Previously, we reported two types of neutral ceramidase in mice, one solubilized by freeze-thawing and one not. The former was purified as a 94-kDa protein from mouse liver, and cloned (Tani, M., Okino, N., Mori, K., Tanigawa, T., Izu, H., and Ito, M. (2000) J. Biol. Chem. 275, 11229–11234). In this paper, we describe the purification, molecular cloning, and subcellular distribution of a 112-kDa membrane-bound neutral ceramidase of rat kidney, which was completely insoluble by freeze-thawing. The open reading frame of the enzyme encoded a polypeptide of 761 amino acids having nine N-glycosylation sites and one possible transmembrane domain. In the ceramidase overexpressing HEK293 cells, 133-kDa (Golgi-form) and 113-kDa (endoplasmic reticulum-form) Myc-tagged ceramidases were detected, whereas these two proteins were converted to a 87-kDa protein concomitantly with loss of activity when expressed in the presence of tunicamycin, indicating that the N-glycosylation process is indispensable for the expression of the enzyme activity. Immunohistochemical analysis clearly showed that the ceramidase was mainly localized at the apical membrane of proximal tubules, distal tubules, and collecting ducts in rat kidney, while in liver the enzyme was distributed with endosome-like organelles in hepatocytes. Interestingly, the kidney ceramidase was found to be enriched in the raft microdomains with cholesterol and GM1 ganglioside.

Over the past decade, sphingolipids and their metabolites have emerged as a new class of lipid biomodulators of various cell functions (1, 2). Ceramide (N-acylsphingosine; Cer), a common lipid backbone of sphingolipids, functions as a second messenger in a variety of cellular events including apoptosis and cell differentiation (3, 4). Sphingosine (Sph) has bifunctional effects on cell growth, i.e. it exerts mitogenic (5) and apoptosis inducing (6) activities, depending on the cell type and cell cycle. Sph-1-phosphate (SIP) was found to function as an intra- and intercellular second messenger to regulate cell growth (7), motility (8), and morphology (9). Interestingly, SIP inhibits the apoptosis induced by Cer and Fas ligand (10), indicating that the balance of Cer/Sph/SIP affects cell phenotype.

Ceramidase (CDase, EC 3.5.1.23) is an enzyme that catalyzes hydrolysis of the N-acyl linkage of Cer to produce Sph, which can be phosphorylated to SIP by sphingosine kinase (11). Sph is not produced by de novo synthesis (12), and thus the activity of CDase is crucial not only for switching off the Cer-induced signaling but also for generation of Sph and SIP. CDase is classified into two categories: acid and neutral/alkaline enzymes depending on pH optimum. Acid CDase is thought to be a housekeeping enzyme to catalyze Cer in lysosomes. The enzyme was purified from human urine (13), and cDNA encoding the enzyme was isolated from cDNA libraries of human (14) and mouse (15). A deficiency of the enzyme could cause Farber disease in which Cer is accumulated in lysosomes (16). Neutral/alkaline CDase seems to change the balance of Cer/Sph/SIP in response to various stimuli including cytokines and growth factors, and could modulate the sphingolipid-mediated signaling. For example, the activity of membrane-associated neutral CDase was shown to be up-regulated by platelet-derived growth factor in rat glomerular mesangial cells (17), and the enzyme activity was modulated in a bimodal manner by interleukin-1β in rat hepatocytes (18), resulting in a decrease of Cer concomitantly with an increase of Sph. However, the biological function of the enzyme is still not clear. Recently, cDNAs encoding sphingomyelinase, Sph kinase (19), and SIP receptors (Edg family) (20) have been successively cloned. The functions of sphingolipids are now open for elucidation at the molecular level.

