A neutral ceramidase homologue from *Dictyostelium discoideum* exhibits an acidic pH optimum

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Ceramidases (CDases) are currently classified into three categories (acid, neutral and alkaline) based on their optimal pHs and primary structures. Here, we report the first exception to this rule. We cloned the CDase cDNA, consisting of 2142 nucleotides encoding 714 amino-acid residues, from the slime mould, *Dictyostelium discoideum*. The putative amino-acid sequence indicates 32–42 % identity with various neutral CDases, but does not show any similarity to the acid and alkaline CDases, indicating the enzyme should be classified as a neutral CDase. However, overexpression of the cDNA in *D. discoideum* resulted in increased CDase activity at an acidic, but not a neutral pH range. Knockout of the gene in slime mould eliminated CDase activity at

acidic pH. The recombinant enzyme expressed in the slime mould was purified and then characterized. Consequently, the purified CDase was found to exhibit the maximal activity at approx. pH 3.0. The singular pH dependency of slime mould CDase is not derived from the specific post-translational modification in the slime mould, because the enzyme showed an acidic pH optimum even when expressed in Chinese hamster ovary cells, whereas rat neutral-CDase exhibited a neutral pH optimum when expressed in slime mould.

Key words: ceramidase, ceramide, *Dictyostelium discoideum*, pH dependency, sphingolipid.

INTRODUCTION

Sphingolipids are a class of lipids that have ceramide (Cer) as a basic frame. In recent years, sphingolipids have attracted attention as a biomodulator of various cell functions [1–3]. Ceramidase (CDase; EC 3.5.1.23) is an enzyme that hydrolyses the *N*-acyl linkage between sphingosine (Sph) and fatty acid moieties in Cer [4]. The activity of CDase is thought to be crucial for switching off Cer-induced signalling, but also for the generation of Sph and possibly Sph-1-phosphate. Therefore, CDase could be a key enzyme in regulating the signalling system that is mediated by sphingolipids.

In the past few years, molecular cloning of CDase has been performed extensively. Human [5] and mouse [6] CDases with acidic pH optima (acid CDase; optimum pH = 4.0) and bacterial [7], *Drosophila melanogaster* [8], mouse [9], rat [10] and human [11] CDases with a neutral to alkaline pH optima (neutral CDase; optimum pH = 6.5–8.5) have been cloned. CDases with extremely alkaline pH optima (alkaline CDase; optimum pH = 9.5–10.0) were cloned from yeast [12,13] and humans [14]. These cloning studies clearly indicated that CDases with different pH optima represent completely different primary structures and could be generated from different ancestral genes. Thus, CDases are now classified into three families based not only on their pH optima, but also on their primary structures [15].

Here, we describe the molecular cloning and characterization of the CDase from a slime mould, *Dictyostelium discoideum*, which is a simple amoeboid eukaryote and proliferates in a haploid unicellular form. Surprisingly, the CDase of slime mould exhibited pH optima in an extremely acidic range, although the

sequence is homologous with neutral CDase but not acid enzyme. This is the first reported exception to the correlation between the primary structure and pH optimum of CDase.

EXPERIMENTAL

Materials

The cDNA library of *D. discoideum* and Chinese hamster ovary cells that express polyoma LT antigen (CHOP cells) [16] were gifts from Dr H. Yasukawa (Toyama University, Toyama, Japan) and Dr K. Nara (Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan) respectively. The expression vector pcDNA3.1/MyC-His(+) was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Glycopeptidase F and DNA-modifying enzymes were obtained from Takara Bio Inc. (Otsu, Japan). Horseradish peroxidase-labelled anti-rabbit IgG, HiTrap Q HP, HiTrap chelating, HiLoad 16/60 Superdex 200 pg, HiTrap Phenyl Sepharose HP and ECL Plus were purchased from Amersham Bioscience (Piscataway, NJ, U.S.A.). Pre-coated Silica Gel 60 thin-layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany). [¹⁴C]Cer [17], [¹⁴C]glycosphingolipids [18] and C12–4-nitrobenzo-2-oxa-1,3-diazole (NBD)–Cer [19] were prepared as described previously. All other reagents were of the highest purity available.

