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Beta-glucosidase 1 (GBA1) is a second bile acid β-glucosidase in addition to β-glucosidase 2 (GBA2). Study in β-glucosidase deficient mice and humans

Klaus Harzer [a], Yotam Blech-Hermoni [b], Ehud Goldin [b], Ursula Felderhoff-Mueser [c], Claudia Igney [d], Ellen Sidransky [b], Yildiz Yildiz [e,*]

[a] Neurometabolic Laboratory, Klinik für Kinder- und Jugendmedizin, University of Tübingen, Tübingen, Germany
[b] Medical Genetics Branch, National Human Genome Research Institute, NIH, Bethesda, MD, 20892-4405, USA
[c] Kinderklinik, University of Essen, Essen, Germany
[d] Siemens MRT Group, Erlangen, Germany
[e] Department of Internal Medicine I, University Clinic of Bonn, Bonn, Germany

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ABSTRACT

Beta-glucosidase 1 (GBA1; lysosomal glucocerebrosidase) and β-glucosidase 2 (GBA2, non-lysosomal glucocerebrosidase) both have glucosylceramide as a main natural substrate. The enzyme-deficient conditions with glucosylceramide accumulation are Gaucher disease (*GBA−/−* in humans), modelled by the *Gba−/−* mouse, and the syndrome with male infertility in the *Gba2−/−* mouse, respectively. Before the leading role of glucosylceramide was recognised for both deficient conditions, bile acid-3-*O*-β-glucoside (BG), another natural substrate, was viewed as the main substrate of GBA2. Given that GBA2 hydrolyses both BG and glucosylceramide, it was asked whether *vice versa* GBA1 hydrolyses both glucosylceramide and BG. Here we show that GBA1 also hydrolyses BG. We compared the residual BG hydrolysing activities in the *GBA1−/−*, *Gba1−/−* conditions (where GBA2 is the almost only active β-glucosidase) and those in the *Gba2−/−* condition (GBA1 active), with wild-type activities, but we used also the GBA1 inhibitor isofagomine. GBA1 and GBA2 activities had characteristic differences between the studied fibroblast, liver and brain samples. Independently, the hydrolysis of BG by pure recombinant GBA1 was shown. The fact that both GBA1 and GBA2 are glucocerebrosidases as well as bile acid β-glucosidases raises the question, why lysosomal accumulation of glucosylceramide in GBA1 deficiency, and extra-lysosomal accumulation in GBA2 deficiency, are not associated with an accumulation of BG in either condition.

1. Introduction

Following the description of a non-lysosomal glucocerebrosidase [1] in addition to the lysosomal glucocerebrosidase long known to be deficient in Gaucher disease (glucosylceramide lipidosis), several steps were required to clarify the present definition of these two enzymes as β-glucosidase 2 (GBA2; OMIM # 609471) and β-glucosidase 1 (GBA1; OMIM # 230800, 230900, 231000), respectively [2,3]. The identity of the non-lysosomal glucocerebrosidase [1] to an independently described extra-lysosomal enzyme which hydrolysed bile acid–3-*O*-β-glucosides (BG) [4] was not clear. However, this situation changed when this enzyme also named 'microsomal β-glucosidase' or 'bile acid β-glucosidase' [4] was cloned [5]. This was followed by the generation of a 'bile acid β-glucosidase'-deleted mouse model in which extra-lysosomal accumulation of glucosylceramide was shown, and in which this accumulation was attributed to a deficiency in the deleted enzyme, with the recombinant wild-type of this enzyme, then named GBA2, being shown to hydrolyse glucosylceramide in different expression systems [3]. The phenotype of this *Gba2−/−* mouse presenting with male infertility [3] was completely different from that of the earlier known 'Gaucher mouse' (*Gba1−/−*) [6], which had both with visceromegaly and neurological symptoms (as in type 2 of human Gaucher disease, *GBA1−/−*; *GBA1* as a provisional gene symbol instead of the official *GBA1*).

