O-Glycosylation of Mucin-like Domain Retains the Neutral Ceramidase on the Plasma Membranes as a Type II Integral Membrane Protein

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**FOOTNOTES**

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2 The abbreviations used are: CDase, ceramidase; Cer, ceramide; FBS, fetal bovine serum; GFP, green fluorescent protein; HRP, horseradish peroxidase; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PNA, peanut agglutinin; SM, sphingomyelin; Sph, sphingosine; S1P, sphingosine-1-phosphate.
SUMMARY

Ceramidase is a key enzyme involved in regulating cellular levels of ceramide, sphingosine, and possibly sphingosine-1-phosphate, and thus could modulate sphingolipid signaling. Here we report that O-glycosylation of the mucin-like domain of neutral ceramidases was required for localization to the surface of plasma membranes. The deduced amino acid sequences of the mammalian enzymes contain a serine-threonine-rich domain (mucin box) which follows the signal/anchor sequence, whereas those of bacterial and invertebrate enzymes completely lack a mucin box, suggesting that the specific domain has been acquired during evolution. In HEK293 cells overexpressing ceramidase, the enzyme was not only secreted into the medium after cleavage of the NH₂-terminal signal/anchor sequence, but also localized at the plasma membrane as a type II integral membrane protein. Lectin blot analysis using peanut agglutinin revealed that the mucin box of the enzyme is highly glycosylated with O-glycans. Interestingly, a mutant lacking the mucin box or possible O-glycosylation sites in the mucin box was secreted into the medium, but not localized at the surface of the cells. Furthermore, a mucin box-fused chimera GFP, but not GFP itself, with the signal/anchor sequence was distributed on the surface of the cells. These results suggest that O-glycosylation of the mucin box retains proteins on the plasma membranes. We also found that the 112-kDa membrane-bound enzyme from mouse kidney is O-glycosylated whereas the 94-kDa soluble enzyme from liver is not. These results clearly indicate that posttranslational modification of
the enzyme with O-glycans is tissue specific and helps the enzyme to localize at the surface of plasma membranes as a type II membrane protein.
INTRODUCTION

Sphingolipids have emerged as a multifunctional lipid biomodulator within or among cells. Ceramide (N-acylsphingosine; Cer), a common lipid backbone of sphingomyelin (SM) and glycosphingolipids, has been shown to mediate many cellular events including cell growth arrest, differentiation, and apoptosis (1, 2), possibly regulating various cytoplasmic proteins such as protein kinases C-ζ (3), -α, and -δ (4), and protein phosphatases 1 and 2A (5). Sphingosine (Sph), the N-deacylated product of Cer, exerts mitogenic and apoptosis-inducing activities, depending on the cell type and cell cycle (6, 7). Sph is known to be a potent inhibitor of protein kinase C (8) and an activator of 3-phosphoinositide-dependent kinase 1 which is thought to be occasionally localized at the inner plasma membrane (9). Notably, Sph can be phosphorylated to yield Sph-1-phosphate (S1P), which regulates cell proliferation (10), motility (11) and morphology (12). In contrast to Cer and Sph, S1P appears to act extracellularly by interacting with cell surface G protein-coupled receptors, the EDG family (13).

Ceramidase (CDase; EC 3.5.1.23), an enzyme that catalyzes hydrolysis of the N-acyl linkage of Cer to produce Sph, has been classified into three types mainly based on catalytic pH optima, i.e. acid, neutral and alkaline. Neutral CDases, which have an optimum pH of 6.5-8.5, have been cloned from bacteria (14), drosophila (15), mouse (16), rat (17), and human (18). Interestingly, phylogenetic analysis revealed that the three CDase isoforms having different pH optima may be derived from different ancestral genes (16). Mammalian neutral
CDase seems to regulate the balance of Cer/Sph/S1P in response to various stimuli including cytokines (19, 20) and growth factor (21) and thus could modulate sphingolipid-mediated signaling. Furthermore, the fact that Sph is not produced by de novo synthesis (22) implies a significant role for CDase in the generation of Sph and possibly S1P.

We found that neutral CDases of bacteria (14) and drosophila (15) were released from cells as a soluble form, while those of mammalian origins were mainly recovered in membrane fractions (19). Recently, it was found that the intracellular distribution of the mammalian enzyme was cell/tissue-specific. In rat kidney, neutral CDase was mainly localized at the apical membrane of proximal tubules, distal tubules, and collecting ducts, while in liver the enzyme was distributed among endosome-like organelles in hepatocytes (17). Human neutral CDase was exclusively localized to mitochondria in HEK293 and MCF7 cells when overexpressed as a fusion construct with green fluorescent protein (GFP) at the NH$_2$-terminus of the enzyme (18). Furthermore, both neutral and acid CDases were found to be released by murine endothelial cells (23). However, the molecular mechanism by which neutral CDases are localized to different organelles is not well understood.

This paper clearly shows that O-glycosylation of the mucin-like domain (mucin box) was required to retain neutral CDases on the plasma membranes as a type II integral membrane protein. It was found that the domain was occasionally lost by posttranslational processing, resulting in a different localization of the enzyme. These findings facilitate the understanding of the mechanism for cell/tissue-specific
localization of neutral CDases and provide some insight into sphingolipid metabolism at the cell surface or in the extracellular milieu.
EXPERIMENTAL PROCEDURES

Materials - CHOP cells and anti-Rab6 antibody were kindly provided by Dr. K. Nara (Mitsubishi Kagaku Institute of Life Sciences, Japan) and Dr. Shigeyasu Tanaka (Shizuoka University, Japan). HRP-labeled anti-mouse IgG antibody was purchased from Nacalai Tesque (Japan). Cy3-labeled anti-mouse IgG antibody, anti-flag M2 antibody, benzyl-GalNAc, brefeldin A, and cytochalasin D were obtained from Sigma. ECL plus, HRP-labeled and Cy3-labeled anti-rabbit IgG antibodies were from Amersham Pharmacia Biotech. Anti-myc and anti-GFP antibodies were purchased from Invitrogen Co. Anti-neutral CDase antibody was raised in a rabbit using the recombinant rat CDase as the antigen (17). C12-NBD-Cer was prepared as described in (24). HEK293 cells (JCRB9068, established by Graham, F. L.) were obtained from the Human Science Research Resource Bank. All other reagents were of the highest purity available.