In the past few years, molecular cloning of neutral/alkaline CDases, one of the missing links of sphingolipid signaling, has been performed in mice (21), human (22), bacteria (23), and yeast (24). In mice, we found two types of neutral CDase, one solubilized by freeze-thawing and the other not. The former was purified as a 94-kDa protein from mouse liver (25), and the cDNA encoding the enzyme was cloned (21). In the present paper, we report the purification, characterization, and cDNA cloning of a 112-kDa membrane-bound CDase of rat kidney, which was absolutely resistant to extraction with freeze-thawing and had an optimum pH of 6–7. It is worth noting that
neutral/alkaline CDase of human brain is specifically localized in mitochondria, suggesting the existence of a Cer pool in this organelle (22). On the other hand, we show here using a specific antibody against the neutral CDase that the enzyme was mainly localized at apical membranes of proximal tubules, distal tubules, and collecting ducts in rat kidney, while in rat liver the enzyme was distributed with endosome-like organelles in hepatocytes. Furthermore, the kidney CDase was recovered in the detergent-insoluble, cholesterol, and GM1-enriched fractions by sucrose density gradient centrifugation, suggesting that the enzyme is present in the raft microdomains.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal antibody YA30 against Lgp85 (Limp-2) and CHOP cells were kind gifts from Dr. K. Akasaka (Fukuyama University, Japan) and Dr. K. Nara (Mitsubishi Kasei Institute of Life Sciences, Japan), respectively. Horseradish peroxidase-labeled anti-mouse IgG and anti-rabbit IgG were purchased from EY Laboratories and Santa Cruz Biotechnology, respectively. DEAE-Sepharose FF, phenyl-Sepharose 6FF, chelating Sepharose FF, HiTrap lentil lectin, HiTrap ConA, HiLoad 16/60 Superdex 200 pg, Percoll, ECL plus, FITC-labeled anti-mouse IgG, and Cy3-labeled anti-rabbit IgG antibody were from Amersham Pharmacia Biotech. Precoated Silica Gel 60 TLC plates were obtained from Merk (Germany). Amplex Red (N-acetyl-3,7-dihydroxyphenoxyxone) and cholesterol oxidase were from Molecular Probe and Toyobo Co. (Japan), respectively. Various [³²P]Cers and C12-NBD-Cer were prepared as described in Ref. 26. HEK293 cell (JCRB9068, established by F. L. Graham) was from the Human Science Research Resource Bank. All other reagents were of the highest purity available.

**CDase Assay**—CDase activity was measured using C12-NBD-Cer as a substrate (25). Briefly, 550 pmol of C12-NBD-Cer was incubated at 37 °C for 30 min with an appropriate amount of the enzyme in 20 μl of 25 mM Tris-HCl buffer, pH 7.5, containing 0.25% Triton X-100. The reaction was stopped by heating in a boiling water bath for 5 min. After being dried up with a Speed Vac concentrator (Savant Instruments, Inc.), the sample was dissolved in 30 μl of chloroform/methanol (2/1, v/v), and applied to a TLC plate, which was developed with chloroform, methanol, 25% ammonia (90/20/0.5, v/v). The NBD-dodecanoic acid obtained after digestion with lysylendopeptidase AP-1 showed high identity to the rat liver neutral CDase (21), and thus we designed two primers based on the nucleotide sequence of the mouse enzyme. PCR using sense (5'-AGGAAATGTTGCTAATGTGC-3') and antisense primers (5'-GTGACACGTCCTCCGAGATG-3') was performed with the cDNA using the gene-specific primer for rat kidney (Takara Shuzo Co., Otsu, Japan) as a template in a GeneAmp PCR System 9700 (Applied Biosystems) using AmpliTaq Gold (Applied Biosystems). The cycling parameters for PCR were 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s, and the cycle number was 40. After this amplification, a 325-base pair PCR product containing the sequence of rat CDase was obtained. To obtain the full-length cDNA encoding the rat CDase, colony hybridization was performed using a PCR-dense pair PCR product as a probe after concentration of the CDase cDNA with a CloneCapture™ Selection Kit (CLONTECH). The probe was labeled with [α-³²P]dCTP using Ready-To-Go DNA labeling kit (Amersham Pharmacia Biotech). Colony hybridization was carried out by the standard method (28). Nucleotide sequences were determined by the dideoxynucleotide chain termination method with a Bigdyte Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and a DNA Sequencer (model 377A, Applied Biosystem).

**Preparation of Recombinant CDase**—A DNA fragment encoding the open reading frame of rat CDase was prepared by PCR using 5’ primer containing a HindIII site (5’-AGTGGCCTATCCAGCACAAGTTGAGC-3’) and 3’ primer containing a XhoI site (5’-GGCCGCTGCAGATCCGAGCCTTCAAGAGGAGAA-3’) and the cloned rat cDNA (pLPKCD) as a template. The PCR product was inserted into the HindIII and XhoI sites of pET23b vector (Novagen) with a COOH-terminal histidine tag. Escherichia coli strain BL21(DE3) was transformed with the construct in the presence of ampicillin (100 μg/ml). To obtain the recombinant CDase, 2 ml of overnight culture was induced by 100 μM of LB in the presence of 0.5 mM IPTG and incubated at 37 °C. When the OD600 reached 0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and incubated for no more than 5 h at 37 °C. Cells were harvested by centrifugation at 5,000 × g for 10 min at 4 °C, and the pellet was suspended in 10 mM Tris-HCl buffer, pH 7.5, containing 1% Triton X-100 and 1 mM EDTA. After centrifugation at 15,000 × g for 10 min, the pellet (inclusion bodies) was lyzed by
sonication in 50 mM Tris-HCl buffer, pH 7.5, containing 8 mM urea. Recombinant protein was purified using a HiTrap chelating column (Ni²⁺) according to the manufacturer's instructions. Purified protein was dialyzed against distilled water before being used for immunization. From a rabbit immunized with the purified recombinant CDase, antiserum was obtained and purified using a HiTrap Protein A column according to the manufacturer's instructions.

