Purification and Characterization of Recombinant, Human Acid Ceramidase: Catalytic Reactions and Interactions With Acid Sphingomyelinase

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**Running Title:** Acid Ceramidase Purification and Characterization
Human acid ceramidase was overexpressed in Chinese hamster ovary cells by amplification of the transfected, full-length cDNA. The majority of the overexpressed enzyme was secreted into the culture media and purified to apparent homogeneity. The purified protein contained the same 13 (α) and 40 (β) kDa subunits as human acid ceramidase from natural sources, had an acidic pH optimum (4.5), and followed normal Michaelis-Menten kinetics using [14C]- and BODIPY-labeled C12 ceramide as substrates. Deglycosylation studies showed that the recombinant enzyme contained mostly “high mannose” type oligosaccharides, and that two distinct β-subunits were present. Amino acid sequencing of these subunit polypeptides revealed a single N-terminus, suggesting that the ~2-4-kDa molecular mass difference was likely due to carboxy-terminal processing. The purified enzyme also catalyzed ceramide synthesis in vitro using [14C]-labeled C12 fatty acid and sphingosine as substrates. Surprisingly, we found that media from the overexpressing hamster cells had increased acid sphingomyelinase activity, and that this activity could be co-precipitated with acid ceramidase using anti-ceramidase antibodies. Overexpression of acid ceramidase in normal, human skin fibroblasts also led to enhanced acid sphingomyelinase secretion, but this was not observed in Niemann-Pick disease cells. RNA studies showed that this increased activity was not due to overexpression of the endogenous, acid sphingomyelinase gene. Uptake studies using mouse macrophages revealed rapid internalization of the acid ceramidase activity from the hamster cell media, but not acid sphingomyelinase. These studies provide new insights into acid ceramidase and the related lipid hydrolase, acid sphingomyelinase.
INTRODUCTION

Human acid ceramidase (AC\(^1\); \(N\)-acylsphingosine deacylase, EC 3.5.1.23) catalyzes the hydrolysis of ceramide to sphingosine and fatty acid. Ceramide serves as the precursor for most sphingolipids, and is a signaling molecule that induces apoptosis in a number of different cell types (reviewed in 1,2). In contrast, sphingosine is converted into sphingosine-1-phosphate, another important cell signaling lipid that is “anti-apoptotic” and can counteract the effects of ceramide (reviewed in 3,4). The only source of sphingosine in vertebrate cells is through ceramide hydrolysis, while ceramide can be generated by synthetic pathways or through the degradation of sphingomyelin or glycosphingolipids (5-7).

Several ceramidases have been described in vertebrates that function at varying pHs and presumably reside in distinct intracellular locations. An acidic ceramidase (i.e., AC) was first described and partially purified by Gatt in 1963 (8). In 1972, patients with the lipid storage disorder, Farber disease (Farber Lipogranulomatosis), were found to have a marked deficiency of this enzymatic activity (9). In 1995, human AC was purified to apparent homogeneity from urine (10), and was found to be a \(\sim50\)-kDa polypeptide that consisted of two subunits, \(\alpha\) (\(\sim13\)-kDa) and \(\beta\) (\(\sim40\)-kDa). Processing studies performed in human skin fibroblasts revealed that human AC was first synthesized as a \(\sim55\)-kDa precursor that was proteolytically processed into the two subunits within late endosomes or lysosomes. Most of the enzyme was found within cells, presumably in acidic compartments, but small amounts of the precursor and a partially processed 47-kDa form were found extracellularly. The \(\alpha\)-subunit contains five
$N$-linked oligosaccharide chains, several of which are of the “complex” type (i.e., resistant to digestion with endoglycosidase H [Endo H]) (11). Kinetic analysis has revealed that $N$-lauroylsphingosine (C12 ceramide) is the best in vitro substrate for this enzyme (e.g., 10).

In 1996, the full-length cDNA encoding human AC was obtained and found to encode the complete 55-kDa precursor polypeptide (12). Comparison of this sequence with that of several cloned neutral and alkaline ceramidases (13-15) confirmed that these were distinct enzymes encoded by different genes. The complete genomic sequence encoding human AC also has been isolated and found to reside on the short arm of chromosome 8 (16). To date, more than a dozen mutations causing Farber disease have been found within the AC gene (e.g., 12,17). In addition, the AC gene has been disrupted in mice by gene targeting and found to cause an early embryonic lethal phenotype, further indicating the importance of this enzyme in vertebrate development (18).

In this manuscript we report purification and characterization of recombinant, human AC from the media of genetically engineered Chinese hamster ovary (CHO) cells. We find that many properties of the recombinant enzyme are similar to those previously reported for enzyme obtained from natural sources, and that in addition to ceramide degradation, recombinant AC could catalyze ceramide synthesis using free fatty acid and sphingosine as substrates. Of note, the pH optima for the degradative and synthetic reactions were distinct. In addition, we report that overexpression of human AC in CHO cells or human skin fibroblasts leads to elevated acid sphingomyelinase (ASM) activity in the media. Additional studies revealed that the secreted AC from the overexpressing CHO media existed in two molecular forms, one associated with ASM and the other not.
EXPERIMENTAL PROCEDURES

