
METABOLISM AND BIOENERGETICS:
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Characterization and Subcellular Localization of Murine and Human Magnesium-dependent Neutral Sphingomyelinase*

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Sphingomyelinases (SMases) catalyze the hydrolysis of sphingomyelin, an essential lipid constituent of the plasma membrane, lysosomal membranes, endoplasmic reticulum, and the Golgi membrane stacks of mammalian cells. In this study, we report the biochemical and functional characterization and subcellular localization of magnesium-dependent nSMase1 from overexpressing human embryonic kidney (HEK293) cells. Site-directed mutagenesis of conserved residues probably involved in the enzymatic sphingomyelin cleavage as well as the removal of one or both putative transmembrane domains lead to the complete loss of enzymatic activity of human nSMase1 expressed in HEK293 cells. Polyclonal antibodies raised against recombinant mammalian nSMase1 immunoprecipitated and inactivated the enzyme in membrane extracts of overexpressing HEK293 cells and different murine tissues. Cell fractionation combined with immunoprecipitation studies localized the nSMase1 protein predominantly in the microsomal fraction. The enzyme colocalized with marker proteins of the endoplasmic reticulum and the Golgi apparatus in immunocytochemistry. Anti-nSMase1 antibodies did not affect the nSMase activity in the plasma membrane fraction and membrane extracts from murine brain. Our study leads to the conclusion that nSMase1 is one of at least two mammalian neutral sphingomyelinases with different subcellular localization, tissue specificity, and enzymatic properties.

Sphingomyelin is ubiquitously present in eukaryotic cells distributed in a gradient fashion from membranes of the endoplasmic reticulum, Golgi apparatus, and lysosomes to the plasma membrane which contains 70–90% of total cellular sphingomyelin. Sphingomyelinases (SMases¹; EC 3.1.4.12) cat-

alyze the hydrolysis of sphingomyelin to ceramide and phosphocholine. Ceramide is further degraded to sphingosine and fatty acid by ceramidase(s). Since their discovery 3 decades ago, several SMase isoforms, including alkaline SMase (1, 2), lysosomal acidic SMase (aSMase) (3, 4), secreted Zn²⁺-dependent acidic SMase (5), membrane-bound Mg²⁺-dependent SMase (nSMase) (6–8), and cytosolic Mg²⁺-independent SMase (9), have been described. These enzymes differ in their subcellular localization, tissue specificity, and enzymatic properties, especially pH optimum (10).

aSMase and nSMases release ceramide in the “sphingomyelin cycle” (11, 12). Ceramides are regarded as lipid “second messenger” molecules involved in numerous and very divergent cellular functions (10), e.g. in the regulation of cell differentiation (13), apoptosis induced by tumor necrosis factor α (12, 14) and Fas (15), and stress-induced apoptosis (16–18). Therefore, increasing attention has been focused on neutral magnesium-dependent sphingomyelinases and their participation in a variety of cellular processes in the recent past; however, their molecular characterization has resisted all attempts.

In order to define the controversially discussed functions of SMases in cellular processes (19–22), the isolation and molecular characterization of these enzymes is urgently needed.

Recently, we described the cloning of the first mammalian neutral sphingomyelinase (nSMase1) (23). nSMase1 is a member of an extensive Mg²⁺-dependent phosphodiesterase family. The murine nSMase1 (mnSMase1) cDNA encodes a 419-amino acid residue protein, and the human enzyme, hnSMase1 (47.6 kDa), contains 423 residues. Both proteins show no obvious signal sequence. The hydrophobicity plot reveals two transmembrane domains in the C-terminal part and suggests a bitopic membrane topology with the N and C termini facing the cytosol.

We describe in this report the expression of tagged nSMase1 in human embryonic kidney (HEK293) cells, the affinity purification of the enzymatically active protein to apparent homogeneity, and subsequent biochemical characterization as well as studies on potential posttranslational modifications and site-directed mutagenesis of conserved residues believed to be essential for catalytic activity. The enzymatic properties of the affinity-purified nSMase1 differ clearly from a recently published nSMase purified from rat brain (24).

Polyclonal antibodies were raised against complete and truncated murine and human recombinant nSMase1 protein expressed in *Escherichia coli* in order to determine the subcellular distribution and tissue specificity of nSMase1. The subcellular localization of nSMase1 was established biochemically, by immunoprecipitation studies and immunofluorescence microscopy of transfected embryonic fibroblasts. These approaches underscore that the nSMase1 is predominantly localized in the membranes of the endoplasmic reticulum.

Anti-nSMase1-specific antibodies inactivated and precipi-

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¹ The abbreviations used are: SMase, sphingomyelinase; nSMase, neutral sphingomyelinase; nSMase1 and nSMase2, first and second neutral sphingomyelinase, respectively; aSMase, acidic sphingomyelinase; mnSMase and mnSMase1, murine nSMase and nSMase1, respectively; hnSMase1, human nSMase1; aa, amino acid; PBS, phosphate-buffered saline; HEK293, human embryonic kidney cells 293; DTT, dithiothreitol; ER, endoplasmic reticulum; Bip, immunoglobulin heavy chain binding protein; TM, transmembrane helix; EMFI, exponentially growing mouse primary embryonic fibroblast; PAGE, polyacrylamide gel electrophoresis.

tated the enzymatic activity from extracts of overexpressing HEK293 cells, different murine tissues, and microsomal fractions from murine liver homogenate but did not affect nSMase activity in brain extracts and plasma membrane fractions. We proved that the residual activity is contributed by at least one distinct nSMase2, residing in the plasma membrane and strongly expressed in brain.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection of HEK293 Cells—Human embryonic kidney 293 (HEK293) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 μ g/ml streptomycin, 100 units/ml penicillin, and 1 mM sodium pyruvate at 37 °C in a humidified incubator containing 5% CO₂. Stably transfected clones were selected under 1 mg/ml Geneticin (G418; Life Technologies, Inc.). Transiently transfected cells were used 48 h after electroporation.

Measurement of Sphingomyelinase Activity—nSMase activity was determined with *N*-[¹⁴CH₃]sphingomyelin as substrate. Crude membrane extracts from tissues or HEK293 cells were mixed with 100 mM Tris-HCl, pH 7.4, 5 mM DTT, 0.05% Triton X-100, 5 mM MgCl₂, 1× Complete™ without EDTA (protease inhibitor set; Roche), and 10 nmol (60,000 dpm) of *N*-[¹⁴CH₃]sphingomyelin in a total volume of 200 μ l. After a 15–30-min incubation at 37 °C, 100 μ l of H₂O was added, and unreacted substrate was removed by extraction with 800 μ l of CHCl₃/CH₃OH (2:1, v/v). 100 μ l of the aqueous phase containing the enzymatically released *N*-[¹⁴CH₃]phosphocholine was counted in a liquid scintillation counter.

