THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 17, pp. 11253–11259, April 25, 2008

Autoproteolytic Cleavage and Activation of Human Acid Ceramidase*

Received for publication, November 8, 2007, and in revised form, January 24, 2008 Published, JBC Papers in Press, February 14, 2008, DOI 10.1074/jbc.M709166200

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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. We propose the following mechanism for AC self-cleavage and activation. Asp-162 likely forms a hydrogen bond with Cys-143, initiating a conformational change that allows Arg-159 to act as a proton acceptor. This, in turn, facilitates an intermediate thioether bond between Cys-143 and Ile-142, the site of AC cleavage. 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;[3] *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 nucleophilic 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 hydrolyase 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

* This research was supported by Grant R01 DK54830 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Portions of this work were performed in partial fulfillment of the requirements of Tel Aviv University for a Ph.D. thesis.

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[3] The abbreviations used are: AC, acid ceramidase; Ntn, N-terminal nucleophile; CHO, Chinese hamster ovary; MMTS, methyl methanethiosulfonate; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; Bis/Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.

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 Farber disease mutations on AC folding and processing 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 0.1 M 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 semidry 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 IgM 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 ng 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 subcloned 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 Optimem 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 media. After 24 h, the treated cells were harvested, centrifuged at 800 $\times$ *g* for 5 min at 4 °C, and kept at −20 °C. For protein extraction from 239T cells, cell pellets were lysed with the CelLytic reagent (Sigma) and centrifuged (18,000 $\times$ *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 (Sigma). 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 $\times$ *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 (BetaBasic-18, 4.6 $\times$ 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 was 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): $r_1/r_2 = 2^p$. For a first order reaction, if [C1]:[C2] = 1:2, then r_1:r_2 = 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, $ln(dC)$, as a function of incubation time, dt, ($k = ln(dC)/dt$).

AC Processing at Different pH Values—Culture media from cells overexpressing recombinant AC was collected after 24 h of confluent culture and dialyzed into a buffer containing 150 mM

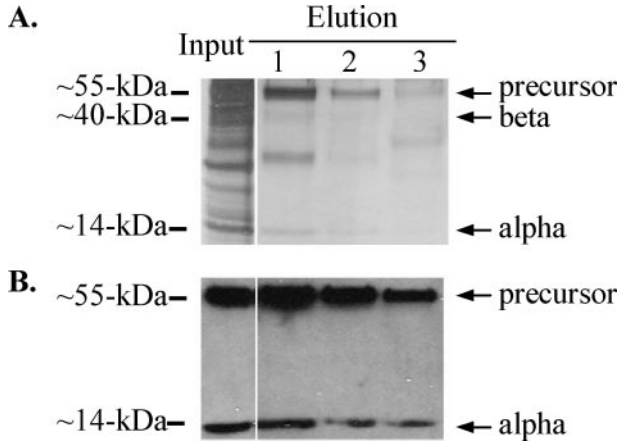


FIGURE 1. **Silver-stained SDS-PAGE (*A*) and Western blot (*B*) analysis of purified recombinant human AC.** *Input*, 400 μg of total protein from CHO6 cell lysates. *Elution fractions 1–3*, 10% v/v of protein purified using anti-AC antibody-magnetic bead conjugates (see "Experimental Procedures"). AC precursor (55-kDa), β (40-kDa), and α subunits (14-kDa) are indicated. The precursor and α subunit were confirmed by Western blotting using mouse monoclonal antibody against the AC α subunit (*B*). Other bands evident in the silver-stained gels were IgG and protein A, as revealed by Western blotting (data not shown). A representative purification experiment is shown.

