Involvement of Neutral Ceramidase in Ceramide Metabolism at the Plasma Membrane and in Extracellular Milieu*

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Neutral ceramidase is a type II integral membrane protein, which is occasionally secreted into the extracellular milieu after the processing of its N-terminal anchor (Tani, M., Iida, H., and Ito, M. (2003) J. Biol. Chem. 278, 10523–10530). We found that when overexpressed in CHO cells, neutral ceramidase hydrolyzed cell surface ceramide, which increased in amount after the treatment of cells with bacterial sphingomyelinase, leading to an increase in the cellular level of sphingosine and sphingosine 1-phosphate. On the other hand, knockdown of the endogenous enzyme by siRNA decreased the cellular level of both sphingolipid metabolites. The treatment of cells with bovine serum albumin significantly reduced the cellular level of sphingosine, but not sphingosine 1-phosphate, generated by overexpression of the enzyme. The cellular level of sphingosine 1-phosphate increased with overexpression of the cytosolic sphingosine kinase. These results suggest that sphingosine 1-phosphate is mainly produced inside of the cell after the incorporation of sphingosine generated on the plasma membranes. The enzyme also seems to participate in the hydrolysis of serum-derived ceramide in the vascular system. Significant amounts of sphingosine as well as sphingosine 1-phosphate were generated in the cell-free conditioned medium of ceramidase transfectants, compared with mock transfectants. No increase in these metabolites was observed if serum or bacterial sphingomyelinase was omitted from the conditioned medium, suggesting that the major source of ceramide is the serum-derived sphingomyelin. A sphingosine 1-phosphate receptor, S1P3, was internalized much faster by the treatment of S1P3-overexpressing cells with conditioned medium of ceramidase transfectants than that of mock transfectants. Collectively, these results clearly indicate that the enzyme is involved in the metabolism of ceramide at the plasma membrane and in the extracellular milieu, which could regulate sphingosine 1-phosphate-mediated signaling through the generation of sphingosine.

Several lines of evidence indicate that ceramide (Cer) and its metabolites, sphingosine (Sph) and sphingosine 1-phosphate (S1P), are potential lipid mediators controlling various cellular functions such as proliferation, migration, apoptosis, and endocytosis (1–3). Cer has been shown to be a potential mediator for apoptosis in response to cytokines, antigens, anticancer drugs, and environmental stress inducers, possibly through the regulation of various cytoplasmic proteins such as protein kinases C-ζ (4), -α, and -δ (5), and protein phosphatases 1 and 2A (6). Sph is also known to be a regulator for various cytoplasmic protein kinases; an inhibitor for protein kinase C (7), and an activator for 3-phosphoinositide-dependent kinase 1 (8) and Sph-dependent protein kinase 1 (9). S1P is a ligand for the G protein-coupled, plasma membrane-spanning receptors S1P1–5 (10). S1P-mediated signaling regulates the migration and proliferation of endothelial cells and smooth muscle cells, being involved in the formation and maintenance of the vascular system. S1P has been also implicated as an intracellular second messenger in the regulation of a variety of cellular processes, including cell proliferation, differentiation, migration, and apoptosis (3). Intracellular S1P seems to be generated through phosphorylation of Sph by Sph kinase, which is mainly located in the cytosol (3). However, the molecular mechanism by which Sph is supplied to Sph kinase has not yet been clarified. In the vascular system, platelets are thought to be a source of S1P because they contain a very large amount of S1P because of the strong activity of Sph kinase and a lack of S1P lysase, and are known to release S1P when stimulated (11). Alternatively, it was reported that intracellular S1P (12, 13) and Sph kinase (14) are released from cells through unknown mechanisms.

Sph is considered to be exclusively generated from Cer through the action of ceramidase (CDase, EC 3.5.1.23) because de novo synthesis of Sph has been ruled out experimentally because of the specificity of Δ4 desaturase, which acts on dihydroCer to convert Cer but not on dihydrosph (15). To date, acid, neutral, and alkaline CDases have been identified, which are clearly distinguished by catalytic pH optimum, primary structure, and localization (16). However, which CDase species are involved in the generation of S1P and S1P-mediated signaling remains to be elucidated.

Neutral CDase, which shows an optimal pH of 6.5–8.5 (except the enzyme from slime mold whose pH optimum is 3.5–4.0), has been cloned from bacteria (17), slime mold (18), fruit fly (19), zebrafish (20), mouse (21), rat (22), and human (23). Murine neutral CDases are localized at the plasma membrane as a type II integral membrane protein and occasionally released from cells after processing of the N-terminal anchoring region (24). O-Glycosylation of a mucin-like domain (mucin box) near the N terminus was found to be required for retention of the enzyme on the plasma membrane (24). The localization of human neutral CDase is controversial, i.e. a form lacking the N-terminal 13 amino acids was exclusively found in mitochondria (23) whereas the full-length enzyme was found in plasma membranes or in the medium when modified Eagle’s medium; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; HPLC, high performance liquid chromatography; SCR, scrambled sequence; HA, hemagglutinin.
Neutral Ceramidase-mediated S1P Generation and Signaling

expressed in HEK293 cells (25). Recently, it was reported that targeted expression of neutral CDase rescued the retinal degeneration in arrestin and phospholipase C mutants of Drosophila through modulation of the endocytosis of rhodopsin in photoreceptors (26, 27). We also found that the gene knockdown of zebrafish neutral CDase during embryogenesis resulted in a defect of blood cell circulation (20). It should be noted that neutral CDase activity was regulated by several cytokines and growth factors (28, 29).

