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The Extended Family of Neutral Sphingomyelinases

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ABSTRACT: The neutral sphingomyelinases (N-SMases) are considered major candidates for mediating the stress-induced production of ceramide, and N-SMase activity has been identified, characterized, and cloned from bacteria, yeast, and mammalian cells. Although the level of identity between these enzymes is low, a number of key residues thought to be involved in metal binding and catalysis are conserved. This has led to the suggestion of a common catalytic mechanism, and thus, these enzymes are considered to form an extended family of N-SMases. Despite considerable research into N-SMase activity in cell culture and various tissues, the lack, until recently, of molecular identification of specific N-SMase enzymes had precluded specific insights into the regulation, physiological, and pathological roles of these proteins. In this review, we summarize, for the first time, current knowledge of the N-SMase family, focusing on cloned members from bacteria, yeast, and mammalian cells. We also briefly consider the major future directions for N-SMase research which promises highly significant and specific insight into sphingolipid-mediated functions.

Ceramide is well-established as a bioactive molecule implicated in processes such as the cellular responses to stress, cell growth, and apoptosis (reviewed in ref *1*) and as a source of other lipid second messengers such as sphingosine and sphingosine 1-phosphate (S1P).[1] The sphingomyelinase (SMase)-mediated hydrolysis of sphingomyelin (SM) has emerged as a major pathway of stress-induced ceramide production, and currently, five types of SMases have been described and classified according to optimum pH and metal ion dependence (reviewed in ref *2*). Of these, the magnesium (Mg^{2+})-dependent neutral SMases have emerged as major candidates for mediating this pathway. Here, we will review current knowledge of the emerging extended N-SMase family of enzymes in bacteria, yeast, and mammalian tissue from an overview of structure and catalysis to specific properties, roles, and regulation of these enzymes in physiological and pathological contexts.

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[1] Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer's disease; Asp, aspartate; CL, cardiolipin; CSS1, can't stop synthesizing cell wall; ER, endoplasmic reticulum; Glu, glutamate; Gly, glycine; GlcT-1, ceramide glucosyltransferase-1; IL, interleukin; IPS, inositol phosphosphingolipids; ISC1, inositophosphosphingolipid phospholipase C; N-SMase, neutral sphingomyelinase; PG, phosphatidylglycerol; PLL, P-loop-like; PM, plasma membrane; PS, phosphatidylserine; S1P, sphingosine 1-phosphate; SM, sphingomyelin; SMase, sphingomyelinase; TNF, tumor necrosis factor.

THE NEUTRAL SPHINGOMYELINASE FAMILY

Neutral sphingomyelinase (N-SMase) activity was first described in fibroblasts from patients with Niemann-Pick disease, who exhibit deficiencies in the previously described acid SMase (*3*). Subsequent characterization indicated that this enzyme was the product of a distinct gene, had an optimum pH of 7.4, was dependent on Mg^{2+} ions for activity,

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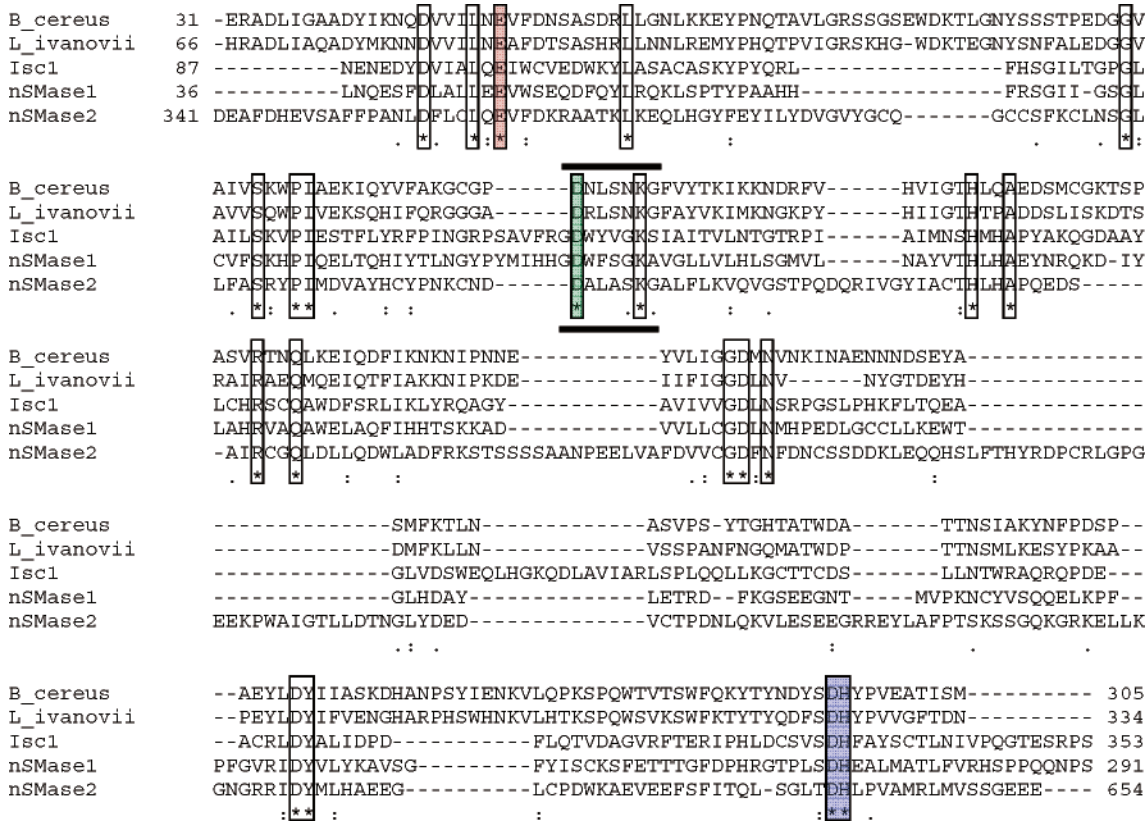

