The Cellular Trafficking and Zinc Dependence of Secretory and Lysosomal Sphingomyelinase, Two Products of the Acid Sphingomyelinase Gene*

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The acid sphingomyelinase (ASM) gene, which has been implicated in ceramide-mediated cell signaling and atherogenesis, gives rise to both lysosomal SMase (L-SMase), which is reportedly cation-independent, and secretory SMase (S-SMase), which is fully or partially dependent on Zn\(^{2+}\) for enzymatic activity. Herein we present evidence for a model to explain how a single mRNA gives rise to two forms of SMase with different cellular trafficking and apparent differences in Zn\(^{2+}\) dependence. First, we show that both S-SMase and L-SMase, which contain several highly conserved zinc-binding motifs, are directly activated by zinc. In addition, SMase assayed from a lysosome-rich fraction of Chinese hamster ovary cells was found to be partially zinc-dependent, suggesting that intact lysosomes from these cells contain subsaturating levels of Zn\(^{2+}\). Analysis of Asn-linked oligosaccharides and of N-terminal amino acid sequence indicated that S-SMase arises by trafficking through the Golgi secretory pathway, not by cellular release of L-SMase during trafficking to lysosomes or after delivery to lysosomes. Most importantly, when Zn\(^{2+}\)-dependent S-SMase was incubated with SMase-negative cells, the enzyme was internalized, trafficked to lysosomes, and became zinc-independent. We conclude that L-SMase is exposed to cellular Zn\(^{2+}\) during trafficking to lysosomes, in lysosomes, and/or during cell homogenization. In contrast, the pathway targeting S-SMase to secretion appears to be relatively sequestered from cellular pools of Zn\(^{2+}\); thus S-SMase requires exogenous Zn\(^{2+}\) for full activity. This model provides important information for understanding the enzymology and regulation of L- and S-SMase and for exploring possible roles of ASM gene products in cell signaling and atherogenesis.

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**SMases** (SM phosphodiesterase, EC 3.1.4.12) have been implicated in a wide variety of physiologic and pathophysiologic processes, including lysosomal hydrolysis of endocytosed SM (1, 2), ceramide-mediated cell signaling (3, 4), membrane vesiculation (5, 6), alterations in intracellular cholesterol trafficking (5, 7–9), and atherogenesis (10–13). One type of mammalian SMase is a magnesium-dependent, membrane-bound neutral SMase, and Tomiuk et al. (14) have recently reported the cloning of an enzyme that has several properties in common with this SMase. Two other types of mammalian SMases are lysosomal SMase (L-SMase) and secretory SMase (S-SMase), both of which arise from the “acid SMase” or “ASM” gene (15, 16). Both enzymes are soluble hydrolases that function optimally at acid pH in a standard in vitro micellar assay (16, 17), although we have shown that S-SMase can hydrolyze physiologic SM-containing substrates at neutral pH (Ref. 18 and see below). Both L- and S-SMase are absent from the cells of patients with types A and B Niemann-Pick disease, which is due to mutations in the ASM gene, and from the cells of ASM knock-out mice (16).

S-SMase may have significant physiologic roles, since extracellular SM hydrolysis may be involved in some or all of the non-lysosomal processes listed above. For example, several lines of evidence have implicated extracellular SM hydrolysis in atherogenesis. First, treatment of LDL with SMase in vitro leads to LDL aggregation (10, 11), which is a prominent event during atherogenesis (19–21) and one that leads to massive macrophage foam cell formation (10, 11, 22–24). Second, aggregated LDL from human and animal atherosclerotic lesions shows evidence of hydrolysis by extracellular SMase, and LDL retained in rabbit aortic strips ex vivo is hydrolyzed by an extracellular, cation-dependent SMase (12). Third, S-SMase, a leading candidate for this arterial wall enzyme, is secreted by macrophages (16) and endothelial cells (25), cell types found in atherosclerotic lesions. Fourth, S-SMase is able to hydrolyze the SM in atherogenic lipoproteins at neutral pH (18). Other possible roles for S-SMase may be in ceramide-mediated cell signaling (26–30), perhaps after re-uptake of the secreted enzyme into endosomal vesicles; in extracellular sphingomyelin

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1 The abbreviations used are: SMase, sphingomyelinase; S-SMase, secreted sphingomyelinase; ASM, acid sphingomyelinase; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; endo H, β-endo-N-acetylglucosaminidase H; HI-FBS, heat-inactivated fetal bovine serum; LDL, low density lipoprotein; L-SMase, lysosomal sphingomyelinase; PBS, phosphate-buffered saline; PG, mencllin, streptomyacin, and glutamine; PAGE, polyacrylamide gel electrophoresis; SM, sphingomyelin; TIMP-1, tissue inhibitor of metalloproteinase-1; BSA, bovine serum albumin.
catabolism after nerve injury and during demyelination (16, 31–33); and in defense against viruses, many of which are enriched in SM (34, 35) and can be inactivated by treatment with SMase in vitro.²

L- and S-SMase are very similar proteins. Previous work from our laboratories has shown that cells transfected with an ASM cDNA overexpress both L-SMase and S-SMase (16), indicating that S-SMase does not arise by alternative processing of the ASM gene. In addition, antibodies made against L-SMase recognize S-SMase, demonstrating that the common mRNA is translated in the same reading frame, and the molecular weights of the enzymes on Western blot are similar (see Ref. 16 and below). Nevertheless, S-SMase requires exogenously added Zn²⁺ for activation in in vitro assays, whereas L-SMase isolated from cell or tissue homogenates does not (16). In fact, the lack of stimulation of L-SMase by any cations and its lack of inhibition by EDTA has led to a long-standing body of literature labeling L-SMase as a “cation-independent” enzyme (1).