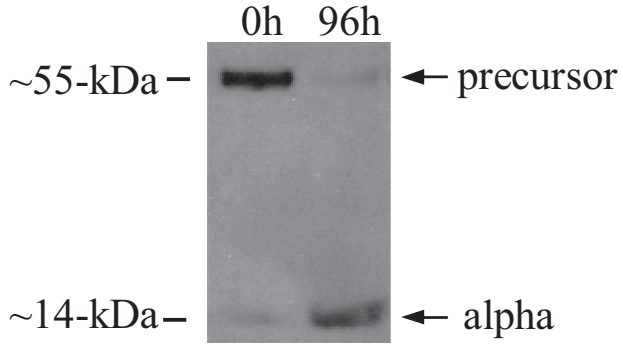


FIGURE 2. **Self-processing of purified recombinant human AC.** Western blot of elution fraction 2 from Fig. 1, before and after incubation at 37 °C for 96 h. Western blotting was performed using a mouse monoclonal antibody against AC α subunit. A representative experiment is shown.

NaCl, 10% glycerol, 0.1% Igepal, and 100 mM citrate/phosphate buffer, pH 4.5, or 100 mM Hepes buffer, pH 7.5. Samples were dialyzed using dialysis cassettes with a molecular mass cut-off of 7 kDa (Pierce). The dialyzed samples were incubated for 4–24 h at 37 °C and subjected to SDS-PAGE separation and Western blotting using mouse monoclonal IgM against AC (specific for the α subunit). Changes in precursor and processed protein were expressed as the relative density of the precursor and α subunit bands on Western blot as determining using the ImageJ software.

Inhibition of AC Processing and Activity—AC processing was followed for 24 h in the presence of 0.1 mM methyl methanethiosulfonate (MMTS) to test the effect of this reversible cysteine protease inhibitor on the transition of precursor into the dimer form. AC activity also was measured, as described above, in the presence of 0.1, 5, or 10 mM MMTS to test its effect on ceramide hydrolysis.

RESULTS

Protein Expression and Purification—To test the possibility of AC self-cleavage, we purified human AC from the media of overexpressing CHO cells using a new method designed for purification of mainly the precursor form (Fig. 1*A*). 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. 1*A*). Three AC-related bands were identified, representing the AC precursor, β and α subunits at apparent molecular masses of ~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 a monoclonal antibody specific for the AC α subunit confirmed that the purification process resulted in highly enriched fractions of pure AC, mostly in the precursor form (Fig. 1*B*). Having the majority of the purified AC in the precursor form allowed us to follow changes in AC cleavage by comparing the levels of precursor and α subunit (the latter representing the processed form of AC) over time.

Autocatalytic Processing of AC—AC contains a conserved domain characteristic of the Ntn hydrolase superfamily (17). To test the possibility of AC autocatalytic processing, similar to Ntn hydrolases, we incubated pure AC (Fig. 1, *Elution fraction 2*) at 37 °C and pH 7, conditions favorable for proteolysis. Samples at time 0 and 96 h were then analyzed by SDS-PAGE and Western blotting using a monoclonal antibody recognizing the AC precursor and α subunit. The results revealed a marked decrease in the amount of precursor and a corresponding increase in the α subunit after the incubation period (Fig. 2).

Site-directed Mutagenesis—To further investigate the precursor processing mechanism, as well as to characterize the catalytic site, we generated point mutations in the AC protein. Human AC processing into the α and β subunits involves proteolytic cleavage between amino acid residues 142 and 143. Cysteine 143 at the N terminus of the β subunit is a conserved cleavage site for AC and AC-like proteins from *Caenorhabditis elegans* through human, as shown in Fig. 3*A*. It is also conserved among many members of the Ntn hydrolase superfamily (17). Since there are currently no data regarding AC structure, we modeled the tertiary structure of the AC β subunit using the crystal structure of cholylglycine hydrolase (PDB code: 2bjg), one member of the Ntn hydrolase family, as a template (Fig. 3, *B* and *C*). 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 thus polarize it to become a nucleophile or to stabilize the tertiary structure to allow the angle of the peptide bond to be exposed to nucleophilic attack. In addition, those candidates should be conserved in AC from different species and in other members of the Ntn hydrolase family.

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

A.