Plasmid Construction - The vector pcDNA3.1/Myc-His(+) containing a full-length rat neutral CDase gene (pcDNAkCD) was constructed as described previously (17). To generate a construct expressing the GFP-fused neutral CDase, the vector pcDNAkCD was treated with KpnI and XhoI, and subcloned into pBluescript II SK (Stratagene). The product of the full-length neutral CDase gene digested with KpnI and SmaI was cloned into pEGFP-N2 (CLONTECH). A mutant which lacks the mucin-like domain (∆mucin) was constructed by fusing the NH₂-terminal fragment of Met1-Lys42 with the COOH-terminal fragment of Asn79-Thr761. The NH₂-terminal
fragment was amplified by PCR using a 5’ primer with a KpnI restriction site (5’-AGGGTACCGAAATGGCAGATGCAAAGCGAACCTTCTCC-3’), and a 3’ primer, (5’-ACACCAATGATGATGACCCACGACTGATTTTTTGCGATGGTGTC CC-3’). The COOH-terminal fragment was amplified with a 5’ primer (5’-GGGACCCTGAAAACCACAACAAAAACTTCAGTTGGCTACTACATTGGTGT-3’), and a 3’ primer with a XhoI restriction site (5’-GCCGCTCGAGGATGACTGACAAATTTCAAAAGGGAAGAAGGAAGAGCCGACACCC TCCTCC-3’) and a 3’ primer with a XhoI restriction site (5’-GCCGCTCGAGGATGACTGACAAATTTCAAAAGGGAAGAAGGAAGAGCCGACACCC TCCTCC-3’). These fragments were extended with Pyrobest DNA polymerase (Takara Shuzo Co.) and subcloned into the vector pcDNA3.1/Myc-His(+) (Invitrogen Co.). To obtain wild-type and Δmucin CDases tagged with flag epitope (MDYKDDDDK) at the NH2-terminus, DNA fragments of the flag-tagged CDases were amplified by PCR using a 5’ primer with a KpnI restriction site (5’-AGGTGGTACCATGACTACAAAGACGATGACAAGGCAAAGCGAACCTTCTCC-3’) and a 3’ primer with a XhoI restriction site (5’-GCCGCTCGAGGATGACTGACAAATTTCAAAAGGGAAGAAGGAAGAGCCGACACCC TCCTCC-3’) and subcloned into the vector pcDNA3.1/Myc-His(+). The mucin box-fused chimera GFP with signal/anchor sequence (S-M-GFP) and GFP with signal/anchor sequence (S-GFP) were designed using the two sequences of the NH2-terminal fragments of the enzyme, Met1-Gln78 and Met1-Lys42, respectively. Each sequence was amplified by PCR using a 5’ primer with a KpnI restriction site (5’-AGGGTACCGAAATGGCAGATGCAAAGCGAACCTTCTCC-3’) and a 3’ primer with a BamHI restriction site (5’-CCACGGATCCCCTGAGAGGGAGGGAGGTCTGG-3’) or (5’-TTCCGGATCCCTTGTGTTTTGCATGGTTCCTCC-3’). The amplified fragments were subcloned into pEGFP-N2. Construction of a mutant (Muc-Ala), in
which all Ser and Thr residues of mucin box were replaced with Ala residues, was described below. NH2-terminal fragment (Met1-Pro62) was amplified with a 5’ primer with a KpnI restriction site (5’-AGGGTACCCGAATGGCAAGCGAACCCTTCTCC-3’), and a 3’ primer (5’-TGGAGCTGCCTGGGCGGCGTCCCTGCGCGGCTGCAACCCAATGATTC CCTGCATCTTT-3’). COOH-terminal fragment (Asp43-Asn330) was amplified with a 5’ primer (5’-AAGATGCAGGGAATCATTGGGTTGCAGCCGCAGGACCCGCAGCCGCCCAGGCAGCTCCAGCCGCACCGCTAGAAGCCAGCGGCTCCTCCTCCCTCCC GCCTCAG-3’), and a 3’ primer (5’-GCAGGCTTTGCTTCATCAA-3’). These fragments were extended with Pyrobest DNA polymerase, digested with KpnI and BamHI, and subcloned into pcDNAkCD. The sequences of these constructs were verified with a DNA sequencer (Applied Biosystems Japan; model 377).

CDase Assay - CDase activity was measured using C12-NBD-Cer as a substrate as described in (25).

Cell Culture and cDNA Transfection - HEK293 cells, human embryonic kidney cells, were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 60 µg/ml of kanamycin in a humidified incubator containing 5% CO2. CHOP cells, chinese hamster ovary cells that express polyoma LT antigen to support the replication of eukaryotic expression vectors (26), were grown in α-minimal essential medium supplemented with 10% FBS, 100 µg/ml of streptomycin and 100 units/ml of penicillin in a humidified incubator containing 5% CO2. cDNA transfection was carried out using LipofectAMINE™ Plus (Invitrogen Co.) according to
the instructions of the manufacturer.

Preparation of Culture Supernatants and Cell Lysates - At 18 h after transfection with the CDase cDNA, the medium was replaced with serum-free Opti-MEM (Invitrogen Co., 500 µl/well on 24-well plates) and cultured for an additional 24 h. The cell culture medium was collected and subjected to centrifugation at 13,000 x g for 5 min. The supernatant, supplemented with a 1/10 volume of 200 mM Tris-HCl, pH 7.5 containing 1% Triton X-100 and 3.3 µg/ml of proteinase inhibitors (leupeptin, pepstatin, chymostatin), was used as cell supernatant. Cells attached to the culture plate were rinsed with PBS and then lysed by adding 200 µl of 10 mM Tris-HCl, pH 7.5, containing 0.5% Triton X-100 and 3.3 µg/ml of the proteinase inhibitors described above. Lysates were collected by pipette and used as cell lysates.