Despite the widespread interest in mammalian SMases in general and in products of the ASM gene in particular, little is known about cellular itineraries of L-SMase and S-SMase or about the mechanism for their apparent difference in zinc dependence. For example, does S-SMase arise by release or exocytosis of L-SMase from lysosomes or by a separate trafficking pathway, and how could two enzymes that are so similar differ in their requirement for zinc? In this report, we show that S-SMase is secreted through a non-lysosomal secretory pathway, and we present evidence that both forms of the enzyme are zinc-activated. According to our model, L-SMase is exposed to cellular Zn²⁺ during trafficking to lysosomes, in lysosomes, and/or during cell homogenization. Most likely, the Zn²⁺ dependence of L-SMase has been overlooked because it is already saturated with Zn²⁺ upon isolation from cell homogenates and thus does not respond to exogenous Zn²⁺ at the time of assay. Furthermore, as is the case with known zinc metalloenzymes (cf. Ref. 36), the Zn²⁺ cannot be stripped from L-SMase by simple exposure to EDTA. In contrast, the pathway targeting S-SMase to secretion appears to be relatively sequestered from cellular pools of Zn²⁺. Thus, this enzyme requires Zn²⁺ during subsequent in vitro assay. The information in this report should prove useful for future studies that explore the enzymology, regulation, and functions of these important SMases.

EXPERIMENTAL PROCEDURES

Materials—The Falcon tissue culture plasticware used in these studies was purchased from Fisher. Tissue culture media and other tissue culture reagents were obtained from Life Technologies, Inc. Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT) and was heat-inactivated for 1 h at 65 °C (HI-FBS). [9,10-³H]Palmitic acid (56 Ci/mmol) was purchased from NEN Life Science Products, and [N-palmitoyl-9–10-³H]phosphatidylcholine was synthesized as described previously (16, 57–58). N,N-Dimethylformamide, 1,3-dicyclohexylcarbodiimide, N-hydroxysuccinimide, and N,N-diisopropylethylamine were purchased from Aldrich. Precast 4–20% gradient polyacrylamide gels were purchased from NOVEX (San Diego, CA). Nitrocellulose was from Schleicher & Schuell. Rabbit anti-FLAG-tagged S-SMase was kindly provided by Dr. Henri Lichenstein (Amgen, Boulder, CO). FLAG-tagged S-SMase was purified by anti-FLAG immunofinity chromatography from the conditioned medium of CHO cells transfected with a human ASM-FLAG cDNA. FLAG-tagged L-SMase was purified from a cell homogenate and was kindly provided by Dr. Stuart Kornfeld (Washington University, St. Louis). Sphingosylphosphorylcholine, 1,10-phenanthroline, and all other chemicals and reagents were from Sigma, and all organic solvents were from Fisher.

Cells—Monolayer cultures of J774.A1 cells (from the American Type Culture Collection, see Ref. 40) were grown and maintained in spinner culture with DMEM/HI-FBS/PSG as described previously (9, 40). Human dermis (R496L mutation (41)) were grown in DMEM/HI-FBS/PSG. CHO-K1 cells were grown in Ham's F-12 containing 10% HI-FBS and PSG. CHO cells stably transfected with ASM cDNA² were maintained in DMEM/HI-FBS/PSG (16). Cells were plated in 35-mm (6-well) or 100-mm dishes in media containing HI-FBS for 48 h. The cells were then washed 3 times with PBS and incubated for 24 h in fresh serum-free media (1 and 6 ml per 35-mm and 100-mm dishes, respectively) containing 0.2% BSA. This 24-h conditioned medium was collected for SMase assays.

Harvesting of Cells and Conditioned Media—Following the incubations described above and in the figure legends, cells were placed on ice, and the serum-free conditioned medium were removed. The cells were washed with ice-cold 0.25 m sucrose and scraped into 0.3 and 3.0 ml of this sucrose solution per 35- and 100-mm dishes, respectively. Unless indicated otherwise, the scraped cells were disrupted by sonication on ice using three 5-s bursts (Branson 450 Sonifier), and the cellular homogenates were assayed for total protein by the method of Lowry et al. (42) and for SMase activity as described below. The conditioned media were spun at 800 × g for 5 min to pellet any contaminating cells and concentrated 6-fold using a Centricon 30 (Amicon; Beverly, MA) concentrator (molecular weight cut-off = 30,000). For the experiment in Fig. 5, CHO-K1 cells were incubated in 100-mm dishes in serum-free media and washed as described above. Cells were then scraped in 5 ml of 0.25 m sucrose and broken open under 500 p.s.i. of nitrogen pressure for 1.5 min using a nitrogen cell disruption bomb (Parr Instruments, Moline, IL). Following disruption, a portion of the cells was subjected to brief sonication as described above; this portion of cells is referred to as the cell homogenate. The remainder of disrupted cells was spun at 1300 × g for 5 min to pellet any remaining intact cells and nuclei. This post-nuclear supernatant was collected, and the volume was increased to 15 ml with 0.25 m sucrose and then spun at 24,000 × g for 30 min. The pellet from this centrifugation was suspended in 1 ml of 0.25 m sucrose and sonicated as above, and this material, as well as the cell homogenate, was assayed for SMase activity.

SMase Assay—As described previously (16), the standard 200-µl assay mixture consisted of up to 40 µl of sample (conditioned media or homogenized cells; see above) and a volume of assay buffer (0.1 m sodium acetate, pH 5.0) to bring the volume to 160 µl. The reaction was initiated by the addition of 40 µl of substrate (50 pmol of [³H]sphingomyelin) in 0.25 m sucrose containing 3% Triton X-100 (final concentration of Triton X-100 in the 200-µl assay mix = 0.6%). When added, the final concentrations of EDTA and Zn²⁺ were 5 and 0.1 m, respectively, unless indicated otherwise. The assay mixtures were incubated at 37 °C for no longer than 3 h and then extracted by the method of Bligh and Dyer (43). The reaction mixture was harvested, evaporation under N₂, and fractionated by TLC using chloroform/methanol (95:5). The ceramide spots were scraped and directly counted to quantify [³H]ceramide. Typically, our assay reactions contained approximately 20 µg of cellular homogenate protein and a volume of conditioned medium derived from a quantity of cells equivalent to approximately 50 µg of cellular protein.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Protein samples were boiled in buffer containing 1% SDS and 10 m/M dithiothreitol for 10 min, loaded onto 4–20% gradient polyacrylamide gels, and electrophoresed for 50 min at 35 mA in buffer containing 0.1% SDS (SDS-PAGE). Following electrophoresis, some gels were fixed in methanol/glacial acetic acid/water (5:2, v/v) and then silver-stained using reagents from Bio-Rad. Other gels were electrotransferred (100 V for 1.5 h) to nitrocellulose for immunoblotting. For immunoblotting, the nitrocellulose membranes were incubated with 5% Carnation nonfat

² S. L. Schissel and I. Tabas, unpublished data.