FIGURE 1: Extended N-SMase family. Shown is an alignment of the catalytic regions of N-SMases from (accession numbers in parentheses) *B. cereus* (GenBank entry CAA31333), *L. ivanovii* (GenBank entry Q9RLV9), Isc1 from *Sa. cerevisiae* (GenBank entry P40015), and human nSMase1 (GenBank entry O60906) and nSMase2 (GenBank entry Q9NY59). Residues conserved across all family members are boxed. These include (*B. cereus* numbering) Glu-53 (red), Asp-126 (green), and Asp-295 and His-296 (both blue), critical for Mg^{2+} binding and catalytic activity. The predicted P-loop-like domains of the family are highlighted with black bars [aligned with MAFFT version 5.8 (*87*), available at http://us.expasy.org/tools].

and was particularly enriched in brain (*4*). However, it was some 20 years before a major breakthrough came with the cloning of the first N-SMase family members from *Bacillus cereus* and *Staphylococcus aureus* (*5*, *6*). Via the investigation of homology to these enzymes, N-SMases were identified in other bacteria such as *Listeria ivanovii* (*7*), and subsequently, bacterial SMase sequences were used to identify the yeast N-SMase homologue, ISC1 (*8*), and the mammalian N-SMase homologues, nSMase1 and nSMase2 (*9*, *10*). Together, these enzymes comprise the larger N-SMase family.

The level of identity between bacterial, yeast, and mammalian family members is low, being approximately 20% between nSMase2 and the SMase from *B. cereus* (*10*). However, an alignment of the sequences indicates a number of conserved residues throughout the family (Figure 1), particularly in the catalytic region of the enzymes, leading to the suggestion of a common catalytic mechanism. Interestingly, a recently cloned nSMase3 bears little sequence similarity to nSMase1 or -2, although the high degree of evolutionary conservation reported for nSMase3 suggests it may comprise a unique and distinct N-SMase (*11*). Consequently, as nSMase3 does not belong to the extended N-SMase family described here, it is beyond the scope of this review and will not be discussed in detail. However, it is noteworthy that this correlates with the reported existence of multiple N-SMase activities in mammalian tissues (*12*).

CATALYSIS AND STRUCTURE

The reaction catalyzed by N-SMases closely follows that of the phospholipases in that a phosphodiester bond is hydrolyzed, removing a soluble molecule from the insoluble lipid. To date, both SMase types C and D have been found, with the N-SMase family belonging to the former type (cleaving between the phosphate and the lipid). In contrast, SMase D enzymes generate ceramide 1-phosphate and choline and have been found only in spider venom and a few bacteria (*13*).

Catalysis. Sequence searches and alignments (Figure 1) have shown that N-SMases across the phyla share the same basic residues, and domain analysis of the N-SMase family shows a conserved catalytic domain (*10*). These include histidines (His), important in the acid−base function, a glutamate (Glu), essential for Mg^{2+} binding, and several aspartates (Asp) for substrate recognition. Investigation of the catalytic mechanism of this group of proteins has mostly focused on the soluble *B. cereus* SMase. Initial chemical modifications of *B. cereus* SMase suggested the use of acidic residues in the catalytic process (*14*), and subsequent mutational experiments revealed that Asp-295 was essential for hydrolytic activity while Asp-126 and -156 were involved in substrate recognition (*15*). Via identification of these residues, the SMase could be reasonably aligned with mammalian DNase1 (*15*), revealing conservation of two histidine residues catalytically important for the DNase phosphoester bond hydrolysis. This prompted homology

modeling of *B. cereus* SMase that was confirmed by demonstrating that mutation of these two histidines (His-134 and His-252) abolished activity (*16*). While the dependence of the reaction on Mg^{2+} ions had been reported previously (*6*), further analysis demonstrated the presence of two Mg^{2+}-binding sites (one with high affinity and one with low affinity) (*17*), and the Mg^{2+}-liganding residue Glu-53, found in the high-affinity site, was identified (*18*). Inhibition of catalytic activity has been demonstrated for Ca^{2+}, Sr^{2+}, Zn^{2+}, EDTA, and the unusual phosphate analogue BeF_2 (*19*, *20*). However, while low concentrations of Zn^{2+} (micromolar) activated the enzyme, at high concentrations (millimolar), competitive inhibition occurred (*21*). Thus, it was postulated that Zn^{2+} binding to the high-affinity site activates the enzyme and, conversely, binding to the low-affinity site inhibits the enzyme.

Crystal Structures. Recently, two crystallographic structures of bacterial SMases from *L. ivanovii* (*22*) and *B. cereus* (*23*) were determined. Both structures show the same fold as DNase I, confirming original modeling predictions (*18*), and the similarity between the two crystal structures is good (0.71 Å rmsd for 269 C_α atoms). Neither crystal structure contains the SM bound, although the *B. cereus* structure does show the lipid modeled, and the *L. ivanovii* structure shows a buffer phosphate bound in the active site (*22*, *23*).

