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Saposins (*sap*) A and C activate the degradation of galactosylceramide in living cells

Klaus Harzer[a,*], Barbara C. Paton[b], Helen Christomanou[c], Martine Chatelut[d], Thierry Levade[d], Masao Hiraiwa[e], John S. O'Brien[e]

[a]*Institut für Hirnforschung, Universität Tübingen, D-72070 Tübingen, Germany*
[b]*Department of Chemical Pathology, Women's and Children's Hospital, North Adelaide, Australia*
[c]*Laboratory of Neurochemistry and Molecular Biology, Athens, Greece*
[d]*Laboratoire de Biochimie, 'Maladies Métaboliques', INSERM U.466, C.H.U. Rangueil, Toulouse, France*
[e]*Department of Neurosciences, University of California-San Diego, Center for Molecular Genetics, La Jolla, CA, USA*

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Abstract In loading tests using galactosylceramide which had been labelled with tritium in the ceramide moiety, living skin fibroblast lines derived from the original prosaposin-deficient patients had a markedly reduced capacity to degrade galactosylceramide. The hydrolysis of galactosylceramide could be partially restored in these cells, up to about half the normal rate, by adding pure saposin A, pure saposin C, or a mixture of these saposins to the culture medium. By contrast, saposins B and D had little effect on galactosylceramide hydrolysis in the prosaposin-deficient cells. Cells from β-galactocerebrosidase-deficient (Krabbe) patients had a relatively high residual galactosylceramide degradation, which was similar to the rate observed for prosaposin-deficient cells in the presence of saposin A or C. An SV40-transformed fibroblast line from the original saposin C-deficient patient, where saposin A is not affected, showed normal degradation of galactosylceramide. The findings support the hypothesis, which was deduced originally from in vitro experiments, that saposins A and C are the in vivo activators of galactosylceramide degradation. Although the results with saposin C-deficient fibroblasts suggest that the presence of only saposin A allows galactosylceramide breakdown to proceed at a normal rate in fibroblasts, it remains to be determined whether saposins A and C can substitute for each other with respect to their effects on galactosylceramide metabolism in the whole organism.

Key words: Saposin A; Saposin C; Galactosylceramide; β-Galactocerebrosidase; In vivo

1. Introduction

The degradation of sphingolipids by lysosomal lipid hydrolases is facilitated by sphingolipid activator proteins, with the enzymatic deglycosylation of monohexosyl and small oligosaccharyl ceramides (for review, see [1]) and the desulfation of sulfogalactosyl ceramide (sulfatide [2]) being dependent on activator proteins. In addition, the enzymatic hydrolysis of ceramide to sphingosine and fatty acid is also mediated by an activator protein [3].

Two genes encoding sphingolipid activator proteins have been identified: the gene for prosaposin and one for the GM2 ganglioside activator [1]. Prosaposin is a precursor protein giving rise to four saposin (*sap*) activators, *sap* A, B, C and D, through proteolytic cleavage [4]. Two mechanisms of action appear to be involved in sphingolipid activator function. Some activators mobilize glycosphingolipids by loosening the sphingolipid's ceramide moiety within the membrane, thereby making the sphingolipid's glycosyl groups more accessible to sphingolipid hydrolases [5]. Other activators (e.g. *sap* C) bind to a given lipid hydrolase (e.g. glucosylceramide-β-glucosidase = β-glucocerebrosidase), rather than the sphingolipid, and the ensuing activator/enzyme complex is more effective at hydrolyzing its sphingolipid substrate [6].

