The Reverse Activity of Human Acid Ceramidase*

Received for publication, March 31, 2003, and in revised form, May 14, 2003
Published, JBC Papers in Press, May 22, 2003, DOI 10.1074/jbc.M303310200

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An overexpression system was recently developed to produce and purify recombinant, human acid ceramidase. In addition to ceramide hydrolysis, the purified enzyme was able to catalyze ceramide synthesis using [14C]lauric acid and sphingosine as substrates. Herein we report detailed characterization of this acid ceramidase-associated “reverse activity” and provide evidence that this reaction occurs in situ as well as in vitro. The pH optimum of the reverse reaction was ~5.5, as compared with ~4.5 for the hydrolysis reaction. Non-ionic detergents and zinc cations inhibited the activity, whereas most other cations were stimulatory. Of note, sphingomyelin also was very inhibitory toward this reaction, whereas the anionic lipids, phosphatidic acid and phosphatidylserine, were stimulatory. Of various sphingosine stereoisomers tested in the reverse reaction, only the natural, d-erythro form could efficiently serve as a substrate. Using d-erythro-sphingosine and lauric acid as substrates, the reaction followed normal Michaelis-Menten kinetics. The $K_m$ and $V_{max}$ values toward sphingosine were 23.75 μM and 208.3 pmol/g/h, respectively, whereas for lauric acid they were 73.76 μM and 232.5 pmol/g/h, respectively. Importantly, the reverse activity was reduced in cell lysates from a Farber disease patient to the same extent as the acid ceramidase activity. Furthermore, when 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)) (NBD)-conjugated lauric acid and sphingosine were added to cultured lymphoblasts from a Farber disease patient in the presence of fumonisin B (1), the conversion to NBD-ceramide was reduced ~30% when compared with normal cells. These data provide important new information on human acid ceramidase and further document its central role in sphingolipid metabolism.

Ceramide is an important cellular lipid involved in signal transduction and the biosynthesis of complex sphingolipids (1, 2). It can be hydrolyzed into sphingosine (Sph), a more important signaling lipid, by the activity of ceramidases. Sph and sphingosine 1-phosphate, a phosphorylated derivative of Sph, exert a variety of effects on cell growth and differentiation (3, 4). Because ceramide degradation is the only catabolic source of Sph, ceramidase activity is considered a rate-limiting step in determining the intracellular levels of this compound (5-7).

Acid ceramidase (N-acylsphingosine amidohydrolase (EC 4.5.29), AC) is one of several enzymes responsible for ceramide degradation within mammalian cells (8). Based on its in vitro pH optimum of ~4.5, the hydrolytic activity of this enzyme is thought to occur within lysosomes and/or late endosomes. An inherited deficiency of AC activity results in the lipid storage disorder, Farber disease, characterized by progressive joint pain, lipid accumulation in various tissues, and early death (9). In 1995, human AC was purified to apparent homogeneity from urine (10). It was found to be a heterodimeric enzyme containing two subunits, α (~13 kDa) and β (~40 kDa), both of which resulted from cleavage of a ~55-kDa precursor polypeptide. The full-length human and murine AC cDNA and genomic sequences have been cloned and characterized (11-13), and several point mutations in the human AC gene have now been found in Farber disease patients (11, 14). In addition, insertional mutagenesis of the mouse AC gene led to an early embryonic lethal phenotype, indicating that AC activity is essential for mammalian development (15).

In the 1960s, Gatt first reported that partially purified AC preparations carried out ceramide synthesis using free fatty acids and Sph as substrates (i.e. the ceramidase-associated “reverse reaction”) (8, 16). However, because these early studies did not use highly purified enzyme, the question of whether a single protein could catalyze the hydrolysis and reverse reactions remained unclear. Recent studies using several cloned neutral and alkaline ceramidases have confirmed these early observations and revealed that these enzymes, which are distinct from AC, also can catalyze both reactions in vitro (i.e. ceramide hydrolysis and synthesis) (17-22). However, based on the acidic pH optimum of AC and the fact that de novo ceramide synthesis is not thought to occur within lysosomes, it has remained unclear whether AC could catalyze the reverse reaction.

Recently, we established a Chinese hamster ovary (CHO) cell line overexpressing the full-length, human AC cDNA, and purified the recombinant enzyme to apparent homogeneity from the culture medium. In the course of these studies we deter-

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* This work was supported in part by National Institutes of Health Grant R01 DK54830, Grant 6-FY-00-241 from the March of Dimes Birth Defects Foundation, and Grant 5R03 TW 01372 from the Fogarty International Center. 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.

¶ Supported by a postdoctoral fellowship from the Japan Society for the Promotion of Science.