**Cell Culture and cDNA Transfection—**CHOP cells, Chinese hamster ovary cells that express polycoma LT antigen for supporting efficient replication of eukaryotic expression vectors (29), were grown in a 24-well plate with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in a humidified incubator containing 5% CO₂ at 37 °C. HEK293 cells, human embryonic kidney cell, were grown in a Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 60 µg/ml kanamycin in a humidified incubator containing 5% CO₂. HEK293 cells, human embryonic kidney cell, were grown in a Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 60 µg/ml kanamycin in a humidified incubator containing 5% CO₂. CDase transfection was carried out using LipofectAMINE Plus (Life Technologies, Inc.) according to the instructions of the manufacturer. To obtain Myc-tagged CDase, cDNA encoding the CDase was subcloned into pcDNA3.1/Myc-His(+) vector (Invitrogen Co.) by PCR using a 5' primer with a KpnI restriction site (5'-AGGTTACGAAATTGCGAAGCACTTCTC-3') and a 3' primer with a XhoI restriction site and disrupted stop codon (5'-GC-GCCGCTGAGCAGTGAGAATACATTGAGAAAGA-3'). Cells were treated with tunicamycin to block the N-glycosylation of neutral CDase. Myc-tagged CDase was purified from the culture supernatant and was added to the culture 72 h after transfection of CDase gene and cells were harvested after 12 h.

**Protein Assay, SDS-PAGE, and Western Blot—**Measurement of protein was determined by the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as standard. SDS-PAGE was carried out according to the method of Laemmli (30). Protein transfer onto a polyvinylidifluoride membrane was performed using TransBlot SD (Bio-Rad) according to the method described in Ref. 31. After treatment with 3% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (T-TBS), the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. After another wash with T-TBS, the ECL reaction was performed for 2–3 min as recommended by the manufacturer. To obtain Myc-tagged CDase, the samples were stained with silver solution (Fig. 2A). Among several staining bands, a 75 kDa band was identified as the CDase (Fig. 2A). The specific activity increased 3,640-fold in the microsome fraction (27,000 g) as judged by CDase assay. After purification using chromatography on DEAE-Sepharose FF, phenyl-Sepharose 6FF, chelating Sepharose FF, HiTrap lentil lectin, and HiLoad Superdex 200 pg, the CDase was purified using a MonoQ HR5/5 column.

**RESULTS**

**Purification of Membrane-bound CDase from Rat Kidney—**As shown in Fig. 1, the CDase of rat liver was solubilized from the membrane fraction by freeze-thawing (A), whereas the enzyme of rat kidney was completely resistant to extraction with freeze-thawing (B). This discrepancy was also observed when the neutral CDase was extracted from mouse liver and kidney (25). The neutral CDase of mouse liver was easily solubilized by freeze-thawing, and purified as a 94-kDa protein, and thus cDNA encoding the enzyme was cloned (21). However, the CDase resistant to solubilization by freeze-thawing had yet to be purified from mouse liver or kidney, because of the limited amount of the enzyme. In this study, we found that the same polymer fraction (27,000 × g pellet) of rat kidney contained a large amount of membrane-bound neutral CDase which was solubilized with a mixture of 1% Triton X-100 and 1% Tween 20 with high yield as described under "Experimental Procedures." Following purification using chromatography on DEAE-Sepharose FF, phenyl-Sepharose 6FF, chelating Sepharose FF, HiTrap lentil lectin, and HiLoad Superdex 200 pg, 48 µg of CDase was obtained from 106 rat kidneys with 3% recovery (Table 1). The specific activity increased 3,640-fold in the polymer fraction (27,000 × g pellet) of rat kidney. The CDase activity was eluted in fractions 78–84 as shown by shadow in Fig. 2A. Aliquots of the fractions were subjected to SDS-PAGE followed by staining with silver solution (Fig. 2B). Among several staining bands, a 112-kDa protein is likely to be the CDase (Fig. 2B), since the elution profile of the CDase activity from the gel filtration most
Neutral Ceramidase of Rat Kidney

