Saposin C mutations in Gaucher disease patients resulting in lysosomal lipid accumulation, saposin C deficiency, but normal prosaposin processing and sorting

Anna M. Vaccaro¹, Marialetizia Motta¹, Massimo Tatti¹, Susanna Scarpa², Laura Masuelli², Meenakshi Bhat³, Marie T. Vanier⁴, Anna Tylki-Szymanska⁵ and Rosa Salvioli¹,*

¹Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Roma, Italy, ²Department of Experimental Medicine, University of Rome ‘La Sapienza’, Roma, Italy, ³Centre for Human Genetics, Bangalore, India, ⁴INSERM Unit 820, UCBI, Lyon-Est, Medical School, and Hospices Civils de Lyon, Lyon, France and ⁵Instytut-Pomnik Centrum Zdrowia Dziecka, Warsaw, Poland

Received March 2, 2010; Revised April 26, 2010; Accepted May 12, 2010

Gaucher disease (GD) is characterized by accumulation of glucosylceramide (GC) in the cells of monocyte/macrophage system. The degradation of GC is controlled by glucosylceramidase (GCase) and saposin (Sap) C, a member of a family of four small glycoproteins (Saps A, B, C and D), all derived by proteolytic processing of a common precursor, prosaposin (PSAP). Saps contain six cysteine residues, forming three disulfide bridges, that affect their structure and function. Sap C is an essential activator of GCase and its deficit impairs the GCase activity causing GD. In the present study the biological properties of cells from four recently described GD patients carrying mutations in the Sap C domain of the PSAP gene have been characterized. Two patients had mutations involving a cysteine residue, whereas the other two had a L349P mutation. It was found that: (i) in the four Sap C-deficient cells PSAP was normally processed and sorted, the lack of Sap C being mainly due to the Sap C instability in late endosomal/lysosomal environment; (ii) the decrease/absence of Sap C affected the GCase intracellular localization; (iii) the lowest level of Sap C and enhanced autophagy were observed in the cells, which carried a Sap C mutation involving a cysteine residue; (iv) the four Sap C-deficient fibroblasts stored GC, ceramide and cholesterol, the last two lipids being clearly localized in lysosomes; (v) a correlation was observed between the type of Sap C mutation and the Gaucher phenotype: apparently, mutations involving cysteine residues lead to a neurological variant of GD.

INTRODUCTION

Gaucher disease (GD), the most common sphingolipidosis, is characterized by the accumulation of glucosylceramide (GC) in the lysosomes of the cells of the monocyte/macrophage system. The degradation of GC is controlled by two proteins, namely the lysosomal enzyme glucosylceramidase (GCase) and saposin (Sap) C (1,2). Although most cases of GD are due to mutations within the GBA gene that codes for GCase, only seven patients with normal enzyme levels and mutations in the PSAP gene which encodes for Sap C have been described to date (3–11). The latter condition is rare, but most likely underdiagnosed, due to the lack of awareness and of easy biochemical testing.

Sap C is a member of a family of four small lysosomal glycoproteins (Saps A, B, C and D), all derived by proteolytic processing from a common precursor protein, prosaposin (PSAP), encoded by the PSAP gene on chromosome 10. Saps are essential cofactors for the lysosomal SLs degradation and can result in

*To whom correspondence should be addressed at: Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Roma, Italy. Tel: +39 0649902416; Fax: +39 0649387087; Email: rosa.salvioli@iss.it

© The Author 2010. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
conditions mimicking either GD (mutations in the Sap C domain), or metachromatic leukodystrophy (mutations in the Sap B domain), or Krabbe disease (mutations in the Sap A domain). The complete deficiency of PSAP results in a devastating, early infantile and rapidly fatal neurovisceral disease with a complex multiple SL storage (12,13).

All Saps contain about 80 amino acids and have six identically placed cysteine residues forming three disulfide bridges (14,15). The localization of the disulfide bridges has been well established, the first two half-cysteine residues being disulfide linked to the last two, and two half-cysteine residues centrally placed being linked to each other. The disulfide bonds, which establish interactions between distant parts of the Sap C molecule, play a major role in stabilizing its conformation. The conservation of the cysteine residues along the sequence of the four Saps indicates the importance of the disulfide arrangement for their functions. Disulfide cross-linking renders the structure of Saps remarkably stable to heat, acid environment and proteolytic degradation.

The main clinical manifestations of GD are hepatosplenomegaly, anemia, thrombocytopenia, bone lesions, pulmonary hypertension and, in few cases, neurological involvement. Three major clinical subtypes (16) have been defined, based on the onset and severity of the phenotype. Type 1 (GD-1), known as non-neuronopathic form, type 2 (GD-2) with a rapidly progressive neurological course and type 3 (GD-3) with a more slowly progressive neurological deterioration.