Glucosylceramide is a substrate of both GBA1 and GBA2, and thus the question was raised as to whether BG, another natural metabolically important β-glucoside, is not only a substrate of GBA2 but also of GBA1, i.e., the latter enzyme is a second bile acid β-glucosidase in addition to GBA2. The question was answered by analysing the residual β-glucosidase activities towards BG in tissues and fibroblasts both *in vitro* and *in situ*, in materials from

Abbreviations: BG, bile acid-3-*O*-β-glucoside; GBA1, β-glucosidase 1, lysosomal-glucocerebrosidase; GBA2, β-glucosidase 2, non-lysosomal glucocerebrosidase; LABG, lithocholic acid-3-*O*-β-glucoside.

* Corresponding author at: University Clinic of Bonn, Department of Internal Medicine I, Sigmund-Freud-Str. 25, 53105 Bonn, Germany. Fax: +49 228 287 14698.
E-mail address: yildiz.yildiz@ukb.uni-bonn.de (Y. Yildiz).

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the *GBA1*−/−, *Gba1*−/− and *Gba2*−/− conditions and wild-types, and by degrading BG with pure recombinant GBA1. The existence of two bile acid β-glucosidases raises additional questions regarding why the two substrates, glucosylceramide and BG, differ in their contributions to the biochemical phenotypes in the studied enzyme-deficient conditions, with lysosomal glucosylceramide accumulation in the *GBA1*−/− and *Gba1*−/− conditions, extra-lysosomal glucosylceramide accumulation in the *Gba2*−/− condition [3,7], but no accumulation of BG in either condition according to present knowledge. In particular, bile acid metabolism is essentially normal in *Gba2*−/− [3].

2. Materials and methods

2.1. Patients

The phenotypes and genotypes of the Gaucher patients studied are listed in Table 1. Informed consent was provided under a clinical protocol approved by the Institute Review Board. We included patients with low residual glucocerebrosidase activity, in particular the Gaucher type 2 patient G1 [8].

2.2. Materials

The following reagents were procured from commercial sources: isofagomine D-tartrate (No. I816010, Toronto Research Chemicals, Toronto, Canada), sodium taurocholate (No. 86340 Sigma–Aldrich, Taufkirchen, Germany), and glucosyl-(stearoyl-1-[^{14}C])ceramide with a specific radioactivity of 2035 dps/nmol (No. ARC-1331, Biotrend, Köln, Germany). Lithocholic acid β-3-*O*-[U-^{14}C]glucoside with a specific radioactivity of 629 dps/nmol was a gift from F. Dallacker, Department of Organic Chemistry, Technical University of Aachen, Germany. Pure recombinant GBA1 was pooled from small traces of one of the industrial preparations for Gaucher enzyme replacement therapy that remained in the ampoules that had been therapeutically used strictly as prescribed. These ampoules, otherwise handled as waste, were a generous gift from a Gaucher patient who was responding well to therapy.

2.3. Methods

2.3.1. Crude tissue preparation

Frozen human and murine liver and brain samples (from stocks stored at −70 °C) were homogenised in 10 volumes (w/v) of water, put through three cycles of freezing and thawing, and centrifuged at 500*g* (3 min) to remove non-homogenisable material. The supernatant, i.e., uniform homogenate was used in the enzyme assays. Solubilisation of the enzymes was by the chemicals present in the assays.

2.3.2. Preparation of cultured fibroblasts

Murine or human primary (up to 10th passage) fibroblasts were cultured to early confluency, and then placed in fresh RPMI medium containing 10% calf serum. After 24-h incubation they were washed with saline and given a final wash with water (10 s). The cell layer was homogenised (briefly sonicated) in 0.4 ml water in the culture flask, and the homogenate was used for the enzyme assays. This method led to similar specific activities as those in homogenates of trypsin-harvested cells.

2.3.3. Protein determinations

Were performed using the Lowry technique; bovine serum albumin was the standard.