Fig. 1. Solubilization of neutral CDase from rat microsome fractions by freeze-thawing. The mirosome fractions of rat liver and kidney were prepared as described under “Experimental Procedures.” Each fraction (about 1–2 mg of protein) was suspended in 100 μl of 0.25 M sucrose containing protease inhibitors (2 μg/ml leupeptin, 1 mM phenylmethylsulfonfluoride, and 1 mM EDTA), frozen in ethanol with dry ice, and then thawed at 37 °C. After the procedure had been repeated for the number of times indicated, aliquots of 100 μl were withdrawn and centrifuged at 105,000 × g for 60 min. The supernatant obtained was used as the soluble fraction. The precipitate was suspended in 100 μl of 20 mM Tris-HCl buffer, pH 7.5, containing 0.2% Triton X-100 and then used as the insoluble fraction (membrane fraction). CDase activity was evaluated as described under “Experimental Procedures.” A, rat liver; B, rat kidney. M, activity of the insoluble fraction; C, activity of the solubilized fraction. Values are the mean for duplicate determinations.

TABLE I

Purification of a neutral CDase from rat kidney

| Steps                          | Total activity | Specific activity | Recovery | Purification |
|-------------------------------|----------------|------------------|----------|--------------|
|                               | milliunits     | mg               | milliunits/mg | % | fold |
| 27,000 g pellet               | 3000           | 5700             | 0.53     | 100          | 1 |
| Detergent extract             | 1140           | 3000             | 0.38     | 38.0         | 0.71 |
| DEAE-Sepharose FF             | 820            | 1210             | 0.68     | 27.3         | 1.28 |
| Phenyl-Sepharose 6FF          | 1100           | 810              | 1.4      | 36.7         | 2.64 |
| Chelating-Sepharose FF        | 860            | 24               | 35.8     | 28.7         | 67.5 |
| HiTrap lentil lectin          | 148            | 0.76*            | 195      | 4.9          | 368 |
| Superdex 200 pg               | 91.7           | 0.048*           | 1910     | 3.1          | 3694 |

* Estimated by SDS-PAGE.

Neutral Ceramidase of Rat Kidney—Characterization of the rat CDase was conducted using the enzyme preparation after HiLoad Superdex 200 pg chromatography. The enzyme exhibited a pH optimum at 6–7, although the pH dependence of the enzyme was quite broad and about 50% activity was observed at pH 8–9 (Fig. 3A), indicating that the enzyme should be classified as a neutral or neutral/alkaline CDase. The activity was completely inhibited by Hg2⁺, whereas Zn2⁺ and Cu2⁺ inhibited the activity by 80% (Fig. 3B). In contrast to the bacterial CDase (36), the rat CDase was not activated by Ca2⁺, EDTA, Mn2⁺, and Mg2⁺ had little effect on the rat CDase. The enzyme activity was greatly enhanced by addition of detergents such as sodium cholate and sodium taurodeoxycholate. The optimum concentration of detergents differed markedly depending on the detergent used. For sodium taurodeoxycholate and sodium cholate, the optimum concentrations were found to be 0.1–0.2 and 0.4–2%, respectively, which increased the enzyme activity about 4–5-fold in comparison with that in the absence of the detergent (Fig. 3C). Triton X-100 at 0.1–0.2% also enhanced the enzyme activity by about 2-fold, although the detergent showed an inhibitory effect beyond the optimum concentration. The substrate specificity of the CDase was examined at pH 7.0 using various 14C-labeled Cers (Table II). Among various Cers tested, N-lauroylsphingosine (C12:0/d18:1) was most efficiently hydrolyzed by the enzyme followed by N-palmitoylsphingosine (C16:0/d18:1) and N-stearoylsphingosine (C18:0/d18:1). Cers containing sphinganine (d18:0) and phytosphingosine (t18:0) as a long chain base were somewhat resistant to the enzyme. It is of note that glycosphingolipids such as GalCer, sulfatide, and GM1a or sphingomyelin were not hydrolyzed by the enzyme.

NBD-N-dodecanoylsphingosine (C12-NBD-Cer) was hydrolyzed much faster than N-lauroylsphingosine (C12:0/d18:1), indicating that attachment of NBD to the fatty acid residue at the α-position increased the susceptibility of the enzyme to the substrate.

