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Review

New insight into the structure, reaction mechanism, and biological functions of neutral ceramidase☆

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ABSTRACT

Ceramidase (CDase) is an enzyme that hydrolyzes the *N*-acyl linkage between the sphingoid base and fatty acid of ceramide. These enzymes are classified into three distinct groups, acid (Asah1), neutral (Asah2), and alkaline (Asah3) CDases, based on their primary structure and optimum pH. Acid CDase catabolizes ceramide in lysosomes and is found only in vertebrates. In contrast, the distribution of neutral and alkaline CDases is broad, with both being found in species ranging from lower eukaryotes to mammals; however, only neutral CDase is found in prokaryotes, including some pathogenic bacteria. Neutral CDase is thought to have gained a specific domain (mucin box) in the N-terminal region after the vertebrate split, allowing the enzyme to be stably expressed at the plasma membrane as a type II membrane protein. The X-ray crystal structure of neutral CDase was recently solved, uncovering a unique structure and reaction mechanism for the enzyme. Neutral CDase contains a zinc ion in the active site that functions as a catalytic center, and the hydrolysis of the *N*-acyl linkage in ceramide proceeds through a mechanism that is similar to that described for zinc-dependent carboxypeptidase. This review describes the structure, reaction mechanism, and biological functions of neutral CDase in association with the molecular evolution, topology, and mechanical conformation. This article is part of a Special Issue entitled New Frontiers in Sphingolipid Biology.

1. Introduction

Ceramide is synthesized in the endoplasmic reticulum (ER) and transported by the ceramide-transfer protein CERT to the *trans*-Golgi membrane [1] where it is converted to sphingomyelin (SM) by SM synthase-1, 2 [2,3]. Alternatively, ceramide can be transported to the *cis*-Golgi membrane and converted to glucosylceramide (GlcCer) by UDP-glucose ceramide glucosyltransferase-1 [4]; GlcCer is further converted to various glycosphingolipids (GSLs) in the *medial*/*trans*-Golgi apparatus. SM and GSLs are then sorted into the outer leaflet of the plasma membrane *via* the *trans*-Golgi network (TGN); these sphingolipids are concentrated into specific microdomains on the plasma membrane, so-called lipid rafts, with sterols [5]. Although free ceramide can also migrate to the plasma membrane and intracellular organelles, this transport system has not been fully elucidated.

The catabolism of ceramide in mammals occurs continuously in lysosomes through the activity of ceramidase (CDase), which catalyzes the hydrolysis of the *N*-acyl linkage between the sphingoid base and fatty acid under acidic conditions [6,7]. Dysfunction of the human gene encoding lysosomal acid CDase leads to typical lysosomal sphingolipidosis, termed Farber disease, in which ceramide accumulates in the lysosomes [8,9]. At the end of the 1960s, Gatt and Yavin reported non-lysosomal CDase activity in rat brain and obtained the partially purified enzyme, which showed an optimal pH of approximately 7 in the presence of cholate [10]. However, the enzyme was difficult to purify because it appeared to be a membrane-bound protein; thus, the molecular characterization and physiological function of non-lysosomal CDase remained unresolved for many years [11]. In the mid-1990s, Sandhoff and associates succeeded in purifying lysosomal acid CDase from human urine [12] and then cloned the gene using its partial amino acid sequence [13]; the mouse homolog was subsequently cloned by Schuchman's group [14].

