Molecular Cloning, Characterization, and Expression of a Novel Human Neutral Sphingomyelinase*

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Neutral sphingomyelinase (N-SMase) has emerged as an important cell membrane-associated enzyme that participates in several signal transduction and cell regulatory phenomena. Using expression cloning, we have identified a 3.7-kilobase pair cDNA transcript for N-SMase whose open reading frame predicts a 397-amino acid polypeptide. Transfection of COS-7 cells with cDNA for N-SMase resulted in a marked increase in N-SMase activity. Recombinant N-SMase (r-N-SMase) had the following physical-chemical properties. Mg\(^{2+}\) activated and Cu\(^{2+}\) inhibited the activity of r-N-SMase. In contrast, diethiothreitol did not alter the activity of the enzyme. Of several phospholipids examined, sphingomyelin was the preferred substrate for r-N-SMase. The apparent molecular mass of r-N-SMase derived from COS-7 cells was \(\sim 90\) kDa, similar to the native neutral sphingomyelinase prepared from human urine. However, upon expression in Escherichia coli, the apparent molecular mass of the recombinant enzyme was \(\sim 45\) kDa. We speculate that this apparent difference in recombinant enzymes derived from COS-7 and E. coli cells may be due to extensive post-transcriptional changes. r-N-SMase has numerous post-transcriptional modification sites such as phosphorylation sites via protein kinase C, casein kinase II, tyrosine kinase, and cAMP- and cGMP-dependent protein kinases as well as sites for glycosylation and myristoylation. Amino acid sequence alignment studies revealed that r-N-SMase has some similarity to acid sphingomyelinase and significant homology to the death domains of tumor necrosis factor-α receptor-1 and Fas/Apo-I. We believe that the molecular cloning and characterization of N-SMase cDNA will accelerate the process to define its role as a key regulator in apoptosis, lipid and lipoprotein metabolism, and other cell regulatory pathways.

Type C sphingomyelinases (sphingomyelin phosphodiesterase, EC 3.1.4.12) are a group of phospholipases that catalyze the hydrolytic cleavage of sphingomyelin to ceramide and phosphocholine (1). Neutral sphingomyelinase from human urine and cultured human kidney proximal tubular cell membranes has an apparent molecular mass of \(92\) kDa and neutral pH optima and is heat-unstable. This enzyme is associated with the cell membrane in tissues and cultured cells (1–4).

In cultured mammalian cells, the addition of diverse agonists, i.e. vitamin D\(_3\), tumor necrosis factor-α (TNF-α), interferon-γ, and nerve growth factor, results in the activation of N-SMase and the consequent production of ceramide. Ceramide and its higher homologs have been shown to serve as lipid second messengers that lead to diverse cell regulatory phenomena, such as differentiation, proliferation, and programmed cell death or apoptosis (1, 4–6). Activation of N-SMase by TNF-α in human skin fibroblasts results in not only the hydrolytic cleavage of sphingomyelin, residing on the cell surface, but also the mobilization of cholesterol to the interior of the cell. Such cholesterol is esterified by the action of fatty-acyl-coenzyme A acyltransferase to form cholesteryl esters (1, 7). In a human hepatocyte cell line, TNF-α-induced N-SMase activation and ceramide production led to the maturation of sterol regulatory element-binding protein-1 and a subsequent increase in LDL receptor mRNA expression (8). Interestingly, this phenomenon is not accompanied by apoptosis and occurs in a sterol-independent fashion. Thus, LDL receptors may be up-regulated via this cascade of reactions involving N-SMase independent of the presence of sterols in the culture medium. These studies suggest that N-SMase may be involved in the regulation of lipid and lipoprotein metabolism and sterol influx (7, 8). Rabbit skeletal muscle has been shown to contain at least two kinds of N-SMase. These are the classical 92-kDa Mg\(^{2+}\)-dependent N-SMase and a Mg\(^{2+}\)-independent 53-kDa protein. The localization of N-SMase in skeletal muscle transferase tubule membrane is in agreement with the production of the sphingomyelin-derived second messenger, sphingosine, in such tubules. Since sphingosine has been shown to modulate calcium release from sarcoplasmic reticulum membranes, these studies imply that N-SMase/sphingosine signaling systems may be a physiologically relevant mechanism of regulation of Ca\(^{2+}\) levels in skeletal muscle and may well be involved in muscle contraction (9). In additional studies, we have shown that Sindbis virus entry into cells triggers apoptosis by activating sphingomyelinase and the release of ceramide. Collectively, these studies imply that N-SMase may play a central role in diverse cell regulatory phenomena. Nevertheless, the evidence accumulated so far in support of this view has been largely indirect and has recently been the subject of rigorous debate (11), particularly in regard to the controversial role of ceramide in apoptosis (12).

