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Caenorhabditis elegans Contains Two Distinct Acid Sphingomyelinases*

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Mounting evidence supports a role for acid sphingomyelinase (ASM) in cellular stress signaling. Only murine and human sphingomyelinases have been defined at the molecular level. These enzymes are the products of a conserved gene and at the amino acid level share 82% identity. In this study, we show that the nematode *Caenorhabditis elegans* possesses two ASMs, termed ASM-1 and ASM-2 encoded by two distinct genes, but lacks detectable neutral sphingomyelinase activity. The *C. elegans* ASMs are about 30% identical with each other and with the human and murine enzymes. The conserved regions include a saposin-like domain, proline-rich domain, and a putative signal peptide. In addition, 16 cysteines distributed throughout the molecules, and selected glycosylation sites, are conserved. The expression of these genes in *C. elegans* is regulated during development. Asm-1 is preferentially expressed in the embryo, whereas asm-2 is predominantly expressed in postembryonic stages. When transfected as Flag-tagged proteins into COS-7 cells, ASM-1 is found almost entirely in a secreted form whereas only 20% of ASM-2 is secreted. Only the secreted forms display enzymatic activity. Furthermore, ASM-2 requires addition of Zn^{2+} to be fully active, whereas ASM-1 is active in the absence of cation. *C. elegans* is the first organism to display two ASMs. This finding suggests the existence of an ASM gene family.

The sphingomyelin pathway is an ubiquitous signaling system linking a specific set of cell surface receptors and environmental stresses through to the intracellular signaling apparatus (1). This pathway is initiated by hydrolysis of the phospholipid sphingomyelin (*N*-acylsphingosine-1-phosphocholine) which is preferentially concentrated in the plasma membrane of mammalian cells (2). Sphingomyelin hydrolysis occurs via the action of sphingomyelin specific forms of phospholipase C, termed sphingomyelinases (sphingomyelin phosphodiesterases; EC 3.1.4.12). Ceramide, generated by this reaction, acts as a second messenger and initiates cell type specific signaling.

Sphingomyelinases are categorized as alkaline, neutral, or acid according to their pH optima. Alkaline sphingomyelinase is found mostly in intestinal mucosa and bile (3–5). Alkaline sphingomyelinase possesses an optimum pH at 9 and is Zn^{2+}- and Mg^{2+}-independent. Alkaline sphingomyelinase may play a role in the digestion of dietary sphingomyelin. A neutral sphingomyelinase is found in many, if not all, tissues. The neutral sphingomyelinase is membrane-bound, Mg^{2+}-dependent, and displays a pH optimum of 7.4 (6). The gene encoding the mammalian neutral sphingomyelinase is yet to be identified. In addition to the membrane-bound form, a cytosolic cation-independent form of neutral sphingomyelinase has been described (7).

ASM,[1] possessing optimum pH between 4.5 and 5.5, is found in all mammalian cells (8). The human and murine ASMs are the products of a conserved gene and at the amino acid level share 82% identity. The hASM gene encodes a 629-amino acid glycoprotein with a calculated molecular mass of 64 kDa (9). Human ASM contains a signal sequence at the N terminus comprised of five leucine-alanine repeats. This signal peptide may present a hydrophobic core and play a role in targeting the ASM into the endoplasmic reticulum. There are six potential *N*-linked glycosylation sites (amino acids 86, 175, 335, 395, 503, and 520, respectively) in hASM. Site-directed mutagenesis studies show that five of these sites (amino acid 86, 175, 335, 395, and 520) are glycosylated in COS-1 cells (10). Elimination of any of these sites results in various degrees of decreased enzyme activity. A similar result is found in the murine ASM (11). An N-terminal region of the hASM (residues 89–165) is found to be highly homologous to the sphingolipid activator protein saposin and may possess lipid-binding and/or sphingomyelinase activator properties (12). Within this saposin-like domain are six cysteine residues (residues 89, 92, 120, 131, 157, and 165) that may participate in formation of intramolecular disulfide bridges. A proline-rich domain (amino acid 182–197) is located immediately after the second glycosylation site and may act as a flexible hinge region linking the activation domain (saposin-like domain) and the C-terminal putative enzymatic domain.