We independently purified CDase from the opportunistic bacteria *Pseudomonas aeruginosa* [15] and cloned its gene [16]. Interestingly, the predicted amino acid sequence of CDase was not homologous with that of the human and mouse lysosomal acid CDases. Both the native CDase purified from *P. aeruginosa* and the recombinant CDase expressed in *Escherichia coli* showed an optimum pH of approximately 8.0–8.5 [16], and thus we named the protein neutral CDase (the enzyme was initially named alkaline CDase but was later re-named to distinguish it from the actual alkaline CDase described later). Following the purification of neutral CDase from *P. aeruginosa*, we purified neutral CDase homologs from mouse liver (soluble form) [17] and rat kidney (membrane-bound form) [18] and then cloned the genes responsible for each enzyme activity [18,19]. Hannun and associates have also

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purified a neutral CDase from rat brain [20], which is likely the same enzyme previously described by Gatt [7], and the human homolog was also cloned and characterized by two different laboratories [21,22]. These mammalian neutral CDases demonstrate high sequence similarity with *Pseudomonas* CDase but not with lysosomal acid CDase. Over the past decade, many neutral CDase homologs have been cloned from such organisms as the fruit fly [23], zebrafish [24], slime mold [25], red flour beetle [26], rice small brown planthopper [27], rice [28], wheat [29], filamentous fungus [30] and such pathogenic bacteria as *Dermatophilus congolensis* [31] and *Mycobacterium tuberculosis* [32]. The overall primary structure of neutral CDase is evolutionally conserved from bacteria to humans (Fig. 1A), whereas the distribution of acid CDase is restricted to vertebrates (Fig. 1B).

Obeid, Mao, and associates cloned a phytoceramide-specific CDase from budding yeast [33,34], and the enzyme was named alkaline CDase due to its alkaline pH optimum (pH 8.5–9.5). The mouse and human homologs of alkaline CDase were also cloned by the same group [35–37]. Three different human alkaline CDases, which are encoded by three different genes (ACER1–3), and their mouse homologs have been characterized to date [38]; however, these alkaline CDases are not homologous with the acid or neutral CDases and form an independent family (Fig. 1C).

Three different CDase families (acid, neutral, and alkaline), which are distinguished by both the pH optimum and primary structure, have been established (Fig. 1). The acid, neutral, and alkaline CDases are also named Asah1, Asah2, and Asah3, respectively. This review article will address the structure, reaction mechanism, and function of neutral CDase (Asah2).

Ceramide and their metabolites, sphingosine and sphingosine 1-phosphate, participate in a variety of cellular events as lipid mediators [39,40]. The cellular generation of sphingosine and most likely sphingosine 1-phosphate (S1P) depends on the action of CDase; thus, the enzyme is an important modulator for sphingolipid-mediated signaling. The mammalian CDases and their roles in regulating cellular ceramide, sphingosine, and S1P have been extensively reviewed [38].

2. Molecular evolution of neutral CDase acquiring a mucin box

Neutral CDase is widely distributed in microbes, invertebrates, plants, and mammals (Fig. 1A). The properties of neutral CDases from different origins are summarized in Table 1. Neutral CDase is a secreted protein in bacteria [15], slime molds [25], and fruit flies [23], whereas the enzyme is mainly membrane-bound and occasionally found in soluble form in zebrafish [24], mice [19], rats [18], and humans [21,22]. A distinct difference between the vertebrate and invertebrate/bacterial enzymes was found in the NH_2-terminal region: neutral CDases after the vertebrate split contain a serine/threonine/proline-rich domain (mucin box) that follows the signal/anchor sequence at the NH_2 terminus, whereas those of bacteria and invertebrates lack a mucin box (Fig. 2). When expressed in HEK293 or CHOP cells, both wild-type and mutant rat neutral CDase with a deleted mucin box were released into the medium; however, the level of release of the mutant CDase was much higher than that of the wild-type CDase (Fig. 3A). In contrast, the cell-surface expression of wild-type CDase was much stronger than that of mutant CDase (Fig. 3B). The mucin box is highly glycosylated with *O*-glycans, and the amount of secreted alanine-mutant CDase, with all the Ser and Thr residues in the mucin box replaced with Ala, was much higher than that of the wild-type enzyme, suggesting that *O*-glycosylation of the mucin box is required to stably retain CDase at the plasma membrane (Fig. 3C). Furthermore, green-fluorescent