Molecular Cloning, Sequencing, and Alignment of Rat Neutral CDase—The four peptide sequences (C1–4 in Fig. 4A) were determined using the purified 112-kDa protein by protein sequencer after digestion with lysylendopeptidase as described under “Experimental Procedures.” We found that the four peptide sequences of the rat CDase were homologous to the se-
Sequence of mouse neutral CDase (21); C1 for amino acid 223–245 in the mouse enzyme, C2 for amino acid 261–273, C3 for amino acid 601–619, and C4 for amino acid 701–729. Therefore, we designed two primers based on the nucleotide sequence of mouse CDase (sense primer corresponding to amino acid 223–230 of the mouse enzyme and antisense primer corresponding to amino acid 325–331) and performed PCR amplification using a rat kidney cDNA library. The 325-base pair amplified fragment was then used as a probe for colony hybridization to screen for the cDNA encoding the CDase from a rat kidney cDNA library. Finally, the clone (pAPkCD) containing the full-length cDNA encoding the CDase was obtained. Fig. 4A shows the cDNA and deduced amino acid sequences of the neutral CDase from rat kidney. The pAPkCD contained one open reading frame of 2283 base pairs coding 761 amino acids, 86 amino acid residues of which matched the amino acid sequence of the purified CDase (Fig. 4A, C1–4). The predicted molecular mass and pI of the enzyme were 83,483 and 6.55, respectively, judging from the deduced amino acid sequence. The open reading frame of pAPkCD contained nine potential N-glycosylation sites (Fig. 4A, underlines). This result is well consistent with the fact that the CDase is highly glycosylated with N-glycans.

Fig. 2 Purification of rat neutral CDase. A, gel filtration chromatography of the neutral CDase on Superdex 200 pg. Neutral CDase activity was measured using C12-NBD-Cer as a substrate as described under “Experimental Procedures.” B, SDS-PAGE of each fraction showing CDase activity on Superdex 200 pg. Aliquots (100 μl) of each fraction were subjected to SDS-PAGE and the proteins were stained with silver staining solution. C, elution profiles of a 112-kDa protein and neutral CDase activity on Superdex 200 pg. The 112-kDa protein on SDS-PAGE (B) was quantified by on a TLC chromatogram at 540 nm and plotted with the activity of neutral CDase. D, SDS-PAGE showing the gel-purified 112-kDa protein. Fraction 82 of A was subjected to SDS-PAGE under the nonreducing conditions. The gel was cut into 2-mm slices without staining, and the CDase activity was extracted and determined as described under “Experimental Procedures.” The fraction containing the highest CDase activity was applied to SDS-PAGE under reducing conditions and stained with silver staining solution. E, SDS-PAGE of the purified neutral CDases from rat kidney (lanes 1 and 2) and mouse liver (3 and 4). Fig. 3 General properties of rat neutral CDase. A, optimum pH of the rat neutral CDase. The enzyme activity was measured using 100 pmol of [14C]Cer (C16:0/d18:1) as a substrate in 20 μl of 150 mM GTA buffer at different pH values containing 0.25% Triton X-100. The incubation was carried out using 5 microunits of the CDase at 37 °C for 1 h. Effects of cations (B) and detergents (C) on CDase activity. The CDase activity was measured using C12-NBD-Cer as a substrate as shown under “Experimental Procedures” except that each reaction mixture contained 5 μM of the cation indicated (B) or various detergents at the concentrations indicated (C). In C, △, sodium cholate; ○, Triton X-100; □, taurodeoxycholate.
Computer analysis using a PSORT revealed the presence of one endoplasmic reticulum transitional signal sequence at amino acids 1–36, a signal peptidase cleavage site at amino acids 36–37 (arrowhead), one possible transmembrane domain at amino acids 502–518 (box), and a di-Leu signal at amino acids 740–741 (shading) (Fig. 4). In addition, putative phosphorylation sites for casein kinase II (amino acids 10–13, 259–262, 261–264, 466–469, 565–568, 566–569, 611–614, and 757–760), and protein kinase C (amino acids 134–136, 148–150, 200–202, 252–254, 261–263, 428–430, 431–433, 466–46, and 529–531) were found in the sequence (Fig. 4A). Hydropathy analysis indicated the presence of two prominent hydrophobic regions.

**TABLE II**

| Substrate Structure (fatty acid/long-chain base) | Hydrolysis % |
|-----------------------------------------------|--------------|
| N-Lauroylsphingosine C12:0/d18:1              | 16.4         |
| N-Palmitoylsphingosine C16:0/d18:1           | 14.9         |
| N-Stearoylsphingosine C18:0/d18:1            | 11.1         |
| N-Palmitoylnonacosane C16:0/d18:1            | 7.3          |
| N-Palmitoylphytosphingosine C16:0/t18:0      | 5.5          |
| N-Stearoylphytosphingosine C18:0/t18:0       | 1.7          |
| NBD-N-dodecanoylsphingosine C12:0/d18:1      | 32.6         |
| GalCer                                        | 0            |
| Sulfatide                                     | 0            |
| GM1a                                          | 0            |
| Sphingomyelin                                  | 0            |

