Identification of a novel amidase motif in neutral ceramidase

Sehamuddin GALADARI*[1,2], Bill X. WU*[2], Cungui MAO†, Patrick RODDY*, Samer EL BAWAB‡ and Yusuf A. HANNUN*[3]

*Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, U.S.A., †Department of Medicine, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29425, U.S.A., and ‡Merck-Santé, CardioMetabolic Research, Chilly-Mazarin Research Center, 4 ave. F. Mitterrand, 91380 Chilly-Mazarin, France

Neutral CDases (ceramidases) are newly identified enzymes with important roles in cell regulation, but little is known about their catalytic mechanisms. In the present study the full-length human neutral CDase was cloned and expressed in the yeast double-knockout strain *Δypc1Δydc1*, which lacks the yeast CDases YPC1p and YDC1p. Biochemical characterization of the human neutral CDase showed that the enzyme exhibited classical Michaelis–Menten kinetics, with an optimum activity at pH 7.5. Activity was enhanced by Na^+ and Ca^{2+}. Mg^{2+} and Mn^{2+} were somewhat stimulatory, but Zn^{2+}, Cu^{2+} and Fe^{2+} inhibited the enzyme. Dithiothreitol and 2-mercaptoethanol dose-dependently inhibited neutral CDase. In order to identify which amino acids were involved in the catalytic action of neutral CDase, the purified enzyme was subjected to chemical modifications. It was observed that the serine residue modifier di-isopropyl fluorophosphate dose-dependently inhibited activity, implicating a serine residue in the catalytic action. From an alignment of the sequences of the neutral CDases from different species, all conserved serine residues were selected for site-directed mutagenesis. Of the six aligned serine residues that were mutated to alanine, only the S354A mutant lost its activity totally. Ser^{354} falls within a very highly conserved hexapeptide sequence GDVSPN, which itself was in the middle of a larger conserved sequence, namely NXGDVSPNXXG$^{P}/_{X}$XC. Moreover, mutations of Asp^{352} and Cys^{362} in the consensus sequence to alanine resulted in loss of activity of neutral CDase. Hence the present study identified a novel amidase sequence containing a critical serine residue that may function as a nucleophile in the hydrolytic attack on the amide bond present in ceramide.

Key words: catalytically important serine residue, neutral ceramidase, novel amidase motif, sphingosine, dihydroCDase (YDC1p), phytoCDase (YPC1p).

INTRODUCTION

CDase (ceramidase; EC3.5.1.23) cleaves the N-acyl linkage of ceramide into sphingosine (SPH) and non-esterified fatty acid. CDase exerts important functions in the regulation of its substrate ceramide, its immediate product SPH, or the downstream metabolite sphingosine 1-phosphate (S1P). It is currently understood that the major pathway for the formation of SPH is via the degradation of ceramide and not from the *de novo*-synthesis pathway [1,2]. This underscores the role of CDase in regulating the levels of ceramide, SPH and/or S1P, which are important bioactive lipids [3]. To date, and on the basis of their catalytic pH optima and primary sequence, at least three different types of CDases have been reported: a lysosomal acid CDase, alkaline CDases and a neutral CDase.

Acid CDase is a lysosomal enzyme, a defect in which underlies the human disorder Farber's disease (disseminated lipogranulomatosis) [4]. This enzyme has a pH optimum of 4–5, is glycosylated, has an apparent molecular mass of 50 kDa and it prefers ceramide as a substrate over dhCer (dihydroceramide). Recently, two yeast (*Saccharomyces cerevisiae*) alkaline CDases, namely YPC1p (phytoCDase) and YDC1p (dihydroCDase), were cloned and characterized [5,6]. YPC1p and YDC1p have pH optima at approx. 9 and have predicted molecular masses of 36.4 and 37.2 kDa respectively. A human homologue of YPC1p has been cloned and characterized [7]. It has a pH optimum of 9.5 and a substrate preference for phytoceramide. Recently, a mouse alkaline CDase (maCER1) was also identified [8]. maCER1 hydrolysed D-*erythro*-ceramide but not D-*erythro*-dihydroceramide or D-*ribo*-phytoceramide.

A membrane-bound non-lysosomal neutral CDase having a broad pH optimum has been purified to homogeneity and biochemically characterized from various mammalian tissues, including mouse liver and rat brain, kidney and intestine [9–11]. Using peptide sequences obtained from the purified rat brain enzyme, the human neutral CDase was cloned [12]. A homologous gene was independently purified and cloned from bacteria [13]. These efforts allowed the identification of a family of homologous genes from many species, including the fruitfly *Drosophila* [11,14]. It is noteworthy that phylogenetic analysis reveals that the three CDase families having different pH optima may be derived from different ancestral genes [15].

The rat brain neutral CDase has an apparent molecular mass of 90–95 kDa under reducing conditions and a pI (isoelectric point) of $\approx$6.5. The enzyme exhibited Michaelis–Menten kinetics in a Triton X-100/ceramide mixed-micellar assay. Moreover, anionic phospholipids, such as PA (phosphatidic acid), PS (phosphatidylserine) and CL (cardiolipin; diphosphatidylglycerol) stimulated the activity, whereas SPH acted as a competitive inhibitor [10]. Rat brain neutral CDase has a preference for ceramide

Abbreviations used: CDase, ceramidase; CL, cardiolipin (diphosphatidylglycerol); DEPC, diethyl pyrocarbonate; DFP, di-isopropyl fluorophosphate; dhCer, dihydroceramide; DTT, dithiothreitol; FAAH, fatty acid amide hydrolase; IL-1*β*, interleukin-1*β*; maCER1, mouse alkaline CDase; 2-ME, 2-mercaptoethanol; mito-CDase, mitochondrial CDase; NBD, 7-nitrobenz-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; PA, phosphatidic acid; PDGF, platelet-derived growth factor; phytoCer, phytoceramide; PS, phosphatidylserine; S1P, sphingosine 1-phosphate; SPH, sphingosine; YDC1p, dihydroCDase; YPC1p, phytoCDase.

[1] Present address: Department of Biochemistry, Faculty of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates.
[2] These two authors contributed equally to this work.
[3] To whom correspondence should be addressed (email hannun@musc.edu).