Cell culture of *D. discoideum*

The wild-type axenic strain *D. discoideum* Ax2 were grown at 20 °C in HL5 medium [20].

Abbreviations used: CDase, ceramidase; Cer, ceramide; CHOP cell, Chinese hamster ovary cells that express polyoma LT antigen cell; DDBJ, DNA Data Bank of Japan; GalCer, galactosylceramide; GM1a, Gal β 1-3GalNAc β 1-4(NeuAc α 2-3)Gal β 1-4Glc β 1-1'Cer; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; Sph, sphingosine; TLC, thin-layer chromatography.

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The nucleotide sequence reported for the *Dictyostelium discoideum* ceramidase is available on the DNA Data Bank of Japan (DDBJ).

Preparation of recombinant mouse neutral-CDase and generation of polyclonal antibody

The vector pAPLCD [9], which contains a full-length mouse neutral-CDase cDNA, was treated with *EcoRI* and *HindIII*, and then subcloned into pTV118N (Takara Bio Inc.). The fragment of the mouse neutral-CDase cDNA derived from digestion with *EcoRI* was cloned into a pET23b vector (Novagen, Madison, WI, U.S.A.) with a C-terminal histidine tag. *Escherichia coli* strain BL21 (DE3) was transformed with the construct. The recombinant CDase was purified from the insoluble fraction (inclusion bodies) using a HiTrap chelating column (Ni^{2+}) according to the manufacturer's instructions. The recombinant protein that was obtained was used for immunizing rabbits.

Protein assay, SDS/PAGE and Western blotting

Protein content was determined by the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, U.S.A.), using bovine serum albumin as a standard. SDS/PAGE was carried out according to the method of Laemmli [23]. Western-blot analysis was carried out as described in [24] using antibodies against mouse neutral-CDase.

Expression of the *D. discoideum* CDase in mammalian cells

CHOP cells were grown in a α -minimal essential medium in the presence of 10 % (v/v) fetal bovine serum, 100 $\mu\text{g}/\text{ml}$ of streptomycin and 100 units/ml of penicillin in a humidified incubator containing 5 % CO_2 . cDNA transfection was carried out using LipofectAMINETM Plus (Invitrogen) according to the manufacturer's instructions. At 24 h after transfection, cells were harvested and suspended in 10 mM Tris/HCl (pH 7.5) containing 0.05 % (v/v) Triton X-100 and the CDase activity in cell lysates was measured.

RESULTS AND DISCUSSION

cDNA cloning of a neutral CDase homologue from slime mould, *D. discoideum*

Using the BLAST program, DDBJ databases were searched for a sequence that was homologous with the *Pseudomonas aeruginosa* neutral-CDase. We found that a *D. discoideum* protein (accession number U82513) with unknown function was highly homologous with the bacterial enzyme. PCR was performed using primers designed from the nucleotide sequence of U82513 and *D. discoideum* Lambda Zap library as a template. The obtained PCR product was used as the probe in plaque hybridization. The screening of 4.35×10^5 bacteriophages gave four positive clones. One plasmid, designated pSDCD, with a 2261-bp insert contained a full-length cDNA encoding a putative CDase. The open reading frame of the *D. discoideum* CDase encoded 714 amino acids and contained nine potential *N*-glycosylation sites. The CDase has a predicted molecular mass of 78 434 Da and an isoelectric point of 4.95, based on the deduced amino-acid sequences. The *D. discoideum* CDase exhibits sequence identity with neutral CDases from various sources; 36.4 % identity with *P. aeruginosa* neutral-CDase, 32.2 % with *Mycobacterium tuberculosis* neutral-CDase, 40.3 % with *D. melanogaster* neutral-CDase, 40.6 % with mouse neutral-CDase, 41.7 % with rat neutral-CDase, and 42.3 % with human neutral-CDase (Figure 1). However, the protein is not homologous with acid or alkaline CDases. In contrast with mammalian neutral-CDases [24], the *D. discoideum* enzyme does not have a Ser/Thr-rich mucin-like domain downstream from the N-terminal hydrophobic

region. This is also a characteristic of other invertebrate and bacterial neutral-CDases.

