Molecular Cloning of the Full-length cDNA Encoding Mouse Neutral Ceramidase

A NOVEL BUT HIGHLY CONSERVED GENE FAMILY OF NEUTRAL/ALKALINE CERAMIDASES*

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We report here the molecular cloning, sequencing, and expression of the gene encoding the mouse neutral ceramidase, which has been proposed to function in sphingolipid signaling. A full-length cDNA encoding the neutral ceramidase was cloned from a cDNA library of mouse liver using the partial amino acid sequences of the purified mouse liver ceramidase. The open reading frame of 2,268 nucleotides encoded a polypeptide of 756 amino acids having nine putative N-glycosylation sites. Northern blot analysis revealed that the mRNA of this ceramidase was expressed widely in mouse tissues, with especially strong signals found in the liver and kidney. The ceramidase activity of lysates of CHOP cells increased more than 900-fold when the cells were transformed with a plasmid containing the cDNA encoding ceramidase. We also cloned the ceramidase homologue from the cDNA library of mouse brain and found that the sequence of the open reading frame, but not the 5'-noncoding region, was identical to that of the liver. Interestingly, phylogenetic analysis of various ceramidases clearly indicated that neutral/alkaline ceramidases form a novel but highly conserved gene family that is evolutionarily different from lysosomal acid ceramidases.

Over the past decade, ceramide (Cer), sphingosine (Sph), and Sph 1-phosphate have emerged as a new class of lipid biomodulators of various cell functions (1–3). Sph has been shown to inhibit the activities of several protein kinases including protein kinase C (4) and calcium/calmodulin-dependent protein kinases (5). Cer and Sph induce apoptosis in several cell lines (6, 7). In contrast, Sph 1-phosphate, which is produced by neutral CDase and CDase with activity at neutral to alkaline pH; acid CDases and neutral/alkaline CDases (14). A genetic deficiency of acid CDase causes Faber disease, in which Cer is accumulated in lysosomes (15). Recently, an acid CDase was purified from human urine (16), and the cDNA clone has been isolated from a cDNA library of human fibroblast (17) and mouse brain (18). CDase with activity at neutral to alkaline pH has been proposed to function in signal transduction pathways to produce Sph and might be Sph 1-phosphate. Actually, several lines of evidence indicate that neutral/alkaline CDases regulate the cell proliferation induced by growth factors (19) and the cytotoxic P450 2C11 expression by interleukin-1β (20). However, the precise metabolic and biological roles of neutral/alkaline CDases of eukaryotes are not entirely clear, since these enzymes have not yet been clarified at the molecular level. On the other hand, an alkaline CDase of prokaryotes was purified from Pseudomonas aeruginosa (21), and the gene encoding the enzyme was cloned (22). This bacterial CDase was proposed to be a possible cause of Cer deficiency in atopic dermatitis (23).

Recently, we purified a novel neutral CDase from the membrane fractions of mouse liver (24). The final preparation showed a single protein band corresponding to a molecular mass of 94 kDa on SDS-polyacrylamide gel electrophoresis. This CDase seems to be a glycoprotein with N-glycans (24). Nonlysosomal CDase with a neutral to alkaline pH optimum was also purified from the rat brain (25).

This paper describes the molecular cloning, sequencing, and expression of the gene encoding the neutral CDase of mouse liver and brain and clearly indicates the presence of a novel gene family of neutral/alkaline CDases whose genetic information, which is clearly distinguished from that of acid CDases, is evolutionary conserved in organisms from bacteria to mammals.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB037181 (mouse brain) and AB037111 (mouse liver).

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§ The abbreviations used are: Cer, ceramide; Sph, sphingosine; CTDase, ceramidase; HPLC, high performance liquid chromatography; NBD, nitrobenzo-2-oxa-1,3-diazole; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s).

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Fig. 1. Nucleotide and predicted amino acid sequences (A) and hydrophobicity plot (B) of the mouse neutral CDase. A, the deduced amino acid sequence of the CDase is shown in one-letter symbols below the nucleotide sequence. Amino acids determined by peptide sequencing are underlined. Amino acid residues are numbered beginning with the first methionine, and the translation termination codon is denoted by an asterisk.

Numbers to the right of the sequence correspond to amino acids (lower) and nucleotides (upper). Possible sites of phosphorylation by...
reagents were of the highest purity available.

**Amino Acid Microsequencing—**Neutral CDase was purified from the membrane fraction of mouse liver, and its amino acid sequences were determined after digestion with Lys-C as described previously (24).

**Molecular Cloning and DNA Sequencing—**General cloning techniques were essentially as described by Sambrook et al. (25). Nucleotide sequences were determined by the dideoxynucleotide chain termination method with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) and a DNA Sequencer (model 373A, PE Biosystems).