The first cases of GD due to Sap C deficiency displayed a type 3 phenotype (4,7). More recently, four new patients were identified. Although one of the cases also showed a type 3 phenotype (10), two adult siblings presented a clear non-neuronopathic form (9). The definitive clinical phenotype of the last case could not yet be assessed, due to her young age.

Having the unique opportunity to perform a comparative biochemical characterization in these four cases, we have investigated the biological consequences of the C mutations.

RESULTS

Prosaposin level and processing in Sap C-deficient fibroblasts

To investigate whether the PSAP mutations identified in four GD patients affected the PSAP stability, the level of PSAP in the fibroblast homogenates was evaluated. Genotypes and phenotypes of the patients (P1, P2, P3 and P4) as well as the location of the mutations in the Sap C protein are summed up in Figure 1. Western blot analysis revealed that the PSAP protein was present in all the Sap C-deficient fibroblasts, being significantly reduced (≏50% of control) in P1, P2 and P3 and quite normal in P4, as shown in Figure 2A. This result is in agreement with the presence of mutations (M1L and M1V) abolishing protein production on one PSAP allele of P1, P2 and P3.

Since the mutations in the Sap C domain of the PSAP gene do not prevent the formation of the PSAP protein, it was investigated whether these mutations might influence its processing and secretion. The cells were labeled for different periods of time and both the cell lysate and the growth medium were immunoprecipitated with an antibody which recognizes PSAP and Sap C. After 1 h of pulse, the band of the precursor PSAP (about 65–70 kDa) was present in control and Sap C-deficient fibroblasts (Fig. 2B, upper panel). After 5, 24 and 48 h of pulse, large and increasing amounts of Sap C were immunoprecipitated from control fibroblasts, whereas Sap C was poorly detected in P1 and P2 and almost absent in P3 and P4 fibroblasts. To analyze the presence of Saps other than Sap C, cell lysates were subsequently immunoprecipitated with an antibody which recognizes both PSAP and Sap B. Comparable amounts of Sap B were detected in control and Sap C-deficient fibroblasts (Fig. 2B, lower panel).

To investigate whether the mutated PSAPs present in the fibroblasts were also secreted from the cells, the growth medium was immunoprecipitated with an antibody which recognizes PSAP and Sap C. After 1 h of pulse, the band of the precursor PSAP (about 65–70 kDa) was present in control and Sap C-deficient fibroblasts (Fig. 2B, upper panel). After 5, 24 and 48 h of pulse, large and increasing amounts of Sap C were immunoprecipitated from control fibroblasts, whereas Sap C was poorly detected in P1 and P2 and almost absent in P3 and P4 fibroblasts. To analyze the presence of Saps other than Sap C, cell lysates were subsequently immunoprecipitated with an antibody which recognizes both PSAP and Sap B. Comparable amounts of Sap B were detected in control and Sap C-deficient fibroblasts (Fig. 2B, lower panel).

To check whether the other Saps present in the Sap C-deficient fibroblasts were correctly localized in lysosomes, confocal microscopy experiments have been performed. As shown in Figure 3, both Saps B and D colocalized with lysosomal-associated membrane protein 1 (Lamp1), a typical
lysosomal marker, indicating a normal transport and proteolysis of PSAP in lysosomes. As expected, Sap C was not detected in the mutant cell lines.

**GCase mass, activity and localization in Sap C-deficient fibroblasts**

It has been reported that Sap C not only activates GCase (by promoting the association of the enzyme with anionic phospholipid-containing membranes) (17) but also stabilizes it, protecting from proteolytic digestion (18). In order to check whether the absence of Sap C might affect the GCase stability, the amount of GCase protein in the four Sap C-deficient patients has been estimated by western blot analysis. Figure 4A shows that the level of the enzyme protein and the pattern of its molecular isoforms cross-reacting with a monoclonal anti-GCase antibody in the four mutant cell lines was similar to that in the control. These results indicate that in human cells Sap C is not essential for the stability of GCase.

The enzyme activity was also measured in fibroblasts homogenates, utilizing either the natural, [3H]GC, or the artificial, 4-methylumbelliferyl-β-D-glucopyranoside (4-MU-Glc) substrates. As shown in Figure 4B, in all four patients, the activity of GCase was in the low part of the normal range with both substrates, indicating that the enzyme is potentially active.

We have previously shown that Sap C mediates the interaction of GCase with lysosomal membranes (17,19–22). This consideration prompted us to examine by confocal microscopy the GCase intracellular distribution in the Sap C-deficient fibroblasts. As shown in Figure 5, LysoTracker Red, a fluorescent probe that accumulates in acidic organelles such as mature lysosomes, completely colocalizes with GCase in normal fibroblasts. In contrast, in the mutant fibroblasts, a large percentage of GCase was present in vesicles negative for LysoTracker, indicating that part of GCase resided in less acidic structures.

