Published in final edited form as:
Biochim Biophys Acta. 2008 September ; 1781(9): 424–434. doi:10.1016/j.bbalip.2008.06.002.

Ceramidases: regulators of cellular responses mediated by ceramide, sphingosine, and sphingosine-1-phosphate

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Summary

Ceramidases catalyze hydrolysis of ceramides to generate sphingosine (SPH), which is phosphorylated to form sphingosine-1-phosphate (S1P). Ceramide, SPH, and S1P are bioactive lipids that mediate cell proliferation, differentiation, apoptosis, adhesion, and migration, likely by controlling hydrolysis of ceramides and generation of SPH and S1P. Presently, 5 human ceramidases encoded by 5 distinct genes have been cloned: acid ceramidase (AC), neutral ceramidase (NC), alkaline ceramidase 1 (ACER1), alkaline ceramidase 2 (ACER2), and alkaline ceramidase 3 (ACER3). Each human ceramidase has a mouse counterpart. AC, NC, and ACER1–3 have maximal activities in acidic, neutral, and alkaline environments, respectively. ACER1–3 have similar protein sequences but no homology to AC and NC. AC and NC also have distinct protein sequences. The human AC (hAC) was implicated in Farber disease, and hAC may be important for cell survival. The mouse AC (mAC) is needed for early embryo survival. NC is protective against inflammatory cytokines, and the mouse NC (mNC) is required for the catabolism of ceramides in the digestive tract. ACER1 is critical in mediating cell differentiation by controlling the generation of SPH and S1P and that ACER2's role in cell proliferation and survival depends on its expression or the cell type in which it is found. Here, we discuss the role of each ceramidase in regulating cellular responses mediated by ceramides, SPH, and S1P.

Keywords

apoptosis; bioactive lipid; cell adhesion; differentiation; growth arrest; proliferation

Introduction

Ceramidases comprise a heterogeneous family of enzymes whose main function in cells is to break down ceramides to SPH (2-amino-4-octadecene-1, 3-diol). SPH is an 18-carbon amino alcohol with an unsaturated hydrocarbon chain (Figure 1). Its two natural analogues, dihydrosphingosine (DHS) and phytosphingosine (PHS), both have a saturated hydrocarbon chain, and PHS has one additional hydroxyl group. SPH are mainly found in higher eukaryotes (such as humans and animals) and PHS is chiefly detected in lower eukaryotes (such as yeast,

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fungi, and plants), whereas DHS exists in both higher and lower eukaryotes [1,2]. In yeast or mammalian cells, DHS is synthesized *de novo* through a series of enzymatic reactions starting with the condensation of serine and palmitoyl-CoA [1]. After its formation, DHS is acylated by various fatty acids to form dihydroceramides through the action of dihydroceramide (ceramide) synthases [3]. In mammalian cells, a double bond is then introduced between C4 and C5 of the DHS moiety in dihydroceramides to generate ceramides with the SPH moiety [4]. Ceramides are then incorporated into complex sphingolipids such as sphingomyelins or are hydrolyzed to form SPH through the action of ceramidases [5,6]. The direct conversion of DHS to SPH is not found in mammalian cells. In mammalian cells, therefore, SPH is not synthesized *de novo* but it is generated from ceramides by ceramidases. In addition, SPH can also be derived from sphingosine-1-phosphate (S1P) through the action of S1P phosphatases [7].

In yeast cells, DHS is mainly hydroxylated to form PHS which is acylated by various fatty acids to form phytoceramides [2], suggesting that PHS can be synthesized *de novo* in yeast cells. The direct conversion of DHS to PHS has not been demonstrated in mammalian cells. Recently, the mouse enzyme, Des2, has been reported to have C-4 hydroxylase activity, which induces a hydroxyl group onto the C4 position in the DHS moiety of dihydroceramides to form phytoceramides [8]. Phytoceramides can be hydrolyzed to form PHS through the action of a ceramidase [9], indicating that, like SPH, PHS may only be generated from the hydrolysis of phytoceramides and not from *de novo* biosynthesis in mammalian cells. These observations suggest that the generation of SPH or PHS is controlled only by ceramidases in mammalian cells.

After its generation, SPH is phosphorylated to form S1P through the action of SPH kinases. Because the phosphorylation of SPH is the only pathway for the formation of S1P, cellular S1P is highly dependent on the availability of SPH generated by ceramidases, suggesting that ceramidases are critical in regulating not only the hydrolysis of ceramides but also the generation of both SPH and S1P in cells. Therefore, the action of a ceramidase may lead to an alteration in cellular ceramides, SPH, and S1P, thereby controlling cellular responses mediated by these bioactive lipids. This review will address biochemical properties of the mammalian ceramidases and their roles in regulating cellular ceramides, SPH, and S1P and highlight some of the biological roles of these enzymes.

1. Role of ceramide, sphingosine, and S1P in cellular responses

As bioactive lipids, both ceramide and S1P have been extensively reviewed [10–16], whereas SPH has been briefly discussed. Therefore, this review will briefly touch on cellular effects of ceramide and S1P and will go more in-depth into the cellular effects of SPH.

1.1 Role of ceramide in cellular responses

In mammalian cells, ceramides are a major precursor of complex sphingolipids, which are important constituents of the cell membrane. In addition, numerous studies have depicted ceramides to be bioactive lipids that mediate various cellular processes, such as cell growth arrest, differentiation, and apoptosis (Reviewed in [14,17]). Studies from various laboratories indicate that cellular ceramides are increased in mammalian cells challenged with various stressful stimuli, such as pro-apoptotic cytokines (TNF-α [18], IL-1β [19], FAS ligand, and interferon γ [20]), cancer chemotherapeutic agents (etoposide and doxorubicin) [21], UV [22] and ionizing-irradiation [23], vitamins or derivatives (vitamin D [24,25] and retinoic acid [26]), and serum-deprivation [27]. Blocking increases in ceramide inhibits cell-growth arrest and/or apoptosis in response to these stressful stimuli. In contrast, addition of exogenous cell-permeable, short-chain ceramides (D-e-C_2-ceramide or D-e-C_6-ceramide) induces cell-growth

arrest and/or apoptosis [28]. These observations suggest that an increased generation of ceramide mediates cell-growth arrest and/or apoptosis in response to stressful stimuli.

