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Molecular Cloning and Characterization of a Secretory Neutral Ceramidase of *Drosophila melanogaster*[1]

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We report here the molecular cloning and characterization of the *Drosophila* neutral ceramidase (CDase). Using the BLAST program, a neutral CDase homologue (AE003774) was found in the *Drosophila* GenBank and cloned from a cDNA library of *Drosophila* imaginal discs. The open reading frame of 2,112 nucleotides encoded a polypeptide of 704 amino acids having five putative *N*-glycosylation sites and a putative signal sequence composed of 23 residues. When a His-tagged CDase was overexpressed in *D. melanogaster* Schneider's line 2 (S2) cells, the enzyme was continuously secreted into the medium through a vesicular transport system. Treatment of the secretory 86.3-kDa CDase with glycopeptidase F resulted in the generation of a 79.3-kDa protein, indicating that the enzyme is actually glycosylated with *N*-glycans. The enzyme hydrolyzed various *N*-acylsphingosines but not galactosylceramide, GM1a or sphingomyelin, and exhibited a peak of activity at pH 6.5–7.5, and thus was classified as a neutral CDase. RNAi for the enzyme remarkably decreased the CDase activity in a cell lysate as well as a culture supernatant of S2 cells mostly at neutral pH, indicating that both activities were derived from the same gene product.

Key words: ceramidase, *Drosophila melanogaster*, RNAi, sphingolipids.

Ceramide (Cer) has been implicated as a novel lipid modulator in signal transduction pathways involved in cell growth, differentiation, and apoptosis (*1, 2*). The metabolic product of Cer, sphingosine-1-phosphate (S1P), which is produced through phosphoryration of sphingosine (Sph) by sphingosine kinase, promotes the proliferation and migration of endothelial cells through the activation of G protein–coupled receptors, the Edg family (*3–5*). Sph and S1P seem to also function as intracellular signaling molecules (*3*), although the mechanism underlying the functions has yet to be clarified.

Ceramidase (CDase, EC 3.5.1.23), which catalyzes the hydrolysis of the *N*-acyl linkage of Cer to produce Sph, is a critical enzyme for regulation of the contents of Cer, Sph, and possibly S1P. It is noteworthy that Sph is not produced through *de novo* synthesis (*6*) but rather is thought to be produced from Cer through the action of CDase. Three CDase isoforms, which mainly differ in the catalytic pH optimum, have been reported. A CDase with an acidic pH optimum (acid CDase) is responsible for Farber disease, in which Cer is accumulated in lysosomes due to a genetic deficiency of the enzyme (*7*). Recently, an acid CDase was purified from human urine (*8*), and the cDNAs encoding the enzyme were cloned from human and mouse (*9, 10*). Alkaline CDase, which prefers phyto-Cer over normal Cer containing sphingenine and shows an extremely alkaline pH optimum (9.0–9.5), was found in yeast and human (*11–13*). Neutral CDase seems to regulate the balance of Cer/Sph/S1P in response to various stimuli including cytokines and growth factors, and could modulate the sphingolipid-mediated signaling. Actually, neutral CDase was shown to be regulated by growth factors and cytokines (*14–16*). In the past few years, the molecular cloning of neutral CDases, one of the missing links of sphingolipid signaling, has been performed in mouse (*17*), rat (*18*), human (*19*), and bacteria (*20*).

It was found that the intracellular distribution of the mammalian neutral CDase differed in a cell/tissue-specific manner. 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 with endosome-like organelles in hepatocytes (*18*). Human neutral CDase was exclusively localized to mitochondria in HEK293 and MCF7 cells when overexpressed as a fusion protein with green fluorescent protein (GFP) (*19*). Furthermore, the enzyme was found to be released by murine endothelial cells (*21*), although the characterization of a secretory enzyme has yet to be performed.

Drosophila melanogaster, as an animal model for which a genomic sequence is available, has been shown to possess sphingolipids (*22*) that seem to be essential for morphogen-

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Abbreviations: Cer, ceramide; CDase, ceramidase; dsRNA, double-stranded RNA; FBS, fetal bovine serum; GalCer, galactosylceramide; GM1a, Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ1,1'Cer; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; PAGE, polyacrylamide gel electrophoresis; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; Sph, sphingosine; S1P, sphingosine-1-phosphate; TLC, thin-layer chromatography.

esis; a mutant lacking *lcb2*, the gene encoding a subunit of serine palmitoyltransferase, exhibited an abnormal morphology of alae (*23*).

In this paper, we report the cloning, sequencing and characterization of a secretory CDase of *D. melanogaster*.