The investigation of activator-deficient sphingolipidoses (for review, see [1]) has helped to elucidate the role of sphingolipid activator proteins in vivo, and has revealed that some activators do not always manifest the expected effects, as predicted from in vitro studies, in vivo. For example, in vitro studies indicated that *sap* A [7], *sap* B [8], *sap* C [7–10] and *sap* D [7,11] can stimulate sphingomyelin hydrolysis. However, studies on the human cases of prosaposin deficiency (Prosap-d) [12–14], as well as the Prosap-d mouse [15], showed normal levels of sphingomyelin in various tissues, suggesting that saposins are not required for sphingomyelin hydrolysis in vivo. These findings were supported by studies on Prosap-d fibroblasts which failed to show any defect in sphingomyelin degradation per se in lipid loading studies [12] or in sphingomyelinase assays [12,14,16]. Clearly, the function of a given activator should always be checked in in vivo systems to confirm or clarify any conclusions drawn from in vitro investigations.

Of the four saposins, the crucial role of *sap* B in the solubilization of some sphingolipid substrates (e.g. sulfatide and globotriaosylceramide [17]) has been clearly established (for review, see [18]), and recently the role of *sap* D in the activation of ceramide hydrolysis by acid ceramidase has been recognized [3,16,19].

The involvement of *sap* C in the deglucosylation of glucosylceramide by β-glucocerebrosidase has been supported by the finding that a genetic deficiency of *sap* C (SapC-d) resulted in a glucosylceramide storage disease resembling a neurologic form of Gaucher disease [20]. Meanwhile, in vitro studies indicated that *sap* C also activated the degalactosylation of galactosylceramide (gal-cer) [21] and lactosylceramide [22]. The dependence on one or more saposins for the degalactosylation of lactosylceramide in vivo was clearly established in studies on the Prosap-d patients, where all four saposins are absent [12–14].

So far, *sap* A has only been characterized in vitro. This

*Corresponding author. Fax: (49) (7071) 94-3893.

Abbreviations: gal-cer, galactosylceramide; Prosap-d, prosaposin deficient/deficiency; *sap* A, *sap* B, *sap* C, *sap* D, saposins A, B, C and D, respectively; SapC-d, *sap* C deficient/deficiency

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saposin was found to have similarities to *sap* C, including a mutual specificity for glucosylceramide and gal-cer hydrolysis [21], but differed, to some extent, from *sap* C in its binding to a lipid hydrolase [6].

Whether *sap* C, *sap* A, or other activator factors, also control the degradation of gal-cer in vivo has not yet been established. Only the enzymic factor (β-galactocerebrosidase) involved in gal-cer degradation has a proven role in gal-cer turnover in vivo, as evidenced by its deficiency in Krabbe disease.

To study the role of sphingolipid activators in the degradation of gal-cer in vivo, we undertook loading tests in fibroblast lines derived from patients with deficiencies in *sap* A and/or *sap* C. Cells from the original SapC-d patient [20] and from the Prosap-d patients [12,13] were available. A marked reduction in the in vitro activity of β-galactocerebrosidase in the Prosap-d patients [12,14,16] had already provided indirect evidence of an impairment in gal-cer catabolism.

The present study reports the results of gal-cer loading tests in Prosap-d, SapC-d and other cell lines. Our data provide evidence that *sap* A and *sap* C can activate gal-cer hydrolysis in vivo. The question remains open whether only one or both of these saposins is necessary to maintain adequate gal-cer turnover in the living organism.

2. Materials and methods

2.1. Cell lines

Control, Prosap-d, SapC-d, β-galactocerebrosidase-deficient (early infantile Krabbe disease), β-galactosidase-deficient (early infantile GM1 gangliosidosis) and ceramidase-deficient (infantile Farber disease) cells were grown from the stocks of frozen skin fibroblasts in our diagnostic laboratory. Some of these cell lines were also used after they were SV40-transformed. Transformation did not affect the biochemical characteristics of the Prosap-d cells [16], and retention of the *sap* C deficiency in transformed SapC-d cells was confirmed immunologically (Christomanou, unpublished) by double immunodiffusion [20].

2.2. Activator proteins

The preparation of crude *sap* C used in these studies was a post-DE52 column, concanavalin A-binding fraction [23] containing *sap* C and minor components of *sap* A, B and D. Pure saposins, *sap* A, B, C and D, were prepared from Gaucher disease spleen as described [24] and their purity was checked by SDS-PAGE and reverse phase HPLC (C4 column). Immunoblotting using monospecific antibodies [3,7,11,21,25] confirmed that there was no cross-contamination with other saposins in these preparations.