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§ The abbreviations used are: Sph, sphingosine; NBD, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)); AC, acid ceramidase; CHO, Chinese hamster ovary; CL, cardiolipin; PS, phosphatidylserine; PA, phosphatidic acid; SPM, sphingomyelin; MES, 2-[N-morpholino]ethanesulfonic acid; PBS, phosphate-buffered saline; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; BODIPY, 4,4-difluoro-4-bora-3a,4a-
diaza-s-indacene.

¶¶ He, X., Okino, N., Dhami, R., Dagan, A., Gatt, S., Schulze, H., Sandhoff, K., and Schuchman, E. H. (2003) J. Biol. Chem., in press.
mined that purified AC could indeed catalyze ceramide synthesis in vitro using lauric acid and Sph as substrates. To better understand the reverse activity of human AC and to determine whether this activity occurred in situ, as well as in vitro, we have now investigated the biochemical and mechanistic characteristics of the AC-associated reverse reaction using purified, recombinant AC and cultured cells obtained from Farber disease patients.

**EXPERIMENTAL PROCEDURES**

Materials—[1-14C]Lauric acid (55 mCi/ml), [3-3H]D-erythro-sphingosine (20 Ci/mmol), and [laurol-1-14C]D-erythro-sphingosine (i.e. 14C-labeled C12 ceramide, 55 mCi/ml) were from American Radiolabeled Chemicals, Inc. (St. Louis, MO), BODIPY- and NBD-conjugated C12 fatty acid were purchased from Molecular Probes, Inc. (Eugene, OR). BODIPY-conjugated C12 ceramide was synthesized as previously described (23). Other lipid standards were from Matreya, Inc. (State College, PA). TLC plates (TLC LK6 D Silica Gel 60) were purchased from Whatman (Clifton, NJ). Tissue culture media and reagents were purchased from Invitrogen (Carlsbad, CA). Tissue culture plastic ware and all organic solvents were purchased from Fisher Scientific Co. (Springfield, NJ), except for an 8-well Lab-Tek II glass chamber slide thawn in a humidified 5% CO₂ atmosphere at 37 °C. Human Epstein-Barr virus-transformed lymphoid cell lines were derived from a normal individual or from a patient with Farber disease and routinely grown in RPMI 1640 medium containing L-glutamine (4 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (10%). For in situ experiments, lymphoid cells (5 × 10⁶ cells/ml in RPMI 1640 media supplemented with 10% fetal calf serum) were incubated with 20 μM NBD-C12-fatty acid and 10 μM Sph for 2 h. In the final assay mixture only a small amount of Triton X-100 (0.05%) was included to solubilize the substrates.

**Reverse AC Assay**—Unless otherwise noted, reverse AC activity was determined using the purified, CHO-derived AC as the enzyme source. A stock solution was prepared containing [14C]lauric acid (1 mmol/l), Sph (0.5 mmol/l), and Triton X-100 (1%). BODIPY and NBD-conjugated C12 ceramide were used as substrates.

**RESULTS**

**EFFECTS OF pH AND DETERGENTS**—Previous studies had indicated that purified, recombinant human AC could carry out ceramide synthesis in vitro using [14C]lauric acid and Sph as substrates and that the pH optimum for this reaction was ~5.5, as compared with 4.5 for the hydrolysis reaction. The effects of various detergents on the reverse reaction were next examined. As shown in Fig. 1, the reaction proceeded most efficiently in the absence of detergents. Triton X-100 and Igepal CA-630, both non-ionic detergents, inhibited the enzyme activity in a concentration-dependent manner. Taurocholate, an anionic detergent, strongly inhibited the enzyme activity at low concentrations, but the activity was partially restored at concentrations around 0.2%. Based on the findings, in the final assay mixture only a small amount of Triton X-100 (0.05%) was included to solubilize the substrates.

**EFFECT OF CATIONS**—The addition of CaCl₂, MgCl₂, and NaCl to the reaction mixtures increased AC reverse activity moderately (Fig. 2A). In contrast, ZnCl₂ was very inhibitory toward this reaction. The addition of EDTA (10 mM) did not affect the reverse activity. To further investigate the salt dependence of this reaction, the activity was measured in the presence of increasing concentrations of NaCl. As shown in Fig. 2B, NaCl activation was concentration-dependent, with ~2-fold activation seen at ~150 mM. Therefore, in the final assay mixture the NaCl concentration was maintained at 150 mM, close to the physiologic salt concentration.

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Kinetics of the Reverse Activity

To study the kinetics of the reverse reaction, the activity was measured using various amounts of each substrate (Fig. 3). For these studies, the concentration of either lauric acid (Fig. 3A) or Sph (Fig. 3B) was fixed at 200 μM in the reaction mixture and that of the other was increased. As can be seen, the Lineweaver-Burk plots of these data were linear and followed normal Michaelis-Menten kinetics. The apparent $K_m$ and $V_{max}$ values of the reverse reaction for Sph were 23.75 μM and 208.3 pmol/μg/h and for lauric acid were 73.76 μM and 232.5 pmol/μg/h.

