Nuclear localization of neutral sphingomyelinase 1: biochemical and immunocytochemical analyses

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SUMMARY

To examine the intracellular localization of neutral sphingomyelinase 1 (nSMase 1), a rabbit polyclonal antibody was raised against a recombinant form of the enzyme expressed in *E. coli*. It has been reported that, in rat liver or in ascites hepatoma AH7974, high activity of neutral sphingomyelinase (SMase) is found at the plasma membrane, with a lesser but significant amount in nucleus and cytoplasm. The biochemical properties, dithiothreitol requirement and high salt concentration dependency, of cloned and expressed nSMase 1 resemble those of previously described nuclear neutral SMase of AH7974. The present study was therefore focused on the nuclear localization of this enzyme. Western blotting of subcellular fractions using anti-rat nSMase 1 antibody revealed most nSMase 1 to be associated with the nuclei and some with microsomes, but not with plasma membranes. Consistently, neutral SMase activity in nuclear extract was immunoprecipitated by the antibody, while that of plasma membranes was not. The results indicate that nSMase 1 mainly resides in the nucleus and may thus differ from neutral SMase in plasma membrane. On gel-filtration column chromatography of nuclear extract, the profile of neutral SMase activity corresponded well with immunoreactive protein bands on western blotting, suggesting that a large part of nuclear neutral SMase may be nSMase 1. Removal of the nuclear envelope by treatment with Triton X-100 did not significantly decrease the amount of nuclear nSMase 1, and western blotting of subnuclear fractions (i.e. nuclear envelope, chromatin, and nuclear matrix) revealed nSMase 1 signal exclusively in the nuclear matrix. Immunocytochemistry with AH7974, as well as rat fibroblast cell line 3Y1, demonstrated nSMase 1 to be localized mainly in the nucleus, with some in the cytoplasm. Moreover, immuno-electron microscopy clearly showed the signal of nSMase 1 to be more dense in the nucleus than in the cytoplasm of AH7974.

Key words: Neutral sphingomyelinase, Intracellular localization, Rat ascites hepatoma AH7974, Nuclei, Immunocytochemistry

INTRODUCTION

Sphingomyelinase (SMase) catalyzes the hydrolysis of sphingomyelin to yield ceramide and phosphocholine. In mammalian cells, at least five types of the enzyme have been described: acidic, Zn^{2+}-dependent and -independent; neutral, Mg^{2+}-dependent and -independent; and alkaline SMase (Duan et al., 1995; Okazaki et al., 1994; Quintern et al., 1989; Schissel et al., 1996; Schuchman et al., 1991). Tomiuk et al. has recently cloned human and mouse nSMase 1 genes by searching for sequences that are homologous to highly conserved sequences among prokaryotic SMases (Tomiuk et al., 1998). Mouse nSMase 1 is localized in kidney most predominantly (Tomiuk et al., 2000). They also cloned another species, nSMase 2, which is mainly localized in Golgi apparatus of brain cells (Hofmann et al., 2000). Chatterjee et al. found a new, distinct species of neutral SMase localized in human placenta, lung and liver (Chatterjee et al., 1999). These plural neutral SMases presumably have different functions, depending on their intracellular localization, cell type, or organ, in response to different signals (Levade et al., 1999; Wiegmann et al., 1994; Zhang et al., 1997a).

Ceramides formed through activation of SMase may function as second messengers in mediating cell growth, differentiation, stress responses and apoptosis (Gallardo et al., 2000; Hannun and Luberto, 2000; Sawai and Hannun, 1999; Toman et al., 2000). Signal pathways downstream of ceramide are presently under extensive study (Dobrowsky and Hannun, 1992; Lee et al., 1996; Richter and Ghafourifar, 1999; Spiegel and Milstien, 2000; Zhang et al., 1997b) but SMase may be important in initiating the first step in signal transduction. Therefore, the elucidation of its biochemical properties as well as its regulation is crucial to understanding this processes.

To ascertain the details of biochemical and molecular properties of nSMase 1 we have cloned the rat nSMase 1 gene (GenBank accession no. AB047002) (Mizutani et al., 2000). This gene was chosen because we have engaged in studying neutral SMase of rat ascites hepatoma AH7974 as well as

normal rat liver for a number of years (Tamiya-Koizumi and Kojima, 1986; Tamiya-Koizumi et al., 1989b; Tsugane et al., 1999). Although neutral SMase is most abundant on plasma membranes (Hostetler and Yazaki, 1979), a significant part of the activity is associated with nuclear fractions (Albi and Magni, 1997; Alessenko and Chatterjee, 1995; Tamiya-Koizumi et al., 1989b). Interestingly, we have observed that apoptosis of hepatocytes in rat liver in vivo might be triggered by activation of a nuclear neutral SMase and nuclear ceramidase (Tsugane et al., 1999). Further, nuclear SMase is activated in apoptotic human erythro-myeloblastic cells after the ionizing irradiation (Jaffrezou et al., 2000). These results may indicate that the nuclear neutral SMase plays an important role in apoptotic signal transduction. The biochemical properties of nuclear neutral SMase resemble those of the plasma membrane-bound enzyme but differ in some points, such as stimulation by both dithiothreitol (DTT) and high concentration of salts (Tamiya-Koizumi et al., 1989b) as well as the effects of phosphatidylserine (Tamiya-Koizumi and Kojima, 1986). Therefore, it is intriguing to speculate whether one of these enzyme species might correspond to the newly cloned rat nSMase 1.

Rat nSMase 1 is composed of 422 amino acids (Mizutani et al., 2000) that share 87.6 and 79.0% identity with mouse and human nSMase 1, respectively. Expressed in *E. coli* and purified to homogeneity, it was found to catalyze sphingomyelin hydrolysis at neutral pH in a Mg^{2+}-dependent manner, requiring Triton X-100, DTT and KCl for full activity (Mizutani et al., 2000) and sharing many properties with the previously described neutral SMase in AH7974 nuclei (Tamiya-Koizumi et al., 1989b).

In the present study, we produced a rabbit polyclonal antibody against the recombinant rat nSMase 1 to analyze its intracellular localization. It has been reported that a high activity of neutral SMase is present in plasma membranes, whereas acidic SMase is found in cytoplasmic lysosomes in rat liver cells (Hostetler and Yazaki, 1979). Previously we showed that the Mg^{2+}-dependent, neutral SMase exists in the nucleus as well as in the plasma membrane of rat ascites hepatoma AH7974 (Tamiya-Koizumi et al., 1989b). The nuclear form resembles the plasma membrane form, but they are not identical in enzymological characteristics (Tamiya-Koizumi et al., 1989b). In order to investigate their relation to the newly cloned rat nSMase 1, we examined the subcellular fractions of AH7974 cells by western blotting and immunoprecipitation using the anti-rat nSMase 1 antibody. This and immunocytochemical analyses demonstrated nSMase 1 to exist predominantly in the nucleus and to some extent in the cytoplasm in AH7974 cells.