1.2 Role of SPH in cellular responses

In cells, ceramides can be further metabolized to SPH [5,6,29]. Research indicates that SPH is also a mediator of cell-growth arrest and apoptosis (Reviewed in [30–32]). Lepine *et al.* [33, 34] revealed that treatment with dexamethasone increased the generation of SPH in thymocytes, and inhibition of an increase in SPH attenuated dexamethasone-induced apoptosis of these cells. Suzuki *et al.* [35] showed that serum-deprivation increased SPH in 3T3/A31 cells, and blocking the generation of SPH attenuated the serum-deprivation-induced apoptosis. Cuvillier *et al.* [36] treated MCF-7 cells with doxorubicin and found that cellular SPH was elevated; treatment with exogenous SPH potently induced apoptosis of these cells. Ohta *et al.* [37] then showed that that phorbol 12-myristate 13-acetate (PMA) induced the generation of SPH in HL60 prior to apoptosis, and addition of exogenous SPH potently induced apoptosis of these cells in the presence of fumonisin B, a ceramide synthase inhibitor, which blocks the conversion of cellular SPH to ceramide, suggesting that SPH induces apoptosis of HL60 cells on its own, and not by its conversion to ceramide. This group also suggested that TNF-α induces the generation of SPH in neutrophils, and addition of exogenous SPH also induced apoptosis of this cell type [38]. Cuvillier *et al*. reported that treatment of Jurkat cells with an anti-FAS antibody increased generation of SPH prior to cytochrome c release, activation of caspases-2, -3, -6, -7, -8, and apoptosis, and addition of exogenous SPH induced apoptosis of these cells [39], suggesting that SPH may be implicated in FAS-mediated cell apoptosis. Studies by Krown *et al.* indicated that TNF-α markedly increased the generation of SPH in rat cardiomyocytes in culture, and addition of exogenous SPH potently induced the apoptosis of these cells [40]. The similar results were observed in rat skeletal muscle cells [41] and in the mouse liver [42]. These results strongly suggest that SPH is a second messenger of various agonists that induce cell-growth arrest and apoptosis.

SPH has also been reported to have anti-tumor activity in certain animal models. Schmezl *et al.* [43] showed that Apc Min/+ mice fed diets supplemented with sphingolipids have fewer tumors in all intestinal regions. Schmezl's laboratory also reported that SPH was the major digestive product of sphingolipids and that treatment with SPH caused both growth arrest and apoptosis in two human colon cancer cell lines in culture (SW480 and T84). More recently, Kohno [44] demonstrated that knockout of the mouse Sphk1 inhibits small intestinal tumor-cell proliferation in Apc Min/+ mice due to an increase in SPH but not a decrease in S1P. These studies suggest that an increased generation of SPH inhibits the proliferation of tumor cells *in vivo*.

Similar to SPH, DHS also induces cell-growth arrest and apoptosis when either applied exogenously [45] or endogenously accumulated by an inhibition of its conversion to dihydroceramide [46–48]. The role of PHS in mediating various cellular responses has been extensively studied in Baker's yeast, *Saccharomyces cerevisiae* (Reviewed in [49–52] whereas its role in cellular responses in mammalian cells has only recently been described. Addition of exogenous PHS induces the differentiation and growth arrest of epidermal keratinocytes by activating PPAR-γ [53] PHS also causes the apoptosis of Jurkat cells through the mitochondrial pathway [54], or sensitizes Jurkat cells to TRAIL-induced apoptosis [55] although its role as a second messenger has yet to be conclusively proven.

SPH was initially found to compete with diacylglycerol for binding to protein kinase C (PKC) and thus inhibited *in vitro* activity of PKC [56]. Later, endogenous SPH was reported to also potently inhibit PKC cellular activity [57]. Because activation of many PKC isoforms has been implicated in cell proliferation and survival, inhibition of PKC by SPH may be associated with its inhibitory effects on cell proliferation or survival.

Interestingly, SPH was found to activate the C-terminal-half kinase domain of PKCδ, PKCδ KD, which was termed SPH-dependent kinase (SDK) [35]. SDK can phosphorylate certain isoforms of the 14-3-3 protein family [58] although it is unclear whether SDK-mediated phosphorylation of these proteins is associated with SPH-induced cellular responses. SPH has been shown to increase *in vitro* activity of the 85-kDa isoform of the cytosolic diacylglycerol kinase (DGK) but inhibit the activity of the 150-kDa isoform [59]. DGK activation by sphingosine has been demonstrated in cells [60]. Because this activation decreases the levels of diacylglycerol (DAG) on one hand and increases those of phosphatidic acid (PA) on the other hand, sphingosine may modulate cellular responses to both bioactive lipids.

In addition to PKC, SPH has been shown to inhibit other kinases, such as v-Src or c-Src tyrosine kinase [61], Ca^{2+}/calmodulin-dependent protein kinase [62], mitogen activated kinase (MAPK), ERK1/2 [63], and protein kinase B, AKT [64].

Apart from protein targets, SPH has been shown to alter the integrity of intracellular membrane systems. It has been shown that SPH markedly increases the permeability of lysosomes and induces the release of lysosomal cysteine proteases, such as cathepsin B, D, or L to the cytosol, leading to activation of the lysosomal pathway of apoptosis [65]. SPH also increases the permeability of mitochondria, resulting in the release of cytochrome C, which in turn activates the intrinsic pathway of apoptosis [36]. Our recent studies showed that endogenous SPH generated in HeLa cells treated with D-e-C_6-ceramide causes Golgi fragmentation, leading to cell-growth arrest and apoptosis [66]. Similarly, we showed that an increased generation of SPH by overexpressing the alkaline ceramidase 2 (ACER2) in HeLa cells also causes Golgi fragmentation, growth arrest, and apoptosis [67]. Golgi fragmentation has been observed to be an early event during FAS-induced apoptosis [68]. SPH was found to be increased in response to FAS prior to apoptosis of Jurkart cells [39]. These observations suggest that SPH-induced fragmentation of the Golgi complex may be associated with cell apoptosis in response to FAS. In several studies, the Golgi complex was suggested to also be disassembled or fragmented during apoptosis in some neurodegenerative disorders such as amyotrophic lateral sclerosis [69], Alzheimer's disease [70], and Parkinson's disease [71]. Thus, it would be appropriate and interesting to investigate whether increased generation of SPH is implicated in Golgi fragmentation in neurodegenerative diseases.

The integrity of the Golgi complex is important for its function as a major organelle for protein trafficking and posttranslational modifications such as glycosylation [72,73]. We showed that SPH-induced Golgi fragmentation inhibits the trafficking of proteins from the ER to the Golgi complex [66]. One of such proteins is β1 integrin, a major subunit of integrin receptors that mediate cell-cell or cell-extracellular matrix adhesion. β1 integrin is a highly glycosylated protein [74,75], and is synthesized as a polypeptide, which is partially glycosylated in the ER to generate the precursor of the integrin β1 subunit. This precursor is then transported to the Golgi complex where it is further fully glycosylated to form the mature β1 integrin. The glycosylation of β1 integrin in the Golgi has been shown to be important for its binding to the extracellular matrix [74] and its trafficking to the cell surface to function as a cell-adhesion receptor [66,76–78]. We showed that increased generation of SPH in the Golgi complex in HeLa epithelial cells inhibits the formation of the mature form of β1 integrin, leading to defects in cell adhesion and anoikis, a subtype of programmed cell death that is due to loss of cell contact with the extracellular matrix [66]. Therefore, SPH induces growth arrest, differentiation, apoptosis, or anoikis by acting on various intracellular targets (Figure 2).