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over dhCer and, of the four possible stereoisomers of ceramide, only the natural D-*erythro*-C_{18}-ceramide isomer was used by the enzyme as substrate [16].

Neutral CDase appears to regulate changes in ceramide/SPH/S1P in response to various stimuli, including cytokines and growth factors. For instance, it has been reported that PDGF (platelet-derived growth factor) up-regulates neutral CDase activity in rat mesangial cells [17], and the enzyme activity was modulated in a bimodal manner by IL-1β (interleukin-1β) in rat hepatocytes [18], leading to a decrease in ceramide concomitantly with an increase in SPH. It was shown that neutral CDase activity increased and ceramide level decreased after IL-1β stimulation of rat mesangial cells [19], whereas NO led to degradation of neutral CDase [20,21]. In mesangial cells, neutral CDase could also mediate the effect of advanced glycation end-products (generated during chronic hyperglycaemia) on cell proliferation [22]. In *Drosophila*, mutation of neutral CDase causes synaptic dysfunction with impaired vesicle fusion and trafficking [23]. Moreover, targeted expression of neutral CDase rescued *Drosophila* mutants from retinal degeneration [24]. In zebrafish (*Danio rerio*), neutral CDase is important in embryo development [25].

Our understanding of the catalytic mechanism of neutral CDase is still deficient. Since neutral CDases act by attacking the amide bond between the fatty acid and the sphingoid base in ceramide, one would envisage that their mechanism of action might be similar to other amidases, and the nucleophile responsible for attack on the amide bond may be similar. However, neutral CDases do not align with known amidases, nor do they appear to contain the amidase signature sequence $GSSG(X)_{14}GXD(X)_3S(X)_3PX$ found in FAAH (fatty acid amide hydrolase) and other amidases, including some proteases [26–28]. Moreover, neutral CDases do not show significant sequence similarity to other enzymes, including acid and alkaline CDases [15], implying that neutral CDases define a unique enzyme family with a distinct enzymatic mechanism.

In the present study we found a highly conserved hexaresidue core that was conserved among the different neutral CDases. We report the identification of this core as a new amidase sequence containing a proposed serine nucleophile. This finding enhances our mechanistic understanding of this group of important regulatory enzymes in sphingolipid biosynthesis and sphingolipid-mediated signal transduction.

EXPERIMENTAL

Materials

All chemicals were from Sigma (St Louis, MO, U.S.A.), unless otherwise stated. D-*erythro*-C_{12}-NBD-ceramide (D-*erythro*-C_{12}-7-nitrobenz-2-oxa-1,3-diazolylceramide) was kindly provided by the Lipidomics Core Facility at the Medical University of South Carolina. NBD-C_{12} fatty acid was purchased from Molecular Probes (Eugene, OR, U.S.A.) and Triton X-100 from Pierce (Rockford, IL, U.S.A). Synthetic oligonucleotides were from IDT (Integrated DNA Technologies) (Coralville, IA, U.S.A). Anti-His_6 monoclonal antibody was from Novagen (Madison, WI, U.S.A) was reconstituted in sterile double-distilled deionized water at a concentration of 0.2 μg/μl. Goat anti-mouse horseradish-peroxidase-conjugated secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.).

Cloning of the human neutral CDase

Using the advanced BLAST program, a genomic DNA sequence encoding the human mito-CDase (mitochondrial CDase) was identified [12]. Another ATG codon upstream of the translational initiation site proposed for mito-CDase was found in this genomic DNA sequence which was not detected in the original sequencing of the cDNA. Immediately upstream of this ATG codon, an in-frame stop codon was found. In order to verify that this sequence is a new translational initiation site, we designed primers that correspond to this region. The forward primer:

5′-CCATCTCTGCTGTACCTGAGAAGAAATGGCC-3′

and the reverse primer:

5′-GGGCCGAAAACTTCAACATTGATAAATCACT-3′

and a human liver cDNA library (Stratagene) were used. Using a Biometra T3 thermocycler, PCR amplification was performed at a denaturing temperature of 94 °C for 1 min, followed by annealing at 65 °C for 1 min and extension for 2.5 min at 72 °C for a total of 33 cycles. The amplified fragment was purified on a 1.5%-(w/v)-agarose gel and subcloned into TOPO® blunt-end cloning vector (Invitrogen). Sequencing confirmed this new full-length coding sequence.

Expression of the human neutral CDase in yeast

In order to generate the construct pYES2-CDase that expresses the human neutral CDase under the control of the *Gal1* promoter, the open reading frame of the full-length human neutral CDase gene was amplified by PCR using previously described conditions [12]. The forward primer:

5′-CGGGGTACCCATGGCCAAGCGCACCTTC-3′

and the reverse primer:

5′-GCTCTAGACTAATGATGATGATGATGATGATGATGATG-AATAGTTACAACTTCAAAAGCCGGGGA-3′

were used. In addition, the reverse primer contained His_9 to help with its detection. The underlined sequences in the forward and reverse primers are the restriction sites of KpnI and XbaI respectively. The PCR amplified product was digested with the restriction enzymes KpnI and XbaI and cloned into the KpnI and XbaI sites of the vector pYES2 (Invitrogen), thus expressing CDase under the control of the promoter *Gal1*. All constructs were confirmed by sequencing. The construct pYES2-CDase/His_9 or the empty vector pYES2 was transformed into the yeast strain *Δypc1Δydc1* by the lithium acetate method, as previously described [29]. The yeast double-knockout strain *Δypc1Δydc1*, which lacks the yeast CDases YDC1p and YPC1p, had been generated previously [6]. The strain containing pYES2-CDase/His_9 was grown and maintained in synthetic complete medium (SC-Ura^-) with the omission of uracil. Expression of the CDase was induced by 2 % (w/v) galactose in SC-Ura^- medium.

