Combined saposin C and D deficiencies in mice lead to a neuronopathic phenotype, glucosylceramide and α-hydroxy ceramide accumulation, and altered prosaposin trafficking

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Saposins (A, B, C and D) are ~80 amino acid stimulators of glycosphingolipid (GSL) hydrolases that derive from a single precursor, prosaposin. In both humans and mice, prosaposin/saposin deficiencies lead to severe neurological deficits. The CD−/− mice with saposin C and D combined deficiencies were produced by introducing genomic point mutations into a critical cysteine in each of these saposins. These mice develop a severe neurological phenotype with ataxia, kyphotic posturing and hind limb paralysis. Relative to prosaposin null mice (~30 days), CD−/− mice had an extended life span (~56 days). Loss of Purkinje cells was evident after 6 weeks, and storage bodies were present in neurons of the spinal cord, brain and dorsal root ganglion. Electron microscopy showed well-myelinated fibers and axonal inclusions in the brain and sciatic nerve. Marked accumulations of glucosylceramides and α-hydroxy ceramides were present in brain and kidney. Minor storage of lactosylceramide (LacCer) was observed when compared with tissues from the prosaposin null mice, suggesting a compensation in LacCer degradation by saposin B for the saposin C deficiency. Skin fibroblasts and tissues from CD−/− mice showed an increase of intracellular prosaposin, impaired prosaposin secretion, deficiencies of saposins C and D and decreases in saposins A and B. In addition, the deficiency of saposin C in CD−/− mice resulted in cellular decreases of acid β-glucosidase activity and protein. This CD null mouse model provides a tool to explore the in vivo functional interactions of saposins in GSL metabolism and lysosomal storage diseases, and prosaposin’s physiological effects.

INTRODUCTION

Mammalian prosaposin encodes in tandem four small acidic activator proteins (saposins) with specificities for glycosphingolipids (GSLs) hydrolases (1–4). Saposins are processed from prosaposin in the late endosome and lysosome (5–7), and their stability is maintained by three disulfide bridges (8). They are essential for the activity of specific lysosomal GSL hydrolases (9). The saposins enhance the activities of their respective cognate lysosomal enzymes by several mechanisms to achieve optimal in vitro and in vivo degradation of GSLs (9–11). Saposin B in a dimeric shell structure facilitates the partial extraction of GSLs from membranes and presents sulfatides or globotriosylceramide to arylsulfatase A or α-galactosidase A, respectively (11–13). Saposin C promotes acid β-glucosidase (GCase) activity by inducing a conformational change in the enzyme at acidic pH (14,15), and also has protective effects for GCase against proteolytic digestion (16). Both saposin B and C have in vitro activation effects on GalCer-β-galactosidase and GM1-β-galactosidase, as well as the β-galactosidase for hydrolysis of lactosylceramide (17). In vitro, saposins A and C enhance GCase activity and negatively charged phospholipids are required for these actions, but they have different membrane interaction modes (15,18).
In vivo, saposin A and C specifically enhance the activities of galactosylceramidase and GCase, respectively (19). Saposin D enhances acid ceramidase degradation of ceramide (20). Recently, the saposins’ lipid binding properties were shown to be critical to NKT cell development and function in the immune response system (21,22). Extracellularly, intact prosaposin has ex vivo or in vivo functions as a neurite outgrowth or nerve regeneration factor, respectively (23,24). The secreted form of prosaposin has been postulated to have a role in spermatogenesis (3,25).

The physiological importance of the prosaposin locus has been demonstrated by the genetic deficiencies of individual saposins or prosaposin that lead to various GSL storage diseases (26–29). In humans, saposin B deficiency leads to sulfatide accumulation and a metachromatic leukodystrophy-like disease, similar to that resulting from arylsulfatase A deficiency (30). Saposin C deficiency leads to a Gaucher-like disease with neurological manifestations because of diminished glucosylceramide cleaving activity in the cells (31,32). A complete deficiency of prosaposin in humans led to the storage of multiple GSLs in a variety of organs (26,28).

A mouse model of saposin A deficiency was analogous to a late onset, chronic form of globoid cell leukodystrophy (33) with some phenotypic and biochemical abnormalities of Krabbe disease. Deficiency of saposin D in mice causes a loss of Purkinje cells and urinary system defects (34), and does not mimic the embryonic lethality of complete acid ceramidase deficiency (35). Targeted disruption of prosaposin in the mouse leads to a complex neurodegenerative phenotype with severe leukodystrophy, and neuronal and microglial accumulations of GSLs (27). The critical roles for saposins in GSL metabolism are highlighted by the extensive GSL storage in the CNS and visceras in the respective human and mouse deficiencies (26,27). However, simple summation of individual saposin deficiencies does not predict the phenotype observed in total prosaposin/saposin deficiency, implying significant functional interactions among the various saposins.

To explore the in vivo effects and interactions of the saposins, a series of saposin mutant mice were created with single saposin or double saposin deficiencies. Here, mice with combined deficiencies of saposins C and D were characterized for prosaposin processing, GSL metabolism and phenotypic development.

