Saposins (*sap*) A and C activate the degradation of galactosylsphingosine

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Abstract As previously shown for [^{3}H-galactosyl]ceramide, the breakdown of [^{3}H-galactosyl]sphingosine was reduced in prosaposin-deficient skin fibroblast homogenates. Galactosylsphingosine hydrolysis was also deficient in cell homogenates from Krabbe's disease (β-galactocerebrosidase-deficient) patients, but not acid β-galactosidase-deficient patients. Moreover, hydrolysis of galactosylsphingosine in the prosaposin-deficient cell homogenates could be partially restored by adding pure saposin A or C, thereby identifying these saposins as essential facilitators of galactosylsphingosine hydrolysis. By contrast, saposins B and D had little effect on galactosylsphingosine hydrolysis in the prosaposin-deficient cells. The reduced galactosylsphingosine turnover in prosaposin-deficiency suggests that there could be a pathogenetic cerebral accumulation of galactosylsphingosine in this disorder. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

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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 and the desulfation of sulfogalactosyl ceramide being dependent on activator proteins (for review, see [1]). In addition, the enzymatic hydrolysis of ceramide to sphingosine and fatty acid is also mediated by an activator protein [2,3].

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 (e.g. *sap* B) mobilise glycosphingolipids by loosening the sphingolipid's ceramide moiety within the membrane, thereby making the sphingolipid's glycosyl groups more accessible to sphingolipid hydrolases [1]. 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 hydrolysing the sphingolipid substrate [1,5].

The investigation of activator-deficient sphingolipidoses (for review, see [1]) has helped to elucidate the metabolic role of sphingolipid activator proteins. For example, the crucial role for *sap* B in the solubilisation of some sphingolipid substrates (e.g. sulfatide and globotriaosylceramide) has been clearly established (for review, see [1]). Studies on *sap* B- and *sap* C-deficient cases, as well as the prosaposin-deficient (Prosap-d) patients [3,6–9], where all four saposins are absent, have highlighted the dependence of hydrolytic degradation of a number of sphingolipids on one or more saposins (for reviews, see [1,4]). In particular, analysis of the Prosap-d cells provided evidence for the crucial role of saposins in the degalactosylation of lactosylceramide [6] and galactosylceramide (GC) [9], thereby supporting the hypothesis that *sap* A and/or *sap* C are especially required for the hydrolysis of the monohexoside GC [10].

In Krabbe's disease, where the activity of β-galactocerebrosidase (GALC) is deficient, the degradation of both GC and *lyso*-GC (galactosylsphingosine = psychosine; PS), which lacks GC's fatty acid moiety, is defective [11,12]. Given that *sap* A and/or *sap* C activate the degalactosylation of GC by GALC [9], we wondered whether these saposins might also assist degalactosylation of PS by this enzyme. We have utilised Prosap-d cells to investigate this possibility.

2. Materials and methods

2.1. Cell strains

Control, Prosap-d [6–9,13,14], GALC-deficient (early infantile and late onset [15] Krabbe's disease) and acid β-galactosidase (ABG)-deficient (early infantile G_{M1}-gangliosidosis and juvenile Morquio-IVB syndrome [16]) cells were grown from stocks of frozen skin fibroblasts in our diagnostic laboratory in Tübingen, Germany. Clinical information for the relevant patients is provided in Table 1.

2.2. Cell culture and preparation of cell homogenates

Before 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) foetal calf serum under an atmosphere of 5% CO_2. Prior to performing the enzyme assays, the cell monolayers were washed twice with 0.9% (w/v) NaCl, and then 500 μl H_2O was added to each 25 cm^2 flask. After freeze–thawing twice, the cellular material was collected by scraping and the suspension was stirred for 2 min to give a homogeneous suspension.