	Cleavage site ↓
Human AC (Q13510)	....YELFTICTSI....
Monkey AC (Q60HH4)	....YEFFTLCTSI....
Mouse AC (Q9WV54)	....YELFTMCTSI....
Human AC -like protein (Q02083)	....YESSVFCTSI....
Mouse AC -like protein (Q9D7V9)	....YEASAFCTSI....
Rat AC -like protein (Q5KTC7)	....YEASAFCTSI....
C elegans AC (O45686)	....YEIFTVCTSV....
C elegans uncharact. prot. (Q09551)	....YEMSRFCTSI....

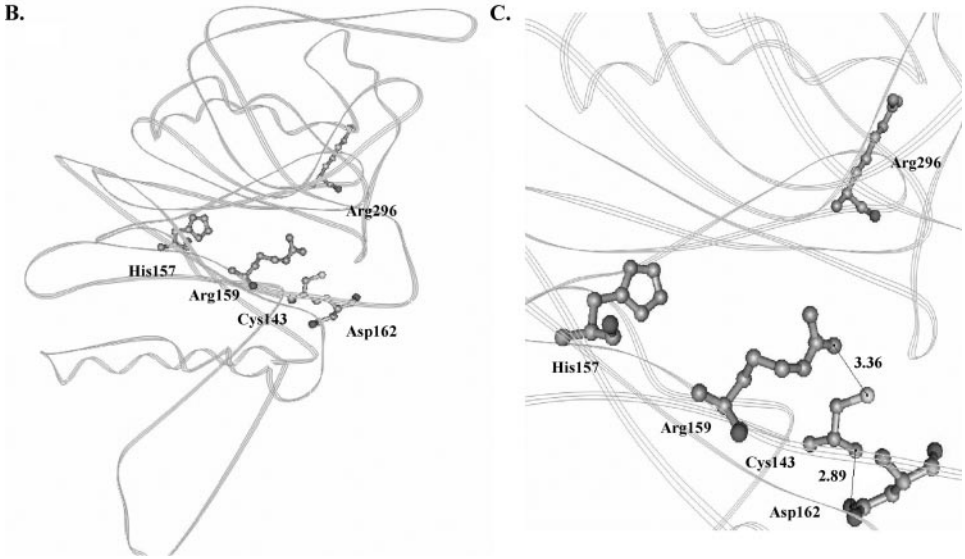


FIGURE 3. **Predicted AC cleavage site and structural model based on cholylglycine hydrolase.** Cluster alignment of the cleavage site sequence for several AC family proteins is shown (*A*) using T-COFFEE Version_1.41, CPU = 13.45 s, SCORE = 48, Nseq = 8, Len = 406. *uncharact. prot.*, uncharacterized protein. *B* and *C*, a model of AC structure based on cholylglycine acylase and data obtained from QuickPhyre, Job code: a60be35ee0e734a0, SCOP Code c2bjfa, E-value 4.6e-08, Identity 13%, Estimated Precision 100%; the predicted β 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.

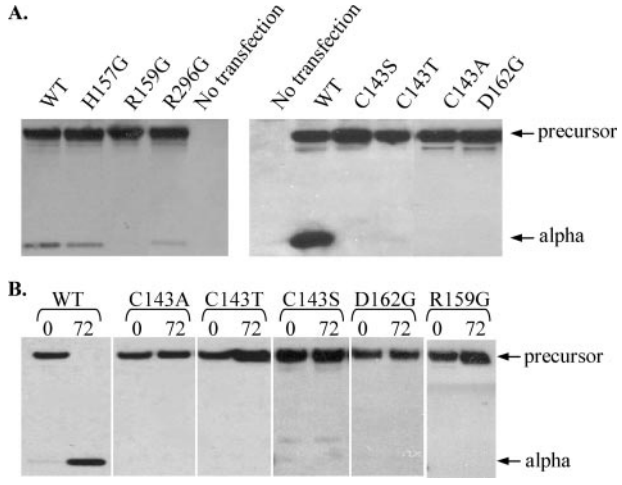


FIGURE 4. **Site-directed mutagenesis and AC processing.** Western blot analysis using mouse monoclonal antibody against the AC α subunit. *A* and *B*, 293T17 cell extracts were analyzed, 24 h after transfection with wild-type (*WT*) and mutant AC cDNAs (*A*) and before and after incubation at 37 °C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

