Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 331 (2005) 37–42
BBRC
www.elsevier.com/locate/ybbrc

Subcellular localization of human neutral ceramidase expressed in HEK293 cells ☆

Young-ha Hwang, Motohiro Tani, Tetsuto Nakagawa, Nozomu Okino, Makoto Ito *

Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

Received 7 March 2005
Available online 30 March 2005

Abstract

We previously reported that rat and mouse neutral ceramidases were mainly localized to plasma membranes as a type II integral membrane protein and partly detached from the cells via processing of the N-terminal/anchor sequence when expressed in HEK293 cells [M. Tani, H. Iida, M. Ito, *O*-glycosylation of mucin-like domain retains the neutral ceramidase on the plasma membranes as a type II integral membrane protein, J. Biol. Chem. 278 (2003) 10523–10530]. In contrast, the human homologue was exclusively detected in mitochondria when expressed in HEK293 and MCF7 cells as a fusion protein with green fluorescent protein at the N-terminal of the enzyme [S.E. Bawab, P. Roddy, T. Quian, A. Bielawska, J.J. Lemasters, Y.A. Hannun, Molecular cloning and characterization of a human mitochondrial ceramidase, J. Biol. Chem. 275 (2000) 21508–21513]. Given this discrepancy, we decided to clone the neutral ceramidase from human kidney cDNA and re-examine the intracellular localization of the enzyme when expressed in HEK293 cells. The putative amino acid sequence of the newly cloned enzyme was identical to that reported for human neutral ceramidase except at the N-terminal; the new protein was 19 amino acids longer at the N-terminal. We found that the putative full-length human neutral ceramidase was transported to plasma membranes, but not to mitochondria, possibly via a classical ER/Golgi pathway and localized mainly in plasma membranes when expressed in HEK293 cells. The N-terminal-truncated mutant, previously reported as a human mitochondrial ceramidase, was also weakly expressed in HEK293 cells but mainly released into the medium possibly due to the insufficient signal/anchor sequence.

Keywords: Sphingolipid; Ceramide; Ceramidase; Plasma membrane; Mitochondria

Ceramidase (EC 3.5. 1.23, CDase), an enzyme that catalyzes the hydrolysis of the *N*-acyl linkage of ceramide, is classified into three groups based on optimal pH and primary structure; acid, neutral, and alkaline enzymes [1]. Acid CDase functions in the catabolism of ceramide in lysosomes and a genetic mutation in it causes Farber disease in which ceramide is accumulated in lysosomes [2]. Alkaline CDase was recently reported to be present in the ER where it possibly functions to regulate the amount of ceramide and its metabolites [3]. Neutral CDase, which has an optimal pH of 6.5–8.5, has been cloned from bacteria [4], *Drosophila* [5], zebrafish [6], mouse [7], rat [8], and human [9]. Recently, the enzyme was found to be involved in angiogenesis during zebrafish embryogenesis [6] and functions of photoreceptors in *Drosophila* [10].

The primary structure of neutral CDase is highly conserved from bacteria to humans. However, there is a clear difference in the molecular architecture between invertebrate and murine neutral CDases. The latter possesses a 'mucin box,' which is a Ser/Thr/Pro-rich domain

☆ *Abbreviations:* CDase, ceramidase; GFP, green fluorescent protein; hCD, human neutral ceramidase; HRP, horseradish peroxidase; ΔN hCD, N-terminal 19 amino acids truncated human neutral ceramidase; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; PNA, peanut agglutinin.

* Corresponding author: Fax: +81 92 642 2898.
E-mail address: makotoi@agr.kyushu-u.ac.jp (M. Ito).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.03.134

glycosylated with *O*-glycans, while the former does not. The domain is not found in the bacterial CDase either. We previously concluded that glycosylation of the domain with *O*-glycans is necessary to retain the enzyme on the plasma membrane as a type II integral protein, because a mucin box-deleted mutant or Ala-replaced mutant enzyme, in which all Ser/Thr residues in the domain were replaced with Ala and thus no glycosylation occurred in the domain, was found to be released into the medium whereas the wild-type enzyme was mainly distributed on the plasma membranes when expressed in HEK293 and CHOP cells [11]. Invertebrate and bacterial enzymes were exclusively a secreted protein [4,5]. In contrast to neutral CDases from mouse and rat, the human homologue was reported to be localized exclusively to mitochondria when it was expressed in HEK293 and MCF7 cells with green fluorescent protein (GFP) fused to its N-terminal [9].