**Lipid accumulation in Sap C-deficient fibroblasts**

It is expected that GCase, although present at almost normal levels in the four Sap C-deficient fibroblasts, might be unable to hydrolyze GC due to the lack of Sap C. To verify this concept, the GC catabolism was examined by metabolic labeling the cells with [14C]serine. Compared with control fibroblasts, increased retention of labeled GC (at least 5-fold increase) in the four Sap C-deficient cell lines was observed (Fig. 6A). In control lipid extracts GC was below the level of visual detection. Unexpectedly, also the amount of ceramide (Cer) was dramatically increased. The densitometric analysis showed that the intensity of GC and Cer bands with a monoclonal anti-GCase antibody in the four mutant cell lines was similar to that in the control. These results indicate that in human cells Sap C is not essential for the stability of GCase.

The enzyme activity was also measured in fibroblasts homogenates, utilizing either the natural, [3H]GC, or the artificial, 4-methylumbelliferyl-β-D-glucopyranoside (4-MU-Glc) substrates. As shown in Figure 4B, in all four patients, the activity of GCase was in the low part of the normal range with both substrates, indicating that the enzyme is potentially active.

We have previously shown that Sap C mediates the interaction of GCase with lysosomal membranes (17,19–22). This consideration prompted us to examine by confocal microscopy the GCase intracellular distribution in the Sap C-deficient fibroblasts. As shown in Figure 5, LysoTracker Red, a fluorescent probe that accumulates in acidic organelles such as mature lysosomes, completely colocalizes with GCase in normal fibroblasts. In contrast, in the mutant fibroblasts, a large percentage of GCase was present in vesicles negative for LysoTracker, indicating that part of GCase resided in less acidic structures.

**Figure 2.** PSAP level and processing in Sap C-deficient fibroblasts. (A) Western blot analysis of PSAP in control and Sap C-deficient fibroblasts. Identical amounts of homogenates (10 µg of protein) were loaded in each lane. The blots were reprobed for β-actin as a loading control. (B) Control and Sap C-deficient fibroblasts were labeled with [35S]methionine–cysteine for the indicated times (pulse experiments). Cell lysates were immunoprecipitated with the anti-Sap C (upper panel) and subsequently with the anti-Sap B antibodies (lower panel). (C) The media from the pulse experiments were also immunoprecipitated with the anti-Sap C antibody. Immunoprecipitates were separated by SDS-PAGE and visualized by fluorography. The numbers on the left refer to the molecular mass of standards (in kDa).

**Figure 3.** Intracellular localization of Saps in Sap C-deficient fibroblasts. The fibroblasts from P3 were double-immunostained for Sap B and Lamp1 (upper panel) or Sap D and Lamp1 (middle panel) or Sap C and Lamp1 (lower panel). The right panels show the merged images. Saps B and D completely colocalize with Lamp1, an endolysosomal marker. No stain was observed with the anti-Sap C antibody, confirming the absence of this Sap. Scale bars, 10 µm.
were at least four to five times stronger in Sap C-deficient fibroblasts than in control fibroblasts (Fig. 6B).

To confirm the storage of Cer, immunofluorescence experiments were performed utilizing an anti-Cer monoclonal antibody, which has been previously used to demonstrate Cer accumulation in vesicles of Cftr-mice epithelial cells (23). In the control fibroblasts the Cer level was below the detection limit, whereas in all the Sap C-deficient fibroblasts strongly fluorescent vesicular structures were observed (Fig. 6C).

It has been found that free cholesterol (Chol) accumulates together with SLs in several lysosomal diseases (24). This observation prompted us to examine the intracellular distribution of Chol in the four Sap C-deficient fibroblasts. As shown in Figure 7A, increased filipin fluorescence was observed in each of these cell lines. To better characterize the Chol-containing vesicles, the P3 fibroblasts were also immunostained with a specific mouse monoclonal antibody anti-Lamp1, a marker of late endosomal–lysosomal organelles. The complete colocalization of Chol and Lamp1 indicates that free Chol accumulated in late endosomes/lysosomes. To check whether also SLs resided in the same organelles, the P3 fibroblasts were stained with filipin and immunostained with the antibody anti-Cer. Figure 7B shows that Cer and Chol colocalize in the endo/lysosomal system.