ASMs are found in cellular and secreted forms in mammalian cells (13). This appears to reflect post-translational processing of the primary gene product. The secreted form of ASM is Zn^{2+} stimulated. In contrast, the lysosomal form is not Zn^{2+} stimulable, which may result from acquisition of cellular Zn^{2+} during the lysosomal targeting process (13). Mammalian ASM is subject to substantial post-translational proteolysis. Sandhoff and co-workers (14) found that the sphingomyelinase gene product manifests an apparent molecular mass of 75 kDa in SDS-PAGE, and is subsequently processed to a 72-kDa form or alternately to a 57-kDa form. All three forms are found in COS-1 cell lysates. Tabas and co-workers (15) found that macrophages, fibroblasts, and endothelial cells secrete sub-

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[1] The abbreviations used are: ASM, acid sphingomyelinase; hASM, human ASM; PAGE, polyacrylamide gel electrophoresis; NPD, Niemann-Pick disease; PCR, polymerase chain reaction.

stantial amounts of the high molecular weight form of ASM. Secretion of this form is increased by cytokine stimulation, perhaps by increasing the flux of the ASM precursor through the Golgi-secretory pathway. There is emerging evidence suggesting that caveolae, putative plasma membrane microdomains, may be important sites for ligand-induced sphingomyelin hydrolysis via ASM (16).

ASM has been shown to be involved in cytokine and cellular stress signaling cascades. A number of studies showed that CD95 and the 55-kDa tumor necrosis factor receptor activate ASM during induction of apoptosis, perhaps via a mechanism requiring the death domain of these receptors (17, 18). *In vitro*, lymphoblasts from patients with Niemann-Pick disease (NPD), a genetic deficiency of ASM, and *in vivo*, endothelium from ASM knock-out mice, fail to generate ceramide and undergo apoptosis upon radiation exposure (19). Furthermore, retroviral transfer of the hASM cDNA into NPD lymphoblasts restored ASM activity into the normal range, and conferred radiation-induced ceramide generation and apoptosis. Similarly, Debatin and co-workers (18) found that NPD fibroblasts were deficient in UV- and anthracycline-induced ceramide generation and apoptosis, and linked ceramide generation to transcriptional up-regulation of CD95 ligand (18). In this regard, Dong and co-workers (20) found that ASM activity is essential for UV-induced c-Jun kinase activation, which may be involved in transcriptional regulation of the apoptotic response. It should be noted, however, that ceramide can also signal apoptosis in a transcriptionally independent fashion since ceramide signals mitochondrial permeability transition, reactive oxygen species generation and release of apoptosis initiating factor in cytoplasts from murine 2B4.11 T cells (21). This latter event is inhibited by anti-apoptotic Bcl-2 family members. Whether the secreted form of ASM may play a role in stress signaling is presently uncertain (22).

The nematode *Caenorhabditis elegans* is an attractive model for studying signal transduction. *C. elegans* and mammalian cells share numerous genes encoding proteins involved in conserved signal transduction pathways. Some of these pathways, such as the those involved in vulval development or programmed cell death, are well defined in *C. elegans*. However, the existence of a sphingomyelinase signaling system has not yet been demonstrated. In fact, no gene or biochemical activity corresponding to a *C. elegans* sphingomyelinase has been described. In this study, we show that *C. elegans* possesses two distinct, evolutionarily related, ASMs.

EXPERIMENTAL PROCEDURES

Materials

DNA restriction and modifying enzymes were purchased from Boehringer Mannheim (Indianapolis, IN). AmpliTaq DNA polymerase and rTth DNA polymerase, XL were from Perkin-Elmer (Branchburg, NJ). Genomyx Δ-Genozyme Cycle Sequencing Kit was from Genomyx (Foster City, CA). Random Primers DNA Labeling System was from Life Technologies, Inc. (Gaitherburg, MD).

Methods

Growing and Handling C. elegans—Bristol (N2) *C. elegans* was used throughout these studies and maintained as described previously (23). Briefly, worms were grown at 22 °C in 60-mm plates containing 3 ml of nematode growth medium agar carrying a lawn of OP50, a leaky uracil-requiring strain of *Escherichia coli*. Worms were harvested by washing them off the plates with ice-cold 0.1 M NaCl. Dead worms were separated by centrifugation in 30% (w/w) sucrose at 600 × *g* for 5 min. Live worms were resuspended in 0.1 M NaCl at 22 °C for 30 min to allow the complete digestion of bacteria remaining in the nematode guts and then washed three times with 0.1 M NaCl. Generally, 60 mg of worms can be recovered from a 60-mm plate.

Cell Culture—COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, Inc., Gaitherburg, MD) at 37 °C in a 5% CO_2 atmosphere.