In this study, we found that mouse neutral CDase is involved in the metabolism of Cer at the plasma membrane and in the extracellular milieu. Sph generated in the plasma membrane by the CDase was promptly incorporated into the cells and then phosphorylated by cytosolic Sph kinase while Sph generated in the serum by the secreted CDase was then phosphorylated by serum-derived Sph kinase. These two pathways for the generation of S1P involving neutral CDase are demonstrated for the first time in this study.

EXPERIMENTAL PROCEDURES

Materials—CHO cells were kindly provided by Dr. K. Nara (Mitsubishi Kagaku Institute of Life Sciences). Horseradish peroxidase-labeled anti-mouse IgG antibody and o-phthalaldehyde (OPA) were purchased from Nacalai Tesque. Doxorubicin and Triton X-100 were obtained from Sigma, EGL plus from Amersham Biosciences, Bacillus cereus sphingomyelinase from Funakoshi, [14C]palmitic acid and [14C]stearic acid from Amersham Biosciences Radiolabeled Chemicals, anti-Myc antibody from Invitrogen, anti-HA antibody and rhodamine-labeled anti-mouse IgM from Santa Cruz Biotechnology, anti-Cer monoclonal antibody 15B4 from Boicyte Biotech, and dimethylsphingosine from BIOMOL. Anti-Sph kinase type I antibody was raised in a rabbit using the recombinant mouse Sph kinase as an antigen (30). C12-NBD-Cer was prepared as described (31). All other reagents were of the highest purity available.

CDase Assay—The activity of neutral CDase was measured using C12-NBD-Cer as a substrate (32).

Plasmid Construction—To obtain a mouse neutral CDase tagged with Myc at the C terminus (wild-type CDase), cDNA encoding the CDase was subcloned into the vector pcdNA3.1/Myc-His (+) (Invitrogen) by PCR using a 5′ primer with a KpnI restriction site (5′-AGGGTACCCGAATTCCGGAAGGAACCTTCTCC-3′) and a 3′ primer with a XhoI restriction site and a disrupted stop codon (5′-GGCGTCTGGAGTAGTGTGACAATTTCAAGGGGAAG-3′). To obtain a CDase mutant, which possesses the secretory signal sequence of the mouse Vh chain instead of the signal-anchor sequence (SecCD), a DNA fragment of the CDase lacking the signal/anchor sequence and containing a sequence encoding a Myc-His epitope and a stop codon at the 3′-end, was amplified with a 5′ primer with a NotI restriction site (5′-CCACATGCGAAACGGAGAACCTTCTCC-3′) and a 3′ primer with a NotI restriction site (5′-GGCGTCTGGAGTAGTGTGACAATTTCAAGGGGAAG-3′). These fragments were digested with PstI and NotI, and subcloned into the vector pCMV/Myc/ER (Invitrogen). The sequences of these constructs were verified by a DNA sequencer (Applied Biosystems Japan; Model 377).

RNA Interference—Gene silencing of the neutral CDase in mouse B16 cells was performed using the RNAi-Ready pSIREN-DNR vector (Clontech) according to the instructions of the manufacturer. The sequence-specific DNA oligonucleotide was selected from mouse neutral CDase cDNA as described below. Mouse neutral CDase starting 721 nucleotides from the initiation codon, TCCACAGTCAAGAGAGGAC, and a scrambled sequence (SCR) for the control experiment, GATGACAGTCAAGAGGACC, were used. The sequences were evaluated against the data base using the NIH BLAST program to test for specificity. To express siRNA, B16 cells were transfected with the target sequence inserted in pSIREN-DNR using Lipofectamine™ PLUS (Invitrogen) according to the instructions of the manufacturer.

Cell Culture and cDNA Transfection—CHO cells, Chinese hamster ovary cells that express polyoma LT antigen to support the replication of eukaryotic expression vectors (33), were grown in a minimal essential medium (MEM) supplemented with 10% FBS, 100 μg/ml of streptomycin, and 100 units/ml of penicillin in a humidified incubator containing 5% CO2. CHO cells, Chinese hamster ovary cells, and B16 melanoma cells were grown in Ham’s F-12 medium or Dulbecco’s modified Eagle’s medium (DMEM), respectively, each supplemented with 10% FBS, 100 μg/ml of streptomycin, and 100 units/ml of penicillin. cDNA transfection was carried out using Lipofectamine™ PLUS according to the instructions of the manufacturer. CHO-SPHK1 and CHO-S1P1 cells, which are transfectants of CHO cells stably expressing mouse Sph kinase type 1 (SPHK1) and S1P1, respectively, were established as described previously (30, 34).

