutation of all potential palmitoylation sites resulted in a dramatic reduction in the plasma membrane distribution and an increase in the punctate structures. The palmitoylation-deficient mutant was directed to lysosomes and rapidly degraded. Palmitoylation had no effect on enzyme activity but affected membrane-association properties of the protein. Finally, the catalytic region of nSMase2 where palmitoylation occurs was found to be localized at the inner leaflet of the plasma membrane. In summary, the results from this study reveal for the first time the palmitoylation of nSMase2 via thioester bonds and its importance in the subcellular localization and stability of this protein.

Ceramide, a bioactive sphingolipid, is involved in the regulation of diverse cellular functions, including cell growth, apoptosis, and differentiation (1). Sphingomyelinase (EC 3.1.4.12, SMase), an enzyme that catalyzes the hydrolysis of the phosphodiester bond of sphingomyelin to produce ceramide and phosphocholine, has emerged as a major pathway of stress-induced ceramide production (2). To date, three classes of SMase, acid, neutral, and alkaline, have been identified that are clearly distinguished by catalytic pH optimum, cation dependence, primary structure, and subcellular localization (3, 4).

The Mg2+-dependent neutral SMases (nSMases) have emerged as major candidates for mediating ceramide-induced signal transduction (5). Recent advances have resulted in molecular identification of at least three distinct nSMases in mammals, nSMases1, -2, and -3 (6–8). nSMase1 was the first identified mammalian nSMase, which was cloned by remote sequence similarity with bacterial SMase (6). Although this enzyme exhibits in vitro SMase activity (6), cells overexpressing it did not show changes in sphingomyelin or ceramide metabolism (9), and the nSMase1 knock-out mouse appeared to exhibit a normal phenotype (10).

nSMase2 has been also cloned by data base search using bacterial SMase genes (7). The enzyme is a membrane-bound protein and has two highly hydrophobic segments near the NH2-terminal region, both of which are thought to function as transmembrane domains. In contrast to nSMase1, nSMase2 possesses in vivo SMase activity when overexpressed in mammalian cells (11). A number of studies using culture cell lines have focused on potential signaling roles of nSMase2. Studies in MCF-7 cells have shown that nSMase2 is up-regulated during cell growth and is required for cells to undergo confluence-induced cell cycle arrest (12). Interestingly, nSMase2 had been previously isolated as a confluence-induced gene in rat 3Y1 fibroblasts (13). nSMase2 has also been implicated in signal transduction events in response to cytokines (14–16), oxidative stress (17), or amyloid β-peptide (18). In addition, the nSMase2 knock-out mice developed growth retardation that remained throughout development (19). In an independent study, Aubin et al. (20) identified a deletion in the gene encoding nSMase2 in the fragilis ossium or “fro” mouse, a model of osteogenesis imperfecta.

Bacterial SMase, which has remote sequence similarity with eukaryotic nSMases, has provided important information on the catalytic mechanism of the enzyme with preservation of key catalytic amino acid residues between the bacterial and eukaryotic enzymes (21–23). Site-directed mutagenesis analyses of this mutant protein; PBS, phosphate-buffered saline; SNARE, soluble NSF attachment protein receptors.

□  □

□  □

□  □

□  □

□  □
mammalian nSMase1 and the yeast nSMase, Isc1p, revealed that these key amino acid residues are essential for catalytic activity of the enzyme as well as bacterial SMases (24, 25). In contrast to the accumulated evidence for the catalytic mechanism of nSMases from molecular aspects, there is little information about other primary features, post-translational modifications, or subcellular localization and topology of the protein.

nSMase2 strictly localized at the plasma membrane in the confluence phase of MCF-7 cells and in primary hepatocytes when overexpressed (12, 14); however, there is no information on the mechanisms that determine this localization, except for the presence of putative transmembrane regions at the NH₂ terminus. In this study, we found that nSMase2 is palmitoylated via thioester bonds, and this post-translational modification has crucial roles for determination of subcellular localization and life span of the protein. This is the first report of post-translational analysis of nSMase2. The significance of this modification is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—[choline-methyl-14C]Sphingomyelin was provided by Dr. Alicia Bielawska (Medical University of South Carolina, Charleston, SC). [9,10-3H]Palmitic acid (50 Ci/mmol) was from American Radiolabeled Chemicals, Inc. The scintillation mixture Safety Solve was from Research Products International. Other chemicals were from Sigma. Culture media were obtained from Invitrogen.