MATERIALS AND METHODS

Preparation of nuclei

Nuclei were prepared from rat ascites hepatoma AH7974 cells as described (Tamiya-Koizumi et al., 1989b). AH7974 cells (20 g wet weight) were swollen in 10 volumes of a hypotonic solution containing 2 mM $CaCl_2$, 1 mM $NaHCO_3$, 1 mM $NaHSO_3$, 1 mM benzamidine and 0.1 mM phenylmethylsulfonylfluoride, and disrupted with three strokes of a tight-fitting Dounce homogenizer (Kontes Glass Co., USA). The homogenate was immediately made isotonic by adding sucrose to 0.25 M and centrifuged at 800 ***g*** for 10 minutes to obtain crude nuclei (HITACHI, R20A2 rotor). After washing by centrifugation in 0.25 M sucrose containing 2 mM $CaCl_2$, the crude nuclei were re-suspended in 50 volumes of 2.1 M sucrose containing 2 mM $CaCl_2$ and centrifuged at 48,000 ***g*** for 60 minutes. Purified nuclei, obtained as pellets, were washed twice with 0.25 M sucrose containing 5 mM $MgCl_2$ by centrifugation at 800 ***g*** for 10 minutes. The purity of the nuclei was estimated to be more than 92%, based on electron microscopy and activities of marker enzymes, 5′-nucleotidase and glucose-6-phosphatase, for plasma membranes and microsomes, respectively (Tamiya-Koizumi et al., 1989a).

Preparation of other subcellular fractions

Plasma membranes were prepared from rat ascites hepatoma AH7974 cells as described previously (Koizumi et al., 1976). Mitochondria were obtained by centrifuging the post-nuclear supernatant containing 0.25 M sucrose at 8000 ***g*** for 10 minutes (HITACHI, R20A2 rotor). Pellets were used as the crude mitochondrial fraction. The resulting supernatant was ultracentrifuged at 105,000 ***g*** for 60 minutes (Beckman L70, rotor 50.2Ti) and the pellets obtained were used as microsome.

Preparation of denuded nuclei and subnuclear fractions

Nuclear envelope-depleted nuclei (denuded) were prepared as described previously (Izaurralde et al., 1988; Payrastre et al., 1992). Highly purified nuclei (corresponding to 10 mg DNA) were suspended in 4 ml of 1% Triton X-100 solution containing 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 1 mM DTT for 7 minutes on ice and then centrifuged at 650 ***g*** for 5 minutes. The resultant precipitate was saved as the denuded nuclei fraction and the supernatant as the nuclear envelope fraction for further analyses. Subnuclear fractions were prepared from highly purified nuclei as described (Smith and Berezney, 1982).

Nuclear matrix was prepared by digesting purified nuclei (2.5 mg DNA/ml) with 35 units/ml of bovine pancreatic DNase 1 (Takara Shuzo Co., Kyoto, Japan) at 4°C for 16 hours in 0.25 M sucrose containing 10 mM $MgCl_2$ and Tris/HCl (pH 7.5). The digested nuclei were washed four times with 10 mM Tris/HCl (pH 7.5) containing 0.2 mM $MgCl_2$, and three times with 10 mM Tris/HCl (pH 7.5) containing 0.2 mM $MgCl_2$ and 2 M NaCl, and centrifuged. Supernatant from the first wash with 2 M NaCl was saved as the chromatin fraction. The final pellet was considered to correspond to the high-salt nuclear matrix-1 (Smith and Berezney, 1982).

Solubilization of neutral SMase from nuclei and plasma membranes

Neutral SMase was solubilized from purified nuclei of AH7974 as described (Tamiya-Koizumi et al., 1989b) and from plasma membranes of the same cells essentially in the same way. Purified plasma membranes were sonicated at 0°C for 30 seconds in 10 volumes of 0.1% Triton X-100, 10 mM Tris/HCl (pH 7.5), 0.2 mM $MgCl_2$ and 0.5 mM DTT. The concentration of Triton X-100 was then raised to 1.0%, kept at 4°C overnight, the membrane fraction was centrifuged at 30,000 ***g*** for 10 minutes. The resulting supernatant containing solubilized enzyme was used for the immunoprecipitation experiments. The nuclear extract containing solubilized neutral SMase was also subjected to gel-filtration column chromatography using Sephacryl S-300.

Assay of neutral SMase activity

Activity of neutral SMase was measured in a reaction mixture (50 μl) containing 100 mM Tris/HCl, pH 7.4, 1.2 M KCl, 0.05% Triton X-100, 10 mM $MgCl_2$, and 1 mM DTT, 0.18 nmol of [N-methyl-14C] sphingomyelin (20,000 cpm) (Amersham Pharmacia Biotech), diluted with 0.93 nmol of unlabeled sphingomyelin (Sigma). Incubation was carried out at 37°C for 30 minutes and the reaction was terminated by the addition of 200 μl of chloroform-methanol (2:1, v/v). After

centrifugation, 80 μl of the upper phase, containing released [14C]phosphocholine, was adsorbed onto a glass microfiber filter (GF/C 24, Watman) and dried. Radioactivity was measured in a liquid scintillation spectrometer with a toluene-based scintillator (Scintiblender I, Nacalai Tesque, Kyoto, Japan). Background activities with EDTA were subtracted from apparent activities with crude samples in order to eliminate the effects of other species of enzymes such as acid SMase.

Expression and purification of rat nSMase 1

A cDNA for rat nSMase 1 was subcloned in *Nde*I/*Bam*HI sites of the *E. coli* expression vector pET-15b, and a histidine-tagged rat nSMase 1 (His-nSMase 1) was expressed in *E. coli* BL21 (DE3) (Novagen). His-nSMase 1 was purified from transformed *E. coli* cells using ProBond™ (Invitrogen, Groningen, Netherlands) according to the manufacturer's instructions. Glutathione S-transferase (GST)-fused rat nSMase 1 was prepared as described previously (Mizutani et al., 2000).

Production of polyclonal antibody against rat nSMase 1

An anti-rat nSMase 1 polyclonal rabbit antibody (anti-rnSMase 1 antibody) was raised with 4 intracutaneous injections of 200 μg each of purified His-nSMase 1 mixed with an equal volume of incomplete adjuvant at 2-week intervals into three Japan White rabbits. Antibody was purified from the IgG fraction by an immunoaffinity column chromatography using purified His-nSMase 1-immobilized CNBr-activated Sepharose™ 4B (Amersham Pharmacia Biotech).

