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Identification of the Non-lysosomal Glucosylceramidase as β-Glucosidase 2*

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The primary catabolic pathway for glucosylceramide is catalyzed by the lysosomal enzyme glucocerebrosidase that is defective in Gaucher disease patients. A distinct non-lysosomal glucosylceramidase has been described but its identity remained enigmatic for years. We here report that the non-lysosomal glucosylceramidase is identical to the earlier described bile acid β-glucosidase, being β-glucosidase 2 (GBA2). Expressed GBA2 is identical to the native non-lysosomal glucosylceramidase in various enzymatic features such as substrate specificity and inhibitor sensitivity. Expression of GBA2 coincides with increased non-lysosomal glucosylceramidase activity, and GBA2-targeted RNA interference reduces endogenous non-lysosomal glucosylceramidase activity in cells. GBA2 is found to be located at or close to the cell surface, and its activity is linked to sphingomyelin generation. Hydrophobic deoxynojirimycins are extremely potent inhibitors for GBA2. In mice pharmacological inhibition of GBA2 activity is associated with impaired spermatogenesis, a phenomenon also very recently reported for GBA2 knock-out mice (Yildiz, Y., Matern, H., Thompson, B., Allegood, J. C., Warren, R. L., Ramirez, D. M., Hammer, R. E., Hamra, F. K., Matern, S., and Russell, D. W. (2006) *J. Clin. Invest.* 116, 2985–2994). In conclusion, GBA2 plays a role in cellular glucosylceramide metabolism.

In higher eukaryotes, glucosylceramide is the precursor of glycosphingolipids, a complex group of ubiquitous membrane lipids, including gangliosides and globosides. Steady-state levels of these lipids are the result of ongoing synthesis and degradation. A well defined pathway for their degradation is the intra-lysosomal stepwise removal of terminal carbohydrate moieties. The penultimate product of this catabolic pathway is glucosylceramide that subsequently is converted to free glucose and ceramide by the action of the lysosomal acid β-glucosidase, named glucocerebrosidase (EC 3.2.1.45).

The importance of intra-lysosomal glycosphingolipid catabolism is illustrated by the existence of inherited lysosomal storage disorders in which specific glycosphingolipids accumulate as the consequence of an inherited defect in some lysosomal glycosidases (1). Deficiency of glucocerebrosidase is the cause for the most common lysosomal storage disorder named Gaucher disease (2, 3). This disorder is characterized by the accumulation of glucosylceramide laden tissue macrophages (Gaucher cells). In most other cell types of Gaucher patients, despite a severe deficiency in glucocerebrosidase activity, no obvious accumulation of glucosylceramide is notable. This finding points to the existence of an alternative catabolic pathway (2, 3). Indeed, a glucosylceramidase distinct to glucocerebrosidase has been detected in various tissues and cell types (4). The activity of this enzyme, named non-lysosomal glucosylceramidase (NLGase),[2] is not deficient in patients with Gaucher disease and is not located in lysosomes but near the cell surface (4). Contrary to glucocerebrosidase, this enzyme behaves as an integral membrane protein. It also differs from glucocerebrosidase in enzymatic features like specificity toward artificial substrates and inhibitors (4, 5). For example, NLGase is not able to hydrolyze artificial β-xylosidic substrates, contrary to glucocerebrosidase. Another striking difference in this respect is the irreversible inhibition of glucocerebrosidase by conduritol B epoxide (CBE) and the relative insensitivity of NLGase for this compound. NLGase is extremely sensitive to inhibition by hydrophobic deoxynojirimycin (DNM) analogues (5). The IC_{50} for *N*-(5-adamantane-1-yl-methoxy)pentyl-DNM (AMP-DNM) is ~1 nM (5). The two enzymes also differ in response to hydrophobic agents. Glucocerebrosidase is potently stimulated by negatively charged bile salts and phospholipids, whereas NLGase is not. The enzymatic activity of NLGase in membranes is completely lost upon exposure to various kinds of detergents (4). This inactivation has seriously hampered the attempts to purify the enzyme. Suitable conditions for re-activation of detergent-solubilized NLGase could not be established. So far the only tools for studying NLGase were very sensitive, low nanomolar inhibitors consisting of *N*-alkylated deoxynojirimycins linked to a large hydrophobic moiety such as adamantane or cholesterol (5). It is relevant to note that the enzyme is also inhibited by *N*-butyl-deoxynojirimycin (*N*-butyl-DNJ, IC_{50} 200 nM). This compound is a recently registered

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[2] The abbreviations used are: NLGase, non-lysosomal glucosylceramidase; CBE, conduritol B epoxide; DNM, deoxynojirimycin; AMP-DNM, *N*-(5-adamantane-1-yl-methoxy)pentyl-DNM; DNJ, deoxynojirimycin; GFP, green fluorescent protein; 4-MU, 4-methylumbelliferyl; RNAi, RNA interference; DRM, detergent-resistant membrane; MES, 4-morpholineethanesulfonic acid; GBA1–3, β-glucosidases 1–3; shRNA, short hairpin RNA; C6-NBD-glucosylceramide, 6-[*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3 diazol-4-yl)amino-dodecanoyl]sphingosyl-β-D-glucoside; PC, phosphatidylcholine.

drug (Zavesca, Actelion) for the treatment of type 1 Gaucher disease (6–8). The therapeutic action of Zavesca is ascribed to its ability to inhibit the synthesis of glucosylceramide by glucosylceramide synthase (IC_{50} 50–80 μM) (6). So-called substrate reduction therapy of Gaucher disease implies chronic administration of Zavesca (100 mg three times daily), resulting in average plasma concentrations of ~5 μM. Such concentrations of *N*-butyl-DNJ should only partially inhibit glucosylceramide synthase but completely abolish NLGase activity (5, 6). In view of this, increased insight into the physiological relevance of the non-lysosomal glucosylceramidase is of additional interest.

