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], Droso phila 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, Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1′Cer; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; Sph, sphingosine; TLC, thin-layer chromatography.

1 To whom correspondence should be addressed (e-mail makoto@agr.kyushu-u.ac.jp). The nucleotide sequence reported for the Dictyostelium discoideum ceramidase is available on the DNA Data Bank of Japan (DDBJ).

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cDNA cloning and sequencing

A *D. discoideum* nucleotide sequence (accession number U82513) homologous with *Pseudomonas aeruginosa* CDase was found in the DNA Data Bank of Japan (DDBJ) database using the BLAST program. We designed two primers based on the nucleotide sequence of U82513. PCR using a sense primer 10S (5′-TTATCATGTGGGTITTTTAT-3′) and an antisense primer 357A (5′-GGACCTGAAATGAAAGTGTTGT-3′) was performed with the *D. discoideum* Lambda Zap library as a template. The cycling parameters for PCR were 40 cycles of 94 °C for 30 s and 72 °C for 30 s. After amplification, a 367-bp product containing the sequence of *D. discoideum* CDase was obtained.

To obtain the full-length cDNA encoding the CDase, plaque hybridization was performed using the 32P-labelled 367-bp PCR product as a probe.

Construction of expression and disruption vectors

To generate a polyhistidine-tagged *D. discoideum* CDase expression vector (pEXDH5), cDNA encoding the CDase with a polyhistidine tag was subcloned into the vector pEX by PCR using a 5′-primer with a BamHI restriction site (5′-AAGGATCC-AAAATGCAAAGATCAATAGATTAT-3′) and a 3′-primer with a XhoI restriction site, a polyhistidine tag and a disrupted stop codon (5′-AATCTCATAGTTGTTGTTGTTTGTGTGTTTG-AACATTTAATCTTTGGA-3′). A chimaeric protein containing the signal anchor sequence of rat neutral CDase and catalytic region of *D. discoideum* CDase (pcDNA rs-dCD) was constructed by fusing the N-terminal fragment (Met1-Lys42) of rat neutral CDase with the C-terminal fragment (Phe24–Gln714) of *D. discoideum* CDase. The N-terminal fragment was amplified by PCR using a 5′-primer with a BamHI restriction site (5′-AGAGGATCCGAAATGGCAAAGCGAACCTT-3′) and a 3′-primer with a XhoI restriction site, a polyhistidine tag and a disrupted stop codon (5′-AGAGGATCCGAAATGGCAAAGCGAACCTT-3′) and a XhoI restriction site, a polyhistidine tag and a disrupted stop codon (5′-AGAGGATCCGAAATGGCAAAGCGAACCTT-3′) and a XhoI restriction site, a polyhistidine tag and a disrupted stop codon (5′-AGAGGATCCGAAATGGCAAAGCGAACCTT-3′) and a XhoI restriction site, a polyhistidine tag and a disrupted stop codon (5′-AGAGGATCCGAAATGGCAAAGCGAACCTT-3′). A chimaeric protein containing the signal anchor sequence of rat neutral CDase and catalytic region of *D. discoideum* CDase (pcDNA rs-dCD) was constructed by fusing the N-terminal fragment (Met1-Lys42) of rat neutral CDase with the C-terminal fragment (Phe24–Gln714) of *D. discoideum* CDase.

Overexpression and knockout strain of the CDase gene

To obtain a knockout strain 10G, the disruption vector was treated with ScaI and NotI. This fragment was introduced into Ax2 cells by electroporation. To obtain the CDase-overexpressing strains, the expression vectors pEXDH5 or pEXkCD were introduced into Ax2 or 10G cells. Electroporation was performed with a Gene Pulser™ (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The selection was carried out in medium HL5 containing 20 µg/ml of geneticin for the overexpression strain [21] and 10 µg/ml of blasticidin S for the knockout strain [22] respectively.

CDase assay

CDase activity was measured using C12-NBD-Cer as a substrate. Briefly, 200 pmol of C12-NBD-Cer were incubated at 37 °C for 30 min with an appropriate amount of the enzyme in 20 µl of 100 mM acetate buffer (pH 5.5) containing 0.05 % (v/v) Triton X-100. The reaction was stopped by heating in boiling water for 5 min. After drying in a Speed Vac concentrator, the sample was dissolved in 12 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, by vol.). The released NBD-dodecanoic acid and the remaining C12-NBD-Cer were quantified with a Shimadzu CS-9300 chromatoscanner (Shimadzu, Kyoto, Japan). In some cases, 100 pmol of [14C]Cer was used instead of the fluorescent Cer as a substrate. The released [14C]-labelled fatty acid and the remaining [14C]Cer were separated by TLC and quantified with a BAS1000 imaging analyser (Fuji Film, Tokyo, Japan). The percentage hydrolysis was calculated as follows:

\[
\text{percentage hydrolysis} = \frac{\text{released fatty acid} \times 100}{\text{released fatty acid} + \text{remaining substrate}}
\]