2.3. Activator proteins

The preparation of crude sap C used in these studies was a post-DE52 column, Con A-binding fraction [17] containing sap C and minor components of sap A, B and D. Pure saposins, sap A, B, C and D, were prepared from Gaucher's disease spleen as described [10,18] and their purity checked by SDS–PAGE and reverse phase

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Abbreviations: ABG, acid β-galactosidase; GALC, β-galactocerebrosidase; GALC-GC, galactosylceramide-β-galactosidase; GALC-PS, psychosine-β-galactosidase; GC, galactosylceramide; Prosap-d, prosaposin deficient/deficiency; PS, psychosine (galactosylsphingosine); *sap* A, B, C and D, saposins A, B, C and D, respectively

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high performance liquid chromatography (C4 column). Immunoblotting using monospecific antibodies (rabbit polyclonal anti-sap A [10], anti-sap B [19] and anti-sap D [19] and mouse monoclonal anti-sap C [20]) indicated that the specific preparations were not contaminated with other saposins, and this was confirmed by N-terminal sequence analysis. Saposins were either added directly to the enzyme assays or, alternatively, the saposin was added to the cell culture medium at the indicated concentration 4 days prior to harvesting the cells.

2.4. Assay of psychosine-β-galactosidase (GALC-PS) activity

Radioactive PS was prepared from [^{3}H]GC [21] by alkaline hydrolysis [22]; the GC having been labelled with tritium in the galactose moiety using the tritium–borohydride procedure [23]. The [^{3}H]PS, which had a specific radioactivity of 10^4 dps/nmol, was purified from the deacylated preparation by preparative thin layer chromatography (TLC). For each reaction, 33 pmol of the stock PS solution was dried in a vial and then dissolved in 50 μl buffer solution containing 0.3 M acetate, 0.016 M phosphate and 0.012 M citrate (sodium salts) adjusted to pH 4.5. From 20 to 70 μg protein of fibroblast homogenate in 100 μl water was carefully admixed and the assay incubated for 4 h at 37°C. The reaction was stopped by adding 150 μg unlabelled galactose in 75 μl water. The assay volume was reduced to 40 μl, 80 μl methanol was added and the mixture completely applied to a silica-gel TLC plastic sheet (Macherey and Nagel, Düren, Germany, number 805013). The sheet was chromatographed (16 cm) using chloroform/methanol/water (14/6/1 by volume). The dried sheet was then deactivated in a refrigerator at 6°C for 20 min, and the cold sheet was chromatographed a second time with the same solvent mixture. The dried sheet was analysed with a linear radioscanner for radioactivity, and the peaks corresponding to the enzymatically released galactose and undegraded PS substrate were identified. Iodine vapours were additionally helpful in localising the radioactive galactose, as there was sufficient unlabelled galactose present to give an iodine-positive spot. The galactose and PS spots were cut out, along with the plastic backing sheet, and the radioactivity determined by liquid scintillation counting. In control cell assays, about 15% of the radioactivity from the added PS substrate was found in the galactose fraction.

2.5. Assay of galactosylceramide-β-galactosidase (GALC-GC) activity

GALC activity was determined using [^{3}H]GC as described [24].

3. Results

When compared to control cells, the GALC-PS activity was almost completely deficient in cell lysates derived from infantile Krabbe's patients, and it was also profoundly deficient in cells from two late onset Krabbe's patients (numbers 1 and 2), with cells from the third late onset patient (No. 3) having a somewhat higher residual activity (Table 2). By contrast, in ABG-deficient cells the GALC-PS activity was within the normal range (G_{M1}-gangliosidosis patient) or relatively high (patient with Morquio IVB syndrome).

The activity of GALC-PS was also profoundly deficient in cells from the Prosap-d patient [7,13] and foetus [8] (Table 2). Cell preparations from the foetus (patient cells could not be studied) showed a dramatic, approximately six fold, increase in GALC-PS activity on addition of *sap* A or C to the assay (for details, see Table 2), whereas in normal cell preparations, the GALC-PS activity was increased by a factor of only 1.3 on the addition of 5 μg *sap* C (Table 2).

The GALC-GC activity was characteristically deficient in all Krabbe's disease cell strains, with cells from the late onset patients showing generally higher residual activities than those from infantile patients (Table 2). A reduced GALC-GC activity was also confirmed for Prosap-d cell lysates (Table 2). Whereas addition of *sap* C to the GALC-GC assay had minimal impact on substrate turnover (see also Section 4), prior addition of *sap* C to the cell culture medium (30 μg crude *sap* C/ml) slightly increased (2.6 fold) the GALC-GC activity in the cell lysates (Table 2). Unfortunately, we were unable to do comparable experiments to assess the impact of pre-culturing the Prosap-d cells with *sap* C-supplemented media on the enzyme's GALC-PS activity, due to insufficient stocks of the saposin.