Sphingomyelinase Assay—To measure ASM activity, cells or worms were suspended in 0.2% Triton X-100 and disrupted at 4 °C using a hand held homogenizer (Kontes, Vineland, NJ) for 2 min. Cells debris was removed by centrifugation at 600 × *g* for 5 min. Lysates were incubated in ASM assay buffer (250 mM sodium acetate, 0.1% Triton X-100, pH 5.0) containing various concentrations of [*N-methyl*-^{14}C]sphingomyelin (47 mCi/mmol, Amersham Life Science, Arlington Heights, IL) in a final volume of 60 μl at 37 °C for 1 h. Radioactive phosphorylcholine was extracted with 200 μl of chloroform:methanol (2:1, v/v) into the upper aqueous phase and analyzed by scintillation counting. To measure neutral sphingomyelinase activity, cells or worms were suspended in a buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA, 5 mM dithiothreitol, 0.1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 30 mM *p*-nitrophenyl phosphate, 10 mM β-glycerophosphate, 750 μM ATP, 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin, and 0.2% Triton X-100), and homogenized and centrifuged as above. Lysates were incubated in neutral sphingomyelinase assay buffer (20 mM HEPES, pH 7.4, 1 mM $MgCl_2$, 0.1% Triton X-100) containing various concentrations of [*N-methyl*-^{14}C]sphingomyelin, and sphingomyelinase activity was determined as described for ASM assay.

Molecular Cloning of C. elegans ASM Genes—By searching the *C. elegans* genomic sequence data base for homologs of the hASM gene, we identified two regions of genomic DNA that showed significant sequence similarity to hASM. These regions were contained within two predicted genes, B0252.2 (*asm-1*) and ZK455.4 (*asm-2*), defined by the *C. elegans* sequencing project at Washington University. The cDNA of *asm-1* and *asm-2* were cloned by reverse transcription-PCR with primer pairs flanking the predicted start and stop codons. Briefly, total RNA from mixed stages of N2 *C. elegans* was purified using RNAzol (Biotecx, Houston, TX) according to the manufacturer's protocol. First strand cDNA was synthesized by reverse transcriptase using oligo(dT) as primer (32) and then used as template in PCR using primers flanking the predicted start and stop codon (*asm-1*, GAAACTCTGGAAATGAGGATAA and TGTACAAGGTCTTTGAAATGAT; and *asm-2*, TGAAAATACATCTGAGAA and ATGCAGCAGCCTCTCATTATT). The PCR products were fractionated in agarose gels, excised, and extracted using the QIAEX GEL Extraction Kit (QIAGEN, Chatsworth, CA). The resulting DNA was cloned into pCRII using the TA cloning system from Invitrogen (San Diego, CA). The DNA inserts were sequenced using SP6 and T7 primers (24).

Construction of Mammalian Expression Vectors of asm-1 and asm-2—The Flag epitope tag was introduced at the C terminus of *asm-1* cDNA by PCR using T7 primer as the 5′ primer and CGGGATCCTACTTGTCATCGTCGTCCTTGTAGTCAAAAGTTGGACAAAG as the 3′ primer. The PCR products generated from pCRII/*asm-1* were digested with *Bam*HI and *Not*I, and the resulting fragment was subcloned into the mammalian expression vector pCEP4 (Invitrogen, San Diego, CA). For constructing the *asm-2* expression vector, 5′ primer (GGGGTACCGCCACCATGCAGCAGCCTCTCATTATT) containing the *asm-2* start codon and 3′ primer (CCGCTCGAGTCTACTTGTCATCGTCGTCCTTGTAGTCTTTTGTTAAATGATCA) with the Flag sequence following the C terminus sequence of *asm-2* were used in PCR to generate the Flag-tagged *asm-2*. The Flag-*asm-2* sequence was subcloned into pCEP4 using *Xho*I and *Kpn*I sites.

Expression of ASM Genes in C. elegans—Mixed stages worms and eggs, prepared by the method of Sulston and Hodgkin (25), were used for isolation of total RNA. For Northern blot analysis, 30 μg of RNA was fractionated on a 1.0% agarose gel in the presence of formaldehyde. The gel was stained with ethidium bromide to determine the adequacy of RNA loading. The RNA was transferred to a BA-85 nitrocellulose membrane (Schleicher and Schuell, Keene, NH) with 10 × SSC. Hybridization was carried out using ExpressHyb from CLONTECH Laboratories (Pine Alto, CA) with [^{32}P]dCTP-labeled full-length *asm-1* and *asm-2* cDNA as probes. The membrane then was exposed to x-ray film at −80 °C. Molecular weights of the asm transcripts were determined by comparison to RNA molecular weight markers (Life Technologies, Inc., Gaithersburg, MD).