Materials

Lauryl-1-14C]D-erythro sphingosine ([14C]-labeled C12 ceramide), [1-14C]lauric acid, and [choline methyl-14C] sphingomyelin were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). BODIPY-conjugated C12 sphingomyelin and C12 fatty acid were from Molecular Probes, Inc. (Eugene, OR). BODIPY-conjugated C12 ceramide was synthesized as previously described (19). Standard tissue culture media and other tissue culture reagents were purchased from either Life Technologies (Baltimore, MD) or Gibco-BRL (Gaithersburg, MD), with the exception of heat inactivated, fetal bovine serum (FBS), which was obtained from Hyclone Laboratories (Logan, UT), and UltraCULTURE medium without serum supplements, from BioWhittaker, Inc. (Walkersville, MD). Tissue culture plasticware and all organic solvents were purchased from the Fisher Scientific Co. (Springfield, NJ). Concanavalin A Sepharose, Blue Sepharose, and Superose 12 were purchased from Amersham Biosciences (Piscataway, NJ). Precast 4-20% gradient polyacrylamide gels and a Colloidal Blue Staining Kit were from Invitrogen (Carlsbad, CA). Nitrocellulose membranes were from Schleicher and Schuell (Keene, NH). A cDNA probe for glyceraldehyde-3 phosphate dehydrogenase (G3PDH) was obtained from Clontech Laboratories Inc. (Palo Alto, CA). A commercial, adenoviral vector (Type 2) was from Life Technologies (Baltimore, MD). Peptide-N-glycosidase F (PNG F), Endo H, and sialidase A (neuraminidase) were purchased from Prozyme Inc. (San Leandro, CA). A protein determination kit was purchased from Bio-Rad (Hercules, CA). All other biochemical reagents were from the Sigma Chemical Company (St. Louis, MO).
Construction of CHO Cells Overexpressing Human AC—A full-length, human AC cDNA (11) was inserted into the p91023(B) mammalian expression vector (20,21). The vector was then transfected into dihydrofolate reductase negative (dhfr) DG44 Chinese hamster ovary (CHO) cells, and stably transfected clones were selected for their ability to grow in DMEM medium containing 10% FBS, but lacking hypoxanthine (0.05 mM) and thymidine (0.008 mM). Non-transfected DG44 cells require these supplements to grow. Over 250 surviving colonies were isolated in this manner, expanded, and then plated in medium containing 0.02 M methotrexate. After 2 weeks, surviving clones were isolated, expanded, and placed into medium containing 0.1 M methotrexate. A similar selection/expansion procedure was used to eventually isolate 5 clones that survived in medium containing 20 M methotrexate. Of these, one clone, designated CHO6, was selected for further analysis based on its high AC activity. Parental CHO cells were maintained in DMEM medium containing 10% FBS, hypoxanthine (0.05 mM), and thymidine (0.008 mM).

Purification of Human AC from the Overexpressing CHO6 Medium—The procedure described below summarizes the typical purification of human AC from 250 ml of CHO6 medium containing 5% FBS. The medium was centrifuged at 1000 g to remove debris, concentrated 20 times using a 100-kDa cut-off ultrafiltration membrane (Amicon Inc., Beverly, MA) to reduce serum components (e.g., BSA), and stored at 4°C before processing through the following purification steps. Prior studies had revealed that more than 90% of the AC activity was retained by these filters, consistent with the fact that the native molecular weight of the recombinant enzyme was ~150-kDa (see “Results”).
Step 1: Concanavalin A-Sepharose (Con A)—The concentrated medium was slowly applied to a 10 ml Con A column that had been equilibrated with 10 bed volume of Con A wash buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, 0.1% Igepal CA-630, and 0.02% NaN₃, pH 7.2). The column was attached to an AKTA FPLC (Amersham Biosciences, Piscataway, NJ). The washing, pre-elution, and elution steps were carried out with Con A wash buffer, pre-elution buffer (Con A wash buffer containing 10 mM methyl-D-glucopyranoside), and elution buffer (Con A wash buffer containing 1 M methyl-D-glucopyranoside), respectively, and monitored using a UV detector. The flow rates were maintained at 2, 1 and 0.5 ml/min during the washing, pre-elution, and elution steps, respectively. Fractions from the elution step containing the highest AC activity were pooled, concentrated to less than 10 ml using a 100-kDa ultrafiltration membrane, and then diluted and re-concentrated three times in the presence of 20 mM Tris-HCl, pH 7.2 at 4°C (for buffer exchange).

Step 2: Blue Sepharose—The concentrated Con A eluant was loaded onto a Blue Sepharose column (5 ml) that had been equilibrated with washing buffer (20 mM Tris-HCl, pH 7.2, and 0.1% Igepal CA-630). Bound proteins were eluted from the column using a NaCl gradient (from 0 to 1 M) in washing buffer. The flow rates were maintained at 2, 0.3 and 0.5 ml/min during the washing, loading, and elution steps, respectively. Two peaks of AC activity were obtained from this column (see “Results” and Fig. 7), and each was concentrated to less than 2 ml using a 100-kDa ultrafree centrifugal column (Millipore Corp., Bedford, MA). The “low salt” fraction was routinely used for further purification and thus diluted and re-concentrated three times in the presence of 20 mM Tris-HCl, pH 7.2 at 4°C for buffer exchange and subsequent gel filtration.
Step 3: Superose 12 Gel Filtration—The concentrated, “low salt” material from the Blue Sepharose column was loaded onto a 106 ml Superose 12 column (1.6×53 cm, XK16/70, Amersham Biosciences) equilibrated with 5 bed volume of running buffer (20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.02% NaN₃, 0.1% Igepal CA-630). The flow rate was maintained at 0.2 ml/min. The eluate was collected in 1 ml fractions after the void volume (37 ml). Fractions containing the highest AC activity were pooled, washed and concentrated three times with 20 mM Tris-HCl, pH 7.2 using a 100-kDa ultrafree centrifugal column at 4°C, and concentrated to a final protein concentration above 1 mg/ml.