**Cell Culture and cDNA Transfection**—MCF-7 cells were cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO₂ incubator. For cDNA transfection, MCF-7 cells were plated in 6-well plates or 35-mm glass-bottom microwell dishes at a density of 100,000 cells/well and cultured for 24 h. Transfections were done using Effectene transfection reagent (Qiagen) as recommended by the manufacturer. After transfection, cells were cultured for an additional 24 h and used for activity assays, immunoblotting, and immunofluorescence analyses. To obtain tetracycline-inducible stably cell lines, MCF-7 cells were first transfected with pcDNA6/TR vector (Invitrogen) and selected in RPMI 1640 medium supplemented with 10% FBS and 7 μg/ml blasticidin (Invitrogen). The blasticidin-resistant cells were then transfected with the expression construct pcDNA4-NSM2 or pcDNA4-Cys3A5A and selected in RPMI 1640 medium supplemented with 10% FBS, 100 μg/ml Zeocin (Invitrogen), and 7 μg/ml blasticidin. The antibiotic-resistant clones were screened for expression of nSMase2 by immunofluorescence analysis using anti-Myc antibody. A representative clone, termed NSM2-Tet-On or CYS3A5A-Tet-On, was chosen and maintained in RPMI 1640 medium supplemented with 10% Tet System Approved FBS (Clontech) and 7 μg/ml blasticidin.

**nSMase Activity**—MCF-7 cells transfected with plasmids were washed twice with PBS and lysed by sonication in buffer containing 25 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture (Roche Diagnostics). Post-nuclear supernatants were prepared by centrifugation at 600 × g for 10 min and used for enzyme assays. One hundred μM [choline-methyl-14C]Sphingomyelin (10 cpm/pmol) was incubated at 37 °C for 30 min with an appropriate amount of cell lysate in 100 μl of reaction mixture (100 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 2.5 mM diethiothreitol, 0.2% Triton X-100, and 50 μM phosphatidylserine). The reaction was stopped by adding 500 μl of chloroform/methanol (2:1, v/v), and the resulting 180-μl upper phase was mixed with 4 ml of Safety Solve (Research Products International) for liquid scintillation counting.

**Preparation of Rabbit Polyclonal Antibody against nSMase2**—The antibody was prepared by the Medical University of South Carolina antibody facility. Briefly, a synthetic oligopeptide corresponding to the 335–353 amino acids (RRRHPDEAFD-HEVSAFF) of the human nSMase2 (this sequence is identical to the 335–351 amino acids of the mouse enzyme) was used for immunization. Antiserum was affinity-purified over a cyanogen bromide-activated agarose column bound with the same oligopeptide.

**Protein Determination, SDS-PAGE, and Western Blotting**—Protein content was determined by the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as a standard. Samples for gel electrophoresis were combined with 5 × SDS sample buffer containing 5% 2-mercaptoethanol and separated by SDS-PAGE. For Western blotting, following separation by SDS-PAGE, proteins were electrotransferred to a nitrocellulose membrane. The membrane was blocked with PBS, 0.1% Tween 20 (PBS-T) containing 3% dried milk. Proteins were identified by incubating with anti-V5 (0.2 μg/ml; Invitrogen) or anti-glyceraldehyde-3-phosphate dehydrogenase (1 μg/ml; Ambion) in 3% dried milk/PBS-T at 4 °C for overnight. Secondary antibodies, horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch) diluted 1:10,000, were incubated in 3% dried milk/PBS-T at room temperature for 1 h. Finally, proteins were visualized using enhanced chemiluminescence (Pierce) with exposure to CL-X Posure™ film (Pierce).

**Immunoprecipitation**—The cells were lysed with 100 μl of 20 mM Tris-HCl (pH 7.4) containing 1% SDS and 1% 2-mercaptoethanol and were boiled for 5 min. Samples were centrifuged at 10,000 × g for 5 min, and the supernatant was mixed with 1 ml of 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100. The mixture was incubated with 1 μg of anti-V5 antibody at 4 °C for 12 h, and then 10 μl of protein A/G-agarose (Santa Cruz Biotechnology) was added, and the mixture was incubated at 4 °C for 3 h. The precipitate was spun down by centrifugation, washed with PBS five times, and then suspended in 20 μl of SDS sample buffer. After boiling for 5 min, the sample was subjected to SDS-PAGE, followed by Western blotting analysis as described above.