Expression of C. elegans ASMs in COS-7 Cells—COS-7 cells were transfected using LipofectAMINE (Life Technologies, Inc.) according the manufacturer's protocol. At 48 h post-transfection, cell culture medium was centrifuged at 16,000 × *g* for 10 min, and the secreted Flag-tagged ASM was immunoprecipitated. The cells were lysed in RIPA buffer (25 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 0.1% SDS, and 0.1% deoxycholate) containing the protease inhibitor mixture tablet Complete (Boehringer Mannheim, Indianapolis, IN). The homogenate was spun at 16,000 × *g* for 10 min and the supernatant was collected for immunoprecipitation. Flag-tagged ASM-1 and ASM-2 proteins were immunoprecipitated from the

medium or cell lysate using the anti-Flag M2 affinity gel (Scientific Imaging System, New Haven, CT) at 4 °C for 2 h. The beads were washed six times with Nonidet P-40 buffer (25 mM Tris, pH 7.5, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 2 mM EDTA) containing the protease inhibitor mixture tablet. Beads then were resuspended in sphingomyelinase assay buffer to measure acid or neutral sphingomyelinase activities. To estimate the protein content in the enzymatic reaction, the immunoprecipitate was fractionated on SDS-PAGE, transferred to nitrocellulose membrane, and stained with colloidal silver. Then the protein content was estimated by comparison to known quantities of bovine serum albumin standard.

RESULTS

C. elegans Contains Acid but Not Neutral Sphingomyelinase—Prior studies demonstrated that sphingomyelin accounts for 8% of the total phospholipid in *C. elegans*, suggesting that enzymes capable of sphingomyelin hydrolysis might exist in these animals. Table I shows, using an assay optimized for the mammalian enzyme, that mixed stages *C. elegans* possesses ASM activity. The specific activity of the *C. elegans* ASM was similar to that of hASM found in HL-60 cells. This ASM activity is from *C. elegans* and not due to contamination with *E. coli*, since *E. coli* does not manifest any sphingomyelinase activity under our experimental conditions (data not shown). Furthermore, Zn^{2+} (0–10 mM) did not significantly increase ASM activity in lysates of N2 mixed stages worms (data not shown). In contrast, *C. elegans* did not display neutral sphingomyelinase activity in lysates (Table I) or in isolated microsomal membrane whether or not ATP, Mg^{2+}, or various protease or phosphatase inhibitors were included in the reaction buffer (data not shown).

TABLE I

C. elegans possesses acid but not neutral sphingomyelinase activity

Lysates were prepared from mixed stages *C. elegans* (N2) and HL-60 cells, and sphingomyelinase activity was determined at pH 5.0 and pH 7.4 as described under "Experimental Procedures."

Enzyme source	Sphingomyelinase activity	
	pH 5.0	pH 7.4
	nmol/mg/h	
C. elegans	11.7 ± 1.0	0
HL-60	5.7 ± 0.1	1.1 ± 0.2

Cloning and Sequence Analysis of Sphingomyelinase Genes in C. elegans—We found two regions in the *C. elegans* genomic DNA containing deduced open reading frames with significant identity to the human and murine ASM genes. These regions, found in cosmid B0252.2 located on chromosome II and in cosmid ZK455.4 located on chromosome X, were designated as *asm-1* and *asm-2*, respectively. The cDNAs of *asm-1* and *asm-2*