Here we describe that the GBA2 gene encodes the non-lysosomal glucosylceramidase. The identification of the corresponding gene offers novel tools to study the physiological importance of extra-lysosomal glucosylceramide catabolism. During the final preparation of the manuscript Yildiz and coworkers reported that GBA2 knock-out mice are deficient in a glucosylceramidase activity (9). They elegantly demonstrated that GBA2 deficiency results in impaired spermatogenesis in mice and hypothesize that GBA2 might be responsible for the non-lysosomal glucocerebrosidase activity reported by us in the 1990s. Our results are completely in line with this hypothesis.

EXPERIMENTAL PROCEDURES

Cloning—The complete open reading frames for klotho (NM_153683.2), KLPH/LCTH (NM_207338.1), β-klotho (NM_175737.2), and GBA2 (human NM_020944.2, mouse NM_172692.1) were amplified from human and mouse tissue, using specific primers containing restriction sites and an extended Kozak sequence in front of the start codon. PCR products were subsequently cloned in the pGEM-T vector (Promega, Madison, WI), and their sequence were verified. For expression studies in mammalian cells the appropriate fragments were subcloned in pCDNA-3.1 (Invitrogen) using the added restriction sites. Fusion proteins, containing the complete GBA2 fused to Myc/His, were generated by cloning the full-length cDNA of GBA2 without stop codon in-frame with the Myc/His sequence of pCDNA3.1-Myc/His (A+, Invitrogen). Fusion proteins containing the complete GBA2 fused to either the C terminus or the N terminus of GFP were generated by cloning of the full-length GBA2, with or without stop codon (depending on the site of the GFP fusion) in the pEGFP-N3 or pEGFP-C3 vectors (Clontech, Palo Alto, CA).

A di-leucine motif close to the transmembrane in GBA2 was changed to a di-alanine motif by PCR with a primer that contained the changed codons. A unique HindIII site upstream of the mutated codons allowed subsequent exchange of the sequence verified mutated PCR fragment with the wild-type GBA2 rendering a full-length GBA2 with the mutated di-alanine motif.

Transient Transfections—Transient transfection experiments in either COS-7 cells or the mouse melanoma cell-line MEB-4 were performed using Fugene-6 transfection reagent (Roche Applied Science) according to the manufacturer's protocol.

Enzyme Activity Assays—As convenient source of enzyme, water extracts of Gaucher disease spleens were generated by homogenization of 10 g of tissue in 30 ml of water (4 °C) using an Ultra-Turrax and sonication followed by centrifugation for 20 min at 15,000 × *g*. Membrane suspensions were prepared by resuspending the pellet in 30 ml of 50 mM potassium phosphate buffer (pH 5.8) and centrifugation (15 min, 15,000 × *g*). This procedure was repeated two times.

The activity of the non-lysosomal glucosylceramidase with the artificial 4-methylumbelliferyl-β-glucoside (4-MU-β-glucoside, Sigma) substrate, and the natural substrate was determined as described previously (4, 5). Briefly, either membrane suspension, total cellular lysate, or intact cells were incubated with 3.7 mM 4-MU-β-glucoside in McIlvaine buffer (0.1 M citrate and 0.2 M phosphate buffer), pH 5.8, with or without preincubation for 30 min at 4 °C with 1 mM CBE (Sigma) to inhibit the lysosomal glucocerebrosidase. Assays were incubated at 37 °C and stopped by the addition of glycine/NaOH (pH 10.6). The amount of liberated 4-MU was determined with a PerkinElmer Life Sciences LS30 fluorometer.

6-[*N*-Methyl-*N*-(7-nitrobenz-2-oxa-1,3 diazol-4-yl)aminododecanoyl]sphingosyl-β-D-glucoside (C6-NBD-glucosylceramide) was synthesized exactly as described (5, 10). *In vitro* analysis of glucosylceramidase activity was determined with C6-NBD-glucosylceramide as substrate exactly as described (4, 5).

In vivo analysis of glucosylceramidase activity in intact cells was determined with C6-NBD-glucosylceramide as substrate as described (4, 5). Briefly, cells were preincubated with or without 1 mM CBE for 30 min, washed, and incubated for the indicated time in 500 μl of medium with or without 1 mM CBE and 1 nmol of C6-NBD-glucosylceramide. The cells were harvested followed by lipid extraction, and separation by thin-layer chromatography (11). C6-NBD lipids were quantified using a Molecular Dynamics Typhoon phosphorimaging device.

RNA Interference—Suitable 21-mer sequences for RNA interference were selected using the mouse GBA2 sequence and the Reynolds algorithm (12). Subsequently oligonucleotides were ordered (RNAi1, 5′-GATCCCGGGAAGGACCTGAACCTGAAGAAGCTTGTTCAGGTTCAGGTCCTTCCTTTTTT-3′ and 5′-CTAGAAAAAAGGAAGGACCTGAACCTGAACAAGCTTCTTCAGGTTCAGGTCCTTCCCGG-3′; RNAi2, 5′-GATCCCGGTCATTGCAGACCAATTTAGAAGCTTGTAAATTGGTCTGCAATGACTTTTTT-3′ and 5′-CTAGAAAAAAGTCATTGCAGACCAATTTACAAGCTTCTAAATTGGTCTGCAATGACCGG-3′; and RNAi3, 5′-GATCCCGGATCATGTTTGGAGCTAAAGAAGCTTGTTTAGCTCCAAACATGATCTTTTTT-3′ and 5′-CTAGAAAAAAGATCATGTTTGGAGCTAAACAAGCTTCTTTAGCTCCAAACATGATCCGG-3′) annealed and cloned into the GeneEraser short hairpin vector (Stratagene, La Jolla, CA). For RNAi experiments the short hairpins were transiently expressed in COS-7 cells co-transfected with the mouse GBA2-Myc for analysis of activity toward the mouse GBA2. This was followed by analysis of enzyme activity and protein expression using Western blot and the anti-Myc antibody compared with a negative short hairpin-expressing cells.

For RNAi on endogenous GBA2 the short hairpins were transiently expressed in the mouse melanoma cell line MEB-4 followed by analysis of enzyme activity using the 4-MU-β-glucoside substrate, and enzyme levels were compared to negative short hairpin-expressing cells.