The *B. cereus* SMase structure has been determined in the presence of various divalent cations relevant to SMase activity, resulting in three structures that contain Ca^{2+}, Co^{2+}, and Mg^{2+}. Of these, the Co^{2+} and Mg^{2+} structures are known to be active whereas the Ca^{2+} one is inhibited (*19*). Interestingly, only the Co^{2+} structure had three metal-binding sites, two in the proposed active site (site A) and one on the periphery of the protein (site B) (Figure 2), whereas the Ca^{2+}- and Mg^{2+}-bound protein was reported to exhibit electron density suitable for a single cation at site A. The conserved residues Glu-53, His-151, Asp-195, and His-296 are visible. Interestingly, Glu-53, His-151, and Asp-195 are at the C-terminal ends of a parallel β-strand, whereas His-296 is on a long loop between strands β14 and β15. Furthermore, Asp-126 and -156, implicated in substrate recognition (*17*), are on the two loops not visible in these crystal structures, implying a high degree of variation within the crystal structure; in both the Ca^{2+}- and Co^{2+}-bound structures, two of four chains are missing these loops. These two loops are located at metal site A, indicating that this region is the active site. In the *L. ivanovii* structure, both of these loops are observed and show a more closed confirmation of the active site, whereas in the bacterial SMase structure, the residues adjacent to the missing loops suggest they have folded out to open the active site prior to substrate binding.

So far, the crystal structures have confirmed the enzymology and homology modeling of the bacterial SMases. These results have been successfully transferred to N-SMases from higher organisms, thus demonstrating the conservation of the key catalytic residues, consistent with a common catalytic mechanism.

BACTERIAL SPHINGOMYELINASES

Cloning and Properties. Pathogeneic bacteria produce a variety of structurally heterogeneous molecular species of phospholipases that exert cytotoxicity to host cells and are

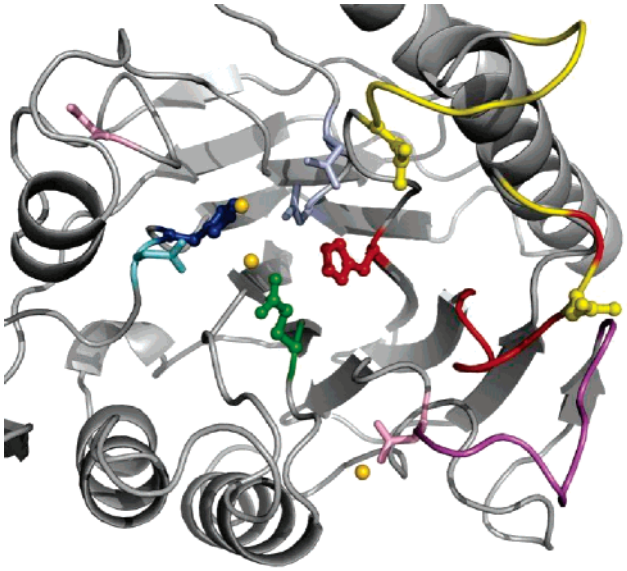

FIGURE 2: Crystal structure of *B. cereus* sphingomyelinase. The cobalt-bound crystal structure (*23*) was created using PyMOL (*88*) and is colored gray with cobalt ions shown as gold spheres, the solvent-exposed loop colored orange, and the potential P-loop-like domain (*48*) colored red/yellow. Residues predicted in the original homology model (*16*) are shown as colored side chains as follows: yellow for substrate recognition (Asp-126 and Asp-156), green for metal binding (Glu-53), blue for general base in hydrolysis (His-296), light blue for phosphate group interaction (Asp-195 and Asn-197), pink for hydrogen bonds to His (Asp-100 and Asp-233), and cyan for stabilization of the pentacovalent transition state (Asp-295). Ball-and-stick side chains are residues for which the function has been tested, whereas stick side chains have not been tested.

implicated in the pathogenesis of diseases caused by such bacteria (*24*). The gene for SMase, a member of these phospholipases C, has been identified from a number of bacteria such as *S. aureus* (*5*), *B. cereus* (*6*), *L. ivanovii* (*7*), *Leptospira interrogans* (*25*), and *Pseudomonas* sp. strain TK4 (*26*). Interestingly, whereas mammalian N-SMases are membrane-bound proteins, the bacterial SMases are secretory proteins released from cells into the media (*24*). The molecular mass of bacterial SMases varies from 36−38 kDa for *B. cereus*, *S. aureus*, and *L. ivanovii*, all with a cleavable secretory signal sequence at their N-termini (*5*−*7*), to 58.1 and 63.3 kDa for the TK4 strain and *Le. interrogans*, respectively, due to the presence of additional sequences at their C-termini (*25*, *26*).

N-SMase activity was also identified in *Heliobacter pylori* and *Mycobacterium tuberculosis* (*27*−*29*). However, as these activities were found in cell lysates, it is unclear if these enzymes are secretory proteins. Although the genes have yet to be identified, there is some evidence to suggest these enzymes can be categorized into the bacterial SMase gene family. Antiserum against the purified *B. cereus* SMase recognized the purified 32 kDa protein from *H. pylori*, suggesting a common antigenic epitope in both proteins (*27*). Additionally, in the *M. tuberculosis* database, a hypothetical protein that is significantly similar to N-SMase from *Schizosaccharomyces pombe* was found, although it is unclear whether this sequence encodes the observed N-SMase activity (*29*).