RESULTS

Generation of saposin CD −/− mice

The mutation of selected cysteines destroys a single disulfide bridge in saposins C and D, and leads to instability and deficiency of these saposins (33,34). The fifth cysteines of saposin C (Cys → Pro) and D (Cys → Ser) were mutated in the targeting vector (Fig. 1A). The recombinant ES cells were screened by PCR and confirmed by Southern blot analysis (Fig. 1B). The mutations in individual saposins were validated by DNA sequencing. To remove the neo gene in the targeting vector, heterozygous F1 mice were bred with Zp3-Cre transgenic mice that delete floxed neo in the female germline (see Materials and Methods). Subsequently, the Cre transgene was eliminated by crosses into wild-type C57BL/6J mice (Fig. 1C). No differences in phenotype or histology have been observed between CD −/− and CD −/−, neo mice. The resulting CD −/− and CD +/+ or CD +/− mice were analyzed in all studies. Both sexes were used in the analyses and no differences in phenotype or biochemistry were found. Prosaposin null (PS −/−) mice (27) and saposin D mutant (D −/−) (34) mice were included in this study for some of the analyses.

Phenotypes of CD −/− mice

Mendelian ratios of CD −/− mice were obtained from the heterozygote crosses. The saposin CD −/− mice grew normally during the first 3 weeks after birth and showed onset of neurological deficits beginning at 4 weeks (~28 days). After 5 weeks, the mutant mice started losing weight. Their rear legs were spread out, and there was an ataxic gait and uncoordinated walking. By 6–7 weeks, tail arching and kyphotic posturing developed as did hind limb paralysis (Fig. 2A). Compared with the prosaposin knock out mice (PS −/−), CD −/− mice had an extended life span (Fig. 2B) and delayed onset of the neurologic phenotype (28 versus 20 days) (Table 1). The polyuria noted in the D −/− mice was not present in CD −/− mice (34). CD −/− mice did not reproduce. No differences of phenotype were observed between sexes.

Histological studies

As early as 10 days, the neurons of the spinal cord and dorsal root ganglia had inclusions and these lesions progressed significantly by 7.5 wks (Fig. 3 and Supplementary Material, Fig. S1). Similar inclusions in neurons and neuronal degeneration were observed in the cortex and thalamus (Fig. 3 and Supplementary Material, Fig. S1). Macrophage/microglial cell activation was assessed using anti-CD68 antibody. CD68 is an intracellular membrane glycoprotein that is expressed in quiescent and activated tissue macrophages (36). CD68 positive cells were present in restricted regions of the brain including the thalamus, hippocampus, cerebellum and cerebral cortex (Fig. 3). In cerebellum, CD68 signals were detected in white matter at 10 days, and progressed in most areas of the cerebellum until 7.5 weeks. At 10 days, CD68 signals were present in the sciatic nerve of CD −/− mice. The spinal cord stained positive for CD68. The CD −/− brain stem had a few CD68 positive cells by 7.5 weeks. The liver and other visceral tissues of CD −/− had CD68 staining similar to wild-type controls. While in PS −/− mice, engorged CD68 positive macrophages were in the liver and spleen by 25 days (Supplementary Material, Fig. S1).

The brain stem of CD −/− mice was relatively normal and this contrasts with severe involvement of this area in PS −/− (Fig. 4A). The PS −/− mice had severe ballooning of brain stem neurons and a marked infiltration by CD68 positive macrophage/microglial cells. Partial loss of Purkinje cells was evident in cerebellar folia III in the CD −/− mice at about 7 weeks (Fig. 4B) using anti-calbindin antibody as a Purkinje cell marker. The gross morphology of CD −/− kidneys, livers and lungs was normal. Approximately 40% of the CD −/− mice had heavily pigmented parts of spleen.
that contained melanin with dark brown precipitates in H&E section.

Electron micrographs revealed normal morphology of the CD2/2 liver and kidney. Inclusions were present in cerebral neuronal cell bodies and axons of CD2/2 mice (Fig. 5). The inclusions were a heterogeneous complex mixture of vesicular bodies and lamellar structures. The density of the bodies varied from electron dense material with occasional stacks of lamellar material, to vesicular bodies with more electron lucent amorphous material as well as many bodies with a mixture of electron dense and electron lucent material (Fig. 5C and D). The inclusion material had a membranous appearance that was in round or irregular shaped bodies. Axonal degeneration was characterized by large distended axonal processes (axonal spheroids) filled with complex mixture of vesicular bodies in both myelinated and unmyelinated axons. Breakdown and focal disruptions of the myelin sheath was evident in some areas of axonal degeneration.
Expression of myelin basic protein in CD −/− mice

Myelin basic protein (MBP) is a component of the myelin sheath. Brain MBP levels in CD −/− and D −/− were compared with those in PS −/− mice by quantitative immunobLOTS. The MBP reaches mature levels by 3 weeks (37,38). Relative to WT, MBP levels in PS −/− brains were reduced to~17% of WT levels (Fig. 6), whereas in CD −/− and D −/− brains the decreases were much less drastic and at older ages, i.e. ~45% of WT levels. This result was consistent with the EM findings and indicated that myelin degeneration was severe in PS −/− brain, but much less affected so in CD −/− mice.