³ X. He, S. R. P. Miranda, A. Dagan, S.Gatt, and E. H. Schuchman, submitted for publication.
dry milk in buffer A (24 mM Tris, pH 7.4, containing 0.5 mM NaCl) for 3 h at room temperature. The membranes were then incubated with rabbit anti-FLAG-tagged S-SMase polyclonal antisera (1:2000) in buffer B (buffer A containing 0.1% Tween 20, 3% nonfat dry milk, and 0.1% bovine serum albumin) for 1 h at room temperature. After washing four times with buffer A containing 0.1% Tween 20, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) for 1 h in buffer B at room temperature. The membranes were subsequently washed twice with 0.3% Tween 20 in buffer A and twice with 0.1% Tween 20 in buffer A. Finally, the blots were soaked in the enhanced chemiluminescence reagent (NEL Life Science Products) for 2 min and exposed to x-ray film for 1 min.

**Glycosidase Treatments**—We followed the procedure described by Hurvitz et al. (44). CHO-K1 cells were incubated overnight with serum-free medium (CHO-S-SFM II from Life Technologies, Inc.). Fifty µg of 30-fold-concentrated conditioned medium and 50 µg of cell homogenate were diluted 1:1 (v/v) with 50 mM sodium acetate buffer, pH 5.0, containing 2% SDS and 20 mM p-mercaptoethanol (44). One set of aliquots of the diluted conditioned medium and cell homogenate was treated for 16 h at 37 °C with 4 milliunits of endo H. Another set of aliquots was diluted another 15-fold with 50 mM sodium phosphate buffer, pH 7.2, containing 1% Nonidet P-40 and treated for 16 h at 37°C with 100 milliunits of peptide-N-glycanase F. The endo H digest and a trichloroacetic acid pellet of the peptide-N-glycanase F digest (44) were boiled in SDS-sample buffer and then electrophoresed and immunoblotted as described above.

**Zinc-Chelate Chromatography**—We used a modification of the method of Hortin and Gibson (45). Packed 1-ml columns of chelating Sepharose 6B (iminodiacetic acid coupled to agarose gel via a hydrophilic spacer; from Amersham Pharmacia Biotech) were washed with 10 mM sodium acetate buffer, pH 6.0, containing 10 mM EDTA, to leave the column uncharged, or containing 60 mM ZnCl₂, to charge the column with Zn²⁺. The columns were then equilibrated with 50 mM Hepes buffer, pH 7.4, containing 50 mM NaCl. One-ml samples of a 1:1 (v/v) mixture of this equilibration buffer and unconcentrated conditioned medium from ASM-transfected CHO cells (above) were loaded onto the columns and incubated for 15 min at room temperature. The columns were then washed with 7.5 ml of 50 mM Hepes, pH 7.4, containing either 100 mM NaCl or 1 x 10⁻⁷ M NaCl, which was collected as 10 0.75-ml fractions. Aliquots of each of the fractions were spotted on nitrocellulose using a slot-blot apparatus and then immunoblotted using goat anti-human L-SMase polyclonal antiserum as described above.

**Statistics**—Unless otherwise indicated, results are given as means ± S.D. (n = 3); absent error bars in the figures signify S.D. values smaller than the graphic symbols.

**RESULTS**

**Zn²⁺ Requirement for S-SMase Does Not Involve a Zn²⁺-dependent Cofactor**—We sought to address how L- and S-SMase acquire their apparent differences in zinc dependence. One explanation would be that the secreted form requires a Zn²⁺-dependent cofactor. Because many lysosomal enzymes undergo proteolytic activation (46), an obvious candidate for a Zn²⁺-dependent cofactor would be a zinc metalloproteinase. Five sets of results, however, ruled out this possibility. First, Zn²⁺-activated S-SMase can be subsequently inactivated by Zn²⁺-chelation (see below); reversibility of Zn²⁺-induced activation is not consistent with proteolytic activation. Second, inhibitors of zinc metalloproteinases, such as tissue inhibitor of metalloproteinase-1 (TIMP-1) (47) and two different thiol-based peptide inhibitors, HS-CH₂-R-(CH₂-C=CH(CH₃)=C)-Phe-Ala-NH₂ and HO-CH₂-C=CH₂-(CH₂-CH(CH₃)=C)-naphthyl-Ala-NH₂ (CH₂-CH₂-NH₂-NH₂ (48), did not affect the ability of Zn²⁺ to activate the S-SMase (data not shown). Third, mammalian zinc metalloproteinases require Ca²⁺ as well as Zn²⁺ for activity (49), whereas Ca²⁺ is not a requirement for the activation of S-SMase (16). Fourth, comparison of Zn²⁺-activated S-SMase from CHO cells with that of the intracellular (lysosomal) enzyme by immunoblot analysis showed that the activated secreted form had a somewhat higher, not lower, apparent molecular weight (see control data in Fig. 3, below); in addition, S-SMase not activated with Zn²⁺ had the same apparent molecular weight as Zn²⁺-activated S-SMase (data not shown). Finally, we attempted to chelate Zn²⁺ away from each enzyme in vitro to determine the effect on catalytic activity. In fact, the conclusion by others (54–59) that L-SMase is a cation-independent enzyme is based partly on the observation that EDTA does not inhibit activity. Many zinc metalloenzymes, however, bind the metal very tightly and thus require more potent chelation, such as by long term incubation with the Zn²⁺ chelator 1,10-phenanthroline (36). To begin, we conducted our studies with S-SMase, which we know binds and is directly activated by Zn²⁺.