TABLE 1
Candidate β-glucosidase genes

Name	Glycosyl hydrolase family	Transmembrane domain	Tissue expression	4MU-β-D-glucosidase activity[a]	References
Cytosolic β-glucosidase (GBA3)	1	−	Most	+	19
Lactase phlorizin hydrolase	1	+	Small intestine	+	20
Klotho	1	+	Most	ND[b]	21
Klotho-like 1 (β-Klotho)	1	+	Most	ND	22
Klotho-like 2	1	+	Most	ND	23
Bile acid β-glucosidase (GBA2)	NC[c]	+	Most	+	24–26

[a] 4-MU-β-D-glucosidase activity according to the cited literature.
[b] ND, not determined.
[c] NC, not classified.

Subcellular Localization—GBA2-GFP or GFP-GBA2 fusion proteins or GFP alone was/were expressed in COS-7 cells grown on coverslips. Living cells were subsequently analyzed using Confocal laser scanning microscopy on a Leica instrument (Leica Microsystems, Heidelberg, Germany).

Protease Protection—For protease protection experiments intact COS-7 cells expressing GBA2-GFP or GFP-GBA2 fusion proteins were treated with increasing concentrations of Proteinase K (Roche Applied Science) ranging from 0 to 10 mg/ml for 40 min at 30 °C. The reaction was stopped by adding a saturated phenylmethylsulfonyl fluoride solution (1:100), followed by sample buffer. SDS-PAGE and Western blot analysis using an anti-GFP antibody 1:2500 (AbCam, Cambridge, UK) was employed to detect the protected fragments.

DRM Isolation—Detergent-resistant membrane (DRM) fractions were prepared as described previously (13, 14). Briefly, COS-7 cells expressing Myc-tagged GBA2 were homogenized at 4 °C with 1% (v/v) Triton X-100 or 1% (v/v) Brij-35 or 1% (v/v) Lubrol in MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl), supplemented with mammalian protease inhibitor mixture (Sigma). The lysate was adjusted to 80% sucrose in MES-buffered saline, overlaid with a 30–5% sucrose step gradient, and centrifuged for 18 h at 36,000 rpm in a Kontron TST41.14 rotor at 4 °C. Subsequently, for SDS-PAGE, either equal volumes from gradient fractions were used, or protein concentrations were first quantified to determine the volume required for 10 μg of protein. Western blot analysis was performed by transfer of protein to polyvinylidene fluoride membranes. Primary antibodies, anti-Myc (Cell Signaling Technology, Danvers, MA) or anti-Cav-1 (BD Transduction Laboratories, Franklin Lakes, NJ) as positive DRM control were used at 1:5000 and 1:2500 dilutions, respectively, for detection of the aforementioned proteins. Horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution, Bio-Rad, Hercules, CA) were used in combination with a chemiluminescent substrate.

Synthesis of Inhibitors and Analysis—All reagents and solvents were used as received. *N*-[5-(Adamantan-1-yl-methoxy)pentyl]-1-deoxynojirimyci (5), *N*-butyl-1-deoxynojirimycin (5), 2,3,4-*O*-tribenzyl-D-xylose (15), *N*-butyldeoxygalactonojirimycin, and 5-(adamantane-1-yl-methoxy)-1-pentanal (5) were synthesized as described previously (5). The synthesis of *N*-butyl-1,5-dideoxy-1,5-imino-xylitol was accomplished as follows. Treatment of 2,3,4-*O*-tribenzyl-D-xylopyranose with excess butylamine, one equivalent of *p*-toluenesulfonic acid, and 3-Å molecular sieves in toluene at 90 °C for 2 h produced the hemiaminal (16). The crude hemiaminal intermediate was reduced with lithium aluminum hydride in tetrahydrofuran to provide the 1,5-aminoalcohol, which was cyclized (17) under Mitsunobu conditions. The benzyl protecting groups in the cyclized product were removed by palladium on charcoal-catalyzed hydrogenolysis in the presence of hydrochloric acid to provide *N*-butyl-1,5-dideoxy-1,5-imino-xylitol. The initial synthetic steps for the preparation of *N*-[5-(adamantan-1-yl-methoxy)pentyl]-1,5-dideoxy-1,5-iminoxylitol were analogous to the steps described above but employing allylamine in the synthesis of the hemiaminal. After Mitsunobu-mediated cyclization, the allyl group of the endocyclic amine was isomerized and cleaved by heating in Me_2SO with potassium butoxide (18) followed by treatment with aqueous hydrochloric acid to afford 2,3,4-*O*-tribenzyl-1,5-dideoxy-1,5-iminoxylitol. Treatment of this product with 5-(adamantane-1-yl-methoxy)-1-pentanal under the agency of sodium cyanoborohydride (5) and acetic acid was followed by removal of the benzyl protecting groups by palladium on charcoal-catalyzed hydrogenolysis in the presence of hydrochloric acid to give *N*-[5-(adamantan-1-yl-methoxy)pentyl]-1,5-dideoxy-1,5-iminoxylitol.

For inhibitor analysis, cellular extracts were preincubated with the indicated concentrations of the DNM-type inhibitors, and activity was measured as described above in the presence of the inhibitor. Enzyme activities were related to those in the absence of the inhibitors.