Western blot analysis

Partially purified enzyme and crude extracts were subjected to SDS-PAGE, transferred to PVDF membranes (Immobilon™, Millipore), and then reacted with the anti-rnSMase 1 antibody. A mouse anti-KDEL monoclonal antibody (StressGen), that recognizes the glucose regulation protein (Grp78), was used to stain microsomes/endoplasmic reticulum (ER). The reactive protein bands were visualized with horseradish peroxidase-conjugated goat IgG against rabbit or mouse IgG (Biosource). Sizes of the reactive proteins were estimated with molecular mass markers (Nacalai Tesque). After several washing steps, protein bands were visualized with Immuno Star Reagents (Wako, Tokyo, Japan).

Immunoprecipitation

Aliquots of extracts (20 μl) were incubated with various amounts of anti-rnSMase 1 antibody on ice for 3 hours. Antigen-antibody complexes were adsorbed on 25 μl of the protein A-Sepharose™ CL-4B (Amersham Pharmacia Biotech) by keeping on ice for 36 hours, and then removed by centrifugation. The remaining nSMase activity in each supernatant was assayed to determine reactivity with the anti-rnSMase 1 antibody.

Immunofluorescence microscopy

AH7974 cells were washed with PBS and smeared on a poly-L-lysine (Sigma)-coated slide glasses. They were fixed with 3% paraformaldehyde in PBS for 15 minutes on ice and then treated with 0.5% Triton X-100 for 20 minutes at room temperature. Normal rat fibroblast 3Y1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. After washing with PBS, they were fixed with 3% paraformaldehyde in PBS for 30 minutes on ice and then treated with 0.5% Triton X-100 in PBS for 10 minutes at room temperature. After blocking with 3% bovine serum albumin in PBS for 1 hour, fixed cells were incubated with the anti-rnSMase 1 antibody for 2 hours at room temperature. The cells were then incubated with the secondary antibody that was labeled with FITC (green fluorescence). Mouse anti-KDEL monoclonal antibody was used to stain cytoplasm in the same way as described for the anti-rnSMase 1 antibody. After washing, cells were incubated with Cy3-conjugated second antibody (red fluorescence) for 30 minutes at room temperature. Nuclear DNA was stained with DAPI. Triple staining with these two antibodies and DAPI was also performed. Staining signals were detected under a fluorescence microscopy or confocal laser microscopy (BioRad, MRC-1024), and whole cell contours were observed under a phase-contrast microscopy.

Electron microscopy

The nucleus and plasma membrane of AH7974 cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 3 hours on ice. After washing over night in the cacodylate buffer, samples were post-fixed with 1% osmium tetraoxide in distilled water for 3 hours on ice. After dehydration in a graded series of ethanol, samples were finally embedded in epoxy resin (Epon 812). Ultra thin sections were obtained on a LKB-8800 ultramicrotome sections and then stained with 2% uranyl acetate in distilled water for 10 minutes and lead-citrate for 5 minutes. These sections were viewed under a transmission electron microscope JEOL JEM-2000EX at 100 kV.

Immuno-electron microscopy

AH7974 cells were fixed in 0.1 M sodium cacodylate containing 4% paraformaldehyde and 0.5% glutaraldehyde (pH 7.4), dehydrated in a graded series of ethanol, and immersed in a mixture of Lowincryl

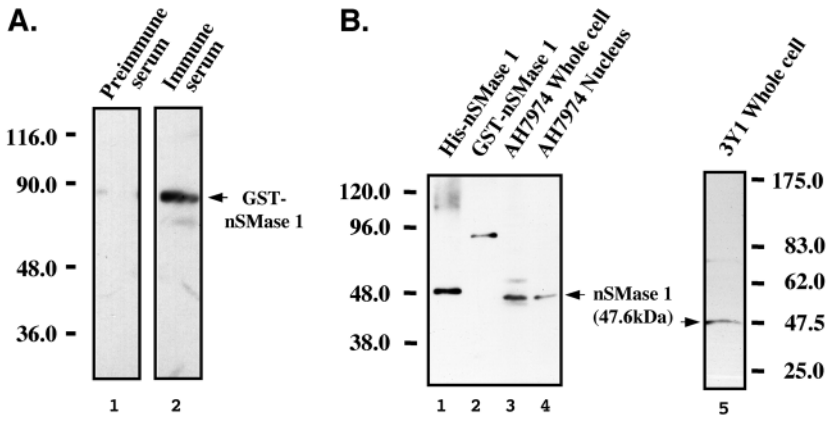

Fig. 1. Specific detection of nSMase 1 by western blotting using a rabbit polyclonal antibody. (A) Detection of GST-tagged nSMase 1 expressed in *E. coli* with immune rabbit serum. Lysate of *E. coli* expressing GST-tagged recombinant nSMase 1 was electrophoresed on 8% SDS-polyacrylamide gel, transferred to a membrane, and probed with immune rabbit serum (dilution 1:1000, lane 2), as described in Materials and Methods. An immunoreactive protein band was detected at 76.1 kDa, the predicted value for the GST-tagged nSMase 1. As a control, preimmune rabbit serum was used (lane 1). (B) Western blot analysis of the whole cell lysates and a nuclear extract using an affinity-purified anti-rnSMase 1 antibody. Whole cell lysates of AH7974 (lane 3) and 3Y1 (lane 5) were analyzed by western blotting using an affinity-purified antibody. Histidine-tagged nSMase 1 (49.4 kDa, lane 1) and GST-conjugated nSMase 1 (76.1 kDa, lane 2) were also stained as positive controls. Nuclear extract of AH7974 was also analyzed (lane 4). The arrow indicates the immunoreactive band detected at 47.6 kDa, corresponding to the predicted value for the full-length rat nSMase 1.