To resolve the controversy over the intracellular localization of murine and human neutral CDases, we cloned a neutral CDase from human kidney cDNA and re-examined its intracellular localization when expressed in HEK293 cells.

Materials and methods

Materials. HEK293 cells (JCRB9068) were obtained from the Human Science Research Resource Bank. HRP-labeled anti-mouse IgG antibody was purchased from Nacalai Tesque (Kyoto, Japan). Anti-*myc* antibody and the vector pcDNA3.1/Myc-His (+), MitoTracker Red CMXRos, and Alexa Fluor 488 anti-mouse IgG (H + L) were from Invitrogen. Human kidney cDNA was obtained from Clontech. ECL plus and HRP-labeled PNA lectin were from Amersham Biosciences and Seikagaku (Tokyo, Japan), respectively. Anti-mouse neutral CDase antibody was raised in a rabbit using the recombinant mouse neutral CDase as the antigen [8]. C12-NBD-ceramide was prepared as described in [12]. All other reagents were of the highest purity available.

Cloning of human neutral CDase. Taking into consideration the genome sequences of human neutral CDase (hCD) (Accession No. AL450382 in GenBank), we identified another candidate for the initiation Met located 19 amino acids upstream of the N-terminal Met of a hCD previously reported [9]. To obtain the full-length sequence, PCR was performed using the human kidney cDNA as a template. The sense primer (5′-TTCTTCCATCTCTGCTGTACCTGAG-3′) was designed based on the sequence upstream of the new candidate for the initiation codon, and the antisense primer (5′-GTGTGTGCTATGTCAATGCTCTTCA-3′) was based on the internal sequence of the enzyme. PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems) using AmpliTaq Gold (Applied Biosystems). The parameters for PCR were 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 150 s. As a result, a 0.7-kb fragment was obtained and then two more sets of PCR were further performed using a sense primer (5′-AGGTCCTGCAGGATATTTCCAGTA-3′) designed based on the sequence of the 0.7-kb fragment and an antisense primer (5′-ACAAGTGCTATTGGCGTTATCACA-3′) based on the internal sequence of the hCD, and a sense primer (5′-GCAGCCTTTGCTTCATCAA-3′) and an antisense primer (5′-TCACTAAATAGTTACAACTTCAAAAGCC-3′) both of which were based on the internal sequence of the enzyme. Consequently, 0.5 and 1.3-kb fragments were amplified, respectively. On assembling the 0.7, 0.5, and 1.3-kb fragments, a possible full-length sequence of hCD was obtained (full hCD), which is composed of 2346 bp encoding 782 amino acids. The sequence was verified with a DNA sequencer (model 377, Applied Biosystems).

Construction of full hCD and its deletion mutant (ΔN hCD). A cDNA fragment encoding the open reading frame of full hCD was prepared by PCR using a sense primer containing a *Kpn*I site (5′-GGGTACCGAAATGGCCAAACGCACCTT-3′) and an antisense primer containing an *Xho*I site (5′-GCTCGAGAATAGTTACAACTTCAAAAGCCGG-3′) and the cloned full hCD as a template. To obtain a hCD deleted of 19 amino acids (ΔN hCD), a sense primer containing a *Kpn*I site (5′-GGGTACCATGAGTGCCATCACAGTGG-3′) and cloned full hCD as a template were used. The PCR products were inserted into the *Kpn*I and *Xho*I sites of a pcDNA3.1/Myc-His (+) vector with a C-terminal *myc* tag.