Preparation of the Conditioned Medium—CHO cells were grown on 6-well plates for 24 h and transfected with plasmid containing the CDase cDNA. At 18 h after transfection, the medium was replaced with serum-free MEM (2 ml/well on 6-well plates), and the cells were cultured for an additional 24 h. The cell culture medium was collected by pipette and subjected to centrifugation at 10,000 g for 5 min. The supernatant was used as a conditioned medium.

Measurement of Sph and S1P by HPLC—The amounts of Sph and S1P were measured with HPLC (Agilent 1100 series, Agilent Technologies) after derivatization with o-phthalaldehyde (OPA) essentially as described (35).

Extraction and Analysis of Radioisotope-labeled Cer—Cells labeled with [14C]palmitic acid or [14C]stearic acid were harvested by centrifugation (120 × g for 5 min) and washed with PBS. Lipids were extracted with 500 μl of methanol with sonication. After centrifugation (10,000 × g for 5 min), the supernatants obtained were dried under N2 gas, dissolved in 20 μl of chloroform/methanol (2/1, v/v), and then applied to the TLC plate, which was developed with chloroform/methanol/25% ammonia (90/20/0.5, v/v). Radiolabeled Cer separated on the TLC plate was visualized with an imaging analyzer (FLA5000 model, Fuji Film, Japan), and the photostimulated luminescence of the band was quantified.

Sph Kinase Assay—A Sph kinase assay was performed according to the method of Olivera et al. (36) with slight modifications. In brief, an appropriate amount of FBS was incubated with 1 μCi of [γ-32P]ATP (PerkinElmer Life Sciences) and 50 μM Sph at 37 °C for 30 min in 100 μl of reaction mix (20 mM Tris-HCl buffer, pH 7.5, containing 0.25% Triton X-100, 5 mM MgCl2, 12 mM β-glycerophosphate, 0.25 mM EDTA, and 1 mM sodium pyrophosphate). The reaction was terminated by the addition of 375 μl of chloroform/methanol/concentrated HCl (100:200:1), and lipids were extracted by the method of Bligh and Dyer (37). A portion of the lipid extract was applied to a TLC plate, which was then developed in butanol/acetate acid/water (3/1/1, v/v). Radioactive bands were visualized and quantified using an imaging analyzer. One enzyme unit was defined as the amount capable of forming 1 μmol of S1P per min. A value of 10−7 units of enzyme was expressed as 1 nanounit in this study.
Neutral Ceramidase-mediated S1P Generation and Signaling

FIGURE 1. Neutral CDase-dependent hydrolysis of cell surface Cer generating Sph and S1P. A, CHOP cells were transfected with the CDase cDNA or empty vector (Mock). Three hours after transfection, cells were incubated with α-MEM supplemented with 10% FBS containing 0.2 μCi of [3H]palmitic acid, and cultured for an additional 20 h. Then, the cells were treated with 100 milliunits/ml of SMase from E. cereus for 12 h. Lipids were extracted from cells, separated by TLC, and then analyzed with an imaging analyzer as described under “Experimental Procedures.” Data were averaged from four independent experiments with S.D. Statistical analysis was done using Student’s t-test, and the p values obtained are indicated. B, immunocytochemical analysis of Cer in the plasma membranes of CHOP cells using a specific anti-Cer antibody. At 24 h after transfection, cells were treated with 100 milliunits/ml of SMase for 12 h. Cells were then fixed, permeabilized, and immunostained with the anti-Cer monoclonal antibody 15B4 followed by rhodamine-labeled anti-mouse IgM. The immunofluorescence was examined under a confocal laser-scanning microscope. Arrows indicate Cer at plasma membranes. C, effects of neutral CDase expression on the amount of Sph and S1P in CHOP cells after SMase treatment. CHOP cells were transfected with the CDase cDNA or empty vector (Mock). Twenty-four hours after transfection, the medium was replaced with the serum-free-conditioned medium from CDase or mock transfectants, and then cells were treated with 100 milliunits/ml of SMase for the indicated periods. Cells were harvested and subjected to HPLC analysis for measurement of the amounts of Sph and S1P as described under “Experimental Procedures.” Data were averaged from three independent experiments with S.D.

RESULTS

Hydrolysis of Cell Surface Cer by Neutral CDase and Subsequent Generation of Sph and S1P—Because the catalytic domain of neutral CDase faces the extracellular space (24), cell surface Cer is expected to be hydrolyzed by the enzyme. However, no significant increase in Sph was observed when mouse neutral CDase was overexpressed in CHOP cells, possibly because of the limited amount of free Cer in the outer leaflet of plasma membranes. Thus, bacterial sphingomyelinase (SMase) was used as a tool for hydrolysis of the cell surface SM to increase the amount of free Cer in the plasma membranes (39). After treatment of the cells with bacterial SMase, a marked increase in the level of free Cer was observed in both CHOP cells transfected with a neutral CDase cDNA (CDase transfectants) and cells transfected with a vector without the CDase gene (mock transfectants) (Fig. 1A). Expectedly, the amount of free Cer in CDase-overexpressing cells was found to decrease about 30% compared with that in mock transfectants, indicating cell surface Cer was hydrolyzed by neutral CDase expressed in the CHOP transfectants. This was confirmed with an immunocytochemical method using a specific anti-Cer monoclonal antibody, 15B4, which was successfully used for the detection of cell surface Cer (40–42). The amounts of cell surface Cer in CDase transfectants, which increased significantly after treatment with bacterial SMase (Fig. 1B, panels a versus c), were reduced much faster than those in mock transfectants (Fig. 1B, panels c versus d). Simultaneously, the amounts of cellular Sph and S1P in CDase transfectants increased much faster than those in mock transfectants (Fig. 1C). The increase in these metabolites occurred transiently, peaking at 0.5–1 h (Fig. 1C).