**In Vivo [3H]Palmitic Acid Labeling**—MCF-7 cells grown on a 6-well plate were transfected with plasmids and incubated for 24 h at 37 °C. Culture medium was then changed to 1.5 ml of serum-free RPMI 1640 medium. After a 1-h incubation at 37 °C, cells were labeled with 100 μCi of [3H]palmitic acid (50 Ci/mmol; American Radiolabeled Chemicals, Inc.) at 37 °C for 3 h. Cells were washed twice with PBS, and V5-tagged nSMase2 was immunoprecipitated using anti-V5 antibody and protein A/G-agarose as described above. Immunoprecipitates, suspended in SDS sample buffer containing 1% 2-mercaptoetha-
nol, were boiled for 3 min, separated by SDS-PAGE, and visualized by autoradiography using the fluorographic reagent ENHANCE\textsuperscript{TM} (PerkinElmer Life Sciences). 1% of respective cell lysates was analyzed by Western blotting with anti-V5 antibody as described above.

Preparation of Soluble and Membrane Fractions—MCF-7 cells transfected with plasmids were washed twice with PBS, suspended in buffer A (20 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 \times protease inhibitor mixture (Roche Diagnostics)), and sonicated. After removal of cell debris by centrifugation at 500 \times g for 5 min at 4 °C, cell lysates were centrifuged at 100,000 \times g for 90 min at 4 °C. Occasionally, the cell lysates were treated with an equal volume of buffer A containing 1% Triton X-100, 1% Tween 20, or 0.5% deoxycholate for 1 h on ice before centrifugation. The resulting supernatant and pellet were used as soluble and membrane fractions, respectively.

Immunofluorescence and Confocal Microscopy—Transfected cells were cultured on cover glass and then fixed with 3% paraformaldehyde in PBS for 15 min. After being rinsed with PBS and 50 mM NH\textsubscript{4}Cl in PBS, cells were permeabilized by 0.1% Tween 20, or 0.5% deoxycholate for 1 h on ice before centrifugation. The proteins were labeled with primary antibodies and then with secondary antibody at room temperature for 1 h. All confocal images were taken with a laser-scanning confocal microscope (LSM 510 Meta; Carl Zeiss, Thornwood, NY). Each microscopic image is representative of 20 fields over at least three experiments, and all images were taken at the equatorial plane of the cell. Raw data images were cropped in Adobe Photoshop\textsuperscript{TM} 7.0 for publication.

Plasmid Construction—Wild-type mouse nSMase2 tagged with V5 at the COOH terminus (pcEF6NSM2) was constructed by PCR using a 5′ primer with an XhoI restriction site (5′-TCCGCTCAGAATGTTTGGTACACGAC-3′) and a 3′ primer disrupted stop codon (5′-CGCCTCCTCTCTCCCTTGACGACA-3′) and subcloned into pcEF6/V5-His-TOPO vector (Invitrogen). To generate a construct expressing the GFP-fused nSMase2 (pcDNA4-NSM2) or Cys3A5A mutant (pcDNA4-Cys3A5A) was constructed by PCR using a 5′ primer containing the XhoI restriction site (5′-GGGTTACCATGTTTGGTACACGACCCCTTCTC-3′) and a 3′ primer with an XhoI restriction site disrupted stop codon (5′-TTCTCGCTAGATGCTCTCTCTTTCTCCCTTGACGACA-3′) and subcloned into pcDNA4/TO/myc-HisA (Invitrogen). Point mutants of nSMase2 were constructed by fusing the NH\textsubscript{2}- and COOH-terminal fragments of the enzyme. The NH\textsubscript{2}-terminal fragments were amplified by PCR using a 5′ primer containing the sequence of pcEF6/V5-His TOPO vector (5′-TAATACGACTCACTATAGGG-3′), 3′ primers (see supplemental Table 1), and pcEF6NSM2 as a template. The COOH-terminal fragments were amplified by PCR using 5′ primers (see supplemental Table 1) and a 3′ primer (5′-CCGCTCCTCTCTCCCTTGACGACA-3′). These fragments were extended with Pj50\textsuperscript{TM} DNA polymerase (Invitrogen), digested with SpeI and EcoRI, and subcloned into pcEF6NSM2. The sequences of all constructs were verified with an ABI377 DNA sequencer.