MATERIALS AND METHODS

Materials—*D. melanogaster* Schneider's line 2 (S2) cells and the *Drosophila* expression system were obtained from Invitrogen (USA). The cDNA library of *Drosophila* imaginal discs was kindly donated by Dr. Y. Nishida (Nagoya University, Nagoya). ECL plus, Hi-trap Q and chelating columns were purchased from Amersham Pharmacia Biotech (UK). Precoated Silica Gel 60 TLC plates were obtained from Merck (Germany). Brefeldin A was purchased from Sigma (USA). Various [^{14}C]Cers and C12-NBD-Cer were prepared as described in Refs. *24–26*. All other reagents were of the highest purity available.

Molecular Cloning and DNA Sequencing—General cloning techniques were carried out essentially as described by Sambrook *et al.* (*27*). Nucleotide sequences were determined by the dideoxynucleotide chain termination method with a Bigdye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, USA) and a DNA Sequencer (model 377A, PE Biosystems; USA).

Isolation of a cDNA Clone Encoding Full-Length Drosophila CDase—To obtain a DNA fragment encoding *Drosophila* CDase, PCR amplification was performed using sense and antisense oligonucleotide primers based on the genomic sequence of *D. melanogaster* (AE003774), which is homologous to the mouse neutral CDase (AB037111): the sense primer (5′-TTGTGGTGGAGGATGAAAAG-3′) and antisense primer (5′-ATGAGGGCGAACGATTGATG-3′) were used for PCR with the cDNA library of *Drosophila* imaginal discs as a template. The PCR was conducted in a GeneAmp PCR System 2400 (PE Biosystems) for 40 cycles (each consisting of denaturation at 95°C for 20 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s) using AmpliTaq Gold (PE Biosystems). An amplified 335-bp PCR product containing the putative *Drosophila* CDase sequence was subcloned into the pGEM T-easy vector (Promega, USA), and then sequenced. A clone containing a full-length CDase cDNA was isolated from the cDNA library of *Drosophila* imaginal discs by colony hybridization using the 335-bp PCR product as a probe. The probe was labeled with DIG using a DIG-DNA labeling and detection kit (Roche, USA) according to the manufacturer's instructions. Colony hybridization was performed according to standard procedures (*27*). Finally, a clone encoding the whole *Drosophila* CDase sequence was isolated, and the plasmid in the clone was designated as pNBfCD.

Cell Culture—S2 cells were grown at 25°C in Schneider's *Drosophila* medium (SDM) in the presence of 10% fetal bovine serum (FBS), penicillin G (50 units/ml), and streptomycin sulfate (50 μg/ml).

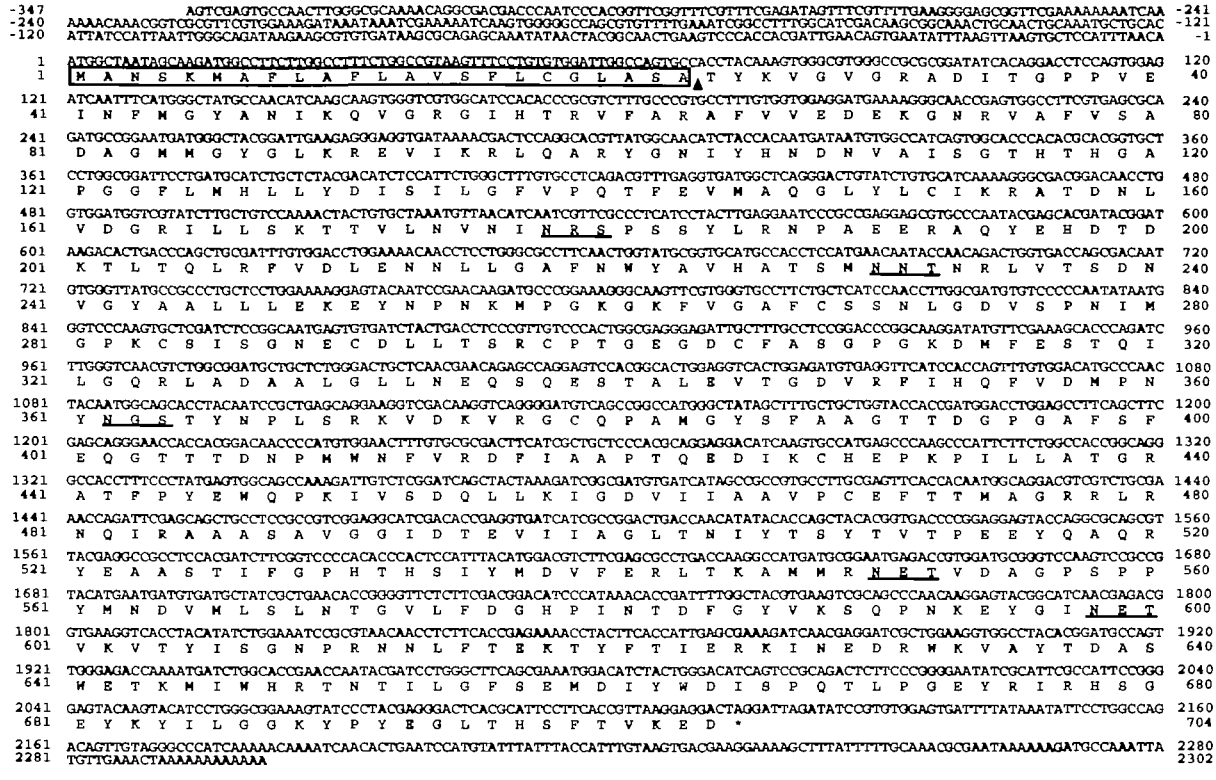
Transient Expression of His-Tagged Drosophila CDase in S2 Cells—To obtain the His-tagged *Drosophila* CDase (pAcfCD), cDNA encoding the CDase was subcloned into pAc5.1 (Invitrogen, USA) by PCR using the sense primer with an *Eco*RI site (5′-TGAATTCATGGCTAATAGCAAGATGGCC-3′), and the antisense primer with a *Xho*I site and a disrupted stop codon (5′-ACTCGAGGTCCTCCTTAACGGTGAAGGA-3′). For transient expression, S2 cells (3×10^6/well), after seeding into 6-well plates containing SDM in the presence of 10% FBS and incubation at 25°C for 6 h, were transfected with 19 μg of pAcfCD or empty vector by calcium phosphate transfection according to the manufacturer's instructions. After incubation at 25°C for 1 day, the cells were washed twice with SDM containing 10% FBS, and then incubated in fresh medium. After incubation at 25°C for 5 days, the cells were collected by centrifugation at 130 ×*g* for 5 min, washed with PBS, and then lysed by the addition of 200 μl of 10 mM Tris-HCl buffer, pH 7.5, containing 0.1% Triton X-100.