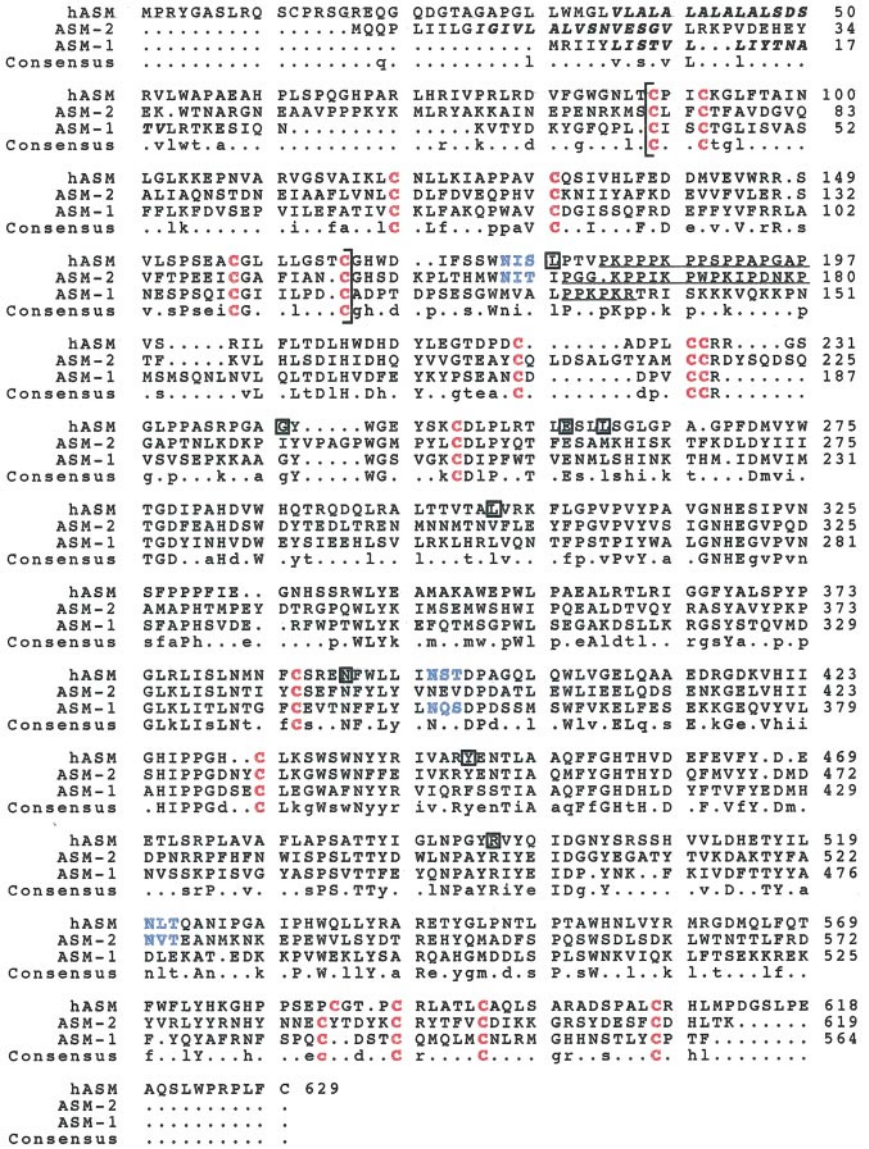

FIG. 1. **Comparison of *C. elegans* and human ASMs.** The derived amino acid sequences of ASM-1 and ASM-2 are aligned with the sequence of hASM by the Clustal W. program (30). Identical amino acids are designated in *uppercase*, whereas those conserved between two sphingomyelinases are designated in *lowercase*. Conserved cysteine residues are shown in *red*. Putative N-terminal signal peptide sequences are in *bold italic*. The saposin-like domains are *bracketed*. Potential proline-rich domains are *underlined*. The conserved glycosylation sites are shown in *light blue*. The *boxed* amino acid residues in the hASM are mutated in NPD and conserved in one or both *C. elegans* ASMs (31).

were cloned by reverse transcription-PCR as described under "Experimental Procedures."

The *asm-1* and *asm-2* cDNAs contain single large open reading frames which encode polypeptides of 564 and 619 amino acids, respectively (Fig. 1). Several lines of evidence suggest that these two coding sequences are complete. 1) Northern blot analysis using *asm-1* or *asm-2* cDNA as probes identified both 1.8 and 2.0 kilobase mRNAs, respectively, which correspond to the lengths of the cloned cDNAs (see below, Fig. 2); 2) Western blot analysis using a polyclonal antibody generated in rabbits against full-length hASM, shows that the endogenous ASM-1 and ASM-2 in *C. elegans* exhibit the same size as their recombinant counterpart expressed in COS-7 cells (data not shown); 3) reverse transcription-PCR of the *asm-1* mRNA indicated that the 5′ end of this transcript is trans-spliced to a SL1 leader sequence 12 bases upstream of the putative start codon (data not shown). Although *asm-2* RNA was not trans-spliced to SL1 or SL2, there was no other potential start codon in the genomic sequence upstream of the predicted initiating methionine (data not shown). Comparison of the cDNA and genomic sequences revealed that both *asm-1* and *asm-2* consist of 11 exons.

The proteins encoded by *asm-1* and *asm-2* are 32% identical with each other. Furthermore, ASM-1 and ASM-2 share 30 and 33% identity with hASM, respectively. ASM-1 and ASM-2 share several functional domains with hASM. As in the hASM, there is a putative secretory signal peptide at the N terminus of

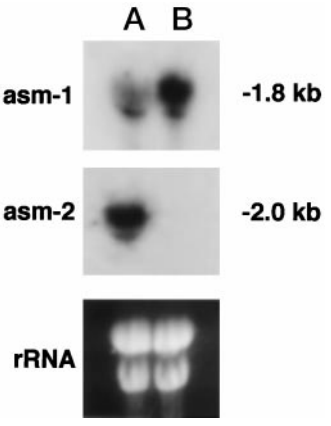

FIG. 2. **Sphingomyelinase gene expression in *C. elegans*.** Thirty μg of total *C. elegans* RNA from mixed stages (*A*) and embryos (*B*) was electrophoresed in 1.0% agarose gel and transferred to nitrocellulose membrane. For Northern analysis, RNA was probed with the indicated cDNA probes and subjected to autoradiography. Data represent one of two experiments.