AC and Protein Assays—Throughout the purification procedure, AC activity was detected using a modified, fluorescence-based HPLC method (22). The standard 10 μl AC assay mixture consisted of 5 μl of enzyme source and 5 μl of 0.2 M citrate/phosphate buffer (pH 4.5) containing 200 mM BODIPY-conjugated C12 ceramide, 300 mM NaCl, 0.1% BSA, and 0.2% Igepal CA-630. Assays were carried out at 37°C for 30 min. After the reactions were complete, 5 μl of the assay mixtures were removed and pipetted into 95 μl ethanol, mixed, and then centrifuged for 5 min for 10,000×g. The supernatants were then transferred to a Waters glass sampling vial, and 5 μl (2.5% of the original reaction mixture) was auto-sampled by a WIPS 712 (Waters Corp., Milford, MA) autosampler onto an HPLC equipped with a reverse-phase column (BetaBasic-18, 4.6×30 mm, Keystone Scientific Inc., Bellefonte, PA), and eluted isocratically with methanol:water (95:5 v/v) at a flow rate of 1 ml/min. Fluorescence was quantified using a Waters 474 (Waters Corp., Milford, MA) fluorescence detector set to excitation and emission wavelengths of 505 and 540 nm, respectively. The undigested substrate and
product (i.e., BODIPY-conjugated C12 ceramide and fatty acid, respectively) peaks were identified by comparing their retention times with standards, and the amount of product was calculated using a regression equation that was established from a standard curve using BODIPY-conjugated C12 fatty acid.

For certain experiments, AC activity also was detected using a radiolabeled substrate ([14C]-labeled C12 ceramide). The reactions were prepared and carried out as described above for the BODIPY-conjugated substrate. After the reactions were complete, the assay mixtures were spotted onto a thin layer chromatography (TLC) plate, and the product, [14C]-lauric acid, was separated from the [14C]-labeled C12 ceramide substrate using a solvent consisting of chloroform:methanol:28% ammonium hydroxide (90:20:0.5, v/v). The TLC plate was exposed to a PhosphorImager screen that was subsequently scanned by a PhosphorImager system (Storm 860, Molecular Dynamics, Sunnyvale, CA). The undigested substrate and product were identified by co-migration with standards, and the signal was quantified using the Imagequant software. Total protein was determined using the Bio-Rad protein assay kit according to the manufacturer’s instructions.

ASM Assays—The standard 10 µl ASM assay mixture consisted of 5 µl of enzyme source and 5 µl of assay buffer (200 mM BODIPY-conjugated C12-sphingomyelin, 0.1 mM ZnCl2, 0.2% Igepal CA-630, 0.2 M sodium acetate buffer, pH 5.0) (23). Assays were generally carried out at 37°C for 1 h. After the reactions were complete, 5 µl of the assay mixtures were removed and quantified as described above for AC. The undigested substrate and product (i.e.; BODIPY-conjugated sphingomyelin and ceramide, respectively) peaks were identified by comparing their retention times with standards,
and the amount of product was calculated using a regression equation that was established from a standard curve using BODIPY-conjugated C12 ceramide.

ASM activity also was determined using choline-radiolabeled [14C] sphingomyelin as a substrate. Other components of the reaction mixtures were the same as those described above for BODIPY-conjugated sphingomyelin. Following a 1 h incubation at 37°C, the samples were spotted onto a TLC plate and dried. The product and substrate ([14C] sphingomyelin and [14C] choline phosphate, respectively) were separated using a running buffer of chloroform/methanol/H2O (5:4:1, v/v). The radioactive signals were visualized and quantified using a Storm 860 PhosphorImager system.

**AC Reverse Reaction (Ceramide Synthesis) Assay**—The reverse activity of purified AC was measured using [1-14C]lauric acid and sphingosine as substrates. The standard reaction mixture contained 5 µl of enzyme source and 5 µl of 200 mM citrate/phosphate buffer (pH 6.0), containing 200 µM [14C]-lauric acid, 100 µM sphingosine, and 0.1% Triton X-100. The reactions were incubated at 37°C for 30 min, and then terminated by spotting onto a TLC plate and drying immediately using a hairdryer. The product, [14C]-ceramide, was separated from the substrates using a running buffer of chloroform:methanol:28% ammonium hydroxide (90:20:0.5, v/v). The TLC plate was exposed to PhosphorImager screen that was subsequently quantified by a PhosphorImager system (Storm 860). The radioactive product ([14C]-ceramide) and substrate ([14C]-lauric acid) were identified by co-migration with standards, and the signals were quantified by the Imagequant software.

**SDS-PAGE Analysis**—Samples were boiled in loading buffer (12 mM Tris-HCl, pH 6.8, containing 0.5% SDS, 5 mM β-mercaptoethanol, 5% glycerol, and 0.02%
bromophenol blue) for 10 min, loaded onto 4~20% gradient polyacrylamide gels, and electrophoresed in an XCELL II mini-cell apparatus (Novex, San Diego, CA) for 1 h. The voltage was maintained at 150 V and the running buffer contained 0.1% SDS. Following electrophoresis, the gel was stained using either Colloidal Blue or Silver according to the manufacturer’s instructions.

**Glycosylation Studies**—To evaluate the effects of various glycosidases on the purified AC, 5 μg of the enzyme was added to a 25 μl reaction mixture containing Endo H (5 mU), PNG F (5 U), or sialidase (5 mU) in 50 mM sodium phosphate buffer (pH 5.5, 7.5, and 5.0, respectively). The purified AC was first denatured by heating at 100°C for 5 min in the presence of 0.1% SDS and 50 mM 2-mercaptoethanol, and then incubated at 37°C for 3 h after adding Endo H and PNG-F. The heat denaturation step was omitted for the sialidase reaction. After digestion with the individual glycosidases, the samples were boiled in SDS/dithiothreitol (DTT), electrophoresed on 4-20% Tris-Glycine polyacrylamide gels, and stained with Colloidal Blue.

**Northern Blot Analysis**—To investigate the expression levels of the ASM mRNAs in the parental and transfected CHO cells, total RNA was isolated from CHO and CHO6 cells using the RNeasy mini kit, separated by formaldehyde-denatured agarose gel electrophoresis, and transferred to Zeta-Probe GT Blotting membranes. Hybridization was performed using a cDNA fragment from murine ASM (nucleotides 1-283) (24) in QuickHyb hybridization solution at 68°C for 1.5 h. This region of the mouse ASM sequence has greater than 90% nucleotide identity with the hamster ASM sequence (24 and unpublished result), facilitating identification of the endogenous hamster ASM RNA. The hybridized membranes were washed with 2XSSC/0.1% SDS at room temperature for
15 min and then with 0.5X SSC/0.1% SDS at 58°C for 15 min. The radioactive, hybridized signals were visualized and quantified using the STORM imaging analyzer. The Northern blots were also hybridized with a human housekeeping cDNA fragment (G3PDH) as a control.