Expression of neutral CDase in HEK293 cells. HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 60 μg/ml kanamycin and 10% fetal bovine serum (FBS) in a humidified incubator containing 5% CO_2. The cells were seeded at 5×10^4 cells/well of a 24-well plate. cDNA transfection was carried out using LipofectAMINE and PLUS reagents (Invitrogen) according to the instructions of the manufacturer. Transfection with the vector alone (pcDNA3.1/Myc-His (+)), or a vector containing a full hCD sequence or a ΔN hCD sequence was performed using 2 μl PLUS reagent, 1 μl LipofectAMINE reagent, and 0.25 μg of each plasmid per well. At 24 h after transfection with the cDNA, the medium was replaced with serum-free Opti-MEM (Invitrogen) and cultured for an additional 24 h. The cell culture medium was collected and subjected to centrifugation at 13,000*g* for 5 min to remove cell debris. The supernatant obtained was added to a 1/10 volume of 200 mM Tris–HCl buffer, pH 7.5, containing 1% Triton X-100 and 3.3 μg/ml of proteinase inhibitors (leupeptin, pepstatin, and chymostatin), and then subjected to the enzyme assay as a cell supernatant. Cells attached to the culture plate were rinsed with PBS and then lysed by adding 200 μl of 10 mM Tris–HCl buffer, pH 7.5, containing 0.5% Triton X-100 and 3.3 μg/ml of the proteinase inhibitors. Lysates were collected by pipette and used as a cell lysate.

CDase assay. The enzyme activity was measured at pH 7.5 using C12-NBD-ceramide as a substrate as described previously [7]. Briefly, 900 pmol C12-NBD-ceramide 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 1% sodium cholate. The reaction was stopped by adding 100 μl chloroform/methanol (2/1, v/v) and then the lower phase was applied to a TLC plate, which was developed with chloroform/methanol/25% ammonia (90/20/0.5, v/v). The TLC plate was analyzed and quantified using a Shimadzu CS-9300 chromatoscanner (excitation 470 nm, emission 525 nm, Shimadzu, Kyoto, Japan).

Protein assay, SDS–PAGE, and Western and lectin blottings. Protein content was measured using the bicinchoninic acid protein assay (Pierce) with bovine serum albumin (BSA) as a standard. SDS–PAGE was carried out according to a method of Laemmli [13]. Protein was transferred onto a PVDF membrane using Trans-Blot SD (Bio-Rad) according to a method described previously [14]. After treatment with 5% 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 horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h. For lectin blotting, the membrane was treated with 5% BSA in T-TBS for 1 h and incubated with the HRP-conjugated peanut agglutinin (PNA) lectin for 2 h at room temperature. After another wash with T-TBS, the ECL reaction was performed as recommended by the manufacturer, and chemiluminescent signals were visualized on an ECL Mini-camera (Amersham Biosciences).

Immunoprecipitation of neutral CDase. Anti-mouse neutral CDase antibody at a dilution of 1:100 was conjugated with 10 μl protein A–agarose (Santa Cruz Biotechnology) in 100 μl reaction buffer (10 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl, 1% Triton X-100,

and 0.1% BSA) at 4 °C for 2 h. After five washes with reaction buffer, the precipitated protein A–agarose was resuspended in 900 μl reaction buffer, to which 100 μl of denatured CDase was added, and then incubation was conducted at 4 °C for 18 h. Before immunoprecipitation, CDase was denatured by boiling for 5 min in 100 μl of 20 mM Tris–HCl buffer, pH 7.5 containing 1% SDS and 1% 2-mercaptoethanol. The precipitate was spun down by centrifugation, washed with reaction buffer five times, and then suspended in 20 μl SDS sample buffer (20 mM Tris–HCl buffer, pH 7.5, containing 1% SDS, 1% 2-mercaptoethanol, and 10% glycerol). After boiling at 100 °C for 5 min, the sample was subjected to SDS–PAGE, followed by Western blotting as described above.

Immunocytochemistry. Transfected cells were cultured on cover glass and then fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. After being rinsed with PBS and 50 mM NH_4Cl 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 fluorescence-labeled secondary antibody at room temperature for 2 h. Immunostained samples were examined with a confocal laser-scanning microscope (Digital Eclipse C1, Nikon, Tokyo, Japan).