Expression and processing of prosaposin/saposins in CD −/− and D −/− mice

The effects of mutations on prosaposin and saposins expression were investigated at RNA and protein level. RT–PCR analyses showed that prosaposin mRNAs in CD −/− mouse livers and brains were expressed at WT control levels (Fig. 1D). Quantitative RT–PCR demonstrated that prosaposin mRNAs levels in CD −/− liver and brain were not different from those in wild-type controls (Supplementary Material, Fig. S2). The protein levels of prosaposin and saposin D in CD −/− and D −/− mice were determined by western blot using anti-mouse saposin D antibody that also reacts with prosaposin. Saposin D was not detected in the liver, brain (Fig. 7A) or fibroblasts (data not shown) from CD −/−, D −/− or PS −/− mice. Saposin C was not detected in CD −/− fibroblasts using anti-mouse saposin C antibody (Fig. 7B). The levels of remaining saposins in CD −/− tissues were assessed with anti-mouse saposin A or saposin B antibodies. Saposin A and B proteins were produced, but their levels were reduced relative to WT (Fig. 7C). Saposin A and B levels were slightly altered in D −/− mice. Compared with WT, CD −/− and D −/− had 1.73–5.4-fold increases of prosaposin protein in the brain or liver. Prosaposin is N-glycosylated. To assess the attached oligosaccharide nature, the fibroblast lysates from WT and CD −/− mice were treated with Endo H and visualized by western bloting, using anti-mouse saposin D antibody (Fig. 7D). Complex oligosaccharides are resistant to Endo H cleavage and the proportions of sensitive to non-sensitive prosaposins differed between WT and CD −/− cells. A much greater proportion of the prosaposin in CD −/− cells was Endo H sensitive than in WT cells, indicating less complex oligosaccharide modification of CD −/− prosaposin. This result implies a greater amount of high mannose oligosaccharides on the CD −/− prosaposin and retention in the ER. However, not all CD −/− prosaposin was ER retained since a significant amount was Endo H resistant and, therefore, processed to contain complex oligosaccharides and targeted to lysosome possibly via mannose-6 phosphate receptor (39).

To clarify whether prosaposin and saposins in CD −/− fibroblast cells were in the correct cellular compartments, localization studies using immunofluorescence were performed (Fig. 8A and B) with rabbit anti-mouse saposin A or anti-mouse prosaposin antibodies. The anti-saposin A antibodies showed granular signal in WT cells that co-localized with the late endosome/lysosomal marker, Lamp1 (Fig. 8A). Similar co-localization was found in D −/− and CD −/− cells, but the signals were lower and less complete co-localization was evident in CD −/− cells compared with those in WT or D −/− cells. With anti-prosaposin antiserum, very low-level signals for prosaposin were detected in WT cells (Fig. 8B). Much more intense prosaposin signals were present in CD −/− and D −/− cells, and the signals significantly co-localized with the ER marker, calreticulin (Fig. 8B). These results are consistent with the greatest amount of prosaposin being retained in the ER in CD −/− cells.

To evaluate prosaposin processing and secretion, fibroblasts from CD −/−, D −/−, and WT mice were labeled with [35S]cysteine/methionine for 1 hour and chased for 0, 1, 4 and 24 h. The cell lysates and media were treated with goat anti-mouse prosaposin antibody that immunoprecipitates both prosaposin and saposins. In WT cells, most prosaposin was processed to saposins within an hour (Fig. 8C). Similar timing for prosaposin processing to saposin appearance was observed in D −/− cells, but more prosaposin remained at 0, 1 and 4 h, and some was present at 24 h. In CD −/− cells, prosaposin was processed slowly to saposins and low
levels of saposins A and B (saposins in Fig. 8C, also see Fig. 7C) were produced by 24 h. Prosaposin was present in the media of WT and D −/− cells, but was absent in the media from CD −/− cells (Fig. 8D). This result showed an impaired processing of prosaposin in CD −/− fibroblasts and the combined mutations on saposin C and D disrupted secretion of prosaposin protein.

GSL analyses

In CD −/− mice, GC accumulated to much greater extent than in WT mice in the liver (Fig. 9A), cerebrum (Fig. 9B), lung and kidney (data not shown), and to comparable levels as in PS −/− tissues. Compared with age-matched PS −/− mice, CD −/− mice accumulated much less LacCer in the liver (Fig. 9A), cerebrum (Fig. 9B) and lung (data not shown). Also, GalCer in CD −/− mice was at WT levels in the cerebrum, whereas it was greatly decreased in PS −/− mice.

In the cerebrum and kidneys from CD −/− mice, ceramide amounts were significantly increased (Fig. 10). In WT mice, ceramide levels were from mice harvested at 4, 7 and 13 wks and no differences were evident. In CD −/− and PS −/− brain, but not D −/−, increases (1.5–2.5-fold) of non-hydroxy-Cer (NFA-Cer) were found. The brain of CD −/− mice had ~3-fold increase in C24-Cer, and C24:1-Cer were ~2-fold increased in CD −/− and D −/− mouse brains (Fig. 10A). Marked increases (5–70-fold) in α-hydroxy-ceramide (HFA-Cer) levels were present in CD −/− and D −/− brains (Fig. 10A, bottom panel). C20–C24 HFA-Ceramides showed the major accumulations, whereas C18, C24:1 and C26-ceramides showed lesser increases. These accumulations were always about 2-fold greater in CD −/− mice than in D −/− mice. In PS −/− brains, the fold increases over WT were much less, ~3-fold. These results indicate that saposin C in the brain has an effect on HFA-Cer degradation in vivo, and indicates a synergistic interaction of saposin C and D in the hydrolysis of HFA-Cer in CD −/− mice. By LC/MS, sphingomyelin levels were not altered in CD −/− or D −/− mouse brains.