Antibody Production and Immunoprecipitation Studies—Polyclonal antibodies were raised in rabbits against purified, recombinant AC (i.e., the LSP fraction from Blue Sepharose, see Fig. 7). For immunoprecipitation studies, CHO6 medium was mixed 1:2 with binding buffer (150 mM NaCl, 1% Igepal CA-630, 1 mM PMFS, and 50 mM Tris-HCl, pH 8.0), and then 30 μl of either rabbit preimmune serum or anti-AC serum was added and incubated for 1 h at 4°C. 50 μl of a 50% slurry containing protein A Sepharose and binding buffer was then added to the mixtures and rocked gently for an additional 1 h at 4°C. The immune complexes were centrifuged (13,000xg) and washed three times with binding buffer, and the final pellets were then resuspended in appropriate buffers for the determination of individual enzyme activities (see Table 2).

Overexpression of Human AC in Skin Fibroblasts — The full-length, human AC cDNA was inserted into a commercial, adenoviral vector (Type 2), and recombinant virus was prepared according to the manufacturer’s instructions. One day prior to infection, human skin fibroblasts (from a normal individual or patient with Type A Niemann-Pick disease (NPD) were subcultured at a density of 1.0 x 10^5 cells/well in 12-well culture plates. Cells were infected using 0.25 ml of medium (DMEM with 10% fetal bovine serum) containing mock virus (control) or the AC recombinant virus. After incubation at 37°C for 1 h, an additional 0.5 ml of medium was added to each well. Two days after infection, the cells were washed and 0.6 ml of fresh UltraCULTURE medium without
serum supplements was added. After 3 days, the culture media was collected and the AC and ASM activities were determined as described above.

*Enzyme Uptake Studies*— Macrophages were obtained from the lung airspaces of normal and ASM knockout (ASMKO) mice as previously described (25), and were cultured in RPMI media containing 10% FBS at 37°C. The cells were incubated overnight, washed 3X with PBS, and then fresh media was mixed (1:1, v/v) with conditioned CHO6 or parental CHO media and added to the culture flasks. These conditioned medias were obtained by growing CHO6 or parental CHO cells for 48 h prior to the macrophage uptake studies. The macrophages were grown in the presence of these conditioned media (or standard RPMI medium as a control) for an additional 72 h at 37°C, and then harvested and centrifuged at 3000xg. After washing the cells several times with PBS, AC and ASM activities were determined in the cell homogenates as described above.
RESULTS

Overexpression and Purification of Human AC—The human AC cDNA was overexpressed in CHO cells using a methotrexate gene amplification system (20,21), and the recombinant enzyme was purified from the culture media of the overexpressing cells. Table 1 shows a typical purification from 250 ml of media. AC specific activity was enriched ~60-fold in the final preparations and ~2 mg of pure enzyme was obtained. Notably, AC activity was eluted from the Blue Sepharose column in two fractions (see below and Fig. 7), but unless otherwise mentioned, the analyses described within were performed using the “low salt fraction” (Fig. 7) only. Fig. 1 shows SDS-PAGE analysis at each step of the purification. In the final Superose 12 eluant (lane 4), only three bands were visible using Colloidal Blue or Silver\(^2\) staining, corresponding in molecular mass to the AC \(\alpha\) and \(\beta\)-subunits (~13 and 40-kDa, respectively), and precursor protein (~55-kDa) (10). The \(\beta\)-subunit band had a broad appearance, suggesting that it was glycosylated. Deglycosylation analysis using PNG F or Endo H confirmed this observation, and revealed that two peptide backbones were present for the precursor protein and \(\beta\)-subunit (Fig. 2). This analysis also revealed that the oligosaccharide chains on the \(\beta\)-subunit of recombinant AC were of the “high mannose” type (Endo H sensitive). Consistent with this observation, digestion with sialidase had no effect on recombinant AC (lane 4). Amino acid sequencing of the two \(\beta\)-subunit backbones revealed a single \(N\)-terminus, identical to that previously published for AC from human urine (10). This suggested that the different molecular masses were likely due to carboxy-terminal processing.
Kinetic Analysis of Recombinant Human AC—Numerous assay procedures have been developed to determine AC activity in vitro (e.g., 26-28), and previous studies have shown that the activity was highly dependent on the type of substrate used and specific assay conditions (e.g., detergents, salts, etc). We evaluated several assay conditions using the purified recombinant, human AC as the enzyme source and BODIPY-conjugated or [14C]-labeled C12 ceramide as substrates, and arrived at an optimal reaction buffer that was suitable for both substrates. The standard reaction mixtures contained 0.1 M citrate/phosphate buffer, pH 4.5, enzyme source, 100 μM substrate, 150 mM NaCl, 0.05% BSA, and 0.1% Igepal CA-630. All subsequent experiments were carried out using these conditions unless otherwise noted.

As shown in Fig. 3, kinetic analysis using the radioactive and fluorescent substrates revealed normal Michaelis-Menten profiles, and the K_m and V_max values were 389 μM and 27,778 nmol/mg/h, respectively for the [14C]-labeled substrate, and 413.2 μM and 2000 nmol/mg/h, respectively for the BODIPY-conjugated substrate. As shown in Fig. 4A, the pH optimum for this degradative reaction was ~4.5.