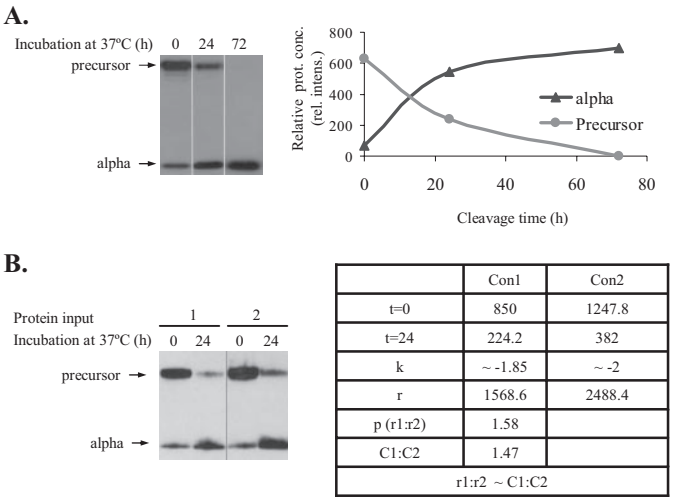
For the purpose of mutagenesis, we subcloned the human AC cDNA into the pcDNA/HisMaxTOPO vector and introduced single nucleotide changes that altered the specific amino acids. The wild-type and mutant cDNAs were then transiently transfected into 293T17 cells, and the transfection efficiency, as well 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. 4*A*, 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. 4*B*). 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. 5*A*, the *in vitro* half-life for AC cleavage from precursor to α subunit was about 17 h (Fig. 5*A*). To deter-



	Con1	Con2
t=0	850	1247.8
t=24	224.2	382
k	~ -1.85	~ -2
r	1568.6	2488.4
p (r1:r2)	1.58	
C1:C2	1.47	
r1:r2 ~ C1:C2		

FIGURE 5. **Kinetic analysis of AC processing.** *A* and *B*, processing of AC from CHO6 cell culture media, incubated at 37 °C for 24- and 72-h intervals (*A*), or processing of pure AC incubated for 24 h (*B*). SDS-PAGE and Western blotting analysis was performed using mouse monoclonal antibody against the AC α subunit. The relative density of the AC precursor and α subunit bands on Western blots was determined and presented as a progress curve of protein processing, as a function of incubation time (*A*). *Relative prot. conc.*, relative protein concentration; *rel. intens.*, relative intensity. The calculation of the reaction order at two different input concentrations is shown in *B*. Representative experiments are shown.