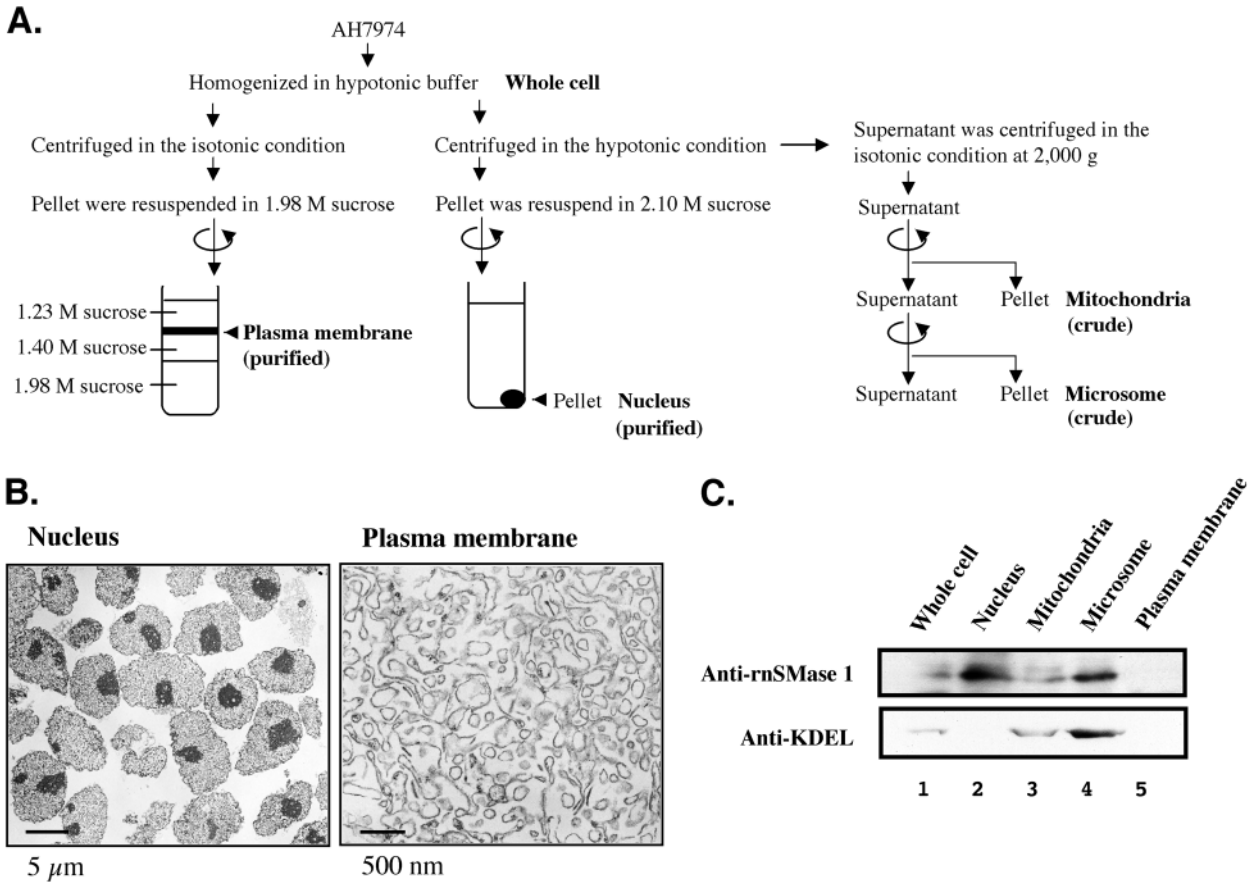

Fig. 2. Detection of nSMase 1 in subcellular fractions of AH7974 cells. (A) Scheme for isolating subcellular fractions of AH7974 cells (for details, see Materials and Methods). Pellets obtained by centrifugation's at 8000 ***g*** and 105,000 ***g*** were designated as mitochondrial and microsomal fractions, respectively. They may contain some of other organelle but were not purified further. Nucleus and plasma membrane were highly purified by this protocol, as shown in the electron microscopic photographs (B). (C) Subcellular fractions of AH7974 (50 μg protein each) were subjected to western blotting with the anti-rnSMase 1 antibody. An immunoreactive protein band was detected at 47.6 kDa. The microsomal marker Grp78 was also detected with an anti-KDEL antibody in the same way. Extracts of whole cells and organelle showed the bulk activities of neutral SMases (nmol/mg protein/hour) as follows: whole cell, 17.7; nucleus, 5.80; mitochondria, 20.4; microsome, 26.3; plasma membrane, 22.2.

K4M resin (Polyscience Inc.) and 94% ethanol (1:1) for 1 hour. Samples were embedded in the resin, which was then polymerized by irradiating ultraviolet light for 48 hours at −20°C. Ultra-thin sections (0.1 μm thick), prepared with LKB Ultramicrotome 8800 and mounted on nickel 200 grids, were then treated with anti-rnSMase 1 antibody for 1 hour, goat anti-mouse IgG conjugated with gold colloid (10 nm diameter) for 1 hour, successively. After the immunoreactions, the sections were stained with 2% uranyl acetate for 5 minutes, and examined under JEOL JEM-2000EX electron microscope.

Expression of enhanced green fluorescent protein (GFP)-conjugated nSMase 1

cDNA for rat nSMase 1 was inserted into *Eco*RI site of pEGFP-C1 (Clontech, UK), a vector for expressing enhanced GFP-fusion protein. 3Y1 cells were cultured for 24 hours in 24-well microplates, and then transformed with pEGFP-C1 harboring rat nSMase 1 cDNA. The plasmid DNA (0.4 μg) was dissolved in 25 μl of serum-free medium, which was mixed with 0.6 μl of FuGene^{TM} 6 Transfection Reagent (Roche, USA) and stood for 15 minutes, and then added to cell cultures, according to the manufacturer's protocol. After 48 hours, cells on plates were examined under a fluorescence microscope (BioRad, MRC-1024). Whole cell contours were observed under a phase-contrast microscopy.

RESULTS

Specificity of the antibody

Western blot analysis showed that immune rabbit serum raised against recombinant rat nSMase 1 specifically recognized GST-fused rat nSMase 1 in the crude extract of *E. coli* (Fig. 1A). Polyclonal rabbit antibody (anti-rnSMase 1 antibody), which was purified by an affinity column conjugated with antigen, strongly reacted with histidine-tagged nSMase 1 and GST-conjugated nSMase 1 (Fig. 1B, lanes 1,2). With the whole cell lysates of AH7974 ascites hepatoma cells and rat fibroblast 3Y1, this antibody stained a protein as essentially a single band at 47.6 kDa, corresponding to the molecular mass of full length rat nSMase 1 (Fig. 1B, lanes 3,5). It also showed the nSMase 1 signal with the extract of purified nuclei from AH7974 cells (Fig. 1B, lane 4). From these results, we conclude that our polyclonal antibody is highly specific to rat nSMase 1.