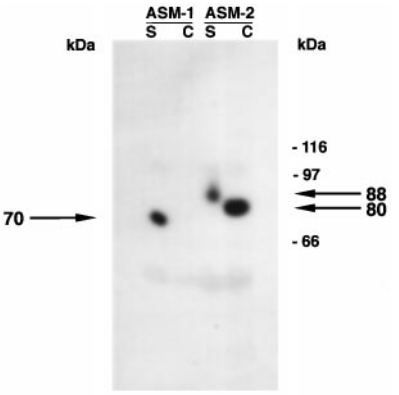

FIG. 3. ***C. elegans* ASMs are expressed in both secreted and cellular forms in COS-7 cells.** COS-7 cells were transfected with pCEP4/*asm-1* or pCEP4/*asm-2*, and after 48 h were homogenized in buffer as described under "Experimental Procedures." ASM, quantitatively immunoprecipitated from cell lysate (*C*) and culture medium (*S*) using an agarose-conjugated anti-Flag antibody, were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Flag antibody as described under "Experimental Procedures." Data represent one of five experiments.

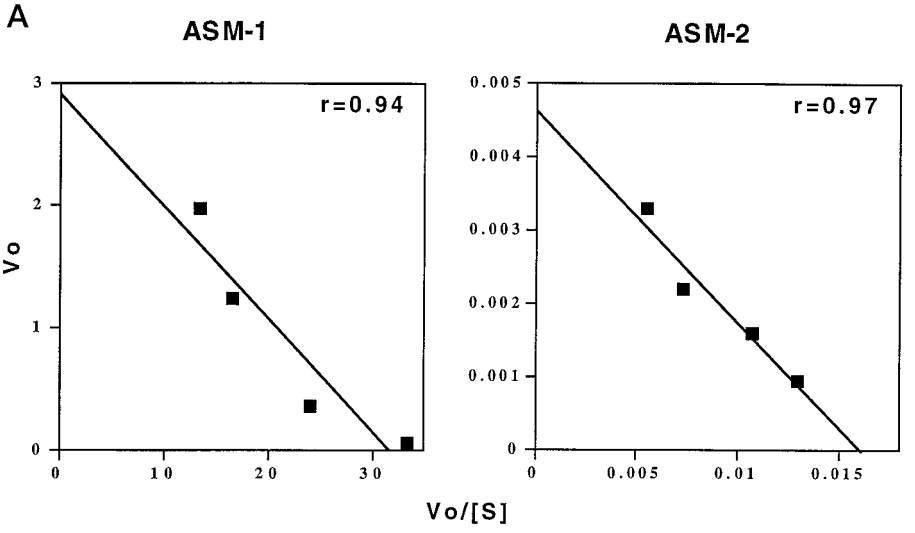

FIG. 4. **The putative *C. elegans* sphingomyelinase gene products possess ASM activity when expressed in COS-7 cells.** COS-7 cells were transfected with pCEP4/*asm-1* or pCEP4/*asm-2* and 48 h post-transfection sphingomyelinase gene products were immunoprecipitated from medium as in Fig. 3. The immunoprecipitate was washed 6 times with Nonidet P-40 buffer containing 2 mM EDTA. Sphingomyelinase activity was determined at pH 5.0 in the presence of 2.0 mM $ZnCl_2$ as described under "Experimental Procedures." Data are presented using the Eadee-Hofstee transformation. The units of velocity (V_o) are millimole/mg of protein/h, and substrate concentration (*S*) is millimolar. These data represent one of two similar experiments.

B

	Vmax (mmol/ mg/h)	Km (μM)
ASM-1	2.9	92
ASM-2	4.8×10^{-3}	288

TABLE II
ASM-1 and ASM-2 are acid sphingomyelinases

COS-7 cells were transfected with pCEP4/ASM-1 or pCEP4/ASM-2. At 48 h post-transfection, Flag-ASM-1 and Flag-ASM-2 were immunoprecipitated from cell culture medium with anti-Flag-agarose and washed 6 times with Nonidet P-40 buffer containing 2 mM EDTA. Sphingomyelinase activity was determined at various of pH in the presence of 2.0 mM $ZnCl^{2+}$ as described under "Experimental Procedures."

	pH 5.0		pH 7.4		pH 9.0	
	V_{max}[a]	K_m[b]	V_{max}	K_m	V_{max}	K_m
ASM-1	2.9	92	0	NA[c]	0	NA
ASM-2	4.8×10^{-3}	288	0	NA	0	NA

[a] V_{max} units are mmol/mg/h.
[b] K_m units are μM.
[c] NA, not applicable.