Effects of Down-regulation of Endogenous Neutral CDase on the Amounts of Cellular Sph and S1P—To verify whether endogenous neutral CDase is involved in the generation of Sph and S1P by the hydrolysis of cell surface Cer, siRNA was used to suppress the activity of the endogenous enzyme. The effectiveness of the neutral CDase siRNA was first examined using neutral CDase overexpressors. Mouse B16 melanoma cells were simultaneously transfected with mouse neutral CDase cDNA and neutral CDase siRNA. For the control experiment, SCR siRNA was co-transfected with the CDase cDNA. As shown in Fig. 2A, the increase of neutral CDase activity in the CDase transfectants was strongly suppressed by co-transfection with the CDase siRNA, but not with the SCR siRNA. Furthermore, the CDase siRNA effectively inhibited the activity of the endogenous neutral CDase as expected (Fig. 2B), while acid SMase activity was not affected by the transfection with the CDase siRNA under the conditions used (data not shown). We then examined the effects of the CDase siRNA on the cellular levels of Sph and S1P during the treatment of cells with bacterial SMase. Interestingly, production of Sph and S1P in B16 cells was significantly reduced by CDase siRNA, but not SCR siRNA, during SMase treatment (Fig. 2C). Collectively, not only the overexpressed neutral CDase but also the endoge-
Neutral Ceramidase-mediated S1P Generation and Signaling

FIGURE 2. Effects of neutral CDase siRNA on the generation of Sph and S1P in B16 cells. A, mouse B16 melanoma cells were co-transfected with mouse neutral CDase cDNA and control siRNA (SCR siRNA) or siRNA specific to mouse neutral CDase (neutral CDase siRNA). Twenty-four hours after transfection, cells were harvested and subjected to an assay of neutral CDase activity using C12-NBD-Cer as a substrate. Data were averaged from three independent experiments with S.D. B, suppression of endogenous neutral CDase activity by siRNA. B16 cells were transfected with neutral CDase siRNA or SCR siRNA. At 24 h in the siRNA transfection, cells were transfected again with the same vector and cultured for an additional 24 h. Cells were harvested and subjected to an assay of neutral CDase activity. Data were averaged from three independent experiments with S.D. Statistical analysis was done using Student’s t test, and the p values obtained are indicated. C, B16 cells were transfected with siRNA as described in B. The medium was replaced with serum-free DMEM, and then cells were treated with 100 milliliters/ml of SMase for the periods indicated. Cells were harvested and subjected to HPLC analysis for measurement of the amount of Sph and S1P as described under “Experimental Procedures.” Data were averaged with S.D. from three independent experiments.

Effects of Localization of Neutral CDase on the Generation of Sph and S1P—To disclose the effects of the subcellular localization of neutral CDase on the generation of Sph and S1P, two constructs were generated: a secretable mutant CDase (SecCD), which has a secretory signal sequence instead of the signal/anchor sequence, and an ER-retainable mutant CDase (ERCD), which has an ER retention signal at the C terminus (Fig. 3A). These constructs were tagged with Myc at the C terminus. The mouse neutral CDase (wild-type CDase) was detected as two protein bands having a molecular mass of 123 kDa (a mature form) and 107 kDa (a developing form in the ER) by Western blotting using anti-Myc antibody when expressed in CHO cells (Fig. 3B). The mature form was also detected in the culture medium (Fig. 3B). The signals for wild-type CDase were detected in ER/Golgi compartments as well as on plasma membranes by fluorescent microscopy (Fig. 3C). On the other hand, SecCD was exclusively released into the medium (Fig. 3B), and no signals were detected on the plasma membranes under impermeable conditions (Fig. 3C), indicating that the mutant enzyme was not retained on the cell surface. In contrast to SecCD, ERCD was detected in the cell lysate as a 107-kDa ER-developing form but not in the medium (Fig. 3B), and exclusively localized to the ER and not present on the cell surface (Fig. 3C). ERCD showed strong activity in the cell lysate but not in the medium (Fig. 3B). Using these mutant and wild-type CDases, the generation of cellular Sph and S1P was examined after treatment of the cells with SMase. A significant increase in the amounts of Sph and S1P was observed in the cells transfected with wild-type CDase and SecCD, but not ERCD, compared with mock transfectants (Fig. 3D). These results indicate that plasma membrane-bound and secreted neutral CDases, but not the ER-retainable enzyme (ERCD), are involved in the generation of Sph by hydrolysis of Cer at the outer leaflets of plasma membranes possibly leading to an increase in the cellular S1P level. It is worth noting that ERCD did not participate in the generation of Sph and S1P although it exhibited strong activity in the cells (Fig. 3B).