Existence of nSMase 1 in nucleus of rat ascites hepatoma cell AH7974

To analyze the subcellular distribution of nSMase 1, we

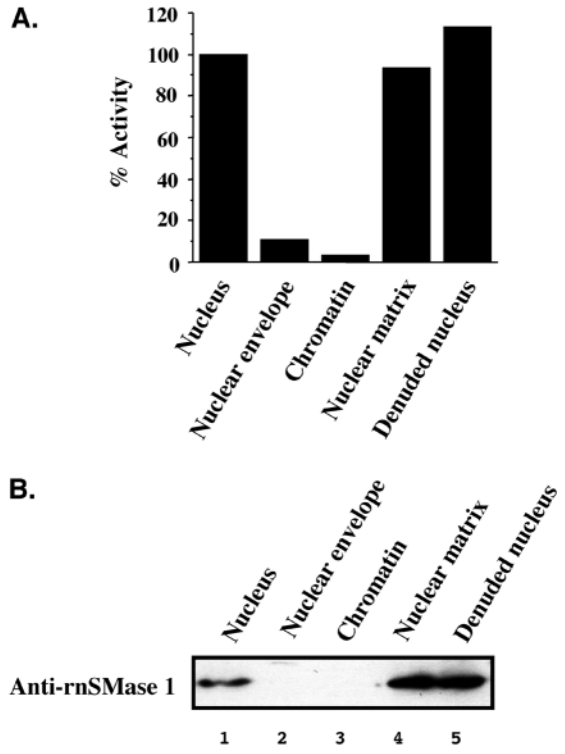

Fig. 3. Detection of nSMase 1 in subnuclear fractions of AH7974 cells. (A) Neutral SMase activity in subnuclear fractions, such as nuclear envelope, chromatin, nuclear matrix, and denuded nucleus were measured. 10 µl aliquots of each sample (4 ml) were assayed for neutral SMase activity. Activities were relative to that in the intact nucleus, which was taken as 100%. (B) Western blotting of subnuclear fractions were carried out using the anti-rnSMase 1 antibody with aliquots of subnuclear fractions corresponding to 75 µg DNA, as indicated. Immunoreactive protein bands were detected at 47.6 kDa.

fractionated AH7974 cells into purified nuclei, crude mitochondria, crude microsome and purified plasma membrane, by the procedure summarized in Fig. 2A (for more details, see Materials and Methods). The purities of nuclei and plasma membrane fractions were examined by electron microscopy (Fig. 2B). These fractions were subjected to western blotting. As shown in Fig. 2C, the polyclonal antibody clearly stained the protein in both nuclei (lane 2) and microsomes (lane 4) at the molecular weight corresponding to that of rat nSMase 1 (47.6 kDa). It was remarkable that the lysate of plasma membrane, which contained high neutral SMase activity among fractions (legend for Fig. 2), did not show any nSMase 1 signals (Fig. 2C, lane 5). Mitochondria showed a faint signal of nSMase 1 (Fig. 2C, lane 3). This could be due to trace amounts of microsomes existing in the crude mitochondrial fraction that was detected by the anti-KDEL microsome marker (Fig. 2C, lane 3). Results of western blotting described here strongly suggest that rat nSMase 1 is localized in the nucleus as well as in the ER/microsome. It is also suggested that the plasma membrane enzyme differs to that in nucleus or microsome.

Tomiuk et al., have suggested that mouse nSMase 1 is mainly localized in cytoplasm, especially on the endoplasmic reticulum (ER) in mouse liver (Tomiuk et al., 2000). To rule out the possibility that our purified nuclei were associated with microsomes as a part of the outer nuclear envelope, we prepared denuded nuclei by treatment with Triton X-100. The content of neutral SMase activity in intact nuclei was not decreased significantly by this treatment (Fig. 3A) and western blotting of the denuded nuclei showed that the nSMase 1 signal did not decrease in intensity (Fig. 3B, lane 5). These results, combined with the high purity of nuclei (Fig. 2B), strongly suggest that the enzyme activity and western blot signal in nuclei were not due to contaminating microsome/ER, and that the nSMase 1 exists inside the nucleus of the AH7974 cell.

Association of nSMase 1 with the nuclear matrix

On further fractionation of nuclei into nuclear envelope and nuclear matrix fractions, as well as a DNase I-digested supernatant containing dissolved chromatin proteins, almost all neutral SMase activity was detected in the nuclear matrix (Fig. 3A). Western blotting demonstrated strong signals for nSMase 1 in the nuclear matrix fraction, whereas the nuclear envelope and chromatin fraction were negative (Fig. 3B, lanes 2-4). These results, which point to a nuclear matrix localization, are consistent with our previous observation that a large part of nuclear neutral SMase activity existed as particle-bound (presumably nuclear matrix) and cannot be solubilized efficiently by the conventional extraction with high salt buffer (Tamiya-Koizumi et al., 1989b).

Identification of the nuclear neutral SMase as the nSMase 1

Activities in extracts from the highly purified nuclei and the plasma membrane were immunoprecipitated with various amount of the antibody and the remaining neutral SMase activities in supernatants were measured, in comparison with that of purified rat nSMase 1 (Fig. 4A). In nuclear extract precipitated with antibody, the reactivity was of lower efficiency compared with purified His-nSMase 1. From these results, we estimated that 50% or more of the nuclear neutral SMase activity is accounted for by nSMase 1. By contrast, the enzyme in the plasma membranes was not immunoprecipitated by this antibody (Fig. 4A). The neutral SMase activity in the microsome fraction was immunoprecipitated at a much lower efficiency (approximately 20%; data not shown).

To examine whether nSMase 1 is identical to the previously described nuclear neutral SMase of AH7974 cells (Tamiya-Koizumi et al., 1989b), we partially purified the neutral SMase from nuclear extracts by gel-filtration through a Sephacryl S-300 column. Active fractions were subjected to western blotting using anti-rnSMase 1 antibody. As shown in Fig. 4B, the signal for nSMase 1 coincided well with the activity peak. These results indicate that a large part of the neutral SMase activity in AH7974 nucleus is attributable to nSMase 1.

Immunocytochemical study

AH7974 cells were immunostained with anti-rnSMase 1 and anti-KDEL antibodies. The precise location of nuclei could be shown by DNA staining with DAPI and cell contours were delineated by phase-contrast microscopy. As shown in Fig. 5B, almost all nuclei were stained with anti-rnSMase 1 antibody. Faint staining was recognized in some parts of the cytoplasm, but no staining signal was evident in the plasma membrane. Merging the ER/microsome staining with anti-KDEL antibody