Enhanced autophagy in Sap C-deficient cells

Lipid lysosomal storage may lead to a dysregulation or a block of autophagy, which in turn has been proposed as a crucial component in the pathogenesis of several lysosomal diseases. Abnormal autophagy has been described in Niemann–Pick C1 (25), multiple sulfatase deficiency (26), Pompe disease (27), mucopolysaccharidosis VI (28) etc. In order to monitor whether also the four Sap C-deficient fibroblasts presented a defective autophagic degradation, we assessed by western blotting the level of the microtubule-associated protein 1 light chain 3 (LC3), a highly specific autophagosomal marker. During autophagosome formation, the LC3-I cytosolic form is converted into lipid conjugated LC3-II form, whose amount correlates with the number of autophagosomes (29,30). We analyzed the level of LC3-II isoform both in cells grown in normal medium (basal autophagy) and in starved cells (induced autophagy). In non-starved cells, a high level of LC3-II was observed in cells from P3. Following starvation, LC3-II levels rose in all the cells, the increase being more dramatic in P3 and P4 compared with control fibroblasts (Fig. 8A).

Enhanced autophagy may be due to either increased autophagosome formation or to decreased autophagosome degradation. To distinguish between these two possibilities, cells were incubated with E64d and pepstatin A, which are inhibitors of lysosomal proteases. As shown in Figure 8A, the levels of LC3-II dramatically increased in presence of protease inhibitors. In P3 and P4 cells, the level of LC3-II was 6–7-fold higher than in control fibroblasts treated in the same way, whereas a lower increase was observed in P1 and P2. This is consistent with the notion that fusion of autophagosomes with lysosomes is not blocked in Sap C-deficient fibroblasts and that increased autophagy may be at least partially due to augmented autophagolysosome formation (31).

Enhanced autophagy in the cells treated with the protease inhibitors was confirmed by electron microscopy. This analysis demonstrated an increased number of autophagic vesicles in P3 and P4 cells compared with control fibroblasts after treatment with E64d and pepstatin A (Fig. 8B). Autophagic vesicles appeared as non-homogeneous electron-dense vacuoles, sometimes with the aspect of multilamellar bodies.
DISCUSSION

Past work has evidenced the essential role played by Sap C in modulating the GCase function (17,19–22). Mutations in the Sap C molecule can impair the GCase activity causing GD. Only few cases of Sap C-deficient GD have been described up to now and the biological consequences of a decrease/absence of Sap C are poorly known. In the present study the properties of cells from four recently described patients with GD caused by mutations in the Sap C domain of the \textit{PSAP} gene have been characterized. The level of Sap C was dramatically reduced, but the fibroblasts from the four patients were able to produce PSAP and Saps (except Sap C) at almost normal levels. These results confirm that 'in vivo' GCase can express its activity only in the presence of Sap C, whose function cannot be substituted by other Saps.

In human cells, PSAP is synthesized as a 55 kDa protein in the endoplasmic reticulum, post-translationally modified to a 65 kDa glycosylated form and further glycosylated in the Golgi compartment to a 70 kDa product. The 65 kDa form is sorted to the lysosomes where it is converted into four small proteins, Saps A, B, C and D (molecular weight 10 kDa), whereas the 70 kDa form is sorted as such into the external medium. Our present results show that PSAPs carrying different mutations in the Sap C domain (Fig. 1) were sorted to the lysosomes, where they were converted into Saps as indicated by the lysosomal localization of mature Saps B and D. Moreover, none of the PSAP mutations involving the Sap C domain prevented the secretion of intact PSAPs from the cells (Fig. 2C). These results indicate that the processing and sorting of the mutated PSAPs were similar to those of control PSAP and that the dramatically decreased level or absence of Sap C in the cells was due to the instability of the Sap C protein in the endolysosomal environment. Interestingly, PSAP, Saps A, B and D were present at almost normal levels also in a Sap C-deficient mouse containing a knock-in of a cysteine to proline substitution in Sap C (32). On the other hand, no storage cells were found in the visceral organs of the Sap C-deficient mouse, whereas ‘Gaucher cells’ (enlarged macrophages containing undigested GC) were observed in P1, P2, P3 and P4 (9–11). More recently, other types of Sap C-deficient mice have been generated (33). These mice developed a neurodegenerative disease, but were not an appropriate model of human GD. Their tissues did not show accumulation of GC or infiltration of ‘Gaucher cells’. Most likely, it depends on the difference between humans and mice in SL metabolism, as suggested by the authors (33).
Sap C, like the other three Saps (A, B and D), contains six cysteine residues, forming three disulfide bridges. It was previously shown that the capacity of Sap C to interact with membranes and to stimulate GCase is greatly reduced after disruption of the disulfide bonds (14). The cells from P3 and P4 carry mutations that involve cysteine residues and prevent the correct formation of one of the three disulfide bridges in the Sap C molecule. Interestingly, no Sap C was present in P3 and P4 cells, whereas a very low, but detectable amount of residual Sap C could be observed in the fibroblasts from P1 and P2, where no cysteine residues were mutated. These results strongly suggest that the disulfide structure is essential not only for the activity, but also for the ‘in vivo’ stability of Sap C.