Purified AC Can Catalyze Ceramide Synthesis In Vitro—Studies performed in the 1960s using partially purified AC obtained from brain homogenates had suggested that this enzyme could synthesize ceramide in vitro using free fatty acids and sphingosine as substrates (29). Several recent studies using purified neutral or alkaline ceramidases have also shown that these enzymes can carryout this “reverse reaction” in vitro (30-34). We therefore evaluated whether recombinant AC could catalyze ceramide synthesis using [14C]-lauric acid and sphingosine as the substrates. As shown in Fig. 4B, purified AC could synthesize ceramide efficiently using the same assay conditions developed for
ceramide degradation (see “Experimental Procedures”), except that the pH optimum for the synthetic reaction was ~5.5-6.0, as compared to 4.5 for the degradative reaction (panel A).

Overexpression and Secretion of AC Leads to Enhanced Secretion of ASM—

Ceramide and sphingomyelin metabolism are highly interrelated. We were therefore interested in determining whether overexpression of the human AC cDNA in CHO cells would lead to overexpression of the endogenous, hamster ASM gene, and/or whether the human AC and hamster ASM might co-purify with one another. As shown in Fig. 5A, CHO6 cells that were genetically engineered to overexpress human AC released large amounts of ASM activity into the culture media. This increased ASM activity was not due to overexpression of the endogenous, hamster ASM gene, as revealed by Northern blot analysis (Fig. 5B).

To determine whether this observation was restricted to CHO cells, we next overexpressed the human AC cDNA in normal human skin fibroblasts or skin fibroblasts from a patient with Niemann-Pick disease who lacked ASM activity. As shown in Fig. 6, overexpression of AC in normal skin fibroblasts also led to increased secretion of both AC and ASM activities into the culture media. In the media of NPD cells, however, only AC activity was detected, indicating that the source of ASM activity was the endogenous ASM polypeptide.

We next used anti-AC antibodies for immunoprecipitation studies from the CHO6 media. As shown in Table 2, the immune complexes formed using anti-AC serum had ~60-fold increase in AC activity compared to control complexes formed using preimmune serum. Importantly, these same complexes had ~36-fold increased ASM
activity, revealing that the interaction of AC and ASM was specific enough to permit co-precipitation. We also found that the β-galactosidase activity was increased ~100-fold in these immune complexes, while the activities of several other enzymes, including two lipid hydrolases, β-galactosidase and β-glucosidase, were not.

To further investigate the AC-ASM interaction, AC was purified from the CHO6 media as described in the “Experimental Procedures” using a salt gradient to elute the enzyme from Blue Sepharose. As shown in Fig. 7A, two peaks of AC activity were eluted from this column at low and high salt concentrations, respectively (designated LSP and HSP in Fig. 7). We routinely used the “low salt fraction” for the characterization experiments described above. Interestingly, however, ASM activity was associated with the high salt, but not the low salt, fraction. These results suggested that the secreted AC in the overexpressing CHO media existed in two molecular forms, one associated with ASM and the other not.

As shown in Fig. 7B, when the low and high salt fractions were further purified individually using Superose 12 and analyzed by SDS-PAGE under reducing conditions, the same staining pattern was observed and only AC-associated polypeptides were observed. This result suggested that the amount of ASM polypeptide in the high salt fraction was very low (below detection using Colloidal Blue or Silver), and that the specific activity of this enzyme must therefore be very high. Attempts to visualize the hamster ASM in the high salt fraction by Western blotting were unsuccessful since the anti-human ASM antibodies did not cross react with the hamster enzyme. Of particular interest, analysis of the Superose 12 elution profiles showed that the low and high salt fractions had similar native, molecular weights, both greater than 150-kDa. This
suggested that under these running conditions both AC forms existed in high molecular weight complexes.

Enzyme Uptake Studies Using Cultured Macrophages—We next used murine macrophages to evaluate uptake of the secreted AC and ASM activities. Our choice of macrophages was based on the facts that: 1) the overexpressed, secreted AC protein contained “high mannose” type oligosaccharides (see Fig. 2), and 2) previous data had shown that purified, human ASM could be readily taken up these cells. As shown in Fig. 8 (control), under standard growth conditions (see “Experimental Procedures”), macrophage extracts from normal mice had detectable levels of ASM activity, but very low levels of AC activity. When the macrophages were grown in conditioned media from parental CHO cells, there was no increase in the amount of either enzyme activity. This was consistent with the fact that parental CHO cells secrete little, if any, AC (see Fig. 5), and suggests that the secreted, hamster ASM likely contains “complex” type oligosaccharides that are not efficiently taken up by mannose receptors on macrophages. When the cells were grown in conditioned media from CHO6 cells (containing high levels of both AC and ASM activities, see Fig. 5), a dramatic increase in AC activity was observed, but not ASM. This result confirmed the data in Fig. 2 showing that secreted AC contained “high mannose” type oligosaccharides, and suggested that the form of AC in the CHO6 media that was associated with ASM (i.e., “high salt” Blue Sepharose fraction) could not be internalized by these cells, while free AC could.
DISCUSSION

In this study we report the purification and characterization of recombinant, human AC from the media of genetically engineered CHO cells. It has generally been found that overexpression of human lysosomal enzymes in this cell type leads to their selective secretion into the culture media (e.g., 20,21), and such was the case for human AC. Most of the physical characteristics of recombinant, human AC obtained from the culture media were similar or identical to the human enzyme purified from urine. Indeed, SDS-PAGE analysis revealed nearly identical $\alpha$ and $\beta$-subunits (~13 and 40-kDa, respectively), and small amounts of an ~55-kDa precursor protein. Subsequent amino acid sequencing confirmed the identity of these AC-specific polypeptides.