1.3 Role of S1P in cellular responses

S1P was initially identified as a mitogen in fibroblast cells [79]. S1P was suggested to stimulate DNA synthesis in fibroblast cells through an unknown intracellular target(s) although none have been identified [80]. The mitogenic effect of S1P has been observed in various cell types

[81–83]. S1P is also a potent suppressor of apoptosis in various cell types [84]. These observations suggest that S1P is a universal pro-proliferative and pro-survival bioactive lipid although it induces growth arrest [85], differentiation [86], and apoptosis of certain cell types [87]. In addition, S1P is also an important regulator of cell adhesion and migration [88,89], and cell adherens junction assembly [90]. Along with the findings that S1P is a ligand for 5 G protein-coupled receptors ($S1P_{1–5}$), S1P has been found to play an essential role in various biological processes, such as cardiovascular development [91,92], angiogenesis [93,94], immunity [95–97], reproduction [98], and central nervous system development [99].

2. Protein sequences, chromosomal locations, tissue distributions, and cellular localizations of ceramidases

Table 1 summarizes current knowledge about protein sequences, chromosomal locations, tissue distributions, and cellular localizations of the 5 human ceramidases. This review will discuss them briefly.

2.1 Human acid ceramidase (hAC/ASAH1)

hAC is synthesized as a polypeptide of 395 amino acids (~53–55 kDa), which is processed into a mature heterodimeric enzyme (hAC) composed of an α (13 kDa) and a β subunit (40 kDa) [100]. Recent studies suggest the hAc polypeptide undergo self cleavage to form 2 subunits [101]. Complete deglycosylation reduces the apparent molecular weight of the β subunit to 28 kDa, but does not alter the α subunit [102,103], suggesting that the β subunit of hAC is highly glycosylated. The hAC gene is located on chromosome 8 [104]. The tissue distribution of hAC mRNA remains unknown, and hAC is localized to the lysosomes [103] or is secreted extracellularly [105]. The mouse AC (mAC/Asah1) is synthesized as a polypeptide of 394 amino acids [106]. Similar to its human counterpart, the mAC polypeptide is processed into a mature protein with α and β subunits, and its gene is also located on chromosome 8. mAC mRNA is ubiquitously and highly expressed in kidney, lung, heart, and brain but at relatively low levels in spleen, skeletal muscle, and testes [106].

2.2 Human neutral ceramidase (hNC/ASAH2)

hNC was initially cloned as a protein of 763 amino acids [29]. Later, hNC was cloned by a different group as a protein of 782 amino acids [107], which was 19 amino acids longer at the N-terminal. It is unknown whether the short hNC (hNC-S) and the long hNC (hNC-L) represent two endogenous protein products resulting from alternative translational initiations. The overexpression of hNC-L results in two isoforms with different apparent molecular masses of 142 and 118 kDa [107]. Northern blot analysis indicated that hNC is ubiquitously expressed [29]. The hNC gene is located on chromosome 10 (10q11.21), and hNC-L was mainly found in the plasma membrane [107]. The mouse NC (mNC/Asah2) is a highly glycosylated protein of 756 amino acids [108], and the glycosylation results in an increased apparent molecular mass of 95 kDa [109]. Northern blot analysis showed that mNC mRNA is ubiquitously expressed at high levels in the kidney, liver, and heart, and expressed at medium levels in the brain and lung, with low levels being expressed in the spleen, skeletal muscle, and testes [108]. mNC is mainly localized to the plasma membrane [110], and its gene is located on chromosome 19. The rat NC (rNC/Asah2) is a 761-amino acid protein with a predicted molecular weight of 83.4 kDa [111]. Western blot analysis revealed that when overexpressed in HEK293 cells, rNC, which is mainly localized to the plasma membrane [111], also generates 2 isoforms with molecular masses of 113 and 133 kDa, [111]. Nine glycosylation sites were predicted in this protein. Deglycosylation by glycopeptidase F converted the 133-kDa isoform to an 87-kDa polypeptide. Blockade of glycosylation by tunicamycin converted both isoforms into the 87-kDa polypeptide, suggesting that rNC is also highly glycosylated.

2.3 Human alkaline ceramidase 1 (ACER1/ASAH3/haCER1)

ACER1 is a 264-amino acid protein with a predicted molecular weight of 31 kDa [112]. Western blot analysis revealed that the apparent molecular mass of ACER1 is as predicted [112], suggesting that posttranslational modifications, if any, do not significantly alter the molecular mass of this protein. ACER1 is predicted to have several putative transmembrane domains, and it is localized to the ER [112]. Northern blot analysis showed that ACER1 mRNA is highly expressed in the skin but has no detectable expression in other tissues. With qPCR, ACER1 mRNA was found to be modestly expressed in other tissues. ACER1 is expressed in epidermal keratinocytes but not in dermal fibroblast cells, and the ACER1 gene is located on chromosome 19 (19p13.3). The mouse alkaline ceramidase 1 (Acer1/Asah3), a 273-amino acid protein, has nearly identical protein sequence to its human counterpart [113] (Figure 3). The gene of Acer1 is located on chromosome 17. Similar to the human ACER1, Acer1 is localized to the ER and is mainly expressed in the skin [113].

2.4 Human alkaline ceramidase 2 (ACER2/ASAH3l/haCER2)

ACER2 is 275-amino acid protein with a predicted molecular weight of 31.3 kDa [67]. Western blot analysis showed that ACER2 has an apparent molecular mass close to the predicted one [67]. ACER2 has a high degree of similarity in protein sequence to ACER1 (Figure 3), with has several putative transmembrane domains and is localized to the Golgi complex [67]. Northern blot analysis showed that ACER2 mRNA is ubiquitously expressed but at higher levels in the placenta. The gene of ACER2 is located on chromosome 9 (9p22.1). The mouse alkaline ceramidase 2 (Acer2/Asah3l/maCER1) was previously cloned by a different group as the product of a gene (*CRG-L1*) whose expression is upregulated in liver carcinomas [114]. Acer2 has a nearly identical protein sequence to its human counterpart (Figure 3), but its cellular localization and tissues distribution remain unclear. The gene of *Acer2* is located on chromosome 4.