In our past work, we have shown that Sap C perturbs the organization of lipids in lysosomal membranes and mediates the interaction of GCase with lipid surfaces containing appropriate amounts of anionic phospholipids (17,19–22). It can thus be expected that a decrease in the Sap C level may affect the GCase interaction with and/or localization on membranes. Actually, the cells from P1, P2, P3 and P4 contained an almost normal level of GCase protein, but the lack of Sap C affected the intracellular localization of the enzyme, which partly resided in organelles less acidic than mature lysosomes. This finding confirms that Sap C plays an important role in the localization/interaction of the enzyme with the lysosomal membranes.

As shown by our and other laboratories, GCase alone cannot physically interact with its natural substrate, the membrane-embedded GC. Only Sap C, by perturbing the membrane bilayer, can help to expose the embedded GC, promoting in this way its contact with GCase and its enzymatic hydrolysis (17,19–22,34). In the four Sap C-deficient cell lines, GCase was potentially active, namely it was able to hydrolyze ‘in vitro’ both artificial (4-MU-Glc) and natural ([^3H]GC) substrates in the presence of activators such as detergents. The enzyme activity could not be expressed ‘in vivo’ in the absence of Sap C as demonstrated by the GC accumulation observed in the serum of P1 and P2 (9). After long-term ^14C-serine loading, storage of GC was clearly visible in the four Sap C-deficient fibroblasts (Fig. 6A). Several authors have observed that, in GD caused by mutations in the GBA gene, GC accumulates in macrophages, but rarely in cultured fibroblasts (35). The lack of GC storage in some GD fibroblasts was explained by the low but still significant residual activity of the mutated GCases. Otherwise, it was proposed that the uncleaved GC in GD fibroblasts was transferred from lysosomes to other cell compartments where it was further processed, being used as substrate for the synthesis of more complex glycolipids (36,37). The mechanism

![Figure 7](https://academic.oup.com/hmg/article/19/15/2987/2527156)
responsible for the extralysosomal transport of undegraded GC in non-macrophage cells is poorly known and the molecules involved in this process remain unidentified (37). The evident GC storage in fibroblasts from P1, P2, P3 and P4 suggests that not only the GC degradation, but also the intracellular transport of uncleaved GC is impaired in the absence of Sap C. A possible role of Sap C in the lysosomal lipid transport was suggested by recent findings showing that Sap C can extract a variety of lipids from intralysosomal membranes for loading onto CD1b, a glycoprotein that binds and presents lipids to T lymphocytes (38). It is tempting to hypothesize that Sap C might play a more general role in the transport of lipids from lysosomal membranes to other lipid-binding proteins or vesicles.

Also a considerable amount of Cer was stored in late endosome/lysosomes of the four Sap C-deficient fibroblasts. The Cer and GC storage looks like that previously observed in PSAP-deficient fibroblasts, where all the four Saps are missing (12,13). Sap D is the saposin that is thought to be involved in the lysosomal Cer degradation by enhancing the activity of lysosomal ceramidase (39). In a mouse model of Sap D-deficiency, Cer accumulates in the kidney and brain, but not in the cultured fibroblasts (40). In the fibroblasts of P1, P2, P3 and P4, Sap D cannot be responsible for the Cer accumulation since it is clearly present in the lysosomes (Fig. 3). The most likely explanation for the Cer lysosomal storage in Sap C-deficient cells is that ‘in vivo’ Sap C might play an important role in Cer degradation and/or lysosomal transport.

Free Chol accumulates together with SLs in several lysosomal diseases (24). Accordingly, we found storage of Chol in the lysosomes of P1, P2, P3 and P4 fibroblasts. Although the Sap C-deficient cells carried distinct mutations, no significant differences in the lipids stored in the four cell lines were observed. Of course, in cell types more relevant than fibroblasts in the physiopathology of GD, the lipid pattern can be differently modulated by the Sap C deficiency.
Lysosomal storage diseases such as GD are characterized by the accumulation of undegraded material in lysosomes, which become large and dysfunctional. It has been reported that the reduced function of lysosomes leads to autophagy alterations, suggesting that common mechanisms are downstream of different genetic defects (25–28). A variety of methods have been described to monitor abnormal autophagy. Among them, immunoblotting of LC3-II, a highly specific marker for autophagosome, is the most widely used. Under basal growth conditions, the highest level of LC3-II was observed in all cells, but the up-regulation of autophagy was especially evident in P3 and P4. Treatment of the cells with inhibitors of lysosomal proteases resulted in a similar increase in the LC3-II levels, indicating that autophagosomes–lysosomes fusion is not completely blocked in mutant cells and that the autophagy up-regulation, confirmed also by electron microscopy, might depend on alternative mechanisms.