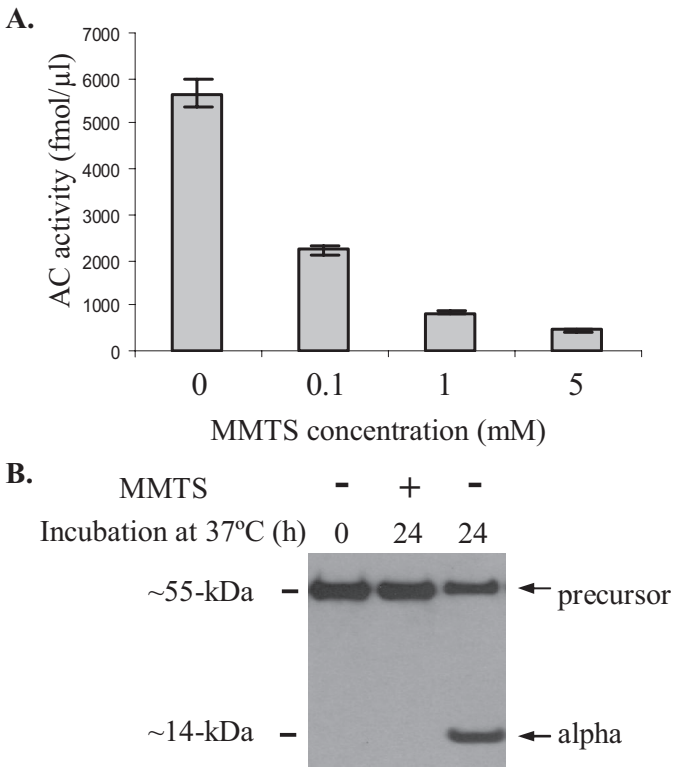


FIGURE 6. **Effect of MMTS on AC activity and processing.** *A*, AC activity was measured in the presence of 0.1, 5, or 10 mM MMTS. *B*, AC processing was followed for 24 h at 37 °C in the presence of 0.1 mM MMTS and analyzed by SDS-PAGE and Western blotting using mouse monoclonal antibody against AC α subunit Representative experiments are shown.

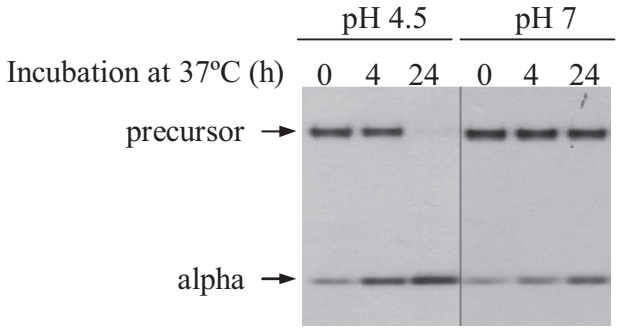


FIGURE 7. **Effect of pH on AC processing.** CHO6 cell culture media were dialyzed into citrate-phosphate buffer, pH 4.5, or Hepes buffer, pH 7. AC processing at the different pH values was monitored at 37 °C during 4- and 24-h intervals, and samples were analyzed by SDS-PAGE and Western blotting using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

mine the reaction order, we then followed the processing of AC at two different input concentrations (Fig. 5*B*) and calculated the reaction rates as described under "Experimental Procedures." We observed that increasing the initial concentration of AC precursor led to a corresponding increase in the reaction rate (Fig. 5*B*). Therefore, the reaction was classified as first order. This type of kinetics is characteristic of autoproteolysis and further defines AC processing as a self-cleavage event that does not involve other proteases.

Inhibition of AC Activity and Processing by the Cysteine Protease Inhibitor MMTS—As part of our hypothesis, we propose that Cys-143, when exposed on the N terminus of the β subunit after cleavage, serves as the active site for ceramide hydrolysis. To test this concept, we monitored AC activity in the presence of increasing concentrations of the cysteine protease inhibitor MMTS (Fig. 6). We also followed AC processing in the presence of MMTS for 24 h. As shown in Fig. 6*A*, AC activity was inhibited by MMTS in a dose-dependent manner. Western blotting analysis further revealed that a 24-h incubation of AC with 0.1 mM MMTS inhibited processing completely (Fig. 6*B*). Taken together, these results confirm that one or more cysteines are involved in both AC autoproteolytic processing and ceramide hydrolysis. Due to its high conservation and location at the N terminus of the β subunit, Cys-143 stands out as a potential candidate for both of these functions.

AC Processing at Different pH Values—The translocation of AC into lysosomes is thought to be a prerequisite for precursor processing. However, ceramide signaling is thought to occur at the cell membrane or other non-acidic compartments. Thus, 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." 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.

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 these data, we propose a mechanism to describe AC autocatalytic transformation from a precursor into active enzyme (Fig. 8). The process could be initiated by conformation change and/or removal of a physiological inhibitor. Based on our model, Asp-162 is located 2.89 Å from Cys-143, allowing formation of a hydrogen bond (Fig. 3*C*). The formation of this bond stabilizes a new conformation that is favorable for the amino group of Arg-159, which is located 3.36 Å from Cys-143, to accept a proton from the side chain sulfydril. The latter becomes a polar nucleophile and can form a thioether intermediate bond with the carbon atom of the carboxyl group of Ile-142. The hydrolysis of the thioether bond is catalyzed by a water molecule, resulting in cleavage of the peptide bond between Cys-143 and Ile-142. Cys-143, exposed on the N terminus of the β subunit, remains a powerful nucleophile and thus may potentially serve as an active site for ceramide hydrolysis.