Preparation of yeast microsomes

Yeast cells were washed twice with ice-cold double-distilled deionized water and suspended in yeast homogenization buffer (25 mM Tris/HCl, pH 7.4, containing 1mM PMSF and 20 μg/ml each of the protease inhibitors chymostatin, leupeptin, antipain and pepstatin). The cell slurry was homogenized three times for 40s with 1 min chilling on ice between each homogenization. Acid-washed glass beads (Sigma) and a Mini-bead-beater-8 (Biospec Products) set at maximum speed were used. The homogenate was centrifuged at 700 ***g*** for 5 min at 4 °C, and the resulting supernatant was transferred to a new tube. This was further centrifuged at 1400 ***g*** and for 5 min at 4 °C to remove cell debris. Finally, in order to pellet the membrane fraction, the supernatant was centrifuged at 36 500 rev./min for 1 h at 4 °C in a Beckman TLX-100 table-top ultracentrifuge using a TLA45 rotor. The

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membrane fraction was rinsed gently with homogenization buffer and resuspended in an appropriate assay buffer. Protein concentrations were determined by Bradford assay using a kit from Bio-Rad.

Purification of rat brain neutral CDase

Neutral CDase was purified to homogeneity as previously described [10]. Briefly, the enzyme was extracted from the 10 000 ***g*** pellet with Triton X-100. This extract was then applied to a Q-Sepharose anion-exchange-chromatography column, followed by columns of Blue-Sepharose, Phenyl-Sepharose and MonoS cation-exchange resin. Using this protein-purification protocol, the specific activity was increased about 20 000-fold, and the protein, when subjected to SDS/PAGE and silver staining, appeared as a single band in the first fractions of the last column.

Neutral CDase enzyme assays and biochemical characterization

Enzyme activity was measured using D-*erythro*-C_{12}-NBD-ceramide at a final concentration of 100 μM (2.08 mol %) in a 25 mM Tris buffer, pH 7.5, containing 0.3 % Triton-X-100 final in a total volume of 100 μl. The enzymatic reaction was started by adding 60 μg of yeast microsomal enzyme (unless otherwise stated). For the pH-optimum determination, the substrate was dissolved in the following buffers: pH 4–5.5, 100 mM acetate buffer; pH 6–7, 100 mM phosphate buffer; pH 7–7.5, 100 mM Hepes buffer; and pH 8–10 ,100 mM glycine buffer. A comparative study was done at neutral pH values between those of Tris and Hepes buffers and there were no significant differences (results not shown). The enzymatic reaction was continued for 1 h at 37 °C, and it was stopped by addition of chloroform/methanol (1:1, v/v). The mixture was left at room temperature for 5 min, after which it was centrifuged and the organic phase was taken and dried using a SpeedVac (Savant) evaporator. Lipids were dissolved in 25 μl of chloroform/methanol (2:1, v/v), and 15 μl was spotted on to TLC plates. The product NBD-C_{12}-fatty acids were separated from the substrate by developing the plate in chloroform/methanol/25 % ammonium hydroxide (90:30:0.5, by vol.) solvent system. The TLC plate was scanned using PhosphorImager (Storm 860) system set at fluorescence mode. The NBD-fatty acid product was identified by comparison with NBD-C_{12}-fatty acid standards and was analysed using ImageQuant™ software. The specific activity was determined using standard curves with known concentrations of NBD-C_{12}-fatty acid. When using the C_{16} ceramide as substrate, the reaction conditions were the same as those described above, except that CDase activity was determined by the release of sphingoid bases from ceramide. The reactions were stopped by extraction with chloroform and methanol. Sphingoid bases in the lipid extracts were determined by HPLC with D-*erythro*-C_{17}-sphingosine as an internal standard, as described in [30].

Reverse CDase activity assay

The assay was performed as described in [31], with slight modifications. Briefly, the substrates, [^{3}H]palmitic acid and SPH, were dried and then resuspended in 100 μl of reaction buffer [100 mM Hepes buffer (pH 7)/0.3 % Triton X-100]; 60 μg yeast lysate was used for each reverse action. The enzymatic reaction was carried out at 37 °C for 1 h. The lipids were extracted and loaded on to TLC plates as described above. Labelled S1P was visualized by autoradiography, and densitometric analysis was performed using ImageJ software.

Chemical modification

Amino-acid-side-chain modifications were carried out as previously described [32–34] with minor changes. Briefly, aliquots of 200 μl of purified rat brain neutral CDase were pre-incubated with freshly prepared solutions of DFP (di-isopropyl fluorophosphate), DEPC (diethyl pyrocarbonate) and NEM (*N*-ethylmaleimide) at different final concentrations. The modification reactions were carried out in 100 mM phosphate buffer, pH 7.5, and at 37 °C, for 15 min (unless otherwise stated in the manufacturer's protocol). Control reaction mixtures contained either chemical modifiers and no enzyme or no modifiers but with the enzyme. To determine the effect of pre-incubation of ceramide on the DFP inhibitor, yeast lysates were first preincubated with 500 μM D-*erythro*-C_{12}-NBD-ceramide for 30 min on ice, and then incubated with or without 100 μM DFP for another 15min at 37 °C. The enzyme solutions were freed from unchanged chemical modifiers by size-specific filtration using Centricon protein concentrators (Millipore). The concentrates were diluted with neutral CDase assay buffer, and the activity was measured as described above.

Table 1 Synthetic oligonucleotides used for site-directed mutagenesis

The mutated nucleotides are underlined and in bold.

Mutant (forward)	Primer sequence (5′–3′)
S258A	GTGCAGATCAACAGA**GCC**CCGTATTCTTACCTTC
S354A	AACCTAGGAGATGTG**GCC**CCCAACATTCTTGGA
S374A	TGTGATAACGCCAAT**GCC**ACTTGTCCCATTGGT
S396A	CAGGATATGTTTGAC**GCC**ACACAAATTATAGGA
S595A	CGATATGAGGCAGCA**GCC**ACAATTTATGGACCG
S729A	GGACTCCTGGGTCTG**GCC**AATGCAACAGTGGAA
D352S	TCATCAAACCTAGGA**GGT**GTGTCCCCCAACATT
C362A	ATTCTTGGACCACGT**GCC**ATCAACACAGGAGAG

Preparation of neutral CDase mutants

The coding sequence for human neutral CDase was inserted in the pYES2 vector as described above. Mutagenesis of CDase to alter individual residues to other amino acids was performed by PCR of CDase in the pYES2 vector using the QuikChange site-directed mutagenesis kit (Stratagene). The PCR products were transformed into Epicurian Coli XL-1-Blue supercompetent cells (Strategene) and then sequenced at the DNA Sequencing Facility of the Medical University of South Carolina to confirm the expected mutants. PCR primers containing the mutations are listed in Table 1.