pH-dependency of the slime mould CDase

Interestingly, the cell lysate of a wild strain of *D. discoideum* showed CDase activity only in the acidic pH range, with maximum activity at approx. pH 3.0 (see inset to Figure 2). A stable transfectant of the slime mould that overexpresses the *D. discoideum* CDase was established and CDase activity of the cell lysate was measured at different pHs. The CDase activity was found to increase drastically compared with that of a wild strain when the activity was measured at pH 3.0, but not in the pH range 6.5–8.5, the latter being the optimum pH range for neutral CDases that have been reported to date (Figure 2). Furthermore, a knockout of the CDase gene in *D. discoideum* eliminated the CDase activity at acidic pH (Figure 2). These results strongly suggest that the neutral CDase homologue of slime mould possesses an acidic optimum pH.

Next, we purified the *D. discoideum* CDase from a CDase-overexpressing transfectant to determine the optimum pH of the purified enzyme. The final preparation, which was purified 170-fold with an overall yield of 34 %, gave a single protein band corresponding to a molecular mass of 93-kDa on SDS/PAGE under reducing conditions (Figure 3A; lane 1). Treatment of the 93-kDa protein with glycopeptidase F resulted in generation of an 81-kDa protein (Figure 3A, lane 2), indicating that the CDase is glycosylated with *N*-glycans. Both 93- and 81-kDa proteins were specifically detected by Western blotting using an antibody against mouse neutral-CDase (Figure 3B). Judging from the deduced molecular mass (78 434 Da), the enzyme seems to be a monomeric polypeptide without subunit structure. In contrast, the acid CDase is composed of α and β subunits [5]. The optimum pH of the purified CDase was found to be approx. pH 3.0 when both C12-NBD-Cer (Figure 4A) and [^{14}C]Cer ([^{14}C]C12:0/d 18:1) (Figure 4B) were used as substrates. In contrast, the most favourable pH range for the purified mouse neutral-CDase was 7.0–7.5 under the same conditions used (results not shown).

Effects of host cells on pH dependency of the enzyme

Two experiments were designed to examine whether the singular pH dependency stemmed from the specific post-translational modification in slime mould. The constructs used in these experiments are illustrated in Figure 5(A). First, a rat neutral-CDase construct (pEXkCD) was expressed in a CDase-knockout strain of *D. discoideum* and the optimum pH of the recombinant CDase was examined. It was found that the pH dependency of the rat CDase expressed in slime mould is very similar to that in CHOP cells, the optimum pH range being 6.0–7.0 (Figure 5B). Secondly, a *D. discoideum* CDase construct (pcDNA rs-dCD) was expressed in CHOP cells. In this experiment, the signal sequence of slime mould CDase was replaced by that of rat CDase (Figure 5A). When the chimaeric protein was expressed in CHOP cells, a significant increase in CDase activity was observed at pH 3.0 (Figure 5C). The activity at a neutral, but not acidic, pH was also observed under these conditions when a mock transfectant was used as the enzyme source, indicating that the activity at neutral pH shown in Figure 5(C) was due to the endogenous CDase of CHOP cells. Therefore, *D. discoideum* CDase showed an acidic pH optimum when expressed in slime mould, as well as in mammalian cells, whereas rat CDase exhibited a neutral pH optimum, even when expressed in slime mould. These results indicate that the singular pH dependency of *D. discoideum* CDase does not stem from the specific post-translational modification in slime mould.

Figure 2 pH dependency of *D. discoideum* CDase

CDase activities of cell lysates from a wild strain (Ax2) (○), a CDase-overexpressing strain (Ax2/pEXDH5) (●), and a CDase-knockout strain (10G) (▲) of *D. discoideum*, respectively. The CDase activity was measured using 200 pmol of C12-NBD-Cer as a substrate in 20 μ l of 150 mM glycine/HCl buffer (pH 2.0–3.5) or GTA buffer (50 mM 3,3-dimethylglutaric acid, 50 mM Tris/HCl, 50 mM 2-amino-2-methyl-1,3-propanediol; pH range, 3.0–10.0) at the indicated pHs containing 0.05 % (v/v) Triton X-100. The reaction was carried out at 37 °C for 30 min. Units of CDase was determined by the method described in the Experimental section. mU, m-units.