In Fig. 1A, conditioned medium from J774 macrophages was incubated with either EDTA or Zn²⁺ (1st two bars) and then assayed for S-SMase activity. As we have reported previously (16), the S-SMase is markedly activated by Zn²⁺. We then took an aliquot of Zn²⁺-activated S-SMase and incubated it for 18 h with EDTA plus 1,10-phenanthroline in an attempt to chelate the enzyme-bound Zn²⁺. As shown in the 3rd bar in Fig. 1A, this treatment resulted in an approximately 50% loss of activity; treatment with EDTA alone did not affect enzyme activity (data not shown). Finally, the partially inactivated S-SMase was dialyzed against Zn²⁺-containing buffer (4th bar), which restored activity to the original level observed when Zn²⁺ was added initially to the conditioned medium (compare 4th and 2nd bars in Fig. 1A).

**Evidence for Direct Activation of L-SMase by Zn²⁺**—Despite the long-standing tenet that L-SMase is a cation-independent enzyme (2), several lines of evidence initially suggested to us that L-SMase was a zinc-activated enzyme. First, L-SMase and S-SMase come from the same gene and same mRNA in the same reading frame (16), and S-SMase binds and is directly activated by Zn²⁺ (above). Second, there are seven aminocyl sequences in the enzyme that are homologous to Zn²⁺-binding sequences in known zinc metalloenzymes (50), including one sequence that is very similar to that in another phosphodies- terase enzyme (Table I). Histidine residues in two of these sequences (His-425 and His-575) are highly conserved and are sites of mutations in Niemann-Pick disease. Moreover, His-421 is conserved in mouse ASM and in a homologue of ASM in Caenorhabditis elegans that is zinc-dependent but not in an ASM homologue in C. elegans that is zinc-independent (51). Third, L-SMase shares two other properties of known zinc metalloenzymes, namely inhibition by phosphate ions (1, 2), which are thought to block the Zn²⁺-binding pocket(s) in zinc metalloenzymes (52) and inhibition by high concentrations (e.g., 6 mM) of ZnCl₂ (49, 53).

To test directly whether L-SMase is a zinc-activated enzyme, we attempted to chelate Zn²⁺ away from each enzyme in vitro to determine the effect on catalytic activity. In fact, the conclusion by others (54–59) that L-SMase is a cation-independent enzyme is based partly on the observation that EDTA does not inhibit activity. Many zinc metalloenzymes, however, bind the metal very tightly and thus require more potent chelation, such as by long term incubation with the Zn²⁺ chelator 1,10-phenanthroline (36). To begin, we conducted our studies with S-SMase, which we know binds and is directly activated by Zn²⁺.
TABLE I
Comparison of amino acid sequences of portions of L/S-SMase with known Zn\(^{2+}\)-binding consensus sequences of zinc-metalloenzymes

| Enzyme                          | Amino acid sequence                                      |
|---------------------------------|---------------------------------------------------------|
| L/S-SMase (282)                 | (H)(X)\textsubscript{b}-(H)(X)\textsubscript{33}E       |
| L/S-SMase (421)                 | (H)(X)\textsubscript{b}-(H)(X)\textsubscript{15}E       |
| Astracin zinc-protease gene family | (H)(X)\textsubscript{b}-(H)(X)\textsubscript{15}E       |
| cGMP-specific phosphodiesterase | (H)(X)\textsubscript{b}-(H)(X)\textsubscript{15}E       |
| Thermolysins and B. cereus neutral protease | (H)(X)\textsubscript{b}-(H)(X)\textsubscript{15}E       |
| L/S-SMase (221)                 | C-(X)\textsubscript{103}-C-(X)\textsubscript{23}P       |
| Aspartate transcarboxamylase     | C-(X)\textsubscript{103}-C-(X)\textsubscript{23}P       |
| L/S-SMase (136)                 | (H)(X)\textsubscript{b}-(E)(X)\textsubscript{142}H       |
| L/S-SMase (459)                 | (H)(X)\textsubscript{b}-(E)(X)\textsubscript{115}H       |
| Carboxypeptidase A and B        | (H)(X)\textsubscript{b}-(E)(X)\textsubscript{132}H       |
| L/S-SMase (575)                 | H\textsuperscript{3}{-(H)}-(X)(X)\textsubscript{30}H       |
| DD carboxypeptidase             | (H)(X)\textsubscript{b}-(X)\textsubscript{16}H          |
| L/S-SMase (457)                 | H\textsuperscript{3}{-(H)}-(X)(X)\textsubscript{30}H       |
| \(\beta\)-Lactamase             | H\textsuperscript{3}{-(H)}-(X)(X)\textsubscript{30}H       |

a The number in parentheses represents the position of the first amino acid (H or C) of the SMase sequences displayed.

b X refers to any amino acid.

This histidine residue is also conserved in mouse ASM and in the C. elegans ASM that is zinc-dependent but not in the C. elegans ASM that is zinc-independent.

d These histidine residues are sites of mutations in Niemann-Pick disease.

Next, we examined cellular (i.e. lysosomal) SMase\(^4\) (Fig. 1B). As reported previously by others (1, 2) and by us (16), and in contrast to the situation with the secreted enzyme, L-SMase in cell homogenates shows maximal activity without added Zn\(^{2+}\) and is not inhibited by EDTA (1st three bars of Fig. 1B). We then incubated the original, active cellular enzyme (i.e. not exposed to exogenous Zn\(^{2+}\) in vitro) with EDTA plus 1,10-phenanthroline for 8 h (4th bar). This treatment resulted in almost total inactivation of the enzyme, and substantial activity was restored by dialyzing against Zn\(^{2+}\) (5th bar) or by directly adding back excess Zn\(^{2+}\) (not shown).\(^5\) The actual degree of inhibition by chelation and re-activation by Zn\(^{2+}\) dialysis differed somewhat between the secreted and cellular SMases, perhaps due to a lesser stability of the cellular enzyme under the incubation conditions employed. Nonetheless, the overall patterns of inhibition and reactivation shown in Fig. 1, together with the lines of evidence mentioned earlier, provide strong evidence that L-SMase, like S-SMase, is a zinc-activated enzyme.