Western-blot analysis

Microsomal lysates were prepared as described above. They were solubilized in SDS/PAGE sample buffer and boiled for 10 min. The boiled lysates were then applied, under reducing conditions, to an SDS/7.5 %-(w/v)-polyacrylamide gel and transferred to a PVDF membrane overnight in transfer buffer containing 20 % (v/v) methanol. Blots were probed using monoclonal antibodies against His_6, followed by goat anti-mouse horseradish-peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and visualized using ECL® (enhanced chemiluminescence) reagent (Amersham Biosciences).

RESULTS

Characterization of the expressed human neutral CDase

Previous studies characterized the purified rat brain CDase. In order to determine important catalytic residues for the enzyme, a heterologous expression system was required where the properties of CDase could be determined with high specificity. Thus, in order

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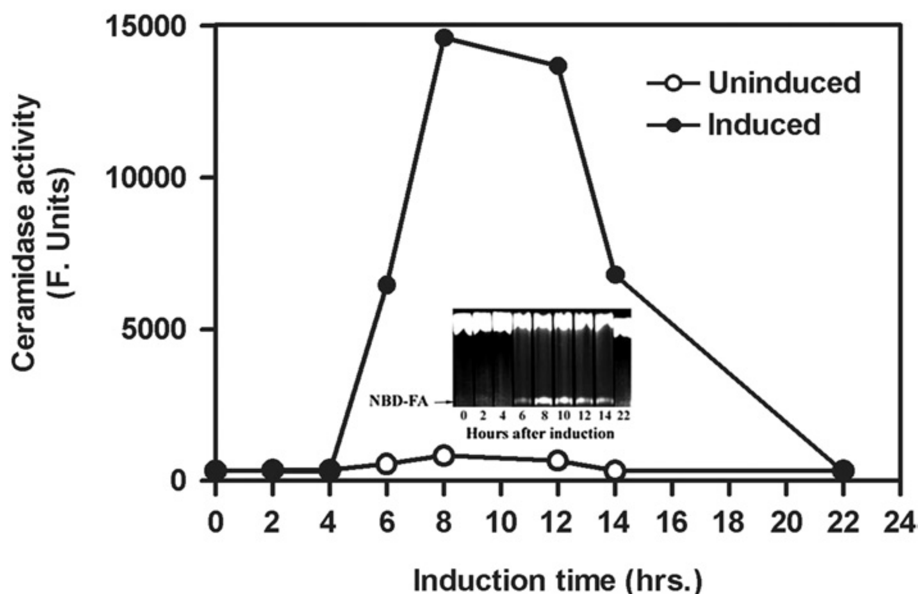

Figure 1 Time course of induction of human neutral CDase

The neutral CDase was cloned into the pYES2 vector and the construct was transformed into the yeast double-knockout strain *Δypc1Δydc1*, which lacks the yeast CDases. Gene expression was induced by growth in galactose medium, and at different time points the culture was stopped and homogenates were assayed for CDase activity using D-*erythro*-C_{12}-NBD-ceramide as substrate. The average values from two independent experiments were plotted. Abbreviation: F. Units, arbitrary fluorescence units.

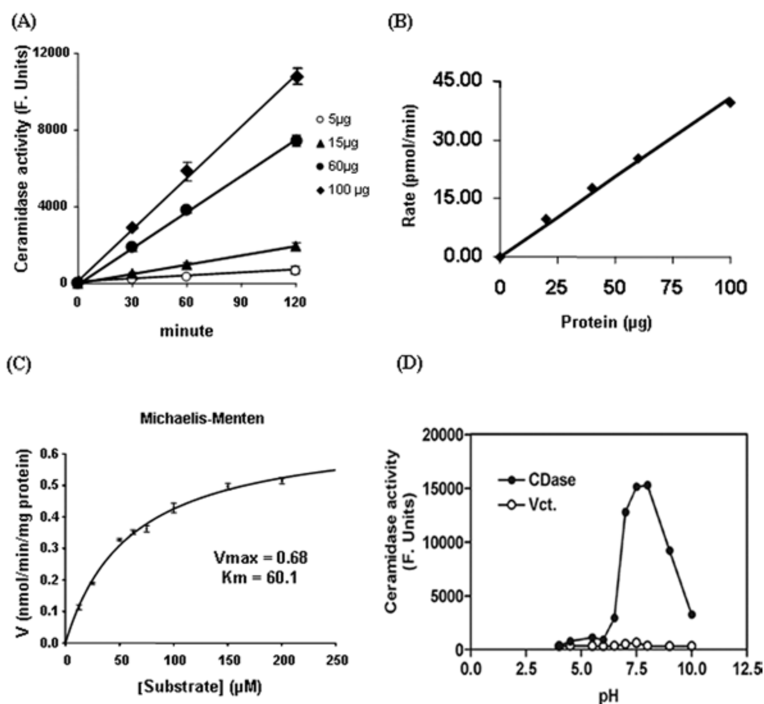

Figure 2 Biochemical characterization of the human neutral CDase expressed in yeast

(**A**) The effect of different amounts of yeast protein on CDase activity at different times. (**B**) Different amounts of human neutral CDase were assayed by incubation at 37 °C for 1 h as described in the Experimental section. For (**C**) and (**D**), 60 μg of yeast microsomal protein was used for CDase activity assay and the reaction time was 1 h. (**C**) Michaelis–Menten representations for CDase activity toward increasing concentrations of NBD-C_{12}-ceramide. (**D**) pH optimum determination. The buffers used are described in the Experimental section. The results shown are representative of triplicates from at least two independent experiments. Abbreviation: F. Units, arbitrary fluorescence units.

