Posttranslational Regulation of Acid Sphingomyelinase in Niemann-Pick Type C1 Fibroblasts and Free Cholesterol-enriched Chinese Hamster Ovary Cells*

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Niemann-Pick type C disease is characterized by the accumulation of cholesterol and other lipids within the lysosomal compartment, a process that is often accompanied by a reduction in acid sphingomyelinase activity. These studies demonstrate that a CHO cell mutant (CT-60), which accumulates lysosomal cholesterol because of a defective NP-C1 protein, has approximately 5–10% of the acid sphingomyelinase activity of its parental cell line (25-RA) or wild type (CHO-K1) cells. The cholesterol-induced reduction in acid sphingomyelinase activity can be reproduced in CHO-K1 cells by incubation in the presence of low density lipoprotein (LDL) and progesterone, which impairs the normal egress of LDL-derived cholesterol from the lysosomal compartment. Kinetic analysis of sphingomyelin hydrolysis in cell extracts suggests that the CT60 cells have a reduced amount of functional acid sphingomyelinase as indicated by a 10-fold reduction in the apparent Vmax. Western blot analysis using antibodies generated to synthetic peptides corresponding to segments within the carboxyl-terminal region of acid sphingomyelinase demonstrate that both the CT60 and the LDL/progesterone-treated CHO-K1 cells possess near normal levels of acid sphingomyelinase protein. Likewise, Niemann-Pick type C fibroblasts also displayed normal acid sphingomyelinase protein but negligible levels of acid sphingomyelinase activity. These data suggest that cholesterol-induced inhibition is a posttranslational event, perhaps involving cofactor-mediated modulation of enzymatic activity or alterations in acid sphingomyelinase protein trafficking and maturation.

Sphingomyelin is an important cellular phospholipid that is structurally similar to phosphatidylcholine with a few significant exceptions (1). The distinctive structural features of sphingomyelin impart unique biological characteristics including an extremely high affinity for cholesterol (1–3). Consequently, cholesterol and sphingomyelin have a similar subcellular distribution (2, 4, 5) with both lipids concentrated in the plasma membrane (4).

Because of the colocalization and affinity of cholesterol and sphingomyelin, manipulations that directly impact the metabolism of one lipid generally affect events involved in the homeostasis of the other lipid. For example, enrichment with sphingomyelin up-regulates cholesterol synthesis in skin fibroblasts (6) and alters the rate of cholesterol absorption in intestinal cells (7). Depletion of plasma membrane sphingomyelin stimulates low density lipoprotein (LDL) uptake (8), induces cholesterol internalization, or stimulates cholesterol efflux to the culture medium if cells are grown in the presence of appropriate cholesterol acceptors (9). Conversely, incubation of cells with 25-hydroxycholesterol (10) or enrichment of cells with cholesterol using cholesterol/cyclodextrin complexes (11) stimulates endogenous sphingomyelin synthesis, an event that appears to be related to the phosphorylation status and subcellular localization of oxyysterol binding protein (12).

There are two distinct phosphodiesterases that are responsible for the hydrolysis of sphingomyelin to yield ceramide and phosphocholine (13). One of these enzymes functions at neutral pH (14), whereas the other, a separate gene product (15, 16), functions optimally at pH 4–5, requires intrinsic zinc for activity (17), and resides primarily within the lysosomal compartment (18, 19). There is convincing evidence from the Niemann-Pick group of diseases that the lysosomal hydrolase plays an important role in the intracellular relationship between cholesterol and sphingomyelin metabolism. In the type I forms of Niemann-Pick disease the absence of functional acid sphingomyelinase (aSMase) results in the accumulation of numerous lipid species within the lysosomal compartment, including sphingomyelin, bis(monoacylglycerol)phosphate, several glycosphingolipids, and cholesterol (20). In the type II form of Niemann-Pick disease, exemplified by Niemann-Pick disease type C (NP-C), cells are unable to release lipoprotein-derived free cholesterol from the lysosomal compartment because of a defect in the NP-C1 protein or possibly a separate gene product, NP-C2, which yields an identical phenotype. Despite the presence of a normal aSMase gene in NP-C fibroblasts, aSMase activity is reduced by as much as 80% (18, 20, 21) when these cells are grown in medium containing 10% fetal bovine serum (FBS). However, removal of lysosomal cholesterol by growth in medium containing lipoprotein-deficient serum (LPDS) restores aSMase activity to normal levels (21, 22). This suggests that the elevated lysosomal cholesterol concentration of NP-C cells down-regulates or inhibits aSMase activity.

