Molecular Cloning and Characterization of a Human Mitochondrial Ceramidase*

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We have recently purified a rat brain membrane-bound nonlysosomal ceramidase (El Bawab, S., Bielawska, A., and Y. A. Hannun (1999) J. Biol. Chem. 274, 27948–27955). Using peptide sequences obtained from the purified rat brain enzyme, we report here the cloning of the human isoform. The deduced amino acid sequence of the protein did not show any similarity with proteins of known function but was homologous to three putative proteins from Arabidopsis thaliana, Mycobacterium tuberculosis, and Dictyostelium discoideum. Several blocks of amino acids were highly conserved in all of these proteins. Analysis of the protein sequence revealed the presence at the N terminus of a signal peptide followed by a putative myristoylation site and a putative mitochondrial targeting sequence. The predicted molecular mass was 84 kDa, and the isoelectric point was 6.69, in agreement with rat brain purified enzyme. Northern blot analysis of multiple human tissues showed the presence of a major band corresponding to a size of 3.5 kilobase. Analysis of this major band on the blot indicated that the enzyme is ubiquitously expressed with higher levels in kidney, skeletal muscle, and heart. The enzyme was then overexpressed in HEK 293 and MCF7 cells using the pcDNA3.1/His-ceramidase construct, and ceramidase activity (at pH 9.5) increased by 50- and 12-fold, respectively. Next, the enzyme was characterized using lysate of overexpressing cells. The results confirmed that the enzyme catalyzes the hydrolysis of ceramide in the neutral alkaline range and is independent of cations. Finally, a green fluorescent protein-ceramidase fusion protein was constructed to investigate the localization of this enzyme. The results showed that the green fluorescent protein-ceramidase fusion protein presented a mitochondrial localization pattern and colocalized with mitochondrial specific probes. These results demonstrate that this novel ceramidase is a mitochondrial enzyme, and they suggest the existence of a topologically restricted pathways of sphingolipid metabolism.

The lipid mediator ceramide has been suggested to play a critical role in cell growth, differentiation, and apoptosis (1, 2). Several mechanisms are involved in the regulation of cellular ceramide levels, which include activation of sphingomyelinas, activation of the de novo synthetic pathway, and inhibition of ceramidas (CDase).1 Ceramidas hydrolyze ceramide to form sphingosine, which in turn can serve as a substrate for sphingosine kinase, resulting in the formation of sphingosine-1-phosphate. Ample evidence suggests distinct functions for these sphingolipids (1).

Recent studies are also beginning to suggest a role for ceramidas in regulating the net levels of ceramide in response to stimuli. For example, it has been shown in rat hepatocytes that interleukin 1β at low concentration activates sphingomyelinas and ceramidas, resulting in the formation of sphingosine, whereas high concentrations of interleukin-1β, stimulated only sphingomyelinas resulting in the accumulation of ceramide (3). In rat renal mesangial cells, both tumor necrosis factor α and nitric oxide donors have been shown to stimulate sphingomyelinas, but only nitric oxide donors inhibited ceramidas and resulted in an increase in ceramide levels and the consequent biological effects (4). Also, in smooth muscle cells, oxidized low density lipoprotein has been shown to stimulate sphingomyelinas, ceramidas, and sphingosine kinase, leading to the production of sphingosine-1-phosphate, which these authors suggested promotes the proliferation of these cells (5). Ceramidas have also been shown to be activated in response to platelet-derived growth factor in rat glomerular mesangial cells (6). These studies underscore a key role for ceramidas in regulating cell death and proliferation, in response to various stimuli and in different cell types. However, to date, there has been a paucity of molecular tools to study the function of ceramide and to understand the significance of nonlysosomal enzymes of ceramide metabolism.

We recently purified a rat brain membrane-bound ceramidase with a pH optimum in the neutral to alkaline range (7). In this study, we used peptides obtained from purified rat brain enzyme to clone the human isoform. We also demonstrate using a GEP-ceramidase construct that the enzyme is localized in mitochondria. These results demonstrate significant compartmentation of sphingolipid metabolism and raise important possibilities on direct interaction between ceramide and mitochondria.