**PCR Amplification—**PCR with degenerate oligonucleotides was used to amplify a DNA fragment encoding the CDase. Sense, and antisense oligonucleotide primers were designed using the internal amino acid sequences of the Lys-C digestion product of the purified mouse CDase (C-53, GYLPQGGPVPAGASSNLGVDSPNILPXVV(N/T)GE). PCR using the sense primers (53-S1, 5'-CARGGGNCNTTYYGTNCG-3') and the antisense primers (53-A3, 5'-GGGNCNAADATTNTNGG-3') was performed with a cDNA library from mouse liver as a template in a GeneAmp PCR System 2400 (PE Biosystems) for 40 cycles (each consisting of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and extension at 72 °C for 30 s) using AmpliTaq Gold (PE Biosystems). An amplified 68-bp PCR product was subcloned into the pGEM T-easy vector (Promega), and its DNA sequence was determined. Two anti-sense primers (MA2, 5'-GGTGGACAGCTCTCCAGATG-3'; MA1, 5'-TGTGATTGAGCAAGCTTCTCCTG-3') were synthesized using the sequence of the obtained 68-bp PCR product. Sense (TOut, 5'-TCTGCTCTA-AAGATGCCG-3'; TIn, 5'-TTAATACTAGCTATAGGACG-3') primers were designed using the sequence of pAP3neo vector. The first PCR using the sense (TOut) and the antisense (MA2) primers was performed with a cDNA library from mouse liver as a template for 40 cycles (each consisting of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and extension at 72 °C for 2 min). The second PCR using the sense (MA1) and the antisense (TIn) primers was performed with the 1st PCR product as a template for 40 cycles (each consisting of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and extension at 72 °C for 2 min). Finally, a 332-bp PCR product containing the CDase sequence was obtained, and the DNA sequence was determined.

**Isolation of a cDNA Clone Encoding CDase—**A clone containing full-length cDNA encoding CDase was isolated from a cDNA library of mouse liver by colony hybridization using a 332-bp PCR product as a probe. The probe was labeled with [32P]dCTP using a Ready-To-Go DNA labeling kit (Amersham Pharmacia Biotech). Colony hybridization was performed according to the standard procedure (26). Finally, a clone encoding mouse neutral CDase was isolated, and the plasmid in the clone was designated pAPLCD. A cDNA clone encoding mouse brain CDase was also obtained from a cDNA library of mouse brain by colony hybridization using a 2.7-kb EcoRI fragment of pAPLCD insert as a probe. The probe was labeled with [32P]dCTP using a Ready-To-Go DNA labeling kit (Amersham Pharmacia Biotech).

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**Transient Expression of CDase in CHOP Cells—**CHOP cells (3 x 10⁶ cells/well), Chinese hamster ovary cells that express polyoma LT anti-tyrosine kinase (A), protein kinase C (β), and casein kinase II (γ) are indicated. The putative N-glycosylation (■) and N-myristoylation (▲) sites are indicated, respectively. A hydrophobic motif is shown by boldface letters. B, the deduced amino acid sequence of the CDase was analyzed by the method of Kyte and Doolittle (35) for hydrophobicity plotting. Amino acid residues are numbered beginning with the first methionine.
ured using C$_{12}$-NBD-Cer as a substrate at pH 7.5. The activity of CDase in untransfected CHOP cells and in mock-transfectants was about 20 microunits/mg of protein, while that in cells transfected with pAPLCD was 19,500 microunits/mg at 24 h after transfection, which corresponds to a 970-fold increase in comparison with mock transfectants (Table I).

Expression of Neutral CDase in Mouse Tissues—The distribution of CDase mRNA in adult mouse tissues was analyzed by Northern blotting (Fig. 2). In all tissues tested, a predominant 6.0-kb mRNA was detected, indicating that the neutral CDase is expressed widely in mouse tissues. However, the mRNA expression level differed somewhat among the tissues tested. Strong signals were observed in kidney and liver (Fig. 2B). This result is well correlated with the enzymatic activity in each of the tissues tested using C$_{12}$-NBD-Cer as a substrate at pH 7.5 (24).

Expression of Neutral CDase in Mouse Brain—Bawab et al. (25) reported the purification of a nonlysosomal 90-kDa CDase exhibiting a broad pH optimum (pH 7–10) in rat brain, while the present study showed the expression of neutral CDase in mouse brain by Northern blotting analysis (Fig. 2). Thus, we performed cDNA cloning of the CDase homologue from a cDNA library of mouse brain by colony hybridization using a 2.7-kb EcoRI fragment of pAPLCD as a probe. As a result, one clone was selected, and the plasmid in the clone was designated pSBCD, which consisted of a 440-bp 5'-untranslated sequence, a 2,268-bp open reading frame, 2,107-bp 3'-untranslated sequence, and a 20-bp poly(A).

FIG. 2. Northern blot analysis of mouse neutral CDase. A, poly(A)$^+$ RNA blotting membrane of multiple mouse adult tissues was hybridized with the EcoRI fragment of the pAPLCD insert. Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. B, the relative levels of CDase mRNA expression of various tissues were calculated after taking into account variations in the amount of RNA in each lane, as revealed by hybridization with the β-actin probe.

