Autoproteolytic Cleavage and Activation of Human Acid Ceramidase*

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Herein we report the mechanism of human acid ceramidase (AC; N-acylsphingosine deacylase) cleavage and activation. A highly purified, recombinant human AC precursor underwent self-cleavage into α and β subunits, similar to other members of the N-terminal nucleophile hydrolase superfamily. This reaction proceeded with first order kinetics, characteristic of self-cleavage. AC self-cleavage occurred most rapidly at acidic pH, but also at neutral pH. Site-directed mutagenesis and expression studies demonstrated that Cys-143 was an essential nucleophile that was required at the cleavage site. Other amino acids participating in AC cleavage included Arg-159 and Asp-162. Mutations at these three amino acids prevented AC cleavage and activity, the latter assessed using BODIPY-conjugated ceramide. Hydrolysis of this bond is catalyzed by water. Treatment of recombinant AC with the cysteine protease inhibitor, methyl methanethiosulfonate, inhibited both cleavage and enzymatic activity, further indicating that cysteine-mediated self-cleavage is required for ceramide hydrolysis.

Human acid ceramidase (AC; N-acylsphingosine deacylase; EC 3.5.1.23) hydrolyzes the sphingolipid, ceramide, into sphingosine and free fatty acid. AC is considered a lysosomal enzyme since it has optimal in vitro activity at acidic pH, and most of the lipid storage in Farber disease patients (the genetic disorder resulting from the deficiency of this enzyme) occurs within late endosomes and/or lysosomes (1). It is therefore thought that the main function of AC is to participate in lysosomal membrane turnover. A low level, secreted form of AC also has been described (2–4), although its biological function remains unknown.

In addition to its important housekeeping function in sphingolipid metabolism, AC participates in signal transduction pathways that regulate various physiological and pathological processes. Recently, it was shown that the AC gene (Asah1) is among the first genes expressed in newly formed mouse embryos, and its deficiency causes embryo death at the two-cell stage (5). In addition, many studies have reported the involvement of AC in complex diseases. For example, AC is overexpressed in several types of human cancer (prostate, head and neck squamous cell, etc.) (6, 7), and cancer therapy based on the inhibition of AC activity has recently been proposed (8, 9). Moreover, impaired ceramide metabolism has been implicated in the pathogenesis of diabetes, Alzheimer disease, atherosclerosis, thrombosis, and cardiomyocyte apoptosis (10–14). Although the precise role of AC in these common diseases is unknown, the enzyme is likely to act as a rheostat controlling the levels of ceramide, sphingosine, and sphingosine-1-phosphate in cells and/or extracellular spaces (15).

AC purified from human urine was an ~50-kDa glycoprotein that could be resolved into individual 14- and 40-kDa subunits (α and β, respectively) under reducing conditions (2). The isolation of the full-length cDNAs and genes encoding human and murine AC revealed that the two subunits were derived from a single precursor by proteolytic processing (2). However, the mechanism of AC precursor cleavage remains unclear.

A PSI-BLAST search (16) for the AC sequence revealed high homology with the N-terminal nucleophile (Ntn) hydrolase family (17). The Ntn hydrolases are a diverse superfamily of enzymes that differ in their substrates, but the mechanism of their activation is conserved. Each is synthesized as a preprotein, and an autocatalytic, endoproteolytic process transforms them into mature multimers. An amino group of an amino acid nearby the cleavage site acts as the proton acceptor and activates the nucelophilic thiol in a Cys residue at the cleavage site (or the nucleophilic hydroxyl in a Ser or Thr residue). After the cleavage of the zymogen, the nucleophilic Cys is exposed at the N-terminal side of the β subunit, which serves as the enzyme active site. All known members of the Ntn hydrolase family catalyze the hydrolysis of amide bonds in either proteins or small molecules (18, 19).

Through a combination of protein purification, site-directed mutagenesis, and biochemical studies, the data in this study demonstrate that AC undergoes autoproteolytic cleavage and activation similar to other members of the Ntn hydrolase superfamily. Namely, AC undergoes the transition from an
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inactive precursor into a mature heterodimer through autocatalytic self-cleavage. These studies provide a novel perspective on the function of AC and its regulation. They also provide new insights into the effects of individual Faber disease mutations on AC folding and suggest novel ways of regulating AC activity and sphingolipid signaling. In addition, they should facilitate the design of a new class of AC-specific inhibitors that could potentially be used in cancer and other therapies.