Thus, regulation of aSMase by cholesterol is likely to play an important role in the regulation of cellular sphingomyelin concentrations and, therefore, in the maintenance of cholesterol homeostasis. To gain insight into this process, studies were th...
conducted in a Chinese hamster ovary (CHO) cell mutant, CT60, that displays faulty regulation and maintenance of cholesterol homeostasis (23) because of a defect in the NP-C1 protein (24). The absence of a functional NP-C1 protein in CT60 cells results in accumulation of cholesterol within the lysosomal compartment. A comparison of the CT60 cells and wild type CHO cells reveals that although cholesterol accumulation within lysosomes has a dramatic effect on aSMase activity, there is little change in the quantity of the aSMase protein. This conclusion is corroborated by studies in NP-C1 fibroblasts and normal CHO cells induced to accumulate intralysosomal cholesterol by incubation with LDL and progesterone.

EXPERIMENTAL PROCEDURES

Materials

CT60 and 25-RA cells were kindly provided by Dr. T. Y. Chang (Dartmouth Medical School, Hanover, NH). Wild-type CHO cells (CHO-K1) and normal human skin fibroblasts (GM970) were obtained from American Type Culture Collection (Manassas, VA). Niemann-Pick Type C fibroblasts (GM12) and GM1312 were obtained from Coriell Cell Repositories (Camden, NJ). Bovine [choline-methyl-14C]sphingomyelin (54.5 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). Stigmasteryl was obtained from Steraloids (Wilton, NH). Tissue culture medium and supplements were obtained from Mediatech (Hernando, VA). PBS was obtained from Atlantic Biologicals (Norcross, GA). All other chemicals and reagents were obtained from Sigma, St. Louis, MO. Trizol reagent was obtained from Life Technologies (Palo Alto, CA).

Methods

Growth of Cells—All cell lines were grown as monolayers in tissue culture flasks or dishes in a 37 °C, humidified incubator equilibrated with 5% CO₂. CT60 and 25-RA cells were maintained in Ham’s F-12 medium, which was maintained in 90/10 mix of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium. NP-C and normal human skin fibroblasts were maintained in minimal essential medium with Earle’s salts. All media were supplemented with 2 mM glutamine, 10% (v/v) LPDS, which was obtained by density gradient centrifugation (Kodak) prior to scanning the film. To ensure viability, the cells were counted using a label-free trypan blue dye exclusion assay, and the plating efficiency was determined by the method of Lowry (27) using bovine serum albumin as standard. This method was used only for determination of aSMase activity. For method 2, the cell pellet was disrupted by repeated up and down pipetting with a Pasteur pipette in cell lysis buffer (1% Triton X-100, 50 mM Tris Cl, pH 7.4) containing the following protease inhibitors at the indicated concentration: pepstatin (10 μg/ml), leupeptin (10 μg/ml), aprotonin (1 μg/ml), and phenylmethylsulfonyl fluoride (1 mM). The disrupted cells were incubated on ice for an additional 20 min, and then nuclei were pelleted by centrifugation at 12,000 × g for 5 min. The supernatant was transferred to a new tube and stored at −20 °C.

Activity of Lysosomal Hydrolases—aSMase activity was determined as described by Carre et al. (28). The appropriate volume of disrupted cell suspension was placed in a microcentrifuge tube, and the volume was adjusted to 90 μl with ice-cold water. If method 2 was used for disruption of cells, the volume was adjusted to 90 μl with cell lysis buffer. Unlabeled sphingomyelin in chloroform/methanol (2:1), Triton X-100, and [choline-methyl-14C]sphingomyelin were mixed and evaporated to dryness under a stream of nitrogen. The dried residue was resuspended in 272 mM sodium acetate (pH 5.1). The solution was incubated for 1 h at 50 °C with bath for 1 min and then cooled in an ice bath until the solution became clear. 110 μl of this substrate solution was added to the 90 μl of cell suspension for a final assay volume of 200 μl. The final concentrations in the assay mixture were: 0.5 mM sphingomyelin (0.2 mM/mmol) (except in Fig. 2 where various concentrations were used), 0.1% Triton X-100, 150 mM sodium acetate, and the protein concentration of the samples was included in the assay mixture. The assay mixture was incubated at 37 °C for the indicated time and then cooled in an ice bath for 2 min. 100 μl of 10% bovine serum albumin, 100 μl of 10% trichloroacetic acid, and 800 μl of water were added with vortexing after each addition. The unhydrolyzed sphingomyelin was pelleted by centrifugation at 10,000 rpm for 4 min. 800 μl of the supernatant was removed and analyzed in a liquid scintillation counter for the presence of [choline-methyl-14C]. Activity of N-acetyl-β-glucosaminidase was determined by the method of Findlay et al. (29) using p-nitrophenyl-N-acetyl-β-glucosaminide as substrate.