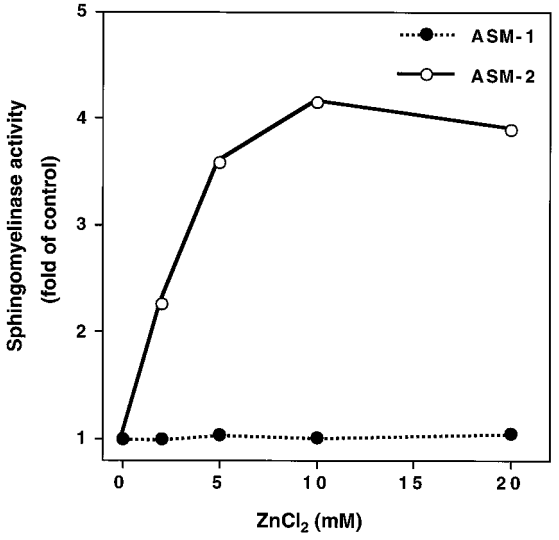

FIG. 5. **Effect of Zn^{2+} on *C. elegans* ASM activity.** ASMs were immunoprecipitated as in Fig. 4 from COS-7 cells transfected with pCEP4/*asm-1* (*closed symbols*) or pCEP4/*asm-2* (*open symbols*). Sphingomyelinase activity was determined with varying concentrations of $ZnCl_2$ as described under "Experimental Procedures." Data are representative of two experiments.

both ASM-1 and ASM-2 (Fig. 1, *italicized*). Furthermore, 16 cysteine residues dispersed throughout the protein sequence are highly conserved between the hASM, ASM-1, and ASM-2 (in *red*). Similar to the hASM, ASM-1 and ASM-2 possess a saposin-like domain (*bracketed*) in the N terminus followed by a proline-rich domain (*underlined*; truncated in ASM-1). Seven putative *N*-linked glycosylation sites are found in ASM-1, whereas five are found in ASM-2. Among these N-linked glycosylation sites, site 351 in ASM-1 and sites 159 and 523 in ASM-2 are conserved with respect to the human sequence.

Developmental Expression of asm-1 and asm-2—Although the human and murine ASM genes have been cloned, their developmental pattern remains largely unknown. To study ASM gene expression in *C. elegans*, we isolated total RNA from wild type (either embryos or mixed stages) animals and performed Northern blot analysis using *asm-1* or *asm-2* cDNA as probe. The *asm-1* probe detected a 1.8-kilobase transcript that was expressed at a higher level in embryos than in the mixed stages samples (Fig. 2, *top*). In contrast, the 2.0-kilobase *asm-2* transcript appears to be preferentially expressed during post-embryonic development, as it was not detectable in embryos (Fig. 2, *middle*). Smaller bands, observed below the primary *asm-1* and *asm-2* bands, may represent alternative spliced transcripts.

ASM-1 and ASM-2 Are Both Secretory Enzymes—In order to

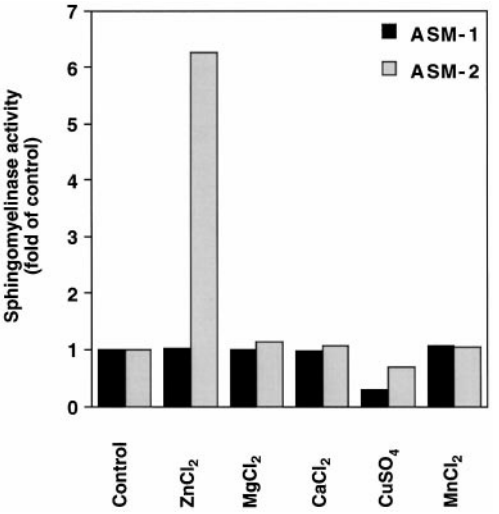

FIG. 6. **Effects of divalent cations on *C. elegans* ASM activity.** ASMs from COS-7 cells, transfected with pCEP4/*asm-1* (*solid bars*) or pCEP4/*asm-2* (*stippled bars*), were immunoprecipitated as in Fig. 4. Sphingomyelinase activity was determined in the presence of 20.0 mM of cations as described under "Experimental Procedures." Data are representative of two experiments.

study whether the gene products of *asm-1* and *asm-2* exhibit sphingomyelinase activity, these cDNAs were cloned into the mammalian expression vector pCEP4 and overexpressed as Flag-tagged proteins in COS-7 cells. Mammalian ASM can be detected as intracellular and secreted forms (13, 14). We therefore checked whether ASM-1 or ASM-2 might also be secreted. Flag-ASM-1 was found exclusively in the cell culture medium by Western blot analysis (Fig. 3). In contrast, only one-fifth of the Flag-ASM-2 was secreted. Similar to the mammalian enzymes, the form of Flag-ASM-2 found in the cell culture medium exhibited higher apparent molecular weight than its intracellular counterpart. Furthermore, the apparent molecular weights in SDS-PAGE of both Flag-ASM-1 and Flag-ASM-2 are higher than their calculated molecular weights, consistent with post-translational modification such as glycosylation.