to characterize the human neutral CDase, we cloned the full-length cDNA, which is identical with the neutral CDase sequence reported recently [35]. The cDNA was cloned into the vector pYES2 under the control of the *Gal1* inducible promoter. The vector pYES2 and the new construct were transformed into the yeast double-knockout strain *Δypc1Δydc1*, which lacks the yeast CDases YDC1p and YPC1p, hence providing an environment for the expression of the human neutral CDase without any

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interference from endogenous enzymes with similar activities. Gene expression was induced by growth in galactose medium, and at different time points the cells were collected and homogenates were assayed for CDase activity using D-*erythro*-C_{12}-NBD-ceramide as substrate. Figure 1 demonstrates that, following induction with galactose, CDase activity became detectable at about 6 h, peaking at 8–10 h, and decreasing thereafter. It is noteworthy that no CDase activity was detectable after induction of the control yeast containing the vector alone. We thus selected 8–10 h as the appropriate induction time for enzyme expression in our further studies. This expression in yeast thus afforded a controlled system for studying mammalian neutral CDases.

Next, the biochemical properties of neutral CDase were examined. Figures 2(A) and 2(B) show the effect of different amounts of yeast protein on CDase activity at different times, indicating the linearity of the reaction during the first 60 min. Figure 2(C) demonstrates that the enzyme exhibited classical Michaelis–Menten kinetics, and with D-*erythro*-C_{12}-NBD-ceramide as substrate, the enzyme displayed an apparent K_m of $60.1 \pm 4.5\ \mu$M and an apparent V_{max} of 0.68 ± 0.15 nmol/min per mg of protein. The human neutral CDase showed a rather broad pH range, with an optimum activity at pH 7.5 (Figure 2D). Moreover, there was no detectable background CDase activity over this broad pH range. Activity was enhanced by increasing NaCl concentration (Figure 3A). Figure 3(B) illustrates that Ca^{2+}, Mg^{2+} and Mn^{2+} somewhat stimulated, and Zn^{2+}, Cu^{2+} and Fe^{2+} inhibited, CDase activity.

The effect of reducing agents upon CDase activity was investigated and DTT (dithiothreitol) dose-dependently inhibited neutral CDase activity (Figure 3C). Although 2-ME (2-mercaptoethanol) at these concentrations was not as effective as DTT, at higher concentration it inhibited the CDase activity to the same extent as DTT (Figure 3C). Therefore the human enzyme, expressed in yeast, exhibited biochemical properties almost identical with those of the purified rat brain enzyme. This concordance in properties also suggested the operation of similar catalytic mechanisms.

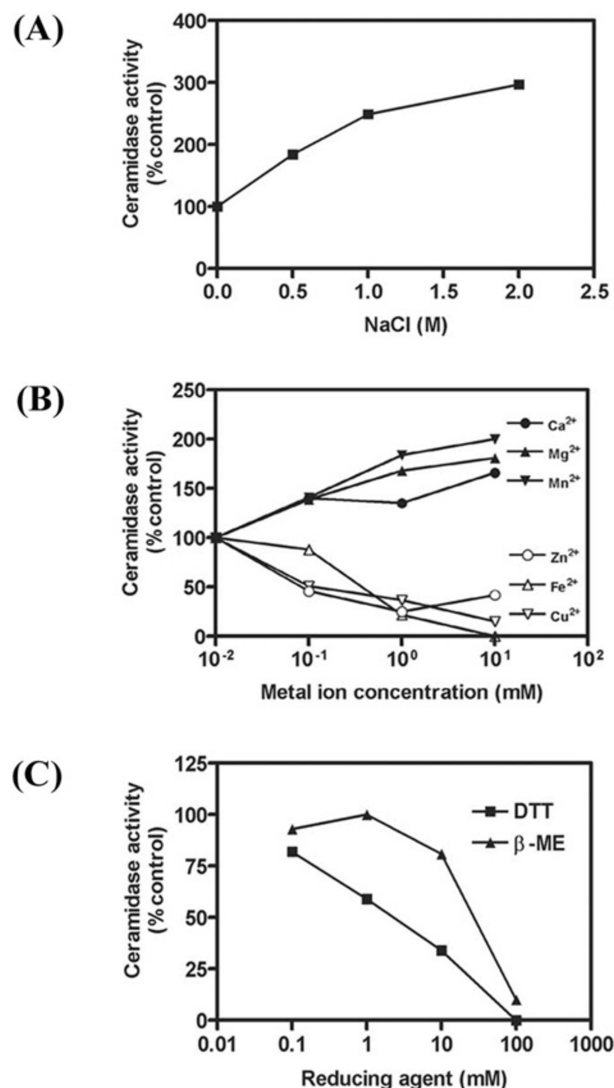

Figure 3 Effects of cations and reducing agents on human neutral CDase expressed in yeast

CDase assays were carried out at 37 °C and for 1 h as described in the Experimental section. (**A**, **B**) Effect of increasing concentrations of the indicated cations on CDase activity. (**C**) Effect of increasing concentrations of reducing agents on CDase activity. Values are the means from two independent experiments. β-ME, 2-ME.

Investigation of the catalytic mechanism of neutral CDase

CDases act by hydrolysing the amide bond between the fatty acid and the sphingoid-base backbone of ceramides. Therefore they are likely to be similar to proteases in their catalytic properties, where the attack is on the peptide bond (an amide bond). With this proviso, and in order to determine the catalytic mechanism of this enzyme, the effects of protease inhibitors on CDase were investigated at their maximal inhibitory range. As seen in Figure 4(A), neither aprotinin nor PMSF, which are known serine-protease inhibitors, at either their usual maximal concentration range or at a concentration 10 times higher than that, affected CDase activity, and neither did leupeptin, a cysteine-protease inhibitor, nor pepstatin, an aspartate-protease inhibitor. Thus none of the protease inhibitors tested exerted inhibitory effects on CDase activity.