Despite these similarities, however, deglycosylation analysis using PNG F and Endo H showed that although the $\beta$-subunit was heavily glycosylated, similar to the “natural” enzyme, the oligosaccharide chains on the recombinant enzyme appeared to be of the “high mannose” type (Endo H sensitive). This is different from the urinary AC, which contained mostly “complex” type sugars (10), and indeed different from most other lysosomal enzymes secreted from overexpressing CHO cells, which generally have a mixture of “complex” and “high mannose” type oligosaccharides (e.g., 20,21,40,41). Of note, however, the glycosylation pattern on at least one other lysosomal enzyme produced in the CHO cell system, $\alpha$-N-acetylglicosaminidase (42), was different from the “natural” form, similar to what we have observed for AC. There are several possible explanations for this finding, but since processing of the AC precursor protein to the $\alpha$ and $\beta$-subunits is thought to occur in late endosomes or lysosomes (10), and the majority
of the recombinant AC obtained from the CHO media had already been processed into the individual subunits, we believe that the presence of “high mannose” type oligosaccharides on the secreted enzyme indicates that it has been trafficked through the late endosome/lysosome compartments prior to its release, where carbohydrate trimming was likely to occur.

Deglycosylation studies also revealed the presence of two distinct polypeptide backbones for the AC precursor and b-subunit. Since only a single N-terminus was obtained for these polypeptides, these results indicated that carboxy-terminal processing of the precursor protein likely occurred prior to its proteolytic digestion into the d and b-subunits. Based on the difference in molecular mass (~2-4-kDa), ~20 to 40 amino acid residues were presumably removed from the carboxy end of the AC precursor during this processing event. Within this region are several potentially important amino acid residues, including two cysteine residues (C388 and C392) that might be involved in intramolecular or intermolecular disulfide bond formation.

Kinetic analysis of the purified, recombinant enzyme was carried out using fluorescent (BODIPY-conjugated) and radioactive ([14C]-labeled) ceramide substrates, and the K$_m$ and V$_{max}$ values were similar to those obtained with the purified urinary enzyme (10). C12 ceramides were used for these analyses since previous studies had revealed that this was an optimal substrate for the in vitro ceramidase reaction (10). The optimal pH for this reaction was ~4.5, similar to what had been previously described for the enzyme from urine. Of interest, while the K$_m$ values using the BODIPY and [14C]-labeled substrates were similar for the recombinant enzyme, the V$_{max}$ value for the radioactive substrate was ~10-fold greater than that for the fluorescent substrate,
indicating a similar binding affinity, but more rapid turnover rate for the radioactive vs. fluorescent substrates.

Over three decades ago it was observed that partially purified AC preparations could carry out ceramide synthesis using free fatty acids and sphingosine as substrates (29). While in these early studies it was unclear whether the distinct synthetic (i.e., “reverse”) and degradative (i.e., “forward”) reactions were being carried out by the same enzyme, recent studies using cloned neutral/alkaline ceramidases have suggested that some of these enzymes also can perform both reactions in vitro (30-34).

We therefore decided to test whether our purified, recombinant human AC could catalyze ceramide synthesis using [14C]-labeled C12 fatty acid and sphingosine as substrates. Indeed, we found that this reaction was quite robust, did not require ATP, and, importantly, occurred at an optimal pH of ~6.0, distinct from the ceramide degrading activity. These results suggested that AC might drive either ceramide degradation or synthesis depending on its subcellular localization and pH. Since both ceramide and sphingosine are important signaling lipids, this result also suggested that AC might play a critical regulatory role in lipid-mediated cell signaling by acting as a “rheostat” controlling the levels of both biologically important lipids in distinct intracellular compartments. Studies are currently underway to further characterize the reverse reaction using purified AC, and to determine if it occurs in vivo.

Surprisingly, we also observed that the conditioned CHO6 media had high levels of ASM activity. This was observed using either BODIPY-conjugated or [14C]-labeled sphingomyelin substrates, and was not due to overexpression of the endogenous, hamster ASM gene. We also found that overexpression of AC in normal, human skin fibroblasts
led to a similar finding, but when the experiments were carried out using skin fibroblasts from a NPD patient, no secreted ASM activity was observed. This latter result revealed that the source of ASM activity was the endogenous, ASM polypeptide.

Importantly, immunoprecipitation studies demonstrated that the ASM and AC activities in the CHO6 media were co-precipitated using anti-AC antibodies, indicating that there was a tight association of these two enzymes. Intriguingly, high levels of β-galactosidase activity also were co-precipitated with the AC and ASM, while the activities of several other lysosomal hydrolases were not. It is notable that there are two lysosomal β-galactosidases present in mammalian cells, and that one of these enzymes hydrolyzes galactose-ceramide to ceramide (i.e., galactocerebrosidase; EC 3.2.1.46). The other β-galactosidase (EC 3.2.1.23) hydrolyzes a variety of glycoconjugates, including the sphingolipid GM₁ ganglioside. Although the assay method we used in the current study did not distinguish between these two activities, a comprehensive analysis of this multienzyme complex and its role in sphingolipid metabolism is currently underway.

Of particular interest, we also found that the AC activity secreted into the CHO cell media could be fractionated on a Blue Sepharose column into two peaks using a salt gradient, one that contained ASM activity and one that did not (high and low salt fractions, respectively; see Fig. 7). This also suggested that the secreted AC existed in two molecular forms, one associated with ASM and the other not. It is notable that a prior publication similarly reported co-purification of human ASM and AC from urine (43), confirming our observations in the CHO cell system. However, despite the marked difference in ASM activity between the two Blue Sepharose fractions, SDS-PAGE
analysis performed under reducing conditions revealed only AC polypeptides. This indicated that the amount of ASM in the high salt fraction was very low (below detection with Colloidal Blue or Silver staining), and that the specific activity of this enzyme must be very high (since the total amount of ASM activity in this fraction was equal to or greater than AC; see Fig. 7). Indeed, a review of the literature revealed that most purified ASM preparations had specific activities that were about 100-fold greater than AC, consistent with this observation (e.g., 21,43).

Enzyme uptake studies using cultured macrophages supported the notion of two distinct AC forms, and suggested that only the AC specific form (i.e., without ASM activity) could be internalized by these cells. Since purified, human ASM is readily taken up by macrophages, and the CHO6 media contained several fold more ASM activity than the parental CHO cells (see Fig. 5), we presume that the reason ASM was not internalized in the present studies is that its association with AC precluded it’s interaction with cellular receptors responsible for uptake.