EXPERIMENTAL PROCEDURES

Antibodies—The following antibody reagents were used: anti-AC goat polyclonal IgG from Santa Cruz Biotechnology, catalog number sc-28486; anti-AC mouse monoclonal IgM from BD Transduction Laboratories, catalog number 612302; donkey anti-goat IgG-horseradish peroxidase conjugate from Santa Cruz Biotechnology, catalog number sc-2020; goat anti-mouse IgG-horseradish peroxidase conjugate from Santa Cruz Biotechnology, catalog number sc-2005.

Protein Expression and Purification—The human AC cDNA was overexpressed in Chinese hamster ovary (CHO) cells using a methotrexate gene amplification system (20), and the recombinant enzyme was purified from the culture media of the overexpressing cells. Polyclonal anti-AC antibody (Santa Cruz Biotechnology) was covalently cross-linked to protein A-coupled magnetic beads (Dynal, Invitrogen) according to the manufacturer’s instructions and incubated with overexpressing (CHO6) media collected after 24 h of culture. AC bound to the antibody–ProteinA-bead complex was eluted using 0.1M citrate-phosphate buffer, pH 4, containing 150 mM NaCl, 0.1% Igepal, 10% glycerol.

In Vitro, Autoproteolytic Cleavage Analysis—Purified AC precursor or cell lysates were incubated at 4 or 37 °C. At various times, an aliquot was withdrawn and subjected to SDS-PAGE and Western blotting as described below.

Western Blot Analysis—Samples were separated by SDS-PAGE using 12% precast NuPAGE Bis/Tris gels under reducing conditions and MES running buffer (Invitrogen) and then transferred onto nitrocellulose membranes (Amersham Biosciences) using a semidy Courier transfer apparatus (Bio-Rad) and NuPAGE-MOPS transfer buffer. For immunoblot analysis, membranes were blocked with Tris-buffered saline/Tween containing 5% dry milk and then incubated with mouse monoclonal IgG against AC (specific for the α subunit). Bound antibodies were recognized by secondary antibodies conjugated to horseradish peroxidase. Detection was performed by an enhanced chemiluminescence (ECL) detection reagent (Amersham Biosciences) using a Kodak M35A X-OMAT processor. Approximate molecular masses were determined by comparison with the migration of prestained protein standards (Bio-Rad).

Silver Staining—For detection of proteins by silver staining, at least 1 μg of total protein from cell lysates or 50 μg of pure protein was subjected to SDS-PAGE separation using 12% precast NuPAGE Bis/Tris gels under reducing conditions and MES running buffer (Invitrogen). The proteins were visualized using a silver staining kit (Owl, Portsmouth, NH) according to the manufacturer’s instructions.

DNA Cloning and Site-directed Mutagenesis—The full-length human AC cDNA was cloned in-frame into the pcDNA4/HisMaxTOPO vector (Invitrogen). To introduce point mutations into the AC cDNA, the QuikChange® site-directed mutagenesis kit from Stratagene was used (catalog number 200518). Primers were constructed for PCR site-directed mutagenesis according to the manufacturer’s instructions. The newly synthesized cDNA constructs were confirmed by sequencing.

Transient Transfection and AC Overexpression—The hAC-pcDNA/HisMaxTOPO constructs were preincubated with the Lipofectamine 2000 (Invitrogen) transfection reagent in Opti-mem media according to the commercial instructions. DNA-Lipofectamine 2000 complexes were then added to 293T cells cultured overnight in 0.5 ml of antibiotic free Dulbecco’s modified Eagle’s medium. After 24 h, the treated cells were harvested, centrifuged at 800 × g for 5 min at 4 °C, and kept at –20 °C. For protein extraction from 293T cells, cell pellets were lysed with the Celllytic reagent (Sigma) and centrifuged (18,000 × g) to obtain protein lysates.

AC Activity Assay—The AC activity assay was performed as described previously (20). Briefly, pure protein or cell lysates were incubated for 22 h at 37 °C with 0.1 ng/μl BODIPY-conjugated C12-ceramide in 0.1 M citrate/phosphate buffer, pH 4.5, 150 mM NaCl, 0.05% bovine serum albumin, and 0.1% detergent Igepal CA-630 (Galg). After the reactions were complete, 5 μl of the assay mixtures was removed and added into 95 μl of ethanol, mixed, and then centrifuged for 5 min at 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 autosampled by a WIPS 712 (Waters) autosampler onto a high-performance liquid chromatograph equipped with a reverse-phase column (BetalBasic-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 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.