In the kidney, NFA-Cer were >2-fold increased in CD −/− and PS −/− (Fig. 10B), whereas they were at WT levels in this tissue from D −/− mice. The CD −/−, D −/− and PS −/− mice had HFA-Cer (C16–C26) accumulation in the kidney. The accumulation of long chain fatty acid ceramide (C20–C24) was more evident than those for short chain lipids. Unsaturated NFA-Ceramides (C18:1, C24:1) were not altered. In CD −/− kidney, HFA-Cer with C22 and C24 fatty acid acyl chains were ~2-fold increased over those in D −/− and PS −/− mice. Thus, both saposin C and D had effects on HFA-Cer metabolism in the kidney. In CD −/− liver, NFA-Cer was increased ~2-fold compared with WT. HFA-Cer was not altered (data not shown).

Decreased GCase in CD −/− mice

Owing to the effects of saposin C on GCase activity and protein levels (16), enzyme activity and western blot analyses were performed in CD −/− tissues. Decreases of GCase activity and protein were found in CD −/− livers (Fig. 11) and kidneys (data not shown). The GCase protein levels were ~37% of WT in CD −/− liver. No changes of GCase activity and protein were found in saposin D −/− liver (data not shown). This result supports the previous finding that saposin C protected the stability of GCase in vivo (16).

DISCUSSION

Saposins are activator proteins for their cognate enzymes in GSL degradation. Understanding of the physiological function for saposins derives from in vitro or ex vivo assays, and more recently, in vivo from newly developed hypomorphic mouse models (40). While the prosaposin null mouse (27) and a few such affected humans (30–32,41) provided some insights into the general function of prosaposin-derived saposins in vitro, the development of single saposin mutant mice has proved challenging (33,34). The impetus to develop single or combination saposin mutant mice was based on the observation that the phenotype of the prosaposin null mice differs significantly from the extrapolated sum of presumed functions of the individual saposins A, B, C and D (20,42,43). For example, the saposin A or D deficient mice manifest late onset of a Krabbe-like demyelinating disease, or ceramide accumulation and bladder defects, respectively (33,34). In comparison, such pathological findings are missing or minor in the prosaposin deficient mouse (27). Similarly, the saposin B or C deficient mice manifest slowly progressive sulphatide or glucosylceramide accumulation (Sun and Grabowski, unpublished data), whereas these are more prominent and rapid in the prosaposin deficient mice (40). Furthermore, prosaposin itself may have significant non-GSL hydrolytic roles in neuritogenesis, T-cell development and lipid transfer (21,22,24,44). To begin dissection of the interactions of the various prosaposin-derived saposins in the GSL pathway,
combined deficiencies of saposin C, the acid β-glucosidase activator, and saposin D, the acid ceramidase activator, were shown to have significantly different histopathological and biochemical effects than might be predicted from the individual saposin C and D deficiencies. In addition, the mutant prosaposin precursor to the altered saposins C and D showed abnormal post-translational processing, probably due to disruption of the folding pathway needed for transport from the ER to the lysosome for production of mature saposins.

The CD−/− mice provided in vivo insights into the role of saposins in lactosylceramide (LacCer) and ceramide hydrolysis in various tissues. Saposins A, B and C have been

![Image of Figure 3](https://academic.oup.com/hmg/article-abstract/16/8/957/553501/169755301)
implicated in the hydrolysis of lactosylceramide in cells (17,42), while in vitro detergent-free liposome systems of saposin B or C suggest their essential role in this hydrolytic reaction (17). The CD \(-/-\) mice had significantly less LacCer accumulation in both CNS and visceral tissues compared with that in the prosaposin null mice (i.e. total saposin deficient mice). Since saposin D has no known function in LacCer hydrolysis, these results suggest that in vivo saposin C is not essential to LacCer degradation, but other saposins could be, i.e. saposin B or A. LacCer does not accumulate to a significant extent in saposin A deficient mice (33). These results imply that saposin B is the major regulator of the \(\beta\)-galactosidase for LacCer hydrolysis (Fig. 12). Importantly, saposin C is essential for degradation of GC in vivo (32,45) and in cell culture (46). As expected, CD \(-/-\) mice accumulated GC. In addition, the lack of saposin C resulted in decreases of GCase protein and activity levels due to the inadequate saposin C protection of GCase from proteolysis (16,47). As a control, the GCase protein and activity decreases were not observed in saposin D \(-/-\) mice.