Generation of Polyclonal Antibodies—Truncated (mnSMase1, aa 1–282; hnSMase1, aa 1–271) or complete nSMase1 cDNAs were fused to a C-terminal c-Myc/His₆ tag derived from the plasmid vector pcDNA3.1/Myc-His (Invitrogen) and expressed in *E. coli* (BL21(DE3)-pLys) using the pET expression system (Novagen). Recombinant protein was purified from inclusion bodies using Talon™ (CLONTECH) according to the manufacturer's instructions. Rabbit anti-nSMase1 antibodies were raised against these different recombinant nSMase1 proteins. Recombinant proteins were coupled to BrCN-activated Sepharose FF (Amersham Pharmacia Biotech) and used for the affinity purification of nSMase1-directed antibodies. Aliquots of the purified antibodies were biotinylated using D-biotinoyl- ϵ -aminocaproic acid-*N*-hydroxysuccinimide (Roche Molecular Biochemicals) following the manufacturer's instructions.

Combined Immunoprecipitation/Immunoblotting—Tissues of 3-month-old CD1 mice or of *asmase*(^{-/-}) mice were homogenized in homogenization buffer (0.25 M sucrose, 10 mM EDTA, 10 mM EGTA, 50 mM Tris-HCl, pH 7.4, 2× Complete™ without EDTA) and centrifuged for 10 min at 900 × *g*. 5 mM DTT was included in the homogenization buffer only in samples used for measurement of sphingomyelinase activity. The sediment was extracted in homogenization buffer, 1% Triton X-100 and centrifuged for 60 min at 100,000 × *g*. Protein concentration in the supernatant was determined by the BCA protein assay (Pierce) with bovine serum albumin as a standard. Aliquots of the extracts (2 mg of protein each) were mixed with 50 μ l of immobilized streptavidin (Roche) and incubated at 4 °C overnight with gentle agitation. After removal of the streptavidin beads, 10 μ l of rabbit anti-nSMase1 serum or preimmune serum was added to the extracts. After incubation for 1 h at 4 °C, the antigen-antibody complexes were precipitated by the addition of 50 μ l of protein G coupled to cross-linked (4%) agarose beads (Sigma). After different washing steps, antigen-antibody complexes were dissociated by heating in SDS-PAGE loading buffer and separated by 10% SDS-PAGE. Western blot analysis was performed with biotinylated purified anti-mnSMase1 antibody (1 μ g/ml) followed by stringent washing and incubation with a streptavidin-horseradish peroxidase conjugate (Sigma). After several washing steps, bands were visualized by chemiluminescence staining using SuperSignalSubstrate (Pierce).

Affinity Purification of nSMase1 Protein from Stably Transfected HEK293 Cells—HEK293 cells stably transfected with the mnSMase1/pcDNA3.1/Myc-His construct were sedimented by centrifugation, resuspended in lysis buffer (50 mM sodium phosphate, 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1× Complete™ without EDTA) and disrupted by repeated freezing and thawing. A crude membrane fraction was obtained by centrifugation for 5 min at 2700 × *g*. Membrane proteins were extracted by the addition of extraction buffer (lysis buffer plus 0.2% Triton X-100). Insoluble components were removed by ultracentrifugation. nSMase protein was purified by metal-chelating chromatography using Talon™ (CLONTECH) and immediately adjusted to 5 mM DTT and 20% glycerol.

***N*-terminal Sequencing of His₆-tagged mnSMase1 from Stably Transfected HEK293 Cells**—mnSMase1 protein was affinity-purified from HEK293 cells grown on 20 150-mm dishes as described above and separated by SDS-PAGE. The nSMase1 band was eluted from the gel and *N*-terminally sequenced by Edman degradation using a gas phase sequencer (model 477A; Applied Biosystems).

Subcellular Fractionation of Mouse Liver Homogenate—Subcellular fractionation of mouse liver homogenate was performed according to the method of Fleischer and Kervina (25). The enzymatic activity of glucose-6-phosphatase was measured following the protocol of Swanson (26) using 100 μ g of protein, and the activity of 5'-nucleotidase was determined as described using 20 μ g of protein (27).

Site-directed Mutagenesis—Modified hnSMase1 cDNAs were obtained by two standard polymerase chain reactions on the basis of the hnSMase1/pRc/CMV construct, each primed with an "external" and a modified "internal" oligonucleotide. The internal primers contained the modified codon and further silent mutations leading to an additional endonuclease recognition site, which was used to fuse the polymerase chain reaction products. The external primers allowed the cloning of the modified cDNAs back into the original expression vector. The numbers in parentheses indicate the position of the oligonucleotide in the human nSMase1-cDNA (s, sense; as, antisense), restriction sites are shown in italics, and bases differing from the original cDNA are depicted in underlined letters. External primers were as follows: 5'-CGT CCC CAC CGC GGC CGT CGC TGG-3' (s289–312) and 5'-CTA TAG AAT AGG GCC CTC TAG ATG CAT G-3' (pRc/CMV as978–1005). Internal primers were as follows: E49Q, 5'-GCT TTG CTC GAG CAA GTG TGG AGT G-3' (s483–508); 5'-C ACT CCA CAC TTG CTC GAG CAA AGC-3' (as483–508); N180H, 5'-GTG GAG ACC TCC ATA TGC ACC CAG A-3' (s878–901), 5'-T CTG GGT GCA TAT GGA GGT CTC CAC-3' (as878–901); H272N, 5'-CCC TCT CTG ATA ATG AAG CGC TGA TG-3' (s1154–1179), 5'-CA TCA GCG CTT CAT TAT CAG AGA GGG-3' (as1154–1179). Truncated constructs devoid of one or both putative transmembrane domains and the C terminus were generated by a single polymerase chain reaction with the oligonucleotide s289–312 (see above) and one of the following primers: –TM, –C terminus (5'-CTC TAG ATT AGC GAG CCT GAG CCA TGC CCA GAC-3'; 1292–1314); –2.TM, –C terminus (5'-CTC TAG ATT ATT CCC CGG CCC CTC CTC CAG CCG-3'; 1385–1407). A construct devoid of the transmembrane domains but including the C-terminal part of hnSMase1 was cloned by the method described above. The internal primers were used to insert a spacer of six amino acids (GPTNPG), which replaces the transmembrane domains between the N- and the C-terminal part of the protein: –TM, +C terminus (5'-GGG CCC ACC AAC CCC GGG CAC GTA CAG GAG GTC AAT GGC-3' (s1474–1494) and 5'-CCC GGG GTT GGT GGG CCC GCG AGC CTG AGC CAT GCC CAG-3' (as1294–1314)).