Bacterial SMases and Hemolysis. Although the SMase-producing bacteria do not synthesize sphingolipids, they have developed mechanisms by which they can utilize host

sphingolipids, possibly to promote their pathogenicity. The most well-established cytotoxic action of bacterial SMases is in their hemolytic activity to erythrocytes (*24*), which all the known bacterial SMases possess. This occurs through hydrolysis of the erythrocyte cell surface SM with studies showing that the susceptibility of erythrocytes from different species correlated with their SM content. Thus, sheep erythrocytes (SM is 51% of total lipid) are most vulnerable to SMase treatment, whereas dog, horse, and guinea pig erythrocytes (11% SM) are virtually resistant (*30*−*32*). Additionally, even after SM hydrolysis takes place at 37 °C, efficient cell lysis occurs only when the erythrocytes are exposed to low temperatures, a phenomenon known as hot−cold hemolysis (*30*, *31*).The mechanism of hot−cold hemolysis was attributed to the generation of fragile erthrocytes after the cleavage of plasma membrane (PM) SM. Thus, when the membrane lipids cool, the phase change may cause stress resulting in the lysis (*30*).

The hemolytic activity of bacterial SMases is increased by cooperative and synergistic interactions among virulence factors secreted by the same bacteria. For instance, *B. cereus* SMase has a synergistic effect in hemolysis in conjunction with hemolysin BL, a three-component pore-forming toxin, and phosphatidylcholine phospholipase C, major membrane-damaging factors released by the bacteria (*33*). These toxins are under the control of PlcR, a pleiotropic regulator of extracellular virulence factor genes, and are expressed simultaneously, suggesting an important role for this synergism during *B. cereus* infection (*34*). Furthermore, this synergistic effect on hemolysis was also reported for proteins from different bacteria. *L. ivanovii*, which has a strong hemolytic effect on blood agar, gives rise to a characteristic shovel patch of cooperative hemolysis (termed CAMP-like hemolysis) when grown close to *Rhodococcus equi* (*35*). However, SMase knockout mutant *L. ivanovii* does not give rise to the shovel-shaped reaction with *R. equi*, indicating the cooperative action of the SMase with other, unknown factors from *R. equi* (*7*, *35*).

Bacterial SMase and Membrane Association. When catalyzing hemolysis, bacterial SMases have been reported to bind to the cell surface (*30*). Very recently, analysis of the crystal structure of the SMases from *B. cereus* and *L. ivanovii* revealed that a unique hydrophobic β-hairpin region located in the C-terminus is important for membrane interaction of the enzymes (*22*, *23*). This structure protrudes into the solvent with two hydrophobic amino acids, located at the apex of the hairpin and exposed to the bulk solvent. Furthermore, replacement of these amino acids with alanine in *B. cereus* SMase weakened binding to SM liposomes and sheep erythrocytes, confirming their importance in membrane−protein interactions (*23*). In contrast, the TK4 and *Le. interrogans* SMases do not appear to contain the β-hairpin structure, suggesting the presence of other membrane association mechanisms. Consistent with this, both these SMases contain an additional 186 amino acids in the C-terminus that are not present in other bacterial SMases (*26*). Furthermore, a deletion mutant of strain TK4 lacking these amino acids lost all hemolytic activity due to the lack of SM hydrolysis in the erythrocyte plasma membrane, despite its still being able to hydrolyze SM in detergent micelles. Thus, the C-terminal region of the TK4 SMase is indispensable for its hemolytic activity, possibly through regulating membrane association (*26*). Finally, it has also been shown that binding of *Bacillus* SMase to erythrocytes during hemolysis requires calcium, although the mechanism by which this occurs remains unclear (*30*).

Bacterial SMase and Cytotoxicity. Multiple studies have demonstrated that bacterial SMases are cytotoxic for several mammalian cell types (*36*). *Staphylococcus* SMase (β-toxin) has been intensively studied in cellular cytotoxicity and pathology. Although found to degrade SM efficiently in human fibroblasts, granulocytes, and lymphocytes without affecting viability (*36*−*38*), the enzyme had potent cytotoxicity to human monocytes, resulting in a rapid loss of cellular ATP (*36*). Interestingly, and unlike in erythrocytes, this difference was not reflected by the SM content of monocytes and granulocytes, reported to be 10.9 and 20.8%, respectively (*39*, *40*).

Although the precise mechanism of the selective cytotoxicity of bacterial SMases remains unclear, a role for the generated ceramide has been suggested. One potential influencing factor may be the speed of metabolizing ceramide. Consistent with this, treatment of B16 melanoma cells with *Bacillus* SMase resulted in an increased level of glycosphingolipid in the plasma membrane because of the action of ceramide glucosyltransferase-1 (GlcT-1), whereas a GlcT-1-deficient mutant was unable to avoid the cytotoxic effects of the SMase, likely due to a lack of ceramide clearance (*41*).

Genetic Knockout of Bacterial SMase. The role of SMases in bacterial virulence is also supported by evidence from gene knockout strains. A SMase knockout strain of *L. ivanovii* was less virulent for mice than the wild-type strain (*7*). Further study in the MDBK epithelial-like cell line found that intracellular proliferation of the mutant strain was impaired, because of an inability of the bacteria to escape from the phagosomal compartment into the cytoplasm where it can actively multiply (*7*, *42*). Finally, the pathogenicity of a SMase knockout mutant strain of *S. aureus* was investigated in an infection model of the mouse mammary gland with recovery of the knockout strain significantly lower than that of the wild type (*43*). Furthermore, this mutant showed less damage in a rabbit corneal infection model, whereas application of purified β-toxin to the eye resulted in edema in the sclera and conjunctivitis (*44*). Taken together, these results indicate the importance of bacterial SMases as a virulence factor in various pathological states.