Results

Alignment of the sequences reported for neutral CDases from mouse [7], rat [8], and human [9] indicated that in the N-terminal region, the human enzyme was shorter than the murine enzymes. Analyzing the genome sequence of the human neutral CDase (hCD), we found an alternative candidate for the initiation codon located 57 bases upstream from that of the hCD cDNA previously reported [9]. Thus, we cloned the cDNA of the whole sequence composed of 2346 bp encoding 782 amino acids in this study. The putative amino acid sequence of the newly cloned full-length hCD (full hCD) was identical to that previously reported as a human mitochondrial CDase (ΔN hCD) [9] except in the N-terminal region, that is, full hCD was 19 amino acids longer at the N-terminal than ΔN hCD. Both full hCD and ΔN hCD seem to possess a 'mucin box' that contains 19 Ser/Thr residues which are possible *O*-glycosylation sites (Fig. 1A). Interestingly, the domain of full hCD was rather longer than those of the mouse and rat enzymes.

To assess the effect of truncation of the N-terminal region of the hCD, expression vectors containing full hCD and ΔN hCD were constructed, both enzymes being designed to be tagged with *myc* at the C-terminal (Fig. 1B). These constructs were transfected into HEK293 cells by lipofection and the CDase activity of cell lysates and cell-free medium was measured using C12-NBD-ceramide as a substrate at pH 7.5. As shown in Fig. 2, the cell lysate of HEK293 cells expressing full hCD showed an increase in enzyme activity of more than 15-fold compared to the mock transfectant and some activity was detected in the medium, although the specific activity of the cell lysate of the full hCD transfectant was about one-fifth of that of the rat neutral CDase transfectant. Very weak but ascertainable activity was detected for the ΔN hCD transfectant, but the truncated enzyme tended to be released into the medium (inset of Fig. 2).

Next, to analyze the expression of the hCD in HEK293 cells at the protein level we performed Western blotting using an anti-*myc* antibody that recognizes the *myc* tag at the C-terminal of each enzyme. As previously reported [11], 133- and 113-kDa proteins, which are a Golgi-mature form and an ER-developing form, respectively, were detected in the lysates of HEK293 cells expressing the rat enzyme (Fig. 3A). In the medium, a

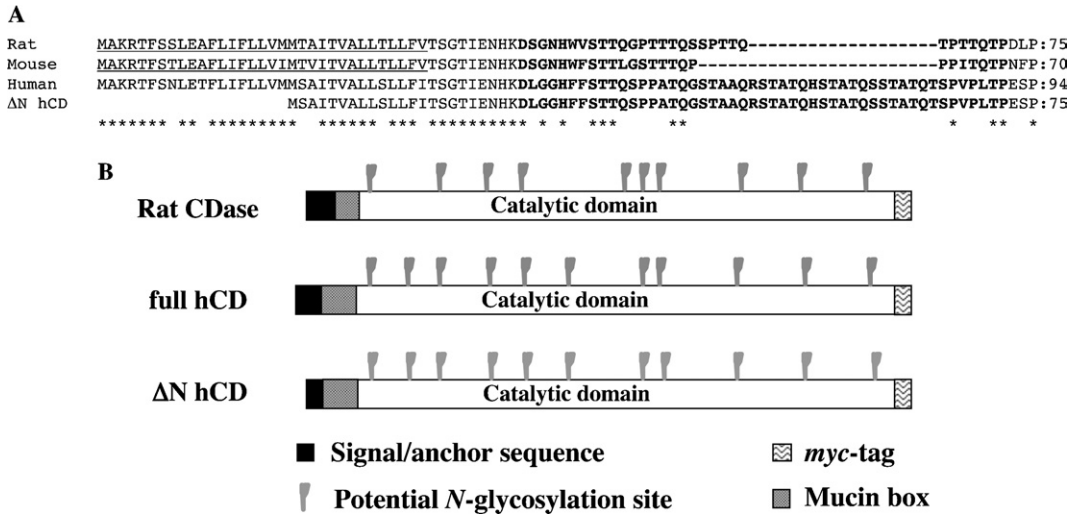

Fig. 1. Comparison of amino acid sequences in the N-terminal region of neutral CDases from rat, mouse, and human and the N-terminal deletion mutant of human neutral CDase. (A) Signal/anchor sequences are underlined and mucin-like domains are in bold. *, identical amino acids among these N-terminal sequences. (B) Schematic structure of neutral CDases and the deletion mutant. Rat CDase, neutral CDase from rat kidney; full hCD, full-length human neutral CDase; ΔN hCD, a deletion mutant in which 19 amino acids were deleted from the N-terminus of full hCD.