RESULTS

Selection of Candidate Genes Coding β-Glucosidases—Attempts to conventionally purify NLGase all failed by the instability of detergent-solubilized enzyme. We therefore analyzed potential β-glucosidase genes as candidate genes for the enzyme (Table 1). NLGase could belong to family 1 of glycosyl hydrolases (www.cazy.org/CAZY/fam/acc_GH.html). This family includes cytosolic β-glucosidase (GBA3, Unigene: Hs.371763), lactase phlorizin hydrolase (Unigene: Hs.551506), klotho (Unigene: Hs.524953), β-klotho (Unigene: Hs.90756), and klotho-lactase phlorizin hydrolase like (Unigene: Hs.585062). The former two enzymes are known to not be identical to NLGase. We cloned and expressed the latter three β-glucosidase-like cDNAs. None of the gene products showed β-glucosidase activity when measured with 4-methylumbelliferyl-β-glucoside (not shown). These negative results led us to consider another known β-glucosidase, the so-called bile acid β-glucosidase (GBA2, Unigene: Hs.443134) (24–26). Initially GBA2 was excluded as a candidate, because it had been reported that this enzyme is expressed specifically in the liver, is inactive toward glucosylceramide, and is sensitive toward CBE, all features clearly distinct from those of NLGase (4, 24). However, more recently it was reported that GBA2 is expressed in

TABLE 2
***In vitro* 4-MU-β-glucoside activity in transfected COS-7 cells**

	CBE-insensitive activity	CBE-sensitive activity
	nmol/mg.h^{-1}	
Mock	1.23 (±0.15)	5.95 (±0.37)
GBA2	7.29 (±2.77)	8.80 (±2.87)

TABLE 3
Enzymatic features of GBA2 and NLGase

Inhibitor	IC_{50}	
	COS_{GBA2}	Gaucher membrane
	μM	
N-Butyl-DNJ (Zavesca)	0.180	0.200
N-Butyldeoxygalactonojirimycin	10	10
N-Butyl-1,5-dideoxy-1,5-iminoxylitol	6.0	6.0
AMP-DNM	0.002	0.003
AMP-DNM-xylitol	0.5	0.8

various tissues and that the corresponding enzyme is relatively insensitive toward CBE, features more reminiscent to those of NLGase (26). We therefore cloned the full-length cDNA of GBA2 and studied the features of its gene product expressed in COS cells.

Enzymatic Activity of Expressed GBA2—Extracts of cells transfected with GBA2 cDNA showed increased activity to 4-methylumbelliferyl-β-glucoside (Table 2). The activity was not affected by CBE, a potent irreversible inhibitor of the lysosomal glucocerebrosidase. Expression of GBA2 with a C-terminal Myc/His tag or with a GFP tag either on the C or N termini also resulted in increased β-glucosidase activity that was insensitive to CBE (data not shown).

Comparison of Enzymatic Properties of GBA2 and NLGase—To further investigate the possibility that GBA2 encodes NLGase, we compared the enzymatic features of NLGase in membranes isolated from Gaucher spleen and expressed GBA2 in transfected COS cells. Earlier experiments had revealed that NLGase is highly sensitive to inhibition by hydrophobic DNMs, in particular AMP-DNM (5). Native NLGase and the expressed GBA2 gene product showed comparable IC_{50} values for all inhibitors tested (Table 3). The inhibitory effect of Triton X-100 and taurocholate on native NLGase and expressed GBA2 was also found to be similar (data not shown).

In Vitro and in Situ Glucosylceramidase Activity of GBA2—NLGase is able to hydrolyze glucosylceramide in the presence of CBE (4). We tested whether GBA2 shows glucosylceramidase activity. Using an *in vitro* assay with C6-NBD-glucosylceramide as substrate, it was observed that extracts of COS cells overexpressing GBA2 are able to hydrolyze glucosylceramide more efficiently compared with extracts of mock transfected cells. This activity was insensitive to CBE (Fig. 1*A*).

In vivo activity assays showed that conversion of C6-NBD-glucosylceramide to C6-NBD-ceramide occurs in COS-7 cells expressing GBA2 or its fusion proteins. Part of the C6-NBD-ceramide formed was rapidly converted to C6-NBD-sphingomyelin (Fig. 1*B*). This phenomenon was also earlier observed for native NLGase (4).

GBA2 RNAi—For a definitive proof that GBA2 is identical to NLGase, we evaluated whether GBA2-targeted RNAi constructs led to knockdown of NLGase activity. We first selected the RNAi sequences using the Reynolds algorithm (12) and the