Substrate Specificity of the Reverse Activity

Fig. 4 shows the substrate specificity of the reverse reaction toward various Sph stereoisomers. Among the stereoisomers tested, only the...
Effects of Lipids—The effects of various phospholipids and sphingolipids on the reverse activity were next investigated and compared with their effects on the hydrolysis reaction (Fig. 5). Fig. 5A shows that the acidic phospholipids, phosphatidylserine (PS) and phosphatidic acid (PA), significantly stimulated the reverse activity. In contrast, cardiolipin (CL), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine, and phosphatidylinositol inhibited this activity. As shown in Fig. 5B, sphingomyelin (SPM) also strongly inhibited the reverse activity, whereas glucosylceramide and C16-ceramide were less effective inhibitors. Notably, of the lipids tested, several were found to have differential effects on the reverse and hydrolysis reactions. For example, PA stimulated the reverse activity, but was inhibitory toward the hydrolysis reaction. SPM and CL, on the other hand, were very inhibitory toward the reverse reaction but stimulated the hydrolysis reaction.

Reverse AC Activity in Farber Disease Cells—To further demonstrate that AC carried out the reverse reaction, we next used normal, human lymphoid cells and lymphoid cells from a patient with Farber disease containing mutations in the AC gene. As shown in Fig. 6A, the reverse activity was reduced in lysates prepared from the Farber disease cells to the same extent as ceramide hydrolysis, further demonstrating that both reactions were catalyzed by the same enzyme.

To investigate whether AC carried out the reverse activity in situ, as well as in vitro, the lymphoid cell lines were incubated with NBD-C12-fatty acid and Sph with and without fumonisin B1. The amount of synthesized NBD-ceramide formed in the cells was then assessed. As shown in Fig. 6B, in the absence of fumonisin B1, the FD cells synthesized about 20% less NBD-ceramide than normal cells. When fumonisin B1 was included to inhibit ceramide synthesis by acyl-CoA-dependent N-acyltransferase (ceramide synthetase), the reduction in the Farber disease cells as compared with normal was ~30%. Note that there was no difference in the amount of ceramide synthesized by normal cells with or without fumonisin B1, indicating that little, if any, NBD-C12-fatty acid received CoA modification and was utilized by ceramide synthetase.

Localization of AC in Cultured Skin Fibroblasts—We next determined the subcellular location of AC in normal, human skin fibroblasts using anti-human AC antibodies in combination with antibodies specific for several subcellular compartments (Fig. 7). As can be seen, most of the AC staining (red) was punctate and perinuclear, indicating that the majority of the enzyme was present in lysosomes or late endosomes. Co-incubation of the anti-AC antibodies with anti-LAMP-2 antibodies confirmed this observation (see yellow, merged image). Under these normal growth conditions, little or no AC colocalized with early endosomes (EEA1) or the endoplasmic reticulum (BiP).

**DISCUSSION**

In the present study we describe characterization of the AC-associate reverse ceramidase activity. Several criteria demonstrate that AC can catalyze ceramide synthesis, as well as degradation. First, highly purified, recombinant AC could synthesize ceramide in vitro using free fatty acids and Sph as substrates. Second, cell lysates prepared from patients with Farber disease containing mutations in the AC gene were deficient in the AC-associated reverse activity to the same extent.
as they were deficient in the hydrolysis reaction. Third, in situ studies using NBD-labeled fatty acid and Sph revealed that the Farber disease cells synthesized ceramide less efficiently than normal cells, confirming the in vitro results. We also demonstrated that this reaction was distinct from the major, de novo ceramide synthesis reaction, because it proceeded in vitro without acyl-CoA, ATP, or Mg^{2+} and was not inhibited by fumonisins B1.  

The genes encoding several ceramidases have been recently cloned from eukaryotic and prokaryotic sources (20, 21, 25–29). Table I compares several of their properties. Among these, all but one (human alkaline ceramidase) can carry out the reverse ceramidase reaction in vitro. The characteristics of these reverse activities have not been fully evaluated, but from the data available some common features are evident: 1) Ceramidase-associated reverse activities proceed in an energy-independent manner and are resistant to fumonisins B1, revealing that the mechanism of the reverse reaction is distinct from that of acyl CoA-dependent N-acyltransferase (ceramide synthetase). 2) The optimum pH of the hydrolysis and reverse reactions are different for each enzyme. In the case of AC, the reverse reaction proceeded at a more alkaline pH than the hydrolysis reaction; for the other ceramidases the reverse reactions preferred a more acidic pH than the hydrolysis reactions. 3) The stereospecificity for the sphingoid base is very strict and the natural, l-erythro form is preferred. 4) In contrast to the sphingoid base, the utilization of fatty acids is quite broad.