2.3. Radioactive gal-cer lipid substrate

The hydroxy fatty acid fraction of galactosylceramides from bovine brain (Sigma-Aldrich, Deisenhofen, Germany, no. C 8752) was catalytically hydrogenated with tritium across the ceramide double bonds and re-purified using two-dimensional thin layer chromatography on silica gel plates. The specific radioactivity was 3000 dps/nmol. This substrate was used for the lipid loading tests (see below) and for the determination of β-galactocerebrosidase activity in vitro [26].

2.4. Cell culturing

Before further use, the fibroblasts were maintained for 1 week in standard medium 199 (C.C. Pro GmbH, D-67433 Neustadt, Germany) with glutamine and 10% (v/v) fetal calf serum under an atmosphere of 5% CO_2.

2.5. Lipid loading tests with gal-cer

Layers of freshly confluent fibroblasts in 25 cm^2 flasks (about 0.4 mg protein) were incubated with 5 ml standard medium to which 13 nmol radioactive gal-cer, solubilized in 75 μl ethanol, was added. In

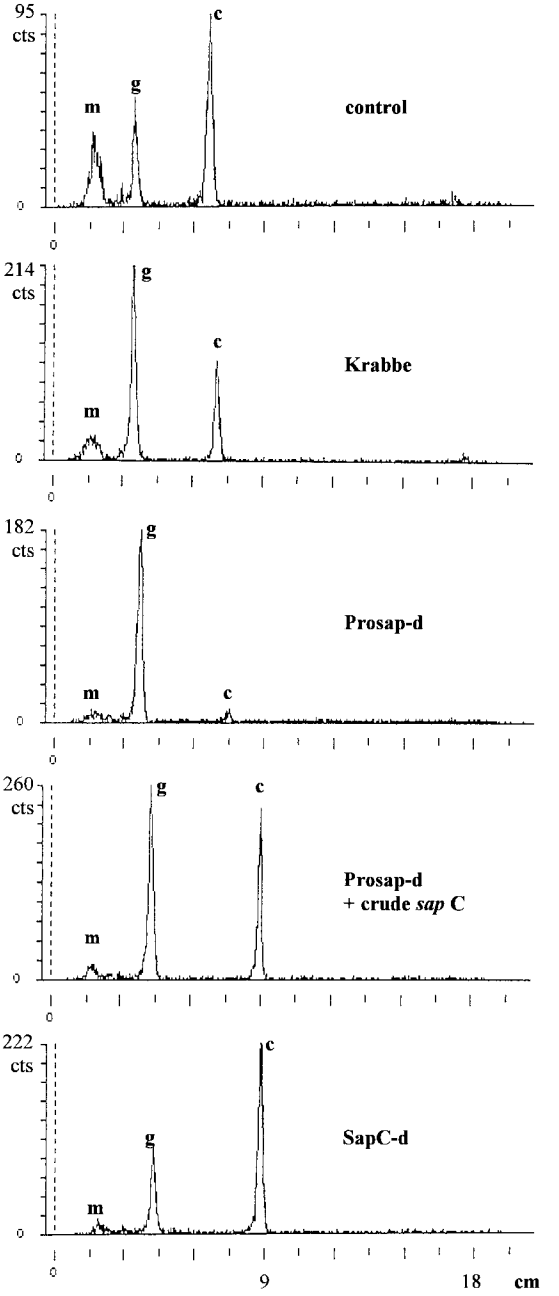

Fig. 1. Thin layer chromatographic analysis of recovered radioactive cellular lipids after loading intact fibroblast cultures with galactosyl [^{3}H]ceramide (gal-cer) for 3 days. The ordinate indicates the relative amount of radioactivity, where cts is counts per 13 min, and the abscissa indicates the distance moved in cm on the thin layer plates. The origin was at 1 cm and the solvent front at 18 cm. Please note that the migration distances for the radioactive lipid peaks vary slightly on different chromatography plates. Peak identification is as follows: g, undegraded gal-cer substrate; c, ceramide; m, metabolites except ceramide, including sphingomyelin and other biosynthesized lipids. Control, untransformed normal control cells; Krabbe, untransformed cells from a Krabbe disease patient; Prosap-d, untransformed cells from the prosaposin-deficient fetus; Prosap-d+crude *sap* C, the same cells with 16 μg crude *sap* C added per ml of medium; SapC-d, SV40-transformed cells from the original *sap* C-deficient patient (corresponds to the middle SapC-d sample in Fig. 2).

some experiments a specified amount (see Fig. 3) of crude *sap* C or pure saposin(s), dissolved in 0.1 ml water, was also added. After incubation for 3 days at 37°C, the cells were washed three times with phosphate-buffered saline and homogenized in 0.1 ml distilled water. One half of each homogenate was applied directly as a narrow streak to a thin layer silica gel plate (Merck, Darmstadt, Germany, no. 5626) and dried. The plate was first chromatographed twice with chloroform/methanol/acetic acid (47:1:2; by volume) to its upper edge, and then with chloroform/methanol/acetic acid/water (34:7:0.8:1; by volume) one third of the way up the plate. Radioscanning of the chromatograms revealed radioactive lipid peaks from the origin upwards in the following order: biosynthesized lipids including sphingomyelin (near the origin), the gal-cer substrate and free ceramide. The silica gel corresponding to each peak was scraped off the plate and the radioactivity determined by liquid scintillation counting. Lipid metabolites were identified according to our earlier experience with lipid loading tests [12,16].