The level of LC3-II in the Sap C-deficient fibroblasts seems to correlate with the PSAP mutational profile and with the patient phenotypes. P1 and P2, who clinically present with the full picture of typical GD-1, have low levels of LC3-II in their cells. Those are the only Sap C-deficient patients known to date not to carry a mutation affecting a cysteine residue. In contrast, the highest level of LC3-II was observed in the cells from P3, the patient displaying a GD-3 phenotype and carrying a mutation that involves a cysteine residue and carrying a mutation that involves a cysteine residue. P3 developed epilepsy from the age of 11 and showed neurological symptoms and intellectual decline by the age of 15 (10).

The fibroblasts from P4, with a deletion of seven amino acid residues, including a cysteine, show a very high level of LC-3 II after starvation or treatment with lysosomal protease inhibitors (induced autophagy). The phenotype of this patient cannot yet be well defined due to her young age (5 years old). Thus far, she does not present neurological manifestations, but the high level of induced autophagy suggests that her condition might deteriorate in the future.

The idea that a Sap C mutation involving a cysteine residue most probably results in a GD-3 phenotype is strengthened by perusal of the genotype and phenotype of two previously described Sap C-deficient patients. Both had a quite similar mutational pattern, with a mutation in the Sap C domain on one PSAP allele, involving a cysteine residue (p.C382F and p.C382G, respectively) and another mutation abolishing the production of all SapS on the other allele. Both cases displayed a GD-3 phenotype; one patient had a neurological onset at the age of about 4 years and died at 14 years of age (3,5), whereas the second patient had a normal development until 8 years and died at 15.5 years of age (4,6–8).

In conclusion, our work indicates that in the four Sap C-deficient cells the lack of Sap C is mainly due to the Sap C instability in the late endosomes/lysosomes. The lowest level of Sap C is observed when the mutation involves a cysteine residue in the Sap C molecule. The decrease/absence of Sap C impairs the ‘in vivo’ activity of GCCase and affects the enzyme intracellular localization. Our results also suggest that Sap C might have a role in the intralysosomal SL transport as indicated by the accumulation of GC and Cer in the lysosomes of the Sap C-deficient cells. Finally, a correlation was observed between the type of mutation and the GD phenotype: apparently, mutations involving cysteine residues and thus affecting the disulfide structure of Sap C lead to a GD-3 phenotype.

**MATERIALS AND METHODS**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), Minimum Essential Medium (MEM), Nu-PAGE gels and electrophoresis reagents were obtained from Invitrogen, Carlsbad, CA, USA. Fetal bovine serum (FBS) was obtained from HyClone, Waltham, MA, USA. Complete™ (protease inhibitor mixture) was obtained from Roche Diagnostics, Mannheim, Germany. Methionine/cysteine-deficient DMEM was obtained from ICN Biomedicals, Irvine, CA, USA. PRO-MIX L-[35S] (70% L-[35S]methionine and 30% L-[35S] cysteine, specific activity >1000 Ci/mmol), L-[3,34C]serine (56 mCi/mmol) and ECL western blotting reagents were from GE Healthcare, Buckinghamshire, UK. Earle’s Balanced Salt Solution (EBSS), Protein A–Sepharose CL-4B, 4-MU-Glc, filipin, E64d and pepstatin A were obtained from Sigma-Aldrich (St Louis, MO, USA). ProLong anti-fade kit and LysoTracker Red DND-99 were obtained from Molecular Probes (Eugene, OR, USA). X-Omat Blue films were from PerkinElmer Life Sciences (Boston, MA, USA). All other reagents were of the purest available grade.

**Cells**

Skin fibroblasts analyzed in this study came from four GD patients carrying mutations in the PSAP gene. Two siblings of Polish origin (P1 and P2, brother and sister) carry a L349P mutation in the Sap C domain on one PSAP allele and a M1L mutation abolishing PSAP production on the other allele. Both show characteristic symptoms of GD-1 (9). The third patient (P3), of French origin, carries a M1V mutation on one allele (again abolishing PSAP production) and a C315S mutation in the Sap C domain on the other allele (10). This patient has a clinical presentation of GD-3. A mutation in the start codon of PSAP, corresponding to the findings in patients P1, P2 and P3, but present on both alleles, was first described in ‘generalized saposin activator deficiency’ (41). The fourth patient (P4) is of Indian Sikh origin and carries a homozygous mutation resulting in the deletion of seven amino acids (342–348) in the Sap C domain. Both parents of P4 are heterozygous for this mutation. This is the first reported case with both alleles carrying a mutation in the Sap C domain (11). This patient, presently 5 years old, shows only systemic manifestations. Control fibroblast cultures were established from skin explants of normal individuals.