Kinetics of AC Processing—The kinetics of AC cleavage were analyzed to determine whether AC processing could be described as a first order reaction, characteristic of self-cleavage. To calculate the reaction order, p, we compared the reaction rate for two concentrations of the precursor (C1 and C2): r1/r2 = 2p. For a first order reaction, if [C1]/[C2] = 1/2, then r1/r2 = 1/2 and p = 1. To calculate the rate of AC cleavage, we used the following equation: r = k[C], where [C] is the concentration of precursor, estimated by Western blot densitometry, and k is the rate constant, represented by the natural logarithm of the precursor concentration, ln(dC)/dt, as a function of incubation time, dC/dt.
RESULTS

Protein Expression and Purification—To test the possibility of AC self-cleavage, we purified recombinant human AC from the media of overexpressing CHO cells using a new method designed for purification of mainly the precursor form (Fig. 1A). For maximal purification, we used magnetic separation technology and a goat polyclonal antibody against the AC protein. Silver staining showed that there was significant enrichment of AC in the elution fractions when compared with the input (Fig. 1A). Three AC-related bands were identified, representing the AC precursor, $\beta$ and $\alpha$ subunits at apparent molecular masses of $\sim$55, 40, and 14 kDa, respectively. Other bands evident in the silver-stained gels represent antibody and protein A leaking from the beads. Western blotting analysis using mouse monoclonal IgM against AC precursor and $\alpha$ subunit (14-kDa) are indicated. The $\alpha$ subunit bands on Western blot as determined using the anti-AC subunit (Fig. 1B). These amino acids are conserved among many members of the Ntn hydrolase superfamily (17). This allowed us to predict amino acids that might be in close proximity to Cys-143 and could also be involved in the catalysis of self-cleavage. Those candidates should be able to accept proton from Cys-143 and could also be involved in the catalysis of self-cleavage. Based on this analysis, likely candidates included: Arg-159, His-157, and Asp-162 (Fig. 3, B and C). These amino acids were therefore mutated to a non-functional Gly, and the effects on processing were analyzed. We also mutated Cys-143 to other nucleophilic amino acids (Thr or Ser) or to the non-functional Ala. By doing this, we aimed to obtain a mature, functionally active AC with a nucleophilic amino acid at the cleavage site other then Cys. This would allow us to test whether Cys-143 is essential for the self-cleavage by AC cleavage by comparing the levels of precursor and $\alpha$ subunit (the latter representing the processed form of AC) over time.

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Fig. 2. Self-processing of purified recombinant human AC. Western blot of elution fraction 2 from Fig. 1, before and after incubation at 37°C for 96 h. Western blotting was performed using a mouse monoclonal antibody against AC $\alpha$ subunit. A representative experiment is shown.
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A. Cleavage site

<table>
<thead>
<tr>
<th>Protein Type</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human AC (Q13510)</td>
<td>YEFTITCSTI...</td>
</tr>
<tr>
<td>Monkey AC (Q60H14)</td>
<td>YEFTTLCSTI...</td>
</tr>
<tr>
<td>Mouse AC (Q968Y54)</td>
<td>YEFTITCSTI...</td>
</tr>
<tr>
<td>Human AC-like protein (Q02083)</td>
<td>YEVSVFCTSI...</td>
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<tr>
<td>Mouse AC-like protein (Q09D7V9)</td>
<td>YEASAFCTSI...</td>
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<tr>
<td>Rat AC-like protein (Q5KTC7)</td>
<td>YEASAFCTSI...</td>
</tr>
<tr>
<td>C elegans AC (O45686)</td>
<td>YEFTVCCTSV...</td>
</tr>
<tr>
<td>C elegans uncharact. prot. (Q09551)</td>
<td>YEEMRFCSTI...</td>
</tr>
</tbody>
</table>

B. Predicted AC cleavage site and structural model based on cholyglycine hydrolase. Cluster alignment of the cleavage site sequence for several AC family proteins is shown (A) using T-COFFEE Version_1.41, CPU = 13.45 s, SCORE = 48, Nseq = 8, Len = 406. uncharact. prot., uncharacterized protein. B and C, a model of AC structure based on cholyglycine acylase and data obtained from QuickPhyre. Job code: a60be35ee0e734a0, SCOP Code c2bjfa, E-value 4.6e-08, Identity 13%, Estimated Precision 100%; the predicted C subunit structure is shown (B) along with magnification of the cleavage site (C). The amino acids in bold were subjected to site-directed mutagenesis. Distance between the atoms also is shown in C, permitting potential hydrogen bonds.

C. Site-directed mutagenesis and AC processing. Western blot analysis using mouse monoclonal antibody against the AC α subunit. A and B, 293T17 cell extracts were analyzed, 24 h after transfection with wild-type (WT) and mutant AC cDNAs (A) and before and after incubation at 37 °C for 72 h (B). Representative experiments are shown.