Figure 3 SDS/PAGE of the purified recombinant *Dictyostelium* CDase

(A) Coomassie Brilliant Blue-staining and (B) Western blotting with anti-mouse neutral-CDase antibody. Lanes 1 and 2 show the native and the deglycosylated forms of the enzyme respectively. Deglycosylation of the enzyme was conducted using glycopeptidase F, as described in the manufacturer's instructions.

Characterization of the *D. discoideum* CDase

As shown above, a *D. discoideum* homologue of the neutral CDase has an acidic optimum pH. Thus, we characterized the enzymic properties of this unique neutral CDase homologue. The *D. discoideum* enzyme hydrolysed various 14 C-labelled Cers, while the enzyme did not hydrolyse glycosphingolipids, such as GalCer and Gal β 1-3GalNAc β 1-4(NeuAc α 2-3)Gal β 1-4Glc β 1-1'-Cer or sphingomyelin (Table 1). Cers that contain sphingenine (d18:1) were hydrolysed more rapidly than those that contain

Figure 4 pH dependency of the purified enzyme

The CDase activity was measured using 200 pmol of C12-NBD-Cer (A) and 100 pmol of [14 C]Cer ([14 C]C12:0/d18:1 sphingosine) (B) as substrates in 20 μ l of 100 mM various buffers at different pHs containing 0.05 % (v/v) Triton X-100. The reaction was carried out at 37 °C for 30 min. The hydrolysis of C12-NBD-Cer or [14 C]Cer was measured by the method described in the Experimental section.

Table 1 Specificity of *D. discoideum* CDase

14 C- or NBD-labelled substrates (100 pmol each) were incubated at 37 °C for 30 min with 3 μ -units of CDase in 50 mM acetate buffer (pH 3.5) containing 0.05 % (v/v) Triton X-100. The released fatty acid and the remaining substrate were separated by TLC using chloroform/methanol/25 % ammonia (90/20/0.5, by vol.) for various Cers and chloroform/methanol/0.02 % CaCl_2 (5/4/1, by vol.) for glycosphingolipids and sphingomyelin as a developing solvent respectively. The extent of hydrolysis (%) was calculated as follows: released fatty acid $\times$ 100/(released fatty acid + remaining substrate).

Substrate	Structure (fatty acid/long-chain base or hydrophilic portion)	Hydrolysis (%)
<i>N</i> -Lauroylsphingosine	C12:0/d18:1	30.3
<i>N</i> -Palmitoylsphingosine	C16:0/d18:1	19.3
<i>N</i> -Stearoylsphingosine	C18:0/d18:1	14.4
<i>N</i> -Palmitoylsphinganine	C16:0/d18:0	15.5
<i>N</i> -Stearoylsphinganine	C18:0/d18:0	10.7
<i>N</i> -Palmitoylphytosphingosine	C16:0/t18:0	1.0
<i>N</i> -Stearoylphytosphingosine	C18:0/t18:0	0.1
NBD- <i>N</i> -dodecanoylsphingosine	NBD-C12:0/d18:1	41.5
GalCer	Gal β -1'-Cer	0
GM1a	Gal β 1-3GalNAc β 1-4(NeuAc α 2-3)Gal β 1-4Glc β -1'-Cer	0
Sphingomyelin	Choline phosphate Cer	0

Figure 1 Alignment of deduced amino-acid sequences of neutral CDases from *D. discoideum*, mouse, rat, human, *P. aeruginosa*, *M. tuberculosis* and *D. melanogaster*

Alignment was performed using the CLUSTAL algorithm [28]. Amino acids identical with *D. discoideum* CDase are indicated by white text on a black background. Gaps inserted into the sequence are indicated by dots. The mucin-like domain, which is present only in vertebrate CDases [24], is indicated by underlined residues.