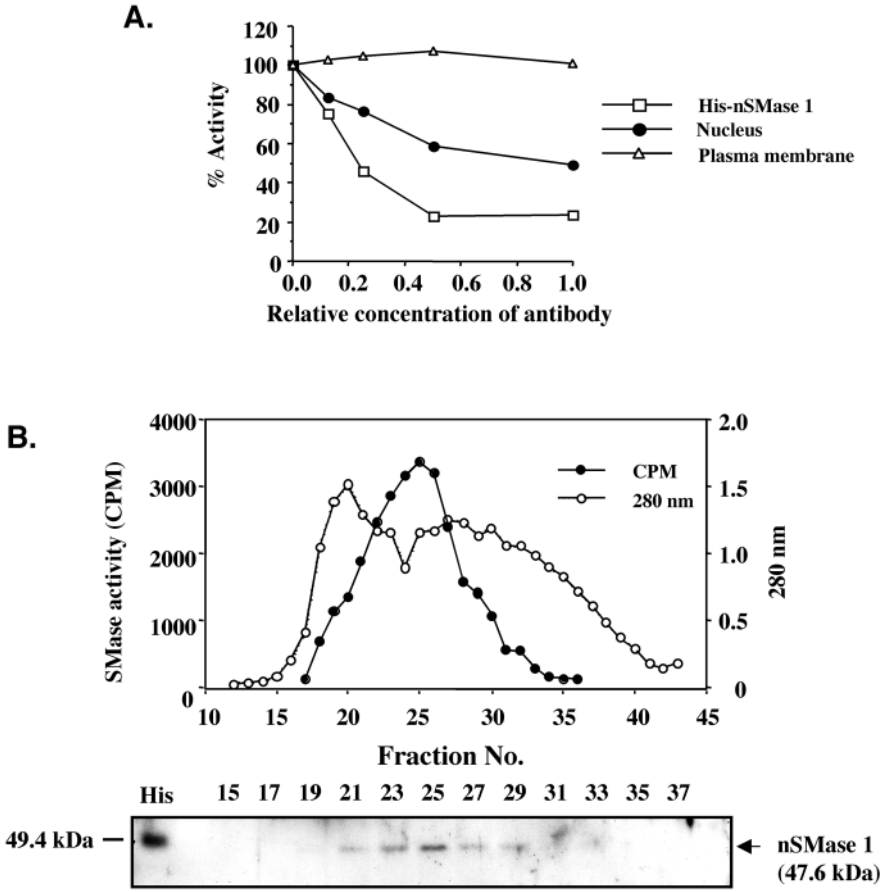

Fig. 4. Identification of nuclear neutral SMase as nSMase 1. (A) Immuno-precipitation of the neutral SMase activity in extracts from nuclei and plasma membranes with anti-rnSMase 1 antibody. Extracts were prepared as described in Materials and Methods. Aliquots (20 μl) containing activity of neutral SMase (2.5 nmol/hour) were mixed with the anti-rnSMase 1 antibody, and antigen-antibody complexes were adsorbed onto protein A-Sepharose beads. After the incubation, the mixture was centrifuged and supernatants were assayed for neutral SMase activity, indicated as% of the control. B. Nuclear extract (10 ml) from AH7974 cells was applied to a Sephacryl S-300 column (1.2 × 85 cm) and 7 ml fractions were collected. Activity of neutral SMase was assayed with 10 μl aliquots of each fraction, and the peak fractions (10 μl each) were subjected to Western blotting using anti-rnSMase 1 antibody. As a molecular size marker, histidine-tagged nSMase 1 (49.4 kDa) was shown in the first lane. Open circles, optical density at 280 nm; closed circles, neutral SMase activity.

with nSMase 1 staining showed little co-localization (Fig. 5G). The isolated nuclei of AH7974 were also positively stained with anti-rnSMase 1 antibody (data not shown). Then, we stained normal rat fibroblast 3Y1 cells in the same manner as AH7974 cells. In this case, again, nuclei were clearly stained with the antibody (Fig. 6B). Pretreatment of the antibody with purified nSMase 1 completely abolished fluorescence signals on nuclei (Fig. 5F; Fig. 6F).

Immuno-electron microscopy

Immuno-electron microscopy of thin sections of AH7974 cell revealed that clusters of gold colloid particles, signals for nSMase 1, were observed on the nucleus more densely than on cytoplasm (Fig. 5H). We counted the number of gold particle clusters (more than 3 particles) in several fields, and found the ratio of nucleus/cytoplasm to be from 2.0 to 5.0. The control staining without the first antibody (anti-rnSMase 1) did not show significant signals (Fig. 5I).

Localization of overexpressed GFP-nSMase 1 in 3Y1 cells

We constructed a recombinant plasmid for expressing GFP-conjugated rat nSMase 1 in mammalian cells. 3Y1 cells were transformed with this plasmid and were examined under a fluorescence microscope. As shown in Fig. 6H, fluorescence due to overexpressed GFP-nSMase 1 was detected in cytoplasm almost exclusively, and was much weaker in nuclei. The cytoplasmic localization of overexpressed GFP-nSMase 1 was also observed with a monkey cell line COS-7 (data not shown). Further, anti-rnSMase 1 antibody strongly stained the cytoplasm of these transformed cells, confirming the cytoplasmic accumulation of overexpressed nSMase 1 (data not shown). These results are in a sharp contrast to the nuclear localization of endogenous rat nSMase 1 (Fig. 5; Fig. 6A-F), and suggest that overexpressed nSMase 1 cannot be transported efficiently into nuclei.

DISCUSSION

In the present study we showed that rat nSMase 1 is localized in the nucleus of rat cells, with a polyclonal antibody raised against a recombinant full-length rat nSMase 1. Western blotting thus provided strong evidence that nSMase 1 is found in nuclei as well as in cytoplasm, but not in the plasma membrane, of rat ascites hepatoma cell AH7974 (Fig. 2C). Furthermore, nuclear nSMase 1 was not removed either by denuding the outer nuclear envelope or by digestion with DNase I followed by washing with low and high salt buffers (Fig. 3B). Immunoprecipitation experiments (Fig. 4A) also supported a nuclear localization and it was remarkable that the activity peak of nuclear neutral SMase from gel-filtration correlated well with the western blotting signal using anti-rnSMase 1 antibody (Fig. 4B). Possible contamination by microsomes in the nuclear fraction could be carefully ruled out by purifying nuclei through high density sucrose, examination by electron microscopy, and with marker enzymes.