2.5 Human alkaline ceramidase 3 (ACER3/PHCA/aPHC)

ACER3, previously referred to as alkaline phytoceramidase (aPHC), was the first alkaline ceramidase to be cloned in mammals [9]. ACER3 is a 267-amino acid protein with a predicted molecular weight of 31.6 kDa. Western blot analysis confirms this apparent molecular mass [9]. ACER3 is homologous to both ACER1 and ACER2 (Figure 3). Similar to ACER1 and ACER2, ACER3 has several transmembrane domains. Northern blot analysis revealed that ACER3 mRNA is highly and ubiquitously expressed with the highest levels in placenta [9]. ACER3 is localized to both the ER and Golgi [9], and the ACER3 gene is located on chromosome 11 (11p13.5). The mouse alkaline ceramidase 3 (Acer3) was also cloned by our group (Figure 3) and was found to have a nearly identical protein sequence to ACER3, along with the same cellular localization as ACER3 (unpublished data). Its tissue distribution is unknown, but the gene of *Acer3* is located on chromosome 7.

3. Biochemical properties of ceramidases

3.1 Acid ceramidase

Crude cell lysates from skin fibroblast cells were shown to have the highest AC activity on D-e-C_{12}-ceramide or D-e-C_{14}-ceramide, a moderate activity on D-e-C_{16}-ceramide, but a very low activity on D-e-C_6-ceramide or D-e-C_{18}-ceramide [115]. Interestingly, when ceramides with the same acyl chain length (C18) but different degrees of unsaturation (0, 1, 2, or 3 doubles) were used as substrates, AC activity increased with an increasing number of double bonds [115]. D-e-C_{12}-ceramide has been shown to be the best substrate for purified hAC [102]. These observations suggest that ceramides with medium acyl chains (medium-chain ceramides) are better substrates for AC than short-chain (≤C6) or long-chain (≥C16) ceramides and that AC

prefers unsaturated over saturated ceramides as substrates. With D-e-C_{12}-ceramide as a substrate, hAC has a pH optimum of 4.5 [116]. No cations are required for hAC activity [102].

3.2 Neutral ceramidase

The substrate specificity of hNC has not been established, but it is known to have a broad pH optimum ranging from pH 7–9 [29]. Long-chain ceramides (D-e-C_{16}-ceramide or D-e-C_{18}-ceramide) were described to be better substrates than medium-chain ceramide (D-e-C_{12}-ceramide) for mNC [109]. mNC also hydrolyzes ceramides more efficiently than dihydroceramides with the same acyl-chain length. Long-chain phytoceramides (D-ribo-C_{16}-phytoceramide or D-ribo-C_{18}-phytoceramide) are poor substrates for the mouse enzyme. These observations suggest that mNC prefers long-chain ceramides as substrates. It was found that purified rNC has a similar K_m and V_{max} for D-e-$C_{16:0}$-ceramide, D-e-$C_{18:0}$-ceramide, and D-e-$C_{18:1}$-ceramide [117]. Interestingly, the rat enzyme has the lowest K_m for D-e-α-hydroxyl-C_{16}-ceramide [117]. These results suggest that rNC prefers long-chain ceramides, especially those with a hydroxyl group, as substrates. Different from hNC, both mouse and rat enzymes have a pH optimum of 7.5. No cations are required for NC activity [117].

3.3 Alkaline ceramidase 1

We reported that the human ACER1 catalyzes the hydrolysis of D-e-$C_{24:1}$-ceramide much more efficiently than D-e-$C_{24:0}$-ceramide, with little or no activity on any other ceramides with an acyl chain length less than C18 [112]. ACER1 also fails to hydrolyze any dihydroceramides or phytoceramides, suggesting that ACER1 prefers very long-chain unsaturated ceramides as substrates. ACER1 has a pH optimum of 8.5 and a low basal activity in the absence of cations [112]. The *in vitro* activity is markedly increased by Ca^{2+} but not other cations [112], indicating that Ca^{2+} is a specific metal ion activator of ACER1. Our unpublished data showed that treatment with thapsigargin, a sarco / endoplasmic reticulum Ca^{2+} ATPase inhibitor, which depletes Ca^{2+} from the endoplasmic reticulum (ER), markedly inhibited the generation of sphingosine in response to ACER1 overexpression, suggesting that cellular activity of ACER1 is regulated by the content of Ca^{2+} in the ER.

3.4 Alkaline ceramidase 2

Our group showed that ACER2 catalyzes the hydrolysis of D-e-$C_{24:1}$-ceramide more efficiently than D-e-$C_{24:1}$-dihydroceramide or D-ribo-$C_{24:1}$-phytoceramide, suggesting that ACER2 prefers ceramides over dihydroceramides or phytoceramides as substrates [67]. Our unpublished data showed that purified and reconstituted ACER2 catalyzes D-e-C_{20}-ceramide or D-e-C_{16}-ceramide much more efficiently than D-e-C_{12}-ceramide or D-e-C_6-ceramide, with no activity towards any dihydroceramides and phytoceramides with saturated fatty acids. This suggests that ACER2 prefers long-chain or very long-chain ceramides as substrates. ACER2 in crude microsomal preparations has a pH optimum between 7.4–8.5 [67]. Interestingly, purified and reconstituted ACER2 has a pH optimum of 9.0. Similar to ACER1, ACER2 requires Ca^{2+} for its optimal activity both *in vitro* and in cells (unpublished data).

3.5. Alkaline ceramidase 3

We previously demonstrated that ACER3 prefers D-*ribo*-C_{12}-NBD-phytoceramide over D-e-C_{12}-NBD-ceramide, or D-e-C_{12}-NBD-dihdyroceramide as substrates [9]. Its substrate specificity for natural ceramides, dihydroceramides, phytoceramides was unknown until was recently found that ACER3 hydrolyzes long-chain unsaturated ceramides (D-e-$C_{18:1}$-ceramide or D-e-$C_{20:4}$-ceramide), phytoceramides (D-ribo-$C_{18:1}$-phytoceramide or D-ribo-$C_{20:4}$-phytoceramide), or dihydroceramide (D-e-$C_{20:4}$-dihydroceramide) efficiently. It fails to hydrolyze ceramides, dihydroceramides, or phytoceramides with saturated fatty acids of any

chain-length or ceramides with unsaturated very long-chain fatty acids, such as D-e-C_6-ceramide, D-e-C_{16}-ceramide, D-e-C_{18}-ceramide, D-e-C_{20}-ceramide, D-e-C_{24}-ceramide, or D-e-$C_{24:1}$-ceramide (unpublished data). It is unclear whether ACER3 hydrolyzes ceramides, dihydroceramides, or phytoceramides with unsaturated short or medium chain fatty acids. These results suggest that ACER3 may only use long-chain unsaturated ceramides as substrates. ACER3 has a pH optimum of ~9.5 [9], and ACER3 activity is markedly stimulated by Ca^{2+} [9].