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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) AF 250847.

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¶ The abbreviations used are: CDase, ceramidase; GEP, green fluorescent protein; RACE, rapid amplification of ends; TMRM, tetramethylrhodamine methylester; CAPS, 3-(cyclohexylamino)propanesulfonic acid; HPLC, high pressure liquid chromatography; EST, expressed sequence tag; PCR, polymerase chain reaction; kb, kilobase.
EXPERIMENTAL PROCEDURES

Materials

Three preparations of 100–120 rat brains each were used. The purified protein from the SDS-PAGE was subjected to SDS-polyacrylamide gel electrophoresis, the gel was stained directly with Coomassie Blue or transferred to nitrocellulose membrane using CAPS buffer, pH 11, as transfer buffer, and the membrane was then stained. The CDase band was excised from the gel or from the membrane and subjected to digestion using AspN. The digest mixture was separated by microcapillary reversed phase HPLC, and selected peptides were submitted to Edman degradation and sequencing.

Cloning of CDase—The sequences of the obtained peptides were used to search the data base of the GenBank™. The peptides identified a putative slug protein (accession no. 2367392) and all DNA sequences were obtained by Edman degradation and sequencing. The peptide sequences obtained from the purified rat brain enzyme were aligned to the peptide sequences deduced from the cloned mitochondrial ceramidase. Amino acids in bold show difference in sequence.

| Peptide | Sequence |
|---------|----------|
| 1 Rat   | QFGDVLQPQAPEYR |
| 2 Rat   | TFGDVLQPQAPEYR |
| Human   | KQELKLPVPVAPGQIS |
| 3 Rat   | KQELKLPVPVAPGQIS |
| Human   | KNKSYLPGGQPFPYAPA |

65 °C in ExpressHyb solution. The radioactive probe was then denatured by boiling for 2 min and added to the blot in ExpressHyb solution. Hybridization was carried out overnight at 65 °C. After washing, the blot was exposed to x-ray film for 5 days at –80 °C.

Transfection—HEK 293 cells and MCF-7 cells were seeded at 10^5 cells/dish. Transfection with vector alone (pcDNA3.1/HisC) or vector containing full-length CDase (pcDNA3.1/HisC-CDase) was performed using Superfect and 3 μg of each plasmid/dish. After 3–4 h of incubation with the mixture, the cells were washed with phosphate-buffered saline and fresh medium was added. After 48 h, CDase activity was measured.

Protein Assay and SDS-Polyacrylamide Gel Electrophoresis—Protein concentration was determined using the Bradford assay. SDS-polyacrylamide electrophoresis was performed according to Laemmli (10).

Western Blot—Cells were scraped in 1 ml of lysis buffer (50 mM Tris, pH 7.4, 5 mM EDTA, 1% Triton X-100, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/mL of leupeptin and aprotinin) and kept on ice for 10–15 min. To remove insoluble material, lysates were centrifuged at 12,000 × g for 15 min. Samples (10 μg of lysates) were then boiled for 5 min, loaded onto a 7.5% SDS-polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose membrane. The GFP-CDase fusion protein was detected by using anti-GFP affinity purified antibody at a dilution of 1:1000 and an anti-rabbit secondary antibody at a dilution of 1:3000.

Immunoprecipitation—For immunoprecipitation, cytosolic lysates were first precleared by incubating with 30 μl of a mixture of protein A/protein G agarose beads for 30 min followed by centrifugation at 12,000 × g for 1 min. The cleared lysates were then rocked in the presence of 5 μg of anti-GFP antibody or 5 μg of control IgG complexed to a mixture of protein A/protein G agarose beads for 2 h of incubation, the beads were centrifuged at 12,000 × g for 10 s, washed twice with 0.5 ml of lysis buffer without protease inhibitors and with only 0.1% Triton X-100 (wash buffer). All steps were carried out at 4 °C. Beads were finally resuspended in wash buffer, and ceramidase activity was measured.