Protein Assay, SDS-PAGE and Western Blotting - Protein content 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 (27). Protein transfer onto a polyvinylidifluoride membrane was performed using TRANS-BLOT SD (Bio-Rad) according to the method described in (28). After treatment with 3% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (T-TBS) for 1 h, the membrane was incubated with primary antibody for 1 day at 4°C. After a wash with T-TBS, the membrane was incubated with HRP-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, and
chemiluminescent signals were visualized on ECL™ Mini-camera (Amersham Pharmacia Biotech).

**Amino Acid Sequencing** - The NH$_2$-terminal sequence of the 94-kDa soluble CDase was determined by the method described previously (25), i.e., the 94-kDa band was visualized by SDS-PAGE with GelCode Blue Stain reagent (Pierce), extracted from the gel and determined by automated Edman degradation using an amino acid sequencer model 477A (PE Biosystems).

**Immunoprecipitation of CDase** - Anti-neutral CDase antibody at a dilution of 1:100 was conjugated with 10 µl of protein A-agarose (Santa Cruz Biotechnology) in 100 µl of reaction buffer (10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Triton X-100 and 0.1% BSA) at 4°C for 2 h. After 5 washes with reaction buffer, 10 µl of protein A-agarose conjugated with antibody was resuspended in 900 µl of reaction buffer, to which 100 µl of denatured CDase was added, and then incubation conducted at 4°C for 18 h. Before immunoprecipitation, CDase was denatured by boiling for 5 min in 100 µl of SDS-sample buffer (20 mM Tris-HCl, pH 7.5 containing 1% SDS and 1% 2-mercaptoethanol). The precipitate was spun-down by centrifugation, washed with reaction buffer 5 times, and then suspended in 20 µl of SDS-sample buffer. After boiling at 100°C for 5 min, the sample was subjected to SDS-PAGE, followed by Western blotting analysis as described above.

**Immunocytochemistry and Fluorescence Microscopy** - Transfected cells were cultured on cover glass and then fixed with 3% paraformaldehyde in PBS for 15 min. After being rinsed with PBS and
50 mM NH₄Cl in PBS, cells were permeabilized, if necessary, by 0.1% Triton X-100 in PBS. After treatment with blocking buffer (5% skim milk in PBS) for 15 min, the samples were incubated with primary antibody (diluted 1:1000 with blocking buffer) at 4°C for 1 day followed by Cy3-labeled secondary antibody at room temperature for 2 h. Immunostained samples were examined with a confocal laser-scanning microscope (Digital Eclipse C1, Nikon, Japan).

Flow Cytometric Analysis - Transfected cells were harvested and incubated with primary antibody (1:1000) in 100 µl of PBS containing 50% FBS on ice for 1.5 h. After being washed twice with PBS, cells were treated with Cy3-labeled secondary antibody in 100 µl of PBS containing 50% FBS on ice for 1 h and then analyzed by flow cytometry (EPICS XL System-IC, Beckman Collter).
RESULTS

Subcellular Localization and Topology of Neutral CDase - The neutral CDase was reported to be localized at apical membranes of rat kidney (17), while the enzyme was actively released from murine endothelial cells (23). We found that the CDase fused with GFP at the COOH-terminus of the enzyme (Fig. 1E, all constructs used in this study are illustrated in Fig. 1) was distributed in ER/Golgi compartments as well as the plasma membranes of HEK293 cells using a confocal laser microscope (Fig. 2A). The fluorescent signal for GFP-fused CDase was partly co-localized with that for Rab6, a marker protein for Golgi apparatus (Fig. 2B). To clarify the topology of the CDase, a double-tagged CDase, with a flag-tag at the NH₂-terminus and myc-tag at the COOH-terminus, was constructed (Fig. 1C) and expressed in HEK293 cells. The flag signal was observed in ER/Golgi compartments as well as on plasma membranes when cells were permeabilized with Triton X-100 (Fig. 2C, a). However, the signal was not detected in intact cells (Fig. 2C, b). On the other hand, the COOH-terminal myc signal was observed on plasma membranes with and without treatment of Triton X-100 (Fig. 2C, c, d). These results indicate that the COOH-terminus of the CDase resides on the extracellular side of the plasma membrane whereas the NH₂-terminus is on the cytoplasmic side. In conclusion, the neutral CDase was expressed on plasma membranes as a type II integral membrane protein in HEK293 cells, being anchored to the membranes with an internal uncleaved NH₂-terminal signal/anchor sequence.

Mucin-like Domain of Mammalian Neutral CDases - Alignment of the deduced amino acid sequences of rat and mouse neutral CDases with
those of Pseudomonas aeruginosa, Mycobacterium tuberculosis, Dictyostelium discoideum, and Drosophila melanogaster neutral CDases, revealed that the rat and mouse enzymes have a Ser/Thr-rich domain (amino acids 43-78) downstream of the NH₂-terminal hydrophobic region. The hydrophobic region, which is a putative signal sequence, was observed in all CDases except that from M. tuberculosis. However, the Ser/Thr-rich domains were exclusively observed in CDases from mammals (Fig. 3A), suggesting this domain has been given to the enzyme during evolution. This domain shows a characteristic mucin-type repeating structure which contains not only Ser and Thr but also Pro and Gln, and thus was tentatively designated the mucin box in this study. Sequences homologous to the rat and mouse mucin box were found in rat sucrase isomaltase, human MUC2 and insect intestinal mucin peptide (Fig. 3B).

