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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 *Arabidospis 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 sphingomyelinases, activation of the *de novo* synthetic pathway, and inhibition of ceramidases (CDase).[1] Ceramidases 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 ceramidases 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 sphingomyelinases and ceramidases, resulting in the formation of sphingosine, whereas high concentrations of interleukin-1β, stimulated only sphingomyelinases 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 sphingomyelinases, but only nitric oxide donors inhibited ceramidases 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 sphingomyelinases, ceramidases, and sphingosine kinase, leading to the production of sphingosine-1-phosphate, which these authors suggested promotes the proliferation of these cells (5). Ceramidases 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 ceramidases 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 GFP-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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[1] The abbreviations used are: CDase, ceramidase; GFP, 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

Human kidney rapid amplification of cDNA ends (RACE) library, human multitissue Northern blot, ExpressHyb solution, pEGFP-C3 vector, RACE DNA polymerase, and anti-GFP polyclonal antibody were from CLONTECH. *Taq* DNA polymerase and T4 DNA ligase were from Roche Molecular Biochemicals. The vector pcDNA3.1/HisC, TOPO TA cloning kit, and Nick translation kit were from Invitrogen. *Kpn*I and *Apa*I restriction enzymes were from Promega. Polyvinylidene difluoride membranes were from Applied Biosystems. Bradford protein assay and gel electrophoresis apparatus were from Bio-Rad. Polyacrylamide gels were from Novex. Superfect was from Qiagen. Mitotracker Red CMXRos and tetramethylrhodamine methylester (TMRM) were from Molecular Probes. α-^{32}P was from Amersham Pharmacia Biotech. [^{3}H]C_{16}-ceramide was synthesized as described (8). Peptide sequences (1 and 2) were obtained at the microchemical facility at Emory University School of Medicine. Peptide (3) sequence and all DNA sequences were obtained at the protein and DNA Sequencing facility of the Medical University of South Carolina.

Methods

Peptide Sequences—Rat brain enzyme was purified as described (7). Three preparations of 100–120 rat brains each were used. The purified protein from the last column was subjected to SDS-polyacrylamide gel electrophoresis, the gel was stained directly with Coomassie Blue or transferred to polyvinylidene difluoride 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 two human ESTs (accession no. AA913512 and AC012131). The following primers were synthesized: forward primer based on the EST AC012131, CTGAGTGGCACTCACACTCATTCAGGT; and the reverse primer based on the EST AA913512, GGCTTCAGAATGTCCTGCTTCCGA. PCR amplification was performed using the human kidney RACE library as a template. A 1.8-kb fragment was obtained. New primers were then designated on the 5′- (reverse, ACCTGAATGAGTGTGAGTGCCACTCAG) and 3′- (forward, TTCGGGGATGTCCTGCAGCCAGCAAAACCTGAATACAG) ends of the 1.8-kb fragment to perform touch down PCR. After two RACE rounds, a 5′-end fragment of 0.7 kb and a 3′-end fragment of 0.6 kb were obtained. Assembling the 1.8-kb fragment and the 5′- and 3′-ends fragments resulted in a fragment of around 2.5 kb, with a putative open reading frame of 2289 base pairs.

Construction of Full-length CDase Vectors—The full-length CDase fragment was generated by PCR using the forward primer ATGAGTGCCATCACAGTGGCCCTTCTC starting at the longest start codon and the reverse primer ACTAAATAGTTACAACTTCAAAAGCCGGG. The forward primer also contained the *Kpn*I site sequence, and the reverse primer contained the *Apa*I site sequence. PCR amplification was performed at a denaturing temperature of 94 °C for 1 min followed by annealing at 65 °C for 2.5 min and extension at 72 °C for a total of 35 cycles. The amplified fragment (2289 base pairs) was separated by electrophoresis on 1.5% agarose gel. After purification, the full-length cDNA was subcloned into TOPO blunt end cloning vector. Sequencing, using T7 and M13 reverse primers of the TOPO inserts, revealed multiple full-length clones in the sense and in the antisense direction.

The sense fragments were named TOPO-CDase and were used to construct the pcDNA3.1/HisC-CDase vector. To this end, TOPO-CDase vector was digested with *Kpn*I and *Apa*I overnight. The resulting fragment was gel-isolated and subcloned into the same sites in pcDNA3.1/HisC vector, the His-tag being at the N terminus of ceramidase.