RESULTS

nSMase2 Is Palmitoylated via Thioester Bonds—Several membrane proteins have lipid modifications, and palmitoylation is one of these important lipid modifications. To determine whether nSMase2 is palmitoylated, MCF-7 cells overexpressing nSMase2 with a V5 tag at the COOH terminus were labeled with [\textsuperscript{3}H]palmitic acid, and the protein was immunoprecipitated using anti-V5 antibody and subjected to SDS-PAGE analysis and fluorography. As shown in Fig. 1A, [\textsuperscript{3}H]palmitic acid was incorporated into a 76-kDa protein that co-migrated with nSMase2 on Western blotting, whereas no labeled proteins were detected in mock-transfected cells. In many cases, cova-

![Palmitoylation of nSMase2 via thioester linkage](image)
lent attachment of palmitic acid into proteins occurs through a thioester bond to Cys residues (26). The thioester is cleaved by treatment with weak nucleophiles such as neutral hydroxylamine. Hydroxylamine treatment removed the entire incorporated label from nSMase2, demonstrating that the acyl moiety on the enzyme was attached via a thioester bond (Fig. 1B).

Identification of Two Palmitoylated Cys Clusters in nSMase2—There is no well defined consensus sequence for palmitoylation other than a requirement for Cys. Palmitoylation frequently occurs at Cys residues arranged in clusters (26). We found three Cys clusters in the mouse nSMase2 amino acid sequence as follows: a cluster containing three Cys residues, Cys125, Cys132, and Cys139 (Cys3 cluster), which are located between the two hydrophobic segments; two Cys residues, Cys12 and Cys132 (Cys4 cluster); and four Cys residues, Cys392, Cys395, Cys396, and Cys400 (Cys5 cluster), which are located in the middle of the putative catalytic region of the protein (Fig. 2A). Many proteins are also palmitoylated at Cys residues near the NH2 terminus (26). In nSMase2 there are two Cys residues, Cys12 (Cys1 position) and Cys387 (Cys2 position) in the NH2-terminal region (Fig. 2A). To determine whether these Cys residues are involved in palmitoylation, we created Cys to Ala mutants of each Cys cluster as well as in combinations (Table 1, all constructs used in this study are shown in Table 1). All mutants were tagged with V5 at their COOH terminus. These mutants were transfected into MCF-7 cells, labeled with [3H]palmitic acid, and analyzed by SDS-PAGE and fluorography or Western blotting using anti-V5 antibody. As shown in Fig. 2B, although all mutant proteins were expressed in the cells, the incorporation of [3H]palmitic acid into each protein was quite different. The mutation of Cys1, -2, or -4 positions to Ala (Cys1A, Cys2A, or Cys4A mutant) did not have significant effects on the incorporation of [3H]palmitate. On the contrary, [3H]palmitoylated Cys3A and Cys5A mutants (mutated Cys3 or Cys5 positions to Ala, respectively) were observed but far less than the wild-type protein. Furthermore, the Cys3A5A combination mutant almost completely lost the incorporation of [3H]palmitate. On the contrary, [3H]palmitoylated Cys3A and Cys5A mutants (mutated Cys3 or Cys5 positions to Ala, respectively) were observed but far less than the wild-type protein. Furthermore, the Cys3A5A combination mutant almost completely lost the incorporation of [3H]palmitoylation (Fig. 2B). These results clearly indicated that nSMase2 is palmitoylated in Cys residues of two Cys clusters of the protein.