3. Results

When gal-cer, labelled with tritium in the ceramide moiety, was loaded on SV40-transformed or untransformed fibroblast cultures for 3 days a proportion (about 7–10%) of the lipid substrate was endocytosed and a portion of this was further metabolized. Gal-cer metabolites and undegraded gal-cer were analyzed radiochemically after thin layer chromatographic separation (Figs. 1–3) and the percentage of endocytosed radioactive gal-cer which was metabolized was taken as a measure of the gal-cer metabolic degradation rate. For cell lines where appreciable amounts of gal-cer were degraded, the interassay variability in degradation rate was generally large, thus preventing the detection of possible small differences in degradation rate between different experimental conditions.

For normal control cells a major part of the endocytosed substrate was degraded, with ceramide, the direct hydrolysis product of gal-cer, usually being the most significant radioactive metabolite. Further metabolites, including radioactive biosynthesized lipids (in particular sphingomyelin), were also found, but their proportion only rarely exceeded that of ceramide. Despite their β-galactocerebrosidase deficiency, Krabbe cells metabolized appreciable amounts of the added gal-cer substrate, with a residual degradation rate about half that of control cells (Fig. 2; see also [27]). To check that the radioactive gal-cer substrate was indeed specific for β-galactocerebrosidase, we also tested the substrate in in vitro assays using homogenates of the same Krabbe cells and found that the residual enzyme activity was less than 3% of the activity in control cells. By contrast to the other cell lines, when Prosap-d cells were challenged with radioactive gal-cer, the cells produced very little labelled ceramide or other metabolites (Figs. 1 and 2), indicating that gal-cer catabolism was severely impaired.

Additional loading experiments were undertaken on Prosap-d cells with different preparations of saposins (crude *sap* C, or pure saposins A, B, C or D) added to the culture medium (Fig. 3). *Sap* B and *sap* D had little effect on the very low rate of gal-cer degradation in Prosap-d cells, stimulating the degradation by a factor of, at most, 1.5. On the other hand, addition of the indicated amounts of crude *sap* C, pure *sap* A or pure *sap* C stimulated gal-cer degradation in these cells 2–5-fold. However, complete correction of the gal-cer breakdown defect (i.e. to the mean rate of degradation found in controls) was not attained in the Prosap-d cells. Also, at the concentration used, addition of a combination of *sap* A with *sap* C was no more effective than adding one or other of these two saposins on their own.