Ceramidase Activity—Ceramidase activity was measured as described in Ref. 7, using [3H]C16-ceramide as substrate in a mixed micelle assay system.

Microscopy—Cells were plated on 35-mm diameter glass coverslips. They were transfected with 1 μg of empty vector or vector-containing ceramidase as described above. After 48 h, the cells were loaded with 25 nM Mitotracker Red for 20 min and then washed with phosphate-buffered saline and fixed. For confocal microscopy, images were collected by Zeiss 410 LSCM system equipped with krypton/Argon laser and a 60 X oil merge lens (N.A 1.4). After 48 h of transfection, cells plated on glass coverslips were mounted on a microscope stage and maintained in phosphate-buffered saline buffer. GFP images were collected by excitation at 488 nm and emission at 516–560 nm. To label mitochondria, cells were subsequently co-loaded with 50 nM TMRM. The TMRM images were then taken by excitation at 568 nm and emission at 590 nm long-path emission filter. To void fluorescent cross-talking, green GFP and red TMRM fluorescence were taken sequentially.

RESULTS

Sequenceing and Cloning of CDase—We have purified to homogeneity a rat brain CDase with a pH optimum in the neutral to alkaline range (7). The scale up of the purification protocol was optimized to obtain high amounts of the protein. In each preparation (100–120 rat brains), 1–10 μg of CDase protein were obtained (visible by Coomassie Blue). After digestion and HPLC separation of the AspN digest, three peptide sequences of 14–17 amino acids (Table I) were obtained. The data base of
The GenBank™ was searched using peptides 1 and 2, and the same human EST sequence (accession no. AA913512) was identified by both peptides. We then searched using the human EST and all three peptides and identified a putative slug protein (accession no. 2367392). The human EST aligned at the C-terminus of the slug protein. Next, the slug protein was used to search the GenBank™ database, and this yielded a human genomic sequence of 15,960 kb (accession no. AC012131), which aligned with a region close to the N-terminus of the slug protein. Thus, by performing this search and based on the slug putative protein sequence, human nucleotide sequences were obtained that were localized close to the N and C terminus of the human protein. Based on these observations, a forward primer from this human genomic sequence (no. AC012131) and a reverse primer from the human EST (no. AA913512) sequence were designed, and PCR was performed using a human kidney library as template. Gel analysis of the PCR reaction showed that a 1.8-kb fragment was amplified, in close agreement to what was expected based on the slug sequence. We isolated, subcloned, and sequenced this fragment and found that it contains the human EST (accession no. AA913512), indicating that this 1.8-kb fragment corresponds to part of the human CDase sequence.

New primers of both ends of the 1.8-kb human fragment were synthesized, and RACE-PCR was performed using a human kidney RACE library as template. After two rounds of PCR, a 5'-end fragment of 0.7 kb and a 3'-end fragment of 0.6 kb were obtained. These fragments were then gel-isolated, subcloned into TOPO vector, and sequenced. The fragments contained the primer sequences, part of the 1.8 kb-fragment, and did not identify any EST in the GenBank™ of known function, indicating that these fragments most probably correspond to the extension of the 5'-end and the 3'-end of the 1.8 kb-human fragment.

The 5'-end fragment contained multiple start ATG codons in frame, and the 3'-end contained two stop codons next to each other. Taking the first ATG (longest) as start codon and the double stop codon at the 3'-end as stop codon, an open reading frame of 2289 base pairs encoding a protein of 84 kDa was predicted.