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 precursor form of AC has no activity as a ceramide hydrolase and that AC processing is a prerequisite of AC activation. Inhibition studies confirmed that the activity of AC as a ceramide hydrolase requires cysteine in the active site as it can be inhibited by the cysteine protease inhibitor MMTS. We attempted to confirm that the position of the cysteine is indeed Cys-143 by substituting Cys-143 to Thr or Ser, assuming that the activity of these mutant proteins would not be affected by MMTS. However, neither of these substitutions permitted precursor processing, supporting the significance of Cys-143 in both AC cleavage and AC activity.

To undergo cleavage, the nucleophilic cysteine should be in a free state, not associated in a disulfide bond. Whether Cys-143 is a free cysteine 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 α 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.

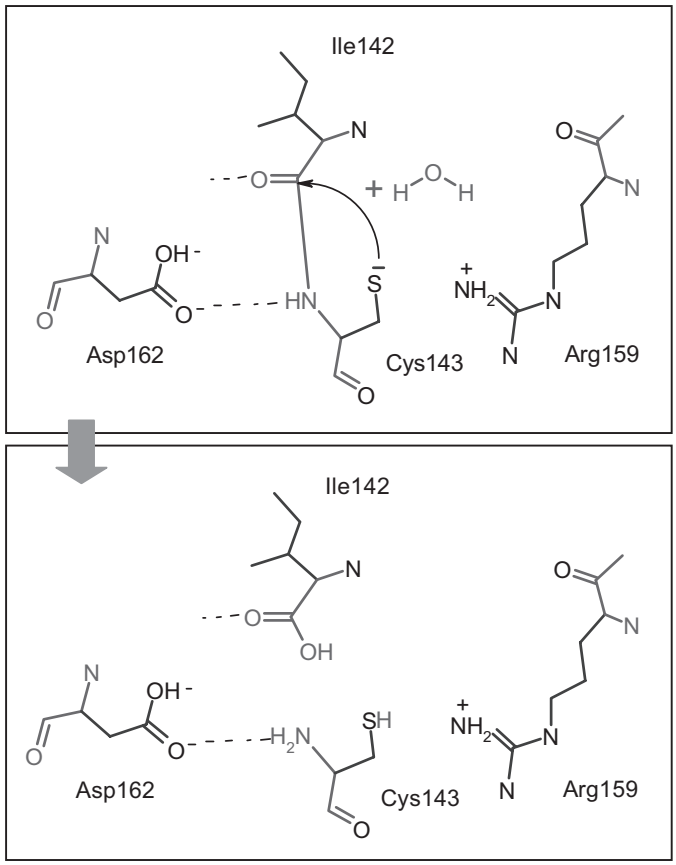


FIGURE 8. **Proposed mechanism of AC processing.** Asp-162, located 2.89 Å from Cys-143, forms a hydrogen bond with the amino group of Cys-143. The formation of this bond stabilizes a new conformation that is favorable for the amino group of Arg-159, which is located 3.36 Å from Cys-143, to accept protons from the side chain sulfydril. The latter becomes a polar nucleophile and can form a thioether intermediate bond with the carbon atom of the carboxyl group of Ile-142. The hydrolysis of the thioether bond is catalyzed by a water molecule, resulting in cleavage of the peptide bond between Cys-143 and Ile-142.

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 *in vitro* at acidic pH, confirming previous reports suggesting that under normal growth conditions, most AC processing likely occurs in acidic organelles (*e.g.* endosomes or lysosomes) (21). However, we also 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*.

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J. Biol. Chem. 2008, 283:11253-11259.
doi: 10.1074/jbc.M709166200 originally published online February 14, 2008