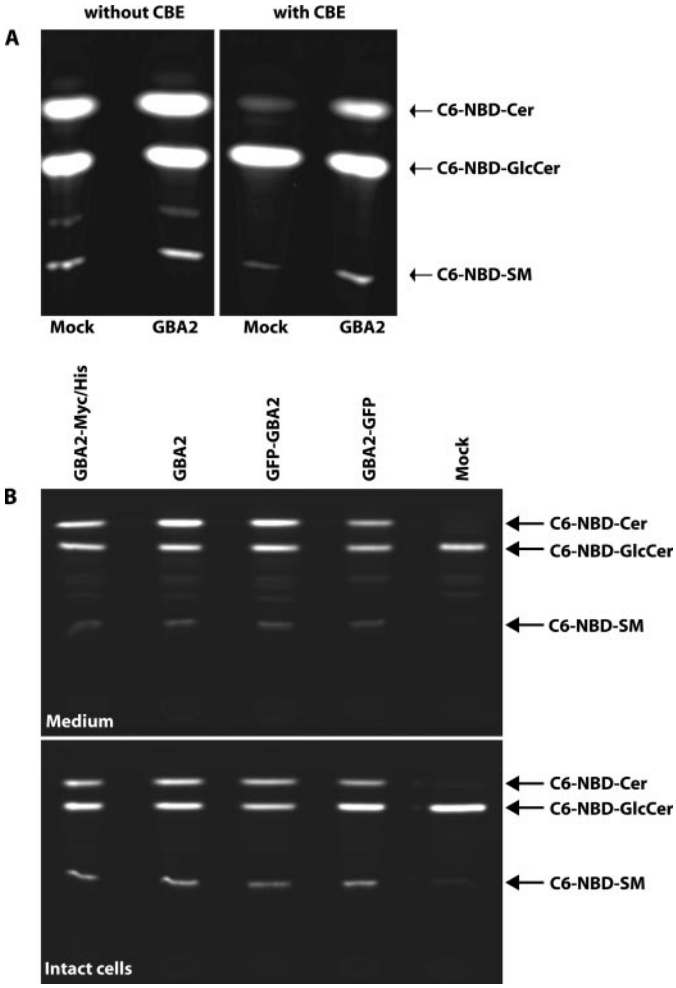

FIGURE 1. ***In vitro* and *in situ* glucosylceramidase activity of GBA2.** *A*, *in vitro* activity of COS-7 cells transfected with GBA2 or empty vector toward C6-NBD-glucosylceramide in the presence or absence of 1 mM CBE. Lysates of cells were incubated with the lipid substrate followed by lipid extraction and thin-layer chromatography analysis. The substrate and the product of the reaction, C6-NBD-ceramide as well as C6-NBD-sphingomyelin (*SM*), are indicated with an *arrow* (see "Experimental Procedures" for details). *B*, *in situ* activity of intact COS-7 cells transfected with empty vector or the indicated constructs. Intact cells were incubated with C6-NBD-glucosylceramide for 2 h in the presence of 1 mM CBE. Medium and cells were collected, followed by lipid extraction and thin-layer chromatography analysis. The substrate and the product of the reaction, C6-NBD-ceramide as well as C6-NBD-sphingomyelin, are indicated with an *arrow* (see "Experimental Procedures" for details).

mouse GBA2 cDNA sequence and subsequently cloned these in a short hairpin vector. Efficacy of the selected shRNAs was checked in COS-7 cells. Cells were co-transfected with both the GBA2-Myc/His and the three different shRNAs. Activity and Western blot analysis revealed that all three shRNAs were able to knock down activity. Compared with a negative control shRNA, the GBA2-targeted shRNAs reduced β-glucosidase activity by 65–80% (Fig. 2*A*). Western blot analysis using anti-Myc antibodies showed reduced GBA2 protein.

Next, the effects of the selected shRNAs on the endogenous NLGase activity in a mouse melanoma cell-line (MEB-4) were examined. This cell line shows a high activity of NLGase. Fig. 2*B* shows the results of a typical experiment. Compared with a negative control shRNA the GBA2-targeted shRNAs resulted in 40–80% inhibition of NLGase activity. Altogether, these findings demonstrate that GBA2 is indeed identical to NLGase.

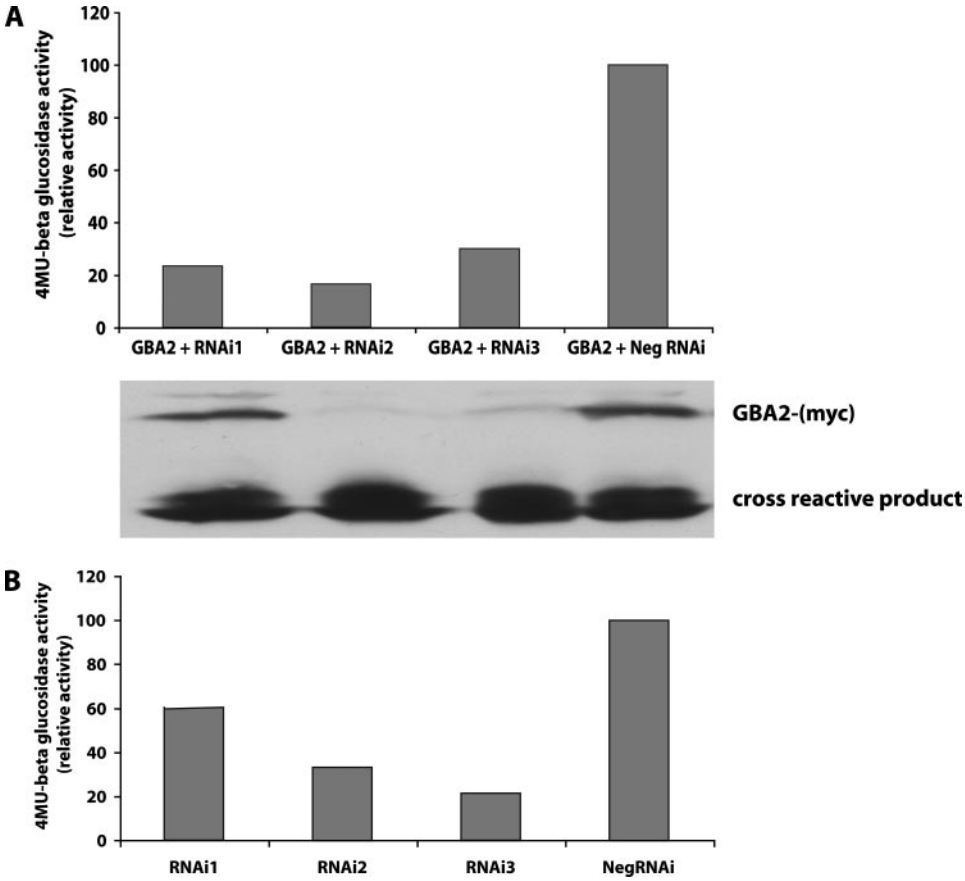

FIGURE 2. **RNA interference of GBA2.** *A*, COS-7 cells were co-transfected with the indicated short hairpin constructs in the presence of the GBA2-Myc construct. Activity of the cell extracts toward 4-MU-β-glucoside was measured and related to that of a negative control short hairpin vector. Western blots analysis was performed in parallel to establish effect on protein expression in the co-transfected cells. *B*, endogenous NLGase activity, in the mouse melanoma cell-line MEB-4 transfected with the indicated short hairpin vectors, was determined toward 4-MU-β-glucoside as substrate and related to that of a negative control short hairpin vector as described under "Experimental Procedures."