Further gal-cer loading experiments were undertaken with

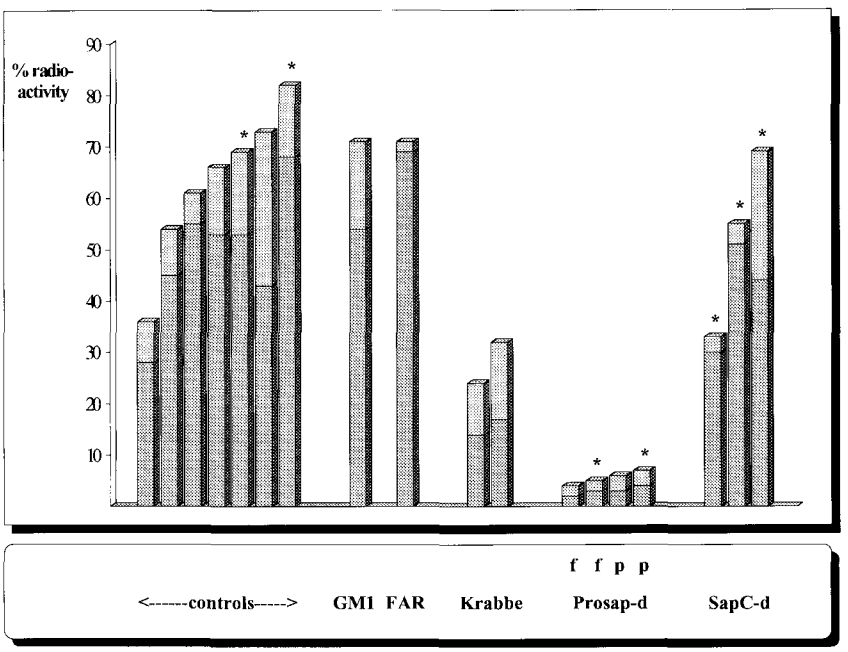

Fig. 2. Degradation of galactosyl [^{3}H]ceramide (gal-cer) in intact fibroblast cultures from normal, sphingolipidosis, and saposin-deficient individuals. The ordinate indicates the amount of radioactivity found in gal-cer metabolites, expressed as a percentage of the total radioactivity in cell bound lipids, after loading the cells with the radioactive lipid for 3 days. Lower column sections = ceramide; upper column sections = other metabolites, including sphingomyelin and other biosynthesized lipids (see m in Fig. 1). The cell lines were as follows: controls, seven normal control cell lines; GM1, GM1 gangliosidosis cells; FAR, Farber disease (ceramidosis) cells; Krabbe, two Krabbe disease cell lines; Prosap-d, prosaposin deficiency, with f and p indicating cells from the fetus and patient (each two experiments), respectively; SapC-d, saposin C-deficient cell line (three experiments). An asterisk denotes SV40-transformed cells.