Expression and Processing of Wild-type and Mucin Box-deleted CDases - To investigate the effect of the mucin box on the expression and processing of the enzyme, two constructs were generated; a wild-type CDase which has the entire sequence of rat neutral CDase and a deletion mutant (Δmucin) which lacks the mucin box from the wild-type CDase. Both constructs have a signal/anchor sequence at the NH₂-terminus and are tagged with myc at the COOH-terminus (Fig. 1. A, B). When the wild-type construct was transfected into HEK293 cells, two protein bands having a molecular mass of 113 kDa and 133 kDa on SDS-PAGE were detected in cell lysate after visualization with anti-myc antibody (Fig. 4A). The 113-kDa and 133-kDa proteins seem to be glycosylated with high-mannose type N-glycans and high
mannose/complex/hybrid type N-glycans, respectively, as reported in (17), suggesting that they are a developing form in the ER and a mature form in the Golgi apparatus, respectively. A single band of approximately 105 kDa was detected in cell lysate when the Δmucin CDase was expressed in HEK293 cells and stained with anti-myc antibody (Fig. 4A). Notably, both wild-type and Δmucin CDases were secreted into the medium (Fig. 4A). To investigate whether a NH₂-terminal signal/anchor sequence was removed by proteolysis, NH₂-terminal flag-tagged wild-type (Fig. 1C) and Δmucin (Fig. 1D) CDases were constructed and expressed in HEK293 cells. Both constructs were also tagged with myc at the COOH-terminus. Western blotting analysis using anti-flag antibody revealed that both overexpressed wild-type and Δmucin CDases were flag-positive in cell lysates, but those in culture media were flag-negative (Fig. 4B). On the other hand, myc-positive CDases were detected in both the lysates and culture media of cells overexpressing wild-type and Δmucin enzymes (Fig. 4B). These results indicate that the NH₂-terminal signal/anchor sequence remains intact before secretion and the enzyme is secreted possibly after cleavage of the signal/anchor sequence, regardless of the presence or absence of mucin box.

O-Glycosylation and Secretion of the CDase - To verify whether or not the mucin box is actually glycosylated with O-glycans, wild-type and Δmucin CDases were subjected to lectin blotting using peanut agglutinin (PNA) which specifically binds to the Galβ1,3GalNAc sequence of O-glycans (29). For wild-type enzyme, the 133-kDa mature form, but not the 113-kDa ER-form, was stained with the lectin whereas no band was
stained for Δmucin CDase (Fig. 5A). Furthermore, treatment of CDase-overexpressing cells with benzyl-GalNAc, an inhibitor of O-glycosylation, resulted in a reduction in the molecular mass of the 133-kDa, but not the 113-kDa, wild-type CDase. The inhibitor did not affect the molecular mass of the Δmucin CDase (Fig. 5B). These results suggest that O-glycosylation crucially occurred in the mucin box of the CDase possibly at the Golgi apparatus. The secreted wild-type CDase was also stained with PNA (data not shown), suggesting that the mucin box is not removed by proteolytic processing of the signal/anchor sequence in HEK293 cells. The wild-type as well as Δmucin CDase was continuously released from cells into the culture medium (Fig. 6A). It should be noted, however, that the Δmucin CDase was found to be secreted much faster than the wild-type enzyme. The secretion of both enzymes was strongly inhibited by brefeldin A and treatment at 5°C but not by cytochalasin D (Fig. 6B), suggesting that the two CDases were processed and secreted by a pathway via ER/Golgi compartments.

**Mucin-box Mediated Cell-surface Expression of Neutral CDase**

To investigate the role of the mucin box in the subcellular localization of the neutral CDase, immunocytochemical analysis of wild-type and Δmucin CDases (Fig. 1A, B), both of which were tagged with myc at the COOH-terminal, was performed. Interestingly, the myc signal of the wild-type CDase was much stronger than that of the Δmucin CDase when the transformed cells were not permeabilized (Fig. 7A, a versus c), although the expression levels of the enzymes were almost the same when cells were permeabilized with Triton X-100 (Fig. 7A, b versus d). Flow cytometric analysis also showed a clear difference in the cell-
surface expression of myc signal of the enzyme due to the presence of a mucin box, i.e. wild-type CDase localizes at the surface of plasma membranes of HEK 293 as well as CHOP cells but Δmucin enzyme does not (Fig. 7B, C).

Significance of O-glycosylation in the Mucin Box for Cell-surface Expression of the CDase - It was revealed by lectin blotting that the mucin box of the CDase was actually glycosylated with O-glycans (Fig. 5). To investigate the significance of O-glycans in the mucin box for cell-surface expression of the CDase, the possible O-glycosylation sites (all Ser and Thr residues) of the mucin box were mutated with Ala (Fig. 8A). When the mutant CDase (Muc-Ala) was transfected into CHOP cells, a single protein band showing 113 kDa was detected in the cell lysate. In contrast to the 133-kDa Golgi form of the wild-type CDase, the Muc-Ala 113 kDa band was not affected when the benzyl-GalNAc, an inhibitor for O-glycosylation, was added into the culture of CHOP cells, indicating the mutant lost the O-glycans as expected (Fig. 8B). Although both wild-type and Muc-Ala mutant CDases were continuously released into the culture medium, the amount of secreted enzyme was significantly increased by the removal of O-glycans from the mucin box (Fig. 8C). In contrast, the cell-surface expression of the enzyme was markedly reduced by removal of O-glycans (Fig. 8D, a versus c). It is noted, however, that the expression of the mutant CDase was almost the same as that of wild-type CDase (Fig. 8D, b versus d). Taken together, it was concluded that the O-glycosylation of the mucin box of the CDase helps the enzyme to localize at the surface of plasma membranes as a
type-II integral membrane protein.