4. Role of ceramidases in regulating cellular responses

4.1 Acid ceramidase

A genetic deficiency in hAC activity causes an accumulation of sphingolipids in lysosomes, leading to a lysosomal storage disorder, Farber disease [118], suggesting that it plays an important role in the catabolism of ceramides in human cells. However, there is an inconsistency about the role of hAC in regulating cellular responses. Several studies with different cell types from Farber disease patients did not find the role of hAC in mediating cellular responses whereas some other studies showed that genetic or pharmacological manipulation of AC activity in tumor cells or primary cultured cells could provoke cellular responses.

Burek *et al.* [119] demonstrated that lymphocytes in Farber disease and normal control cells are equally sensitive to apoptosis in response to various stress stimuli, including staurosporine, anticancer drugs (etoposide and daurorubicin), and γ-irradiation. These authors also showed that Faber disease fibroblasts and normal control cells are equally sensitive to apoptosis in response to DNA-damaging agents (staurosporine, daurorubicin, and doxorubicin). The only difference is that, compared to normal control cells, Farber disease lymphocytes are faster to respond to anti-CD95 antibody-induced apoptosis, but the long-term effects of anti-CD95 antibody are similar. These observations suggest that AC may not mediate apoptosis in fibroblast cells. Consistent with this notion, a careful study by Ségui *et al.* [120] demonstrated that normal and Faber disease lymphoid cells exhibit the same sensitivity to apoptosis in response to TNF-α, the CD95 antibody, anti-CD40 antibodies, daunorubicin, or ionizing radiation although ceramides are markedly elevated in the Farber disease cells. This group also did not observe any differences in apoptosis between normal and Faber disease fibroblasts.

In contrast to the above studies, several other studies suggest that genetic or pharmacological manipulations of AC can significantly affect cell apoptosis in response to various stressful stimuli. Strelow *et al.* [121] demonstrated that overexpression of AC in mouse fibroblast cells-L929 cells inhibits caspase-independent programmed cell death (PCD) in response to human recombinant soluble TRAIL (hTRAIL) and TNF-α, with no effect on caspase-dependent apoptosis, suggesting that AC may mediate a subtype of programmed cell death that is independent of caspases. However, the mechanism by which an increased expression of AC protects cells from PCD remains unclear because the authors did not investigate whether and how AC overexpression regulated ceramides, SPH, or S1P in cells in response to hTRAIL or TNF-α. Thon *et al.* [122] reported that cellular AC overexpression also inhibits caspase-independent PCD in response to murine TRAIL (mTRAIL-R2) without affecting caspase-dependent apoptosis. Elojeimy *et al.* [123] showed that AC is upregulated in head and neck tumors and its expression in head and neck cell lines inversely correlates with the sensitivity of these cell lines to apoptosis induced by an anti-Fas receptor antibody (CH11). This group also showed that RNA interference (RNAi)-mediated knockdown of hAC expression sensitizes head and neck tumor cells to the CH11-induced apoptosis, whereas overexpression of hAC renders tumor cells resistant to apoptosis in response to Fas ligand (FasL). These observations suggest that AC activity modulates caspase-independent PCD or apoptosis in response to cytokines.

Hara *et al.* [124] reported that γ-radiation increased AC protein in human U-87 MG glioblastoma cells in a time-dependent manner. Treatment with N-oleoylethanolamine (NOE), which has been suggested to inhibit AC activity, inhibited the γ-radiation-induced increase in AC protein, resulting in a marked increase in ceramides in U-87 MG cells and cell apoptosis in response to γ-radiation. These results suggest that an increased expression of AC may have a protective role in survival of tumor cells against γ-radiation probably by decreasing the cellular ceramides. NOE was found to inhibit AC activity [125], but this study indicated that NOE also decreased AC protein in cells although the mechanism for this effect remains unclear.

Morales *et al.* [126] demonstrated that daunorubicin (DNR) rapidly activates AC in human (HepG2 cells) or mouse (Hepa1c1c7) hepatoma cell lines as well as in primary cells from murine liver tumors. RNAi-mediated knockdown of AC or inhibition of AC with NOE sensitizes hepatoma cells to the DNR-induced apoptosis whereas treatment with exogenous S1P inhibits the DNR-induced apoptosis. Because AC RNAi or NOE enhances the DNR-induced generation of ceramide in hepatoma cells and NOE also inhibits the DNR-induced generation of S1P, AC activation has a protective role in cell survival against DNR probably by promoting the conversion of pro-apoptotic ceramide to anti-apoptotic S1P in cells. Batra *et al.* [127] found that hypoxia increases AC protein levels and decreases sensitivity of several tumor cell lines to the fenretinide-induced apoptosis, and treatment with NOE sensitizes cells to the fenretinide-induced apoptosis under the condition of hypoxia, suggesting that AC activation may render tumor cells resistance to different chemotherapeutic agents. Interestingly, RNAi-mediated knockdown of AC also inhibits the growth of tumors *in vivo* and enhances DNR tumor therapy, suggesting that inhibition of AC may improve the efficacy of cancer chemotherapy. Saad *et al.* [128] reported that overexpression of hAC in prostate tumor cells (DU145) promotes cell proliferation and suppresses cell apoptosis in response to various apoptotic stimuli, such as doxorubicin, cisplatin, etoposide, gemcitabine or C_6-ceramide. These authors also found that hAC overexpression inhibited the increased generation of ceramides in response to doxorubicin or etoposide, indicating that hAC may protect cells from apoptosis by decreasing ceramides in cells. These observations suggest that regulation of AC can influence cell apoptosis in response to chemotherapeutic agents.

Monick *et al.* [129] showed that AC is highly and constitutively expressed in human alveolar macrophages and its high expression correlates with high levels of SPH in these cells. Unexpectedly, NOE inhibition of AC alone is sufficient to cause apoptosis of alveolar macrophages, suggesting that high AC expression is important for survival of macrophages. Interestingly, the authors showed that addition of exogenous SPH or its analog L-threo-DHS (safingol), which is inert to metabolism in cells, protects cell from apoptosis in response to treatment with NOE, suggesting that SPH may be responsible for cell survival. Surprisingly, addition of SPH to macrophages activates both the PI3K/AKT and ERK pathways whereas NOE inhibits both pathways, suggesting that SPH protects cells from NOE-induced apoptosis likely by activating the AKT and ERK pathways.

Consistent with the notion that AC has a protective role in cell survival, knockout of the mouse AC was found to lead to the death of embryos at a very early stage [130,131]. Addition of exogenous S1P delays the death of embryos, suggesting that inhibition of the generation of S1P is in part responsible for early embryo death caused by knockout of AC [131]. These observations suggest that AC expression is essential for survival of certain cell types.

In addition to cell survival, we recently demonstrated that AC mediates the differentiation of epidermal keratinocytes [112,132]. We showed that AC expression is increased during calciuminduced differentiation of epidermal keratinocytes [112,132], and RNAi-mediated knockdown of AC significantly inhibited the calcium-induced expression of keratin 1 and involucrin, two important proteins highly expressed in differentiated keratinocytes [112].