2.3.4. In vitro enzyme assays

2.3.4.1. Bile acid β-glucosidase. The assay comprised 30–250 μg protein in 70 μl total volume with 80 μM lithocholic acid β-[U-^{14}C]glucoside and 0.15 M sodium acetate, pH 5.5. No $MnCl_2$, EDTA or dithioerythritol [4] were added, because they had no effect on the lithocholic acid β-glucosidase activity in these preparations. After 90-min incubation at 37 °C, the reaction was stopped by the addition of 70 μl methanol. One third of the mixture was applied to a silica gel TLC plate (No. 1.05721, Merck, Darmstadt, Germany). The solvent mixture for chromatography contained chloroform, methanol and water (14:6:1 by volumes). The chromatogram was radioscanned, and the radioactivity peaks corresponding to lithocholic acid β-glucoside and glucose were identified. Enzymatically released glucose was evaluated quantitatively using commercial hard- and software (LB2821, Berthold, Wildbad, Germany). The shift in radioactivity from lithocholic acid β-glucoside to glucose was a measure of bile acid β-glucosidase activity. For assays using the GBA1 inhibitor *isofagomine* (Fig. 1B), a 10 min pre-incubation was performed at 37 °C prior to the addition of the β-glucoside substrate with and without 4.5 (fibroblasts, 2.9) μM isofagomine. For pure *recombinant GBA1* (Table 3), the above assay was modified by increasing the concentration of lithocholic acid β-[U-^{14}C]glucoside up to 175 μM, adding 0.8% (w/v) taurocholate, using 12.5 μg enzyme, and reducing the incubation period to 5 min.

2.3.4.2. Glucosylceramide β-glucosidase (glucocerebrosidase). The assay contained 30–250 μg protein in 70 μl total volume plus 6 μM glucosyl-(stearoyl-1-[^{14}C])ceramide), 0.08 M sodium acetate, pH 5.5, 0.02% triton X-100, 0.4% (w/v) taurocholate and 0.35% heat-inactivated bovine serum albumin. The assay was incubated for 120 min at 37 °C, and the reaction was stopped by the addition of 70 μl methanol. Half of this mixture was applied directly to a silica gel TLC plate (No. 1.05721, Merck). Chromatography was performed using a solvent mixture of chloroform, methanol and acetic acid (47:1:2 by volumes). The chromatogram was radioscanned to quantify the radioactive peaks of glucosylceramide, enzymatically released ceramide and free fatty acid. The shift in radioactivity from glucosylceramide to the breakdown products was a measure of glucocerebrosidase activity. For pure recombinant GBA1 (Table 3), the above assay was modified by increasing the concentration of the glucosylceramide substrate from 6 (radioactive substrate) to 175 μM by adding unlabelled, native glucosylceramide isolated from Gaucher spleen, increasing the taurocholate concentration to 0.8% (w/v), using 12.5 μg enzyme, and reducing the incubation period to 5 min.

Table 1
Different Gaucher disease patients with phenotype and genotype.

No.	Gaucher disease (GD) phenotype	Age at examination/death (years)	*GBA1* genotype	Reference
G1	GD type 2, liver failure, arthrogryposis	Neonatal death	c.1515_1516ins AGTGAGGGCAAT/R120Q	[8]
G2	GD type 2, severe neurological involvement	0.9/0.9	G202R/G202R	
G3	GD type 2	Neonatal death	D409H; H255Q/recNciI	[18]
G4	GD type 3, oculomotor abnormality	1.9/alive	L444P/L444P	
G5	GD type 2, severe neurological involvement	1.2/1.8	Not done	