CDase Assay—CDase activity was measured using C12-NBD-Cer as a substrate as described in Ref. *28*. Briefly, 200 pmol of C12-NBD-Cer was incubated at 37°C for 30 min with an appropriate amount of enzyme in 20 μl of 25 mM Tris-HCl buffer, pH 7.5, containing 0.05% Triton X-100. After being dried with a Speed Vac concentrator, 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 released and C12-NBD-Cer remaining were quantified with a Shimadzu CS-9300 chromatoscanner (Shimadzu, Kyoto). One unit of enzyme was defined as the amount capable of catalyzing the release of 1 μmol of NBD-dodecanoic acid/min under the conditions described above. Values of 10^{-3} and 10^{-6} units of the enzyme were expressed as 1 milliunit and 1 microunit, respectively. To characterize the CDase, various cations at 5 mM, final concentration, were added, or 150 mM GTA buffer (50 mM 3,3-dimethyl-glutaric acid, 50 mM Tris (hydroxymethyl) aminomethane, 50 mM 2-amino-2-methyl-1,3-propanediol) with different pH values was used instead of Tris-HCl buffer, if necessary. At pH 7.5, the apparent CDase activity measured using GTA buffer is much higher than that with Tris-HCl buffer.

Purification of the His-Tagged Drosophila CDase from a Supernatant of S2 Cells—A supernatant of CDase-overexpressing S2 cells was applied to a Hi-trap Q column, pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 0.1% Triton X-100 (buffer A). The enzyme was absorbed onto the column, which was washed with buffer A containing 0.3 M NaCl. The enzyme was then eluted with buffer A containing 1 M NaCl. The fractions showing CDase activity were pooled and then applied to a Hi-trap chelating column (chelated with Ni^{2+}), pre-equilibrated with buffer A. The enzyme was absorbed onto the column, which was washed with buffer A containing 10, 20, and 30 mM imidazol. The enzyme was then eluted with buffer A containing 200 mM imidazole. The purified enzyme was used for the following characterization.

Protein Assay, SDS-PAGE, and Western Blotting—The protein content was determined by means of the bicinchoninic acid protein assay (Pierce, USA) with bovine serum albumin as the standard. SDS-PAGE was carried out according to the method of Laemmli (*29*). Protein was transferred onto a polyvinyldifluoride membrane using TransBlot SD (Bio-Rad, USA) according to the method described in Ref. *30*. After blocking with 3% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (T-TBS) for 1 h, the membrane was incubated with anti-His (C-term) antibodies (Invitrogen, USA) for 16 h at 4°C. After a wash with T-TBS, the membrane was incubated with a