**Cell cultures**

Primary fibroblasts from GD patients (9–11) and from normal subjects were grown in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml of penicillin and 100 μg/ml streptomycin. The fibroblasts were used between passages 4 and 12.
To induce starvation, fibroblasts were cultured under amino acid deprivation in EBSS for 2 h. For drug treatment, cells were incubated for 24 h with the protease inhibitors, E64d (10 μM) and pepstatin A (10 μM). Fibroblasts were harvested after reaching confluence. Cells were suspended in 50 mM phosphate buffer, pH 6.5, containing 0.5% (v/v) Triton X-100 and one tablet of protease inhibitor mixture/50 ml (lysis buffer). After sonication for 20 s, the solutions were centrifuged for 30 min at 20,000g and the supernatants used as fibroblast homogenate.

**Antibodies**

Rabbit polyclonal anti-human Sap C, Sap B and Sap D were prepared as described previously (14). Mouse monoclonal anti-GCase antibody (8E4) was a kind gift from Dr. H. Aerts, E.C. Slater Institute for Biochemical Research, University of Amsterdam, The Netherlands. Anti-actin mouse monoclonal antibody was obtained from Merck Biosciences, Italy. Mouse monoclonal anti-Lamp1 was obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa, USA; anti-Cer mouse monoclonal antibody MAB_0011 was obtained by Glycobiotech GmbH, Germany. Anti-LC3 rabbit polyclonal antiserum was from the MBL International, Woburn, MA, USA.

Secondary antibodies were: goat anti-mouse or anti-rabbit conjugated to Alexa Fluor 488 or 594 (Molecular Probes); Cy3-coupled anti-mouse IgM (KPL, Gaithensburg, MD, USA); HRP-conjugated anti-rabbit or anti-mouse (GE Healthcare).

**GCase assays**

GCase activity was measured utilizing either [3H]GC or 4-MU-Glc as substrates. In the first case, the assay mixture contained, in a final volume of 0.2 ml, 40 μg [3H]GC (2600 dpm/nmol), 0.25% (w/v) sodium taurocholate, 0.05% (w/v) oleic acid, 0.1–0.2 mol/l sodium citrate–phosphate buffer, pH 5.6 (buffer A) and an appropriate amount of enzyme source. Samples were incubated at 37°C for 1 h. The enzymatically released [3H]glucose was measured as described previously (42). In the second case, the assay mixture contained, in the final volume of 0.2 ml of buffer A, 2.5 mM 4-MU-Glc, 0.1% Triton X-100 and 0.25% (w/v) sodium taurocholate. Assay mixtures were incubated at 37°C for 30 min. The extent of reaction was estimated fluorimetrically.

**Western blot analysis**

Fibroblast homogenates were electrophoresed through either a 7.0% or 4–12% sodium dodecyl sulfate (SDS)–polyacrylamide gels (43). After electrophoresis, proteins were electroblotted to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Italy). Membranes were blocked for 1 h with 2% non-fat milk powder in phosphate-buffered saline (PBS) containing 0.1% Tween 20 and incubated for 1 h with specific antibodies. Primary and secondary antibodies were diluted in blocking solution. Immunoreactive proteins were detected by an ECL Advance™ Western Blotting Detection kit, according to the manufacturer’s instructions (GE Healthcare).

**Metabolic labeling of cells and immunoprecipitation**

Prior to being labeled, fibroblasts (grown until near confluence) were washed twice with ice-cold PBS supplemented with 1 mM MgCl2 and 0.1 mM CaCl2. Fibroblasts were then starved for 2 h in methionine and cysteine-free medium containing 4% dialyzed FBS. This medium was replaced with the labeling medium (DMEM lacking methionine and cysteine and supplemented with PRO-MIX 1-[35S], 150 μCi/ml and 4% dialyzed FBS). The cells were pulsed for the indicated periods, detached from dishes by incubation with 0.25% trypsin, collected by centrifugation and lysed in phosphate buffer 50 mM, 0.5% Triton X-100 and protease inhibitors, pH 6.5.

The media from the pulse experiments and cell lysates were then incubated with rabbit pre-immune serum overnight at 4°C and non-specific complexes were precipitated with Protein A–Sepharose CL-4B. The clarified supernatants were then incubated with anti-Sap C antiserum. Cross-reacting material was precipitated with Protein A–Sepharose CL-4B. The cell supernatants were subsequently incubated with anti-Sap B antiserum. Cross-reacting material was precipitated as above. The immunocomplexes were washed four times with PBS containing 1% bovine serum albumin (BSA), 1% Triton X-100, 1% SDS, 0.4% sodium deoxycholate and finally with only PBS. The washed precipitates were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). Labeled proteins were detected by fluorography.