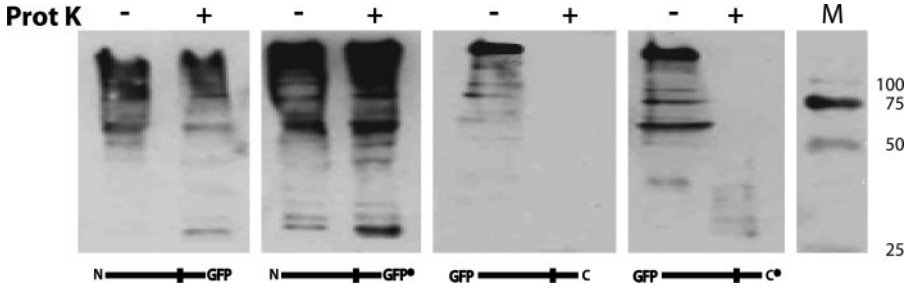

FIGURE 3. **Proteinase K protection assays on intact COS-7 cells expressing GBA2-GFP or GFP-GBA2.** Intact COS-7 cells expressing GBA2-GFP (GFP fused to C terminus of GBA2) or GFP-GBA2 (GFP fused to N terminus of GBA2) fusion proteins were treated with or without Proteinase K (10 μg/ml) for 40 min at 30 °C. The reaction was stopped by adding a phenylmethylsulfonyl fluoride, followed by SDS-PAGE and Western blot analysis using an anti-GFP antibody to detect the protected fragments. Below the blots the used GBA2 fusion proteins are indicated. The *dot* denotes the constructs with the di-leucine motif mutated to a di-alanine motif, and the *rectangle* indicates the position of the putative transmembrane domain. *M* indicates the marker lane (molecular mass in kDa).

Topology of GBA2 in the Membrane—Mattern and coworkers (26) showed that GBA2 is a single pass transmembrane protein with an N-terminal domain of 670 amino acids followed by a transmembrane domain and a C-terminal domain of 220 amino acids lacking a classic signal peptide. To establish its orientation in the membrane we performed protease protection experiments using COS-7 cells expressing GBA2 GFP fusion proteins. Intact cells and cellular lysates were incubated with Proteinase K (10 μg/ml) followed by Western blot analysis using the appropriate antibodies. Fig. 3 shows that C-terminal GFP in GBA2 present in intact COS-7 cells is insensitive to Proteinase K treatment. In sharp contrast, N-terminal GFP in GBA2 present in COS-7 cells is sensitive to the protease. The same observations were made for fusion proteins in which a di-leucine motif was replaced by di-alanine (Fig. 3). These results suggest that the C-terminal part of the GBA2 is cytosolic and that the N-terminal part of the protein is either outside the cells or in the lumen of an organelle easily accessible for Proteinase K.

Subcellular Localization of GBA2—Earlier subcellular fractionation experiments employing Percoll density gradient centrifugation and free-flow electrophoresis indicated that NLGase is located close to the cell surface (4, 5). We therefore investigated the localization of GBA2-GFP fusion proteins in transfected COS cells. GBA2 with enhanced GFP fused either on the C or N termini appeared normal enzymatic activity toward C6-NBD-glucosylceramide substrate (Fig. 1*B*). Confocal microscopy of COS-7 cells expressing GBA2-GFP fusion proteins revealed a predominant localization near the cell surface (Fig. 4).

To test further the localization of GBA2, cultured mouse RAW cells or mouse melanoma MEB-4 cells were incubated in medium containing 4-methylumbelliferyl-β-glucoside in the presence or absence of *N*-butyl-DNJ or CBE. Released fluorescent 4-methylumbelliferon in the medium was monitored. Fig. 5 shows that the *N*-butyl-DNJ-sensitive, CBE-insensitive activity, attributable to GBA2, starts instantly. The *N*-butyl-DNJ-insensitive, CBE-sensitive activity, attributable to lysosomal glucocerebrosidase (GBA1), shows a lag period of ~30 min, probably the time required for sufficient access of substrate to the lysosomes. The rapid accessibility of 4-methylumbelliferyl-β-glucoside in intact RAW and MEB-4 cells suggests that endogenous NLGase is present at or close to the cell surface.

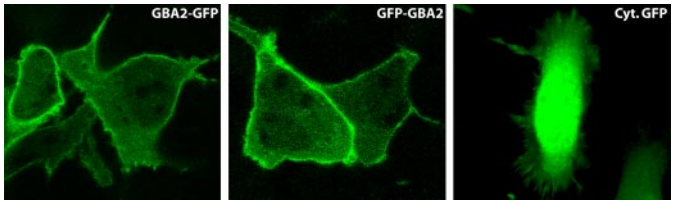

FIGURE 4. **Subcellular localization of GBA2-GFP or GFP-GBA2 fusion proteins.** GBA2-GFP (GFP fused to C terminus of GBA2) or GFP-GBA2 (GFP fused to N terminus of GBA2) fusion proteins or GFP alone were expressed in COS-7 cells grown on coverslips. Living cells were subsequently analyzed for localization of GFP using confocal laser scanning microscopy.

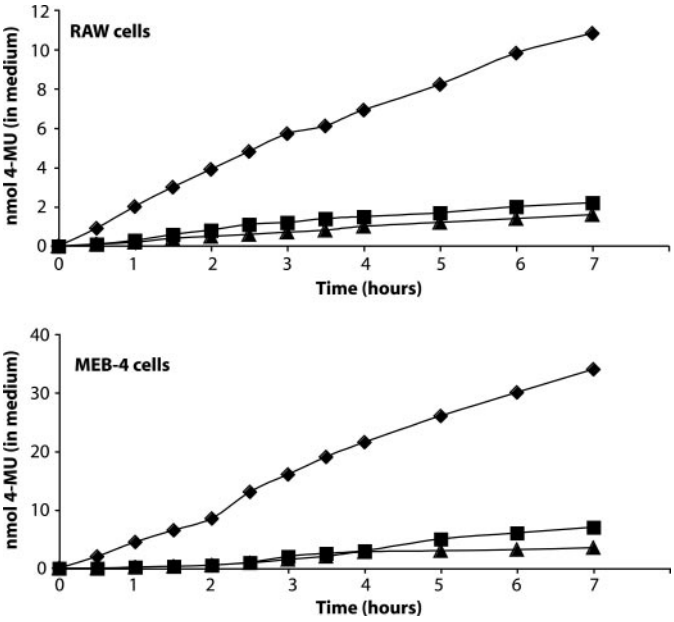

FIGURE 5. ***In situ* β-glucosidase activity in the presence of specific inhibitors.** Mouse RAW or MEB-4 cells were preincubated either in the presence or absence of 1 mM CBE. These cells were subsequently incubated with the artificial substrate 4-MU-β-glucoside in the presence or absence of 5 μM or 10 μM *N*-butyl-DNJ. At the indicated time points the liberated 4-MU in the medium was measured. The *diamonds* indicate the CBE-insensitive β-glucosidase activity attributable to GBA2 (activity measured in the presence of CBE without *N*-butyl-DNJ). The *squares* indicate *N*-butyl-DNJ-insensitive activity attributable to lysosomal glucocerebrosidase (activity measured in the presence of 5 μM *N*-butyl-DNJ). The *triangles* indicate *N*-butyl-DNJ-insensitive activity attributable to lysosomal glucocerebrosidase (activity measured in the presence of 10 μM *N*-butyl-DNJ).