In addition to cell survival and differentiation, AC may have an anti-inflammation role. This notion is supported by a clinic presentation of some patients with Farber disease. It was found that some patients have massive granulomatous infiltrations by accumulating macrophages in liver, spleen, lymphoid tissue, thymus and lungs although how deficiency in AC activity leads to this pathological effect remains unclear [133].

4.2 Neutral ceramidase

Osawa *et al.* [134] found that adenovirus-mediated overexpression of hNC not only blocked TNF-α-induced generation of ceramides in rat primary hepatocytes but also protected cells from TNF-α-induced apoptosis. This study also demonstrated that overexpression of hNC inhibited liver injury and hepatocyte apoptosis in mice treated with D-galactosamine plus TNF-α, and this protective effect was abrogated by the SPH kinase inhibitor N,N-dimethylsphingosine. These results compelled the authors to conclude that the inhibitory effect of hNC on TNF-α-induced apoptosis could be due to not only C_{16}-ceramide reduction but also S1P formation. Because no S1P measurements were performed, it remains unclear whether overexpression of hNC really increased the generation of S1P. Interestingly, overexpression of hNC not only decreased D-e-C_{16}-ceramide but also significantly decreased cellular SPH. Because SPH is the only precursor of S1P, one would predict that S1P would not be increased if SPH was decreased in cells.

Franzen *et al.* [135] demonstrated that treatment with IL-1β upregulates the expression of the NC in mesangial cells, which can be blocked dose-dependently by the p38 MAPK inhibitor SB202190. Co-treatment of cells for 24 h with IL-1β and SB 202190 leads to an increase in ceramide formation, suggesting that NC may play a protective role in mesangial cell survival against inflammatory stimuli by preventing accumulation of ceramides in cells.

Tani *et al.* [136] showed that overexpression of the mouse NC fails to alter the levels of ceramides, SPH, and S1P in CHOP cells unless ceramides are released from sphingomyelin in the plasma membrane or in fetal bovine serum, suggesting that NC may be responsible for the generation of SPH and S1P by controlling the hydrolysis of ceramides in the plasma membrane and the extracellular space. It is unclear whether an increased generation of SPH and S1P by hNC has any effects on cell survival or apoptosis.

Kono *et al.* [137] showed that knockout of the mouse NC impairs the catabolism of ceramides in the intestine, leading to an increase in ceramides but a decrease in SPH in this tissue, suggesting that the mouse NC plays an important role in the catabolism of dietary sphingolipids and regulation of ceramide and SPH in the intestinal tract. Interestingly, inactivation of the NC gene does not cause obvious abnormalities or major alterations in total ceramide in tissues other than the intestinal tract.

4.3 Alkaline ceramidase 1

ACER1 is mainly expressed in the skin [112,132]. *In situ* hybridization revealed that ACER1 mRNA is highly expressed in the upper nucleated layers of the epidermis, but it is undetectable in the basal cell layer. In cell culture, ACER2 mRNA is markedly upregulated in epidermal keratinocytes by 1.2 mM extracellular calcium [112] or the combination of fetal bovine serum, Ca^{2+}, and ascorbic acid [132]. These observations indicate that ACER1 may have an important role in the differentiation of epidermal keratinocytes. Indeed, we demonstrated that RNAi-mediated knockdown of ACER1 significantly inhibits the Ca^{2+}-induced growth arrest of human epidermal keratinocytes and attenuates the Ca^{2+}-induced expression of involucrin and keratin 1 in these cells [112], suggesting that ACER2 upregulation plays an important role in mediating the calcium-induced growth arrest and differentiation of epidermal keratinocytes. Lipid analysis demonstrated that ACER1 overexpression increases the levels of both

sphingosine and S1P with a concomitant decrease in very long-chain ceramides (D-e-C_{24}-ceramide and D-e-$C_{24:1}$-ceramide) in human epidermal keratinocytes whereas its knockdown has opposing effects [112]. As sphingosine has been shown to be an anti-proliferative and pro-differentiating bioactive lipid, its increase may be responsible for ACER1-mediated growth arrest and differentiation of keratinocytes. Although S1P is mitogenic to many cell types, recent studies suggest that S1P has anti-proliferative and pro-differentiating roles in keratinocytes [85,86]. Therefore, an increased generation of S1P may also contribute to ACER1-mediated growth arrest and differentiation of keratinocytes. These studies suggest that ACER1 may have a critical role in regulating cellular responses in keratinocytes by controlling the generation of both sphingosine and S1P. Our unpublished data showed that ACER2 is also expressed in different epithelial cell types, such as human malignant mammary epithelial cells (MCF-7) and malignant cervical epithelial cells (HeLa). It would be interesting to learn whether ACER2 also has a role in the differentiation of other types of epithelial cells.

4.4 Alkaline ceramidase 2

We demonstrated that high ectopic expression of ACER2 causes growth arrest of HeLa cells by generating high levels of SPH in cells whereas low ectopic expression of ACER2 promotes the proliferation of HeLa cells in serum-free medium by elevating S1P without accumulating cytotoxic levels of SPH [67]. These results suggest that the role of ACER2 in cell proliferation and apoptosis may depend on a balance between SPH and S1P in cells. The proliferative effect of low ectopic expression is inhibited by knockdown of S1PR1, a major S1P receptor in HeLa cells, suggesting that an increased expression of ACER2 promotes cell proliferation by activating the S1P/S1PR1 pathway. We found that ACER2 expression is upregulated in HeLa cells in response to serum deprivation, and knockdown of ACER2 inhibits the serum-independent proliferation of HeLa cells and causes apoptosis of cells in serum-free medium [67]. This suggests that ACER2 upregulation has a protective role in cell survival against serum-deprivation.

4.5 Alkaline ceramidase 3

The endogenous substrates of ACER3 were not known until we recently showed that this alkaline ceramidase uses unusual long-chain unsaturated ceramides, dihydroceramides, and phytoceramides, but not common ceramides (D-e-C_{16}-ceramide, D-e-C_{24}-ceramide, and D-e-$C_{24:1}$-ceramide) as endogenous substrates (unpublished data). ACER3 is highly and ubiquitously expressed [9]. In contrast, its endogenous substrates are scarce in most tissues. This inverse correlation between the enzyme and its substrates may suggest that ACER3 may act as a house-keeping enzyme responsible for the catabolism of a specific group of ceramides to generate basal levels of SPH, DHS, or PHS and their phosphates in cells and tissues.