Sites for Neutral CDase-dependent Production of Sph and S1P—To examine whether Sph is generated at the outer leaflet of the plasma membrane by the action of neutral CDase, the amount of Sph in CDase transfectants was examined with or without treatment with 1% fatty acid-free BSA at 4 °C after the SMase treatment (Fig. 4A). The amount of cell-bound Sph was drastically reduced by BSA treatment, possibly because Sph was withdrawn by BSA from the outer leaflet of plasma membranes. The trapped Sph, which was detected in the culture supernatant, increased with time during SMase treatment in the presence of BSA reaching a maximum after 1 h (Fig. 4B). In contrast to Sph, the amount of S1P in CHO cells was not changed by the BSA treatment (Fig. 4B), and no significant release of S1P into the medium was observed during SMase treatment in the presence of 1% BSA (data not shown), indicating that S1P is not generated at the outer leaflet of plasma membrane or not released into the medium under the conditions used in this study, although some other cells have been reported to secrete S1P (12, 13). These results strongly suggest that neutral CDase-mediated generation of Sph, but not S1P, occurs at the outer leaflet of plasma membranes. Although the generation of S1P is closely linked to that of Sph (Figs. 1 and 2), the sites for the generation of these two metabolites seem to be different (Fig. 4, A and B). To disclose where S1P is generated, CHO-SPHK1 cells, which are stable transfectants of CHO cells expressing mouse Sph kinase type I (SPHK1), were employed. Signals for SPHK1 were observed in the cytoplasmic region when CHO-SPHK1 cells were stained with anti-SPHK1 antibody under permeable conditions in the presence of Triton X-100, whereas almost no detectable signals were observed under impermeable conditions (Fig. 4C, panels a versus b), indicating that SPHK1 is mainly distributed in the cytoplasmic region. When CHO-SPHK1 and CHO cells were transfected with neutral CDase cDNA, the increase of S1P in CHO-SPHK1 cells was much greater than that in CHO cells after treatment with bacterial SMase (Fig. 4D), although the amount of Sph generated in both cells was almost the same (Fig. 4D). These results strongly suggested that Sph, generated at the outer leaflet of plasma membranes by neutral CDase, was incorporated into the cells, and part of the Sph was subsequently phosphorylated by the cytosolic Sph kinase.

Involvement of Neutral CDase in Stimulus-induced Cer Metabolism by Doxorubicin—To address whether neutral CDase is involved in the stimulus-induced metabolism of Cer, neutral CDase-overexpressing B16 melanoma cells and mock transfectants were treated with doxorubicin, which is known to induce the accumulation of Cer in some tumor cells (43, 44). As shown in Fig. 5A, the cellular level of Cer was increased by treatment with doxorubicin. Simultaneously, cellular levels of Sph (Fig. 5B) as well as S1P (Fig. 5C) were increased. The increase of Sph and S1P was significantly enhanced in the CDase transfectants compared with mock transfectants (Fig. 5, B and C), suggesting the neutral CDase is involved in the stimulus-induced metabolism of Cer leading to the generation of Sph and S1P.

Hydrolysis of Serum-derived Cer by Neutral CDase—This study presents evidence that neutral CDase was responsible for the hydrolysis of Cer in plasma membranes when the cellular level of Cer was increased by treatment with SMase or doxorubicin. Next, we examined the pos-
Neutral Ceramidase-mediated S1P Generation and Signaling

FIGURE 3. Effects of expression of wild-type and mutant neutral CDases on the generation of Sph and S1P in CHOP cells. A, schematic diagram of cDNA constructs used. Wild-type CDase (mouse neutral CDase tagged with Myc at the C terminus); SecCD (wild-type CDase having the secretory signal sequence of the mouse Vh chain instead of the signal-anchor sequence); ERCD (ER retention signal was added to the C terminus of the wild-type CDase). B, CDase activities and proteins expressed in cells and released in the medium. Cells were transfected with a plasmid vector containing wild-type or mutant CDase cDNAs. The cell lysates and the culture supernatant were subjected to an assay of neutral CDase activity using C12-NBD-Cer as a substrate, and the protein expression was determined by Western blotting using anti-Myc antibody. C, subcellular localization of wild-type and mutant CDases. Cells transfected with a plasmid vector containing wild-type CDase or mutant CDase cDNA were fixed, permeablized with Triton X-100, and stained with anti-Myc antibody followed by Alexa 598-labeled anti-mouse IgG (Molecular Probes). The Myc signal was examined under a fluorescent microscope. D, Sph and S1P contents in mutant CDase-overexpressing CHO cells. Cells were transfected with a plasmid vector containing wild-type CDase or mutant CDase. Twenty-four hours after transfection, the medium was replaced with serum-free-conditioned medium from each transfectant, and then cells were treated with 100 milliunits/ml of SMase for 1 h. Cells were harvested and subjected to HPLC analysis for measurement of the amounts of Sph and S1P. Data were averaged from three independent experiments with S.D. Statistical analysis was done using Student’s t test, and the p values are indicated.