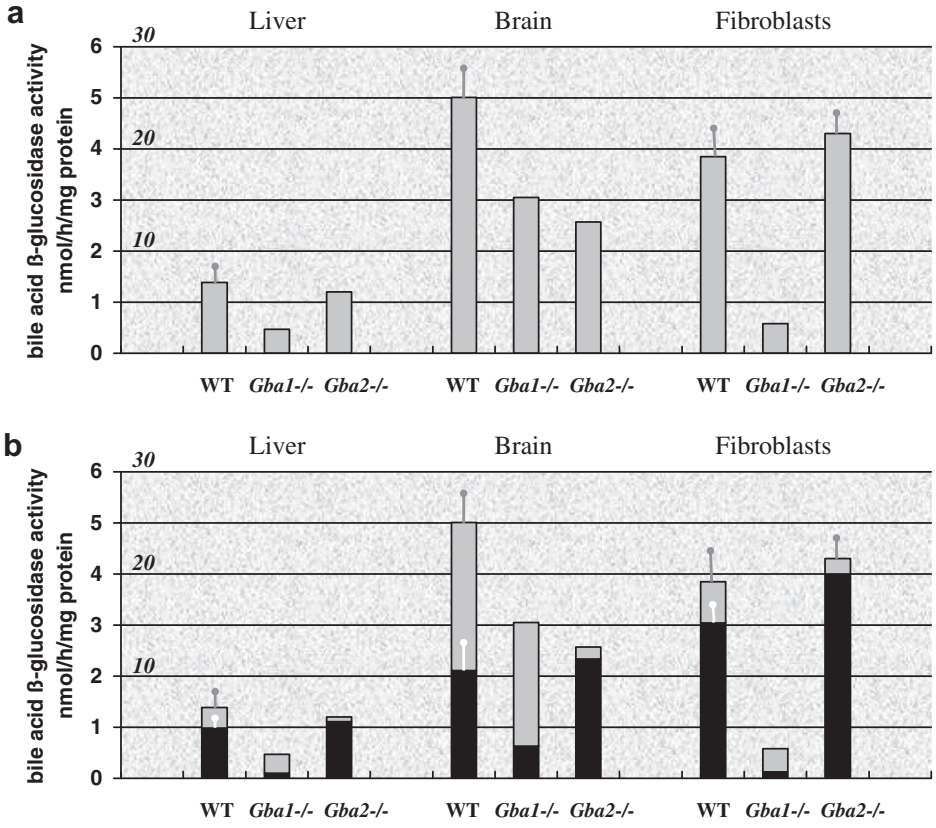

Fig. 1. (A and B) Bile acid β-glucosidase activity in murine tissues and cultured fibroblasts. WT, wild-type; *Gba1*−/− and *Gba2*−/−, the GBA1 and the GBA2 knock-out mouse. Ordinates, nmol lithocholic acid β-glucoside degraded per h and mg protein; numbers in *italics* for fibroblasts. (A) Shows the total activities. These are divided in (B) into the proportions that were inhibited (dark, lower columns sections) or not inhibited (light, upper sections) by 4.5 (in fibroblasts, 2.9) μM of the GBA1 inhibitor isofagomine. Some non-specific inhibition also of GBA2 (see *Gba1*−/− columns; GBA2 active) slightly biases the inhibited proportions (dark column sections) as measures of GBA1 activity in WT. Very small non-inhibited proportions in *Gba2*−/− (GBA1 active) seem to be due to non-GBA1/non-GBA2 β-glucosidases. *Bars* above columns (or above dark column sections) indicate standard deviations ($n = 6$); where bars are absent, the mean of three determinations is given.

2.3.5. In situ enzyme assay: loading of fibroblasts with lithocholic acid β-[U-14C]glucoside

The medium in a 25 cm^2 flask of confluent fibroblasts at about the 10th passage (containing about 0.4 mg protein) was replaced with 4 ml fresh RPMI medium containing 10% foetal calf serum. After a 4-h incubation in a 5% CO_2 atmosphere at 37 °C, 40 nmol (in 75 μl water) lithocholic acid β-[U-14C]glucoside was added to the medium (4 ml). After another 72-h incubation, the medium was removed and saved. A mixture of 40 μl medium and 60 μl methanol was directly applied to a TLC plate (No. 1.05721, Merck), run with a chloroform, methanol and water solvent (14:6:1 by volumes), and the chromatogram scanned to determine the radioactive glucose released into the medium.