Analysis of the protein sequence using the SMART program revealed one transmembrane domain between amino acids 505 and 525 (Fig. 1) and three other putative transmembrane domains (amino acids 176–196, 313–333, 431–451, and 543–563). The sequence also revealed the presence of a signal peptide (amino acids 1–19) and a region of low compositional complexity (amino acids 38–66). This region of low complexity showed some features of a mitochondrial targeting sequence, it was rich in amino acids serine and alanine, contained two positively charged amino acids (arginine and histidine), and did not contain acidic residues (11). Further analysis using the program PSORT at the Expasy Molecular Biology server showed that at a probability of 66% this peptide sequence would localize in mitochondria. Also, we identified a putative myristoylation site (Fig. 1), several putative phosphorylation sites (protein kinase C, cAMP-dependent protein kinase, and casein kinase 2) and putative N-glycosylation sites (not shown).

The CDase amino acid sequence showed no similarity to any...
known mammalian protein. The protein was homologous to three putative proteins from *Arabidopsis thaliana* (accession no. AAD32770), *Mycobacterium tuberculosis* (accession no. CAB09388), and *Dictyostelium discoideum* (accession no. 2367392) (Fig. 2), indicating that these proteins may be ceramidases in those organisms. There were several blocks highly conserved in all of these proteins, and the overall homology between the human and those proteins ranged between 30 and 50%.

**Northern Blot Analysis**—To determine tissue distribution of this ceramidase, we performed Northern blot analysis using the 3′-end of CDase cDNA (0.67 kb) as a probe and a human premade multitissue Northern blot. Fig. 3 shows the presence of a minor high size band at around 7 kb, a major band of 3.5 kb, and two other minor bands of 3.1 and 2.4 kb. The presence of multiple bands could be the result of alternative splicing. The major 3.5-kb ceramidase band was ubiquitously expressed in all tissue represented on the blot, with the highest expression in kidney, skeletal muscle, and heart.

**Overexpression and Characterization of CDase**—HEK 293 cells and MCF7 cells were transfected with empty vector (pcDNA3.1/HisC) or vector containing the full-length CDase (pcDNA3.1/HisC-CDase). Cells were then harvested, and ceramidase activity was measured on the lysates. As shown in Fig. 4A, overexpression of CDase in these cells increased CDase activity (at pH 9.5) 50-fold in HEK 293 cells and 12-fold in MCF7 cells as compared with control empty vector-transfected cells.

To ascertain that the cloned cDNA encodes ceramidase protein, we constructed a GFP-tagged ceramidase, in which the GFP was at the N terminus of ceramidase protein. We then transfected 293 cells with this construct and performed Western blot and immunoprecipitation experiments using GFP antibody. As shown in Fig. 4B, cells overexpressing the fusion protein contain a GFP-positive band at around 123 kDa, this band being absent in control cells transfected with the pEGFP-C3 empty vector. Based on GFP molecular mass (27 kDa), CDase molecular mass was deduced to be around 96 kDa. This was in agreement with 90 kDa mass on SDS-polyacrylamide gel electrophoresis of the rat brain purified enzyme. Further, immunoprecipitation of the fusion protein with anti-GFP antibody increased CDase specific activity by 8-fold in the immunoprecipitant, whereas control rabbit IgG failed to immunoprecipitate any activity. All together, these results clearly indicate that the cloned full-length cDNA encodes the CDase protein. Next, we compared the properties of this human enzyme to the rat brain enzyme. To this end, 293 cells were transfected with the pcDNA3.1/HisC-CDase construct, and characterization experiments were performed using lysates of these overexpressing cells. Fig. 4C shows the pH profile of the human CDase. The enzyme catalyzed the hydrolysis of ceramide in a relatively broad range with a pH optimum between pH 7.5 and 9.5. We also tested the effect of EDTA, MgCl₂, and CaCl₂ (all at 10 mM) and found that they did not affect significantly ceramidase activity. Dithiothreitol at 20 mM was found to inhibit the.
activity by 75% (Fig. 4D). Finally, the predicted isoelectric point value was 6.69. All these properties are in close agreement with the purified rat brain enzyme.