Antibodies and Western Blotting—Antibodies were obtained by inco- lution of rabbit antiserum with synthetic peptides conjugated to keyhole limpet hemocyanin (Lampire Biologicals, Pipersville, PA). The peptides correspond to amino acids 613–627 (K-R-L-Y-R-A-R-E-T-Y-G-L-P-D) of the mature mouse aSMase protein, and the antibodies are denoted as α-613–627 and α-532–545, respectively. The antisera from rabbits injected with peptide 532–545 were affinity purified by passing the rabbit antiserum over an Econo-Pac IgG purification column according to manufacturer’s instructions (Bio-Rad). The isolated IgG fraction was incubated with AffiGel-10 that was previously bound to the synthetic peptide by an overnight incubation with gentle rocking at 4 °C in 0.1 M MOPS, pH 7.5. After an overnight incubation, the antibodies were eluted from the column with 0.1 M glycine, pH 2.8, containing 10% ethylene glycol and immediately neutralized with 1 M Tris-glycine, pH 10.5, dialyzed against PBS, and stored at −20 °C.

Proteins in cell lysates, isolated as described above, were separated by SDS-polyacrylamide gel electrophoresis as described by Laemmli (30) using a 4% stacking and 10% resolving gel, transferred to nitrocellulose (2 h, 100 V), and incubated in 5% nonfat dry milk in PBS, 0.1% Tween 20 at 4 °C overnight. The quality of the transfer was monitored by brief staining with Ponceau S prior to the overnight blocking step. The blot was incubated in a 1:1000 dilution of antiserum (α-613–627 or α-532–545), or 25 μg/ml affinity purified α-532–545 in 2.5% nonfat dry milk in PBS/Tween 20 followed by incubation with a 1:6000 dilution of peroxidase conjugated goat α-rabbit IgG. Bands were detected by chemiluminescence (Renaissance Western blot Chemiluminescence Reagent, PerkinElmer Life Sciences) using Kodak X-OMAT AR film. To ensure that the detection antibodies were binding to the antigens of interest, a preliminary experiment was performed with varying concentrations of cell lysate protein. The band intensity was linear at less than 200 μg of protein/lane. The relative density of the bands within each Western was determined using the Scion Image software (Scion Corporation, Frederick, MD). The scanner (Hewlett-Packard ScanJetADF) was calibrated using a density step gradient (Kodak) prior to scanning the film.
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*ACIDS SPHINGOMYELINASE ACTIVITY IN CHO-K1, 25-RA, AND CT60 CELLS.* CHO-K1, 25-RA, and CT60 cells grown to near confluence in medium containing 10% FBS were harvested according to method 1 and analyzed for asMase activity as described under "Experimental Procedures." Each bar represents the mean ± S.E. of three separate experiments. Bars labeled with different letters are significantly different at the p < 0.05 level.