A



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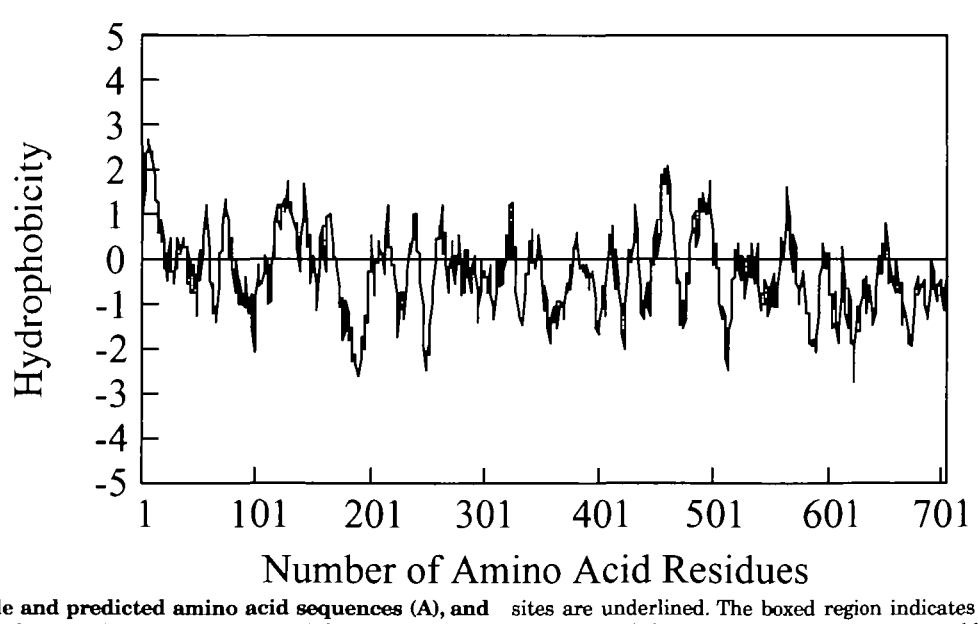

Fig. 1. **Nucleotide and predicted amino acid sequences (A), and hydrophobicity plot (B) of *Drosophila* neutral CDase.** A, the deduced amino acid sequence of the CDase is shown in one-letter symbols below the nucleotide sequence. Amino acid residues are numbered beginning with the first Met, and the translation termination codon is denoted by an asterisk. Five potential *N*-linked glycosylation sites are underlined. The boxed region indicates the putative signal sequence and the arrowhead indicates the possible site of cleavage by signal peptidases. Numbers correspond to amino acids (lower) and nucleotides (upper). B, a hydrophobicity plot of the CDase. It was analyzed by the method of Kyte and Doolittle (*37*).

horseradish peroxidase–conjugated secondary antibody for 2 h. After another wash with T-TBS, the ECL reaction was performed for 2–3 min according to the manufacturer's instructions.

dsRNA Production and Transfection in S2 Cells—The template used for RNA synthesis was the pBlueScriptII SK+ vector (Stratagene, USA) containing the CDase cDNA. Sense and antisense RNA were synthesized using either T7 or T3 RNA polymerase. The resulting transcripts were treated with DNase I. The reaction mixtures were precipitated with ethanol and the precipitates were dissolved in annealing buffer (1 mM Tris-HCl, pH 7.5, containing 1 mM EDTA). Annealing of the single-stranded RNAs was performed by mixing approximately equimolar amounts of sense and antisense RNA. The samples were then boiled for 1 min and allowed to cool at room temperature. Double-strand formation was checked by native agarose gel electrophoresis. The samples were stored at –80°C before use. For transfection with dsRNA, S2 cells (2×10^6/well) were seeded into 6-well plates containing SDM in the presence of 10% FBS. After incubation at 25°C for 6 h, the cells were transfected with 5 μg of dsRNA by the calcium phosphate method.

RESULTS

cDNA Cloning of the Drosophila CDase—Using the BLAST program, FlyBase (http://flybase.harvard.edu:7081/) was searched for sequences homologous to the mouse neutral CDase (AB037111). A *Drosophila* genome sequence (AE003774) was found to be highly homologous to the mouse enzyme at the amino acid level. For cloning of the *Drosophila* neutral CDase, PCR was performed using primers designed from the genome sequence and the cDNA library of *Drosophila* imaginal discs as a template as described under "MATERIALS AND METHODS." Finally, a cDNA clone encoding the *Drosophila* CDase was isolated from the cDNA library by colony hybridization using the PCR product as a DIG-labeled probe. It was found that a plasmid, designated as pNBfCD, contained a 2,649-bp cDNA insert including the entire coding region of *Drosophila* CDase. The open reading frame of the *Drosophila* CDase gene was 2,112-bp long and encoded 704 amino acids (Fig. 1A). The CDase has a predicted pI of 6.14, and a molecular weight of 78,156, judging from the deduced amino acid sequences. The open reading frame of pNBfCD contained five potential *N*-glycosylation sites and a hydrophobic motif composed of 23 amino acid residues near the N-terminus. The presence of the hydrophobic motif was also clearly indicated by hydrophobicity plots (Fig. 1B). The PSORTII program predicted that the *Drosophila* CDase has a possible site of cleavage by signal peptidase between the 23rd and 24th amino acid residues. The *Drosophila* CDase exhibits sequence identity to other neutral CDases; 33.2% for *Pseudomonas aeruginosa*, 28.6% for *Mycobacterium tuberculosis*, 44.5% for mouse, 44.7% for rat, and 43.5% for human, respectively (Fig. 2).