Northern Blot Analysis—RNA from transfected HEK293 cells was prepared following the protocol of Chomczynski and Sacchi (28) and separated on 0.8–1% formaldehyde-agarose gels. After capillary transfer to GeneScreenPlus membranes (NEN Life Science Products), hybridization was performed according to the instructions of the manufacturer. Blots were analyzed with a PhosphorImager 445 SI using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

Metabolic Labeling of HEK293 Cells and Immunoprecipitation—1.5 × 10⁶ transiently transfected HEK293 cells were grown in methionine-free Eagle's medium for 1 h and labeled with 150 μ Ci of L-[³⁵S]methionine (Amersham Pharmacia Biotech) for 4 h at 37 °C. Cells were washed with PBS and resuspended in extraction buffer (0.2% Triton X-100, 10 mM EDTA, 10 mM EGTA, 50 mM Tris-HCl, pH 7.4, 1× Complete™ medium without EDTA). Insoluble material was removed by ultracentrifugation. nSMase1 protein was precipitated from the extracts by the addition of affinity-purified anti-hnSMase1 antibody and protein A-Sepharose (Sigma) and gentle agitation for 1 h at 4 °C in each case. Sepharose was washed stringently and treated with SDS-PAGE loading buffer. Proteins were separated by SDS-PAGE (10%). The gel was dried and analyzed on a PhosphorImager 445 SI (Molecular Dynamics).

Immunofluorescence Microscopy—10⁷ exponentially growing mouse primary embryonic fibroblasts (EMFIs) were electroporated with mnSMase1-pRc/CMV, and 48 h later they were used for immunohistochemistry.

Cells were fixed with 4% paraformaldehyde in PBS and lysed with PBS, 0.5% Triton X-100. After blocking with PBS, 3% bovine serum albumin, cells were incubated with the anti-mnSMase1 antibody together with anti-Grp78 (Bip; Biomol), anti-58K (Clone 58K9; Sigma), or anti- α -tubulin (clone B-5-1-2; Sigma) in PBS, 3% bovine serum albumin, 0.5% Triton X-100. Cells were washed with PBS, 0.5% Triton

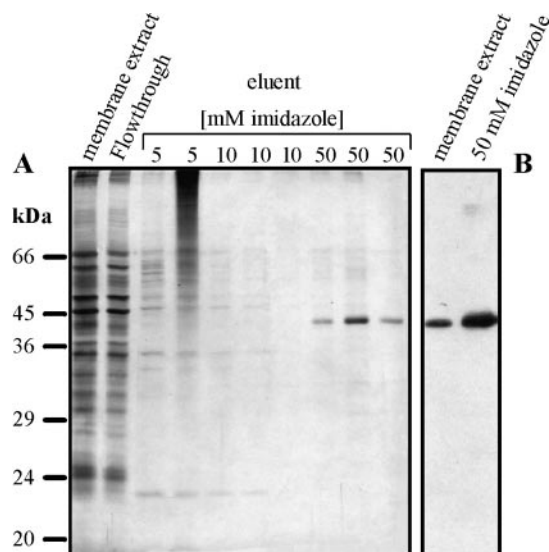


FIG. 1. Affinity purification of His₆-tagged murine nSMase1 protein from stably transfected HEK293 cells. *A*, silver-stained SDS-polyacrylamide gel (10%) of protein fractions from metal-chelating chromatography. Crude membrane extracts were applied to a 1-ml Talon™ (CLONTECH) column, and bound protein was eluted with a stepwise gradient of increasing imidazole concentration. nSMase1 protein eluted from the column at imidazole concentrations above 15 mM. *B*, Western blot analysis of protein extracts before (membrane extract) and after purification (50 mM imidazole). Aliquots of the fractions were separated by (10%) SDS-PAGE, blotted onto nitrocellulose, and treated with affinity-purified anti-mnSMase1 antibodies. Second antibody was anti-rabbit IgG-horseradish peroxidase conjugate. Bands were visualized by chemiluminescence with SuperSignalSubstrate (Pierce).

X-100 and PBS three times each and incubated with a Cy3-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch) and a Cy2-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch), washed and analyzed with a Leica confocal microscope.

Human primary fibroblasts were transiently transfected with hnSMase1-pRc/CMV and hnSMase1-pcDNA3.1/Myc-His (Invitrogen) for 48 h, respectively, and analyzed as described above.

RESULTS

nSMase1 Expression and Affinity Purification of Recombinant nSMase1 and of anti-nSMase1-specific Antibodies—Complete or truncated murine and human nSMase1 cDNAs (23) were fused to an N-terminal Myc-His₆-tag and expressed in *E. coli*. SDS-PAGE of the bacterial homogenate indicated that the recombinant protein was present in inclusion bodies. The tagged nSMase1 protein was solubilized either in 8 M urea or SDS-Triton X-100, adsorbed to a metal-chelating column (Talon®), and desorbed by increasing imidazole concentrations from the thoroughly washed column. All attempts to renature the purified nSMase1 released with 8 M urea from inclusion bodies were unsuccessful. Rabbit anti-nSMase1 antibodies were raised against the different recombinant nSMase1 proteins and purified by absorbing to the immobilized antigen. The antibodies specifically recognize the nSMase1 protein in membrane extracts from stably transfected HEK293 cells (Fig. 1*B*).

Biochemical Characterization of Affinity-purified mnSMase1 from HEK293 Cells—HEK293 cells were stably transfected with nSMase1 cDNA cloned into the eukaryotic expression vectors pRc/CMV and pcDNA 3.1 Myc-His₆. Expression of wild type, mock-transfected cell lines and selected clones was compared on the mRNA level by Northern and on the protein level by Western blot analysis and in addition by the enzyme assay (data not shown). HEK293 cells transfected with the tagged and wild type nSMase1 cDNA expressed the enzyme at a similar rate. Tagged nSMase1 was purified to apparent homogeneity by metal-chelating affinity chromatography from overex-

pressing HEK293 cells (Fig. 1*A*). The flow-through of the affinity column showed no significantly increased nSMase activity compared with membrane extracts from mock-transfected HEK293 cells, demonstrating that the tagged protein is directly responsible for the increased nSMase activity in transfected cells. The enzymatic properties of nSMase1 were unimpaired by the C-terminally fused c-Myc-His₆-tag. We could not detect any other copurified protein compounds. The specific activity of the affinity purified enzyme was enriched 50 times compared with its activity in the crude membrane extract (50 versus 1 μmol of sphingomyelin/h/mg of protein), but the total nSMase activity was reduced dramatically. The loss of activity is caused very likely by the absence of reducing DTT or mercaptoethanol essential in enzyme purification but fatal for the matrix of the affinity column. Increasing purity also reduced the enzyme stability. Reducing agents added after affinity purification were unable to restore the nSMase1 activity. However, the affinity-purified nSMase1 was used for detailed examination of its enzymatic properties.