We rationalized that to unambiguously demonstrate the functional role of N-SMase in the various cell regulatory phenomena discussed above, it was essential to first clone this protein. Therefore, we employed a monospecific polyclonal an-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF069740.

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1 The abbreviations used: TNF-α, tumor necrosis factor-α; TNF-α-R, TNF-α receptor; N-SMase, neutral sphingomyelinase; r-N-SMase, recombinant N-SMase; LDL, low density lipoprotein; PCR, polymerase chain reaction; GST, glutathione S-transferase; bp, base pair(s); kb, kilobase pair(s).

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tibody against human N-SMase and a human kidney cDNA library to pursue expression cloning. In this work, we describe the molecular cloning and characterization of recombinant N-SMase (r-N-SMase) expressed in *Escherichia coli* as well as in cultured COS-7 cells. In work to be published elsewhere, we will show that overexpression of N-SMase results in spontaneous apoptosis in human aortic smooth muscle cells independent of the presence of agonists.

**MATERIALS AND METHODS**

**Isotopes**—[α-^32P]dATP (specific activity of 0.25 Bq/μmol), [α-^32P]dCTP (specific activity of 9.25 Bq/μmol), and N-methyl[^14C]sphingomyelin (specific activity of 1.85 Bq/μmol) were purchased from Amersham Pharmacia Biotech.

**Cells**— *E. coli* strain 1090r was purchased from Life Technologies. Inc. COS-7 cells were purchased from American Type Culture Collection (Manassas, VA). These cells were grown in Eagle’s minimal essential medium containing 10% diazylated fetal bovine serum (HyClone Laboratories, Logan, Utah), penicillin, streptomycin, and nonessential amino acids.

The multiple tissue Northern blot and human kidney library were purchased from CLONTECH (Palo Alto, CA). Restriction endonucleases were purchased from Amersham Pharmacia Biotech. The transient expression vector pSV-SPOT-1, LipofectAMINE®343, and cell culture medium were purchased from Life Technologies, Inc.

**Construction of Sphingomyelinase Expression Cloning—**Bacterial cells and COS-7 cells transformed with N-SMase cDNA were homogenized in Tris/glycine buffer (pH 7.4) containing 0.1% cutscum. The samples were mixed vigorously and sonicated for 10 s. Next, the samples were transferred to a 4 °C incubator and shaken for 2 h. Every hour, the samples were sonicated again on ice and further shaken. Subsequently, the samples were centrifuged at 10,000 × g for 10 min. The supernatants were subjected to secondary and tertiary screening. All positive clones were subjected to subcloning into pBluescript II SK to prepare plasmid pHH1. A plasmid called pH1 was thus constructed.

To transfect COS-7 cells with pH1 and mock vector, we seeded 3 × 10⁶ COS-7 cells/plate in a p100 plate in 8 ml of Dulbecco’s modified Eagle’s medium with 10% diazylated fetal calf serum. Cells were incubated in a 10% CO₂, 37 °C incubator until they were 80% confluent. The cells were then transfected with 1.0 μg/ml purified pH1 using LipofectAMINE®. The medium was then replaced with fresh medium over night incubation. Finally, the cells were harvested at various time points (16, 24, 36, and 48 h post-transfection) by centrifugation at 1500 × g for 10 min, washed with phosphate-buffered saline, and stored frozen at −20 °C. r-N-SMase derived from COS-7 cells was purified as described previously (2) and was employed for detailed characterization studies with regard to pH optima, substrate specificity, requirement for metal ions for activation, and other previously described agonists/antagonists of N-SMase as well as immunoprecipitation with anti-N-SMase antibody and preimmune rabbit serum IgG.