3. Results

3.1. Bile acid β-glucoside (BG) hydrolysing activity of GBA1 in vitro

The following results obtained using the lithocholic acid–3-*O*-β-glucoside (LABG) species of BG as substrate show the role of GBA1 (glucocerebrosidase; glucosylceramide β-glucosidase) also as a bile acid β-glucosidase: (i) In cells and tissues from the *Gba1*−/− mouse, the deficit of BG hydrolysing activity as compared to wild-type reflected the BG hydrolysing activity of GBA1 present in wild-type (Fig. 1A). (ii) In the *Gba2*−/− mouse the high residual level of BG hydrolysis confirms BG hydrolysis by GBA1 (Fig. 1A), given that nearly all other β-glucosidase activity was absent in *Gba2*−/− (for the unexpectedly high residual activity in *Gba2*−/− fibroblasts, see Section 3.3). (iii) In the wild-type mouse, the GBA1 inhibitor isofagomine [9–11] inhibited a high proportion of the total BG hydrolysing activity so that this proportion was essentially attributable to GBA1. (iv) In analogy to the *Gba1*−/− mouse, *GBA1*−/− (human Gaucher patient) cells and tissue homogenates showed reductions of 50–85 % of BG hydrolysing activity compared to normal materials (LABG panels in Table 2), again reflecting the BG hydrolysing activity of GBA1 present in human wild-type. (v) The rapid degradation of LABG by pure recombinant GBA1 indicated the BG hydrolysing activity of this enzyme (Table 3).

3.2. Bile acid β-glucoside (BG) hydrolysing activity of GBA1 in situ

In the loading experiments of fibroblasts with lithocholic acid β-[U-14C]glucoside (Fig. 2), about 4% of the BG substrate (radioactive LABG) was taken up by the cells and partially deglucosylated. The fraction of enzymatically released radioactive glucose that was exported to the culture media during incubation was measured as a signal of LABG metabolising activity *in situ*, whereas the fraction of released glucose which was utilised for radioactive cellular products could not satisfactorily be analysed. Based on the radioactive glucose found in the media, the residual LABG metabolising activity in Gaucher (*GBA1*−/−) fibroblasts from patients G1, G3 and G4 (with 1.8%, 3.7% and 6.5%, respectively, of normal glucocerebrosidase activity *in vitro*; Table 2) was, as a mean, 67% (Fig. 2) of that in normal cells. The difference versus normal cells (see Gaucher and Normal in Fig. 2) was significant (eight assays with three *GBA1*−/− cell lines, and six assays with three control cell lines; *t*-test,

Table 2
Bile acid beta-glucosidase and glucocerebrosidase activity *in vitro* of liver, brain and fibroblasts from Gaucher patients.

	Normal controls			*Gaucher disease* (mean of 3 determinations)			
		nmol/h/mg[a]; mean ± standard deviation		Patient Sample (Table 1)	nmol/h/mg[a]		Mean % deficient GBA1 (reflects % GBA1 in Normal controls)
		LABG[b]	GC[c]		LABG[b]	GC[c]	LABG[b]
Fibro-blasts	(8 expts. in 4 individuals)	14.6 ± 3.5	2.04 ± 0.65	G1	2.19	0.037	85
				G3	2.48	0.075	83
				G4	3.80	0.133	74
Liver	(8 expts. in 2 individuals)	2.47 ± 0.56	0.241 ± 0.07	G2	0.96	0.012	61
Brain	(4 expts. in 2 individuals)	6.16 ± 0.88	0.363 ± 0.19	G5	3.06	0.003	50

[a] Protein-related enzyme activity.
[b] Lithocholic acid β-glucoside as bile acid β-glucosidase substrate.
[c] Glucosylceramide as glucocerebrosidase substrate.

Table 3
Bile acid beta-glucosidase and glucocerebrosidase activity of pure recombinant lysosomal beta-glucocerebrosidase (GBA1).