satility that neutral CDase participates in the hydrolysis of serum-derived Cer in the vascular system. Serum has been reported to contain SM and Cer in a lipoprotein-bound form (45). First, a fluorescent Cer, C12-NBD-Cer, was added to the cell-free supernatant of CDase-overexpressing CHO transfectants (CHO-conditioned medium) to examine whether the secreted CDase hydrolyzes the Cer in the absence of detergents, which are usually used for the hydrolysis of Cer by the enzyme in vitro. As a result, the release of C12-NBD-fatty acid was observed in the conditioned medium of transfectants with wild-type CDase and SecCD cDNAs (Fig. 3A), but not in that of mock transfectants (Fig. 6A). Next, the release of Sph and S1P from fetal bovine serum (FBS) was measured in the conditioned medium during SMase treatment. Significant amounts of Sph as well as S1P were generated in the FBS-containing conditioned medium of SecCD and wild-type CDase transfectants, compared with mock transfectants (Fig. 6B). The increase in both sphingolipid metabolites in SecCD transfectants was much greater than that in wild-type CDase transfectants. This result is consistent with the amount of secreted CDase in the conditioned medium. It is worth noting that no increase in these metabolites was observed if FBS or SMase was omitted from the conditioned medium, suggesting that the major source of Cer is the FBS-derived SM and both Sph kinase and ATP are probably present in FBS. Sph kinase activity was actually detected in the FBS used in this experiment (0.82 ± 0.03 nanounits/ml, n = 3).

Incorporation and Phosphorylation of Sph Generated from Serum-derived Cer—As shown in Fig. 6B, serum-derived Sph, which is generated from serum-derived SM by the combined actions of SMase and neutral CDase, is partly converted to S1P possibly by serum-derived Sph kinase in the cell-free system. In contrast, plasma membrane-derived Sph is converted to S1P by the cytosolic Sph kinase after the incorporation of Sph into the cells (Fig. 4). Here, it was examined whether serum-derived Sph is incorporated into cells and then converted to S1P by cytosolic Sph kinase. When CHO cells were treated with SMase in the SecCD, but not mock, conditioned medium containing FBS, a drastic increase in cellular S1P content was detected. However, if SMase or FBS was omitted, only a negligible amount of cellular S1P was detected (Fig. 7). Interestingly, when CHO-SPHK1 cells, stable transfectants of CHO cells expressing cytosolic Sph kinase (Fig. 4, C and D), were used instead of CHO cells, the amount of cellular S1P increased 3-fold, whereas the amount of S1P in the medium was unchanged (Fig. 7). These results suggest that the serum-derived Sph generated by the actions of SMase and CDase is incorporated into cells, and subsequently phosphorylated by cytosolic Sph kinase, like the plasma membrane-derived Sph.

Neutral CDase-mediated S1P Activation—Recently, it was reported that S1P specifically undergoes internalization from the cell surface to intracellular vesicles after treatment with its ligand S1P (46). Thus, the activation of S1P, could be evaluated based on the degree of the receptor’s internalization. As shown in Fig. 8A, panel a, stably overexpressed S1P, was predominantly distributed in the plasma membrane in the steady state, whereas the receptor was sorted into intracellular vesicles on the stimulation of cells with 10 nM S1P for 10 min after serum starvation for 5 h (Fig. 8A, panel a versus b). The internalization was induced by SMase-treated conditioned medium from not only SecCD transfectants but also mock transfectants, probably because S1P was
Neutral Ceramidase-mediated S1P Generation and Signaling

FIGURE 4. The site for generation of Sph and S1P mediated by neutral CDase in CHOP cells. A, CHOP cells were transfected with neutral CDase cDNA or empty vector. At 24 h after transfection, the medium was replaced with serum-free α-MEM, and the cells were treated with 100 milliunits/ml of SMase for 30 min. Then, cells were washed twice with α-MEM containing 1% or 0% BSA at 4 °C for 5 min, harvested, and subjected to HPLC analysis for measurement of the amounts of Sph and S1P. Data were averaged from three independent experiments with S.D. B, at 24 h after transfection with the CDase cDNA, the medium was replaced with serum-free α-MEM containing 1% or 0% BSA, then cells were treated with 100 milliunits/ml of SMase for the periods indicated, and the culture supernatants were used for the determination of the amount of Sph by HPLC. Data were averaged from three independent experiments with S.D. C, immunocytochemical analysis of CHO cells (mock-transfected cells) and CHO-SPHK1 cells (CHO cells stably overexpressing SPHK1). Cells were fixed, permeabilized with Triton X-100 (+) or not (-), and stained with anti-SPHK1 antibody followed by Alexa 488-labeled anti-rabbit IgG (Molecular Probes). The signal for SPHK1 was examined under a fluorescent microscope. D, the generation of Sph and S1P in CHO-SPHK1 and CHO cells after transfection with a CDase cDNA and CHO cells after transfection with a CDase cDNA or an empty vector. Twenty-four hours after transfection, the medium was replaced with fresh serum-free α-MEM, and then cells were treated with 100 milliunits/ml of SMase for 1 h. Cells were harvested and subjected to HPLC analysis for measurement of the amounts of Sph and S1P. Data were averaged from three independent experiments with S.D. Statistical analysis was done using Student’s t test, and the p values are indicated.