In conclusion, we have developed a system to markedly overexpress and purify recombinant, human AC, and describe many of its properties. We have shown that human AC purified from the media of overexpressing CHO cells can carry out both ceramide degradation and synthesis \textit{in vitro} using radioactive and fluorescent substrates. We have also found that overexpression of AC in cells leads to co-secretion of ASM, suggesting that the two enzymes are capable of forming a complex with one another that is tightly associated and can be co-purified. We are currently investigating whether this complex also forms under natural conditions and determining what other proteins might be present. The results presented clearly confirm the critical role of AC in the regulation
of sphingolipid metabolism, and provide new insights into its catalytic functions and potential interaction with other lipid hydrolases.
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REFERENCES


The abbreviations used are: AC, acid ceramidase; CHO, Chinese hamster Ovary; ASM, acid sphingomyelinase; PNG F, peptide-N-glycosidase F; Endo-H, endo-\(\alpha\)-N-acetylglucosaminidase H; Con A, concanavalin A-sepharose; FPLC, fast protein liquid chromatograph; HPLC, high performance liquid chromatograph; TLC, thin layer chromatograph; PAGE, polyacrylamide gel electrophoresis; NPD, Niemann-Pick disease; LSP, low salt peak; HSP, high salt peak.

unpublished observation
FIGURE LEGENDS

FIG. 1. **Purification of recombinant, human AC from CHO6 medium.** An equal amount of protein (5 μg) from each step of the purification procedure was loaded onto individual lanes to permit direct comparison. All samples were separated by SDS-PAGE using a 4-20% Tris-Glycine gel under reducing conditions. Protein bands were visualized using Colloidal Blue. The migration distances of molecular mass standards (in kDa) are shown to the right. *Lane* 1, CHO6 medium; *lane* 2, Con-A eluate; *lane* 3, Blue Sepharose eluate (“low salt” peak, see text and Fig 7); *lane* 4, Superose 12 eluent.

FIG. 2. **Treatment of recombinant, human AC with Endo H, PNG F, and sialidase.** 5 μg of purified, recombinant human AC (post Superose 12) was treated with Endo H, PNG F, or sialidase as described under “Experimental Procedures”. After digestion, the samples were analyzed by SDS-PAGE using 4-20% Tris-Glycine gel under reducing conditions, and stained with Colloidal Blue. The migration distances of molecular mass standards (kDa) are shown to the right. *Lane* 1, untreated AC; *lane* 2, Endo H treated; *lane* 3, PNG F treated; *lane* 4, sialidase treated.

FIG. 3. **Kinetic analysis of recombinant, human AC.** Purified, recombinant human AC (post Superose 12) was incubated for 20 min at 37°C with varying amounts of fluorescent (BODIPY-conjugated) or [14C]-labeled C12 ceramide substrates. The final reaction
mixtures contained 0.1 M citrate/phosphate buffer, pH 4.5, enzyme source, substrate, 150 mM NaCl, 0.05% BSA, and 0.1% Igepal CA-630. Lineweaver-Burk (double-reciprocal) plots are shown. A, Varying amounts of BODIPY-conjugated C12 ceramide were incubated with pure AC in 10 μl of reaction buffer. B, Varying amounts of [Lauryl-1-\textsuperscript{14}C]D-erythro sphingosine ([\textsuperscript{14}C]-C12 ceramide) were incubated with pure AC in 10 μl of reaction buffer. n=3 for each substrate.

**FIG. 4.** Effect of pH on ceramide hydrolysis and synthesis using pure AC. A, AC hydrolysis reaction was carried at 37°C for 1 h using [\textsuperscript{14}C]-labeled C12 ceramide as the substrate and pure AC (post Superose 12) as the enzyme source. B, The AC “reverse reaction” was carried out using the same conditions as for the hydrolysis reaction, except that instead of [\textsuperscript{14}C]-labeled ceramide, [\textsuperscript{14}C]-labeled C12 fatty acid and sphingosine were used as substrates. Cer, ceramide; FA, fatty acid.

**FIG. 5.** Evaluation of ASM activity and gene expression in CHO6 cell media. A, AC and ASM activities in the media of parental CHO and CHO6 cells are shown. The media was collected and analyzed after 72 h of cell culture. AC and ASM activities were measured at 37°C for 1 h as described under “Experimental Procedures”. Black bars, AC activity; gray bars, ASM activity. Values are expressed as the mean ±SD (n=3). B, RNA was extracted from CHO and CHO6 cells and Northern blot analysis was performed as described under “Experimental Procedures”. The blots were exposed to phosphor imaging screens and the signal intensities quantified. G3PDH was used as a
loading control, and the ASM/G3PDH ratios are shown. *Lane 1*, parental CHO; *lane 2*, CHO6.

**Fig. 6.** Adenoviral vector-mediated expression of human AC in normal and Type A NPD skin fibroblasts. Skin fibroblasts from a normal individual and a patient with Type A NPD were transduced with 0.5 ul of control (empty) recombinant adenovirus and 0.3 and 1.0 ul of a recombinant adenovirus containing the full-length, human AC cDNA (hAC-AV). In panel A, AC activities in the culture media were measured with BODIPY-C12 ceramide 5 days after infection. In, panel B, ASM activities in the same culture media was determined with BODIPY-C12 sphingomyelin. Values are expressed as the mean ±SD (n=3). Details are described in the “Experimental Procedures”.