Saposin D enhances the activity of acid ceramidase (20), but ceramide degradation can be stimulated by saposins A, C or D in vitro and ex vivo (42,48). Saposin D \(-/-\) mice did not exhibit the in utero lethal phenotype of acid ceramidase deficiency in mice, nor do they resemble the human acid ceramidase defects (Farber disease) (34,35,49). Evidently, other saposins could partially compensate for the saposin D effect on acid ceramidase. Ceramide, especially HFA-Cer, levels in CD \(-/-\) mice were much higher than those in saposin D \(-/-\) mice. HFA-Cer accumulation was found mostly in CNS and epidermal tissues. In the brain, HFA-Cer derives to a large extent from degradation of GalCer and sulfatide that contain 40–70% hydroxylated ceramides (50). The balance of saposin C and D function may alter the structure of myelin since the ratio of NFA to HFA of galactosylceramide is known to alter the stability of myelin (38,51). These

Figure 4. Brain stem (A) and cerebellar (B) sections of CD \(-/-\), PS \(-/-\) and WT mice. (A) Brain stem sections from CD \(-/-\) had normal morphology and weak CD68 staining at 7.5 wks (A, left panels). PS \(-/-\) mice at 25 days (A, right panels) had storage in neurons of the brain stem (arrows) and strong CD68 staining (black). (B) Calbindin staining of age matched CD \(-/-\) (left) and WT (right) cerebellum showing loss of Purkinje cells in CD \(-/-\) mouse (7.5 wks).
Figure 5. Ultrastructural CNS of WT and CD−/− mice. Neurons from the cerebral cortex of WT (A) and CD−/− mice (B). The neurons (arrows) in the CD−/− mice show extensive complex storage inclusions. (C and D) Higher magnifications of inclusions shown in (B). The inclusion material is a mixture of electron dense and electron lucent vesicular bodies, multivesicular bodies and lamellar bodies. (E) White matter from a CD−/− mouse with an axonal spheroid (arrow) among normal appearing myelinated and unmyelinated tracts. (F) An axonal spheroid in a longitudinal oriented axon in the white matter associated with focal breakdown (arrows) in the myelin sheath. (G) A higher magnification of the inclusion material in the axon illustrated in (F). The axonal storage material is morphologically similar to the neuronal storage material (C and D). (H) White matter in WT. (I) CD−/− white matter with inclusion material in axons (arrows) and high magnification of the axon in the inset. (J) The granular cell layer of the CD−/− mouse with inclusion material (arrow). The Purkinje cell (P) does not show inclusions. The sciatic nerves in WT (K) and CD−/− (L) showed well myelinated fibers with no degenerative changes. N, nuclei.
results imply a differential efficiency of saposin C or D in the degradation of HFA-Cer or NFA-Cer, and that saposin C plays a significant in vivo role in the hydrolysis of specific ceramide species, i.e. HFA-Cer. Furthermore, these data suggest that these synergistic interactions in the degradation of HFA-Cer reflect the intrinsic ceramide compositions of tissues, since saposins C and D had effects on HFA-Cer in kidney and brain, but not in liver. Results and the in utero lethality of acid ceramidase null mice imply that saposin D may not be an essential activator for NFA-Cer metabolism, but that saposin C stimulates both HFA- and NFA-Ceramide degradation in vivo (Fig. 12).

In addition to the deficiency of saposin C and D, the CD −/− mice had low levels of saposin A and B. The phenotype of the CD −/− mice differs significantly from that of the PS −/− mice, i.e. total saposin deficiency. Specifically, the visceral tissues of CD −/− mice are morphologically normal and have no macrophage activation despite the presence of increased levels of GC and ceramide. In comparison, macrophage activation was observed in visceral tissues from PS −/− mice. CNS demyelination, axonal spheroids and myelin ovoids were also present in PS −/− mice (27,52). In contrast, inclusions in neurons and axons were a major pathologic feature of CD −/− brains without these other findings even at older ages. The storage inclusions in neurons and axons have similar morphological structure but could be derived from different origins. Like PS −/− mice and several other GSL storage diseases (52–55), axonal spheroids were evident in the CD −/− mice. The inclusions in neurons combined with the axonal spheroids result in subsequent disintegration of axon and neuron, thus led to the motor dysfunction and CNS deficit. The major differences in the CNS phenotype of CD −/− and PS −/− was that the latter had weakness in all limbs, whereas the CD −/− mice showed paralysis only in the hind limbs. Thus, we could speculate that the remaining saposin A and B in CD −/− mice could contribute to such differences since they are absent in the PS −/− mice. The CD −/− mice had well-formed myelin fibers and structure in CNS and PNS. Also, GalCer and sulfatide are major GSL components of myelin, and saposins A and B have β-galactosylceramidase and arylsulfatase A, respectively, as cognate enzymes. GalCer and sulfatide were at WT levels in the CD −/− mice. Thus, the low levels of saposin A and B were still above physiological thresholds needed to prevent substrate accumulations. In CD −/− mice, the greatest accumulated CNS ceramides were long chain HFA-Cer that derive from GalCer. Total deficiency of saposins in PS −/− mice led to demyelination with reduced GalCer and sulfatide levels (27). Consequently, a decreased flux of these GSLs through this pathway could explain their lack of accumulation of HFA-Cer in the PS −/− brain. However, the differential GSL flux in the kidney leads to a larger sulfatide load and accumulation in PS −/− mice (27). As a consequence, increased HFA-Cer was present in PS −/−, D −/− and CD −/− kidneys.

Prosaposin is targeted to lysosome by mannose 6-phosphate dependent and independent mechanisms (5,39). A lysosomal targeting sequence of prosaposin was localized to the C terminus of prosaposin and saposin D seems required (56). The processing of prosaposin to saposin occurs in late endosome and lysosome (5–7) and the sequence of each saposin’s cleavage is cell type specific (6). Obliteration of a disulfide bond in saposin D, i.e. saposin D deficiency, led to prosaposin retention in the ER, but did not affect the processing rate. The combined cysteine mutations within saposin C and D of the CD −/− mice led not only to ER retention of prosaposin, but also slowed the processing rate to saposins and decreased/eliminated its secretion. Most likely, the cysteine mutations resulted in prosaposin misfolding and ER trapping.