Recombinant enzyme		LABG[b]	GC[c]
		(nmol/h/mg[a])	
1 unit[d] (12.5 μg protein) reacted for 5 min	Mean of 4 expts.	5428 ± 912[f]	11,054[e] ± 1002[f]

[a] Protein-related enzyme activity.
[b] Lithocholic acid β-glucoside as bile acid β-glucosidase substrate.
[c] Glucosylceramide isolated from Gaucher spleen as glucocerebrosidase substrate.
[d] Unit is μmol p-nitrophenyl-β-D-glucopyranoside hydrolysed per min at 37 °C.
[e] Activity is still much lower than that reported in the literature for recombinant β-glucosidases [11] because the assays were adapted as closely as possible to those used for cell and tissue homogenates in this study, but were not optimised for pure enzyme.
[f] Total deviation.

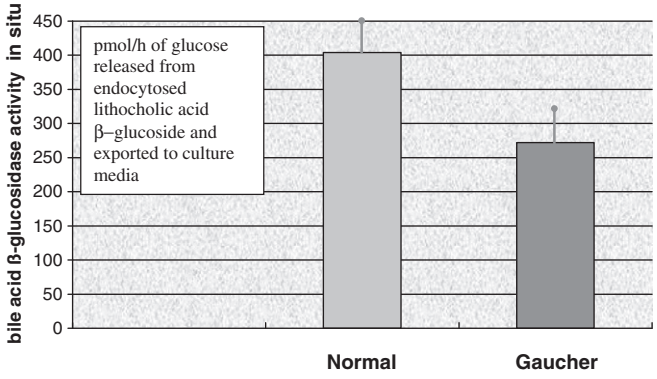

Fig. 2. Metabolism of bile acid β-glucoside (lithocholic acid β-[^{14}C]glucoside) in fibroblasts loaded with this substrate *in situ* and incubated for 72 h. *Normal*, normal control; *Gaucher*, Gaucher disease fibroblast lines. The Gaucher patient cell lines G1, G3 and G4 are compared to control lines. *Bars* above columns show standard deviations of six to eight experiments. Definition of units on the ordinate. The radioactive glucose released from endocytosed lithocholic acid β-glucoside was monitored in terms of its fraction exported during incubation to the culture media (this fraction was taken to be proportional to the total radioactive glucose released intracellularly).

bilateral, $\alpha < 0.2\%$). The 33% deficit of LABG metabolising activity in *GBA1*−/− fibroblasts *in situ* reflected, in addition to the evidence in Section 3.1, the degradation of BG by GBA1 in normal cells. The remaining 67% of activity *in situ* was essentially due to GBA2.

3.3. Minor adverse findings: Incomplete specificity of the chemical inhibition of GBA1; overexpression of GBA1 in the Gba2−/− fibroblasts

According to the fact that 'GBA1 inhibitors' are not absolutely specific [1], it is shown in Fig. 1B that the activity also of GBA2, the almost only β-glucosidase present in the *Gba1*−/− cells, is partially (by about 25%) inhibited by isofagomine (dark sections of *Gba1*−/− columns). However, the small light sections of the *Gba2*−/− columns (the predominant dark sections showing the present GBA1 activity) do not reflect incomplete inhibition of GBA1, but seem to be due to small activities of non-GBA1/non-GBA2 β-glucosidases [12–15]. Fig. 1 shows also that the *Gba2*−/− fibroblast activities are higher than the wild-type fibroblast activities (unexplained GBA1 overexpression in the transgenic cells).