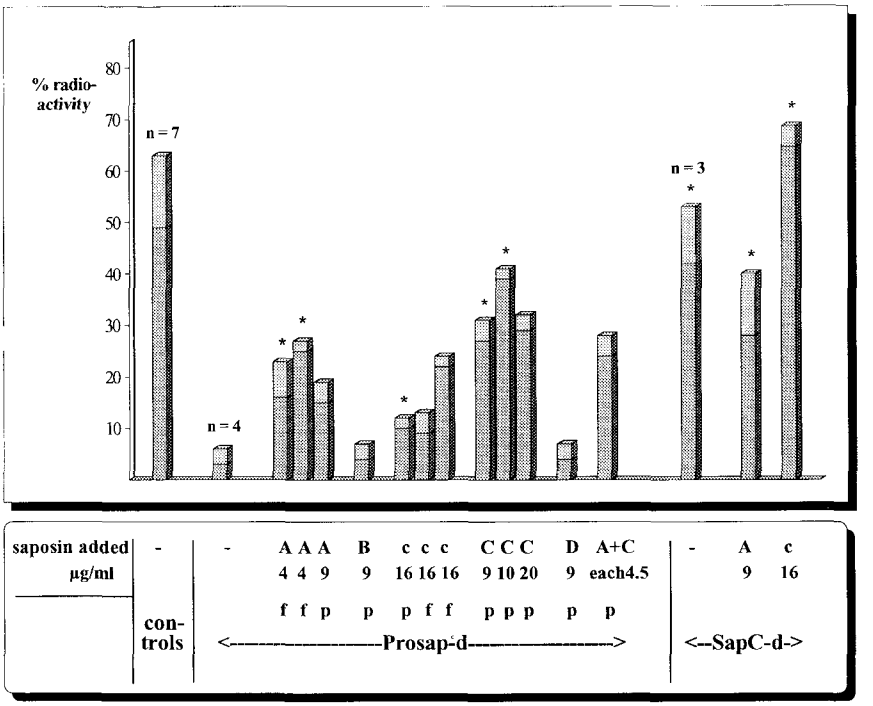

Fig. 3. Degradation of galactosyl [^{3}H]ceramide (gal-cer) in intact fibroblasts in the absence and presence of exogenous saposins. The ordinate indicates the amount of radioactivity found in gal-cer metabolites, expressed as a percentage of the total radioactivity in cell bound lipids, after loading the cells with the radioactive lipid for 3 days. Lower column sections = ceramide; upper column sections = other metabolites (see legend to Fig. 2). Columns marked $n = 7$ (controls), $n = 4$ (Prosap-d), and $n = 3$ (SapC-d) represent mean values for the indicated number of experiments without added saposins, as shown in Fig. 2, for control, Prosap-d and SapC-d cells, respectively. An asterisk denotes SV40-transformed cells. For each cell type, the type of saposin added is indicated under the relevant column as follows: −, no added saposin; A, B, C and D, pure *sap* A, B, C and D, respectively; A+C, a mixture of *sap* A and *sap* C; and c, crude *sap* C. The saposin concentrations used (µg/ml medium) are indicated and the cell types studied are as follows: controls, normal controls; Prosap-d, prosaposin deficiency, with f and p indicating the affected fetus and patient, respectively; SapC-d, saposin C deficiency.

SV40-transformed SapC-d cells, to determine whether the endogenous *sap* A present in these cells was able to sustain normal gal-cer catabolism in the absence of *sap* C. The growth behavior of the SapC-d cell line, as seen under the microscope, varied between experiments and may have contributed to the observed biological variability in gal-cer degradation rate. Nevertheless, the results indicated that gal-cer degradation was not significantly impaired in the SapC-d fibroblasts (the in vitro β-galactocerebrosidase activity for these cells was 59% of that in a transformed control cell line), with the variability in the rate of gal-cer degradation and in the conversion of ceramide to other metabolites being within the range found for control cell lines (Fig. 2). Results for the SapC-d cells in the presence of exogenous crude *sap* C or pure *sap* A were similar to those for unsupplemented SapC-d cells (Fig. 3). Gal-cer degradation was also normal in β-galactosidase-deficient (GM1 gangliosidosis) and ceramidase-deficient (Farber disease) fibroblasts (Fig. 2), however, the latter showed a higher proportion of radioactivity in ceramide.

4. Discussion

Previous investigations of human Prosap-d [14], and of the prosaposin knock-out model [15], have shown that a deficiency of all four saposins (*sap* A, B, C and D) leads to storage of multiple glycosphingolipids, including different galactolipids. Although the disturbed catabolism of lactosylceramide and sulfogalactosylceramide has also been demonstrated in Prosap-d cells in situ [13], the turnover of gal-cer had not been similarly studied in these cells.