**Metabolic labeling of SLs**

Metabolic labeling of SLs was performed as described by Klein et al. (44). Briefly, control and GD fibroblasts were loaded with 2-[3-14C]serine (1 μCi/ml) in MEM containing 0.3% FBS for 24 h. Then, the medium was replaced by a chase medium, containing unlabeled 2-serine (185 nmol/ml) and 0.6% FBS. After 120 h, the cells were washed with PBS, detached from dishes by incubation with 0.25% trypsin and collected by centrifugation. Cell pellets were suspended in 500 μl water and total lipids were extracted with chloroform/methanol/water/pyridine (60:30:6:1, v/v/v/v) for 24 h at 40°C. Phospholipids were degraded by mild alkaline hydrolysis with methanolic sodium hydroxide (100 mM) for 2 h at 37°C and neutralized with acetic acid. Lipid extracts, after desalting by reverse phase chromatography on Strata X 33 μm (Phenomenex, Torrance, CA, USA), were applied on high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany) and developed with chloroform/methanol/0.22% aqueous CaCl2 (60:35:8, v/v/v). Radioactive-labeled SLs were visualized by fluoroaphotography and identified by their Rf values.

**Fluorescence microscopy**

Cells were grown on Labtek chamber slides (Nunc, Naperville, IL, USA) and fixed with 4% paraformaldehyde in PBS.
for 30 min. Cells were then rinsed with PBS, permeabilized 7 min with 0.05% saponin and incubated 2 h with 3% BSA. For intracellular free unesterified Chol staining, fixed cells were incubated 30 min with filipin solution (0.05% in PBS). The cells were observed with a UV 330–380 filter.

For labeling of acidic organelles, living cells were incubated 30 min with 100 nM LysoTracker Red DND-99 in culture medium at 37°C, before fixation.

For double immunostaining, the cells were incubated 1 h with a specific rabbit polyclonal primary antibody (anti-Sap B, anti-Sap C, anti-Sap D), rinsed twice with PBS and incubated 1 h with the secondary anti-rabbit antibody conjugated with Alexa Fluor 594. The cells were then rinsed twice with PBS, incubated 1 h with a specific mouse monoclonal primary antibody (anti-Lamp 1), rinsed twice with PBS and incubated 1 h with the secondary anti-mouse antibody conjugated with Alexa Fluor 488.

Finally, cells were mounted with ProLong anti-fade reagent and the fluorescence was viewed by fluorescence microscopy or by confocal laser-scanning microscopy.

For cellular double staining of Chol and anti-Lamp 1 or anti-Cer, cells were observed with an Olympus BX52 fluorescence microscope equipped with an excitation filter at 490 nm and an emission filter at 525 nm for Alexa Fluor 488 (green), with an excitation filter at 550 nm and an emission filter at 590 nm for Alexa Fluor 594 or Cy3 (red) and with a UV 330–380 filter for filipin (blue). Images were acquired with the IAS 2000 software and elaborated with Adobe Photoshop.

Other apparatus utilized was a Leica TCS SP2, equipped with an argon–krypton laser, double-dichroic splitters (488/568 nm), 520 nm barrier filter for Alexa Fluor 488 (green) and 590 nm barrier filter for Alexa Fluor 594 or Cy3 (red) observations. Image acquisition and processing were conducted by using the SCANware and Multicolor Analysis (Leica Lasertechnik, GmbH, Heidelberg, Germany) and Adobe Photoshop software programs. Signals from different fluorescent probes were taken in parallel, and colocalization was detected in yellow (Saps and Lamp 1), turquoise (Chol and Lamp 1) or in pink (Chol and Cer).

Electron microscopy analysis

Cells were fixed in 2.5% glutaraldehyde in PBS, pH 7.4, at 4°C, then washed in PBS and post-fixed in 1.33% OsO4 for 2 h at 4°C. After several washes in PBS, the cells were dehydrated in graded alcohol, transferred in toluene and embedded in Epon 812 resin (Electron Microscopy Sciences, Hatfield, PA, USA). The resin was allowed to polymerize in a dry oven at 60°C for 24 h. Thin sections were cut with a glass knife on a Reichert microtome, stained with toluidine blue and examined on a Philips electron microscope Morgagni CM10 (Philips, Eindhoven, The Netherlands). Each observation was carried out independently six to seven times per sample (45).

Conflict of Interest statement. None declared.

**FUNDING**

This research was partially funded by a research grant from Shire Human Genetic Therapies to A.M.V.

**REFERENCES**