Figure 6. Western blot of MBP from brain. MBP levels were reduced in CD −/− and D −/− brain, but were much lower in PS −/− mice. Anti-MBP antibody recognizes four bands (14, 17, 18.5 and 21.5 kDa). The 17 and 18.5 kDa bands were not separated under these conditions. The bands were quantified using ImageQuant software and normalized to β-actin for each sample. The data represent the mean ± SE for multiple mice assayed in duplicates and presented as percentage to WT.

Figure 7. Prosaposin and saposin protein levels and glycosylation. (A) No saposin D was detected in CD −/−, PS −/− and D −/− mouse brains and livers by anti-mouse saposin D antibody, but increased prosaposin was present in CD −/− and D −/− tissues. No prosaposin and saposin D presented in PS −/− brain. (B) Saposin C was not detected in CD −/− fibroblasts using anti-saposin C antibody. (C) Saposin A and B levels were decreased in CD −/− liver as detected by anti-saposin A and saposin B antibodies, respectively. Prosaposin or saposin protein levels were normalized to β-actin in the same sample and presented as fold changes relative to WT. The data represent the mean ± SE for three mice assayed in duplicates. (D) Endo H digestion of fibroblast cell lysates. Greater proportion of prosaposin was sensitive to Endo H in CD −/− than in WT lysates.
Figure 8. Cellular localization and processing of prosaposin and saposin proteins. (A) Saposin A (green) was colocalized with lamp1 (red), an endosome/lysosome marker in WT cells and only partial overlap was present in D−/− and CD−/− cells. (B) Prosaposin (green) co-stained with calreticulin (red), an endoplasmic reticulum (ER) marker. WT cells had granular lysosomal localization of saposin A and low levels of prosaposin. D−/− cells showed lysosomal localization of saposin A and small portion of prosaposin retained in ER (calreticulin co-localization in B). In CD−/− cells most saposin A and prosaposin signals were in the ER region (calreticulin co-localization) and small portion of saposin A colocalized with lamp1. Magnifications are 400×. Rabbit anti-mouse saposin A and rabbit anti-mouse prosaposin antibodies (only reacts to prosaposin) were used in the study. (C) Processing of saposins in CD−/−, D−/− and WT fibroblasts. Metabolically labeled cells were chased with non-radioactive media for 0, 1, 4 and 24 h. Cell lysates were immunoprecipitated using goat anti-mouse prosaposin antibody that reacted to both prosaposin and saposins. In WT, prosaposin was processed to saposins within 1–4 h. Saposins in D−/− cell appeared within 1–4 h, but prosaposin was still present at 4 h. Prosaposin was processed at reduced rates in CD−/− cells and only small amounts of saposins were detectable at 24 h. (D) Immunoprecipitation of prosaposin from the media of radiolabeled fibroblast cell using goat anti-mouse prosaposin antibody. Prosaposin was secreted into medium from WT and D−/− fibroblast cells, but not from CD−/− cells. No prosaposin was present in PS−/− cells or medium. Pre, preimmune control serum.
but the misfolded prosaposin did not cause an upregulation of the ER stress response protein (Sun et al., unpublished data). The misfolded prosaposin also had altered sorting to secretory vesicles, since the CD2/2 prosaposin was not secreted at all into the media of cultured cells. Secreted prosaposin has been suggested to promote nerve regeneration (57) and spermato genesis (25), but this has not been proved in vivo. Also, the mechanism of prosaposin secretion remains to be fully clarified. Cells from CD2/2 mice have no extracellular prosaposin and could be useful for studying these aspects of prosaposin’s sorting and secretion, as well as its extracellular function(s) in various tissues.

MATERIALS AND METHODS

Materials
The following were from commercial sources: NuPAGE 4–12% Bis–Tris gel, NuPAGE MES SDS running buffer (Invitrogen, Carlsband, CA). 4-methyl-umbelliferyl-β-D-glucopyranoside (4MU-Glc; Biosynth AG, Switzerland). Sodium taurocholate (Calbiochem, La Jolla, CA). Rat anti-mouse CD68 monoclonal antibodies (Serotec, Oxford, UK). M-PER Mammalian Protein Extraction Reagent, ImmunoPure immobilized Protein G and BCA protein Assay reagent (Pierce, Rockford, IL). Molecular Dynamics Storm 860 scanner (GE Healthcare, Chicago, IL), Hybond™-ECL™ nitrocellulose membrane and ECL detection reagent (Amersham Biosciences, Piscataway, NJ). Anti fade/DAPI, Methyl green, ABC Vectastain and Alkaline phosphatase kit II (Black) (Vector Laboratory, Burlingame, CA). DIG Easy Hyb, Anti-digoxigenin-AP, CDP-Star, Positively charged Nylon membrane and Lumi-film chemiluminescent detection film (Roche Applied Science, Indianapolis, IN), Qiagen Quickchange II XL kit (Qiagen Inc. Valencia, CA). Restriction enzymes and Endo H (New England Labs, Beverly, MA). Rabbit polyclonal to Calreticulin—ER Marker (Abcam InC, Cambridge, MA). Rat anti-mouse Lamp1(MCD107A-D4B) (RDI, Flanders, NJ), Sheep anti-Myelin Basic Protein polyclonal antibody (Chemicon, Temecula, CA). Monoclonal anti-Calbindin D28K (Sigma, St Louis, MO). TOTALLY RNA (Ambion Inc. Austin, TX), High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). FITC conjugated goat anti-rabbit antibody and Rhodamine conjugated goat anti-rat antibody (ICN/CAPPEL, Aurora, OH).