Since it had been predicted that *sap* C [5] or *sap* C and *sap* A [21] would activate gal-cer degradation in vivo, we expected that the Prosap-d fibroblasts would be deficient in gal-cer degradation, and this has now been confirmed. In addition, we were able to show that supplementation of the culture medium with *sap* A or *sap* C, at around 10 µg protein per ml medium, activated the very low gal-cer degradation rate 2–5-fold, up to a value between about 20–50% of the mean control rate, whereas gal-cer degradation in control and Krabbe cells was not affected by addition of these saposins (results not shown). Addition of higher concentrations of these two saposins to Prosap-d cells did not show consistently higher levels of activation, nor, at the concentration used, was a mixture of equal parts of *sap* A and *sap* C more effective than either saposin on its own. *Sap* B and *sap* D gave little stimulation of gal-cer degradation in Prosap-d cells, nor did they further increase metabolic processing of gal-cer in cells that were already stimulated with *sap* A and *sap* C (results not shown). Our results, therefore, provide strong support for the hypothesis that *sap* A and *sap* C are the activators which mediate gal-cer degradation in vivo.

Possible reasons for the failure of added saposins to completely normalize gal-cer metabolism in the Prosap-d cells include the following. First, there may have been insufficient targeting of the activators to the lysosome to allow complete activation. An alternative explanation relates to the very low residual in vitro β-galactocerebrosidase activity in the Prosap-

d cells [12,14,16]. The degradation rate for gal-cer was much lower in Prosap-d than in Krabbe cells with a similarly low enzyme activity, and the addition of *sap* A and *sap* C only corrected the rate in Prosap-d fibroblasts to a value similar to that in Krabbe cells (n.b., it has been shown [28] that even very low enzyme activities can be sufficient to allow considerable sphingolipid breakdown). This raises the possibility that saposins may have an additional role in the biosynthesis, targeting or stabilization of the enzyme protein. A further possible explanation relates to the storage of a number of sphingolipids in Prosap-d [14], as it is conceivable that one or more of these lipids interferes with the interaction between exogenous saposins and β-galactocerebrosidase. Yet another explanation for the difference in degradation rates in the unsupplemented Prosap-d and Krabbe cells, and for the failure to fully correct the defect in the *sap* A- and *sap* C-supplemented Prosap-d cells, is that the enzyme β-galactosidase may also contribute to gal-cer degradation [29], and the putative gal-cer degrading activity of this enzyme could also be compromised in Prosap-d. However, β-galactosidase-deficient (GM1 gangliosidosis) cells showed normal metabolic processing of gal-cer, and addition of *sap* B to *sap* A- and *sap* C-stimulated Prosap-d cells did not increase the gal-cer degradation rate.

Physiological homology for *sap* A and *sap* C was predicted on the basis of their high sequence homology and their effects in vitro [21]. However, some functional heterology was later found when it was shown that these two saposins had distinct binding sites on β-glucocerebrosidase and that they could also act synergistically at certain saposin concentrations [6]. Although we did not observe synergy between *sap* A and *sap* C in their effect on gal-cer hydrolysis in fibroblasts under the conditions used, this does not exclude this possibility under different activator conditions or in different cell types.

The fatal glucosylceramide storage associated with SapC-d [20,30,31] suggests that *sap* A cannot completely substitute for *sap* C in the in vivo situation. However, when we studied gal-cer hydrolysis in SV40-transformed fibroblasts from the same patient no abnormality was found. Although the data indicate that *sap* A is involved in gal-cer hydrolysis in vivo, it remains open to what extent *sap* C is also involved. It is possible that both activators are required in tissues where there is a high substrate load (e.g. for gal-cer turnover in myelinotrophic cells, and in the macrophages for glucosylceramide degradation).

In conclusion, the in situ gal-cer loading tests, using Prosap-d and SapC-d fibroblasts, in the presence or absence of added saposins, has allowed further characterization of the role of saposins in gal-cer metabolism. If and when a patient with an isolated deficiency of *sap* A is identified, it should be possible to further clarify the functional relationship between *sap* A and *sap* C.

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