One enzyme unit was defined as the amount of enzyme capable of catalysing the release of 1 µmol of NBD-dodecanoic acid/min from C12-NBD-Cer under the conditions described above. A reverse hydrolysis reaction was measured using 1 nmol of [14C]lauric acid and 2 nmol of sphingine (d18:1) as substrates instead of C12-NBD-Cer. The remaining [14C]lauric acid and the generated [14C]Cer were separated by TLC and quantified with a BAS1000 imaging analyser. The percentage synthesis was calculated as follows:

\[
\text{percentage synthesis} = \frac{\text{generated [14C]Cer} \times 100}{\text{generated [14C]Cer} + \text{remaining [14C]lauric acid}}
\]

Purification of the recombinant CDase

The CDase-overexpressing strain, 10G/pEXDH5, was cultured at up to 5 × 10^6 cells/ml in HL5 culture medium. The culture fluid was centrifuged at 500 g for 1 min and the cells were resuspended to a concentration of 1 × 10^7 cells/ml with 12 mM phosphate buffer (pH 6.1) and incubated at 20 °C for 24 h with vigorous shaking. The cell suspension was centrifuged for 10 min at 6700 g, and the supernatant (200 ml) was then applied to a HiTrap Q HP column (5 ml) that had been equilibrated with 20 mM Tris/HCl (pH 7.5) containing 0.1% Lubrol PX (buffer A). The enzyme was absorbed on to the column, which was washed with buffer A containing 0.1 M NaCl. The enzyme was then eluted from the column with buffer A containing 0.5 M NaCl (buffer B). The active fractions were pooled, and then applied to a HiTrap Chelating column (chelated with Ni^2+), 1 ml) that had been pre-equilibrated with 200 mM Tris/HCl (pH 7.5) containing 0.1 % Lubrol PX (buffer A). The enzyme was absorbed on to the column, which was washed with buffer A containing 0.1 M NaCl. The enzyme was then eluted with buffer B containing 200 mM imidazole. The active fractions were pooled, and then loaded on to a HiLoad 16/60 Superdex 200 pg column equilibrated with 10 mM Tris/HCl (pH 7.5) containing 150 mM NaCl and 0.1% Lubrol PX at a flow rate of 0.8 ml/min using a BioCAD system. The active fractions were pooled, and then applied to a HiTrap Phenyl Sepharose HP column (1 ml) that had been pre-equilibrated with buffer B containing 2 M NaCl. The enzyme was absorbed on to the column, which was washed with buffer A containing 2 M NaCl, and the enzyme was then eluted with buffer A containing 1 M NaCl.
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 (Ni²⁺) 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 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) 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 × 10⁸ 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 transfec tant 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 transfec tant 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 [¹⁴C]Cer ([¹⁴C]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, whereas rat CDase exhibited a neutral pH optimum, even when expressed in slime mould (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 neutral, but not acidic, pH was also observed under these conditions when a mock transfec tant 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.
<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>Dicyostelium</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td></td>
</tr>
<tr>
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<td>Mycobacterium</td>
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</table>

**Figure 1** For legend, see facing page

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Ceramidase of Dictyostelium discoideum

Figure 2 pH dependency of D. discoideum CDase

CDase activities of cell lysates from a wild strain (Ax2) (H17034), a CDase-overexpressing strain (Ax2/pEXDH5) (H17033), and a CDase-knockout strain (10G) (H17009) of D. discoideum, respectively. The inset shows the CDase activity of cell lysates from a wild strain (Ax2) with a different scale. The CDase activity was measured using 200 pmol of C12-NBD-Cer as a substrate in 20 µl 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 were 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.

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 [14C]Cer (B) as substrates in 20 µl 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 [14C]Cer was measured by the method described in the Experimental section.

Table 1 Specificity of D. discoideum CDase

<table>
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<th>Substrate or hydrophilic portion</th>
<th>Structure</th>
<th>Hydrolysis (%)</th>
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<td>N-Lauroylsphingosine</td>
<td>C12:0/d18:1</td>
<td>30.3</td>
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<tr>
<td>N-Palmitoylsphingosine</td>
<td>C16:0/d18:1</td>
<td>19.3</td>
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<tr>
<td>N-Stearoylsphingosine</td>
<td>C18:0/d18:1</td>
<td>14.4</td>
</tr>
<tr>
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<tr>
<td>Sphingomyelin</td>
<td>Choline phosphate Cer</td>
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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 14C-labelled Cers, while the enzyme did not hydrolyse glycosphingolipids, such as GalCer and Galβ1-3GalNAcβ1-4(NeuAco2-3)Galβ1-4Glcβ1-1′Cer or sphingomyelin (Table 1). Cers that contain sphingenine (d18:1) were hydrolysed more rapidly than those that contain sphinganine (t18:0).
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 \( \omega \)-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 Hg\(^{2+}\), while this concentration of Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\) and EDTA had little effect on the enzyme activity (Figure 6A). Cardiolipin, phosphatidylglycerol and phosphatidylserine (each at 20 \( \mu \)M) 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 \( \mu \)M and 14.2 \( \mu \)mol·min\(^{-1}\)·mg\(^{-1}\) 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.

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The optimum pH of the reverse activity was found to be approx. 5.0 when 14C-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.

We thank Dr H. Yasukawa and Dr K. Nara for providing the *D. discoideum* cDNA library and CHOP cells respectively. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (B) 12140204 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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