**RESULTS**

The CT60 cell line, which contains a disrupted NP-C1 gene, was derived from another CHO mutant, 25-RA. 25-RA cells contain a gain-of-function mutation in the sterol regulatory element-binding protein cleavage activating protein (SCAP) gene (33). The 25-RA cells were derived from wild type CHO cells. Therefore, aSMase activity was measured in cell homogenates from all three cell lines (Fig. 1). In CHO-K1 and 25-RA cells, the rate of aSMase hydrolysis was approximately 1–1.5 nmol/min/mg cell protein. In homogenates from CT60 cells grown under identical conditions, activity was reduced by greater than 85% to 0.17 nmol/min/mg cell protein. The free cholesterol concentration was approximately 3-fold higher in the CT60 cells than in the 25-RA and CHO-K1 cells, and the majority of the excess free cholesterol was located in lysosomes as evidenced by the appearance of punctate, filipin-positive compartments dispersed throughout the cytoplasm (data not shown). Both the CT60 cells and the 25-RA cells had a 4.5-fold greater concentration of esterified cholesterol than CHO-K1 cells because of the presence of the SCAP mutation, which causes an elevated and unregulated rate of endogenous sterol synthesis. However, the 25-RA cells had normal aSMase activity (Fig. 1), indicating that the reduction in aSMase activity of CT60 cells resulted from the accumulation of excess free cholesterol and not from elevated cholesterol ester concentrations. Despite the low aSMase activity of CT60 cells, sphingomyelin hydrolysis was greater than background levels obtained when cell extracts were boiled prior to the assay. Moreover, sphingomyelin hydrolysis in CT60 cell extracts occurred in a concentration- and time-dependent manner, albeit at a much slower rate than seen in the 25-RA cell extracts (data not shown). Thus, the CT60 cells appear to have a normal aSMase gene but display dramatically reduced enzyme activity under normal (10% FBS) growth conditions. There were no differences in the activity of N-acetyl-B-D-glucosaminidase among the three cell lines, indicating that the low aSMase activity of CT60 cells does not reflect a generalized inhibition of lysosomal enzyme activity (data not shown). aSMase activity in CHO-K1 cell homogenates was not reduced by the addition of CT60 cell homogenate, thus ruling out the possibility that CT60 cells possess a soluble factor that inhibits or modifies the enzyme (data not shown).

In cell extracts incubated with varying substrate concentrations, hydrolysis of sphingomyelin reached a plateau at ~200 μM sphingomyelin or less in the CHO-K1 and 25-RA cells (Fig. 2A). In the CT60 cells (Fig. 2A, inset), the concentration re-
required to achieve the half-maximal hydrolysis rate was similar to that seen for CHO-K1 and 25-RA cells. The apparent $K_m$ for the CHO-K1 and 25-RA cells was in the 65 $\mu$m range (Fig. 2B), whereas the apparent $K_m$ for the CT60 cells was approximately 50% lower, indicating that the low aSMase activity of the CT60 cells is not due to the presence of a competitive inhibitor. Increasing the substrate concentration did not restore aSMase activity to the level seen in CHO-K1 or 25-RA cells. The apparent $V_{\text{max}}$ in CT60 cells was nearly 15-fold lower than the $V_{\text{max}}$ in CHO-K1 cells and 9-fold lower in 25-RA cells (Fig. 2B), suggesting that the amount of functional enzyme responsible for hydrolysis of sphingomyelin is considerably less in the CT60 cells than in CHO-K1 and 25-RA cells.

Although the kinetic data suggest that there is less functional aSMase enzyme in the CT60 cells, these data cannot distinguish between lower amounts of the enzyme and potential modification by an effector molecule that functions as a negative allosteric regulator to inactivate the enzyme. To examine this possibility, experiments were conducted to determine whether aSMase activity correlates with aSMase mass. First, however, it was necessary to generate polyclonal antibodies for use in Western blot analysis. One antibody (α-613–627) was made to a synthetic peptide that corresponds to the carboxyl-terminal region of aSMase. It is known that several lysosomal hydrolases become fully active only after removal of several carboxyl-terminal amino acids (34), a terminal processing event that takes place within the lysosomal compartment. Therefore, an additional antibody (α-532–545) was made to an internal sequence. Both antibodies were tested for their ability to recognize aSMase in transfected COS-1 cells, which contain negligible endogenous aSMase activity. COS-1 cells were transfected with aSMase cDNA or control cDNA that encoded for an irrelevant fusion protein consisting of the amino terminus of preprolactin fused to an internal domain of complement C3 (35). The aSMase-transfected COS-1 cells displayed aSMase activity levels that were 35–100-fold greater than the endogenous activity of control-transfected cells (data not shown). Both α-532–545 and α-613–627 detected a prominent protein in the expected molecular mass range (60–70 kDa) in the aSMase-transfected cells (Fig. 3). A band of similar molecular mass was not present in the control-transfected cells verifying that both antibodies specifically recognize aSMase. A higher molecular mass band (approximately 100 kDa), perhaps representing a multimer, was occasionally seen in the transfected cells and in the CHO cells, but it was much less intense than the 60–70-kDa band.