**Fig. 7.** Elution of AC and ASM activities from Blue Sepharose. A, Partially purified AC (post-Con A) from the CHO6 media was loaded onto a 5 ml Blue Sepharose column and the AC and ASM activities were determined in individual fractions eluted using a salt gradient (see “Experimental Procedures” for details). Solid lines, AC activity; dotted lines, ASM activity. LSP, low salt peak; HSP, high salt peak. B, LSP and HSP fractions from the Blue Sepharose column were further purified by gel filtration (Superose 12), and SDS-PAGE analysis was carried out using a 4-20% Tris-Glycine gel under reducing conditions. Protein bands were stained with Colloidal Blue. The molecular masses of the protein standards (kDa) are shown to the right.
FIG. 8. **Uptake of AC and ASM by mouse macrophages.** Alveolar macrophages were obtained from normal mouse lungs and incubated for 72 h with parental CHO or CHO6 media. The cells were then collected, washed extensively, and homogenized in 0.2% Triton X-100. AC and ASM activities were assayed as described under “Experimental Procedures.” Values are expressed as the mean ±SD (n=3).
**Table I**

*Typical purification of recombinant, human AC from the medium of CHO6 cells*

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (nmol/h)</th>
<th>Specific activity (nmol/mg/h)</th>
<th>Enrichment (-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>330.9</td>
<td>11614.6</td>
<td>35.1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>13.1</td>
<td>8457.4</td>
<td>645.6</td>
<td>18</td>
<td>72</td>
</tr>
<tr>
<td>Blue Sepharose</td>
<td>4.3</td>
<td>5172.9</td>
<td>1203.2</td>
<td>34</td>
<td>44</td>
</tr>
<tr>
<td>Superose 12</td>
<td>2.3</td>
<td>4661.6</td>
<td>2026.8</td>
<td>58</td>
<td>40</td>
</tr>
</tbody>
</table>

*a Activity determined using BODIPY-C12 ceramide as a substrate (see “Experimental Procedures”).

*b Starting volume 250 ml

*c Percent of initial activity*
**Table II**

*Immunoprecipitation of lysosomal enzyme activities from CHO6 media using anti-AC antibodies*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Pre-Immune serum (nmol/h/ml&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Anti-AC serum (nmol/h/ml)</th>
<th>Ratio (immune/preimmune)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Ceramidase&lt;sup&gt;22&lt;/sup&gt;</td>
<td>0.65±0.01</td>
<td>35.03±4.23</td>
<td>54.0</td>
</tr>
<tr>
<td>Acid Sphingomyelinase&lt;sup&gt;23&lt;/sup&gt;</td>
<td>0.88±0.06</td>
<td>31.40±3.65</td>
<td>35.7</td>
</tr>
<tr>
<td>β-Glucosidase&lt;sup&gt;35&lt;/sup&gt;</td>
<td>0.22±0.03</td>
<td>0.25±0.03</td>
<td>1.1</td>
</tr>
<tr>
<td>α-Galactosidase&lt;sup&gt;36&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>β-Galactosidase&lt;sup&gt;37&lt;/sup&gt;</td>
<td>0.65±0.04</td>
<td>62.80±5.15</td>
<td>96.6</td>
</tr>
<tr>
<td>α-Iduronidase&lt;sup&gt;38&lt;/sup&gt;</td>
<td>0.80±0.27</td>
<td>0.70±0.16</td>
<td>0.9</td>
</tr>
<tr>
<td>N-Acetylgalactosamine 4-Sulfatase&lt;sup&gt;39&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> per ml of protein A sepharose gel; see “Experimental Procedures” and individual references for specific assay procedures.

Data represents mean of three independent experiments.

<sup>b</sup> Not detectable.
Fig. 1

1 2 3 4

AC precursor
AC β-subunit
AC α-subunit
**Fig. 3**

**A**

\[ Y = 0.014X + 0.000036 \]

\[ K_m = 389 \mu M \]

\[ V_{max} = 27,778 \text{ nmol/mg/h} \]

**B**

\[ Y = 0.0005 + 0.2066X \]

\[ K_m = 413.2 \mu M \]

\[ V_{max} = 2000 \text{ nmol/mg/h} \]
Fig. 4

A

B

Cer

FA

pH

3.0  3.5  4.0  4.5  5.0  5.5  6.0  6.5  7.0

pH

3.0  3.5  4.0  4.5  5.0  5.5  6.0  6.5  7.0
Fig. 5

A

Enzyme Activities in Media (pmol/ml/h)

<table>
<thead>
<tr>
<th>Activity</th>
<th>CHO</th>
<th>CHO6</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASM</td>
<td>2000</td>
<td>10000</td>
</tr>
<tr>
<td>G3PDH</td>
<td>4000</td>
<td>14000</td>
</tr>
</tbody>
</table>

B

Ratio (ASM/G3PDH)

<table>
<thead>
<tr>
<th>Sample</th>
<th>ASM/G3PDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.75</td>
</tr>
<tr>
<td>2</td>
<td>0.72</td>
</tr>
</tbody>
</table>
Fig. 6

**A**

AC activity (pmol/ml/h)

**B**

ASM activity (pmol/ml/h)

- NPD Control
- NPD + 0.3 µl hAC-AV
- NPD + 1.0 µl hAC-AV
- Normal Control
- Normal + 0.3 µl hAC-AV
- Normal + 1.0 µl hAC-AV

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Fig. 7

A

Blue Sepharose Eluant Volume (ml)

AC activity (pmol/ml/h)

ASM activity (pmol/ml/h)

0 20 40 60 80 100 120

0 10 20 30 40 50 60 70 80

LSP HSP

B

LSP HSP

AC precursor
AC β-subunit
AC α-subunit
Fig. 8

A

BC activity (pmol/mg/h)

Control

+ CHO Med

+ CHO6 Med

B

ASM activity (pmol/mg/h)

Control

+ CHO Med

+ CHO6 Med
Purification and characterization of recombinant, human acid ceramidase: Catalytic reactions and interactions with acid Sphingomyelinase
Xingxuan He, Nozomu Okino, Rajwinder Dhami, Arie Dagan, Shimon Gatt, Heike Schulze, Konrad Sandhoff and Edward H. Schuchman

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