Effect of Palmitoylation on the Localization of nSMase2—Generally, protein palmitoylation appears to play an important role in subcellular trafficking of proteins, in modulating protein activity, and/or in protein-protein interactions (26). We initially examined whether palmitoylation affects the subcellular localization of the enzyme in the confluence phase of MCF-7 cells. As reported previously, wild-type nSMase2 was localized at the plasma membrane when overexpressed in the confluent culture of MCF-7 cells. As reported previously, wild-type nSMase2 was localized to the plasma membrane when overexpressed in the confluent phase of MCF-7 cells (Fig. 3A, panel a). Cys1A, Cys2A, and Cys4A mutants showed similar expression patterns to the wild-type protein (Fig. 3A, panels b, c, and e). However, the Cys3A and Cys5A mutants were seen not only in the plasma membrane but also in some punctate structures (Fig. 3A, panels d and f). The Cys3 and -5 double mutant (Cys3A5A) showed a very different distribution, displaying a dramatic reduction in the plasma membrane distribution and an increase in the punc-

---

**TABLE 1**

Identification of the different nSMase2 mutants used in this study

The nomenclature of various mutant proteins, the position of mutations, and the fusion tags present are shown. The mutated amino acids are underlined.

| Name           | Position of mutation (Cys to Ala)                  | Fusion tag (COOH terminus) |
|----------------|---------------------------------------------------|----------------------------|
| Wild type      | None                                              | V5                         |
| NSM2GFP        | None                                              | GFP                        |
| Cys1A          | 10NSALS14                                         | V5                         |
| Cys2A          | 125FAFATANVAL133                                    | V5                         |
| Cys3A          | 132HGCNNFKCL1402                                    | V5                         |
| Cys3A5A        | 132HGCNNFKCL1402                                    | V5                         |
| GFP-tagged Cys3A5A | 132HGCNNFKCL1402                                | V5                         |
| Cys5A          | 391GAHGAANFKALN1402                                  | V5                         |
| Cys3A5A        | 391GAHGAANFKALN1402                                  | V5                         |
| Cys3A-C353A    | 125FAFATANVAL133                                    | V5                         |
| Cys5A-C353A    | 391GAHGAANFKALN1402                                  | V5                         |
| Cys3A-C359A    | 125FAFATANVAL133                                    | V5                         |
| Cys5A-C359A    | 391GAHGAANFKALN1402                                  | V5                         |
| Cys3A-C396A    | 125FAFATANVAL133                                    | V5                         |
| Cys5A-C396A    | 391GAHGAANFKALN1402                                  | V5                         |
tate structures (Fig. 3A, panel g). To eliminate the possibility that this altered localization was somehow caused by the presence of the Ala residues, we also constructed Cys to Ser mutants of each or both Cys clusters. The Ser mutation also caused a dramatic reduction in the \(^3\text{H}\)palmitic acid labeling of the proteins and resulted in identical localization to the Ala mutants (data not shown). To avert the possibility that extreme and transient overproduction of a membrane protein would affect its localization, we also isolated stable transfectant clones using a tetracycline-inducible expression system. As shown in Fig. 3B, tetracycline-induced wild-type protein was strictly expressed in plasma membranes, whereas the Cys3A5A protein hardly expressed in the plasma membranes. To confirm the role of palmitoylation in the subcellular localization, 2-bromopalmitate, an inhibitor of protein palmitoylation (27), was used. As shown in Fig. 3C, treatment with 2-bromopalmitate resulted in a dramatic reduction of the plasma membrane distribution of tetracycline-induced wild-type protein. Taken together, these results indicate that the lack of palmitoylation in nSMase2 greatly affects its subcellular localization.

Identification of Palmitoylated Cys Residues in nSMase2—Next, we investigated which Cys residue is palmitoylated in each Cys cluster of nSMase2. To identify the specific palmitoylated amino acid in the Cys3 position, Cys to Ala mutations of Cys in the Cys3 cluster (Cys54 and Cys59, Cys53 and Cys59, or Cys53 and Cys54) were conducted using the Cys5A quadruple mutant as the receiving strain (Fig. 4A and Table 1). These mutants were overexpressed in MCF-7 cells and labeled with \(^3\text{H}\)palmitic acid for 3 h and then fixed and immunostained with anti-Myc antibody. Results indicate that the three Cys residues in the Cys3 cluster are palmitoylated (Fig. 4A), and thus contribute to the overall palmitoylation. Likewise, Cys to Ala mutations in the Cys5 cluster were introduced in the background of the Cys3A triple mutant (Fig. 4B and Table 1). Point mutation of Cys

\[ Cys^{395} \text{ or } Cys^{396} \] in the
Palmitoylation of Neutral Sphingomyelinase 2

Cys3A mutant showed that these Cys residues were still palmitoylated, whereas mutation of both Cys395 and Cys396 caused almost total loss of palmitoylation (Fig. 4B), indicating that these contiguous Cys residues serve as palmitoylation sites in the Cys5 cluster. In summary, these results suggest that each Cys cluster of nSMase2 is multiply palmitoylated and contributes to the overall palmitoylation.