**FIG. 4.** Nucleotide and deduced amino acid sequences (A) and hydropathy plot (B) of the rat neutral CDase. A, the deduced amino acid sequence of the CDase is shown in single-letter code below the nucleotide sequence. The putative transmembrane domain is boxed, and nine potential O-linked glycosylation sites are underlined. An arrowhead indicates the possible site of cleavage by signal peptidase. The di-Leu signal is shaded. Amino acids determined by peptide sequencing are shown by dotted lines. Numbers correspond to amino acids (lower) and nucleotides (upper). B, the hydropathy plot of the CDase was analyzed by the method of Kyte and Doolittle (47).
phobic segments, one in the amino-terminal region (amino acids 4–31) predicting a putative signal sequence and the other in the middle of the sequence (amino acids 499–518) predicting a possible transmembrane domain (Fig. 4B). Fig. 5 shows the alignment of the deduced amino acid sequences of neutral/alkaline CDases from rat, mouse, human, and bacteria. Alignment of neutral/alkaline CDases was performed using the CLUSTAL algorithm (48). Box 1, endoplasmic reticulum transitional signal sequence of rat and mouse CDases; Box 2, mitochondrial-targeting sequence of human brain CDase. Box 3 and 4, di-Leu sequence of CDases.

Northern Blot Analysis of Rat Neutral CDase—To determine the size and expression of the rat neutral CDase in various tissues, Northern blot analysis was conducted using the EcoRI fragment of pAPkCD as a probe which contained almost the full-length of the CDase cDNA. As shown in Fig. 6, a strong 5.1-kb mRNA signal was detected in brain, kidney, and heart, whereas only weak signals were detected in other tissues including liver. The optimum pH of the recombinant CDase was found to be pH 6–7 (Fig. 7B), which is consistent with the result obtained using the purified enzyme from rat kidney (Fig. 3A). It is interesting to note that the recombinant rat CDase catalyzed the reversible reactions in which the amide linkage of ceramide is cleaved or synthesized (data not shown), as shown in neutral/alkaline CDases from mouse (25), yeast (24), and bacteria (36).

Expression Analysis of Rat Neutral CDase—CHOP cells were transfected with pAPkCD and the CDase activity of cell lysates was measured using C12-NBD-Cer as a substrate at pH 7.5. As shown in Fig. 7A, the CDase activity of the lysate of pAPkCD-transfected cells (pAPkCD) increased more than 9,000-fold in comparison with that of mock transfectants (mock) or untransfected CHOP cells (data not shown). The optimum pH of the recombinant CDase was found to be pH 6–7 (Fig. 7B), which is consistent with the result obtained using the purified enzyme from rat kidney (Fig. 3A). It is interesting to note that the recombinant rat CDase catalyzed the reversible reactions in which the amide linkage of ceramide is cleaved or synthesized (data not shown), as shown in neutral/alkaline CDases from mouse (25), yeast (24), and bacteria (36). To verify whether or not N-glycosylation is essential for the expression of the CDase activity, a Myc-tagged CDase con-
struct was expressed in CHOP cells in the presence or absence of tunicamycin which is a specific inhibitor for N-glycosylation. When the Myc-tagged CDase was expressed in HEK293 cells in the absence of tunicamycin, 1,680 microunits/mg of CDase was detected in cell lysates, concomitantly with the expression of two protein bands of molecular mass 133 and 113 kDa on SDS-PAGE after visualization with anti-Myc antibody (Fig. 7C). Interestingly, CDase activity markedly decreased when the transformation of HEK293 cells was conducted in the presence of tunicamycin (31.4 microunits/mg) (Fig. 7D). The activity was determined using C12-NBD-Cer and the CDase expressed in CHOP cells. The CDase activity was determined using [14C]Cer (C16:0/d18:1) in GTA buffer at different pH values as described under "Experimental Procedures." C, the activity and SDS-PAGE of Myc-tagged CDase of HEK293 cells in the presence or absence of tunicamycin. The rat neutral CDase cDNA was subcloned into pcDNA3.1/Myc-His(+) vector (Invitrogen Co.) and named pcDNAkCD. At 4 h after transfection with pcDNAkCD, the medium was changed to that containing tunicamycin (10 μg/ml), and then cultured at 37 °C for 12 h. Cells were harvested, lysed in standard assay solution, and the activity was determined using C12-NBD-Cer. The lysate was also subjected to the treatments with endoglycosidase H (Calbiochem) and glycopeptidase F as described in manufacturer's instructions. Aliquots of samples were subjected to SDS-PAGE, followed by staining with anti-Myc antibody. D, the effect of cycloheximide on 113-kDa (endoplasmic reticulum form) and 133-kDa (Golgi form) CDases. The medium was changed to that containing cycloheximide (50 μg/ml) at 16 h after transfection of HEK293 with pcDNAkCD, and then cultured in the indicated times. Cells were harvested and the Myc-tagged CDases were detected by Western blotting with anti-Myc antibody.