**Northern Blot Assays**—We selected one set of sequence primers from pBC32-2 (T3-3/473R5) as the reverse transcription-PCR primer to carry out reverse transcription-PCR as described above. A 466-bp specific product was obtained and gel-purified; 50 μg of this product was labeled with 25 μCi of [α-^32P]dATP and 25 μCi of [α-^32P]dCTP using random hexamer primers (Life Technologies, Inc.). The specific activity of this probe was 1.88 × 10⁶ cpm/μg. Next, the multiple tissue Northern blot was prehybridized in 7 ml of hybridization-prehybridization solution at 42 °C for 4 h with continuous agitation. Then, 10 μg of fresh denatured salmon sperm DNA was added. Next, 10 ml of hybridization solution was added and agitated continuously at 50 °C. The blots were washed twice in 2× SSC and 0.1% SDS at room temperature for 30–40 min and then in 0.1× SSC and 0.05% SDS for 40 min at 50 °C. Finally, the blot was exposed overnight to a x-ray film at −70 °C using two intensifying screens. A plasmid containing a β-actin insert was used as a control in these experiments.

**RESULTS**

**Isolation of Human N-SMase cDNA**—Our strategy for the molecular cloning of cDNA for N-SMase involved the use of monospecific antibodies against human urinary N-SMase (2). This rationale stemmed from our previous findings: (i) in human leukemic cells (HL-60), TNF-α-induced apoptosis of N-SMase was abrogated by such antibodies; (ii) in HL-60 cells and aortic smooth muscle cells, TNF-α- and oxidized LDL-induced apoptosis, respectively, was abrogated by anti-N-SMase antibody; and (iii) both TNF-α-induced maturation of sterol regulatory element-binding protein-1 in human liver cells and TNF-α-induced cholesteryl ester synthesis in human skin fibroblasts were abrogated by preincubation of cells with such antibodies (7). We used this antibody to screen the human kidney Agt11 cDNA library. Sixty-three positive clones were obtained by screening 1 × 10⁶ clones. The most intense clones were subjected to secondary and tertiary screening. All positive clones were subjected to PCR to identify their insert size. Finally, a clone containing the longest insert (3.7 kb) called λ32-1 was subjected to subcloning into pBluescript II SK to prepare plasmid λ32-1 was ligated with a phosphorylated BamHI-BstHI linker containing 18 bp of N-terminal sequence (synthesized at the Core facility at The Johns Hopkins University) and a pGEX4T-1 vector double-digested with BamHI and EcoRI (Amersham Pharmacia Biotech).

To express and purify GST-N-SMase fusion protein, plasmid pJK2 was transformed into E. coli strain HB101/pJK2. A single colony of HB101/pJK2 was grown in 2 × yeast extract/tryptone agarose medium at 30 °C until appropriate cell density (A₆₀₀ = 1.5) was achieved. Isopropyl-β-D-thiogalactopyranoside (0.1 μl) was added to induce fusion protein expression for 2 h. Cells were harvested, and the fusion protein was purified using glutathione-Sepharose 4B chromatography according to instructions provided by the manufacturer (Amersham Pharmacia Biotech). The specific activity of 1.85 Bq/mmol was measured in each fraction.

**Expression Cloning of N-SMase in *E. coli* and Screening of cDNA Library**—The human kidney library was screened according to the manufacturer’s protocol. Briefly, Agt11 phage was plated at 3 × 10⁴ plaque-forming units/150-mm plate on a lawn of *E. coli* strain y1090r. Incubation was carried out at 42 °C for 3.5 h to allow lytic phage growth. Then, a filter was placed on a 5% nonfat dry milk plate for 1 h at room temperature. Subsequently, the filter was incubated with antibody against N-SMase at pH 7.4 for 1 h. The filter was then washed twice with 2× SSC and 0.1% SDS at room temperature for 30–40 min and then in 0.1× SSC and 0.05% SDS for 40 min at 50 °C. Finally, the filter was exposed overnight to a x-ray film at −70 °C using two intensifying screens. A plasmid containing a β-actin insert was used as a control in these experiments.

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mid pBC32-2. The insert was sequenced with Sequenase using T7 and T3 primers by automatic sequencing.