Expression of the Drosophila CDase in S2 Cells—To verify that pAcfCD encodes the neutral CDase, S2 cells were transfected with pAcfCD, and then the CDase activity in cell lysates and culture supernatants was measured using C12-NBD-Cer, as a substrate, and GTA buffer, pH 7.5. The activity of the CDase in cell lysates of untransfected and mock-transfected S2 cells was 0.12 and 0.138 milliunits/mg protein, respectively, while that of pAcfCD-transfected cells was 0.443 milliunits/mg, indicating a 3.2-fold increase in

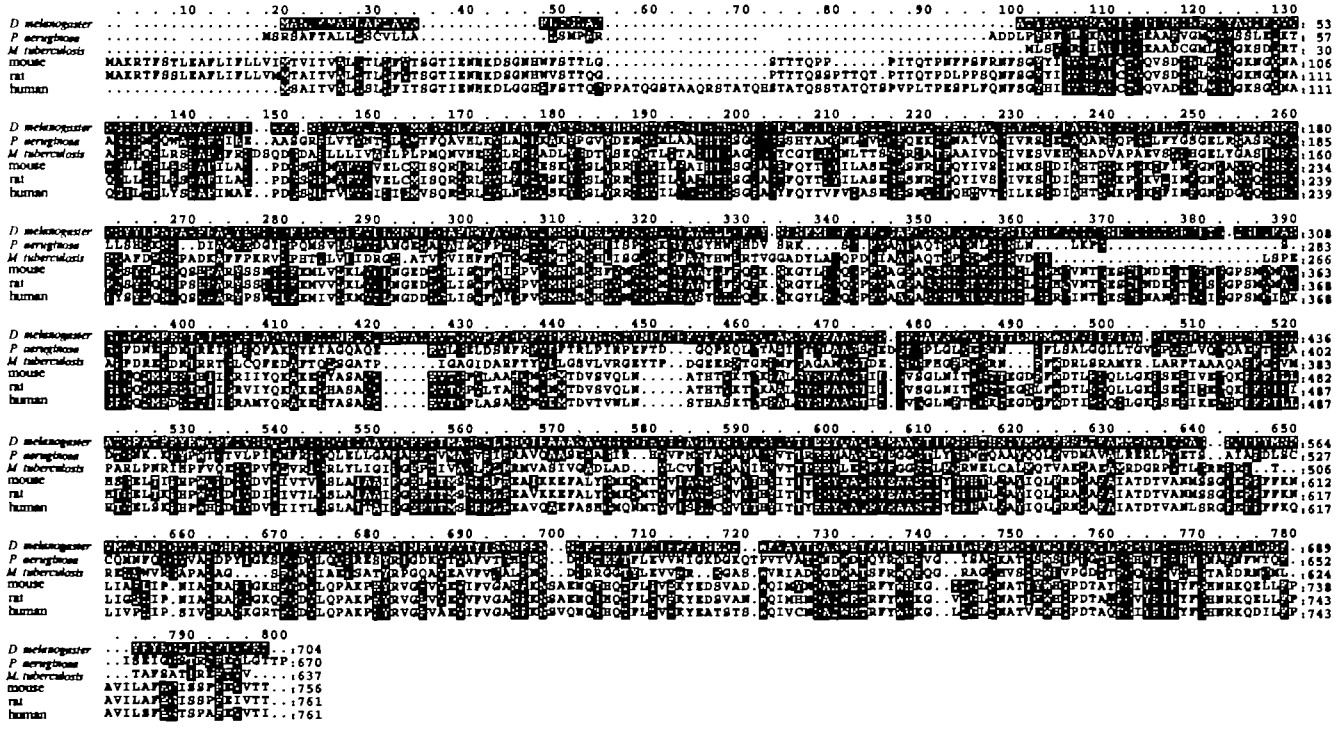

Fig. 2. **Alignment of deduced amino acid sequences of neutral CDases from *Drosophila*, mouse, rat, human, and bacteria (*Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*).** The alignment of neutral/alkaline CDases was performed using the CLUSTAL algorithm (*38*). Amino acids identical to ones in *Drosophila* CDase are indicated by white type on a black background. Gaps inserted into the sequences are indicated by dots.

comparison with the mock-transfectant. Interestingly, the CDase activity was detected in not only the cell lysates but also culture supernatants, and was much stronger in the latter than the former. The CDase activity in culture supernatants of untransfected and mock-transfected cells was 11.9 and 12.1 milliunits/mg cell protein, respectively, while that of pAcfCD-transfected cells was 140 milliunits/mg cell protein, indicating an about 10-fold increase in comparison with mock-transfectants (Table I). These results indicate that the cloned cDNA encodes the CDase, and that endogenous as well as overexpressed CDase was actively released from the cells.