Consistently, immuno-cytochemical study showed that the

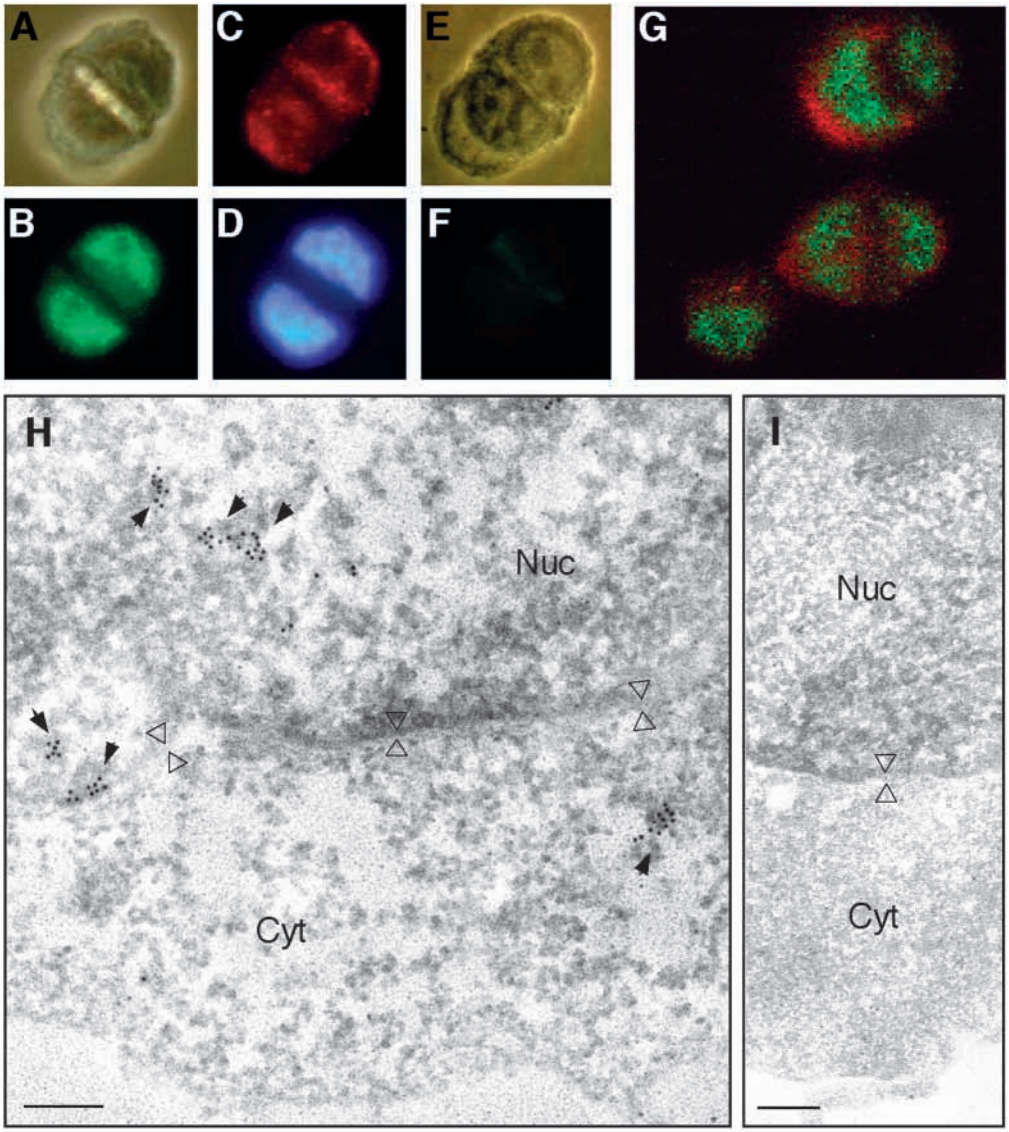

Fig. 5. Immunocytochemistry and immuno-electron microscopy of AH7974 cells. AH7974 cells were stained with anti-rnSMase 1 antibody and the localization of enzyme was observed by immuno-fluorescence microscopy (A-G) and immuno-electron microscopy (H,I) as described in Materials and Methods. (A) Phase contrast of AH7974 cells. (B) Cells in A stained for nSMase 1 with anti-rnSMase 1 antibody (green fluorescence of FITC). (C) Cells in A stained for cytoplasm with anti-KDEL antibody (red fluorescence of Cy^{TM}3). (D) Cells in A stained of nuclear DNA with DAPI. (E) Phase contrast of AH7974 cells. (F) Control staining of cells as in E with the antibody pre-treated with purified rat nSMase 1 at 4°C for 3 hours. (G) Merge of two pictures of confocal laser microscopy stained with anti-rnSMase 1 (green) and anti-KDEL (red). (H) Immuno-electron microscopy of an AH7974 cell with anti-rnSMase 1 antibody. Localization of the enzyme was shown by gold colloid particles (10 nm) conjugated with second antibody. (I) Control staining of immuno-electron microscopy without first antibody. Bar, 200 nm. Abbreviations: Nuc, nucleus; Cyt, cytoplasm; arrows indicate clusters of gold particles, and open arrowheads indicate nuclear

nuclei of AH7974 cells, as well as of 3Y1 rat fibroblasts, were strongly stained with anti-rnSMase 1 antibody, while the cytoplasm was stained only weakly (Fig. 5B; Fig. 6B). Immuno-electron microscopy of the thin sections of AH7974 cells showed gold colloid particles, signals for nSMase 1, to be more dense in the nuclei than in the cytoplasm of AH7974 cells (Fig. 5H). These results strongly support the nuclear localization of nSMase 1 in AH7974 cells.

Our conclusion that nSMase 1 exists in the nucleus is in a sharp contrast with that proposed by other groups (Fensome et al., 2000; Rodrigues-Lima et al., 2000; Tomiuk et al., 2000). Fensome et al., reported fluorescence signals of an GFP-conjugated mouse nSMase 1 localized in ER in NIH 3T3, COS and MDCK cells after transfection or microinjection of a recombinant DNA construct expressing nSMase 1 (Fensome et al., 2000). Rodorigues-Lima et al. demonstrated a GFP-conjugated nSMase 1 in ER and showed that the ER localization depends on the transmembrane region of nSMase 1 (Rodrigues-Lima et al., 2000). Tomiuk et al., showed overexpressed mouse nSMase 1 to be detected in association with ER using a polyclonal antibody against a purified N-terminal fragment of mouse nSMase 1 in a mouse primary embryonic fibroblast that had been transiently transfected with a mouse nSMase 1 expressing vector (Tomiuk et al., 2000).

At present, the reason for the apparent discrepancy in intracellular localization of nSMase 1 is unclear, but it could be attributable to differences in the experimental systems. Overexpression of protein might not always reproduce the intracellular localization of the endogenously expressed protein. Keech et al. have demonstrated that a nuclear autoantigen Ro/SS-A, which lacks NLS, accumulates in cytoplasm but exists in nuclei to a lesser extent, when overexpressed by transfection of human cDNA into human or mouse cells (Keech et al., 1995). In this context, we overexposed GFP-conjugated rat nSMase 1 in 3Y1 cells and observed the fluorescence signal to be much stronger in the cytoplasm than in the nucleus (Fig. 6H). A large amount of overexpressed nuclear protein that lacks a nuclear localization signal (NLS) might accumulate in cytoplasmic ER and exhibit strong fluorescence masking weaker signals in the nucleus.