To investigate which Cys residue in the Cys3 and Cys5 clusters of nSMase2 is multiply palmitoylated and contributes to the overall palmitoylation,

The effects of leupeptin on the intracellular localization of palmitoylation-deficient nSMase2 in confluent culture of MCF-7 cells, the GFP-tagged Cys3A5A mutant was overexpressed in MCF-7 cells, and the co-localization with specific organelle markers was determined by immunofluorescence. In the confluent culture of MCF-7 cells, the Cys3A5A mutant was not co-localized with Golgi (Giantin) but partially with endosome and lysosome markers (Lysotracker and Lamp1) (Fig. 6A).

Next, we investigated whether the mutant is degraded in lysosomes. To clarify the effect of palmitoylation on the half-life of protein, the effects of cycloheximide (CHX), an inhibitor for de novo protein synthesis, were first examined for the stability of the palmitoylation-deficient mutant overexpressed in MCF-7 cells. As shown in Fig. 6B, the Cys3A5A mutant dramatically decreased when de novo synthesis of the protein was arrested by CHX treatment for 5 h compared with the wild-type protein. This decrease of the mutant protein was also confirmed when anti-nSMase2 antibody was used for detection in Western blotting (Fig. 6B). The rapid disappearance of palmitoylation-deficient mutant after treatment of the cells with CHX suggested that they were degraded much faster than the wild-type protein in cells. To investigate how the palmitoylation-deficient mutant is degraded in cells, MCF-7 cells overexpressing either the wild-type nSMase2 or the Cys3A5A mutant were treated with an inhibitor of lysosomal enzymes, leupeptin (inhibitor of Ser and Cys proteases), or an inhibitor of the proteasome MG-132 (28, 29). As shown in Fig. 6C, treatment with MG-132 exerted no notable effects on the content of either protein, whereas treatment with leupeptin resulted in a marked accumulation of the Cys3A5A mutant. These results suggest that the Cys3A5A mutant is degraded by the lysosomal and not the proteasomal pathway.

The effects of leupeptin on the intracellular localization of nSMase2 and the Cys3A5A mutant were next determined by immunofluorescence (Fig. 6D). Cells were transfected with GFP-fused wild-type nSMase2 or GFP-fused Cys3A5A mutant, then treated with leupeptin, and stained with anti-Lamp1 anti-
body. In the absence of leupeptin, the Cys3A5A mutant showed a partial co-localization with the signal for Lamp1, a marker protein for lysosomes, whereas a strong overlap with Lamp1 was observed in the presence of leupeptin. In contrast to the mutant, the wild-type enzyme did not co-localize with Lamp1 even in the presence of leupeptin (Fig. 6D). These results suggest that the palmitoylation-deficient mutant is directed to lysosomes where it undergoes lysosomal degradation in a leupeptin-inhibitable manner.

**Effect of Palmitoylation on the Enzymatic Activity of nSMase2**—Next, it became important to determine whether palmitoylation affects the enzymatic activity of nSMase2. As shown in Fig. 7A, in vitro enzyme assays using \[^{14}C\]sphingomyelin showed that Cys3A, Cys5A, and Cys3A5A exhibited robust and significant nSMase activity (more than 100-fold over endogenous activity in mock transfected cells), all of which corresponded to the expression level of the respective proteins as detected by Western blotting analysis. Interestingly, treatment of the cell lysates with hydroxylamine, which resulted in cleavage of the thioester bond between palmitic acid and the Cys residue (Fig. 1B), had no effect on nSMase activity of wild-type enzyme or the Cys3A5A mutant (Fig. 7B). Furthermore, there was no significant difference in \(K_m\) values of wild-type and Cys3A5A enzymes for sphingomyelin (data not shown). Taken together,
these results indicate that palmitoylation has no significant effect on enzymatic activity of nSMase2.