Table 1
Clinical findings for the patients

	Age in years at diagnosis (last report)	Clinical symptoms
Infantile Krabbe's disease		
No. 1	0.5	Severe leukodystrophic signs with spasticity, increased cerebrospinal fluid protein, reduced nerve conduction velocities (similar to descriptions in [24])
No. 2	0.9	As in No. 1
No. 3	1.1	As in No. 1
No. 4	1.1	As in No. 1
Late onset Krabbe's disease		
No. 1	10 (12)	Severe visual impairment, slightly ataxic movements, reduced nerve conduction velocities, neuro-imaged demyelinating foci
No. 2	4 (17)	Wheelchair-bound patient with contracted joints, atrophic muscles, severe scoliosis, visual impairment, inability to speak but only slightly impaired intellect, neuro-imaged demyelinating foci
No. 3	6 (22)	As in No. 2, but without scoliosis, and with additional symptoms including epileptic seizures, distinct signs of dementia, and swallowing difficulties necessitating tube feeding (see also an earlier report [15])
ABG deficiency		
G_{M1}-gangliosidosis	2	Psychomotor retardation, muscle weakness, hepatosplenomegaly
Morquio IVB syndrome	6	Corneal opacities, dysostosis multiplex, kyphoscoliosis, body length below the 3rd percentile [16]
Prosap-d		
Patient	0.3	Directly after birth there were hyperkinetic motor abnormalities, fasciculation of tongue and face muscles, spontaneous Babinski sign, generalised clonic seizures, hepatosplenomegaly, bone marrow storage cells; child died at 16 weeks [7,8]
Foetus		Pathology of generalised lipid storage [7,8,14]; pregnancy terminated at 20 weeks gestation

Table 2
Activity of specific β-galactosidases in cultured fibroblasts

	Added saposin	GALC-PS pmol/h/mg protein	GALC-GC nmol/h/mg protein
Normal controls			
Mean ± S.D. ($n = 10$)	n.s.a.[b]	20.3 ± 7.9	0.895 ± 0.33
No. 11	n.s.a.[b]	26.9[e]	
No. 11	5 μg pure *sap* C[c]	34.0[e]	
Infantile Krabbe's disease			
No. 1	n.s.a.[b]	0.15	0.033
No. 2	n.s.a.[b]	0.16	0.040
No. 3	n.s.a.[b]	0.50	0.075
No. 4	n.s.a.[b]	0.66	0.027
Late onset Krabbe's disease			
No. 1	n.s.a.[b]	0.99	0.081
No. 2	n.s.a.[b]	1.06	0.101
No. 3	n.s.a.[b]	3.70	0.083
ABG deficiency			
G_{M1}-gangliosidosis	n.s.a.[b]	15.0	0.85
Morquio IVB syndrome	n.s.a.[b]	43.8	0.90
Prosap-d			
Patient[a]	n.s.a.[b]	1.70	0.069
Foetus	n.s.a.[b]	1.57	0.125
Foetus	20 μg crude *sap* C[c]	9.23	
Foetus	5 μg pure *sap* A[c]	9.88	
Foetus	5 μg pure *sap* C[c]	10.2	
Foetus	10 μg pure *sap* C[c]		0.167
Foetus	30 μg/ml crude *sap* C[d]		0.331

Except where otherwise indicated (see below), the results are the mean of triplicate assays. Clinical information for the patients is provided in Table 1.
[a]SV40-transformed cells [13].
[b]n.s.a. indicates no saposin added.
[c]Saposin added to the enzyme assay.
[d]Saposin added to the cell culture medium.
[e]Mean of duplicate assays.

4. Discussion

In previous in vivo studies, we found that the defective turnover of GC in Prosap-d cells could be partially corrected by the addition of *sap* A and/or *sap* C to the culture medium [9]. The present in vitro investigations have clearly established that GALC-PS activity is also deficient in Prosap-d cells. In addition, the hydrolysis of PS could also be increased by supplementation with *sap* A or *sap* C, indicating that the GALC-PS defect in Prosap-d cells largely reflected an absence of saposins rather than a lack of functional GALC enzyme. By contrast, there was only a slight saposin-mediated increase in GALC-PS activity in normal cells.