Figure 5 Host effects on pH dependency of the CDase

(A) The two constructs used in this experiment. (B) Rat CDase activity at different pH values when expressed in *D. discoideum*. (C) *D. discoideum* CDase activity at different pH values, when expressed in CHOP cells. The activity was measured by the method described in the legend of Figure 2.

Figure 6 General properties of *Dictyostelium* CDase

(A) Effects of cations on the CDase activity. Aliquots of 200 pmol of C12-NBD-Cer were incubated with 3 μ-units of the enzyme at 37 °C for 30 min in 20 μl of 100 mM acetate buffer (pH 3.5) containing 0.05 % (v/v) Triton X-100 and 5 mM of the indicated cations. (B) Effects of phospholipids on the CDase. Aliquots of 200 pmol of C12-NBD-Cer were incubated with 3 μ-units of the enzyme at 37 °C for 30 min in 20 μl of 100 mM acetate buffer (pH 3.5) containing 0.05 % (v/v) Triton X-100 and various phospholipids at the indicated concentrations. (C) Lineweaver-Burk plots for the CDase. C12-NBD-Cer at the indicated concentrations were incubated with 3 μ-units of the enzyme at 37 °C for 30 min in 20 μl of 50 mM acetate buffer (pH 3.5) containing 0.05 % (v/v) Triton X-100. (D) pH dependency of the reverse activity. Aliquots of 1 nmol of [¹⁴C]lauric acid and 2 nmol of sphinganine were incubated with 1.5 μ-unit of the enzyme at 37 °C for 30 min in 20 μl of various buffers (100 mM) containing 0.05 % (v/v) Triton X-100 at the indicated pH. The hydrolysis of C12-NBD-Cer and the synthesis of [¹⁴C]Cer were measured by the method described in the Experimental section.

sphinganine (d18:0). Cers that contain phytosphingosine (t18:0) were strongly resistant to hydrolysis by the enzyme. Among various fatty-acid moieties of Cer that were tested, lauric acid (C12:0) resulted in the highest degree of hydrolysis under the conditions used. The fluorescent substrate C12-NBD-Cer, of which NBD is covalently coupled to the amino group of ω-amino-dodecanoic acid, was hydrolysed much faster than native Cers. These substrate specificities are similar to those of neutral CDases of other origins [8,10,25,26]. The effects of various cations and phospholipids on CDase activity were evaluated at pH 3.5 using C12-NBD-Cer as the substrate. The enzyme activity was

greatly inhibited by 5 mM Hg²⁺, while this concentration of Ca²⁺, Mg²⁺, Mn²⁺, Cu²⁺, Zn²⁺ and EDTA had little effect on the enzyme activity (Figure 6A). Cardiolipin, phosphatidylglycerol and phosphatidylserine (each at 20 μM) increased the enzyme activity by 200 %, 170 % and 160 % respectively. However, phosphatidylethanolamine and phosphatidylcholine did not affect the activity (Figure 6B). The apparent K_m and V_{max} values for C12-NBD-Cer were 38.8 μM and 14.2 μmol⁻¹ · min⁻¹ · mg respectively (Figure 6C). As is the case with other neutral CDases, *Dictyostelium* enzyme catalysed the reverse hydrolysis reaction in which a fatty acid is condensed to a Sph base to produce Cer.

The optimum pH of the reverse activity was found to be approx. 5.0 when ^{14}C -labelled lauric acid and sphingenine (d18:1) were used as substrates (Figure 6D).

It is still unclear as to whether *D. discoideum* has genes that are homologous with the acid or alkaline CDases that have been reported to date. However, the protein cloned here seems to be responsible for most, if not all, of the CDase activity in slime mould, because knockout of the gene almost completely eliminated the CDase activity (Figure 2). Recently, it was reported that acid-CDase knockout mice exhibited embryonic fatality [27]. Unexpectedly, however, the disruption of the CDase gene in *D. discoideum* was found to have little effect on phenotype under the present conditions (results not shown), which suggests that the residual CDase activity (corresponding to 0.03% of the activity in the control wild strain) works sufficiently for Cer catabolism in slime mould under the conditions described here.

Further analysis of *D. discoideum* CDase, including X-ray crystallography could provide useful information about the relationship between the structure and pH dependency of CDase.

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