**Amino Acid Sequence of N-SMase**—The 3.7-kb nucleotide sequence of cDNA revealed an open reading frame size of 1197 base pairs, which predicts a 397-amino acid polypeptide. The deduced amino acid sequence of N-SMase is shown in Fig. 1. There are several potential modification sites in this protein. One N-glycosylation site at position 353, one tyrosine phosphorylation site at position 238, and two cAMP- and cGMP-dependent protein kinase phosphorylation sites are presumably located on the exterior. Such sites may be subject to further glycosylation and phosphorylation. The alignment of the death domain of neutral sphingomyelinase with the bacterial N-SMase (see below). The bacterial N-SMase had an activity of 3.9 mU/mg of protein/h. The deduced amino acid sequence of N-SMase indicates the lack of cysteine residues.

To further confirm the cDNA encoding N-SMase, we inserted it into a mammalian cell transient expression vector and transfected it into COS-7 cells. Fig. 4A shows that cells transfected with N-SMase cDNA (pHH1) exhibited a 10-fold increase in N-SMase activity compared with cells transfected with mock vector (pSV-SPT-1) 24 h post-transfection. Measurement of N-SMase activity following sodium lauryl sarcosine gel electrophoresis separation of r-N-SMase showed optimal activity corresponding to an apparent mass of 90–100 kDa and a pl of ~6.55 (2). This difference may be due to multiple post-transcriptional modifications of the mammalian N-SMase compared with the bacterial N-SMase (see below). The bacterial N-SMase was recognized by antibody against human N-SMase (Fig. 3, lane 4). Affinity-purified r-N-SMase expressed in E. coli had an activity of 3.3 µmol/mg of protein/h. The deduced amino acid sequence of N-SMase indicates the lack of cysteine residues.

**Characterization of r-N-SMase from COS-7 Cells**—As shown in **Fig. 5A**, r-N-SMase derived from cells overexpressing the enzyme had a pH optimum of ~7.4. r-N-SMase did not have subcellular enzyme activity in the acidic or alkaline range. Substrate specificity studies revealed that, of several phospholipids investigated, sphingomyelin appeared to be the most preferred substrate (data not shown). Studies on metal ion requirement revealed that r-N-SMase was activable with Mg²⁺ (2.5 mM) (Fig. 5B). In contrast, Cu²⁺ (5 µM) markedly inhibited the activity of the enzyme. Additional studies revealed that
pancreas, liver, lung, and placenta ranged from 2.4 to 9.5 kb in an ascending order. However, Northern blot analysis revealed that the major transcript size of N-SMase expressed in all of the eight human tissues investigated is 1.7 kb. The 1.7-kb transcript may be derived either from different genes or from alternative splicing. A β-actin Northern blot shown at the bottom of Fig. 7 was run to serve as a positive control.

**DISCUSSION**

Using a human N-SMase monospecific polyclonal antibody, we have screened a human kidney cDNA library and have identified a 3.7-kb cDNA transcript. The open reading frame of the cDNA predicts a 397-amino acid polypeptide. When expressed in *E. coli*, it encodes a protein of ~45 kDa based upon SDS gel electrophoretic analysis. When expressed in COS-7 cells, the cDNA conferred to r-N-SMase a 10-fold higher activity relative to mock cDNA-transfected cells and had an apparent molecular mass of ~90 kDa, suggesting multiple post-transcriptional modifications. The presence of numerous phosphorylation sites via the action of protein kinase C, casein kinase II, cAMP- and cGMP-dependent protein kinases, and tyrosine kinase is predictive of the sensitivity of this enzyme to inhibitors of protein kinase C and serine, tyrosine, and casein II kinases (4, 16, 17). The deduced amino acid sequence of N-SMase indicates the lack of cysteine residues. This may explain its insensitivity to reducing agents, i.e. dithiothreitol (Fig. 5B) and β-mercaptoethanol (18, 19). Moreover, inhibition of enzyme activity by glutathione is consistent with previous studies implicating a potential regulatory mechanism for this enzyme (19).