Distribution of the Neutral CDase in Rat Kidney and Liver—We raised anti-neutral CDase antibody (IgG) in rabbit using recombinant neutral CDase as antigen expressed in E. coli as described under "Experimental Procedures." Using this specific antibody and Cy3-labeled anti-rabbit IgG antibody as a second antibody, we examined the distribution of the CDase in the cortex (Fig. 9, A-C) and medulla (Fig. 9D) of rat kidney. Phalloidin-FITC (green) was used to visualize the actin filaments of brush borders in the proximal tubule cells (Fig. 9, B and C, arrowheads). Phalloidin-positive signals were also discernible at the bottom region of urinary tubules (Fig. 9, C and D, double arrows). A strong signal for neutral CDase (red) was observed in the luminal surface in most urinary tubules, such as proximal and distal tubules (Fig. 9, A-C), and collecting
tubules (Fig. 9D). Positive signal for the CDase was, however, hardly detectable in cells of glomerulus (not shown). Counterstaining with FITC-phalloidin revealed that the CDase was localized on top of the microvilli in the proximal tubule cells (Fig. 9, B and C, arrows). In hepatocytes in liver, the CDase positive signal (red) appeared as many dot-like structures that distributed throughout the cytoplasm (Fig. 9, E, center). To specify the organelles containing the CDase in hepatocytes, we performed double immunostaining using monoclonal antibody YA 30 which reacts with LGP85, a marker protein for lysosomes/late endosomes (Fig. 9E, left). The CDase signals (red) were found to be partially co-localized with the signal of LGP85 (green) (Fig. 9E, right, arrows). In summary, in rat kidney the neutral CDase seems to localize to apical membranes of urinary tubule such as proximal tubules, distal tubules, and collecting ducts, while the enzyme is distributed in endosome-like
Neutral Ceramidase of Rat Kidney—

Neutral CDase in Lipid Microdomain Raft of Rat Kidney—
From the brush border of Madin-Darby canine kidney cells, a cholesterol-enriched lipid microdomain raft was isolated as a non-ionic detergent-insoluble fraction using Lubrol 17A17 (34). In this study, we thus prepared a Lubrol-insoluble lipid microdomain from rat kidney and examined whether or not the neutral CDase is associated with the lipid microdomain. As shown in Fig. 10A, neutral CDase activity was found in fractions 6–8, in which free cholesterol and GM1 ganglioside were abundant, indicating that these fractions contain the lipid microdomain raft. Western blotting also confirmed that the CDase was concentrated in fractions 6–8 (B), whereas CD71 (transferrin receptor, a marker for the non-raft membrane fraction) was solely detected in the high density fraction, number 10 (C). In conclusion, the neutral CDase of rat kidney is likely to associate with a lipid microdomain raft in which cholesterol and glycosphingolipid GM1 are enriched.

**DISCUSSION**

Purification, Characterization, and cDNA Cloning of the Neutral CDase from Rat Kidney—Previously, we purified the neutral CDase from mouse liver, which was solubilized from the membrane fraction by freeze-thawing (25). However, the enzyme in mouse kidney was not solubilized by freeze-thawing and therefore had not been purified. In the present study, we succeeded in purifying the membrane-bound neutral CDase from the microsome fractions of rat kidney and cloned the cDNA encoding the enzyme. The rat kidney enzyme was classified as a neutral or neutral/alkaline CDase, based on its optimum pH. The enzymatic properties of the rat kidney CDase are somewhat different from those of mouse liver (25) and rat brain (38) CDases in the cation requirement, substrate specificity, and molecular weight. While the rat brain enzyme was activated by Mn²⁺ (38), the kidney enzyme was not (Fig. 3B).

The mouse liver enzyme hydrolyzed N-palmitoylsphingosine (C16:0/d18:1) most efficiently, the rat kidney enzyme, N-lauroylsphingosine (C12:0/d18:1) (Table II). The molecular mass of the rat kidney CDase was estimated to be 112 kDa on SDS-PAGE, which is clearly different from the neutral/alkaline CDases isolated from other origins: 94 kDa for the enzyme from mouse liver (25), 95 kDa from rat brain (38), 60 kDa from guinea pig skin (39), and 70 kDa from Pseudomonas aeruginosa (36). The molecular mass of acid CDase from human urine was reported to be 50 kDa (13).