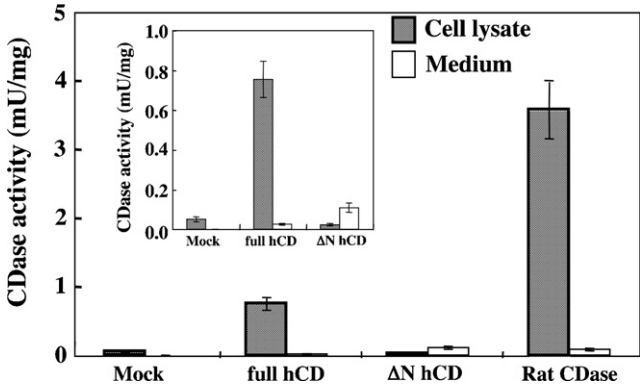

Fig. 2. Neutral CDase activity expressed in HEK293 cells. HEK293 cells were transfected with a plasmid vector containing cDNA encoding full hCD, ΔN hCD or rat CDase sequence, and incubated at 37 °C for 48 h. Cells were harvested and CDase activity in the cell lysate and medium was measured at pH 7.5 using C12-NBD-ceramide as a substrate. Mock represents the transfectant containing vector alone. (inset) The same results of mock, full hCD, and ΔN hCD but the scale of the vertical line is reduced by 1/5.

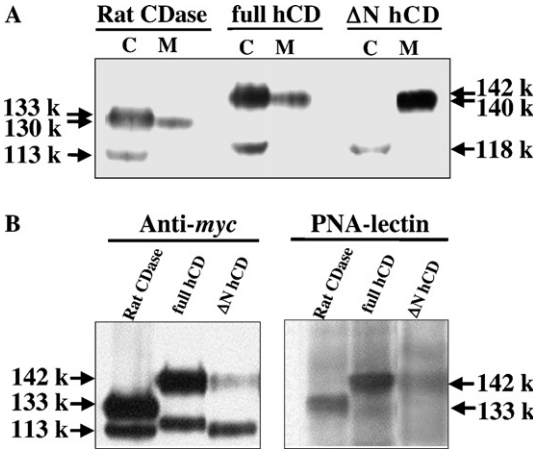

Fig. 3. Western blotting and lectin blotting of neutral CDase expressed in HEK293 cells. (A) Western blotting of neutral CDase. Cells were harvested and proteins in cell lysate and medium were precipitated with trichloroacetic acid (TCA). The TCA-precipitated proteins from 1.2×10^5 cells of rat CDase transfectant, and those from 2.4×10^5 cells of hCD and ΔN hCD transfectants were then subjected to Western blotting using anti-*myc* antibody. C, cell lysate; M, medium. (B) Lectin blotting (right panel) and Western blotting (left panel) of neutral CDase. Neutral CDase was immuno-precipitated with anti-mouse neutral CDase antibody and the immunoprecipitants were treated with *Vibrio cholerae* neuraminidase at 37 °C for 2 h. The neuraminidase-treated immunoprecipitants from 0.8×10^5 cells of rat CDase and full hCD transfectants, and those from 2×10^5 cells of ΔN hCD transfectant were then subjected to lectin blotting and Western blotting. Details are described in Materials and methods.