In the Prosap-d cell lysates, the relative deficiency of GALC-PS activity was similar to that for GALC-GC. In this respect, the defect in GALC activity, as measured in various in vitro assays ([7,8] and present manuscript), was more marked in the Prosap-d lysates than the defect in β-glucocerebrosidase activity [7,8]. This raised the possibility that, in addition to the direct impact of the loss of saposin activation, there may be a secondary impact on the amount of GALC protein (discussed in [8,9]). In the present GALC-PS assays, addition of *sap* A or C led to a large increase in GALC-PS activity, up to values approaching those found for some normal cell lysates, thereby indicating that there was not a major loss of the GALC enzyme. However, due to the wide normal control range for GALC-PS activity, it is unclear whether this represents full recovery of GALC activity, so the possibility that there has been some impact on the amount of GALC protein cannot be completely discounted at this time. Indeed, the increased GALC-GC activity observed after incubating the Prosap-d cells with *sap* C may reflect an impact on the level of GALC protein. A failure to totally restore GALC activity on addition of *sap* A or C could also reflect some other deficiency, such as in the lipid microenvironment, of the in vitro assay system. Earlier studies have indicated that activation of the enzyme requires an acidic (phospho)lipid [25], and the large sphingolipid elevations in the Prosap-d fibroblasts [8] may also impact on the enzyme's activity. In addition, studies on the action of these two saposins indicate that, at least as far as β-glucocerebrosidase activity is concerned, they act synergistically [5,26]. Thus, the absence of the alternative GALC saposin activator (A or C), or indeed *saps* B and D, or perhaps prosaposin itself, may have an additional detrimental effect on GALC function. However, in previous studies we found no further stimulation of GC turnover when *sap* A- or C- treated Prosap-d cells were supplemented with *sap* B or D [9]. It is also possible that the absence of the prosaposin/saposin system suppressed the activity of other potential PS-degrading enzymes, such as ABG, which is known to degrade GC [27–29]. However, the present GALC-PS results with ABG-deficient cells, as well as in vitro studies demonstrating ABG's relative inability to degrade PS [12,27] compared to its appreciable GC-degrading activity [27], do not support the view that PS is hydrolysed by ABG. Compared to the GALC-PS activity, addition of *sap*

C to the in vitro GALC-GC assay reported here had minimal impact on the enzyme's activity. However, this probably reflects the presence of taurocholate in the assay [25]. In earlier studies using a liposomal assay system [8], addition of crude sap C clearly stimulated GALC-GC activity approximately three to five fold in both Prosap-d and normal cell lysates (Paton, unpublished); the fold activation being similar to that reported here for the GALC-PS activity in Prosap-d cell lysates.

It has been postulated that PS has a pathophysiological role in the neurodegeneration observed in Krabbe's disease [11,21,30], with the severe demyelination being attributed to cerebral accumulation of the cytotoxic PS [30,31]. Our results indicate that an accumulation of PS could result not only from a deficiency of the GALC enzyme, but also from a deficiency of *sap* A and/or C; both the enzyme and saposins being essential for its degradation. This view is supported by the recent observation that PS is elevated in a *sap* A-deficient mouse model [32]. Moreover, since the hydrolysis of glucosylceramide by β-glucocerebrosidase is also dependent on *sap* A and/or C [1,4,5,10], we predict that degradation of *lyso*-glucosylceramide (glucosylsphingosine) would also be impaired when these saposins are deficient. Just as PS has been linked to pathogenesis in Krabbe's disease, glucosylsphingosine cytotoxicity is thought to promote the neurodegeneration seen in Gaucher's disease types 2 and 3 [33].

Measuring the activity of GALC-PS in the absence and presence of added *sap* A and/or C should also facilitate the difficult diagnosis of Prosap-d. A deficient GALC-PS activity, which is responsive to added *sap* A/C, should be a characteristic finding in this disease, which has been rarely, and possibly under-, diagnosed in humans. Indeed, human Prosap-d has only recently been described for the second time [34]; the findings essentially confirming those from the original patient and foetal sib [6–8], and also paralleling the analogous findings from a Prosap-d murine model [35].

Until now, the in vivo action of saposins was thought to be directed towards the turnover of hydrophobic, ceramide-based sphingolipids. This is the first report of saposin-mediated hydrolysis of a more polar, natural, ceramide-free *lyso*-sphingolipid substrate, a finding which is consistent with the hypothesis that *sap* A and *sap* C interact with the substrate hydrolysing enzyme [1,4,5].

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