Secretion of CDase from S2 Cells—To elucidate the secretory process, S2 cells were treated with brefeldin A, a fungal metabolite which interferes with the vesicular transport of proteins (*31, 32*), and PDMP, a Cer analog which inhibits the secretion of glycoproteins when used at a high concentration (*33*). Treatment of S2 cells with these reagents strongly inhibited the secretion of CDase, indicating that the CDase is secreted through classical pathway via the endoplasmic reticulum/Golgi compartments (Fig. 3).

Characterization of the Secretory CDase—To characterize the secretory CDase, the His-tagged enzyme was purified from the culture supernatant of pAcfCD-transfected S2 cells using Hi-trap Q and chelating columns. The His-tagged CDase was detected as a single protein band corresponding to a molecular mass of 86.3 kDa on SDS-PAGE after visualization with anti-His antibodies (Fig. 4). The 86.3-kDa protein band was converted to a 83.4 kDa one on treatment with endoglycosidase H, and one of 79.3 kDa with glycopeptidase F, respectively (Fig. 4), indicating that the CDase has high mannose and complex/hybrid type *N*-glycans. The enzyme exhibited a pH optimum of approximately 7–7.5 and thus was classified as a neutral CDase (Fig. 5A). The substrate specificity of the CDase was examined at pH 7.5 using various ^{14}C-labeled Cers (Table II). Among the 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) as a long chain base were somewhat resistant to the enzyme and ones containing phytosphingosine (t18:0) were strongly resistant. It is noteworthy that glycosphingolipids such as GalCer 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 ω-position increased the susceptibility of the enzyme to the substrate. The substrate specificity of *Drosophila* CDase is similar to that of the mouse and rat neutral CDases (*28, 18*).

The enzyme activity was enhanced by the addition of detergents such as Triton X-100 and sodium taurodeoxycholate. The optimum concentrations of detergents differed markedly with the detergent used. For Triton X-100 and sodium taurodeoxycholate, the optimum concentrations were found to be 0.01–0.05 and 0.05–0.1%, respectively, which increased the enzyme activity about 3-fold in comparison with that in the absence of a detergent (Fig. 5B). Sodium cholate at 0.4–0.6% also enhanced the activity by about 1.5-fold.

TABLE I. **Expression neutral CDase cDNA in S2 cells.** S2 cells were transfected with pAcfCD or with pAc5.1 (mock). The activity of the CDase in cell lysates and culture supernatants was measured at pH 7.5 using C12-NBD-Cer, as a substrate, and GTA buffer as described under "MATERIALS AND METHODS." Values are the means ± SD for triplicate determinations.

S2 cells	CDase activity (milliunits/mg cell protein)
Cell lysate	
Untransfected	0.120 ± 0.01
Mock-transfected	0.138 ± 0.01
pAcfCD	0.443 ± 0.05
Cell supernatant	
Untransfected	11.9 ± 3.06
Mock-transfected	12.1 ± 2.82
pAcfCD	140 ± 22.6

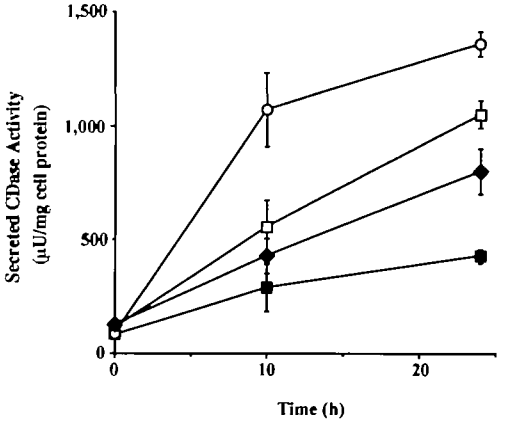

Fig. 3. **Effects of brefeldin A and PDMP on the secretion of neutral CDase from S2 cells.** Cells were cultured at 25°C in SDM containing 10% FBS with brefeldin A or PDMP. At the times indicated, cells were harvested by centrifugation. CDase activity in cell lysates and culture supernatants was measured by the method described under "MATERIALS AND METHODS." ○, control; ◆, 10 μg/ml brefeldin A; □, 50 μM PDMP; ■, 100 μM PDMP.