Detailed characterization of the AC-associated reverse reaction revealed some characteristics that were in common with the AC hydrolysis reaction but also some that were distinct. For example, the activation effect by NaCl and the preference for Sph (d18:1), as compared with sphinganine (d18:0), were shared by the hydrolysis and reverse reactions. In addition, both activities were inhibited by the addition of ceramide to the reaction mixture. Among the important differences were the pH optima for the reverse and hydrolysis reactions (5.5 and 4.5, respectively), suggesting that the two reactions likely occur in different subcellular compartments. Immunostaining using anti-AC antibodies and several subcellular markers revealed that under normal growth conditions most AC was present in lysosomes or late endosomes. In the future it will be interesting to subject cells to various stress stimuli and monitor the effects on AC localization, because, as has been shown previously for the related lipid hydrolase, acid sphingomyelinase, stress stimulation can lead to dramatic and rapid relocalization of enzymes from lysosomes/late endosomes to other cellular compartments (30).

The effect of various lipids on the AC-associated reverse and hydrolysis reactions also was very revealing. CL, PC, LPC, phosphatidylethanolamine, and SPM moderately activated the hydrolysis activity but inhibited the reverse activity at the same concentrations. Indeed, among the various lipids tested, SPM was the most effective inhibitor of the AC-associated reverse activity. CL and SPM also inhibited the reverse reaction associated with rat brain ceramidase (22), and similar to what we have found with AC, CL activated the hydrolysis reaction of that enzyme as well. CL is known to be a major lipid in mitochondria, and it has been suggested that CL might play a role in the regulation of one or more mitochondria-associated

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**Fig. 6.** AC-associated reverse and hydrolysis activities in lymphoid cells. A, cell lysates were prepared from lymphoid cells obtained from a normal individual (Cont) and from a patient with Farber disease (FD) and incubated with NBD-C12 fatty acid (NBD-FA) and Sph for 2 h as described under “Experimental Procedures.” Values are expressed as the mean ± S.D. (n = 3). B, lymphoid cells (5 × 10⁵) from the control individual and Farber disease patient were incubated with NBD-C12 fatty acid (NBD-FA) and Sph for 2 h as described under “Experimental Procedures.” The NBDFA and formed NBD-ceramide (NBD Cer) in the cells were then identified by co-migration with standards following TLC and quantified by fluorography. The data are expressed as the percentage of NBDCer formed relative to NBDFA. Values are expressed as the mean ± S.D. (n = 3). The absolute mean values were Cont, +FB1 7.14 ± 0.15, FD, −FB1 1.59 ± 0.18; Cont, +FB1 7.56 ± 0.40, FD, +FB1 5.22 ± 0.4. FB1, fumonisins B1.

**Fig. 7.** Localization of AC in normal, human skin fibroblasts. Normal, human skin fibroblasts were incubated with anti-AC antibodies (red) in combination with anti-BiP, anti-EEA1, or anti-LAMP-2 antibodies (green). Co-localization is indicated by yellow in the merged image. Original magnification, ×80.
ceramidases. In contrast to CL and SPM, the anionic glycerophospholipid, PA, stimulated the reverse activity ~30%, but inhibited the hydrolysis reaction. Another anionic glycerophospholipid, PS, which is known to activate some neutral sphingomyelinases (31, 32), also activated the AC-associated reverse reaction to about the same extent as PA.

The differential effects of pH and various lipids on the reverse versus hydrolysis reactions may explain, in part, how these activities are regulated in cells. In lysosomes and late endosomes, PA, which stimulates the AC-associated reverse reaction, markedly inhibited the hydrolysis reaction. Notably that PA, which stimulates the AC-associated reverse activity, as well as ceramide hydrolysis. We also suggest that cells from Farber disease patients are deficient in this enzyme that promotes ceramide production.

In conclusion, we have shown that, similar to other cloned ceramidases, highly purified, recombinant AC can carry out ceramide synthesis using free fatty acids and sphingosine as substrates. This reaction is distinct from the activity of ceramide synthetase (acyl CoA-dependent N-acetyltransferase) based on a number of criteria. We have also demonstrated that the AC-associated reverse reaction can proceed in situ and shown that cells from Farber disease patients are deficient in this activity, as well as ceramide hydrolysis. We also suggest that the differential effects of pH and various lipids on the AC-associated reverse and hydrolysis reactions indicate possible mechanisms by which the two activities might be regulated in cells. We believe that the AC-associated reverse reaction represents a "salvage" pathway for ceramide synthesis that is utilized only following cell stress and/or stimulation of signal transduction pathways requiring ceramide. We further believe that the normal subcellular location of AC in lysosomes/late endosomes might be altered under these conditions, similar to acid sphingomyelinase, moving the enzyme into a compartment that promotes ceramide production versus degradation.

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