Localization of Mucin Box-fused GFP - To verify the potential role of the mucin box per se, GFP was used as a reporting molecule, i.e. a mucin box-fused chimera with signal/anchor sequence (S-M-GFP) was constructed (Fig. 1F) and expressed in HEK293 cells. Two major bands of 38 kDa and 57 kDa were detected in cell lysates by Western blotting using anti-GFP antibody (Fig. 9A, lane 3). The 57-kDa, but not 38-kDa, protein was reduced in its molecular mass by treatment with benzyl-GalNAc (data not shown), indicating it was O-glycosylated. The fused protein was found to distribute on the surface of HEK293 cells when stained with anti-GFP antibody (Fig. 9B, b). In contrast, GFP with signal/anchor sequence (S-GFP, Fig. 1G) is not expressed on the cell surface (Fig. 9B, d), although the intracellular expression of S-GFP is comparable to that of S-M-GFP (Fig. 9B, a versus c). Flow cytometric analysis using anti-GFP antibody also revealed that the cell-surface signal of S-M-GFP was much stronger than that of S-GFP and mock transfectants when expressed in HEK293 cells (Fig. 9C). These results clearly indicate that the O-glycosylated mucin box functions as a potential signal to retain proteins on the cell surface.

Mucin Box in Neutral CDases from Mouse Tissues and Serum - It was found that in mouse liver, 72% of neutral CDase activity was recovered in the soluble fraction after freeze-thawing, while in mouse kidney, more than 90% of the activity was recovered in the insoluble fraction (Fig. 10A). The neutral CDase in the kidney insoluble fraction was identified as a 112-kDa protein and that in the liver soluble fraction
as a 94-kDa protein by Western blotting using anti-neutral CDase antibody (Fig. 10B). The 112-kDa CDase was also detected in the insoluble fraction of liver (Fig. 10B). Interestingly, the 112-kDa insoluble CDases from kidney and liver were stained by PNA lectin, whereas the 94-kDa soluble CDase was not, indicating that only the CDases in the insoluble fraction were modified with O-glycans. The NH$_2$-terminal sequence of the 94-kDa soluble CDase was determined by the method described in the previous report (25). The determined NH$_2$-terminal sequence corresponded to FSGYYIGVGRADCTQVSDIN in the deduced sequence (80 –100 in Fig. 3A), indicating that the protein does not possess a signal/anchor sequence or a mucin box. Although the insoluble CDases from both liver and kidney were O-glycosylated, the O-glycan structure of kidney CDase seems to be somewhat different from that of the liver enzyme. This is because sialidase treatment significantly reduced the molecular mass and increased the PNA-reactivity of the liver CDase but had little effect on the kidney enzyme (Fig. 10B). Furthermore, we found that the PNA-positive CDase was also present in mouse serum, which was possibly derived from liver because sialidase treatment of the enzyme reduced its molecular mass and increased its PNA reactivity as in the liver CDase (Fig. 10B). These results strongly suggested that posttranslational modification of the enzyme with O-glycans is performed in a tissue-specific manner and affects the intracellular distribution of the CDase.
DISCUSSION

This study demonstrated that the neutral CDase is sorted by a classical pathway via ER/ Golgi compartments to the plasma membranes where it is expressed as a type II integral membrane protein or alternatively secreted out of cells after proteolytic processing of the NH$_2$-terminal signal/anchor sequence. This report also clearly indicates that the fate of the protein (as a membrane protein or secretion protein) depends on the presence of a mucin box located in the NH$_2$-terminal region of the enzyme. Because, in contrast to the wild-type CDase, the mucin box-deleted mutant CDase does not localize at the surface of cells and is almost entirely secreted. Furthermore, the fact that bacterial and invertebrate neutral CDases which lack the mucin box are secreted without cell-surface expression may support this conclusion. In conclusion, the mucin box acquired by the process of evolution enables the mammalian CDase to localize on the cell surface as a type II integral membrane protein. It should be emphasized that the mucin box-mediated cell-surface localization is not limited to the neutral CDase but occurs in general, because the mucin box-fused chimeric GFP, but not GFP itself, with signal/anchor sequence was found to be exclusively localized at the surfaces of HEK293 cells.

The mechanism by which the mucin box retains CDase on the cell surface is unclear at present. However, the finding made here that replacement of possible O-glycosylation sites (Ser/Thr residues) with Ala greatly increased the secretion and reduced the expression of the CDase on plasma membranes may indicate specific functions of O-glycans, although the structures of O-glycans remain to be elucidated.
A diverse range of membrane proteins of type I or type II topology are occasionally released from the lipid bilayer by proteolysis catalyzed by a group of enzymes referred to as secretases (30). The proteolytic processing of membrane-bound proteins by secretases is well characterized in amyloid precursor protein (31), a type I integral membrane protein, and a Golgi-resident sialyltransferase (32), a type II integral membrane protein. The latter is cleaved in its membrane-anchoring region by β-secretase and secreted out of the cell (32). Thus, it is possible that the neutral CDase is detached from cells after processing of the NH$_2$-terminal signal/anchor sequence by secretases. We indicate the possibility that the mucin box interferes with the action of secretases resulting in the generation of the type II membrane-bound CDase, since the mucin box exists very close to the possible cleavage site of the signal/anchor sequence. However, molecular mechanism for proteolytic processing of the CDase including the participation of secretases remains to be clarified. Another possibility is that the mucin box is a potential signal for the sorting of proteins to plasma membranes without processing of the signal/anchor sequence, although the counterparts for the recognition of O-glycans have yet been characterized.

There have been few reports on the role of the O-glycosylation of secretory proteins. It has been reported that O-glycosylation of the COOH-terminal tandem-repeated sequences regulates the secretion of rat pancreatic bile salt-dependent lipase. The O-glycosylation of the enzyme concealed the Pro, Glu, Ser, and Thr-rich domain (PEST region), which is commonly present in rapidly degraded proteins, resulting in
delivery to a secretion instead of a degradation pathway (33). In polarized cells, the O-glycosylation was also found to be required for apical sorting of sucrase isomaltase (34) and the neurotrophin receptor (35). It was also reported that a mucin-like domain of enteropeptidase directs apical targeting in MDCK cells (36). The neutral CDase was localized at the apical membranes of proximal tubules, distal tubules, and collecting ducts in rat kidney (17) and thus the possible functions of O-glycans in apical sorting should be examined in this CDase.