Physical-chemical characterization of r-N-SMase revealed properties similar to those of the human urinary/kidney N-SMase described by us previously (1, 2, 18). These were a pH optimum of 7.4, requirement for Mg$^{2+}$, heat stability (control activity of 3223 cpm/h; activity after heat treatment at 60°C for 30 min of 330 cpm/h), and detergent for activation and inhibition by Cu$^{2+}$. The amino acid sequence and hydrophathy curve analysis collectively suggest this enzyme to be a membrane-bound Mg$^{2+}$-sensitive N-SMase. Collectively, the physical-chemical properties of r-N-SMase derived from COS-7 cells, including molecular mass, are identical to those of the native enzyme derived from human urine (2). Although nucleotide sequence alignment of N-SMase revealed some similarity to acid SMase (15), it was insensitive to treatment with 5'-adenosine monophosphate (data not shown), a compound that has been shown to inhibit acid SMase activity (2). Although r-N-SMase was recognized (immunoprecipitated) by antibody against N-SMase, but not by preimmune serum IgG, the activity of the enzyme in the immunoprecipitate could not be recovered. Similar observations have been made previously using β-galactosidase and the corresponding antibody. This may be explained on the basis that the polyclonal antibody bound to the active site in N-SMase, rendering it inactive. Application of conditions or procedures such as high salt concentration (4 M KCl), heating, and lowering the pH to 4 to dissociate the enzyme from the immunoprecipitate did not facilitate the recovery of activity, as the enzyme itself was highly sensitive to such treatments. Nevertheless, we demonstrated a dose-dependent increase in the immunoprecipitation of the antigen by anti-N-SMase antibody and a decrease in the activity of N-SMase in the supernatant. In the future, the availability of a monoclonal antibody directed against N-SMase (exclusive of the active site) to immunoprecipitate the enzyme may help retain the activity of the enzyme. The physiological effects of r-N-SMase (data not shown) were also similar to those of human urinary/kidney

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4. D. Usher, personal communication.
N-SMase, such as a dose-dependent increase in cholesteryl ester synthesis and stimulation of the maturation of sterol regulatory element-binding protein-1 and LDL receptor mRNA expression in a continuous line of human hepatocytes (8). Finally, overexpression of r-N-SMase in human aortic smooth muscle cells resulted in apoptosis and augmented oxidized LDL-induced apoptosis in these cells, and antibody against N-SMase abrogated this phenomenon.

The cytoplasmic domain of two cell-surface receptors (Fas/Apo-I/CD95 and TNF-α-R) that share significant homology has been termed the "death domain." These two proteins contain cysteine-rich repeats that are also found in the nerve growth factor family of proteins such as TNF-α-R2, CD30, CD26, CD40, Ox-4, and H-1BB. The ligands for Fas and TNF-α-R are FasL and TNF-α, respectively, and they can induce apoptosis in certain target cells (22, 23). The death domains of TNF-α-R and Fas have been found to interact with several interactive and adaptive proteins that have homology to the death domain and that provide a link to caspase activation and apoptosis (14).

Previous studies have shown that overexpression of Fas/Apo-I or TNF-α-R1 can lead to apoptosis in the absence of ligands due to multi-oligomerization of the death domain (10). Therefore, it was not surprising to find spontaneous apoptosis in cells overexpressing N-SMase that was ligand-independent. This tenet

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5 H. Han, T. C. Wan, and S. Chatterjee, submitted for publication.
is further supported by the alignment of the death domain of N-SMase with the death domains of Fas/Apo-I and TNF-α-R1, which showed close homology (Fig. 2). Indeed, N-SMase and the molecular cloning of these enzymes. It is quite likely that in these two studies. One may be related to strategies used in the overexpression of N-SMase in HEK and U937 cells did not induce apoptosis independent of the presence and/or absence of TNF-α (20). There may be several reasons for the discrepancies in these two studies. One may be related to strategies used in the molecular cloning of these enzymes. It is quite likely that spontaneous apoptosis observed in cells overexpressing N-SMase in our study could be due to the presence of novel death domains absent in other reported N-SMases (20). However, given the complexity of the N-SMase molecule and its vital role in regulating diverse cell signaling pathways, we are not surprised to find different molecular isoforms of N-SMase (a). We predict that, in the future, many additional novel N-SMases will be found that will cleave sphingomyelin but may have diverse biological functions relevant to human health and disease. If so, then such proteins may represent ortholog products of the superfamily of N-SMase genes. Orthologs are defined as genes evolving from a common ancestral gene showing common function (21). We believe that the molecular cloning of N-SMase cDNA will accelerate the process to define its role as a key regulator in apoptosis and other cell regulatory pathways.

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