FIGURE 3. Predicted AC cleavage site and structural model based on cholyglycine hydrolase. Cluster alignment of the cleavage site sequence for several AC family proteins is shown (A) using T-COFFEE Version_1.41, CPU = 13.45 s, SCORE = 48, Nseq = 8, Len = 406. uncharact. prot., uncharacterized protein. B and C, a model of AC structure based on cholyglycine acylase and data obtained from QuickPhyre. Job code: a60be35ee0e734a0, SCOP Code c2bjfa, E-value 4.6e-08, Identity 13%, Estimated Precision 100%; the predicted C subunit structure is shown (B) along with magnification of the cleavage site (C). The amino acids in bold were subjected to site-directed mutagenesis. Distance between the atoms also is shown in C, permitting potential hydrogen bonds.

FIGURE 4. Site-directed mutagenesis and AC processing. Western blot analysis using mouse monoclonal antibody against the AC α subunit. A and B, 293T17 cell extracts were analyzed, 24 h after transfection with wild-type (WT) and mutant AC cDNAs (A) and before and after incubation at 37 °C for 72 h (B). Representative experiments are shown.

Following AC processing in the presence of cysteine protease inhibitors. Mutation at Arg-296, an amino acid that is also conserved in the AC family and in the Ntn hydrolase superfamily, but located far from the cleavage site, served as a negative control.

For the purpose of mutagenesis, we subcloned the human AC cDNA into the pcDNA/HisMaxTOPO vector and introduced single nucleotide changes that altered the specific amino acids. The wild-type and mutant cDNAs were then transiently transfected into 293T17 cells, and the transfection efficiency, as well as the in situ processing of the expressed enzyme, were assessed by subjecting cell lysates to SDS-PAGE and Western blotting using a monoclonal antibody specific for the human AC precursor and α subunit.

As shown in Fig. 4A, the R159G, D162G, C143T, C143S, and C143A mutations each prevented in situ AC precursor cleavage as no α subunit was observed in 293 cell lysates following transient transfection. In contrast, the H157G mutation did not prevent AC processing since the level of α subunit in this cell lysate was similar to that of wild-type. The R296G mutation also underwent processing, although to a somewhat lesser degree than wild type. Substitution of Cys-143 to a different nucleophilic amino acid (e.g. C143S or C143T) also prevented processing.

To test whether the mutations delayed or completely blocked cleavage, we incubated the cell lysates at 37 °C for a total of 72 h (Fig. 4B). No increase in α subunit was observed. Activity assays were also performed on the cell lysates as described previously (2). Each of the mutants at Cys-143, Arg-159, or Asp-162 lacked enzymatic activity. Others had activity comparable with that of wild-type. These results indicate that amino acids Arg-159, Asp-162, and Cys-143 are functional amino acids required for AC proteolytic processing and activity.

Kinetic Analysis of AC Processing—To further characterize AC processing, we examined the kinetics of the transition of AC from a precursor into the α and β subunits. We followed AC processing by monitoring the increase in α subunit at 24- or 72-h intervals, as described above, and plotted the relative density of the bands on Western blots as a function of incubation time.

As shown in Fig. 5A, the in vitro half-life for AC cleavage from precursor to α subunit was about 17 h (Fig. 5A). To deter-
that Cys-143, when exposed on the N terminus of the AC precursor, is a candidate for both of these functions. In our hypothesis, we propose that Cys-143, when exposed on the N terminus of the AC precursor, is a candidate for both of these functions.

We investigated the processing of AC at various pH values. For this purpose, we used media obtained from CHO6 cells that overexpress and secrete human AC and dialyzed into buffers at pH 4.5 or 7, as described under “Experimental Procedures.”

Effect of pH on AC processing. CHO6 cell culture media were dialyzed into citrate-phosphate buffer, pH 4.5, or Hepes buffer, pH 7. AC processing at the different pH values was monitored at 37 °C during 4- and 24-h intervals, and samples were analyzed by SDS-PAGE and Western blotting using mouse monoclonal antibody against the AC α subunit. A representative experiment is shown.

Processing of AC at different pH values was assessed as described above, and the results showed that acidic conditions accelerated the rate of AC processing. However, some precursor processing also occurred at neutral pH (Fig. 7).