YEAST NEUTRAL SPHINGOMYELINASE: ISC1 AND CSS1

Although important for many biological processes in mammalian cells, SM is not present in *Saccharomyces cerevisiae*. Instead, both budding (*Sa. cerevisiae*) and fission yeast (*Sc. pombe*) contain inositol phosphosphingolipids (IPS). The enzyme that catalyzes degradation of IPS in yeast is called inositol phosphosphingolipid phospholipase C with the reaction generating phytoceramide and dihydroceramide. The genes that encode these enzymes are *ISC1* (inositol phosphosphingolipid phopholipase C) for budding yeast and *CSS1* (can't stop synthesizing cell wall) for fission yeast (*8*, *45*).

ISC1

The *ISC1(YER019w)* gene was first identified in 2000 by Sawai et al. representing the first time in *Sa. cerevisiae* that an enzyme involved in the catabolism of complex sphingolipids was identified (*8*). Furthermore, although SM is not the physiological substrate of Isc1, the enzyme has been shown to hydrolyze SM in vitro (*46*). Isc1 is approximately 28% homologous to human N-SMases, and as with other N-SMases, a number of key amino acid residues important for activity are conserved, with mutation of Glu-100, Asp-234, and His-334 completely abrogating Isc1 activity (*47*).

Additionally, Isc1 contains a domain homologous to the P-loop motif of nucleotide-binding proteins, termed the P-loop-like (PLL) domain that has been implicated in catalytic activity (*47*, *48*). Further study of the PLL domain showed that Asp-163 and Lys-168 residues are conserved across the N-SMase family (Figure 1) and both were essential for catalytic activity, while mutation of Gly-162, Gly-167, and Ser-169 also reduced activity and, interestingly, reduced the level of binding of Mg^{2+} to Isc1 (*48*). Taken together, these results indicate that the PLL domain is critical for catalytic activity of Isc1, possibly through a role in substrate and/or Mg^{2+} binding.

In vitro, Isc1 is activated by anionic phospholipids, especially cardiolipin (CL), phosphatidylserine (PS), and phosphatidylglycerol (PG) in a dose-dependent manner, and binding studies revealed selective binding of Isc1 to these lipids (*8*, *47*). Furthermore, PS binding required the C-terminus, suggesting localization of the anionic phospholipid-binding domain to this region (*47*), and when a C-terminal (lipid-binding) fragment was coexpressed with a separate N-terminal (catalytic) fragment, Isc1 activity was reconstituted. Thus, a "tether-and-pull" model for the interaction and stimulation of Isc1 by anionic phospholipids was proposed (Figure 3). In this model, the C-terminus of Isc1 associates with membranes in the presence of PS, CL, and PG (the tether), and as a consequence, the N-terminal catalytic domain is "pulled" to the membrane to interact with lipid substrates. One prediction of this model is that colocalization of PS, CL, and PG and Isc1 might be critical for activation.

Physiological Roles. The deletion of ISC1 from *Sa. cerevisiae* results in slow growth of mutant cells compared to wild-type cells, suggesting the protein is required for optimal growth (*48*). Consistent with these results, when exponentially growing yeast switch from glycolytic to aerobic metabolism occurring when glucose in the media is consumed, termed "diauxic shift", Isc1 activity was found to increase, and a concurrent increase in the amount of phytoceramide was prevented in ΔISC1 cells. Additionally, the enhanced Isc1 activity was independent of translation or transcription (*49*). Microscopy and fractionation studies on Isc1 localization found that the growth-dependent increase in ISC1 activity is accompanied by a relocalization of the protein from the endoplasmic reticulum (ER) (where it resides during logarithmic growth) to the mitochondria (*49*). Thus, both activity and localization of Isc1 are regulated in a growth-dependent manner (Figure 3). This also suggests that Isc1 may play a role in mitochondrial function.

In addition to the slow growth phenotype observed in glucose-containing media, ΔISC1 cells struggle to grow on nonfermentable carbon sources, a defect that resembled that

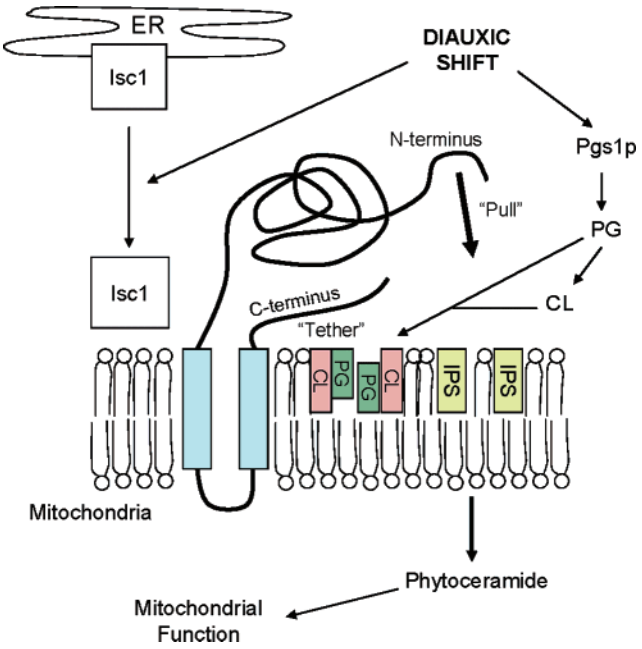

FIGURE 3: Schematic of Isc1 signaling during diauxic shift. Diauxic shift is a switch from glycolytic to aerobic metabolism by exponentially growing yeast, occurring when glucose in the medium is consumed and ethanol produced during the logarithimic growth phase is used as a carbon source. In vivo signaling studies have shown that Isc1 translocates from the ER to mitochondria and the level of production of PG and CL is increased as yeast enters diauxic shift. An in vitro tether-and-pull model suggests that the C-terminus of Isc1 tethers the protein to the membrane through the anionic phospholipid-binding domain and, through intramolecular interactions, the N-terminus is pulled close to the membrane where it has access to the IPS substrate. As a consequence, phytoceramide levels increase and may play a role in mitochondrial function.