The overexpressed recombinant proteins from cell culture medium were immunoprecipitated and the enzymatic activities assayed under various conditions. Eadee-Hofstee transformation of kinetic data shows that at pH 5.0, both ASM-1 and ASM-2 hydrolyzed sphingomyelin to ceramide (Fig. 4). However, sphingomyelinase activity was not found at pH 7.4 or pH 9.0, indicating these two enzymes are ASMs, as anticipated from their primary sequence similarity to hASM (Table II). Whereas the K_m of Flag-ASM-2 is 3-fold higher than that of Flag-ASM-1, the V_{max} of Flag-ASM-1 is 3 orders of magnitude higher than that of Flag-ASM-2 (Table II). It should be noted that these kinetic data were obtained using 2.0 mM $ZnCl_2$. In the presence of 10.0 mM $ZnCl_2$ the V_{max} of ASM-2 is approximately 60% higher.

In contrast, intracellular Flag-ASM-2 did not manifest enzymatic activity, whether in the presence or absence of detergents (Triton X-100, Nonidet P-40, or SDS). To exclude the possibility of a selective inhibitory effect of the sodium acetate buffer used in the assay on the intracellular form of ASM-2, we repeated the assay in a sodium citrate buffer which also did not yield any activity (data not shown). Furthermore, neither ATP, Zn^{2+}, Mg^{2+}, nor Ca^{2+} conferred ASM activity onto the intracellular form of ASM-2. To test whether COS-7 cells contain an inhibitor of ASM-2, we added a lysate of COS-7 cells to the assay containing immunoprecipitated secreted Flag-ASM-2. Whether or not 10 mM Zn^{2+} was included in the buffer, the lysate failed

to inhibit the activity of secreted Flag-ASM-2 (data not shown). In fact, the lysate minimally enhanced ASM-2 activity. Thus, the inability to detect intracellular ASM-2 activity does not result from an endogenous inhibitor in COS-7 cells. These studies suggest that either the intracellular form of ASM-2 is inactive or a necessary co-factor specific for *C. elegans* ASM-2 is absent. Furthermore, at all Zn^{2+} concentrations (0–10 mM), embryo lysates manifested 1.78 ± 0.12 (mean ± S.E.)-fold higher ASM activity than mixed stages lysates, consistent with the apparent specific activities and developmental expression of *asm-1* and *asm-2*.

Metal Ion Requirement—Previous investigations showed that the secreted form of hASM was quite sensitive to EDTA due to Zn^{2+} depletion (26). Characterization of ASM-1 and ASM-2 revealed that Zn^{2+} increased the ASM-2 activity in a dose-dependent manner, but had no effect on ASM-1 (Fig. 5). The effects of four other cations on ASM-1 and ASM-2 activity were tested, none of which showed stimulatory effects (Fig. 6).

DISCUSSION

In this study, we have shown that *C. elegans* possesses two distinct ASMs, encoded by two separate but homologous genes. These enzymes are about 30% identical with each other and with the human and murine enzymes. The expression of these two genes in *C. elegans* is regulated during development. *asm-1* is preferentially expressed in embryos whereas *asm-2* is preferentially expressed during post-embryonic development. This suggests that in addition to any housekeeping function which might be involved in lipid metabolism, these sphingomyelinases may play distinct roles in signaling. *C. elegans* is the first organism to display two ASMs.

Despite extensive investigation into the role of ASM in signaling and NPD, little is known about the relationship between the structure and function of this enzyme. Human and murine ASM share 82% identity, which limits the use of an alignment analysis to define functional domains. Alternately, phospholipases C from in *Staphylococcus*, *Clostridium perfringens*, and *Bacillus cereus,* which exhibit some activity toward sphingomyelin (27–29), share no homology with their mammalian counterparts. The presence of conserved signal peptides, cysteine residues, proline-rich regions, and *N*-linked glycosylation sites between mammalian and nematode enzymes suggest these regions are critical for these enzymes to function as ASMs.

Tabas and co-workers reported that ASM is secreted from various cell types and that secretion is regulated by cytokine stimulation (15). Our data are consistent with their findings, as Flag-ASM-1 and Flag-ASM-2 are both secreted from COS-7 cells. Furthermore, Flag-ASM-2, like the secreted mammalian enzyme, appears to be activated by Zn^{2+}. In contrast, Flag-ASM-1 is fully active upon secretion independent of Zn^{2+} suggesting that activation of Flag-ASM-1 occurs by an alternate mechanism.

Prior to the present investigation, there existed only one ASM gene conserved between mouse and man. The present investigations add two new ASMs, suggesting the possible existence of a gene family. By comparing the action of the two *C. elegans* sphingomyelinases to their mammalian counterpart, it may be possible to delineate important information concerning the relationship of structure to function, as well as mechanisms regulating ASM activity. Finally, *C. elegans* should provide a powerful genetic model to study the role of ASM in signal transduction.

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