The Difference in Requirement for Zn\(^{2+}\) in the in Vitro Assays of L- and S-SMase: Differential Zn\(^{2+}\) Affinity Versus Differential Exposure to Cellular Zn\(^{2+}\) Prior to the Assay—One possible explanation for the difference in Zn\(^{2+}\) requirement in the in vitro assays of L- and S-SMase is that the two enzymes would both be exposed to the same, although limiting, concentration of intracellular Zn\(^{2+}\) but that the lysosomal enzyme would have a higher affinity for the cation, perhaps owing to a difference in post-translational modification. Thus, L-SMase would already have bound Zn\(^{2+}\) at the time of the assay. The secreted enzyme would have lower affinity for Zn\(^{2+}\), and thus excess exogenous Zn\(^{2+}\) would have to be added for activation in vitro.

We sought to estimate the relative Zn\(^{2+}\) affinities of these two enzymes by assaying their inactivation as a function of increasing exposure to metal chelators (36). Therefore, we incubated a cellular homogenate of J774 macrophages and the conditioned medium from these cells with EDTA plus the 1,10-phenanthroline for increasing times at 4 °C and then assayed these two fractions for SMase activity at each time point. As expected (above), both enzymes lost activity with increasing duration of chelation (Fig. 2), whereas incubation in the absence of the chelators for 8 h at 4 °C resulted in no loss of either secreted or cellular SMase activity (not shown). The data show that cellular SMase activity decreased at a greater rate and to a greater extent than secreted SMase activity, which is not consistent with the hypothesis that L-SMase has a higher affinity for Zn\(^{2+}\) than S-SMase.

The other possibility is that both enzymes bind Zn\(^{2+}\) with similar affinities, but only the lysosomal enzyme would be exposed to pools of intracellular Zn\(^{2+}\) prior to the assay; this exposure to Zn\(^{2+}\) could occur during transit to or residence in lysosomes and/or during preparation of the cell homogenate. Indeed, studies in many different cell types have shown that Zn\(^{2+}\) is distributed in various intracellular organelles, including lysosomes (60) and cytoplasmic vesicles (61). This model makes several assumptions and predictions that we tested experimentally. First, the idea that exposure of L-SMase to Zn\(^{2+}\) could occur during transit to or residence in lysosomes assumes that S-SMase does not simply arise by exocytosis of lysosomal vesicles (cf. Ref. 46). To test this important point directly, we obtained data on the carbohydrates of L- and S-SMase. The lysosomal targeting of L-SMase is typical for most lysosomal enzymes as follows: acquisition of Asn-linked high mannose oligosaccharides (44, 62) followed by phosphorylation of some of the mannose residues and shuffling from the trans-Golgi network to early endosomes/late endosomes/prelysosomes via mannose-phosphate receptor-containing vesicles (46, 63–65). In the typical (i.e. non-lysosomal) secretory pathway, however, the original high mannose oligosaccharides on the SMase would be expected to undergo processing to complex oligosaccharides during transit through the Golgi (44, 46, 62, 63). Therefore, we incubated aliquots of conditioned medium and homogenates from untransfected CHO cells with endo H, which is specific for high mannose-type Asn-linked oligosaccharides (66); other aliquots were incubated with peptide-N-glycanase F, which cleaves both high mannose and complex Asn-linked oligosaccharides (66). These incubations were then analyzed by anti-SMase immunoblots. As shown in Fig. 3, S-SMase was completely resistant to endo H but susceptible to peptide-N-glycanase F, indicating the presence of complex-type Asn-linked oligosaccharides. In contrast, L-SMase was susceptible to both glycosidases, which confirms that this form of the enzyme has high mannose-type oligosaccharides. In addition, comparison of the N-terminal amino acid sequences of purified S-SMase and L-SMase from CHO cells transfected with FLAG-tagged ASM revealed that L-SMase, but not S-SMase, underwent N-terminal proteolytic processing typical of lysosomal enzymes (46) (see following section). These data indicate that S-SMase does not arise via exocytosis of lysosomes or vesicles in transit to lysosomes but rather through the typical secretory

\(^4\) Consistent with prior literature (cf. Ref. 2), SMase activity in whole cell homogenates using the standard acidic micellar assay, particularly when EDTA is added, has been equated with “lysosomal” SMase activity. Other types of cellular SMase are not active at acidic pH in this assay, and one of these other SMases also requires Mg\(^{2+}\) for activity (2).

\(^5\) In pilot experiments, we found that 1,10-phenanthroline alone was not as effective as EDTA plus phenanthroline in inhibiting the activity of S- and L-SMase. One possible explanation is that the enzymes bind another divalent cation in addition to Zn\(^{2+}\), and removal of this cation by EDTA facilitates the removal of Zn\(^{2+}\) by 1,10-phenanthroline (cf. Refs. 36 and 53). Whatever the mechanism, the fact that 1,10-phenanthroline alone does not inhibit S- or L-SMase argues against an unlikely alternative interpretation of the data in Fig. 1, namely that 1,10-phenanthroline is a direct SMase inhibitor that becomes inactive as an inhibitor when the compound binds Zn\(^{2+}\).
pathway. These distinctly divergent pathways provide the opportunity for one of the SMase to be exposed to different levels of cellular Zn$^{2+}$ than the other form of the enzyme.

Second, the model implies that it is the sequestration of S-SMase away from Zn$^{2+}$ in the lysosomal pathway, not the oligosaccharide processing of S-SMase per se, that is responsible for the dependence of S-SMase on exogenous Zn$^{2+}$. To test this idea, we looked for a system in which cells secreted S-SMase that was mannose-phosphorylated but not exposed to the lysosomal pathway. We took advantage of the fact that in transfected cells that massively overexpress a lysosomal enzyme, a portion of this partially inactivated conditioned medium was then dialyzed against zinc-containing buffer C (150 mM NaCl, 10 mM Tris-HCl, 1 mM ZnCl$_2$, 0.6% Triton X-100, pH 7.4) for 18 h and then assayed for SMase activity (4th bar). An aliquot of this chelator-treated cell homogenate was then dialyzed against zinc-containing buffer C for 18 h and then assayed for SMase activity (5th bar).