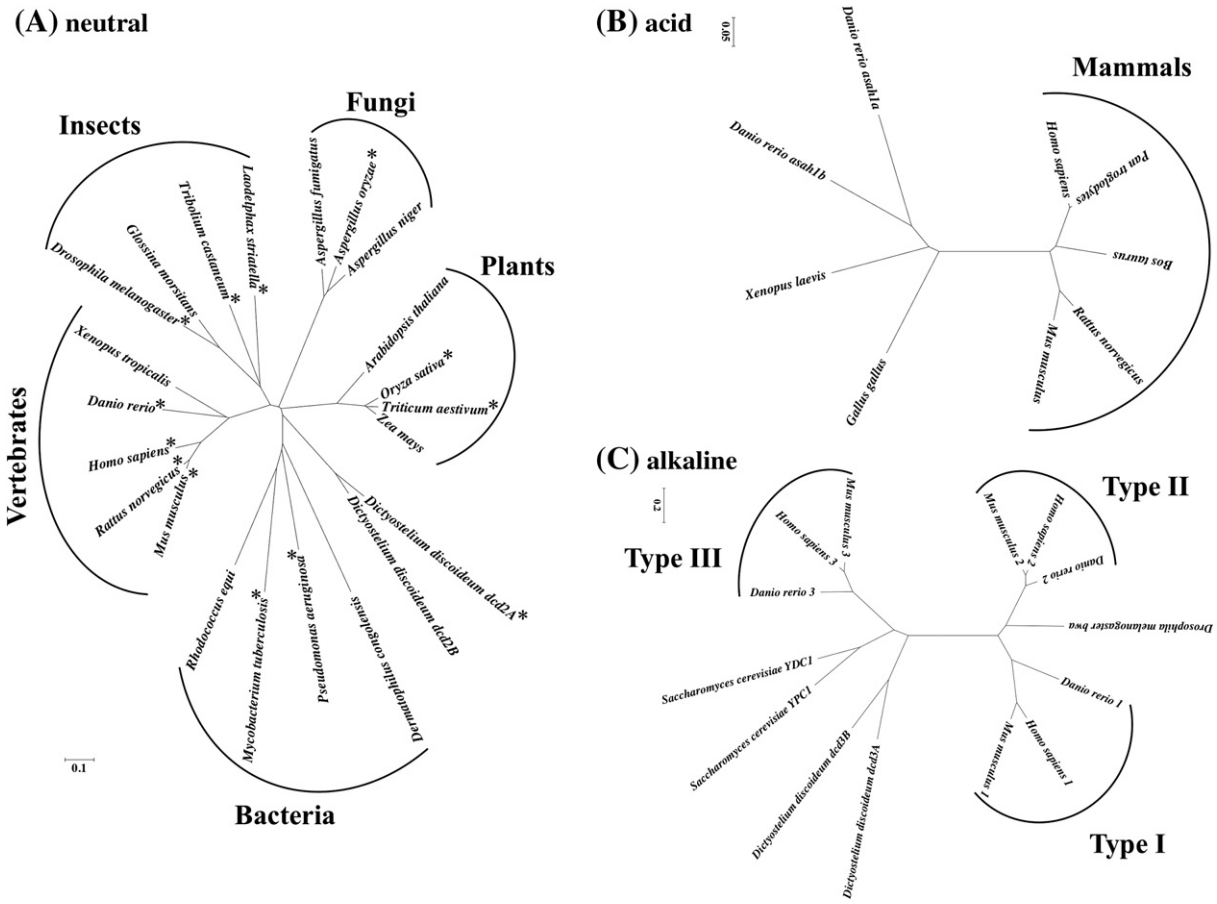

Fig. 1. Phylogenetic trees of neutral (A), acid (B), and alkaline (C) CDases. An unrooted phylogram was constructed with the neighbor-joining method using MEGA5 [79]. The asterisks indicate neutral CDases, the activities of which have been measured and confirmed.