The molecular mass of aSMase is generally reported to be 70 kDa (36–39), and pulse-chase experiments reveal that newly synthesized aSMase is converted to a 70-kDa protein that represents the fully processed form of the enzyme (38, 39). However, Kusada et al. (40) only saw a 58-kDa band in the skin, and some processing studies detect 57- and 52-kDa forms (38, 39). Thus, cells may possess several forms of aSMase that result from numerous processing events that yield aSMase proteins of different sizes. However, the molecular mass of transfected aSMase in COS cells, as well as endogenous aSMase in human skin fibroblasts and CHO-K1 cells, was approximately 67 kDa (Fig. 4), which is in close agreement with the molecular mass (70 kDa) of the fully processed, mature form of the enzyme.

The antibodies were used to compare aSMase mass in CT60 and CHO-K1 cells by Western blot analysis. The intensity of the aSMase protein band in the CT60 cells probed with α-613–627 was approximately 17% less than the band in the CHO-K1 cells (Fig. 5A). The experiment was repeated three times with similar results (data not shown). α-532–545 also detected a band of approximately equal intensity in the CT60 and the CHO-K1 cells (Fig. 5B). Thus, although the activity of aSMase was reduced by greater than 90% in the CT60 cells, there was virtually no corresponding decrease in mass. Moreover, the ability of both antibodies to detect similar amounts of aSMase in the CT60 cells and CHO-K1 cells indicates that the difference in activity between the cell types cannot be attributed to differential processing of the carboxyl-terminal portion of the protein.

As with CT60 cells, two NP-C cell lines, GM 110 and GM 3123, which is known to possess a mutation in the NP-C1 gene (41), express approximately 10% of the aSMase activity of a normal fibroblast cell line (GM 970) (Fig. 6) yet possess abundant aSMase protein (Fig. 6, inset). The small (30%) reduction in aSMase protein in the NP-C cell lines was similar to the CT60 cells, but this difference in mass between normal and NP-C cells did not reach statistical significance (see legend for details).

We cannot eliminate the possibility that the low aSMase activity seen in these cells is a direct consequence of a defective NP-C1 protein rather than a consequence of cholesterol enrichment of the lysosomal compartment. To address this possibility, we incubated CHO-K1 cells in the presence of LDL plus or minus progesterone. Progesterone has been shown to inhibit egress of cholesterol from the lysosomal compartment, resulting in a severalfold increase in lysosomal cholesterol concen-
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Fig. 5. Comparison of aSMase mass in CHO-K1 and CT60 cells. CHO-K1 and CT60 cells grown to near confluence in medium containing 10% FBS were harvested according to method 2 and analyzed for aSMase mass by Western blot using α-532–545 (A) or α-532–545 (B). Each lane represents a single flask of cells from which 10 μg of cell lysate protein was applied to the gel except for A, lane 1, where 50 μg of cell lysate protein was used. In B, COS-1 cells were transfected with aSMase cDNA or control (C3) cDNA as described in the legend to Fig. 3. The cells were harvested according to method 2, and 50 μg of cell lysate protein was analyzed for aSMase mass by Western blotting using α-532–545.

Fig. 6. aSMase activity and mass in normal and NP-C fibroblasts. Normal (GM 970) and NP-C fibroblast cell lines (GM 110 and GM 3123) were grown in medium containing 10% FBS. When the cells were near confluence, they were harvested according to method 2 for analysis of aSMase activity and aSMase mass (inset) by Western blotting using 25 μg of cell protein and α-532–545. aSMase activity and mass were determined in three separate flasks for each cell type. Data for aSMase activity represent the means ± S.D. of these determinations, and the inset shows a representative Western blot. Relative intensities of the bands from the three blots, expressed as means ± S.D., were: GM970, 341.5 ± 38.62; GM110, 234.2 ± 57.99; GM3123, 244.7 ± 51.07. Statistical differences between cell types were determined by one-way analysis of variance, p = 0.072.

In untreated CHO-K1 cells, the free cholesterol concentration was approximately 19 μg/mg cell protein (Fig. 7A, open circle), and the aSMase activity was approximately 4.25 nmol/min/mg protein (Fig. 7B, open circle). Following a 24-h incubation with LDL and progesterone (0 h chase), the free cholesterol concentration reached 37 μg/mg cell protein (Fig. 7A, closed circle), and the aSMase activity was reduced to 0.34 nmol/min/mg protein (Fig. 7B, closed circles). Upon removal of LDL and progesterone (chase), the free cholesterol concentration was reduced to basal levels within 12 h. During this same time period there was a transient increase in esterified cholesterol concentration (closed triangles) that peaked after ~12 h and then slowly declined to near background levels over the next 36 h. There was a gradual decrease in total cholesterol concentration (closed squares) over the chase period reflecting the sum of the changes in free and esterified cholesterol concentrations. Coincident with the reduction in cellular cholesterol concentration was a corresponding increase in the aSMase activity (Fig. 7B, closed circles).