5. Conclusions and future directions

Five mammalian ceramidases have been cloned and biochemically characterized. However, our understanding of their physiological and pathological roles remains inadequate because, except for AC, the other ceramidases have only recently been cloned. Limited studies suggest that these ceramidases have the ability to regulate ceramides, SPH, and S1P by controlling the hydrolysis of cellular ceramides. Due to differences in the substrate specificity, cellular localization, tissue distribution, and expression level, these ceramidases appear to have distinct roles in cellular responses. Current studies suggest that activation or upregulation of AC and NC mainly promotes cell proliferation and survival by attenuating ceramide signaling while augmenting S1P signaling whereas inhibition or down-regulation of these enzymes induces cell growth arrest and/or apoptosis by amplifying ceramide signaling while diminishing S1P signaling. ACER1 appears to have anti-proliferating and pro-differentiating roles in specific cell types by controlling the generation of SPH and/or S1P. ACER2 has dual roles. Its activation

or upregulation promotes cell proliferation and survival by generating S1P while SPH is not aberrantly elevated by its action. On the other hand, ACER2 action may induce cell growth arrest and apoptosis by generating high cellular levels of SPH, whose anti-proliferative and pro-apoptotic effects may exceed the mitogenic and anti-apoptotic role of S1P. Therefore, the role of this ceramidase in cellular responses may be both cell type-specific and stimulus-dependent. Unlike the other four ceramidases that do not catalyze or slightly catalyze the hydrolysis of dihydroceramides and phytoceramides, ACER3 catalyzes the hydrolysis of both unsaturated long-chain dihydroceramides and phytoceramides with a similar efficiency to the hydrolysis of unsaturated long-chain ceramides, suggesting that it has a role in regulating the generation of DHS and PHS and their phosphates, in addition to SPH and its phosphate. This enables us to genetically manipulate the generation of both endogenous DHS and PHS and to define the role of these two sphingoid bases and their phosphates in cellular responses, which have been largely neglected in preceding studies.

With the elucidation of the gene and protein sequences, biochemical properties, cellular localizations, and tissue distributions of the 5 mammalian ceramidases, the physiological or pathological functions of these ceramidases should be revealed more precisely and rapidly. Several new directions should be considered. First, because ceramide and SPH have similar roles in cellular responses and they are metabolically interchangeable, it is important to distinguish the role of ceramide versus SPH in cellular responses to stimuli that increase the cellular levels of both ceramides and SPH. This issue has been addressed by using different ceramidase inhibitors. However, the specificity of these inhibitors has to be validated by purified ceramidases. Furthermore, some of the inhibitors are charged lipids, which may alter the physiochemical properties of cell membranes, thus resulting in non-specific effects. Second, because cDNA for each ceramidase is available, it is possible to increase endogenous SPH or ceramide in different cellular compartments by overexpressing or knocking down a ceramidase, thereby avoiding the use of exogenous short-chain ceramides or sphingoid bases because exogenously added lipids may not always fully mimic endogenous lipids in inducing cellular effects. For instance, exogenous SPH is mainly trapped in acidic membrane compartments, such as endosomes or lysosomes after cell entry [65], whereas endogenous SPH can be generated in different cellular compartments by different ceramidases. Third, RNAi has been used to knock down ceramidase expression to define their role in cellular responses. Although this new technology offers relatively specific means for inhibiting the expression of each ceramidase, small interfering RNAs (siRNAs) may generate unintended (off-target) effects by targeting non-specific genes (see reviews [138–140]). Therefore, measures should be taken to eliminate off-target effects when this new technology is applied to define the role of each ceramidase in cellular responses. Finally, because gene-targeting in mouse models is a powerful tool to define the physiological role of a gene at the animal level, knockout mouse models in which ceramidases are inactivated should be considered. We can also define the complementary or redundant roles among different ceramidases, if there are any, using double, triple, or multiple knockout mouse models.

Acknowledgments

Studies on the role of the acid and alkaline ceramidases in our laboratories are supported by the NIH grant R01 CA104834 (CM) and a VA merit award (LMO).

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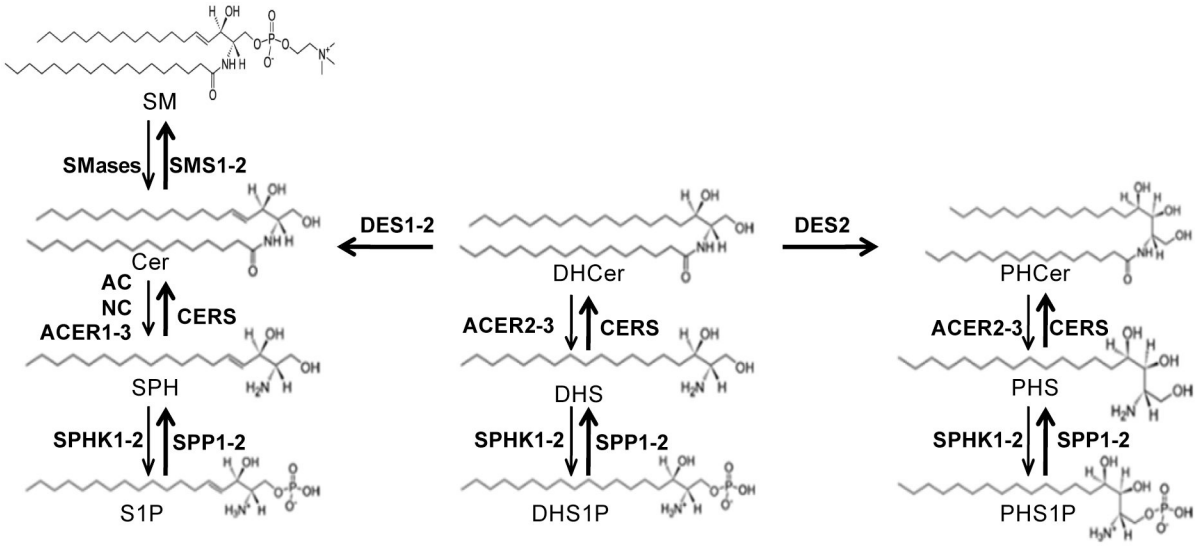

Figure 1. Metabolism of ceramides, dihydroceramides, and phytoceramides in mammalian cells

Cer, ceramide; DHCer, dihydroceramide; PHCer, phytoceramide; SPH, sphingosine; DHS, dihydrosphingosine; PHS, phytosphingosine, SPH1P, sphingosine-1-phosphate; DHS1P, DHS-1-phosphate; PHS1P, PHS-1-phosphate; AC, acid ceramidase; NC, neutral ceramidase; ACER, alkaline ceramidase; SPHK, SPH kinase; SPP, SHP1P (S1P) phosphatase; DES, dihydroceramide desaturase; CERS, ceramide (dihydroceramide) synthase; SMS, sphingomyelin synthase; and SMase; sphingomyelinase.