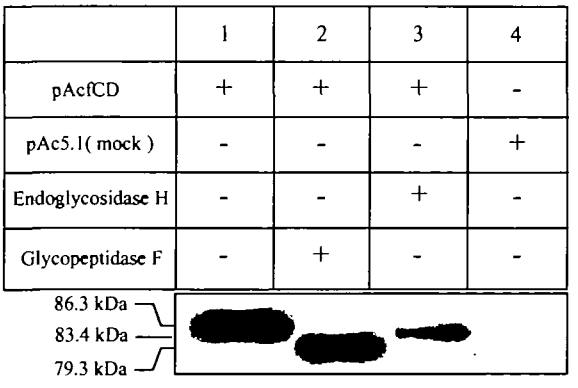

Fig. 4. **SDS-PAGE of the secretory His-tagged CDase of S2 cells.** At 5 days after transfection with pAcfCD or pAc5.1 (mock), the tagged-enzyme was purified with Hi-trap Q and chelating columns as described under "MATERIALS AND METHODS." The purified CDase was subjected to treatment with endoglycosidase H and glycopeptidase F as described in the manufacturer's instruction manual. Aliquots of samples were subjected to 5% SDS-PAGE under reducing condition followed by Western blotting with an anti-His antibody.

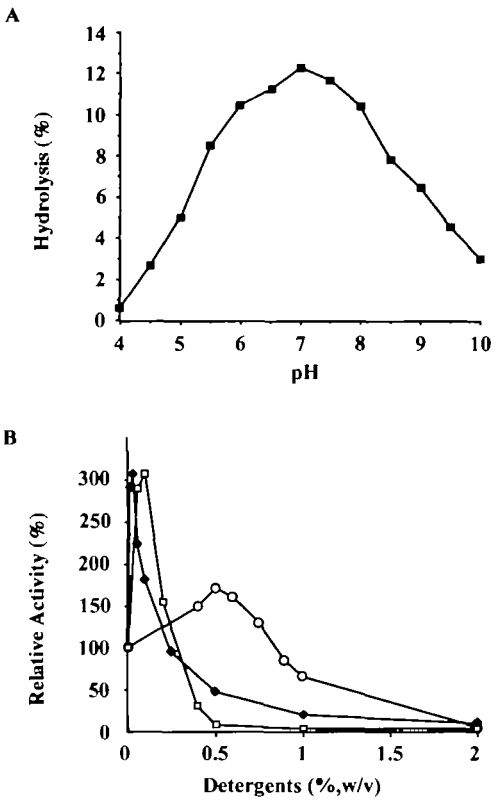

Fig. 5. **General properties of the *Drosophila* neutral CDase.** A: pH dependence of the *Drosophila* CDase. The enzyme activity was measured as described under "MATERIALS AND METHODS" except that 150 mM GTA buffer with different pH values containing 0.05% Triton X-100 was used instead of 25 mM Tris-HCl buffer, pH 7.5. B: Effects of detergents. C: Effects of cations. The CDase activity was measured as described under "MATERIALS AND METHODS" except that each reaction mixture contained various detergents at the concentrations indicated in B or 5 mM of the cations indicated in C. In B, ◆, Triton X-100; □, sodium taurodeoxycholate; ○, sodium cholate.

TABLE II. **Substrate specificity of *Drosophila* neutral CDase.** Various substrates were incubated at 37°C with 2.4 microunits of purified CDase for 30 min in 25 mM Tris-HCl buffer, pH 7.5, containing 0.05% Triton X-100. The extent of hydrolysis of substrates was determined as described under "MATERIALS AND METHODS."

Substrate	Structure (fatty acid/long-chain base)	Hydrolysis (%)
N-Lauroylsphingosine	C12:0/d18:1	29.0
N-Palmitoylsphingosine	C16:0/d18:1	21.7
N-Stearoylsphingosine	C18:0/d18:1	14.8
N-Palmitoylsphinganine	C16:0/d18:0	16.7
N-Stearoylsphinganine	C18:0/d18:0	6.9
N-Palmitoylphytosphingosine	C16:0/t18:0	1.6
N-Stearoylphytosphingosine	C18:0/t18:0	0.8
NBD-*N*-dodecanoylsphingosine	NBD-C12:0/d18:1	36.4
GalCer	Galβ1-1'Cer	0
GMIa	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1'Cer	0
Sphingomyelin	Choline phosphate Cer	0