FIG. 1. Sequential chelation and addition of Zn$^{2+}$ to secreted and intracellular SMase. Serum-free conditioned medium and a cell homogenate from J774 macrophages were prepared as described under "Experimental Procedures." A, conditioned medium (CM) was assayed for SMase activity using $^{35}$Sphingomyelin in Triton X-100 micelles in the presence of either 5 mM EDTA (1st bar) or 0.1 mM ZnCl$_2$ (2nd bar) for 1 h at 37 °C at pH 5.0. An aliquot of the zinc-activated conditioned medium was then incubated for 18 h in the presence of 10 mM EDTA, 0.1 mM 1,10-phenanthroline, and 0.6% Triton X-100 at 4 °C (EDTA PHNANTH) and then assayed for SMase activity (3rd bar). After chelation, a portion of this partially inactivated conditioned medium was then dialyzed against zinc-containing buffer C (150 mM NaCl, 10 mM Tris-HCl, 1 mM ZnCl$_2$, 0.6% Triton X-100, pH 7.4) for 18 h and then assayed for SMase activity (4th bar). B, an aliquot of cell homogenate (Cell Homgn) was assayed for SMase activity in the presence of assay buffer alone (1st bar), 5 mM EDTA (2nd bar), or 0.1 mM ZnCl$_2$ (3rd bar). Another aliquot of cell homogenate was incubated for 8 h in the presence of 10 mM EDTA, 10 mM 1,10-phenanthroline, and 0.6% Triton X-100 (EDTA PHNANTH) at 4 °C and then assayed for SMase activity (4th bar). An aliquot of this zinc-activated conditioned medium was then dialyzed against zinc-containing buffer C for 18 h and then assayed for SMase activity (5th bar).
ceramide/mg protein/h.

100%

conditioned medium were, respectively, 72.0 ± 0.3 pmol

Zn 2

mM EDTA, 10 mM 1,10-phenanthroline, and 0.6% Triton X-100 at

homogenate from J774 macrophages were incubated in the presence of

related SMase by Zn 2

10 mM EDTA, 10 mM 1,10-phenanthroline, and 0.6% Triton X-100 at

4 °C for the indicated times. Each sample was then assayed for SMase

activity in the presence of 5 mM EDTA for 1 h at 37 °C at pH 5.0. The

maximum values (100% on the y axis) for the cell homogenate and conditioned medium were, respectively, 72.0 ± 0.3 and 17.1 ± 0.3 pmol

[3H]Ceramide/mg protein/h.

Fig. 2. Time course of inactivation of secreted and intracellular SMase by Zn 2

+ chelation. Serum-free conditioned medium, pre-activated by incubation with 0.1 mM ZnCl 2 at 37 °C for 10 min, and cell

homogenate from J774 macrophages were incubated in the presence of

10 mM EDTA, 10 mM 1,10-phenanthroline, and 0.6% Triton X-100 at

4 °C for the indicated times. Each sample was then assayed for SMase

activity in the presence of 5 mM EDTA for 1 h at 37 °C at pH 5.0. The

maximum values (100% on the y axis) for the cell homogenate and conditioned medium were, respectively, 72.0 ± 0.3 and 17.1 ± 0.3 pmol

[3H]Ceramide/mg protein/h.

6 Note that treatment with chelators results in almost total inhibition of the internalized, activated S-SMase. This near total effect of chelators is similar to that observed with L-SMase but not with zinc-activated S-SMase from conditioned medium, which is only partially inhibited by chelators (Figs. 1 and 2). Therefore, when S-SMase is delivered to lysosomes, it appears to be converted into a form that allows more complete chelation of its Zn 2

+. It is possible that this conversion is related to the N-terminal proteolytic processing of SMase in lysosomes (cf. Ref. 46 and data in next section).

7 Simply adding S-SMase to cell homogenates after sonication is complete does not reduce the Zn 2

+ dependence of the enzyme (data not shown), suggesting that cellular Zn 2

+ under these conditions is too dilute. To explain this result, we propose that the sequestered pools of Zn 2

+ released during sonication are in close proximity to the lysosomes, which allows exposure of L-SMase to the released Zn 2

+ prior to dissipation of the Zn 2

+ throughout the entire homogenate.

L-SMase Activity from a Lysosome-rich 16,000 × g Pellet of CHO Cells Demonstrates Some Zinc Dependence—The Zn 2

+ dependence of L-SMase and previous work demonstrating discrete intracellular Zn 2

+ pools that can change under certain metabolic conditions (cf. Refs. 70 and 71) led us to consider the hypothesis that Zn 2

+ availability to lysosomes and to L-SMase might be involved in the regulation of this enzyme. A prediction of our hypothesis is that L-SMase may not always be maximally stimulated by intracellular Zn 2

+. In the standard L-SMase assay, cells or tissues are completely homogenized, and the cell homogenate is assayed. As shown in Fig. 5A for CHO-K1 cells disrupted by sonication, the intracellular enzyme is maximally activated, and exogenous Zn 2

+ has no effect (Fig. 5A). To obtain a less damaged lysosomal preparation, a separate aliquot of these CHO cells was disrupted under 500 p.s.i. of nitrogen pressure for 1.5 min, and a 16,000 × g pellet was isolated, which consists of intact lysosomes, as well as mitochondria and peroxisomes (cf. Ref. 59). This 16,000 × g pellet was then sonicated and assayed for SMase activity. Remarkably, under these conditions, the enzyme was only ~50% activated and was substantially stimulated by exogenous Zn 2

+ (Fig. 5A).

To probe this finding further, we isolated a 16,000 × g pellet from CHO cells transfected with FLAG-tagged ASM (see above) and then purified the enzyme by anti-FLAG affinity chromatography, followed by gel filtration and a second round of anti-FLAG affinity chromatography. The purified enzyme migrated as a single band on silver-stained SDS-PAGE slightly below where ASM-S-SMase migrates (data not shown; see Fig. 3). N-terminal amino acid sequence analysis revealed that enzyme began with the sequence GHPARLH, whereas S-SMase, which was purified from the conditioned medium of these cells, began with the sequence HPLSQPGHPARLH. Thus, the enzyme purified from the 16,000 × g pellet meets several criteria for L-SMase as follows: isolation from a lysosome-rich cellular fraction; N-terminal proteolytic processing (46); and more rapid migration on SDS-PAGE than S-SMase, which is due to both proteolytic processing and to differences in oligosaccharide structure (see Fig. 3). We then tested this purified L-SMase for zinc dependence and found that its enzymatic activity was increased 4.7-fold in the presence of Zn 2