Purified nSMase1 showed a neutral pH optimum and no detectable activity against phosphatidylcholine. The enzyme was inactive without magnesium and manganese ions in the assay mixture. Both divalent cations activated the enzyme but showed different kinetics. Mg²⁺ ($K_a = 80 \mu\text{M}$) was effective even at high concentrations up to 10 mM, while Mn²⁺ ($K_a = 15 \mu\text{M}$) inhibited the enzyme at concentrations above 0.5 mM as described before by Hostetler and Yazaki (29) for neutral sphingomyelinase from rat liver homogenate (Fig. 2*A*). It reveals a direct activation by arachidonic acid ($K_a = 20 \mu\text{M}$) (Fig. 2*B*) but no detectable stimulation by the phospholipid phosphatidylserine (10–200 μM) or inhibition at physiological glutathione concentrations as described recently for a brain-specific nSMase (24).

Putative Posttranslational Modifications—Recombinant hnSMase1 and mnSMase1 purified either from *E. coli* or from membrane extracts of HEK293 cells constantly deviated by 6–7 kDa in molecular mass in SDS-PAGE from the calculated sizes of 47.5 and 47.6 kDa for mnSMase1 and hnSMase1 and 50.8 kDa for the tagged murine protein, respectively (Fig. 3). The band at 42 kDa represents mnSMase1, and the faint 80-kDa band represents the dimer (Fig. 3, *A* (lane 2) and *B* (lane 1)). The latter was transformed to the monomeric 42-kDa band by treatment with the reducing agent DTT (Fig. 3*B*, lane 2) and by reductive carboxymethylation (Fig. 3*B*, lane 3). Dimerization through disulfide bonds is obviously an artifact of the purification. This interpretation is supported by the rapid loss of enzyme activity in the absence of reducing SH reagents.

mnSMase1 and hnSMase1 contain one and two putative consensus sequences for *N*-glycosylation. Endoglycosidase H and *N*-glycosidase F treatment did not alter the electrophoretic mobility of the 42-kDa protein band in SDS-PAGE (Fig. 3*C*). Therefore, no evidence for glycosylation of native and mature nSMase1 can be found on the basis of our experiments.

mnSMase1 contains 17 cysteines, eight of which are conserved in hnSMase1. 16 cysteines are located in the putative cytosolic part of the protein and therefore in the reducing environment, which makes disulfide bonds highly unlikely.

Other possible causes for the reduced apparent molecular mass were excluded; Edman degradation of the affinity-purified nSMase1 from HEK293 cells revealed that the translation starts at the first possible methionine in the proposed nSMase1 peptide sequence and that no N-terminal proteolytic truncation had occurred. The successful affinity purification of the C-terminally tagged enzyme protein excludes also a C-terminal proteolysis, which could account for the derived molecular mass of 42 kDa of nSMase1 in SDS-PAGE.

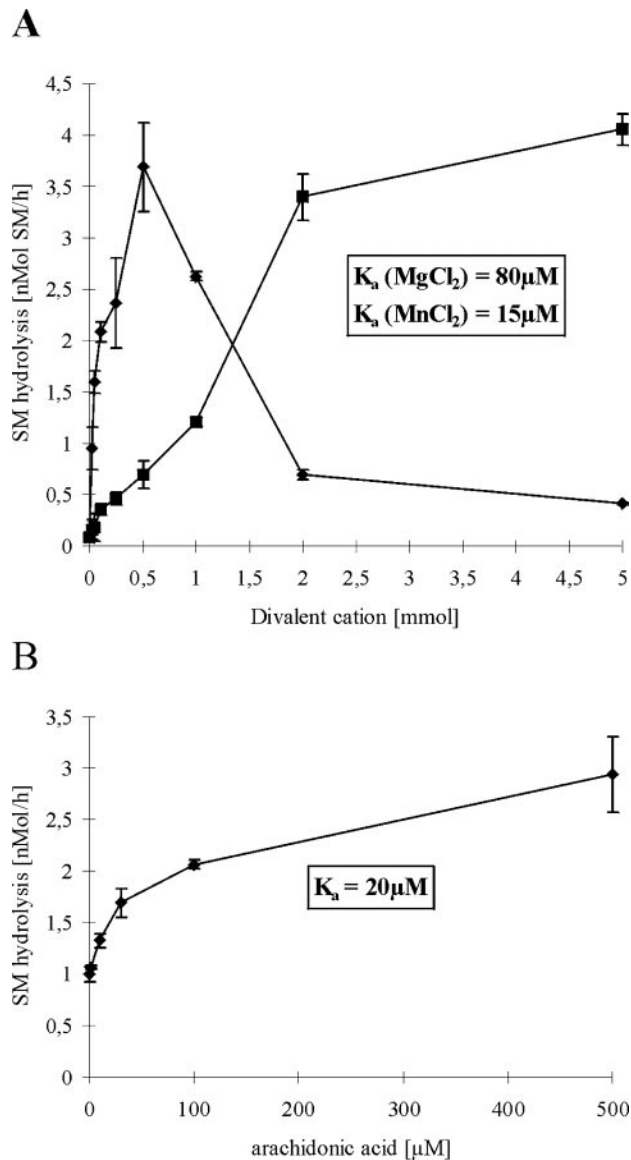


FIG. 2. Influence of divalent cations and arachidonic acid on purified murine nSMase1 from stably transfected HEK293 cells. mnSMase1-Myc-His₆ fusion protein was purified by metal-chelating chromatography from crude membrane extracts from stably transfected HEK293 cells (see Fig. 1). Effectors of nSMase1 activity were added directly to the assay mixture. *A*, effects of divalent cations. \blacklozenge , MnCl₂; \blacksquare , MgCl₂. *B*, activation by arachidonic acid. The results are mean values of three independent measurements \pm S.D. K_m values were calculated from Lineweaver-Burk plots of the data indicated in the figure.

The discrepancy between the calculated and electrophoretic mobility derived molecular mass may be due to a frequently observed unusual SDS binding of the hydrophobic protein domains.