In contrast to our observation, human neutral CDase has been reported to be expressed in mitochondria when overexpressed in HEK 293 and MCF7 cells (18). We found that the deduced NH$_2$-terminal sequence of the human CDase lacks 19 amino acid residues in comparison with those of mouse and rat CDases (16-18). This may result in the generation of an incomplete signal/anchor sequence for ER-targeting, allowing the human CDase to target mitochondria instead of the lumen side of the ER.

Recently, evidence has emerged that the outer leaflet of the plasma membrane is a site of SM metabolism; SM is abundant here particularly in lipid microdomain rafts (37), and sphingomyelinase as well as CDase is actively secreted from endothelial cells (23, 38). Furthermore, acid sphingomyelinase was reported to be secreted and helped to metabolize SM in oxidized lipoproteins (39) and CD95 induces the translocation of acid sphingomyelinase onto the cell surface, resulting in the generation of extracellularly orientated Cer (40). The neutral CDase is expressed at the plasma membrane as a type II membrane protein, the catalytic domain of which is located on
the extracellular side, or else is secreted out of the cell. Importantly, the generation of S1P in the extracellular milieu, which occurs due to the export of the Sph kinase, was reported (41). Very recently, it was found that phorbol 12-myristate 13-acetate (PMA) induced the PKC-dependent translocation of Sph kinase to the plasma membrane, resulting in the extracellular release of S1P (42). Taken together, all metabolic enzymes required for hydrolysis of SM to generate S1P could be present at the outer leaflet of the plasma membrane or in extra milieu, allowing for the formation of an alternative pathway from SM to S1P. In this context, it is important to note that the neutral CDase was actually detected in mouse serum, which may indicate an important role in vascular biology, i.e. regulating the extracellular content of Cer, Sph and possibly S1P, all of which may function in autocrine/paracrine signaling.

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FIGURE LEGENDS

Fig. 1. Schematic diagram of cDNA constructs.

The diagram shows the structures of cDNA constructs used in this study. (A), wild-type CDase (wild-type CDase tagged with myc at the COOH terminus); (B), Δmucin CDase (mucin box-deleted mutant CDase tagged with myc at the COOH terminus); (C), flag-tagged CDase (neutral CDase tagged with flag at the NH2-terminus and myc at the COOH terminus); (D), flag-tagged Δmucin CDase (mucin box-deleted mutant CDase tagged with flag at the NH2-terminus and myc at the COOH terminus); (E), GFP-fused CDase (neutral CDase fused with GFP at the COOH-terminus); (F), S-M-GFP (mucin box with signal/anchor sequence fused with GFP); (G) S-GFP (signal/anchor sequence fused with GFP).

Fig. 2. Immunocytochemical analysis of the neutral CDase overexpressed in HEK293 cells.

(A), HEK293 cells overexpressing GFP-fused neutral CDase. Cells were fixed and examined for GFP fluorescence under a confocal laser microscope. Arrows and an arrowhead indicate the expression of CDase at plasma membrane and ER-Golgi compartments, respectively. (B), HEK293 cells expressing GFP-fused neutral CDase (left) were fixed, and immunostained with anti-Rab6 antibody followed by anti-rabbit IgG-Cy3 (center). Images were merged (right). An arrow indicates the colocalization of neutral CDase and Rab6, a marker protein for the Golgi apparatus. (C), Analysis of membrane topology of neutral CDase. (a), stained with anti-flag antibody after permeabilization with Triton X-100. (b), same as (a) but before treatment with Triton X-100. (c), stained with
anti-myc antibody after permeabilization with Triton X-100. (d), same as (c) but before treatment with Triton X-100. HEK293 cells were transformed with plasmid vector containing flag-tagged CDase cDNA, fixed, and then stained with corresponding antibody before and after treatment with Triton X-100 as described in ‘Experimental Procedures’.

Fig. 3. Alignments of deduced amino acid sequences of neutral CDases based on NH$_2$-terminal regions (A) and mucin-like domains (B). The identification of the mucin box was performed by a BLAST search program (43).

Fig. 4. Expression, processing and secretion of wild-type and mucin box-deleted CDases.

(A), Western blotting of the CDase expressed in HEK293 cells. Cells were transfected with plasmid vector containing wild-type or Δmucin CDase cDNA. The cell lysates and the culture supernatants were separately subjected to SDS-PAGE (7.5% gels), followed by Western blotting using anti-myc antibody. (B), the same experiment was carried out as shown in (A) but cells were transfected with plasmid vector containing flag-tagged wild-type or Δmucin CDase cDNA. The cell lysates and the culture supernatants were separately subjected to Western blotting using anti-myc antibody (upper) or anti-flag antibody (lower). C and M indicate cell lysate and medium (culture supernatant), respectively.

Fig. 5. O-Glycosylation of neutral CDase.
(A), Lectin blotting of wild-type and mucin box-deleted CDases. The lysates of HEK293 cells transfected with plasmid vector containing wild-type or Δmuicn CDase cDNA were subjected to immunoprecipitation using anti-neutral CDase antibody as described under ‘Experimental Procedures’. Thereafter, the immunoprecipitants were subjected to SDS-PAGE, followed by Western blotting using anti-myc antibody or lectin blotting using HRP-labeled PNA. (B), Inhibition of O-glycosylation by benzyl-GalNAc. At 4 h after transfection, the cells were treated with or without 5 mM benzyl-GalNAc in DMEM supplemented with 10% FBS, and then cultured for an additional 20 h. Cells were lysed and subjected to SDS-PAGE, followed by Western blotting using anti-myc antibody.

Fig. 6. Secretion of wild-type and mucin box-deleted CDases.

(A), Time course for the secretion of wild-type and Δmuicn CDases. At 18 h after transfection, the medium was replaced with serum-free Opti-MEM and the cells were incubated at 37°C for the period indicated. The culture supernatant was employed for the determination of neutral CDase activity using C12-NBD-Cer as a substrate and Western blotting with anti-myc antibody. (B), Effects of brefeldin A (BFA), cytochalasin D (CytoD), and temperature on the secretion of CDase. At 18 h after transfection, cells were incubated with 10 µg/ml of BFA or 5 µM CytoD in DMEM supplemented with 10% FBS at 37°C for 1 h. The medium was replaced with Opti-MEM containing the inhibitors at the same concentrations and then cultured at 37°C for an additional 3 h. To examine the effect of temperature, transfected cells were incubated at
5°C for 3 h in fresh Opti-MEM without inhibitors.