130-kDa protein was detected, which was possibly detached from the cell by the processing of the signal/anchor sequence [11]. As expected, the same pattern was observed on the Western blotting using the cell lysate and culture medium of the full hCD transfectant of HEK293 cells (Fig. 3A). However, the molecular mass of corresponding proteins was relatively larger than that of the rat enzyme, suggesting that the human CDase was more glycosylated. In contrast to full hCD, a faint 118-kDa band, which seems to be an ER-developing form, was observed in the cell lysate of the ΔN hCD transfectant whereas a relatively large amount of enzyme showing a 140-kDa band on the Western blotting was detected in the culture medium (Fig. 3A). These results are well consistent with the distribution of CDase activity of full and ΔN hCDs (Fig. 2). To assess the glycosylation of the enzymes with *O*-glycans, the proteins immuno-precipitated with anti-mouse neutral CDase antibody were subjected to lectin blotting using peanut agglutinin (PNA) (Fig. 3B, right panel) that specifically recognizes the core structure of mucin-type *O*-glycans [15]. Using the same sample, Western blotting was also performed with anti-*myc* antibody (Fig. 3B, left panel). For rat neutral CDase, a 133-kDa Golgi mature form, but not the 113-kDa ER-developing form, was clearly detected by HRP-labeled PNA lectin and therefore is surely glycosylated with *O*-glycans. Similarly, a 142-kDa band of full hCD was strongly stained with the lectin while the 118-kDa band was not. Interestingly, the staining of full hCD was much stronger than that of the rat enzyme, indicating that the human enzyme was highly glycosylated with *O*-glycans possibly due to the large number of potential *O*-glycosylation sites (Fig. 1A). This result may explain why the molecular mass of the full hCD was larger than that of the rat enzyme. It is worth noting that a 140-kDa, but not 118-kDa, form of ΔN hCD was also stained with the PNA lectin when the immuno-precipitated sample was applied (Fig. 3B).

Finally, we examined the intracellular distribution of full hCD and ΔN hCD when they were expressed in HEK293 cells using anti-*myc* antibody under a confocal laser microscope, with the rat neutral CDase as a reference. Under impermeable conditions without Triton X-100, signals for full hCD and the rat enzyme were found in the plasma membranes (Figs. 4K and J, respectively) while no signal for ΔN hCD was detected in the plasma membranes under the conditions used (Fig. 4L). It is noted, however, that very weak signal for ΔN hCD was observed in the plasma membranes when the sensitivity of laser microscope was increased (data not shown). After permeabilization of the plasma membranes with Triton X-100, the three enzymes were observed in ER/Golgi compartments (Figs. 4A, D, and G), although the expression of ΔN hCD was much weaker than those of the other two enzymes. It should be emphasized that none of the three enzymes merged with MitoTracker, a specific probe for visualizing mitochondria, indicating that these enzymes were not transported to mitochondria under the conditions used (Figs. 4C, F, and I). In conclusion, full hCD and rat enzyme were mainly transported to plasma membranes, not to