FIG. 3. Alignment of deduced amino acid sequences of mouse neutral CDases, Pseudomonas alkaline CDase, and CDase homologues of M. tuberculosis, D. discoideum, and A. thaliana. Sequences of CDases were aligned using the CLUSTAL algorithm (31). Amino acids identical to mouse CDase are indicated by white type on black background. Gaps inserted into the sequences are indicated by dots.
The amino acid sequence in the open reading frame of pSBCD is identical to that of liver CDase (Fig. 3). It was confirmed that pSBCD actually encoded the neutral CDase, since the definitive CDase activity was detected in the cell lysates of CHOP cells after infection with the pSBCD when the activity was measured using C2-NBD-Cer as a substrate at pH 7.5 (data not shown). Interestingly, however, the sequence of the 5′-noncoding region of the brain CDase is somewhat different from that of liver CDase (data not shown), suggesting that the expression of both CDase genes is regulated by different mechanisms.

Homology of the Deduced Amino Acid Sequence of Mouse Neutral CDase with Those of CDase Homologues and Acid CDases—Fig. 3 shows the alignment of the deduced amino acid sequence of mouse neutral CDase with those of alkaline CDases of *P. aeruginosa* (GenBank™ accession no. AB028646) (22) and *Mycobacterium tuberculosis* (Z95972) (22) and CDase homologues of *Dictyostelium discoideum* (U82513) and *Arabidopsis thaliana* (AB016885). Very recently, we cloned and expressed the *D. discoideum* ceramidase cDNA.2 The similarity in amino acid sequence of neutral/alkaline CDase homologues and acid CDases to mouse neutral CDase was analyzed using CLUSTAL W software (31). It was revealed that mouse neutral CDase exhibited identities of 33.1% for *P. aeruginosa*, 28.5% for *M. tuberculosis*, 38.3% for *D. discoideum*, and 33.7% for *A. thaliana* but no significant similarities for acid CDases of *M. tuberculosis*, 28.5% for *P. aeruginosa* (21) and *A. thaliana* (22) and CDase homologues of *Dictyostelium discoideum* (U82513) and *Arabidopsis thaliana* (AB016885). Very recently, we cloned and expressed the *D. discoideum* ceramidase cDNA.2 The similarity in amino acid sequence of neutral/alkaline CDase homologues and acid CDases to mouse neutral CDase was analyzed using CLUSTAL W software (31). It was revealed that mouse neutral CDase exhibited identities of 33.1% for *P. aeruginosa*, 28.5% for *M. tuberculosis*, 38.3% for *D. discoideum*, and 33.7% for *A. thaliana* but no significant similarities for acid CDases of *M. tuberculosis*, 28.5% for *P. aeruginosa* (21) and *A. thaliana* (22) and CDase homologues of *Dictyostelium discoideum* (U82513) and *Arabidopsis thaliana* (AB016885). Very recently, we cloned and expressed the *D. discoideum* ceramidase cDNA.2

Since the finding of CDase activity in rat brain by Gatt (32), CDases have been found in various mammalian tissues (14), invertebrates (33), and bacteria (21). The primary criterion to distinguish and characterize the CDase isoenzymes are their catalytic pH optimum (14). This study, however, demonstrates for the first time that neutral/alkaline CDases could be distinguished from acid CDases not only by their optimal catalytic pH but by the primary structures of enzyme proteins. It is interesting that the genetic information of neutral/alkaline CDases is conserved in organisms from bacteria to mammals. In conclusion, our study clearly indicates the presence of a novel but highly conserved gene family that includes neutral and alkaline CDases but not acid CDases.

Acid CDase seems to degrade ceramides in lysosomes in the process of recycling membrane lipids through the endolysosomal pathway, whereas the precise metabolic and biological functions of neutral/alkaline CDases have not been fully elucidated. Recently, evidence has emerged to suggest that neutral/alkaline CDases are involved in the regulation of cell proliferation by production of Sph or decrease of Cer (19, 20, 34). Coroneos et al. (19) revealed that the membrane-associated neutral/alkaline CDase could be activated by several growth factors including platelet-derived growth factor but not cytokines, resulting in an increase of Sph with a consequent stimulation of cell proliferation. Genistein, an inhibitor of tyrosine kinase, canceled the activation of the CDase by platelet-derived growth factor, suggesting that the activation of the enzyme involved a tyrosine phosphorylation mechanism. The neutral CDase of rat hepatocytes was activated by interleukin-1β, and the activation also appeared to be regulated by tyrosine phosphorylation (20). It is noteworthy that several phosphorylation sites including a tyrosine-kinase phosphorylation site were found in the deduced amino acid sequence of the mouse neutral CDase (Fig. 1A).

This study reports the first isolation of a full-length cDNA encoding a neutral CDase of mammals. The cDNA encoding neutral/alkaline CDases will be useful for elucidation of the mechanisms by which the intracellular contents of Cer/Sph/Sph 1-phosphate are regulated in cells and how the balance of these sphingolipid metabolites affects cell activities and cell fate. In addition, cDNA sequences of neutral/alkaline CDases reported here could enable one to isolate novel CDase homologues and to generate model animals/plants in which neutral/alkaline CDases are knocked out or overexpressed. The availability of such information should help us to define the possible roles of neutral/alkaline CDases in sphingolipid-mediated signaling and functions.

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Conserved Gene Family of Neutral/Alkaline CDases

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