To construct pEGFPC3-CDase vector, the open reading frame of CDase cDNA was first amplified as described above. The amplified product was then digested by restriction enzymes *Kpn*I and *Apa*I and cloned into *Kpn*I and *Apa*I sites of the vector pEGFPC3, thus generating a GFP tag at the N terminus of ceramidase protein. The sequence and orientation of the fragments were then confirmed by sequencing.

Northern Blot Analysis—Pre-made commercial Northern blot and hybridization solution were used in this experiment. The human EST AA913512 fragment (0.67 kb) was labeled by Nick translation using [^{32}P]dCTP. The labeled fragment was used to probe a human multitissue Northern blot as described (9). Each lane on the blot contained 2 μg of poly(A)$^+$ RNA. The membrane was first prehybridized overnight at 65 °C in ExpressHyb solution. The radioactive probe was then denaturated 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.

TABLE I
Alignment of the sequenced rat brain peptides to the human cloned protein

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	**Q**FGDVLQPAKPEYR
Human	**T**FGDVLQPAKPEYR
2 Rat	KQ**EL**LKPAVIL**A**FEG**I**S
Human	KQ**DI**LKPAVIL**S**FEG**T**S
3 RAT	KN**R**GYLPGQGPFVA**N**FA
Human	KN**K**GYLPGQGPFVA**A**FA

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 a anti-rabbit secondary antibody at a dilution of 1:3000.

Immunoprecipitation—For immunoprecipitation, cell 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. After 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—CDase activity was measured as described in Ref. 7, using [^{3}H]C_{16}-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 microscopy 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