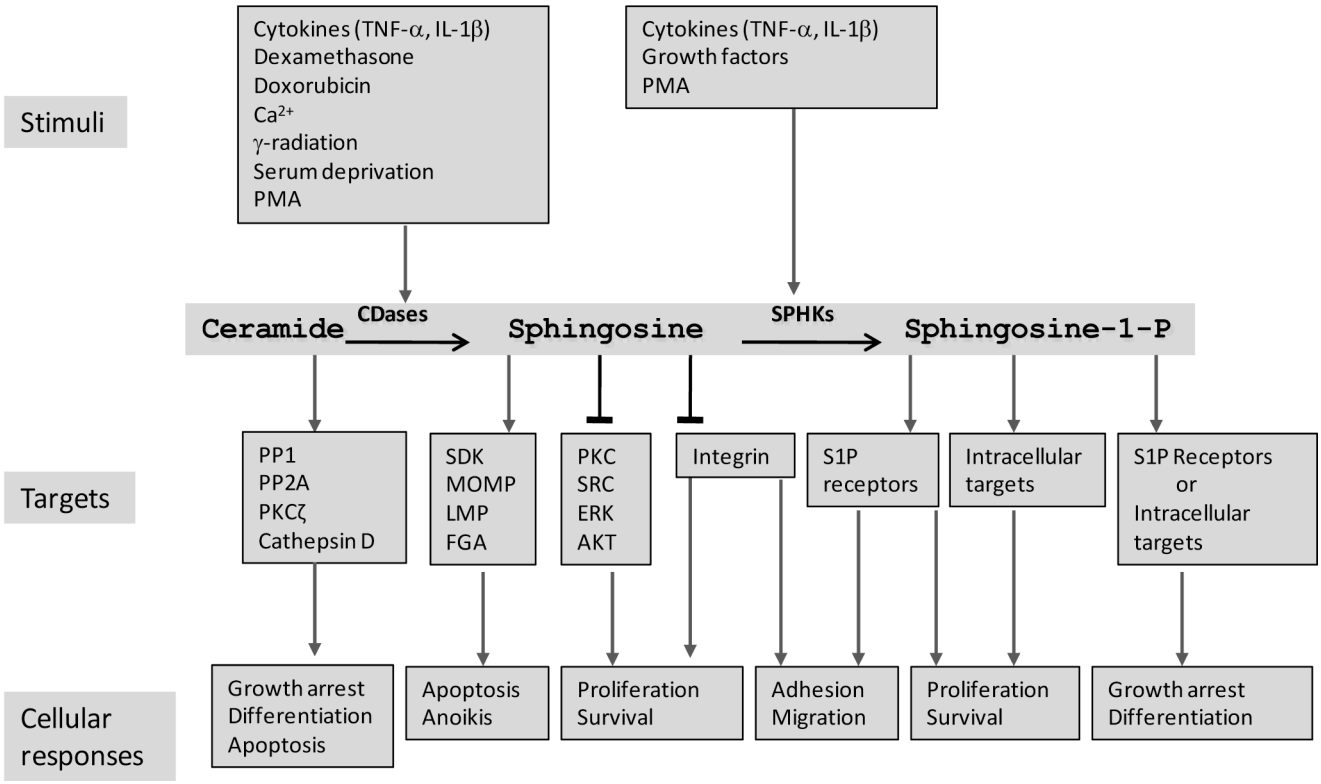

Figure 2. Role of ceramidases in cellular responses

CDases, ceramidases; MOMP, Mitochondrial outer membrane permeabilization; LMP, lysosomal membrane permeabilization; FGA, fragmentation of the Golgi apparatus; S1PR, S1P receptor; SDK, SPH-dependent kinase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PKC, protein kinase C; SRC, Src kinase; ERK, extracellular signal-regulated kinase; AKT, protein kinase B.

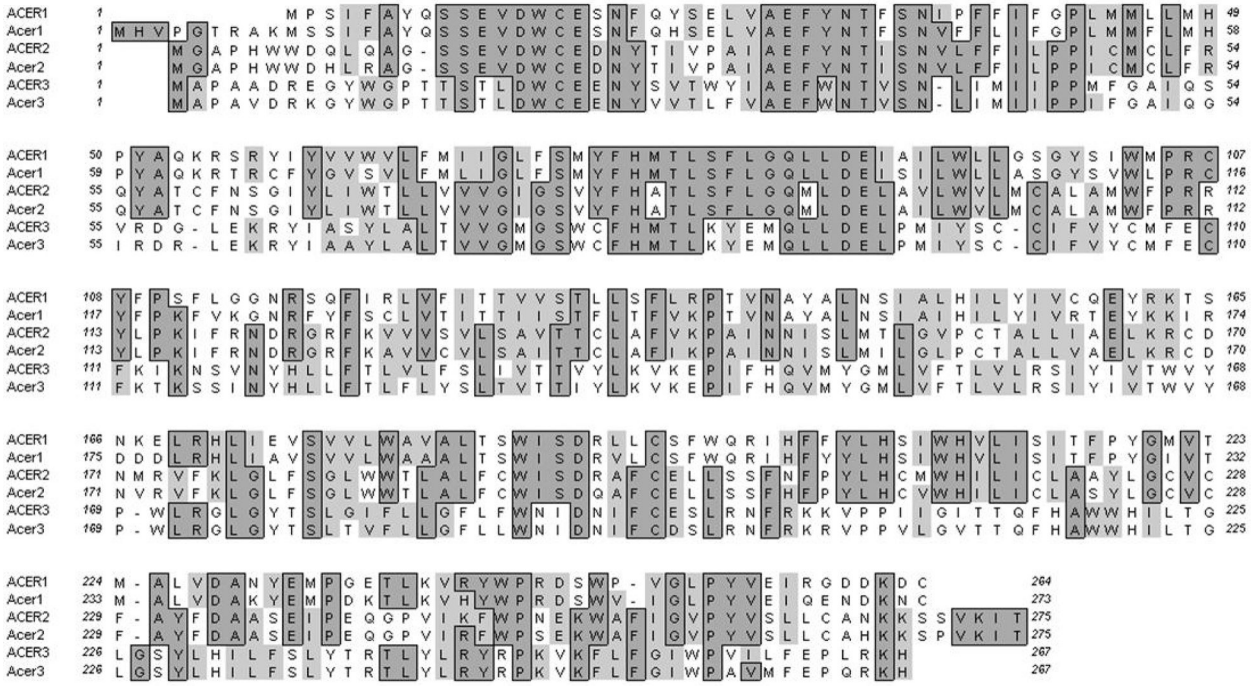

Figure 3. Protein sequences of human and mouse alklaine ceramidases

Dark gray shading indicates identical amino acids among the alkaline ceramidases, and light gray shading indicates similar amino acids among the alkaline ceramidases. Symbols with all uppercase letters represent human enzymes whereas those with the first uppercase letter followed by lowercase letters represent mouse enzymes.