Fig. 7. Cell-surface expression of neutral CDase.

(A), Expression of neutral CDase on the surface (a, c) or inside (b, d). Wild-type- (a, b) and Δmucin-expressing HEK293 cells (c, d) were fixed, permeabilized by Triton X-100 (b, d) or not (a, c), and stained with anti-myc antibody. (B, C), Cell-surface expression of neutral CDase in HEK 293 (B) and CHOP (C) cells. Cells (3 x 10^5) were transfected with plasmid vector containing wild-type or Δmucin CDase cDNA, or empty vector (mock) and then cultured at 37°C for 24 h. Cells were harvested and incubated with anti-myc antibody followed by anti-mouse IgG-Cy3 at 4°C and analyzed by flow cytometry as described under ‘Experimental Procedures’.

Fig. 8. Effects of mutation of possible O-glycosylation sites on the secretion and cell-surface expression of the CDase.

(A), The deduced amino acid sequences of the mucin box in a wild-type CDase and a mutant enzyme disrupting potential O-glycosylation sites (Muc-Ala). All underlined Ser and Thr residues were replaced by Ala as described in ‘Experimental Procedures’. (B), Western blotting of wild-type and Muc-Ala CDases expressed in CHOP cells. At 4 h after transfection with plasmid vector containing wild-type or Muc-Ala CDase cDNA, the cells were treated with (+) or without (-) 5 mM benzyl-GalNAc in DMEM supplemented with 10% FBS, and then cultured for an additional 20 h. Cell lysates were subjected to SDS-PAGE, followed by Western blotting using anti-myc
antibody. (C), Time course for the secretion of wild-type and Muc-Ala mutant CDases in CHOP cells. At 18 h after transfection, the medium was replaced with serum-free Opti-MEM and the cells were incubated at 37°C for the period indicated. The culture supernatants were employed for the determination of neutral CDase activity using C12-NBD-Cer as a substrate and Western blotting using anti-myc antibody. (D) Expression of neutral CDase on the surface (a, c) or inside (b, d) of CHOP cells. Cells expressing wild-type (a, b) and Muc-Ala (c, d) CDases were fixed, permeabilized with Triton X-100 (b, d) or not (a, c), and stained with anti-myc antibody. Arrows indicate the expression of CDase at plasma membrane.

Fig. 9. Expression of mucin box-fused GFP.

(A), SDS-PAGE of the expressed proteins. HEK293 cells were transfected with plasmid vector containing GFP (pEGFP-N2), S-GFP or S-M-GFP cDNA. Cell lysates were subjected to SDS-PAGE (12.5% gels), followed by western blotting using anti-GFP antibody. Lane 1, GFP; lane 2, S-GFP; lane 3, S-M-GFP. (B), Immunocytochemical analysis of S-M-GFP and S-GFP. HEK293 cells overexpressing S-M-GFP (a, b) or S-GFP (c, d) were fixed, and stained with anti-GFP antibody (b, d). The panels (a) and (c) show direct GFP fluorescence. (C), Flow cytometric analysis of cell-surface GFP. HEK293 cells (3 x 10^5) were transfected with plasmid vector containing GFP (pEGFP-N2), S-GFP or S-M-GFP cDNA. At 24 h after transfection, cells were harvested, stained with anti-GFP antibody before a second incubation with anti-rabbit IgG-Cy3 at 4 °C, and analyzed by flow cytometry.
Fig. 10. Mucin box in neutral CDases from mouse tissues and serum.

(A). Solubilization by freeze-thawing of neutral CDase from mouse liver and kidney. The preparation of the membrane fraction and solubilization of the neutral CDase by freeze-thawing were performed according to the method described in Ref. 25. Values are the means for duplicate determinations. (B), Western and lectin blotting of neutral CDase. Each sample was immunoprecipitated and analyzed by Western blotting using anti-neutral CDase antibody or lectin blotting using HRP-labeled PNA. For sialidase treatment, the immunoprecipitants were detached from agarose beads by addition of 10 μl of 100 mM glycine, pH 2.5. The pH of samples was adjusted to 5.0 by addition of 500 mM sodium acetate buffer, pH 5.0, containing 5 mM CaCl₂ and 0.1% Triton X-100. The mixtures were incubated with or without 5 milliunits of sialidase (Vibrio cholera) at 37 °C for 18 h.
Fig. 1. Tani M. et al. TOP

(A) Wild-type CDase

(B) ΔMucin CDase

(C) flag-tagged CDase

(D) flag-tagged
Δmucin CDase

(E) GFP-fused CDase

(F) S-M-GFP

(G) S-GFP

■ Signal/anchor sequence

■ Mucin box

□ flag-tag

Y Potential N-glycosylation site

myc-tag

Neutral CDase

GFP
Fig. 2. Tani M. et al. TOP

(A) GFP-fused CDase

(B) GFP-fused CDase  Rab6  Merged

(C) Triton X-100 (+)  Triton X-100 (-)
Anti-flag

Anti-myc

Fig. 2. Tani M. et al. TOP
**Fig. 3. Tani M. et al.**

**Signal/anchor sequence**

| Gene          | Sequence | Score |
|---------------|----------|-------|
| Rat neutral CDase | HAKRTFSLFLIVMAFLTVAALLLFLVTSGETIENHDGSNHNVSTTGPTQPTSSPTTQPTQTPTPLPSNFQGYIGVGRADCTGVEDINLNGYKGKNGQAGLL | 43 |
| Mouse neutral CDase | HAKRTFSLFLIVMAFLTVAALLLFLVTSGETIENHDGSNHNVSTTGPTQPTSSPTTQPTQTPTPLPSNFQGYIGVGRADCTGVEDINLNGYKGKNGQAGLL | 43 |
| Rat Sucrase isomaltase | MAKSRTSRAFILAD | 43 |
| Mouse Sucrase isomaltase | MAKSRTSRAFILAD | 43 |
| Pseudomonas | VQRTFSRAFLSACLXNLARRR | 58 |
| Mycobacterium | VIQRTFSRAFLSACLXNLARRR | 58 |
| Drosophila | VQRTFSRAFLSACLXNLARRR | 58 |