Sequencing 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

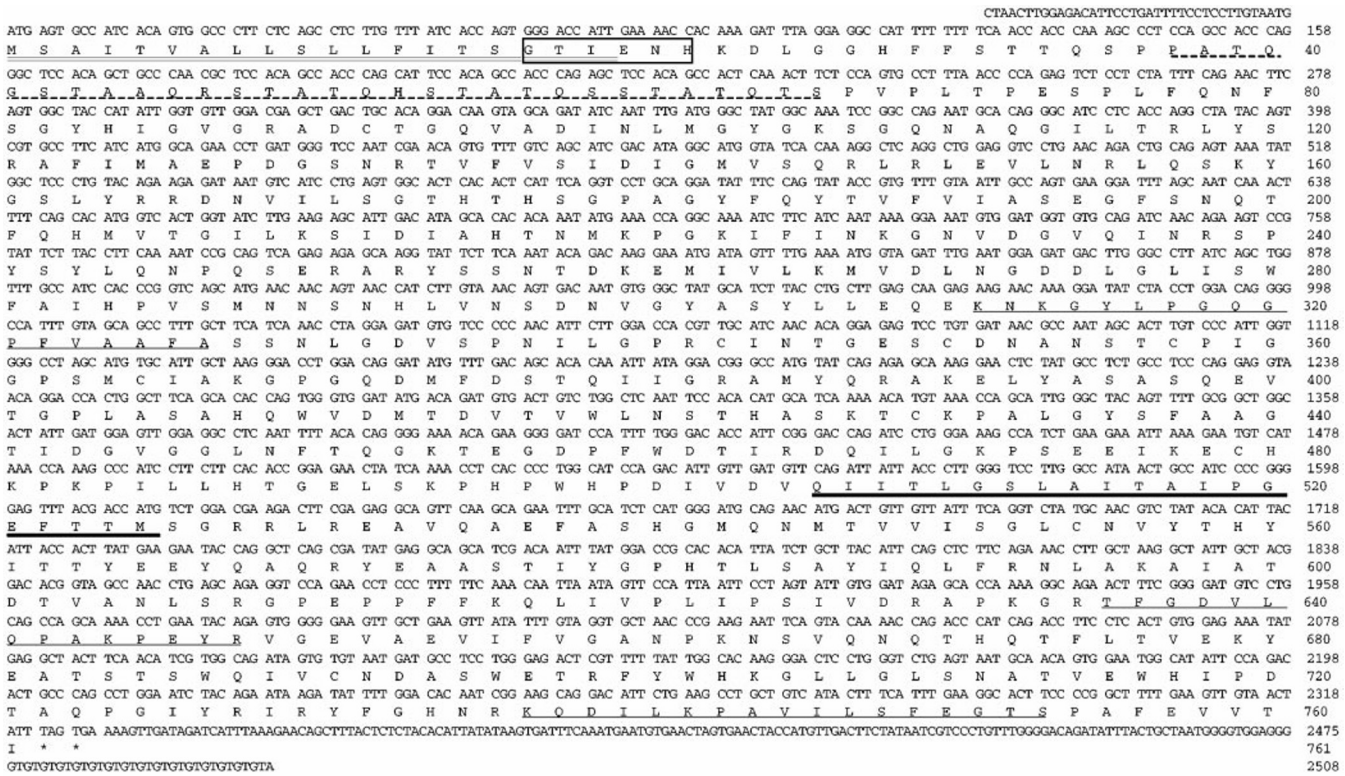

FIG. 1. **cDNA and deduced amino acid sequences of human ceramidase.** The first and second columns indicate the nucleotide and the deduced amino acid sequences, respectively. Nucleotide and amino acid positions are shown on the *right*. Position amino acid 1 refers to the first nucleotide and amino acid of the ceramidase-predicted coding region. Amino acid sequences determined by Edman sequencing of the purified rat brain enzyme (see Table I) are *underlined*. The putative signal peptide (*double underline*), myristoylation site (*boxed*), low complexity signal (- - -), and transmembrane domain (—) are also shown.

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™ Data base, 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.

Primers of both ends, starting at the predicted start and stop codons were made and used to amplify the full-length CDase cDNA from the human kidney library. The full-length CDase cDNA was finally subcloned into pcDNA3.1/HisC and pEGFP-C3 mammalian expression vectors, and the correct sequence and orientation were identified by sequencing. The full-length CDase sequence and the predicted amino acid sequence are shown in Fig. 1. Fig. 1 also shows the position of the sequenced peptides obtained from rat brain, and Table I presents their identity to the cloned human enzyme.

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 futures 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