+ (Fig. 5B). When zinc-activated L-SMase was dialyzed extensively against 10 mM EDTA, which does not remove activating Zn 2

+ from the enzyme (see Fig. 1), and then dialyzed against buffer free of both zinc and EDTA, the enzymatic activity was not changed (data not shown). Thus, the activating effect of zinc cannot be explained by free zinc (i.e. Zn 2

+ not bound to the SMase) affecting the substrate or some other component of the reaction mixture. These data, together with those in Fig. 5A, have two major implications. First, they suggest that L-SMase in these cells encounters subsaturating levels of Zn 2

+ during transit to lysosomes and/or after subsequent storage there, raising the possibility of regulation of L-SMase by intracellular Zn 2

+ availability (see “Discussion”). When cells are disrupted by sonication, we propose that sequestered pools of cellular Zn 2

+ are released, which leads to saturation of the enzyme with Zn 2

+. Second, these data provide further evidence that L-SMase is a zinc metalloenzyme.

Fig. 3. Susceptibility of S- and L-SMase to endo H and peptide-

N-glycanase F. Aliquots of concentrated conditioned media (as a

source of S-SMase) and cellular homogenate (as a source of L-SMase) from CHO cells were incubated in the absence or presence of endo H or peptide-N-glycanase F (PNGase F) for 16 h at 37 °C and then subjected to SDS-PAGE. The electrophoresed proteins were transferred to a nitrocellulose membrane and then immunoblotted using rabbit anti-FLAG-tagged S-SMase antiserum.
Our model to explain the cellular trafficking and apparent difference in Zn\(^{2+}\) dependence of L-SMase versus S-SMase is shown in Fig. 6. Based upon our previous work, the ASM gene gives rise to a common precursor protein (16), which is then modified by typical high mannose oligosaccharide residues (44, 62, 63). We propose that this mannosylated precursor then traffics into either the lysosomal or the secretory pathway. In the lysosomal pathway, the SMase undergoes modification and trafficking that is typical for lysosomal enzymes: acquisition of mannose-phosphate residues by the sequential action of N-acetylglucosamine-1-phosphotransferase and N-acetylglucosamine phosphodiesterase on the mannose residues of the precursor (Fig. 3 and Refs. 44 and 63). Vesicles containing mannose-phosphate receptors then shuttle this modified enzyme to early endosomes or late endosomes/prelysosomes (63, 72, 73), and we propose that at some point along this pathway the enzyme encounters cellular Zn\(^{2+}\) and thus becomes at least partially activated. As mentioned under “Results,” L-SMase, at least in CHO cells, appears to be exposed to subsaturating concentrations of Zn\(^{2+}\) in lysosomes and thus is potentially subject to regulation by changes in Zn\(^{2+}\) availability.

L-SMase has been studied for many years, particularly in the context of its absence in a human disease, namely types A and B Niemann-Pick disease (1, 2). Throughout this period of study, the enzyme has been reported to be cation-independent (1, 2). Although plasma emission spectrometry and x-ray crystallography of large amounts of homogeneously purified L-SMase
will be needed to define the stoichiometry and location of zinc interaction with L-SMase, the data in this report strongly support the conclusion that this enzyme is, indeed, a zinc-activated enzyme. The most compelling data are those in Fig. 1B, Fig. 5, and Table I, especially footnotes d and e. In fact, the information in Table I raises the possibility that some cases of Niemann-Pick disease may be due to mutations in the zinc-binding domain, possibly resulting in defective binding of Zn\(^{2+}\) and thus loss of enzymatic activity. Along these lines, He et al.\(^{3}\) have shown that chelation of Zn\(^{2+}\) from SMase by 1,10-phenanthroline results in defective SM binding to the enzyme. We believe the reason why this fundamental property of this widely studied enzyme has been overlooked is because the enzyme at the time of isolation from whole cell homogenates, which has been the source of L-SMase for the previous studies (54–58), is already tightly bound to Zn\(^{2+}\). In view of the data in Fig. 5, some of this Zn\(^{2+}\) may come from pools of zinc that are released during the homogenization of cells or tissues. Thus, exogenous Zn\(^{2+}\) is not needed for the in vitro assay, and typical short-term EDTA chelation incubations will not strip the enzyme of its metal, similar to findings with other known zinc metalloenzymes (36).

To explain the origin of S-SMase, we propose that a portion of the common precursor, via a potentially regulated process (see below), bypasses N-acetylglucosamine-1-phosphotransferase and thus is directed into the secretory pathway, not the lysosomal targeting pathway (63) (Fig. 6). The difference in susceptibility of S- and L-SMase to endo H (Fig. 3) and the differences in N-terminal proteolytic processing (see "Results") provide direct support for this component of the model. Importantly, our data suggest that SMase in the secretory pathway is not exposed to pools of cellular Zn\(^{2+}\), thus explaining the requirement for exogenously added Zn\(^{2+}\) when the secreted enzyme is assayed in vitro. As mentioned under "Results," however, the subcellular location of Zn\(^{2+}\) may be subject to cell-type variation or regulation (60, 70, 71). For example, recent data from Vallee and colleagues (74) suggest that the redox state of the cell may be an important factor in the transfer of zinc from metallothionein, a cellular zinc reservoir, to intracellular zinc-dependent enzymes. Therefore, it is possible that S-SMase may, under certain circumstances or in certain cell types, be fully or partially Zn\(^{2+}\)-independent. In fact, we have observed that SMase secreted by endothelial cells, unlike that secreted by macrophages (16), is active in the absence of Zn\(^{2+}\) and stimulated only 2-fold by exposure to exogenous Zn\(^{2+}\) (25).