Absence of GBA2 in Detergent-resistant Membranes—The plasma membrane contains so-called lipid rafts or DRMs enriched in cholesterol and glycosphingolipids. We investigated whether GBA2 might reside in lipid rafts. DRM fractions of GBA2-Myc/His-transfected COS cells were prepared using established techniques based on resistance to Triton-X100, Brij-35, or Lubrol at 4 °C and subsequent flotation in sucrose density gradients (13). Equal amounts of the gradient fractions were subjected to SDS-PAGE and analyzed by anti-Myc and anti-CAV1 Western blotting. In contrast to the DRM marker caveolin, GBA2-Myc/His was solely detectable in the non-floating fractions (Fig. 6). This suggests that GBA2 is not located in lipid rafts.

DISCUSSION

Our study indicates that the earlier described non-lysosomal glucosylceramidase, or NLGase, is identical to GBA2, an earlier

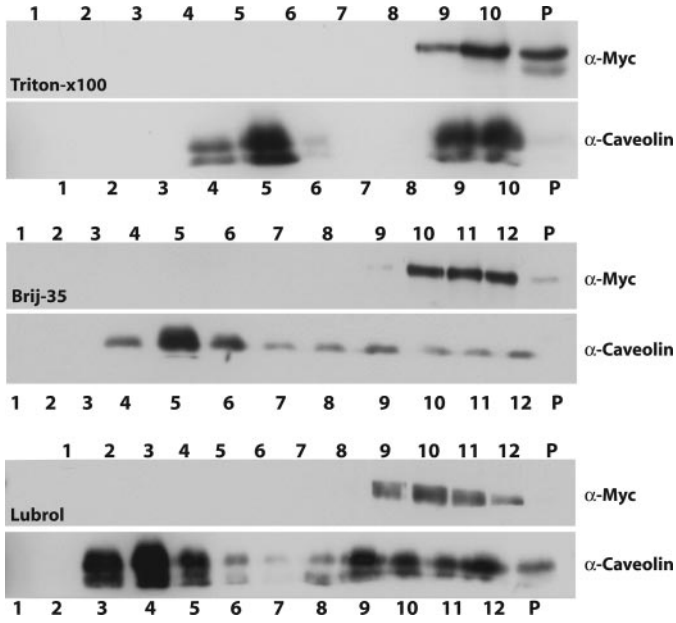

FIGURE 6. **Absence of GBA2 in DRMs.** COS-7 cell extracts, transfected with GBA2-Myc constructs, were prepared with Triton X-100, Brij-35, or Lubrol and fractionated by sucrose-gradient ultracentrifugation as described under "Experimental Procedures." Equal amounts of each fraction were analyzed by SDS-PAGE followed by Western blotting using anti-Myc and anti-caveolin antibodies. Floating fractions (*fractions 3–6*) contain the DRM marker caveolin but fail to show a signal for GBA2-Myc. The signal of GBA2-Myc is found in the non-floating fractions (*fractions 9–12*). *Fraction P* is the pellet fraction from each gradient.

described bile acid β-glucosidase. Several lines of evidence support this. First, COS-7 cells overexpressing GBA2 show an increased glucosylceramidase activity, and GBA2-targeted shRNAs inhibit the endogenous NLGase activity in the mouse melanoma cell-line MEB-4. Second, recombinantly expressed GBA2 and native NLGase are completely identical in enzymatic features such as sensitivity for inhibitory DNM derivatives, insensitivity for inhibition by CBE, as well as inactivation by detergents like Triton X-100 and taurocholate.

The non-lysosomal GBA2 differs in many aspects from the lysosomal glucocerebrosidase (GBA1). GBA2 is an integral membrane protein that looses activity upon membrane solubilization, whereas GBA1 is a soluble protein that weakly associates with membranes and requires negatively charged lipids and the activator protein saposin C for optimal catalytic activity (2–4, 26). Despite the fact that both GBA1 and GBA2 are capable of hydrolyzing artificial β-glucosides and the natural glycosphingolipid glucosylceramide, no homology exists between the proteins. Both glucosylceramidases clearly do not stem from one ancestral gene. This is also reflected in remarkable differences in substrate and inhibitor specificities of GBA1 and GBA2. For example, 4-methylumbelliferyl-β-xyloside is an excellent substrate for GBA1 but not at all for GBA2 (4). Consistently, xyloside analogues of *N*-(5-adamantane-1-yl-methoxy)pentyl-DNM or *N*-butyl-DNJ are far poorer inhibitors of GBA2 than GBA1.

The two glucosylceramidases GBA1 and GBA2 have distinct cellular locations, and the fate of ceramide generated by both enzymes is very different (4). Ceramide formed by GBA1 in lysosomes is further degraded in this compartment into sphin-

gosine and fatty acid (see Ref. 28 for a recent overview). In sharp contrast, we observed that the ceramide generated by GBA2, either in isolated membranes or intact cells can be rapidly converted to sphingomyelin (4). This suggests that GBA2 is in relative close contact with a sphingomyelin synthase. Two such enzymes, SMS1 and SMS2, have been recently described (29). SMS1 was found to be located in the Golgi apparatus, and SMS2 is on the cell surface (29).