**Mucin box**

| Gene          | Sequence | Score |
|---------------|----------|-------|
| Mouse MUC2 | PDGSNRMASFVSVELCMQSRKLRELQKGSQG1YRDNWLSATETHSGPAGFFQYQLILALAEQFSRAFOVYSGIKSIDLNTLCGL67 | 1072 |
| Human MUC2 | PDGSNRMASFVSVELCMQSRKLRELQKGSQG1YRDNWLSATETHSGPAGFFQYQLILALAEQFSRAFOVYSGIKSIDLNTLCGL67 | 1072 |
| E. coli O157 | PDGSNRMASFVSVELCMQSRKLRELQKGSQG1YRDNWLSATETHSGPAGFFQYQLILALAEQFSRAFOVYSGIKSIDLNTLCGL67 | 1072 |
| D. melita | PDGSNRMASFVSVELCMQSRKLRELQKGSQG1YRDNWLSATETHSGPAGFFQYQLILALAEQFSRAFOVYSGIKSIDLNTLCGL67 | 1072 |
| S. enterica | PDGSNRMASFVSVELCMQSRKLRELQKGSQG1YRDNWLSATETHSGPAGFFQYQLILALAEQFSRAFOVYSGIKSIDLNTLCGL67 | 1072 |

**Signal/anchor sequence**

| Gene          | Sequence | Score |
|---------------|----------|-------|
| Rat neutral CDase | HAKRTFSLFLIVMAFLTVAALLLFLVTSGETIENHDGSNHNVSTTGPTQPTSSPTTQPTQTPTPLPSNFQGYIGVGRADCTGVEDINLNGYKGKNGQAGLL | 43 |
| Mouse neutral CDase | HAKRTFSLFLIVMAFLTVAALLLFLVTSGETIENHDGSNHNVSTTGPTQPTSSPTTQPTQTPTPLPSNFQGYIGVGRADCTGVEDINLNGYKGKNGQAGLL | 43 |
| Rat Sucrase isomaltase | MAKSRTSRAFILAD | 43 |
| Mouse Sucrase isomaltase | MAKSRTSRAFILAD | 43 |
| Pseudomonas | VQRTFSRAFLSACLXNLARRR | 58 |
| Mycobacterium | VIQRTFSRAFLSACLXNLARRR | 58 |
| Drosophila | VQRTFSRAFLSACLXNLARRR | 58 |

**Mucin box**

| Gene          | Sequence | Score |
|---------------|----------|-------|
| Mouse MUC2 | PDGSNRMASFVSVELCMQSRKLRELQKGSQG1YRDNWLSATETHSGPAGFFQYQLILALAEQFSRAFOVYSGIKSIDLNTLCGL67 | 1072 |
| Human MUC2 | PDGSNRMASFVSVELCMQSRKLRELQKGSQG1YRDNWLSATETHSGPAGFFQYQLILALAEQFSRAFOVYSGIKSIDLNTLCGL67 | 1072 |
| E. coli O157 | PDGSNRMASFVSVELCMQSRKLRELQKGSQG1YRDNWLSATETHSGPAGFFQYQLILALAEQFSRAFOVYSGIKSIDLNTLCGL67 | 1072 |
| D. melita | PDGSNRMASFVSVELCMQSRKLRELQKGSQG1YRDNWLSATETHSGPAGFFQYQLILALAEQFSRAFOVYSGIKSIDLNTLCGL67 | 1072 |
| S. enterica | PDGSNRMASFVSVELCMQSRKLRELQKGSQG1YRDNWLSATETHSGPAGFFQYQLILALAEQFSRAFOVYSGIKSIDLNTLCGL67 | 1072 |
Fig. 4. Tani M. et al. TOP
Fig. 5. Tani M. et al.
Fig. 6. Tani M. et al. TOP
Fig. 7. Tani M. et al. TOP

(A) Triton X-100 (-)  Triton X-100 (+)

Wild

ΔMucin

(B) HEK293

(C) CHOP

Cy3 fluorescence

Counts

ΔMucin
Mock
Wild

ΔMucin
Mock
Wild
(A) Mucin box

Wild type  43 DSGNHWVSTTQGP TTOSPTTQTPTTPDLPSSQ 78
Muc-Ala  43 DAGNHWV AAAQGP AAAQAPAAQAPAPDLPPAQ 78

(B)

(Benzyl-GalNAc) Wild  Muc-Ala

(C)

Released CDase activity (mU/mg cellular protein)

(D) Triton X-100 (-) Triton X-100 (+)

Wild

Muc-Ala

Fig. 8. Tani M. et al. TOP
**Fig. 9. Tani M. et al.**

(A)  
![Image](595x842.0)  

(B)  

| GFP fluorescence | Anti-GFP |
|------------------|----------|
| S-M-GFP          | ![Image](10 μm) |
| S-GFP            | ![Image](10 μm) |

(C)  

![Histogram](145)  

Counts vs. Cy3 fluorescence.
Fig. 10. Tani M. et al. TOP

(A)

Liver Kidney

|        | Anti-CDase | PNA-HRP |
|--------|------------|---------|
| Kidney (insoluble) | 112 k | ![Image](http://www.jbc.org/Downloaded from) |
| Liver (soluble)     | 94 k   | ![Image](http://www.jbc.org/Downloaded from) |
| Liver (insoluble)   | 112 k  | ![Image](http://www.jbc.org/Downloaded from) |
| Serum (soluble)     | 109 k  | ![Image](http://www.jbc.org/Downloaded from) |