of strains lacking the committed step in PG and CL synthesis (*50*). Further investigation found that the PG and CL biosynthetic pathway was essential for the growth-dependent activation of Isc1 (*50*), thus establishing these lipids as endogenous activators of Isc1 and extending the in vitro studies on direct binding and activation (*47*). Consistent with this, microarray analysis found that a number of aberrant genes in ΔISC1 strains were those involved in carbon source utilization, suggesting that this phenotype may be due to a defect at the genetic level (*51*).

Evidence has also implicated ISC1 in the response of yeast to heat stress. Studies in ΔISC1 strains indicated Isc1 is involved in heat-induced ceramide production. ISC1 deletion was also found to be synthetically lethal in conjunction with a temperature-sensitive mutation in the LCB1 gene (LCB1−100), a strain unable to produce de novo sphingolipids at the restrictive temperature (*51*). Further microarray studies in ΔISC1 strains versus the wild type found 57 aberrant genes, of which eight are involved in meiosis and sporulation, consistent with an observed decrease in the sporulation rate of ΔISC1 cells (*51*).

CSS1

ΔCSS1 mutants of *Sc. pombe* were first characterized as temperature-sensitive mutants with a defect in cell wall formation (*52*) with subsequent analysis indicating an accumulation of cell wall material (such as α- and β-glucans) in the periplasmic space (*45*). Named CSS1 (can't stop

synthesizing cell wall), a comparison with N-SMase proteins from different organisms revealed that Css1 was an Isc1 homologue, confirmed by showing that the expression of Css1 in *Sa. cerevisiae* is able to compensate for the loss of Isc1. Being approximately 37% homologous to nSMase1 and 35% homologous to Isc1, Css1 contains an N-terminal catalytic domain with two putative TM domains in the C-terminus. Furthermore, Css1 appeared to encode the only sphingolipid hydrolyzing activity in *Sc. pombe* (*45*). Investigation of Css1 localization using a C-terminal myc-targeted protein found that the enzyme had a distinct localization to that of Isc1, being found at the plasma membrane and in vesicles of the secretory pathway. Interestingly, sequences in the C-terminus beyond the TM domains were found to be essential for Css1 activity but functioned in a manner independent of membrane targeting (*45*).

The cell wall of *Sc. pombe* is required for the maintenance of cell shape and is remodeled continuously during the cell cycle to allow cell expansion (*53*), suggesting an important role for Css1 as a regulator of *Sc. pombe* growth. Consistent with this, and unlike with ISC1, CSS1 was found to be an essential gene for viability (*45*). Therefore, further investigation of Css1-interacting genes may shed light on the roles of ceramide and sphingolipids in yeast cell function. However, despite this, there has been very little subsequent work on the regulation and physiological roles of Css1.

MAMMALIAN NEUTRAL SPHINGOMYELINASES

Although there is considerable literature on N-SMase activity in general, the relatively recent cloning of mammalian N-SMases means that there is little information concerning the specific functions of each of these enzymes.

Neutral Sphingomyelinase 1 (nSMase1)

In 1998, Tomiuk and colleagues cloned a murine and human Mg^{2+}-dependent N-SMase by pursuing distant homology with bacterial SMases. This protein, nSMase1 (also known as smpd2), had an ORF encoding 419 residues (mouse, 47.5 kDa) and 423 residues (human, 47.6 kDa), respectively, with two putative transmembrane domains at the C-terminus (*9*). Site-directed mutagenesis indicated that two histidine residues, His-136 and His-272 (Figure 1), were essential for catalysis (*54*). Furthermore, nSMase1 activity required reducing agents, and later studies have shown that it is reversibly inhibited by reactive oxygen species and oxidized glutathione whereas it is irreversibly inhibited by peroxynitrite (*54*, *55*). Several groups have established the localization of nSMase1 to the ER (*55*−*57*). However, in hepatoma cells, endogenous rat nSMase1 and overexpressed rat nSMase1 localize to the nuclei and the ER, respectively, suggesting different localization may occur on overexpression (*58*).

Cell Signaling and nSMase1. A number of studies have focused on potential signaling roles of nSMase1. However, whereas nSMase1 exhibits in vitro SMase activity, cells overexpressing the protein did not show changes in SM metabolism, and it was shown that nSMase1 acted in vivo as a lyso-PAF phospholipase C, with lyso-PAF acting as a preferred substrate in vitro (*8*). Consistent with this, stimulation of cells overexpressing nSMase1 with TNF or H_2O_2 (*9*) did not elevate ceramide levels or induce apoptosis. In Jurkat T-cells, the overexpression of catalytically inactive nSMase1 had no effect on CD95-induced ceramide production (*59*). In contrast, it was shown that nSMase1 antisense treatment of T-cell hybridomas inhibited ceramide production in response to T-cell receptor engagement (*60*), although the specificity and effect of the antisense on nSMase1 activity were not evaluated. Importantly, nSMase1 knockout mice showed no lipid storage disease or perturbation in SM metabolism (*61*). Thus, its physiologic roles remain to be determined. Taken together, these results argue against an in vivo role of nSMase1 as an N-SMase. However, the absence of obvious defects in sphingolipid metabolism in nSMase1 overexpression studies and knockout/mutant mice do not rule out more subtle changes.