FIGURE 5. Generation of Cer, Sph, and S1P by treatment of B16 cells with doxorubicin. A, B16 melanoma cells were transfected with doxorubicin. Twenty-four hours after transfection, the medium was replaced with fresh serum-free DMEM containing 0.2 μCi of [14C]stearic acid, and then cells were treated with 0.3 μg/ml of doxorubicin for 18 h. Lipids were extracted from cells, separated by TLC, and analyzed with an imaging analyzer. B and C, B16 cells were transfected with a CDase cDNA or an empty vector. After transfection, the same experiment as in A was performed in the absence of [14C]stearic acid, and cellular amounts of Sph (B) and S1P (C) were determined by HPLC as described under “Experimental Procedures.” Data were averaged from four independent experiments with S.D. Statistical analysis was done using Student’s t test, and the p values are indicated.

present in the FBS (Fig. 8A, panels c and d, and Fig. 8B). However, a clear difference was observed when the conditioned medium was diluted with fresh medium and then subjected to the assay, i.e. a 5-fold dilution caused a 70% reduction in internalization for SecCD-conditioned medium (Fig. 8A, panels e versus f, and Fig. 8B), indicating that the concentration of functional S1P is much higher in conditioned medium of SecCD transfectants than in that of mock transfectants. The addition of 20 μM of N,N-dimethylphosphoglyceride (DMS), a specific inhibitor of Sph kinase, to the SecCD-conditioned medium drastically decreased the amount of S1P produced (Fig. 8G, panel a) and S1P, internalized (Fig. 8C, panel b), confirming that the two are closely related. These findings indicated that neutral CDase regulates the activation of S1P1 through the generation of Sph.

DISCUSSION

In the present study, we showed that neutral CDase is involved in the metabolism of sphingolipids at the outer leaflet of the plasma membrane and in the extracellular milieu. The metabolic pathway from SM to S1P involving neutral CDase is summarized in Fig. 9, in which Cer is supplied from plasma-membrane SM or probably lipoprotein-bound SM in the serum. The former SM is ubiquitous whereas the latter is assumed to be present in the vascular system. In this model, one of the key enzymes is a SMase, which produces Cer from SM, initiating the metabolic cascade. We used bacterial SMase in this study as a tool to convert SM to Cer. Alternatively, acid SMase, distributed in lysosomes and endosomes (47), is a candidate for the Cer-releasing enzyme at the plasma membranes and in the extracellular milieu because several lines of evidence indicate that acid SMase is released from cells under certain conditions through a vesicular transport system (48). For example, acid SMase was actively released from endothelial cells with neutral CDase via a cytokine-regulated mechanism (49, 50). Importantly, the acid SMase hydrolyzes SM in oxidized lipoproteins, being involved in atherogenesis (48). Cell surface lipid microdomain rafts are thought to be the specific sites for the generation of Cer by acid SMase in response to various agonists and stress signals (51). The generation of Cer within rafts induces the coalescence of rafts into larger platforms and subsequently the clustering of CD95 (40), and Fcg receptor II (41). On the other hand, it cannot be ruled out that bacterial SMases are occasionally involved in the hydrolysis of plasma membrane- and/or serum-derived
SM. Bacterial SMases are thought to be a potent virulent factor and play a key role in the lysis of blood cells through hydrolysis of SM located on the outer leaflet of the plasma membranes (52). Very recently, Clostridium perfringens SMase was found to induce hemolysis of sheep erythrocytes by activating the endogenous SM-metabolizing pathway in host cells, by which SM on the cell surface was converted to Cer, Sph and finally S1P (53). Furthermore, Helicobacter pylori SMase was found to induce an increase in the cellular level of Cer and consequently suppress the growth of gastric epithelial cells via activation of JNK kinase (54).

The present study also suggests that neutral CDase functions as a cytoprotective enzyme to reduce the cellular level of Cer when it has increased due to infection by a SMase-producing pathogen or the hydrolysis of SM by other stimuli.

It is worth noting that neutral CDase can hydrolyze plasma membrane-bound Cer and serum-derived Cer in the absence of detergents. The assay of CDase activity in vitro requires anionic or non-anionic detergents such as sodium cholate and Triton X-100, which may solubilize the substrate in aqueous solution (32). The efficient hydrolysis of Cer in lysosomal membranes by acid CDase requires the presence of saposin D, a lysosomal activator protein, which was demonstrated to be an indispensable cofactor for the hydrolysis of membrane-bound Cer in vivo (55). At present, it remains unclear how neutral CDase degrades membrane-bound Cer and lipoprotein-bound Cer in the absence of detergents and whether an activator protein physiologically assists the hydrolysis of Cer by the enzyme in vivo.