According to this model and work by other researchers (46, 63), the key step that would determine the fate of SMase is catalysis of the common mannosylated precursor by N-acetylglucosamine-1-phosphotransferase. Extensive work by Kornfeld and colleagues (75–77) has shown that N-acetylglucosamine-1-phosphotransferase recognizes a particular three-dimensional structure of lysosomal enzyme precursors, and induced modifications that alter this structure can have profound effects on lysosomal enzyme modification and targeting. Moreover, these workers have found that at least one enzyme, bovine DNase I, is a suboptimal substrate for the phosphotransferase, thus presumably giving rise to both intralysosomal and secretory forms (78)\(^a\) If the enzymes that undergo secretion by this mechanism can function at neutral pH (see below) or if the cells are in an acidic environment, this process may enable cells to acquire two groups of functions from a single enzyme, namely functions in lysosomes and functions in the extracellular milieu. In the case of S-SMase, there is an additional requirement for extracellular Zn\(^{2+}\), which is known to exist in sufficient extracellular concentrations in vivo to activate the enzyme (cf. Refs. 16 and 53). Interestingly, we found that certain cytokines increase the secretion of SMase.

\(^a\) S. Kornfeld, personal communication.
from endothelial cells without affecting L-SMase activity, suggesting that the phosphotransferase reaction or perhaps another critical step responsible for determining the fate of SMase may be subject to specific regulation (25). Finally, C. elegans has two separate genes that encode SMases that are highly homologous to mammalian S- and L-SMase; one of these SMases is almost entirely secreted and the other is mostly intracellular (51). Thus, organisms evidently need both intracellular and extracellular SMases; C. elegans has two genes to meet these needs, and it appears as if higher species (i.e. mammals) have evolved the mechanism described above to meet these needs using one gene.

Our current data and previous work by others (79) indicate difficulties with the prior nomenclature of these SMases. First, we now know that both forms of the enzymes are zinc-activated enzymes, and so our previous designation of the secreted form as “Zn-SMase” (16) is obsolete. Second, the “acid SMase” nomenclature reflects the acid pH optima of the lysosomal and extracellular SMases, and so our previous designation of the secreted form as “acid SMase” is obsolete. Second, the “acid SMase” nomenclature reflects the acid pH optima of the lysosomal and extracellular SMases; C. elegans has two genes to meet these needs, and it appears as if higher species (i.e. mammals) have evolved the mechanism described above to meet these needs using one gene.

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REFERENCES

1. Levade, T., Salvayre, R., and Blazy-Douste, L. (1986) J. Clin. Chem. Biochem. 24, 205–220
2. Brady, R. O. (1983) in The Metabolic Basis of Inherited Disease (Stanbury, J. B., Wyngarden, J. B., Fredrickson, D. S., Goldstein, J. L., and Brown, M. S., eds) pp. 831–841, McGraw-Hill Inc., New York
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55. Rao, B. G., and Spence, M. W. (1976) J. Lipid Res. 17, 506–515
56. Yamaguchi, S., and Suzuki, K. (1977) J. Biol. Chem. 252, 3865–3813
57. Callahan, J. W., Shankaran, P., Khalil, M., and Gerrie, J. (1978) Can. J. Biochem. 56, 885–891
58. Bowser, P. A., and Gray, G. M. (1978) J. Invest. Dermatol. 70, 331–335
59. Watanabe, K., Sakuragawa, N., Arima, M., and Satoyoshi, E. (1983) J. Lipid Res. 24, 506–605
60. Bettger, W. J., and O’Dell, B. L. (1981) Life Sci. 28, 1425–1438
61. Danscher, G., Howell, G., Perez-Clausell, J., and Hertel, N. (1985) Histochemistry 83, 419–422
62. Newrzella, D., and Stoffel, W. (1996) J. Biol. Chem. 271, 32089–32095
63. Kornfeld, S. (1987) FASEB J. 1, 462–468
64. Prack, M. M., Rothblat, G. H., Erickson, S. K., Reyland, M. E., and Williams, D. L. (1994) Biochemistry 33, 5049–5055
65. Yamamoto, K. (1994) J. Biochem. (Tokyo) 116, 229–235
66. Ioannou, Y. A., Bishop, D. P., and Desnick, R. J. (1992) J. Cell Biol. 119, 1137–1150
67. Faust, P. L., Wall, D. A., Perara, E., Lingappa, V. R., and Kornfeld, S. (1987) J. Cell Biol. 105, 1947–1945
68. Neufeld, E. F. (1980) Birth Defects 16, 77–84
69. Csermely, P., Fodor, P., and Somogyi, J. (1987) Carcinogenesis 8, 1663–1666
70. Brand, I. A., and Kleineke, J. (1996) J. Biol. Chem. 271, 1941–1949
71. Ludwig, T., Griffiths, G., and Hohlack, B. (1991) J. Cell Biol. 115, 1561–1572
72. Runquist, E. A., and Havel, R. J. (1991) J. Biol. Chem. 266, 22557–22563
73. Jiang, L., Maret, W., and Vallee, B. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3483–3488
74. Baranski, T. J., Cantor, A. B., and Kornfeld, S. (1992) J. Biol. Chem. 267, 23342–23348
75. Cantor, A. B., Baranski, T. J., and Kornfeld, S. (1992) J. Biol. Chem. 267, 23349–23356
76. Dustin, M. L., Baranski, T. J., Sampath, D., and Kornfeld, S. (1995) J. Biol. Chem. 270, 170–179
77. Nishikawa, A., Gregory, W., Frenz, J., Cacia, J., and Kornfeld, S. (1997) J. Biol. Chem. 272, 19408–19412
78. Baranski, T. J., Cantor, A. B., and Kornfeld, S. (1992) J. Biol. Chem. 267, 23342–23348
79. Callahan, J. W., Jones, C. S., Davidson, D. J., and Shankaran, P. (1983) J. Neurosci. Res. 10, 151–163
80. Horinouchi, K., Erlich, S., Perl, D., Ferlinz, K., Bisgaier, C. L., Sandhoff, K., Demick, R. J., Stewart, C. L., and Schuchman, E. H. (1996) Nat. Genet. 16, 288–293
81. Otterbach, B., and Stoffel, W. (1995) Cell 77, 759–803
82. Mukherjee, S., Ghosh, R. N., and Maxfield, F. R. (1997) Physiol. Rev. 77, 759–803
83. Hung, C.-H., Huang, H.-R., Huang, C.-J., Huang, F.-L., and Chang, G.-D. (1997) J. Biol. Chem. 272, 13772–13778
84. Francis, S. H., Colbran, J. J., McAllister-Lucas, L. M., and Corbin, J. D. (1994) J. Biol. Chem. 269, 22477–22480