The hydrolytic action of amidases and proteases has a requirement for a nucleophilic attack at the amide carbonyl atom. This, by analogy, is likely to be the case in the neutral CDase. To investigate this hypothesis, we acquired 12 μg of purified neutral CDase from 55 g of rat brain, and a number of different amino acid side-chain modifiers were evaluated for their effects on the enzyme. Figure 4(B) shows that DFP dose-dependently inhibited neutral CDase activity. Indeed, at 100 μM, DFP decreased CDase activity to 25 % of the control value. This result suggested the presence of a serine nucleophile at the catalytic centre of the enzyme. NEM, a thiol-functional-group modifier that has been used for cysteine side-chain modification, was ineffective towards CDase activity when used at the reported optimum concentration (Figure 4C) [34]. Similarly, DEPC, which is a well-established mild esterifying agent used to inhibit hydrolases, did not inhibit CDase activity when used at previously reported effective concentrations [36].

Inhibition by DFP of neutral CDase activity was further evaluated (in order to implicate serine in catalysis) by examining whether substrate interaction gives protection from the action of DFP. As showed by Figure 4(D), DFP inhibited neutral CDase activity by more than 50 %. However, after pre-incubation of the yeast lysates with the substrate ceramide, CDase activities were similar in both DFP-treated and untreated samples (Figure 4E). These results suggest that DFP and ceramide compete for access at the DFP site of action. Taken together, these results suggested a serine residue as an important one in catalysis.

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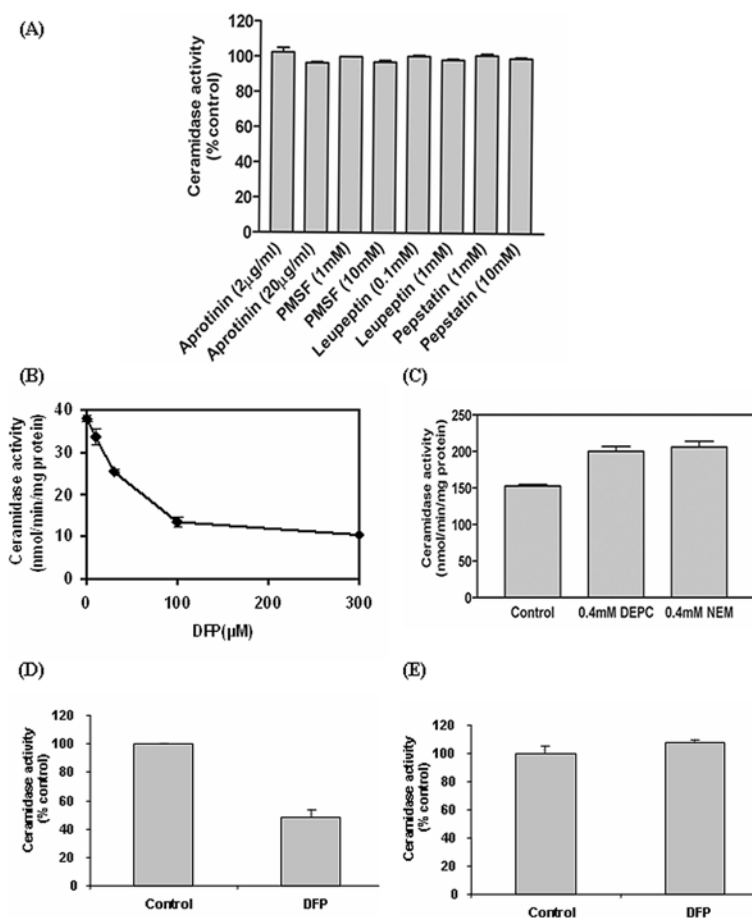

Figure 4 Effects of different protease inhibitors and chemical modifiers on CDase activity

(**A**) The CDase assay was carried out in the presence of different protease inhibitors at the indicated concentrations for 1 h at 37 °C. (**B**–**E**) Chemical-modification reactions were carried out as described in the Experimental section. Control reactions contained no modifiers, but did contain enzyme. (**B**–**C**) Purified rat brain CDase enzyme was used for the assays: (**B**) effect of increasing concentration of DFP upon purified CDase activity; (**C**) effect of DEPC and NEM upon purified CDase activity. (**D**–**E**) Effect of pre-incubation of ceramide on the DFP inhibition of neutral CDase. The human neutral CDase was expressed in yeast and prepared as described in the Experimental section: (**D**) yeast lysates were preincubated with or without 100 μM DFP in 100 mM phosphate buffer, pH 7.5, at 37 °C for 15 min. (**E**) Yeast lysates were first preincubated with (500 μM) D-*erythro*-C_{12}-NBD-ceramide for 30 min on ice, and then incubated with or without 100 μM DFP for another 15 min at 37 °C. The values are expressed as means ± S.D. ($n = 3$). The results are representative of triplicates from two independent experiments.

Sequence alignment and serine mutations by site-directed mutagenesis

Having identified the involvement of a critical serine residue in human neutral CDase, further studies were conducted to establish the role of serine residues and to identify which particular serine residue is involved. By using the ClustalW algorithm and the Vector-NTI software, the sequences of the homologues of neutral CDases from different species were aligned. From this, all conserved serine residues were selected for site-directed mutagenesis, as shown in Figure 5. The aligned serine residues shown in Figure 5 cover the entire sequence of the protein and are not clustered at one region. Different PCR primers were used (listed in Table 1) to generate neutral CDase PCR products, with each containing a specific serine-to-alanine mutation. The PCR products were then cloned into pYES2 vector and the *Δypc1Δydc1* yeast strain was transformed with these constructs. Genes were induced and CDase was prepared and assayed as described above.

Figure 6(A) shows the activity of the serine mutants starting at the N-terminal residue S^{258} up to S^{729} at the C-terminus of CDase, as well as the wild-type enzyme. Activities are expressed as relative to that of the wild-type enzyme. As can be seen, out of the six aligned serine residues that were mutated to alanine, only the S354A mutant lost its activity totally. Of the other mutants, S258A CDase activity was most affected, whereas S396A was the least affected. It is important to note that the differences in catalytic activity were not due to differences in expression levels of these mutants compared with wild-type human neutral CDase, as can be seen in Figure 6(D), which shows a Western-blot analysis of the expression level of the wild-type enzyme as well as the various mutants. The attenuation of activity in some of the other serine mutants could be the result of several factors, but the residual robust activity rules out essential roles in catalysis. Thus only Ser^{354} emerged as a candidate catalytically critical residue.