4. Discussion

Here bile acid β-glucoside (BG [16], used in the form of its LABG subspecies [4]) was shown to be a natural substrate of GBA1 in addition to the glucosylceramide substrate (well known for its fatal accumulation in the GBA1 deficiency Gaucher disease, *GBA1*−/− [1,2,8]). This newly recognised situation, that both GBA1 and GBA2 are not only glucosylceramide β-glucosidases but both also bile acid β-glucosidases, led to the following question: why is glucosylceramide accumulated in both GBA1 and GBA2 deficiency [3,7], whereas BG, according to the present knowledge (including own findings [not shown]), is not accumulated in GBA1 deficiency but also not in GBA2 deficiency where bile acid metabolism was reported not to be impaired (*Gba2*−/− mouse [3])? We hypothesise that BG is sufficiently metabolised in both β-glucosidase deficiencies, and that the cell-compartmental barriers leading to lysosomal glucosylceramide storage in GBA1 deficiency and extra-lysosomal glucosylceramide accumulation in GBA2 deficiency, are of minor importance for BG. If this hypothesis is correct, a possible explanation is that the 'detergent-like' chemical properties of BG (a water-soluble bile acid derivative) might enable this substrate to overcome subcellular barriers, and to join with GBA1, as well as GBA2, for degradation.

To measure the activity of GBA1 as well as GBA2 towards BG in tissue and cell preparations *in vitro*, we developed assays which allowed us to estimate the sum of the activities of GBA1 and GBA2 in the same assay and then to differentiate between the GBA1 and GBA2 proportions of the sum ('total') activity. Assays with homogenates *in vitro* had the advantage that, due to only slightly different pH optima of GBA1 and GBA2 [1], both enzymes were sufficiently active near the optimum of the total activity, while the activity of some non-GBA1/non-GBA2 β-glucosidases with less acidic optima

[12–15], were essentially suppressed. The differentiation of the BG hydrolysing activities of GBA1 and GBA2 was achieved by comparing the total wild-type activity to the residual activities in the GBA1 deficiencies (*GBA1−/−* and *Gba1−/−*), or by using the GBA1 inhibitor isofagomine. Both approaches seemed not to be absolutely exact (see also Section 3.3), but the high percentages of the determined BG hydrolysing activities of GBA1 as compared to normal human cells (Table 2), and the high proportions of isofagomine-inhibitable (GBA1-derived) activity in the murine wild-type and *Gba2−/−* conditions (Fig. 1B) distinctly show GBA1 as a bile acid β-glucosidase. This was confirmed by the results with fibroblasts *in situ* (Fig. 2; where the difference between the residual activity level in Gaucher cells and the normal level is due to GBA1), and independently shown by the BG hydrolysing activity of recombinant GBA1 (Table 3). Of note, the residual activity level in the Gaucher fibroblasts *in situ* (thought to be essentially due to GBA2; Fig. 2) was much higher than the residual levels (again GBA2) in GBA1-deficient fibroblasts *in vitro* (Table 2, Fig. 1). This *in situ*/*in vitro* difference, though not unexpected, requires a separate study for being understood better. Nevertheless, the GBA1 activities *in vitro* as proportions of wild-type BG hydrolysing activity, were characteristically different between fibroblasts, liver and brain (see % values in Table 2; Fig. 1B), and the lowest GBA1 (and, therefore, highest GBA2) proportions were found in brain (see also [5]). For a comparison with the BG hydrolysing activities, the glucosylceramide β-glucoside hydrolysing (glucocerebrosidase) activities *in vitro* were additionally determined in some series of experiments (Tables 2 and 3). The glucocerebrosidase assay requires conditions different from those of the bile acid β-glucosidase assay, partly because of the hydrophobicity of glucosylceramide which also *in vivo* is dependent on cofactors (including saposin C [17]) in its enzymatic degradation. The water-soluble BG substrate may be relatively independent of such cofactors and proved to be particularly useful for determining the GBA1 and GBA2 activities *in vitro*. By contrast, glucosylceramide could not very successfully be used for determining the activity of GBA2 towards this substrate *in vitro*.

In addition to the demonstration of GBA1 as a 'second bile acid β-glucosidase' (GBA2 being the 'first'), the use of the additional natural substrate, BG, allowed us to give preliminary data on the cellular expression of GBA1 und GBA2, in quantitative terms of activity, indicating tissue-specific differences. This study may stimulate further investigations on the very poorly understood properties, including tissue distribution, substrate spectrum, subcellular and cofactor organisation, of the β-glucosidases, also with respect to β-glucosidase-deficient genetic diseases.

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