Effect of Palmitoylation on the Membrane Association Properties of Neutral SMase2—nSMase2 possesses two hydrophobic segments and behaves as an integral membrane protein. Therefore, it became important to determine whether palmitoylation modulates the membrane association properties of nSMase2. To this end, solubilization of the wild-type and mutant proteins was investigated in the presence of various detergents. The wild-type, Cys3A, Cys5A, or Cys3A5A constructs were found in the membrane fraction after fractionation by 100,000 × g centrifugation (Fig. 8). In the presence of 0.25% deoxycholate, half of the wild-type protein was found in the soluble fraction in the wild-type protein, whereas the Cys3A, Cys5A, and Cys3A5A proteins were almost completely solubilized by this detergent. Likewise, treatment with 0.5% Tween 20 resulted in the solubilization of the majority of the Cys3A5A protein in the cell lysates, whereas the wild-type protein still remained in the membrane fraction. All of the proteins examined were almost totally solubilized in the presence of 0.5% Triton X-100 (Fig. 8). These results suggested that palmitoylation affects the membrane association properties of nSMase2.

Proposed Plasma Membrane Topology of nSMase2—Protein palmitoylation via thioester bonds is mostly restricted in the cytoplasmic side of the membranes (26). Because one of the palmitoylation sites in nSMase2 is located in the middle of the catalytic region of the protein, it is likely that the catalytic region of nSMase2 localizes at the inner leaflet of the plasma membrane. The orientation of the COOH terminus was determined by anti-GFP antibody accessibility in permeabilized and unpermeabilized cells overexpressing nSMase2 with a GFP tag at the COOH terminus (Fig. 9A). The signal of anti-GFP antibody was observed on the plasma membrane when cells were permeabilized with 0.1% Triton X-100 after fixation (Fig. 9A, panel b). However, the signal was very weak and hardly detectable in unpermeabilized cells although the protein was clearly detected by GFP fluorescence itself (Fig. 9A, panel a versus d). Thus, although the protein itself was at the plasma membrane, the signal of anti-GFP antibody was not accessible from the exterior of the cell. Taken together, these results suggest that the catal-
hydrophobic segments in its vicinity. In some cases, cytosolic proteins, such as neuronal SNARE protein SNAP-25 (30) or RGS family proteins (31), have palmitoylation of Cys residues in internal sequences that appear to function in anchoring into the membrane, which are soluble protein (discussed below).

Although the catalytic regions of bacterial SMases and mammalian nSMases show low identity, a number of key residues thought to be specifically involved in metal binding and catalysis are well conserved; thus, it appears that they have a similar catalytic mechanism and tertiary structure (6, 7). Very recently, analysis of the crystal structure of the SMases from Bacillus cereus and Listeria ivanovii revealed that a unique hydrophobic β-hairpin region, located in the COOH terminus and protruding into the solvent with the two aromatic amino acid residues, is important for membrane interaction of these enzymes (22, 23). In addition, from the model of binding of Bacillus SMase to the plasma membrane, the solvent-exposed loop, which is located in the middle of catalytic region, was suggested to be the other potential membrane interaction site of the enzyme (22).

From the sequence alignment of nSMase sequences, the β-hairpin region seems not to be conserved in nSMase2; however, interestingly, the position of the Cys5 cluster of nSMase2 corresponds to that of the solvent-exposed loop of Bacillus SMase (22), which may suggest that palmitoylation of the Cys5 cluster regulates the interaction between the catalytic region of the enzyme and the membrane in the vicinity of the substrate sphingomyelin. For further investigation, x-ray crystallography or NMR is required to reveal how the Cys5 palmitoylation contributes to this interaction.

Protein palmitoylation is the addition of palmitic acid through an N-amide (N-palmitoylation) or a thioester bond (S-palmitoylation); the former is observed in the NH$_2$-terminal Cys residue of secretory proteins, and the latter is mostly restricted in the cytoplasmic side of the membranes (26, 32). Because nSMase2 has S-palmitoylation, it is likely that the palmitoylation sites reside on the cytoplasmic side of membranes. Indeed, membrane topology analysis of nSMase2 suggested that the catalytic region of the protein localizes at the inner leaflet of the plasma membrane (Fig. 9). Further characterization of the membrane orientation of nSMase2 and its putative transmembrane domains should be addressed.