Functional Analysis of nSMase1 by Site-directed Mutagenesis of Potential Catalytic Amino Acid Residues and by Truncation of nSMase1—nSMase1 belongs to a large family of phosphodiesterases including mammalian DNaseI, phospholipase C from *S. aureus*, and bacterial sphingomyelinases. They reveal species-independent identical motifs. This suggests their essential function in the catalysis of the hydrolysis of the phosphodiester bonds. Relevant to this study is the prediction of analogous amino acid residues in the hnSMase1 derived from x-ray crystallography of DNaseI (30). They are either involved in Mg²⁺ complexing (Glu⁴⁹) or substrate binding (Asp¹¹¹, Tyr¹⁴¹, and Asn¹⁸⁰) or are essential for acid (His¹⁵¹) and base (His²⁷²) catalysis and the stabilization of the pentavalent tran-

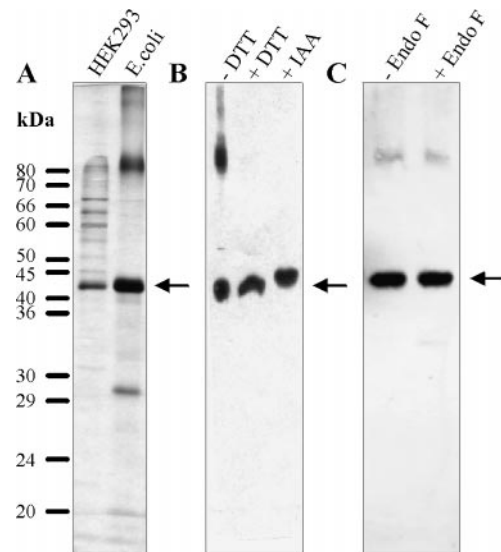


FIG. 3. Putative post-translational modifications of nSMase1 protein expressed in HEK293 cells. *A*, comparative SDS-PAGE of affinity-purified mnSMase from HEK293 cells (lane 1) and *E. coli* (lane 2). Protein bands were visualized by silver staining. *B*, Western blot analysis of mnSMase from stably transfected HEK293 cells after treatment with reducing (DTT) and carboxymethylating (iodoacetamide (IAA)) agents. *C*, same as *B* after treatment with endoglycosidase H/N-glycosidase F. The arrows indicate nSMase-specific bands.

sition state of the phosphorus (Asp²⁵¹). The hnSMase1 constructs for the analysis of the putative functions of the respective amino acid residue side chains are given in Fig. 4.

We also studied the function of the two putative transmembrane (TM) domains. hnSMase1 constructs devoid of the C-terminal part of the protein and one or both TM domains were established as well as a construct with the TM helices replaced by the hexapeptide GPTNPG. The two prolines within the peptide should allow the back-folding of the C-terminal domain into the predicted conformation of the mature nSMase1.

Point-mutated and truncated constructs of hnSMase1 were transiently expressed in HEK293 cells. nSMase1 mRNA was quantitated by Northern blot hybridization analysis (Fig. 5, *A* and *B*). Proteins, immunoprecipitated from membrane extracts of [³⁵S]methionine metabolically labeled HEK293 cells were separated by SDS-PAGE and quantitated by autoradiographic analysis (Fig. 5*C*). Specific nSMase activity was measured in the membrane extracts and the 2700 \times *g* supernatant (Fig. 5, *D.1* and *D.2*). Wild type and the point mutations E49Q, N180H, and H272N showed an almost identical expression on the mRNA and protein level, but only cells transfected with wild type hnSMase1 revealed an increase of nSMase activity. The Northern blot signals of the truncated nSMase1 transcripts were reduced and the protein signals hardly visible in autoradiography. No increase of nSMase activity was measured in cells transfected with this cDNA expression constructs. Furthermore, the truncated nSMase1 proteins were not detectable in immunofluorescence (data not shown).

Tissue-specific Expression of nSMase1 in the Mouse—In a first approach, Western blot analysis with affinity-purified anti-nSMase antibodies produces only weak signals due to the very low nSMase1 protein concentration in the different murine tissues examined. Therefore, an immunoprecipitation step was performed prior to the Western blot analysis; nSMase1 protein was enriched from the solubilized 2700 \times *g* sediment derived from tissue homogenates by immunoprecipitation with mnSMase1-specific affinity-purified antibodies and subsequent adsorption to protein G-Sepharose. The immunocomplex was disrupted by heating in SDS-PAGE sample buffer and sub-

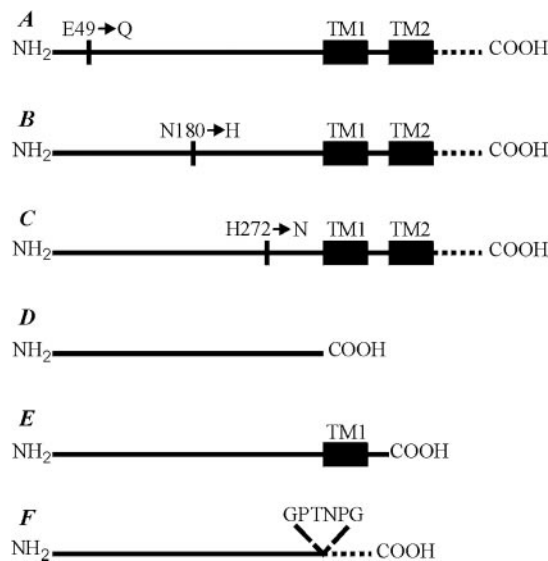


FIG. 4. Schematic view of mutant hnSMase1 proteins. A–C, mutations of amino acids probably involved in enzymatic sphingomyelin cleavage. D, hnSMase1 protein without putative TM domains and subsequent C-terminal part. E, construct without second TM domain and C-terminal part. F, hnSMase1 with TM domains replaced by the linker peptide GPTNPG.

jected to SDS-PAGE and immunoblotting using biotinylated anti-mnSMase antibodies. The signal intensities in Western blot analysis (Fig. 6) corresponded well to those in Northern blot analysis described previously (23). nSMase protein is ubiquitously distributed in all tissues examined with the strongest signal in kidney and of similar size compared with mnSMase1 from transfected HEK293 cells. The absence of a signal in jejunum is probably due to the high proteolytic activity in this tissue. A comparison of the expression in these tissues on the transcriptional (Northern blot signals) and translational level (Western blot signals) with their nSMase activity, however, revealed a striking difference, particularly in the brain homogenate.