**Localization of Ceramidase**—Our previous results of tissue subfractionation, together with the putative mitochondrial targeting sequence suggested the possible localization of this ceramidase in mitochondria. To assess this hypothesis, we transfected MCF7 and HEK 293 cells with the GFP-tagged ceramidase construct. After transfection, cells were stained with Mitotracker Red, a specific mitochondrial probe. In MCF7 and HEK 293 cells, the GFP control signal (empty vector) was diffuse in all compartments (not shown) whereas the GFP-ceramidase signal colocalized with the red mitochondrial probe (Fig. 5A). To further confirm these observations, we performed similar experiments using confocal microscopy. Results in MCF7 and HEK 293 pEGFPC3-Cdase-transfected cells showed a punctuate mitochondrial pattern of the GFP-ceramidase signal (Fig. 5B). The addition of a TMRM mitochondrial probe showed that the ceramidase fusion protein signal colocalizes again with this mitochondrial probe (Fig. 5B), clearly demonstrating that this ceramidase is localized in mitochondria.

**DISCUSSION**

We have cloned and characterized the first mammalian mitochondrial ceramidase. The enzyme has characteristics similar to the rat brain purified enzyme in its estimated molecular mass, isoelectric point, optimum pH, and dependence on cations (7). Protein sequence analysis showed the enzyme is conserved in bacteria, plant, and mammals. While we were preparing this manuscript, Okino *et al.* (12) published the cloning of an alkaline ceramidase from *Pseudomonas aeruginosa* (accession no. 6594292), and Tani *et al.* (13) published the purification of the same protein from mouse liver. The sequence of this protein was also homologous to the *M. tuberculosis* GenBank™ putative protein. These observations indicate that our human clone and the *P. aeruginosa* clone encode the same enzyme.

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*S. El Bawab and Y. A. Hannun, unpublished results.*
In addition, recently Mao et al. (14) reported the cloning of an alkaline ceramidase from the yeast \textit{Saccharomyces cerevisiae}. Two lines of evidence suggest that this yeast enzyme is different from the human mitochondrial ceramidase. First, amino acid comparison showed no homology between the two proteins. Second, the yeast enzyme failed to hydrolyze C$_{16}$-ceramide but rather uses phytoceramide preferentially as a substrate. Further, the whole genome of \textit{S. cerevisiae} has been reported. Very interestingly we could not find any protein or DNA sequence from \textit{S. cerevisiae} homologous to the human, slug, or mycobacterium ceramidase.

It is very intriguing that lower organisms such as \textit{M. tuberculosis} and \textit{P. aeruginosa} harbor the mitochondrial ceramidase-specific gene in their genome, whereas the eukaryotic genome of \textit{S. cerevisiae} does not. It would be interesting to determine if this is related to the pathogenicity of \textit{P. aeruginosa} and \textit{M. tuberculosis}. It is also intriguing to know whether the yeast ceramidase gene is also found in other organisms. At present, the answer to these questions is not clear.

On the other hand, in their reports, Mao et al. (14) and Tani et al. (13) have shown that the yeast ceramidase and the purified mouse ceramidase can also catalyze the reverse reaction by condensing phytosphingosine or sphingosine and a free fatty acid into phytoceramide or ceramide. Both enzymes failed to use fatty acyl-CoA as substrate. Using purified rat brain enzyme we also found that the purified enzyme catalyzes the synthesis of ceramide through a CoA-independent mechanism.\(^3\) These observations raise the important question of the physiological function of these enzymes in cells and their role in ceramide metabolism.

Finally, we present evidence indicating that the human enzyme localizes in mitochondria. This nearly exclusive presence of this ceramidase in mitochondria suggests the existence of a specific pool of ceramide in mitochondria. Given the emerging significance of both mitochondria (15) and sphingolipid metabolism (1, 2) in the regulation of stress and apoptosis, this localization of ceramidase to mitochondria raises possibilities of a specific function of mitochondrial sphingolipids in cell regulation.

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\(^3\) S. El Bawab and Y. A. Hannun, unpublished observations.