Neutral Sphingomyelinase 2 (nSMase2)

Two years after the cloning of nSMase1, the cloning of another mammalian brain-specific, Mg^{2+}-dependent N-SMase, nSMase2 (smdp3), was reported (*10*). Both human and murine ORFs encode proteins of 655 amino acids, resulting in a predicted molecular mass of 71 kDa with two putative transmembrane domains found in the N-terminus, whereas the putative catalytic domain is within the C-terminal region of the protein (*10*). Overexpression of nSMase2 in ΔISC1 yeast cells found that nSMase2 used SM preferentially as a substrate in vitro with no activity against lyso-PAF (*8*, *62*) and was inhibited by the specific N-SMase inhibitor GW4869 (*63*). Furthermore, nSMase2 is activated by PS and other anionic phospholipids, especially CL and phosphatidylglycerol, suggesting the presence of an anionic phospholipid-selective binding domain, similar to Isc1p. This may also have implications for cellular activation of the enzyme as PS is highly enriched in the inner leaflet of the PM whereas large amounts of CL are found in mitochondria.

Studies in MCF7 cells confirmed a role for nSMase2 in SM metabolism as cells overexpressing nSMase2 exhibited clearly lower SM and higher ceramide levels than corresponding vector-transfected cells (*62*). Further analysis of ceramide composition in nSMase2-overexpressing MCF7 cells showed that the enzyme primarily increased levels of very long chain ceramides, which correlated with a decrease in the level of $C_{24:0}$- and $C_{24:1}$-SM species. Importantly, downregulation of nSMase2 using siRNA prevented confluence-induced increases in levels of C24:0 and C24:1 ceramides (*64*). Together, these results confirmed that nSMase2 is an in vitro and in vivo N-SMase and suggest that nSMase2 may have substrate specificity for very long chain SM or may have access to only a specific subset of SM as a substrate.

Localization. The localization of nSMase2 was first described by Stoffel and co-workers, who reported that endogenous and overexpressed nSMase2 colocalized with a Golgi marker in several cell lines (*10*, *65*). Further study in our laboratory showed that nSMase2 in subconfluent cells was primarily intracellular but was found at the PM at regions of cell−cell contact (*64*). Furthermore, at high cell densities, nSMase2 was predominantly detected at the PM, suggesting that confluence can regulate localization of the enzyme (*64*), and such localization at both intracellular and PM sites was supported by other work (*66*). Additionally, a recent report showed that oxidative stress can also regulate nSMase2

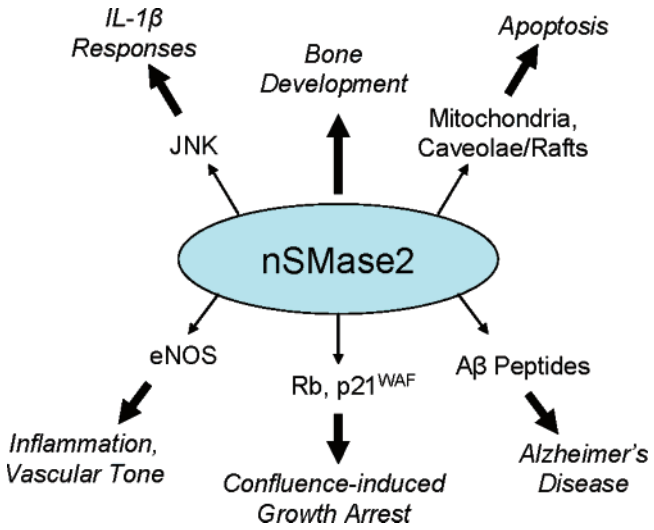

FIGURE 4: Signaling roles of nSMase2. By use of nSMase2 siRNA, nSMase2 knockout mice, and GW4869, a specific inhibitor, a number of downstream targets and physiological roles of nSMase2 are beginning to emerge.

localization. In this study, Levy et al. showed that H_2O_2 induced nSMase2 trafficking to the PM whereas glutathione caused translocation to the perinuclear region (*67*). Taken together, these results suggest that nSMase2 localization is a dynamic process and may be subject to complex regulatory mechanisms.

The C-terminal region of nSMase2 harbors several motifs that may play a role in its localization. For example, the tyrosine-containing sequences $_{529}$FTHY$_{532}$ and $_{581}$YLAF$_{584}$ fit the consensus sequence YXXΦ (where X is any amino acid and Φ is an amino acid with a bulky hydrophobic side chain) that serves as an internalization signal from the PM (*68*). Also of note is the acidic cluster $_{558}$YDED$_{561}$ separated by five amino acids from a dileucine motif, shown to play a role in the endosomal sequestration of several proteins such as the proprotein convertase PC6B, and GLUT-4 (*69*). Further studies will address the role of these motifs in nSMase2 localization.

Cell Signaling and nSMase2. N-SMase activity has been implicated in apoptosis, inflammation, cell growth, and differentiation (reviewed in ref *70*), but very few studies have addressed the specific N-SMase that is involved. We now discuss a few emerging roles for nSMase2 specifically (Figure 4).