To determine whether the reduction in aSMase activity that occurred when cells were grown in the presence of LDL plus progesterone was due to a reduction in aSMase mass, cells were incubated in the presence of LDL with increasing concentrations of progesterone (Fig. 8). Incubation with LDL in the absence of progesterone had little effect on aSMase activity because the free cholesterol liberated by lysosomal hydrolysis of cholesterol ester was not impaired from leaving the lysosomes (data not shown). In fact, LDL concentrations as high as 130 μg/ml reduced activity by only 25%. Incubation in medium containing 10% LPDS (condition 2) increased activity by approximately 30% (compared with 10% FBS; condition 1) presumably because of the absence of an exogenous source of cholesterol. As expected, incubation in the presence of progesterone alone (condition 3) had a minimal effect on activity because there was no exogenous source of cholesterol to accumulate within the lysosomal compartment. However, the addition of LDL and progesterone (conditions 4–7) reduced aSMase activity with maximal inhibition occurring at 5–10 μg/ml of progesterone (conditions 6 and 7). Despite the 4.5-fold reduction in aSMase activity that was seen at this concentration of progesterone, there was not a corresponding decrease in aSMase mass. Thus, like NP-C fibroblasts and CT60 cells, induction of lysosomal cholesterol accumulation in normal CHO cells

Fig. 7. Effect of LDL and progesterone on cholesterol mass and aSMase activity in CHO cells. CHO-K1 cells were plated into T-75 flasks in medium containing 10% FBS. On the following day, the medium was removed and the cells were washed three times with PBS. One flask of cells received fresh medium containing 10% LPDS (open symbols), whereas the remaining flasks received fresh medium containing 10% LPDS, 100 μg/ml LDL, and 10 μg/ml progesterone (closed symbols). After 24 h, the flask of cells on LPDS, and one flask of cells incubated with LDL/progesterone was harvested according to method 1. Data from these cells are shown as the 0 h chase time. The remaining flasks were washed three times to remove residual medium and incubated in fresh medium containing 10% FBS for the indicated period of time. The cell pellets were analyzed for cholesterol mass (A) and aSMase activity (B) as described under "Experimental Procedures." Squares, total cholesterol; circles, free cholesterol; triangles, esterified cholesterol.
CHO-K1 cells were plated in medium containing 10% FBS for analysis of aSMase activity and aSMase mass by Western blotting. After 24 h, the cells were harvested according to method two, washed three times with PBS, fresh medium containing 10% FBS or progesterone was added. On the following day, medium was removed, and cells were rinsed with PBS again. Then, cells were cultured in medium containing 10% FBS or progesterone for 24 h. The cells were harvested again, and the aSMase activity and mass were determined.

Inhibits aSMase activity without markedly altering the amount of aSMase protein.

**DISCUSSION**

**Lysosomal Free Cholesterol Accumulation Reduces Acid Sphingomyelinase Activity but Not Acid Sphingomyelinate Mass**—Cholesterol-mediated regulation of aSMase appears to occur by a novel posttranslational mechanism. This conclusion is derived from the finding that elevated concentrations of intralysosomal cholesterol in CT-60 cells, NP-C fibroblasts, and progesterone-treated CHO-K1 cells virtually abolish aSMase activity but have minimal effects on cellular aSMase protein mass. Several possible explanations for the apparent disparity between aSMase activity and mass were considered. To yield the mature 70-kDa aSMase protein and perhaps an additional, enzymatically active, 57-kDa protein (38, 39, 43) requires the proteolytic cleavage (38, 39, 43) of an amino-terminal fragment (36–39, 43) that includes the signal peptide (37, 43) and addition of N-linked carbohydrates at five or six potential glycosylation sites (36, 38). Mutation of glycosylation sites affects enzymatic activity and can cause protein retention in the Golgi complex or endoplasmic reticulum (ER) (36, 38), association with BiP, and an enhanced rate of degradation (36). The possibility that cholesterol loading results in a gross disruption of aSMase processing or a marked acceleration of degradation is unlikely, however, because the abundance and size of the immunoreactive aSMase protein detected by Western blot analysis was the same in normal and free cholesterol-enriched cells. Nonetheless, it is still possible that intralysosomal cholesterol accumulation induces a selective trafficking or folding defect that dramatically affects aSMase activity.