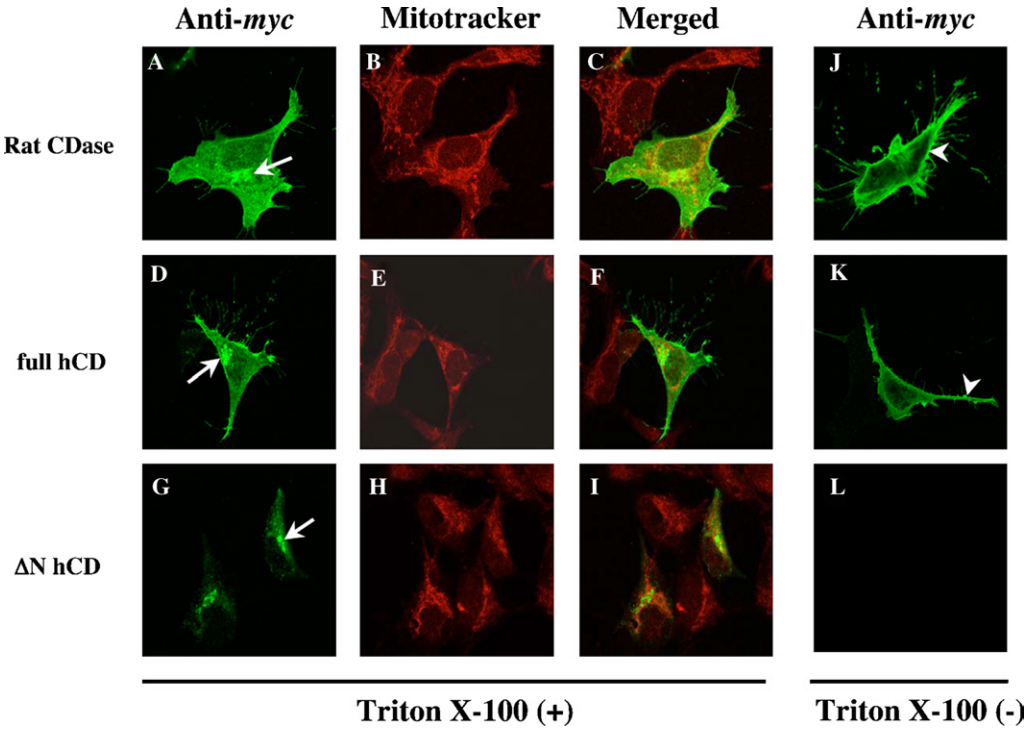

Fig. 4. Subcellular localization of neutral CDase expressed in HEK293 cells. HEK293 cells overexpressing CDase were fixed, permeabilized with Triton X-100 (A–I) or not (J–L), and then stained with anti-*myc* antibody and MitoTracker Red. Cells were observed under a confocal microscope as described in Materials and methods. (A–C and J) rat CDase; (D–F and K) full hCD; (G–I and L) ΔN hCD; (A, D, G, and J–L) visualized with anti-*myc* antibody; (B,E,H) visualized with MitoTracker Red; (C,F,I) merged images produced by superposition of CDase and MitoTracker Red. Arrows and arrowheads indicate the expression of CDase at ER/Golgi compartments and plasma membranes, respectively.

mitochondria, possibly via a classical ER/Gogi pathway and partly released into the medium when expressed in HEK293 cells. ΔN hCD, which lacks 19 amino acid residues in the N-terminal region of full hCD, was mainly distributed in the ER/Golgi compartments and then largely released into the medium.

Discussion

This paper clearly indicates that full hCD behaved similarly to rat neutral CDase [11] when expressed in HEK293 cells. Furthermore, it was found that ΔN hCD, which was previously reported as a human mitochondrial CDase [9], was expressed very weakly in HEK293 cells and largely released into the medium. We assume that ΔN hCD is transported to plasma membranes via an ER/Golgi pathway the same as full hCD but not retained there, detaching from the cells possibly because of the insufficient signal/anchor sequence by which the enzyme is anchored to the membranes. We could not detect either full hCD, ΔN hCD or rat neutral CDase in mitochondria under the fluorescent confocal microscope using MitoTracker as a reference marker under the conditions used in this study. These results are not consistent with a previous report in which human neutral CDase was exclusively found in mitochondria when expressed in HEK293 and MCF7 cells as a fusion protein with GFP [9]. The reason for the difference is unclear at present, but it is likely that the fusion of GFP to the N-terminal seriously affects the intracellular transport of the enzyme.

That both full hCD and ΔN hCD were glycosylated with *O*-glycans (Fig. 3B) may be additional evidence that these enzymes are not localized to mitochondria, because mitochondrial proteins are not usually glycosylated with *O*-glycans [16].

There are several lines of evidence that mitochondria contain ceramide [17] and could be targets in ceramide-mediated apoptosis [18]. Actually, acyl CoA-dependent ceramide synthase was found in not only the ER but also mitochondria-associated membrane (MAM) and purified mitochondrial fractions [19]. Furthermore, activity for the reverse hydrolysis of CDase was detected in a mitochondrial fraction of rat liver though the molecular species of CDase has not yet been clarified [19]. Further study will be necessary to reveal the subcellular distribution of neutral CDase in human tissues. It is worth noting that the rat neutral CDase has been shown to be distributed in the plasma membranes of proximal tubules, distal tubules, and collecting ducts in kidney [8].

Acknowledgment

This work was supported by Grant-in-Aid for Scientific Research on Priority area 12140204 and research B 15380073.