It is possible that localization depends on the physiological state of cells. In this context, it is noteworthy that the nSMase

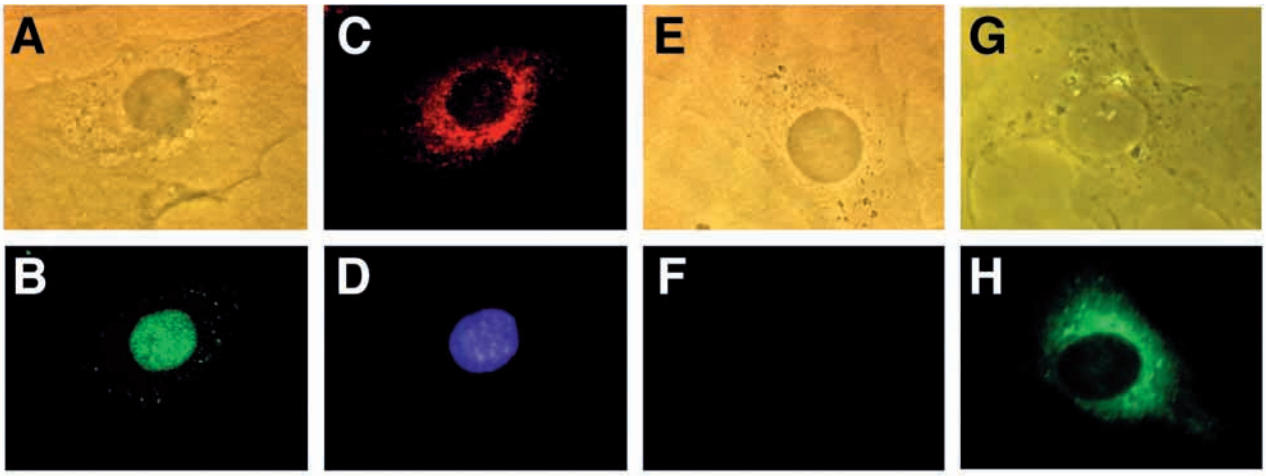

Fig. 6. Comparison between intracellular localization of endogenous nSMase 1 and overexpressed GFP-nSMase 1 in normal rat fibroblast 3Y1 cells. (A) Phase-contrast of 3Y1. (B) Cells in A stained for nSMase 1 with anti-rnSMase 1 antibody (green). (C) Cells in A stained for cytoplasm with anti-KDEL antibody (red). (D) Cells in A stained for nuclear DNA with DAPI. (E) Phase contrast of 3Y1. (F) Control staining of cells in E with the antibody pre-treated with purified rat nSMase 1. (G) Phase contrast of 3Y1 cells in H. (H) Fluorescence of 3Y1 cells transformed with plasmid expressing GFP-nSMase 1 (green).

1 contains a sequence, LLVLHLSGLVL at amino acids 120-130, which is homologous to the nuclear export signal (NES) (Bogerd et al., 1996; Gorlich and Mattaj, 1996; Nigg, 1997), a short leucine-rich motif that has recently been identified as mediating active nuclear export. Although a typical NLS was not identified in nSMase 1, it might be transferred into nuclei by association with other molecules, and into cytoplasm in the NES-dependent manner under certain physiological conditions.

The immunocytochemical experiment most comparable with ours is that by Fensome et al., with detection of endogenous nSMase 1 in ER of PC12 cells using a polyclonal antibody raised against a synthetic 15-mer olygopeptide (amino acids 46-60) (Fensome et al., 2000). At present, we have no explanation for different results regarding localization of the endogenous nSMase 1. It could be due to different conditions for cell fixation or to different cell types, but the conclusions must await further study.

Recently, Sawai et al. have demonstrated that nSMase 1 cleaves lyso-platelet activating factor (lyso-PAF) besides sphingomyelin (Sawai et al., 1999). Since overexpression of this enzyme in HEK293 cells does not increase the intracellular concentration of ceramide but accumulates 1-O-alkyl-sn-glycerol with corresponding decrease of 1-alkyl-2-acyl-sn-glycerophosphocholine, they concluded that it is a lyso-PAF phospholipase C rather than a sphingomyelinase (Sawai et al., 1999).

We have also examined substrate specificity of the purified rat nSMase 1 in vitro, and found it to hydrolyze lyso-PAF but less efficiently than sphingomyelin (Mizutani et al., 2000). Tonnetti et al. suggested the involvement of nSMase 1 in ceramide production in T lymphoid cells (Tonnetti et al., 1999). They showed that T cell receptor-mediated ceramide production in T cell hybridoma 3DO is inhibited by expressing antisense RNA complementary to nSMase 1 cDNA (Tonnetti et al., 1999). This in vivo result, combined with our biochemical data in vitro (Mizutani et al., 2000), indicates that this enzyme acts as neutral SMase. However, under certain conditions, it might hydrolyze other species of lipids such as lyso-PAF.

Regarding the function of nuclear nSMase 1, its subnuclear localization provides clues, the major portion being here found in association with the nuclear matrix rather than the nuclear envelope or chromatin (Fig. 3B). Previously, Ishihara et al., demonstrated that purified nuclei from rat hepatocytes or AH7974 cells contain significant amounts of phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin (Ishihara et al., 1991). These phospholipids are distributed among chromatin, nuclear envelope, and in nuclear matrix (Tamiya-Koizumi, 1996). Since the nuclear matrix is a structure extending throughout the nucleus, the associated nSMase 1 might cleave sphingomyelin in chromatin and nuclear envelope, as well as that in the nuclear matrix itself to generate ceramides. The rat liver nucleus also contains a ceramidase that cleaves ceramide into sphingosine (Tsugane et al., 1999), a functional lipid that mediates apoptotic cell death, presumably by inhibiting a wide variety of enzymes including protein kinase C (Arnold and Newton, 1991; Jefferson and Schulman, 1988) and DNA primase (Simbulan et al., 1994). One of the roles of nuclear nSMase 1 could thus be related to apoptosis. Upon ligation of the portal vein branch of rat liver, a large population of hepatocytes in the ligated lobes rapidly undergoes apoptosis (Tsugane et al., 1999). This process might be triggered by activation of both nuclear sphingomyelinase and ceramidase with concomitant increase in intranuclear concentrations of both ceramide and sphingosine (Tsugane et al., 1999). In this connection, a nuclear neutral SMase was reported to be activated in radiation-induced apoptosis of erythro-myeloblastic cell line, TF-1 (Jaffrezou et al., 2000).

It is also possible that nuclear nSMase 1 is involved in physiological processes in the cell cycle such as the drastic changes that occur in architecture of the nuclear envelope, chromatin and nuclear matrix, especially at the G_2/M transition. Alternatively, nSMase 1 might modify the lipid

environment for DNA polymerase reaction (Yoshida et al., 1989). In this connection, Albi et al., have demonstrated that neutral SMase (Albi and Magni, 1997; Albi et al., 1999; Micheli et al., 1998) and sphingomyelin (Albi and Magni, 1997; Albi et al., 1994; Albi et al., 1999; Micheli et al., 1998) exist in the nuclei of rat liver cells. They showed that activity of the chromatin-associated neutral SMase increases at the beginning of S phase (Albi and Magni, 1997).

Presently it is uncertain how many species of neutral SMases exist in nuclei. To clarify the biological role of nSMase 1, the reported neutral SMase activities that are induced or suppressed under various conditions must be examined regarding their relationships with nSMase 1. The antibody developed here would be a useful tool to analyze this issue and to clarify the novel nuclear lipid metabolism connected with physiological functions.

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