Identification of a conserved catalytically important motif

By focusing more closely on the alignment of the neutral CDases from different species in Figure 5, it was noted that Ser^{354}, which when mutated totally lost neutral CDase activity, falls within a very highly conserved hexapeptide sequence GDVSPN, which itself was in the middle of a larger conserved sequence, namely NXGDVSPNXXG^{P}/$_{X}$XC. This highly conserved sequence could indeed belong to the CDase active site, since it contains the serine nucleophile at its centre. To investigate this possibility, we mutated

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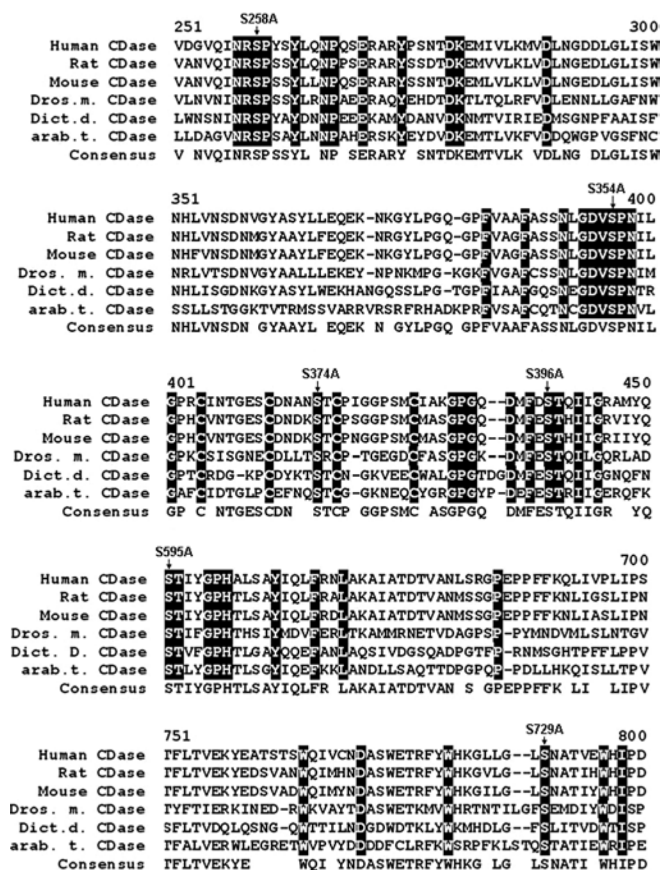

Figure 5 Alignment of neutral CDase from different species

Using the ClustalW algorithm, neutral CDases from different species were aligned and the indicated serine residues were mutated as described in the Experimental section. Identical residues are boxed in black; amino acid numbers of the consensus sequence are shown above the alignment. GenBank accession numbers for neutral CDase are as follows: AF449759 (human), AB057433 (rat), AB037181 (mouse), U82513 (the cellular slime mould *Dictyostelium discoideum*, Dict. d.), AB112076 (*Drosophila melanogaster*, Dros. m.), AB016885 [*Arabidopsis thaliana*, arab. t. (thale cress)].

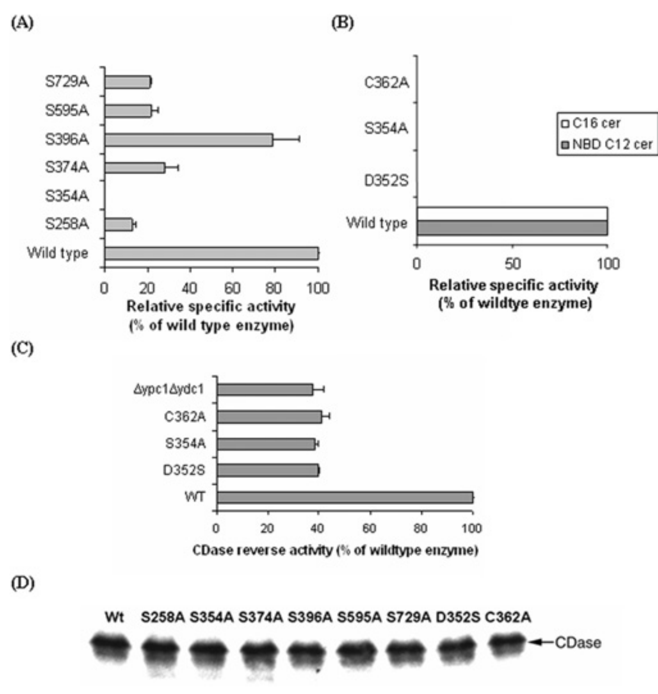

Figure 6 Identification of the hexaresidue core containing a proposed serine nucleophile in neutral CDases

Mutagenesis of CDase to alter individual residues to other amino acids were performed by PCR of wild-type neutral CDase. The PCR products were sequenced, cloned into pYES2 vector, and then transformed into the yeast double-knockout strain *Δypc1Δydc1*. Wild-type and mutant neutral CDase proteins were induced by galactose, and yeast microsomal lysates were subjected to CDase activity, reverse CDase activity and Western-blot assays as described in the Experimental section. (**A**) Effects of mutations in different conserved serines on neutral CDase activity. (**B**) Effect of mutations of on neutral CDase activity using NBD-C_{12}-ceramide or -C_{16}-ceramide as substrate. (**C**) Effect of mutations on reverse CDase activity. (**D**) A representative Western blot showing the expression level of CDase wild-type and mutant proteins. Values are means ± S.D. ($n = 3$). results are representative of triplicates from two independent experiments.