Immunoprecipitation of nSMase1 Activity in Mouse Tissues, Particularly in Brain, Suggests an Additional nSMase2—Immunoprecipitation studies performed with membrane extracts from stably mnSMase-transfected HEK293 cells revealed a specific and quantitative precipitation of nSMase1 activity and protein using purified anti-mnSMase1 antibodies and protein G-Sepharose, subsequently (Fig. 7). An immunoprecipitation study in different murine tissues combined with nSMase activity measurement in the supernatant after the precipitation should answer the question of whether nSMase1 is responsible for the high enzymatic activity in brain despite its low protein amount compared with the other tissues.

mnSMase1-specific antibodies quantitatively precipitate the nSMase1 protein from crude membrane extracts from mouse brain and kidney homogenate (Fig. 8A). 75% of total nSMase activity is inactivated in kidney, therefore corresponding to nSMase1 inhibition (Fig. 8B), but only a minute part of the activity in brain homogenate is inactivated by increasing concentrations of the nSMase1-specific antibody. This suggests a specific sphingomyelinase nSMase2 responsible for the high nSMase activity in brain different from the cloned nSMase1 described here.

nSMase1 Is Located in the Endoplasmic Reticulum—Confocal immunofluorescence microscopy of EMFIs transiently transfected with mnSMase-pRc/CMV indicates that nSMase1 resides predominantly in the ER. Double staining with anti-mnSMase1 antibodies together with anti-Bip (Grp78; residing in the lumen of the ER) antibodies or anti-58K protein (micro-

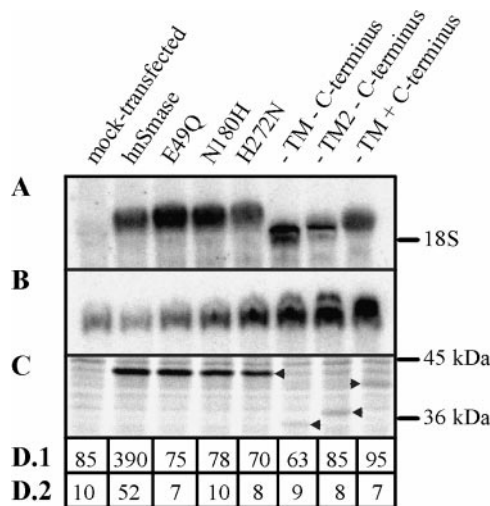


FIG. 5. Complete loss of nSMase1 activity caused by different point mutations and removal of the transmembrane helices. Three point mutations were introduced into the human nSMase1-cDNA (lanes 3–5) and constructs of the nSMase1 cDNA generated, which encode the enzyme protein lacking the transmembrane domains and/or truncated at the C terminus (lanes 6–8). HEK293 cells were transiently transfected with these modified cDNAs and analyzed by Northern blotting, immunoprecipitation, and nSMase activity measurement. Cells transfected with the expression vector (mock-transfected) and the unmodified hnSMase1-cDNA served as a control (lanes 1 and 2). A, Northern blot analysis of total HEK293 cell RNA with hnSMase1-cDNA as a probe. B, same blot as used in A, rehybridized with a 548-bp HindIII/XbaI fragment derived from human GAPDH cDNA. C, immunoprecipitation of the nSMase1 protein from metabolically [³⁵S]methionine-labeled cells. Cells were extracted with a Triton X-100 containing buffer and treated subsequently with affinity-purified anti-hnSMase1 antibodies and protein A-Sepharose. The immunoprecipitate was separated by SDS-PAGE and analyzed autoradiographically. D.1, specific nSMase activity (nmol of sphingomyelin/mg of protein/h) in the 2700 × g sediment of cell homogenates. D.2, specific nSMase activity (nmol of sphingomyelin/mg of protein/h) in the 2700 × g supernatant of cell homogenates. The crude fractionation of the cellular homogenate was performed to detect the expected shift of the nSMase1 protein without transmembrane domains from a membrane-bound to a soluble condition. In –TM –C terminus, the complete C-terminal part of the hnSMase1 protein beginning at the first TM domain (Trp³²²) was deleted; in –TM2 –C terminus, the C-terminal part starting with the second TM domain (Val³⁵³) was removed; and in –TM +C terminus, the two transmembrane-spanning domains of hnSMase1 protein (Trp³²²–Phe³⁷⁴) were replaced by the oligopeptide GPTNPG.

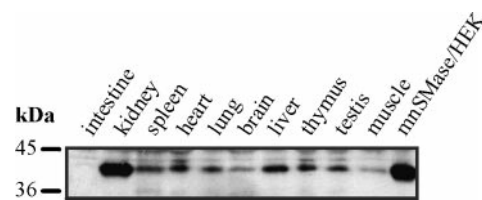


FIG. 6. nSMase1 protein is ubiquitously expressed in all tissues examined. Protein (2 mg) from membrane extracts of different murine tissues was subjected to immunoprecipitation with anti-mnSMase1 serum/protein G-Sepharose. The immunoprecipitate was separated by SDS-PAGE and analyzed by Western blotting using affinity-purified biotinylated anti-mnSMase1-antibodies combined with a streptavidin-horseradish peroxidase conjugate. The blot was developed by using a chemiluminescent peroxidase substrate.

tubule-binding peripheral Golgi membrane protein) antibodies, respectively, show a colocalization most with the ER, but also partially with the Golgi complex. Staining with the cytoskeletal marker α -tubulin shows a different distribution of the proteins (Fig. 9). Neither mock-transfected or untransfected EMFIs nor secondary antibodies alone show nSMase staining (not shown).

Fluorescence of the wild type, c-Myc/His-tagged hnSMase1 fusion protein and the hnSMase1 mutants E49Q, N180H, and

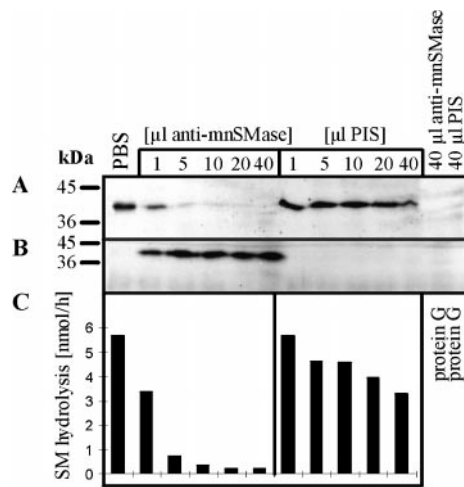


FIG. 7. Anti-mnSMase1 antibodies immunoprecipitate nSMase1 protein and activity from stably transfected HEK293 cells. Membrane extracts of HEK293 cells stably expressing the mnSMase1 cDNA were incubated with increasing amounts of anti-mnSMase1 serum or preimmune serum (PIS) followed by a constant amount of protein G-Sepharose. The protein G-Sepharose/antigen-antibody complex was sedimented by centrifugation. *A*, Western blot analysis of the supernatant after immunoprecipitation. Biotinylated anti-mnSMase1 antibodies and the streptavidin-horseradish peroxidase conjugate was used for immunodetection of nSMase1 protein. Bands were visualized by chemiluminescence with SuperSignalSubstrate (Pierce). *B*, the protein G-Sepharose/antigen-antibody complex was disrupted by heating in SDS loading buffer and subjected to SDS-PAGE and Western blot analysis. Fractions of membrane extracts without added antibody (PBS) (last two lanes) were used as controls. *C*, nSMase activity in the supernatant after immunoprecipitation. nSMase activity was measured in aliquots of the supernatant of the sedimented protein G-Sepharose/antigen-antibody complex.