Instances of selective, cholesterol-mediated alterations in subcellular protein localization have been observed. For example, changes in intracellular cholesterol concentration alter the subcellular distribution of SCAP (44) and oxysterol-binding protein (12), coincident with changes in glycosylation and phosphorylation status, respectively. In addition, other specialized mechanisms appear to play crucial roles in the trafficking of some lysosomal enzymes. Neuraminidase requires protective protein/cathepsin A for transport to lysosomes and conversion to an enzymatically active hydrolase (45). A population of acid β-glucosidase (10–25%) can be selectively retained within the ER through its interaction with egasyn, an ER resident protein (46). Finally, the exit of the β subunit of hexosaminidase from the ER requires formation of an oligomeric complex (47). These examples raise the possibility that cholesterol-mediated disruption of a folding and/or sorting event could result in the improper maturation of active aSMase. This altered trafficking would be specific because it appears that the activities of several mannose-6-phosphate pathway-dependent enzymes are unaffected by cholesterol loading.

One possible domain of aSMase that may play an important role in folding and sorting is the amino terminus, which is homologous to the sphingolipid activator proteins (saposins) (48). Saposin molecules interact with phospholipid bilayers through a disulfide bonded loop termed the "saposin fold" (49). Saposins have a strong preference for phospholipid bilayers that contain anionic phospholipids (50) such as bis(monoacylglycerol)phosphate, which accumulates in NP-C cells (20). Thus, it is possible that saposin domain-mediated interaction of aSMase with the membrane of a prelysosomal compartment plays a crucial role in its trafficking and, perhaps, its enzymatic activation.

**Potential Significance of Cholesterol-mediated Regulation of Acid Sphingomyelinase**—Although the intracellular association of cholesterol and sphingomyelin is well documented and there is emerging evidence of a metabolic relationship, the molecular mechanisms responsible for the apparent coordinated regulation of cellular cholesterol and sphingomyelin concentrations and distribution are poorly understood. Lysosomes, one of several subcellular locations where cholesterol and sphingomyelin metabolism converge, represent a potential site of regulation. Indeed, the data presented here suggest that lysosomal sphingomyelin concentrations, as modulated by aSMase, may play an important role in the maintenance of cholesterol homeostasis. Down-regulation of aSMase by cholesterol within the lysosomal compartment would serve to increase the lysosomal content of sphingomyelin at precisely the time when cellular cholesterol concentrations were increasing by receptor-mediated uptake of lipoproteins. This would provide a rapid and effective method for altering the lysosomal sphingomyelin concentration. The increase in sphingomyelin would, in turn, maintain the proper cholesterol to sphingomyelin ratio within the lysosomal membrane. As cholesterol was...
transported from lysosomes to the plasma membrane and ultimately the ER for esterification by acyl-CoA:cholesterol acyltransferase, the aSMase activity would be restored, and the proper cholesterol to sphingomyelin ratio would be maintained.

In addition to affecting cellular cholesterol metabolism, the cholesterol-mediated regulation of aSMase would also affect the generation of ceramide, a potent lipid second messenger involved in the regulation of cell growth and apoptosis (52, 53). Interestingly, the regulation of cell growth and intracellular cholesterol metabolism are interdependent cellular events (54). Thus, the cholesterol-mediated regulation of aSMase activity may not only be an important player in the maintenance of cholesterol homeostasis but also an important cellular process that links the regulation of cell growth to intracellular cholesterol metabolism.

The precise mechanism(s) by which elevated levels of cholesterol or other lipids regulate aSMase activity will require further study. However, it is evident from the current data that such regulation is not simply achieved by reducing the amount of enzyme by transcriptional or translational means. Rather, elevated intracellular free cholesterol concentrations appear to alter processing and/or trafficking events critical for aSMase activity or induce allosteric changes that render the enzyme inactive. This posttranslational cholesterol-mediated regulation of aSMase appears to be a novel pathway whose elucidation will be critical for fully understanding the complex regulatory pathways that underlie intracellular cholesterol and sphingomyelin homeostasis.

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