DISCUSSION

AC is synthesized as a precursor with an apparent molecular mass of ~55 kDa. It is processed through the vesicular transport system, probably initiated by the presence of an N-terminal signal peptide sequence. When routed into the Golgi, AC undergoes a series of post-translational modifications, including glycosylation and phosphorylation of sugar residues.
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Mature precursor also undergoes cleavage into α and β subunits with apparent molecular masses of 14 and 40 kDa, respectively. Glycosylation of AC seems to be required for precursor processing, probably by controlling proper protein folding (21, 22). Small amounts of AC also are secreted and may be incorporated back into cells and delivered to lysosomes via mannose-6-phosphate receptors present on the cell surface. AC translocation into the lysosomes is thought to be a prerequisite for precursor processing. The presence of mature, cleaved heterodimer in the cell culture media was attributed to acidic proteases that might be secreted into the media (21). To date, there are no data regarding the mechanism of AC cleavage and the potential protease involved, as well as the precise subcellular location of the cleavage event.

Herein we provide new insights into the mechanism of AC processing and activation. Purification of an enriched precursor fraction of AC allowed us to demonstrate that this enzyme is capable of self-cleavage. Site-directed mutagenesis also identified functional amino acids involved in AC self-cleavage. Furthermore, kinetic analysis of AC processing confirmed that AC cleavage is most likely an autoproteolytic event. In addition, inhibition studies showed that Cys-143 is required for both AC processing and AC activity. Finally, we observed that AC cleavage can be accelerated by acidic pH but also occurs at neutral pH.

Based on the results of site-directed mutagenesis, Cys-143, Arg-159, and Asp-162 are the functional triad catalyzing AC cleavage and activation, and for this matter, could be defined as a catalytic center of the enzyme. Mutation at these amino acids resulted in the loss of AC activity, strongly suggesting that the position of the cysteine is indeed Cys-143 by our model of AC self-cleavage only requires that Cys-143 be in a free state in the precursor form. Whether this residue is involved in a disulfide bond after cleavage and thus serves as an active site for ceramide hydrolysis remains unknown, although our model of secondary structure based on sequence homology with other proteins suggests that Cys-143 is far from other cysteines in the β subunit (Fig. 3). It is probable that it also does not form disulfide bonds with the ω subunit as replacing Cys-143 by other amino acids did not affect AC post-translational modifications (e.g. glycosylation); i.e. the precursor showed the same migration pattern on SDS-PAGE as wild-type protein. The full answer could be obtained from solving the crystal structure of the protein.

It is notable that a recent publication examining the structure of recombinant AC purified from insect cells by mass spectroscopy found that Cys-143 was likely involved in a disulfide bond (23). However, in this study, the recombinant AC studied was fully processed into the α and β subunits. In contrast, our studies were performed on a highly enriched precursor form (obtained from the media of CHO cells), and it is important to note that our model of AC self-cleavage only requires that Cys-143 be in a free state in the precursor form. Whether this residue is involved in a disulfide bond after cleavage and thus serves as a nucleophile for ceramide hydrolysis remains a question that awaits the final crystal structure.
Further, we verified that AC processing exhibits the kinetics of a first order reaction, characteristic of any self-cleavage process. This finding is in agreement with our other conclusions about the autoproteolytic activity of AC. Finally, we observed that AC processing occurs most efficiently about the autoproteolytic activity of AC. Finally, we observed that some AC processing proceeds at neutral pH, suggesting that AC activation may occur in other intracellular compartments. To fully investigate this point, in the future, careful subcellular localization studies must be carried out under various growth conditions to assess the state of AC processing in various cell compartments.

In normal cells, the levels of ceramide are carefully regulated. Ceramide may be formed by several pathways, but the only way to degrade ceramide into sphingosine is through the activity of ceramidases. Maintaining a balance between the levels of ceramide, sphingosine, and sphingosine-1-phosphate is an important component of cell survival or death. AC activity cannot be compensated by other ceramidases as deletion of AC is lethal at early stages of embryonic development in the mouse, and reduced AC activity in Farber disease patients often leads to death during early childhood. The data presented here provide new insights into one important mechanism of ceramide regulation: i.e. AC cleavage and activation.

Moreover, in some types of cancer, up-regulation of the AC gene renders the tumors more resistant to ceramide- and/or chemotherapy-induced apoptosis. Thus, inhibition of AC might restore the sensitivity of cancer cells to exogenous ceramides or treatment with chemotherapeutic agents known to produce ceramide (24). Current techniques for AC inhibition are based on using ceramide analogs as inhibitors or anti-AC small interfering RNA. Ceramide analogs, although useful in cell culture, have questionable specificity and may affect multiple cellular pathways (8, 9, 25). Small interfering RNAs have limited stability and inhibit gene expression, not protein function. Based on our results, which support AC autocatalytic self-cleavage, we are now able to identify functional amino acids required for AC activation and are initiating the analysis of a new class of AC inhibitors that act by inhibiting AC processing. Such inhibitors would be potentially more specific and more tolerable when used in vivo.

REFERENCES
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