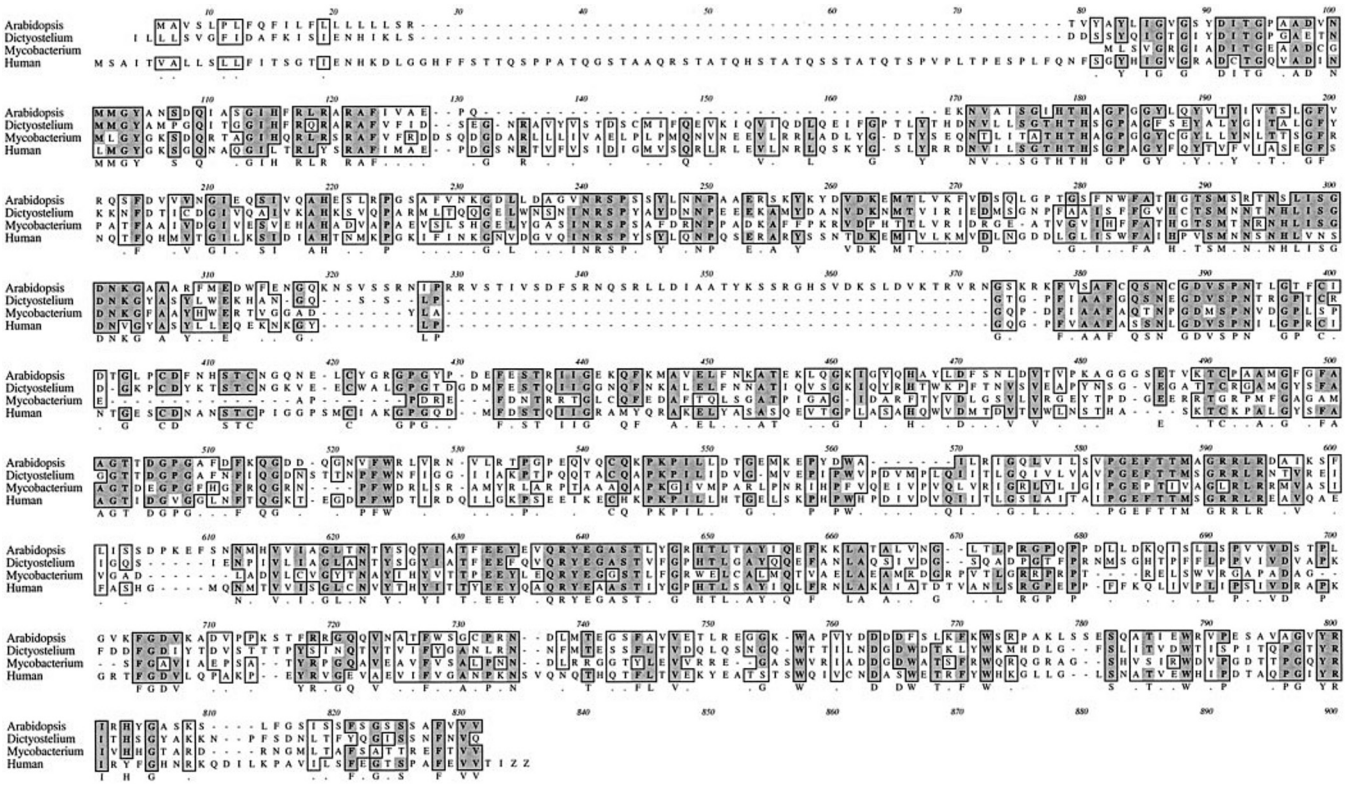

FIG. 2. **Sequence comparison of human ceramidase to putative ceramidases from *A. thaliana*, *M. tuberculosis*, and *D. discoideum*.** Identical amino acids in all four proteins are *shaded*. *Boxed areas* indicate gaps introduced to optimize the alignment. Alignment was performed using the MacVector, Multiple sequence Alignment program.

known mammalian protein. The protein was homologous to three putative proteins from *Arabidospis 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. 4*A*, 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. 4*B*, cells overexpressing the fusion protein contain a GFP-positive band at around 123 kDa, this

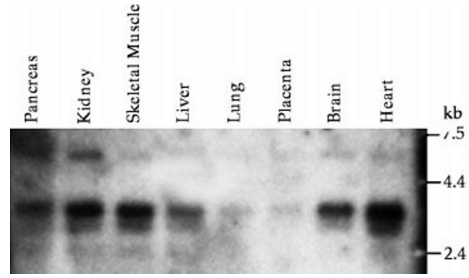

FIG. 3. **Northern blot analysis of poly(A)$^+$ RNAs from human tissues.** The labeled 3′-end of human ceramidase was used to probe a human multiple tissue Northern blot; each lane contained 2 μg of poly(A)$^+$ RNA. Size markers are indicated on the *right*. The major ceramidase band corresponds to a size of 3.5 kb.

band being absent in control cells transfected with the pEG-FPC3 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. 4*C* 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, $MgCl_2$, and $CaCl_2$ (all at 10 mM) and found that they did not affect significantly ceramidase activity. Dithiothreitol at 20 mM was found to inhibit the