S1P functions inside and outside of cells as an important bioactive modulator (3). S1P is a ligand for S1P1–5 which are G protein-coupled membrane spanning receptors possessing ligand binding sites on the extracellular side (10). Because the supply of Sph is the key to the generation of S1P by Sph kinase, CDase is thought to be crucial for S1P-mediated signaling. However, which molecular species of CDase are involved in the generation of S1P and S1P-mediated signaling has not yet been elucidated. Three molecular species of CDase, which differ in pH optimum and intracellular localization, are present in vertebrates (24, 55, 56). This study clearly indicated that neutral CDase is a crucial enzyme for the generation of S1P both inside and outside of cells. Although how Sph is transported from outside to the cytoplasmic region remains to be elucidated, we predict the presence of a novel transporter, which can transport Sph from the extracellular milieu to

FIGURE 6. Metabolism of serum-derived Cer by the neutral CDase secreted in the cell-free conditioned medium. A, TLC showing the hydrolysis of the fluorescent Cer by the neutral CDase secreted in the conditioned medium. Cell-free culture supernatant from wild-type CDase, SecCD, or mock transfectants (conditioned medium) was incubated with C12-NBD-Cer at 37 °C for the periods indicated. The fluorescent substrate was dissolved in Me2SO at a final concentration of 5 μM in 1% Me2SO. Lipids were extracted from the conditioned medium and applied to a TLC plate, which was developed with chloroform/methanol/25% ammonia (90/20/0.5, v/v). B, generation of Sph and S1P from FBS-derived SM in the conditioned medium. The conditioned medium containing 10% or 0% FBS was incubated with or without 100 milliunits/ml of SMase at 37 °C for the periods indicated and subjected to HPLC analysis for measurement of the amount of Sph and S1P. Data were averaged from two independent experiments.

FIGURE 7. Conversion of neutral CDase-mediated FBS-derived Sph to S1P in CHO cells and CHO-SPHK1 cells. CHO cells or CHO-SPHK1 (CHO cells stably expressing SPHK1) cells were grown on 6-well plates for 24 h. In parallel, serum-free-conditioned medium of SecCD or mock-transfected CHO cells was prepared as described under “Experimental Procedures.” The culture media of CHO-SPHK1 and CHO cells were exchanged with 2 ml of SecCD or mock-transfected conditioned medium. Then, 220 μl of FBS (final concentration, 10%) or fresh DMEM was added, and cells were treated with or without 100 milliunits/ml of SMase for 1 h. The amounts of S1P in cells and culture supernatants were separately analyzed by HPLC as described under “Experimental Procedures.” Data were averaged from three independent experiments with S.D.
Neutral Ceramidase-mediated S1P Generation and Signaling

FIGURE 8. Internalization of S1P, mediated by neutral CDase-dependent generation of S1P. A, CHO cells stably expressing HA-tagged S1P1 (CHO-S1P1) were grown on glass coverslips, and then incubated for 5 h with serum-free medium. In parallel, SecCD or mock-conditioned medium containing 10% FBS was treated with 100 milliunits/ml of SMase at 37 °C for 5 h. CHO-S1P1 cells were then stimulated with SMase-treated conditioned medium as described above. The conditioned medium was diluted 2–100-fold with fresh medium before being used for stimulation. After 10 min of stimulation, cells were fixed, permeabilized with Triton X-100, and stained with anti-HA polyclonal antibody. Cells were then observed under a fluorescent microscope.

FIGURE 9. Scheme for metabolic pathway from SM to S1P involving neutral CDase at the outer leaflet of the plasma membrane and in the extracellular milieu.

where extracellular Sph is generated has not yet been elucidated. We present two possibilities for the origin of extracellular Sph, (1) serum-derived Sph, which could be generated from the SM/Cer of lipoproteins, and (2) plasma membrane-derived Sph, which could be detached from the cells in an albumin-dependent manner. Considering the extracellular generation of S1P, how ATP is supplied to the extracellular milieu should be clarified. It should be noted that several studies have indicated that ATP is continually present in the plasma and the extracellular space, and is released from many sources, including sympathetic nerves, activated platelets, inflammatory cells, endothelial cells, and smooth muscle cells (58, 59). Furthermore, a recent report showed that endothelial cells express a functional cell surface H^+–ATP synthase, which actively synthesizes ATP in the extracellular milieu (60).

Sph has been suggested to be an intracellular second messenger since it can regulate intracellular signaling molecules such as protein kinase C (7), 3-phosphoinositide-dependent kinase 1 (8), and Sph-dependent protein kinase 1 (9). It is worth noting that cell surface Sph generated by neutral CDase may act on cytosolic molecular targets, since the Sph seems to be promptly transported to the cytosolic region via an unknown mechanism. In addition, Sph is partly converted to S1P in the cytoplasmic region and extracellular milieu as shown in this study. Disruption of the mouse S1P1 gene revealed that the receptor is required for vascular maturation (61). We found that S1P generated from serum-derived SM through the action of neutral CDase caused the internalization of S1P (Fig. 8). This result strongly suggested that neutral CDase is involved in the generation of S1P and consequently S1P-mediated signaling.

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Neutral Ceramidase-mediated S1P Generation and Signaling

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36600