Asp^{352} and Cys^{362} in the consensus catalytic pentapeptide and generated D352S and C362A individually. The primers shown in Table 1 were used to generate neutral CDase PCR products containing D352S and C362A. The PCR products were then cloned into pYES2 vector, and the *Δypc1Δydc1* yeast strain was transformed with these constructs. Genes were induced, and the CDase enzyme was prepared and assayed as described above. The results showed that these two mutations, though having no effect on the expression levels of neutral CDase (Figure 6D), resulted in a profound loss in activity of neutral CDase activity, as can be seen in Figure 6(B). This implied that the acid side chain of aspartate is required, since replacement of Asp^{352} by a serine residue led to a complete loss of enzyme activity. In addition, the results show that Cys^{362} is also required for enzyme activity, since C362A caused a total loss of neutral CDase activity. We further tested the effects of mutations on reverse CDase activity (Figure 6C). Expression of Asp^{352}, Ser^{354} and Cys^{362} neutral CDase did not exhibit reverse CDase activity over that of the background strain *Δypc1Δydc1*, whereas expression of wild-type neutral CDase actually caused a measurable and significant increase in reverse activity. Thus this motif also seems to play an important role in the reverse CDase activity.

DISCUSSION

In the present study, by using chemical modifications of the purified enzyme, site-directed mutagenesis and sequence alignments, we have identified a novel amidase sequence containing the proposed nucleophile suggested to be responsible for the hydrolytic attack on the amide bond present in ceramide. This novel amidase motif is comprised of a hexapeptide residue core, namely GDVSPN, which is highly conserved among the different neutral CDases. This motif could indeed be the CDase active site, since it contains the serine nucleophile at its centre.

Interestingly, a recent study showed that the *Drosophila slab* gene encodes for neutral CDase. Mutant *slab* exhibits synaptic dysfunction, and the *slab1* mutation results in a single point mutation causing the replacement of Val^{264} with a methionine residue [23]. It is noteworthy that Val^{264} in *Drosophila* CDase is aligned with Val^{342} in human neutral CDase and in the vicinity of the GDVSPN motif.

The possibility that Ser^{354} may function as a nucleophile at the catalytic centre of neutral CDase is supported by multiple lines of evidence, including the present findings. First, the serine-modifying agent DFP dose-dependently inhibited the purified neutral CDase enzyme activity, but cysteine and histidine modifiers were ineffective against CDase activity (although lack of activity of these agents does not rule out participation of cysteine or histidine residues in enzyme action). Secondly,

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pre-incubation with ceramide substrate prevented the action of DFP, demonstrating action of DFP at the substrate-interaction site. Thirdly, using the ClustalW algorithm, the homologues of neutral CDases from different species were aligned, and the aligned serine residues were mutated. When the neutral CDase activity of these mutants were measured, only Ser^{354} lost its activity totally, thus identifying this specific serine residue as the nucleophile. Mutations of the other serine residues, though modulating activity, did not result in an abrogation of total activity, which is a necessary feature for any catalytically critical residue.

In addition, mutation of Asp^{352} and Cys^{362} also totally abrogated activity. This implies that the acid side chain of aspartate is required since replacement of Asp^{352} with a serine residue led to a complete loss of enzyme activity. In addition, Cys^{362} might be also required for enzyme activity, since the C362A mutant was totally without neutral CDase activity. Reducing agents dose-dependently inhibited neutral CDase activity, but NEM did not have any inhibitory effect. This raises the possibility that Cys^{362} may be involved in disulphide-bridge formation since the presence of reducing agents would disrupt such bridges. However, the results do not rule out other roles for this cysteine residue in catalysis. In either case, the mutational studies underscore the significance of the conserved amino acids in this hexapeptide motif.

Neutral CDases act by attacking the amide bond between the fatty acid and the sphingoid base in ceramide; therefore one would envisage that their mechanism of action might be similar to other amidases and that the nucleophile responsible for attack on the amide bond may be similar. However, neutral CDases do not align with known amidases, nor do they appear to contain the fatty amidase signature sequence $GSSG(X)_{14}GXD(X)_3\mathbf{S}(X)_3PX$, found in FAAH and other amidases, including some proteases [26–28], which is comprised of long stretches between the nucleophilic serine residue (shown in bold) and other residues. Indeed, neutral CDases do not show any sequence similarity to other enzymes, including acid and alkaline CDases [15], implying that neutral CDases define a unique enzyme family with a distinct enzymatic mechanism involving the hexapeptide amidase motif.

FAAH is a mammalian integral membrane enzyme responsible for the catabolism of the fatty amide family of endogenous signalling lipids such as the endocannabinoid anandamide. FAAH is the only characterized mammalian member of the amidase signature superfamily of serine hydrolases [37]. Two distinguishing features of FAAH, relative to other amidase-signature-family enzymes, are its integration into membranes and its strong preference for hydrophobic substrates – two properties that are similar to those of neutral CDase. Yet there are no similarities to the neutral CDase amidase motif in FAAH, and nor do potent inhibitors of FAAH inhibit the neutral CDase activity (results not shown).

The other enzyme family to which neutral CDase might have similarity in mechanism is the serine proteases. Almost a third of all proteases can be classified as serine proteases, classified because of the presence of a nucleophilic serine residue at the active site. This family of proteases is well-characterized, with ongoing insight being gained into the different mechanisms by which these enzymes employ serine as a nucleophile to hydrolyse a peptide bond (see [38] for a review). In the present study, serine-protease inhibitors, used at up to 10-fold their effective concentration, were unable to inhibit neutral CDase activity. Moreover, we did not find any sequence alignment within the serine proteases that aligned with the neutral CDase amidase motif. Classically it has been established that serine proteases rely upon the presence of a catalytic triad, consisting of an acidic and a basic residue (usually aspartic acid/histidine) in addition to the serine nucleophile, at their active site. Recent developments have shown that serine proteases can function without the landmark Ser-His-Asp catalytic triad. It has been proposed that catalysis is carried out in these proteases by a Lys-Ser dyad [38]. Indeed other variations on the classical catalytic-triad theme also exist. For instance, penicillin acylase, although not a protease, hydrolyses an amide bond in substrates such as penicillin G, and it seems to use just one residue, the N-terminal serine residue, as its nucleophile [39]. Taken together, our findings imply that the neutral CDase motif is distinct from that of serine proteases and, therefore, indicate that the hexapeptide consensus sequence described here is indeed a novel amidase motif.

In summary, these results identify a highly conserved hexa-residue core as a new amidase sequence containing a highly conserved serine residue, which may function as a nucleophile responsible for the hydrolytic attack on the amide bond present in ceramide.

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