Generating CD mutant mice
The mouse prosaposin genomic DNA clones and targeting vector (OSdupdel) were kindly provided by Dr Kunihiko Suzuki. Construction of the targeting vector is described in Supplementary Material, Methods 1. The Cys mutation breaks one of the three disulfide bridges in selected saposins that results in the deficiency of the individual saposin (33,34). The same strategy was adapted here to make saposin C and D deficiency by creation of Cys mutations in individual saposins. The targeting vector was linearized by NotI and introduced into the ES cell line derived from 129/SvEv mice. Homologous recombinant ES clones were screened by the PCR analysis using the primers outside of the short arm: CDF1 (5’-GCC AGA CCT GTC AGT TTG TGA TGA A-3’), or inside of the short arm: CDF2 (5’-CTG GAG GAG TAT ACG TGC CCA-3’), in combination with the primer in the 5’ region of neo, NEO2R (5’-AAC CAC ACT GCT CGA CAT TGG GTG-3’). The recombinant and WT clones generated 1.1 kb and 0.9 kb PCR products, respectively. The recombinant ES clones were confirmed by Southern blot analysis. The clones were digested by EcoR I and probed with 5’ or 3’ probes. The mutations in the

Figure 9. TLC GSL analyses of liver (A) and cerebrum (B). GC (arrow) accumulation was evident in CD−/− (7 wks) and PS−/− (4 wks) liver and cerebrum. In contrast to PS−/−, a slight increase of LacCer (arrow head) was present in CD−/− livers and cerebrius. CD−/− cerebrum (B) showed additional ceramide bands (small arrowhead). GalCer (small arrow) in cerebrum was decreased in PS−/− and equivalent to WT in CD−/− cerebrum. GC, glucosylceramide; GalCer, β-galactosylceramide; LacCer, lactosylceramide; TriCer, globotriaosylceramide; Sphing, sphingomyelin.
recombinant clone were verified by PCR and sequencing. The correctly targeted clones were used to generate chimeric mice by microinjection into C57BL/6J blastocysts. Electroporation, ES cell culture, selection and chimera breeding were carried out by the Gene-targeted mouse service core at the University of Cincinnati. To remove the neo gene, the heterozygous F1 mice were cross-bred to Zp3-Cre mice [C57BL/6-TgN(Zp3-Cre)93Knw] purchased from The Jackson Laboratory. Zp3 is expressed in the female germline and Cre expression is controlled by the regulatory sequences from mouse (Zp3) gene (58). The resulting CD+/- heterozygous females containing Zp3-Cre were then cross-bred to CD+/- heterozygous males for Cre recombination between two loxP sites in female germline. Zp3-Cre transgene was then removed by breeding the CD+/-, Zp3 with wild-type C57BL/6J mice. The intercross of CD heterozygous without neo gene and without Zp3-Cre generated saposin CD+/- combined mutant. The strain of background for saposin CD+/- is C57BL/6J/129SvEV. The strain background of saposin D+/- is C57BL/6J/129SvEV. The C57BL/6J/129SvEV PS+/- mice were back bred into FVB for 10 generations, i.e. pure FVB background, to obtain larger numbers of offspring. No apparent differences were observed between these two backgrounds in their phenotype, histology or survival. All mice were maintained in the microisolators in accordance with institutional guidelines under IACUC approval at Cincinnati Children’s Hospital Research Foundation. PCR genotyping, Southern blot analysis and RT–PCR were as described in Supplementary Material, Methods 2 and 3. Quantitative RT–PCR (qRT–PCR) was performed as previously described using β-actin as an internal control (59).

Histological studies

The tissues were collected, fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). Karnovsky’s fixative was used for Electron Microscopy study. CD68 monoclonal antibody staining was described previously (47). Calbindin antibody (1/200 in PBS with 5% BSA) was applied to paraffin sections and incubated overnight at 4°C. Detection was performed using ABC Vectastain and Alkaline phosphatase kit II (Black) according to manufacturer's instructions. Histological comparison was performed with standard light microscopy and digitally recorded.