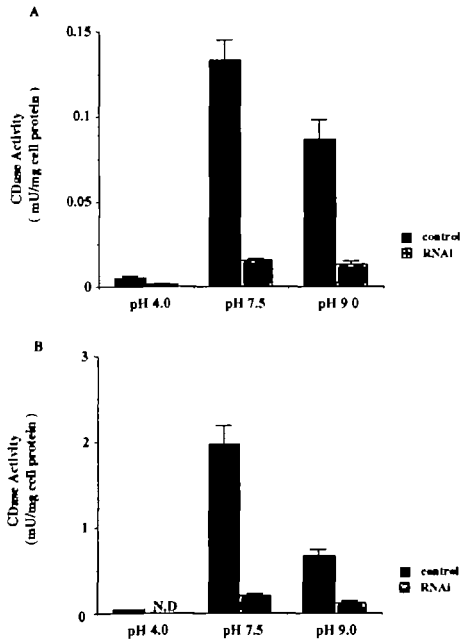

Fig. 6. **Inhibition of neutral CDase activity by RNAi.** S2 cells were transfected with 5 μg of dsRNA corresponding to the neutral CDase cDNA. At 2 days after transfection, cells were harvested, and then the CDase activities of cell lysate (A) and cultured medium (B) were measured at pH 4.0, 7.5, and 9.0, in 150 mM GTA buffer and C12-NBD-Cer as a substrate. The details are given under "MATERIALS AND METHODS."

The enzyme activity was greatly inhibited by Fe^{3+}, Zn^{2+}, and Cu^{2+} at 5 mM (Fig. 5C). In contrast to the *Pseudomonas* CDase (*34*), the *Drosophila* CDase was not activated by Ca^{2+}. EDTA, Ni^{2+}, Mn^{2+}, Mg^{2+}, K^+, and Na^+ had little effect on the *Drosophila* CDase. Notably, the purified *Drosophila* CDase was able to catalyze the reverse hydrolysis reaction, in which NBD-dodecanoic acid was condensed to sphingosine to generate C12-NBD-Cer (data not shown).

Inhibition of the CDase Activity of S2 Cells by RNAi—Double-stranded RNA (dsRNA)-mediated interference (RNAi) of gene expression, a form of post-transcriptional gene silencing, has become widely used for elucidating gene functions (*35, 36*). To clarify whether or not the gene product really functions as a neutral CDase, dsRNA corresponding to the ORF of the CDase was generated and fed to S2 cells. After 2 days the CDase activity in cell lysates and supernatants was measured at pH 4.0, 7.5, and 9.0. For the control cells without RNAi, the highest activity was found at pH 7.5, followed by 9.0 in both cell lysates and culture supernatants, whereas very weak activity was detected at pH 4.0. Notably, the CDase activity of the dsRNA-transfected cells was greatly decreased at pH 7.5 and 9.0 in comparison with that of untransfected cells (Fig. 6, A and B). Interestingly, the activity at pH 4.0 was also decreased by RNAi, suggesting that the activity under acidic conditions was also due to the neutral CDase in S2 cells. These results indicate that the cloned cDNA surely encodes the CDase,

which functions mainly at neutral pH, and almost all of the activity is detached from cells. Unexpectedly, the RNAi for CDase little affected the Cer content when cells were labelled with ^{14}C-palmitic acid as a precursor of sphingolipids (data not shown).

DISCUSSION

This is the first report on the molecular cloning of an invertebrate neutral CDase, it being shown that the *Drosophila* CDase is homologous to the mammalian neutral CDases but lacks a Ser/Thr-rich domain at the N-terminus. This mucin-like domain of the enzyme is a potential *O*-glycosylation site and seems to affect the association of the CDase with plasma membranes in HEK293 and CHOP cells (Tani *et al.*, unpublished results). Since this specific domain was found in the neutral CDases of mammals but not those of bacteria (*20*) and *Drosophila* (this study), it seems to be acquired by the enzyme during evolution.

Human and mouse acid CDases, which reside in lysosomes, function primarily in the degradation of Cer for further catabolism. However, the CDase, which efficiently functions at acidic pH, was not found in the cell lysate and culture supernatant of S2 cells. RNAi for the enzyme remarkably decreased the activity at not only pH 7.5 and 9.0 but also 4.0 (Fig. 6). Furthermore, an acid CDase homologue was not found in the *Drosophila* genomic sequences. These results may indicate that the catabolism of Cer in S2 cells and probably in *Drosophila* is mainly performed at neutral to alkaline pH by the enzyme. On the other hand, RNAi was less effective at pH 9.0 than at pH 7.5 (Fig. 6), suggesting the presence of other CDase species with alkaline pH optima in S2 cells. Unexpectedly, RNAi was found to little affect the morphology or growth of S2 cells under the present conditions (data not shown), suggesting that the residual CDase activity (corresponding to 10% of the control level) is enough for the catabolism of Cer in S2 cells.

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