However, the deduced amino acid sequence from the cDNA encoding the CDase of rat kidney is homologous to that of mouse liver (21) and human brain (22): 92 and 76% identity were found, respectively. The molecular mass estimated from the deduced amino acids was also similar: 83,483 for the rat enzyme (this study), 83,504 for the mouse enzyme (21), and 83,193 for the human enzyme (22). We speculated that this contradiction may stem from cell/tissue/organ-specific post-translational modification of the enzyme including N-glycosylation. It has been reported that the lysosomal proteins were occasionally truncated in the COOH-terminal region (40). Since the anti-Myc antibody reacts with the Myc tag of the CDase at the COOH terminus, we considered that the visualized CDase was not truncated in the COOH-terminal region (Fig. 7C). The possible modification of the enzyme, such as phosphorylation, acylation, and sulfation, should be further investigated and it is of interest that several phosphorylation sites are found in the deduced amino acid sequence of the rat neutral CDase.

To investigate the presence of the human-type CDase homologues in tissues other than human, we performed 5′ rapid amplification of cDNA ends-PCR with a primer designed using amino acid sequence 83–89 from the initiation Met of mouse CDase (21) against cDNA libraries prepared from mRNAs of mouse brain, liver, kidney, and spleen. Four splicing variants were found in mouse brains, 3 variants in liver, 1 variant in kidney, and 2 variants in spleen in which the 5′ noncoding regions were different from each other, although 88 amino acids from the initiation Met in open reading frames were exactly the same. These results strongly suggest that the human-type CDase homologues are not present or not as a major homologue in mice.

Subcellular Localization of Neutral CDase in Rat Tissue—

The liver neutral CDase was efficiently extracted with freeze-thawing whereas the kidney enzyme was not. This paper clearly indicated the reason why, i.e. in hepatocytes the neutral CDase was localized in late endosomes/lysosomes, whereas the enzyme was associated with a lipid microdomain raft on the apical membrane of urinary tubule cells in the kidney. It has been already reported that some lysosomal enzymes could be released from membrane fractions by freeze-thawing (41).

Why does the enzyme distribute in tissue-specific manner? One possible explanation is the tissue-specific expression of a receptor for di-Leu signal, which is a sorting signal for vesicular transport from plasma membrane to endosomes/lysosomes (37) or for targeting to basolateral membranes (42), because a functional di-Leu motif was found in the putative amino acid sequences in CDases of rat and mouse, but not human. Di-Leu receptors are a component of adaptor proteins (AP) associated with clathrin-coated vesicles and the organ-specific expression of AP has been reported (43). It is likely that di-Leu receptors would be expressed in rat hepatocytes but not in rat urinary tubule cells. Thus, in rat hepatocytes the CDase would be sorted from plasma membranes to late endosomes/lysosomes by vesicle transport using the di-Leu motif, whereas in rat urinary tubule cells the CDase would be retained at the apical sites of the plasma membrane possibly due to the lack of a receptor for the di-Leu signal. Tyr signal is also thought to be a conventional sorting signal (44), but is not present in the...
putative amino acid sequence of the rat and mouse neutral CDases. Recently, Bawab et al. (22) reported the presence of a mitochondria-targeting signal in the deduced amino acid sequence of human neutral CDase (Fig. 5) and showed that the overexpressing green fluorescent protein-tagged CDase was exclusively localized to mitochondria in HEK293 and MCF7 cells. However, the rat CDase seems not to be present in mitochondria, since the deduced sequence lacks the mitochondria-targeting signal and the enzyme is highly glycosylated with N-glycans that are not usually present in mitochondrial enzymes (45). It was also revealed in this study that tunicamycin treatment inhibited the generation of matured CDase with full activity, indicating that the N-glycosylation process is indispensable for the expression of the enzyme activity.

The localization of neutral CDase of rat liver is similar, but not identical, to that of LGP85 (Limp-2) which is a marker protein for late endosomes/lysosomes. The enzyme is highly glycosylated with N-glycans that are not usually present in mitochondrial enzymes (46). It is believed that a genetic deficiency of acid CDase could cause Farber disease, since an acid CDase is localized in lysosomes where Cer is accumulated in those with this disease. However, the present study showed that the subcellular localization of neutral/alkaline CDase depends on the cell/tissue/or organ and in hepatocytes, the enzyme actually localizes to late endosomes/lysosomes. Thus, the roles of the neutral/alkaline CDases in the catabolism of Cer in late endosomes/lysosomes and possible participation in Farber disease should be clarified.

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Purification, Characterization, Molecular Cloning, and Subcellular Distribution of Neutral Ceramidase of Rat Kidney
Susumu Mitsutake, Motohiro Tani, Nozomu Okino, Kaoru Mori, Sachiyu Ichinose, Akira Omori, Hiroshi Iida, Takashi Nakamura and Makoto Ito

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