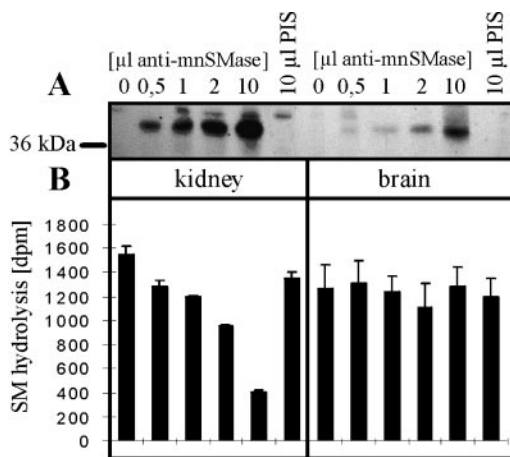


FIG. 8. Anti-mnSMase1 antibodies immunoprecipitate most of the nSMase activity from murine kidney extracts but not from brain extracts. *A*, crude membrane extracts from murine kidney and brain were treated with increasing amounts of anti-mnSMase1 antibody and preimmune serum (PIS). The antigen-antibody complex was precipitated by the addition of protein G-Sepharose and analyzed by Western blotting. *B*, nSMase activity in the supernatant after immunoprecipitation. nSMase activity was measured in aliquots of the supernatant after sedimentation of the protein G-Sepharose/antigen-antibody complex. The results are mean values of three independent measurements \pm S.D.

H272N colocalized with the Bip fluorescence (not shown). This also suggests that nSMase1 resides in the ER but not in the plasma membrane. Pretreatment of the transfected cells with brefeldin A had no influence on the distribution of Bip but had induced the retrograde transport of the 58K protein from the Golgi to the ER (data not shown). The subcellular distribution of nSMase1 remained almost unimpaired by the brefeldin A

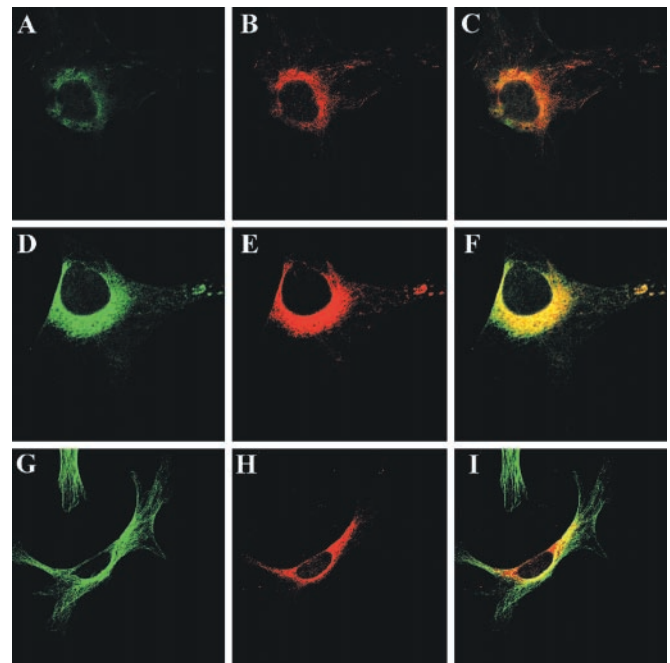


FIG. 9. nSMase1 immunofluorescence mainly stains the endoplasmic reticulum. Transiently transfected EMFIs were stained with anti-mnSMase1 antibody together with anti-58K, anti-Bip, or anti- α -tubulin antibodies, respectively, and corresponding Cy2- and Cy3-conjugated second antibodies and analyzed by confocal microscopy. *A*, anti-58K-Cy2; *D*, anti-Bip-Cy2; *G*, anti- α -tubulin-Cy2. *B*, *E*, and *H*, anti-mnSMase1-Cy3. *C*, *F*, and *I*, superimposition of the Cy2 and Cy3 channels. Yellow color indicates a colocalization.

treatment, which again strongly suggests its predominant localization in ER membranes. The localization of a minor part of the nSMase protein in the Golgi cannot be excluded.

Combined immunoprecipitation and Western blot analysis of protein extracts of subcellular compartments of liver confirmed the assignment of the localization of nSMase1 to the endoplasmic reticulum (Fig. 10B). Cross-contaminations of subcellular fractions were estimated by the activities of compartment-specific marker enzymes. 5'-Nucleotidase activity (Fig. 10C) was highest in the plasma membrane fraction but still detectable in the Golgi fraction also associated with contaminating microsomes, as monitored by glucose-6-phosphatase activity measurement (Fig. 10D).

These results indicate the predominant localization of nSMase1 in the smooth microsomal fraction. The presence in the plasma membrane fraction must be attributed to cross-contaminating ER membranes.

Immunoprecipitation studies with extracts of subcellular fractions of mouse liver homogenate point to the existence of at least two distinguishable nSMase activities located within different subcellular compartments (Fig. 10, E and F); 75–80% of neutral sphingomyelinase activity residing in the microsomal fraction was inactivated by the mnSMase1-specific antibodies, but only 35% of the nSMase activity measured in the plasma membrane fraction is nSMase1-specific, which is contributed by cross-contaminating nSMase1 residing in the ER fraction. This indicates that the anti-nSMase1 antibody is unable to recognize an additional plasma membrane-specific sphingomyelinase (nSMase2). Nonimmunoprecipitable nSMase activity in the Golgi fraction may be contributed by plasma membrane-specific nSMase2 on its translocation pathway from the ER to the plasma membrane or may originate from an additional Golgi-specific nSMase.