Figure 10. LC/MS analyses of ceramides. (A) (upper panel) Non-hydroxy (NFA)-Ceramide C24 was >2-fold increased in CD+/- brains compared with those in the other genotypes. Smaller increases (1.5–2.5-fold) in C16, C18 and C20 ceramides were found in CD+/- and PS-/- mice. Ceramide C24:1 was increased (~2-fold) in CD+/- and D+/- mice. (Lower panel) HFA-Ceramides (C18–C26) were increased to much higher levels (5–70-fold) in CD+/- and D+/- brains, and only slightly increased (~3-fold) in PS-/- mice. The C18–C26 HFA-Ceramides in CD+/- mice were 2–5-fold higher than in D+/- mice. (B) (upper panel) NFA-Ceramide levels were increased in CD+/- and PS-/- kidneys, but not in those from D+/- mice. The increases were significantly greater in PS-/- mice. (Lower panel) HFA-Ceramides (C16, C20, C22 and C24) were greatly increased in kidneys from all three mutant mice with a predominant accumulation of C20–C24 ceramides. CD+/- kidneys had greater C20, C22 and C24 accumulations than those from D+/- and PS-/- mice. Fatty acids are denoted by chain length as C16, C20 or C24, etc. and number of double bonds as C24:1. DHC, dihydroxy. The amounts of ceramide were normalized to phosphate in each sample. The controls had minimal levels of ceramides despite the age difference. Fold change was obtained relative to means of all the control mice at various ages (n = 2–5).
to the manufacturer’s instruction. The slides were counter stained with methylgreen. For saposin protein immunofluorescence staining, the primary fibroblasts were incubated in the rabbit anti-mouse saposin A or rabbit anti-mouse prosaposin antisera (1/200 diluted in PBS with 1% normal goat serum) and rat anti-lamp1 monoclonal antibody (1/20). FITC-conjugated goat anti-rabbit anti-serum) and rat anti-lamp1 monoclonal antibody (1/20) or posin antisera (1/200 diluted in PBS with 1% normal goat serum) were applied to the samples, respectively. The signals were visualized by Zeiss Axiovert 200 microscope equipped with an Apotome.

**Tissue GSL analyses**

The GSLs in the tissue samples (~100 mg wet weight) were extracted as previously described (47). Relative proportions of lipids from the tissue samples were determined by thin-layer chromatography with borate impregnated plates (TLC, 10 cm² Merck HPTLC silica gel 60, 200 μm). The plates were developed in chloroform/methanol/water (65:25:4, v/v/v). GSLs were visualized with primulin spray (100 mg/l in 80% acetone) and blue fluorescence scanning (Storm 860, GE Healthcare).

HFA-Cer and NFA-Cer were analyzed by LC/MS in the Lipidome Core at the University of South Carolina. The ceramide content was normalized to phosphate content in the same sample after lipids extraction and presented as fold changes relative to WT and heterozygous controls. Two to five samples for each genotype were included in the analysis.

**Immunoblots and deglycosylation**

Tissues or cell extracts (50–200 μg) were separated on NuPAGE 4–12% Bis–Tris gel with NuPAGE MES SDS running buffer, and electro-blotted on Hybond™-ECL™ nitrocellulose membranes. The membranes were blocked in 3% BSA for 1 h, followed by incubation overnight with the rabbit anti-mouse saposin D, C, A or B antibodies (1/500 in 0.15% milk and 1% BSA) to detect individual saposins and prosaposin, and the sheep anti-human MBP (1/5000) to detect MBP in the brain tissues. Mouse anti β-actin monoclonal antibody (1/10 000 in 2% w/v dry milk) was applied to detect β-actin. The signal was developed using ECL detection reagent according to the manufacturer’s instructions. Protein concentration was determined using BCA protein Assay reagent. Mouse liver GCase protein detection was described previously (47). The amounts of prosaposin, saposin and GCase proteins were quantitated by ImageQuant software relative to the amount of β-actin in the same sample.

Mouse fibroblast lysate (60 μg) were denatured in 1x Glycoprotein denaturing buffer at 100°C for 10 min. The Endo H (1500 U) deglycosylation (New England labs) was carried on in G5 reaction buffer at 37°C for 8 h following the manufacturer’s instruction. Prosaposin protein was resolved by western blot using anti-mouse saposin D antibody.

The rabbit anti-mouse saposin A, B, C and D antibodies and goat anti-mouse prosaposin antibody were as described (47).

**Metabolic labeling**

Mouse fibroblasts (37°C) were maintained in DMEM +10% FBS +100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. Proteins in the mouse fibroblasts were radiolabeled for 1 h with [35S]cysteine/methionine (150 μCi) and chased with non-radioactive media for the indicated time. The radioactively labeled prosaposin and saposins in the cell lysates were immunoprecipitated using goat anti-mouse prosaposin serum as described (6). Prosaposin in the fibroblast cell medium (24 h labeling) was immunoprecipitated using goat anti-mouse prosaposin serum and ImmunoPure immobilized Protein G. The fibroblasts were matched for passage number and strain.

**Enzyme activity assay**

Tissues collected from CD −/− mice were homogenized and their GCase activities were determined fluorometrically with 4MU-Glc in 0.25% Na taurocholate and 0.25% Triton X-100 as described (60). The assay mixtures were incubated for 60 min (37°C). In all assays, WT control tissues were run in parallel.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.
Figure 12. The in vivo roles of saposin C and saposin D in lysosomal sphingolipids degradation. The current study provides in vivo data that saposin C is essential for degradation of glucosylceramide, but not essential for LacCer degradation. Saposin B compensates for saposin C as a major regulator of the β-galactosidase(s) for LacCer hydrolysis. Saposin C stimulates both NFA-Ceramide and HFA-Ceramide degradation, whereas Saposin D participates in HFA-Ceramide degradation. Saposin D does not appear to be an essential activator for NFA-Ceramide metabolism.

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Conflict of Interest statement. None declared.

REFERENCES