One question occurs as to the interaction of nSMase2 with sphingomyelin, which is mostly localized at the outer leaflet of the plasma membrane (33). Several studies suggested the presence of a sphingomyelin pool at the inner leaflet of plasma membrane (34–37). Furthermore, the sphingomyelin pool, which is involved in ceramide-mediated signaling, is reported to be distinct from that of the outer leaflet of the plasma membrane (36–38). Also, it has been suggested that sphingomyelin in the outer leaflet of the plasma membrane gains access to nSMase by flipping to the inner leaflet in a process of lipid scrambling during the execution phase of apoptosis (39). Further analysis is required for elucidation of the molecular mechanism of hydrolysis of sphingomyelin by nSMase2.

Although the function of palmitoylation of membrane proteins is not fully defined, mutations that eliminate or decrease palmitoylation on various transmembrane proteins result in several types of defects, including inefficient plasma-mem-

DISCUSSION

Although several reports have shown that nSMase2 is involved in many cellular signaling events, there remains a critical need to elucidate various molecular mechanisms that regulate its subcellular localization and hence site of cellular activation. The results from this study revealed that nSMase2 is palmitoylated via thioester linkages. By using site-directed mutagenesis, we identified two palmitoylated Cys clusters as follows: one located between the two hydrophobic segments, and the second one located in the middle of the catalytic region of the protein. Furthermore, this post-translational modification was found to be important for plasma membrane localization, and the mislocalization of the palmitoylation-deficient protein from the plasma membrane resulted in a decrease in the half-life of the protein, mediated by its lysosomal degradation.

Generally, the consensus sequence(s) of palmitoylation are not well defined. However, in many cases, the position of the target Cys residue, which has a possibility of palmitoylation, is close to the NH$_2$ or COOH terminus or transmembrane regions of proteins (26). The Cys3 cluster, which is located close to the putative transmembrane region, exhibits a typical pattern of a palmitoylation site as described above. In contrast, the position of the Cys5 cluster does not fit in any typical case as this cluster is localized in the middle of the catalytic region with no
brane expression and decrease in the life time of the proteins (26). Based on the study of the palmitoylation-deficient mutant, the results from this study clearly showed that loss of palmitoylation of nSMase2 causes the mislocalization from the plasma membrane and its lysosomal degradation. Lysosomal degradation in the absence of proper palmitoylation has been observed previously for other membrane proteins such as CCR5 (40), yeast SNARE Tlg1p (41), or Claudin-14 (42). Recently, it was reported that palmitoylation has a significant role in association of the proteins with lipid microdomains, which are known as rafts, and sometimes the lysosomal targeting of palmitoylation-deficient proteins is caused by dissociation of the protein from lipid microdomains (26). The lipid microdomains can be monitored as ice-cold Triton X-100-resistant membranes (43). However, nSMase2 is mostly solubilized in the presence of 0.5% Triton X-100 even in the wild-type protein (Fig. 8), suggesting that other mechanisms are involved in intracellular trafficking of nSMase2.

For lysosomal degradation, transmembrane proteins, which are distributed in the cytoplasmic side of membranes, require the multisvesicular body pathway to deliver them to the lumen of the endosome; the sorted membrane proteins are incorporated into the multivesicular body before transport to the lysosomal compartment, where degradation occurs (44). Ubiquitination has been shown as an important signal for sorting into the multivesicular body pathway in several cytoplasmic membrane proteins or transmembrane proteins having a cytoplasmic domain (45). For investigation of the mechanism of lysosomal degradation of palmitoylation-deficient nSMase2, the relationship of the palmitoylation and ubiquitination should be addressed.

In conclusion, the results from this study reveal for the first time the palmitoylation of nSMase2 via thioester bonds and its importance in the subcellular localization and stability of this protein. Further studies are needed to address the role of this palmitoylation in nSMase2-mediated signal transduction. Because the thioesterification of Cys residues by palmitic acid is distinct from other lipid modifications, such as prenylation and myristoylation, by its reversibility and its dynamic regulation, distinct from other lipid modifications, such as prenylation and because the thioesterification of Cys residues by palmitic acid is distinct from other lipid modifications, such as prenylation and myristoylation, by its reversibility and its dynamic regulation.