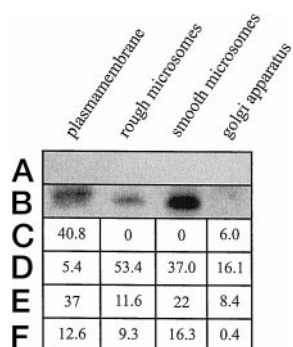


FIG. 10. nSMase1 protein is predominantly located in the smooth endoplasmic reticulum. Subcellular fractions were prepared from mouse liver homogenate by discontinuous sucrose gradient centrifugation. The purity of the subcellular fractions was tested by measurement of marker enzymes for endoplasmic reticulum and plasma membrane. Extracts of the membrane fractions were subjected to combined immunoprecipitation/Western blot analysis and nSMase activity assay after immunoprecipitation. Western blot analysis of subcellular fractions after immunoprecipitation with preimmune serum (A) and anti-mnSMase1 antibodies (B) is shown. C, specific activity (μmol of P_i /mg of protein/h) of plasma membrane marker 5'-nucleotidase. D, specific enzymatic activity (μmol of P_i /mg of protein/h) of the ER marker glucose-6-phosphatase. Shown is specific nSMase activity (nmol of sphingomyelin/mg of protein/h) in membrane extracts after treatment with preimmune serum (E) and in membrane extracts precipitable with anti-mnSMase1 serum (F).

DISCUSSION

In this study, we report the expression, affinity purification, and detailed characterization of the mouse and human nSMase1 enzyme protein expressed in HEK293 cells. Essential for these studies was the availability of nSMase1-specific polyclonal antibodies.

The mammalian nSMase1 is a member of a large family of Mg^{2+} -dependent phosphodiesterases including mammalian DNase1 and *E. coli* exonuclease. The replacement of any of three highly conserved amino acids against structurally related residues within the hnSMase peptide sequence completely abolished the enzymatic activity and provided evidence for the essential function of residues Glu⁴⁹, Asn¹⁸⁰, and His²⁷² in the catalytic cleavage of sphingomyelin. These results suggest the analogy of the active center of eukaryotic nSMase1 to that of bacterial sphingomyelinases, although our experiments cannot rule out the possibility that conformational changes caused by the mutations are responsible for the loss of nSMase activity.

The characterization of the affinity-purified nSMase1 from transfected HEK293 cells led to two important conclusions. First, it was shown that the predicted nSMase protein is directly responsible for the increased nSMase activity in HEK293 cells transfected with the corresponding cDNA. Second, the cloned nSMase1 differs significantly from a recently published nSMase activity from rat brain (no stimulatory effect of phosphatidylserine on enzymatic activity) (24) and, concerning the missing influence of physiological glutathione concentrations (31, 32), from the nSMase activity that has been suggested to be involved in intracellular signal transduction pathways. Additionally, the affinity-purified enzyme showed only a negligible phospholipase C activity as described previously (33).

Pivotal for understanding the function of this nSMase1 is its tissue distribution and subcellular localization. Northern as well as Western blot analyses revealed the ubiquitous distribution of nSMase1 mRNA and protein in all organs with the strongest expression in kidney and weakest in spleen and muscle (23). However, nSMase enzyme activity in brain and jejunum was 500 and 100 times higher than in other tissues. These measurements are inconsistent with the ubiquitous distribution of nSMase1 mRNA and protein in all tissues examined and

raise the possibility of the existence of additional nSMase isoenzymes.

nSMase1, overexpressed in HEK293 cells, is completely inactivated by immunoprecipitation. In mouse kidney extracts, 75% of the total nSMase activity was inactivated by the antibody, while in membrane extracts of total mouse brain the nSMase activity was not affected by the anti-nSMase1 antibody. These results and the results from the biochemical characterization of nSMase1 indicate the existence of at least one other nSMase responsible for the high enzymatic activity in brain.

The subcellular localization of nSMase1 in ER membranes was verified by immunofluorescence studies and analysis of subcellular fractions from mouse liver homogenate. These results suggest the existence of at least two different nSMases located in the plasma membrane and the microsomal fraction. Hostetler and Yazaki (29) described a microsomal nSMase activity in rat liver homogenate slightly different from the enzyme in the plasma membrane fraction. The cloned nSMase1 and this enzyme are probably identical.

The ER localization of nSMase1 raises the question of its function in several cellular processes. Ceramides play a role in vesicle-membrane fusion and endocytosis. Sphingomyelinases induce aggregation and fusion by ceramide release (34). They disturb the bilayer structure in favor of a nonlamellar, micellar phase (35). Membranes of the ER and Golgi complex, especially those of smooth ER, constantly participate in protein and lipid mass transport by local vesicle formation and fusion. It is conceivable that SNARE-mediated vesicle-membrane fusion (36) is facilitated by local activation of membrane-bound nSMases. Kidney cells and jejunum are characterized by their high endocytotic activity. nSMase1 expression is particularly high in these two tissues, supporting this presumption. The high activity of nSMase in gray matter of brain (37) would enable the budding and fusion of neurotransmitter vesicles. A regulatory role of nSMase1 within the maintenance of a constant sphingomyelin content of cellular membranes also cannot be excluded.

Among the proposed functions of nSMases is the release of ceramide in the "sphingomyelin cycle" and its role in signal transduction. Distinct pools of sphingomyelin within a cell coupled to different subcellular sites of ceramide generation seem to be critical concerning their effects on cell fate (38). The proposed transmembrane domains and the residual C-terminal part of mammalian nSMases are absent in the secreted bacterial sphingomyelinases. In order to create a soluble mammalian nSMase for studies of the proposed importance of the subcellular site of nSMase action, truncated nSMase1 expression constructs were generated that lack these domains. The production of nSMase protein lacking the TM domains was decreased, probably due to enhanced degradation or reduced stability. Additionally, these proteins were enzymatically inactive, which made these experiments unnecessary. However, overexpression of the H272N mutant in stably transfected Jurkat cells as a dominant negative system did not affect the kinetics of PARP cleavage (data not shown) as described previously for nSMase1 overexpression (23).

Among the surprising observations were the growth and morphology of nSMase1-overexpressing HEK293 cells (160-fold), which were indistinguishable from wild type and mock-transfected cells. Their sphingomyelin and ceramide content was unchanged. Also, the lipid analysis of the microsomal fraction of the overexpressing cells isolated by gradient centrifugation revealed no reduction of the sphingomyelin concentration (data not shown). This suggests a strongly regulated nSMase1 activity in the ER membranes only constitutively

activated upon detergent extraction. No response to the enhanced sphingomyelinase activity either of a ceramidase that may catalyze the removal of the product of the reaction or of a sphingomyelin synthase that could balance a potential sphingomyelin degradation by nSMase1 was measured.

A comprehensive insight into the structural and cellular functions of nSMase1 will be obtained from a nSMase1-deficient mouse model and the cloning and functional analysis of additional members of the neutral sphingomyelinase family.

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