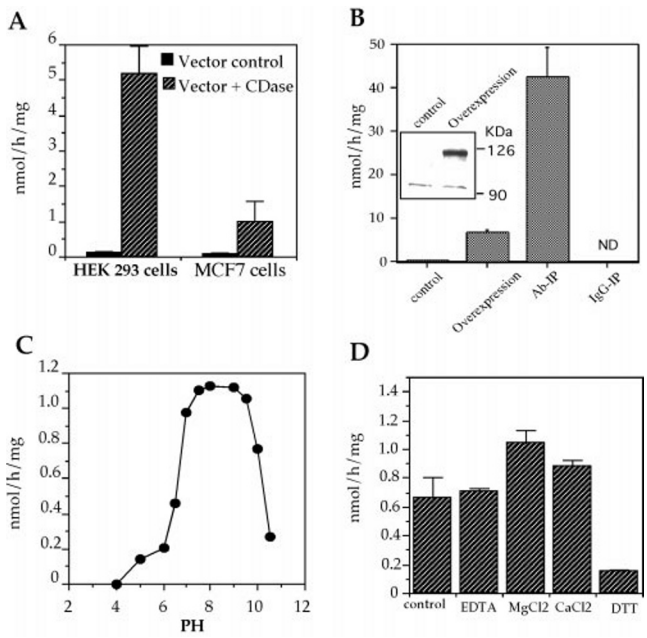

FIG. 4. **Overexpression and characterization of CDase.** *A,* HEK 293 and MCF7 cells were transfected with vector alone (pcDNA3.1/HisC) or vector containing CDase cDNA (pcDNA3.1/HisC-CDase). 48 h after transfection, CDase activity was measured as described under "Experimental Procedures." Data are the mean of three experiments. *B,* cells transfected with empty vector (pEGFPC3, *control*) or vector containing CDase (pEGFPC3-CDase, *Overexpression*) were lysed, and CDase activity was measured on cell lysates. A fraction of the overexpressing cell lysate was also immunoprecipitated with anti-GFP antibody or with normal rabbit IgG as a control. Immune complexes were precipitated by the addition of a mixture protein A/protein G agarose. Ceramidase activity was measured on lysates and immunoprecipitant (*IP*). The *inset* shows a Western blot using GFP antibody of control and overexpressing cells. *Ab*, antibody. *C* and *D*, the activity of CDase was measured in cells transfected with pcDNA3.1/HisC-CDase vector. *C,* the pH was adjusted by the addition of the following buffers at a final concentration of 100 mM: acetate (pH 4 and 5), phosphate (pH 6 and 6.5), Hepes (pH 7–8), glycine (pH 9–10.5). *D,* cations and EDTA were used at 10 mM, and dithiothreitol (*DTT*) was used at 20 mM.

activity by 75% (Fig. 4*D*). 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,[2] 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. 5*A*). 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. 5*B*). The addition of a TMRM mitochondrial probe showed that the ceramidase fusion protein signal colocalizes again with this mitochondrial probe (Fig. 5*B*), 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.

[2] S. El Bawab and Y. A. Hannun, unpublished results.

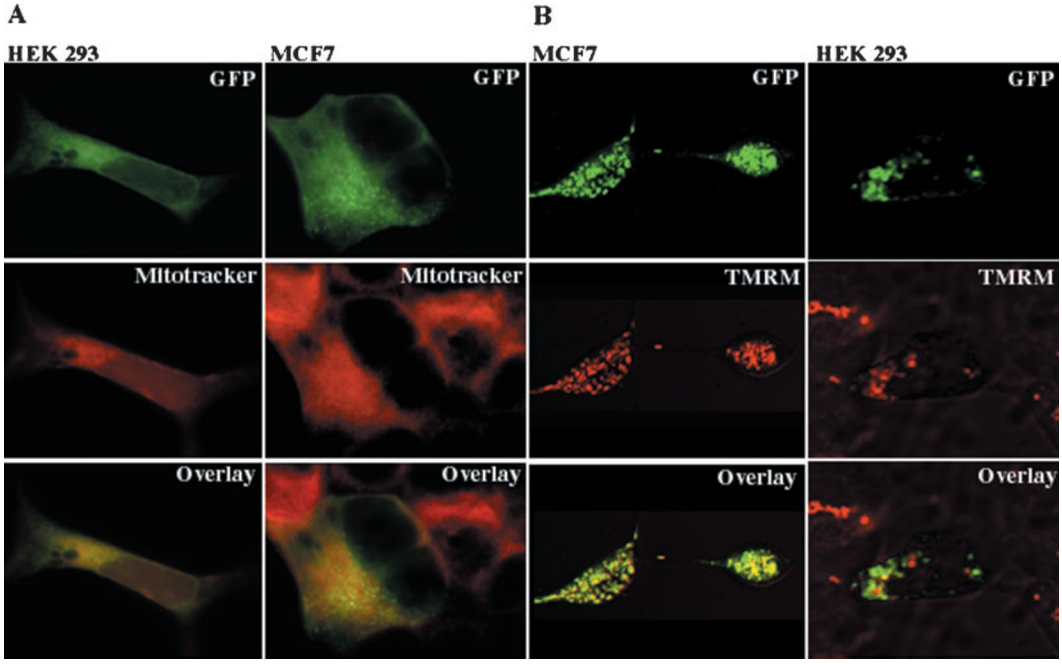

FIG. 5. **Localization of ceramidase.** Cells were transfected with 1 μg of pEGFPC3 empty vector (results not shown) or pEGFPC3-CDase vector containing ceramidase. After 48 h they were stained with specific mitochondrial probes, washed, and then fixed before microscopy observation. *A,* cells were stained with Mitotracker Red and visualized by fluorescence microscopy. *B,* cells were stained with TMRM and visualized by confocal microscopy.

In addition, recently Mao *et al.* (14) reported the cloning of an alkaline ceramidase from the yeast *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 *S. cerevisiae* has been reported. Very interestingly we could not find any protein or DNA sequence from *S. cerevisiae* homologous to the human, slug, or mycobacterium ceramidase.

It is very intriguing that lower organisms such as *M. tuberculosis* and *P. aeruginosa* harbor the mitochondrial ceramidase-specific gene in their genome, whereas the eukaryotic genome of *S. cerevisiae* does not. It would be interesting to determine if this is related to the pathogenicity of *P. aeruginosa* and *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.

[3] S. El Bawab and Y. A. Hannun, unpublished observations.

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