Inflammation and nSMase2. Evidence of a role for nSMase2 in cellular responses to the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is beginning to accrue. Previous research showed that nSMase2 is activated by TNF-α in MCF-7 cells (*64*), and a recent study in HUVEC cells reported that TNF-α activates endothelial nitric oxide synthase through sequential activation of nSMase2 and sphingosine kinase 1, established using siRNA directed against nSMase2 (*71*). The biological relevance of this pathway is evident given the established role of nitric oxide in regulating endothelial physiology and inflammatory processes.

Apoptosis and nSMase2. There is also evidence of a role for nSMase2 in apoptosis. Previous research has reported that the nSMase2 inhibitor GW4869 can attenuate early signs of TNF-stimulated apoptosis in MCF-7 cells such as release of cytochrome *c* from mitochondria (*63*). Also, nSMase2 overexpression in oligodendroma-derived cells increased levels of ceramide in the raft fraction and subsequently increased the level of cell death induced by staurosporine or C2-ceramide (*72*), suggesting that translocation of nSMase2 to caveolae and/or rafts may be important for apoptotic responses. Finally, recent research showed that downregulation of nSMase2 by siRNA completely blocked H_2O_2-induced apoptosis of human aortic endothelial cells. Moreover, this study also showed that GSH specifically inhibits nSMase2 activity, thus identifying nSMase2 as a major candidate for oxidative stress-induced N-SMase activity (*67*). Additional studies (see below) have strongly implicated nSMase2 in the cytotoxic action of amyloid-β peptide.

Cell Growth and nSMase2. Studies in MCF7 cells have shown that nSMase2 is upregulated during cell growth. Using loss-of-function analysis, it was also demonstrated that nSMase2 activity is required for cells to undergo confluence-induced cell cycle arrest (*64*). This role is further supported by the identity of nSMase2 with confluent cell arrest-associated protein-1 (CCA-1), independently isolated from rat 3Y1 cells as a gene whose level of expression is increased upon confluency (*73*).

nSMase2 and JNK. Further insight into downstream effects of nSMase2 has come from studies in primary hepatocytes, where expression of nSMase2 was found to increase the rate of both basal and interleukin (IL)-1β-stimulated JNK phosphorylation in a manner dependent on the ceramide-activated protein phosphatase PP2A (*66*). Thus, nSMase2 may be an important regulator of JNK activation in IL-1β responses.

Alzheimer's Disease and nSMase2. Amyloid-β (Aβ) peptide, a cleavage product of amyloid precursor protein (*74*), is important in the pathogenesis of Alzheimer's disease (AD). Studies have reported that Aβ is toxic to neurons and oligodendrocytes, and more significantly, both pharmacologic and RNAi inhibitors of nSMase2 inhibited the cytotoxic effects of Aβ peptides (*75*−*79*). Additional research has shown that the nSMase2 inhibitor GW4869 decreased both secreted and intracellular Aβ peptide levels, suggesting that nSMase2 may also amplify responses through feedback regulation (*79*), possibly through an nSMase2-mediated decrease in SM levels and/or through production of ceramide, reported to enhance Aβ peptide production in CHO cells (*79*, *80*). Taken together, these studies offer evidence that nSMase2 could play an important role in the pathogenesis of AD and could be a useful therapeutic target in the future. These conclusions are further supported by other studies implicating N-SMase(s) in Aβ-stimulated expression of DP5, a pro-apoptotic protein from the Bcl-2 family, and inducible nitric oxide synthase, pro-apoptotic pathways both in oligodendrocytes (*81*, *82*) and in HIV1-induced apoptosis of neurons (*83*).

In Vivo Models and nSMase2. The recent generation of nSMase2 knockout mice has provided an undoubtedly important tool for future research (*65*). These mice were found to have no significant disruption of lipid storage as with nSMase1 knockout mice but unlike acid SMase knockout mice. Surprisingly, these mice developed growth retardation that remained throughout development and was most visibly manifested in the skeleton with the long bones found to have short stature and deformation (*65*).

Interestingly, in an independent study, Aubin et al. identified a deletion in the gene encoding nSMase2 in the *fragilitas ossium* or "fro" mouse, a model of a severe, often lethal, autosomal recessive form of osteogenesis imperfecta (*84*). The mutation removed the 33 C-terminal amino acids of nSMase2 and led to complete loss of enzymatic activity (*85*). Although it is tempting to speculate that this phenotype can be attributed solely to the lack of nSMase2, it is also possible that other unidentified defects may account for the osteogenesis imperfecta phenotype. However, as both these mutant mice and the nSMase2 knockout mice have related albeit clearly distinct phenotypes (*64*, *84*) and SMase, ceramide, and S1P are all reported inhibitory factors of bone resorption (*86*), this offers strong evidence that nSMase2 may play a role in bone formation and development, possibly through regulating cytokine actions on bone and cartilage.

CONCLUSIONS

Research into the N-SMase family of proteins is currently at a crucial juncture. The cloning of N-SMase proteins from bacteria, yeast, and mammalian cells and the development of knockout strains and animals now provide major tools for their study. We suggest that research should now be directed to exploring the specific mechanisms of regulation of these enzymes, further investigating their roles in a variety of physiological contexts, and to learning more about the proteins themselves. In this sense, care should be taken to consider possible common features across the family as, aside from a common catalytic mechanism, there appear to be some common structural features such as, for example, the presence of an anionic phospholipid-binding domain in Isc1 and nSMase2. We are hopeful, in obtaining a fuller physiological understanding of the extended N-SMase family, this will allow improved understanding how dysregulation of these enzymes or pathways occurs in pathological states and may open avenues of therapeutic treatment.

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