References

[1] M. Ito, N. Okino, M. Tani, S. Mitsutake, K. Kita, in: A.H. Futerman (Ed.), Ceramide Signaling, Landes Bioscience, Kluwer Academic/Plenum Publishers, 2002, pp. 41–48.
[2] M. Sugita, J.T. Dulaney, H.W. Moser, Ceramidase deficiency in Farber's disease (lipogranulomatosis), Science 178 (1972) 1100–1102.
[3] C. Mao, R. Xu, Z.M. Szulc, J. Bielawski, P.K. Becker, A. Bielawska, S.H. Galadari, W. Hu, L.M. Obeid, Cloning and characterization of a mouse endoplasmic reticulum alkaline ceramidase: an enzyme that preferentially regulates metabolism of very long chain ceramides, J. Biol. Chem. 278 (2003) 31184–31191.
[4] N. Okino, S. Ichinose, A. Omori, S. Imayama, T. Nakamura, M. Ito, Molecular cloning, sequencing, and expression of the gene encoding alkaline ceramidase from *Pseudomonas aeruginosa*: cloning of a ceramidase homologue from *Mycobacterium tuberculosis*, J. Biol. Chem. 274 (1999) 36616–36622.
[5] Y. Yoshimura, N. Okino, M. Tani, M. Ito, Molecular cloning and characterization of a secretory neutral ceramidase of *Drosophila melanogaster*, J. Biochem. 132 (2002) 229–236.
[6] Y. Yoshimura, M. Tani, N. Okino, H. Iida, M. Ito, Molecular cloning and functional analysis of zebrafish neutral ceramidase, J. Biol. Chem. 279 (2004) 44012–44022.
[7] M. Tani, N. Okino, K. Mori, T. Tanigawa, H. Izu, M. Ito, Molecular cloning of the full-length cDNA encoding mouse neutral ceramidase: a novel but highly conserved gene family of neutral/alkaline ceramidases, J. Biol. Chem. 275 (2000) 11229–11234.
[8] S. Mitsutake, M. Tani, N. Okino, K. Mori, S. Ichinose, A. Omori, H. Iida, T. Nakamura, M. Ito, Purification, characterization, molecular cloning, and subcellular distribution of neutral ceramidase of rat kidney, J. Biol. Chem. 276 (2001) 26249–26259.
[9] S.E. Bawab, P. Roddy, T. Qian, A. Bielawska, J.J. Lemasters, Y.A. Hannun, Molecular cloning and characterization of a human mitochondrial ceramidase, J. Biol. Chem. 275 (2000) 21508–21513.
[10] U. Acharya, M.B. Mowen, K. Nagashima, J.K. Acharya, Ceramidase expression facilitates membrane turnover and endocytosis of rhodopsin in photoreceptors, Proc. Natl. Acad. Sci. USA 101 (2004) 1922–1926.
[11] M. Tani, H. Iida, M. Ito, *O*-glycosylation of mucin-like domain retains the neutral ceramidase on the plasma membranes as a type II integral membrane protein, J. Biol. Chem. 278 (2003) 10523–10530.
[12] M. Tani, K. Kita, H. Komori, T. Nakagawa, M. Ito, Enzymatic synthesis of omega-amino-ceramide: preparation of a sensitive fluorescent substrate for ceramidase, Anal. Biochem. 263 (1998) 183–188.
[13] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacterio-phage T4, Nature 227 (1970) 680–685.
[14] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[15] R. Lotan, E. Skutelsky, D. Danon, N. Sharon, The purification, composition, and specificity of the anti-T lectin from peanut (*Arachis hypogaea*), J. Biol. Chem. 250 (1975) 8518–8523.
[16] W. Neupert, Protein import into mitochondria, Annu. Rev. Biochem. 66 (1997) 863–917.
[17] D. Ardail, I. Popa, K. Alcantara, A. Pons, J.P. Zanetta, P. Loouisot, L. Thomas, J. Portoukalian, Occurrence of ceramides and neutral glycolipids with unusual long chain base composition in purified rat liver mitochondria, FEBS Lett. 488 (2001) 160–164.
[18] D.K. Perry, J. Carton, A.K. Shhah, F. Meredith, D.J. Uhlinger, Y.A. Hannun, Serine palmitoyltransferase regulates de novo ceramide generation during etoposide-induced apoptosis, J. Biol. Chem. 275 (2000) 9078–9084.
[19] C. Bionda, J. Portoukalian, D. Schmitt, C. Rodriguez-Lafrasse, D. Ardail, Subcellular compartmentalization of ceramide metabolism: MAM (mitochondria-associated membrane) and /or mitochondria, Biochem. J. 382 (2004) 527–533.