All our data suggest that also GBA2 is located at or close to the cell surface. In support of this are the earlier findings with subcellular fractionation experiments and the present results regarding cellular location of GFP-GBA2 fusion proteins (4, 5). It can always be argued that overexpression or construction of a fusion protein changes the natural localization of a protein. However, endogenous GBA2 in intact cells has instantaneous access to the substrate 4-methylumbelliferyl-β-glucoside added in medium, suggesting that its catalytic pocket is in contact with the extracellular space. In line with this, extracellular proteinase K has easy access to the N terminus of GBA2 in intact cells. GBA2 is single pass transmembrane protein (26), and its likely orientation is a cytosolic C terminus and luminal N terminus. Given the absence of a signal peptide, it remains intriguing how GBA2 reaches its final destination and orientation (26). The composition and location of the catalytic pocket in GBA2 are presently unknown. Homology searches with known β-glucosidases have not been informative so far. We only observed a very weak homology of GBA2 with the archeal type glycogen debranching enzyme, the homologous region overlapping with the transmembrane domain of GBA2. It should be noted that it has earlier been demonstrated that GBA2 hydrolyzes glucosylceramide while the lipid is inserted in the membrane bilayer (4). Interestingly, we observed no enrichment of GBA2 in isolated detergent-resistant membrane fractions. This result should be interpreted with caution. It does not exclude the possibility that in intact cells GBA2 is present in semi-ordered microdomains rich in glycosphingolipids.

Deficiency of the lysosomal glucosylceramidase GBA1 (glucocerebrosidase) causes a severe disorder named Gaucher disease. All adult Gaucher patients show some residual glucocerebrosidase activity, and their cellular glucosylceramide accumulation is restricted to tissue macrophages, so-called Gaucher cells. No significant increases in glucosylceramides are detected in other cell types of adult Gaucher patients. Only upon complete inhibition of GBA1 by excess CBE will prominent storage of glucosylceramide occur in cultured fibroblasts. It is possible that in Gaucher patients the non-lysosomal GBA2 is able to partly compensate for the lack of lysosomal glucocerebrosidase activity in cells. GBA2 is ubiquitously present: its mRNA is detected in all tissues, and we earlier observed NLGase activity in all cell types examined (4). Degradation of glucosylceramide by GBA2 leads to further metabolism of the generated ceramide to sphingomyelin. The formation of one sphingomyelin molecule by transfer of the phosphorylcholine moiety from phosphatidylcholine (PC) to ceramide generates one diacylglycerol molecule and consumes one PC molecule. In monocytes/macrophages of Gaucher patients it is found that *de novo* biosynthesis of PC is increased (30). This increased synthesis of PC may be required to replenish the phospholipid consumed in the process of GBA2-mediated glucosylceramide degradation.

It is well established that *N*-butyl-DNJ already inhibits GBA2 at higher nM concentrations (IC_{50} 200 nM) (4, 5). Gaucher patients receiving substrate reduction therapy are daily exposed to three times 100 mg of *N*-butyl-DNJ (Zavesca, Actelion), resulting in low micromolar plasma concentrations (31). Under these circumstances GBA2 should be completely inhibited. A closer examination of the effects of Zavesca treatment on Gaucher patients is therefore of interest. The treatment results, exactly like enzyme replacement therapy, in reversal of clinical symptoms such as hepatosplenomegaly and pancytopenia (31). These clinical improvements coincide with disappearance of storage cells. A characteristic side effect of chronic Zavesca treatment is intestinal complaints. These can be attributed to direct inhibition of *N*-butyl-DNJ of intestinal glycosidases. Otherwise, the chronic Zavesca exposure seems not to cause prominent side effects possibly due to a combined inhibition of glucosylceramide synthesis. Apparently, inhibition of GBA2 activity in humans is not accompanied by overt pathology. Studies by Platt and co-workers (32) have clearly pointed out that *N*-butyl-DNJ causes impairment of spermatogenesis in mice. We have more recently observed a similar effect in mice exposed to AMP-DNM.[3] Such a deleterious effect on spermatogenesis has not been observed with monkeys receiving *N*-butyl-DNJ.[4] In a recent report, Amory *et al.* (27) report that exposure of healthy volunteers to Zavesca did also not affect spermatogenesis. Sperm concentration, motility, or sperm morphology after 6 weeks of therapy were normal. In addition, no changes in acrosome structure or function were observed with treatment, despite low micromolar concentrations of iminosugar in the serum and seminal plasma (27). Apparently glycosylceramide metabolism is less important in spermatogenesis in humans. During the preparation of this report a highly relevant report by Yildiz and co-workers was published (9). These researchers generated mice deficient in GBA2. Knock-out males exhibited impaired fertility. Microscopic examination of sperm revealed large round heads (globozoospermia), abnormal acrosomes, and defective mobility (9). Glycolipids, identified as glucosylceramides by mass spectrometry, accumulated in the testes, brains, and livers of the knock-out mice but did not cause obvious neurological symptoms, organomegaly, or a reduction in lifespan (9). They observed that recombinant GBA2 hydrolyzed glucosylceramide to glucose and ceramide and should be viewed as a glucosylceramidase. They hypothesized that GBA2 could be identical to NLGase as reported by us earlier (4, 5). As we clearly demonstrate in the current study, we agree with this hypothesis. The authors also linked the earlier reports on *N*-butyl-DNJ-induced impairment in spermatogenesis in mice with the phenotype of the GBA2 knock-out males. A small discrepancy may exist in the views on the cellular location of GBA2. In the report by Yildiz *et al.* it is stated that GBA2 is a resident endoplasmic reticulum protein, whereas all our data point to at least a partial location at or near the cell surface

[3] J. M. F. G. Aerts, R. Ottenhoff, C. van Roomen, unpublished observations.
[4] Oxford GlycoSciences, personal communication.

(4, 5, 9). The reason for this discrepancy is not clear at present and warrants further investigation.

A topic not addressed in our investigation is hydrolytic activity of GBA2 toward bile salts. Yildiz and co-workers (9) elegantly show that GBA2-deficient mice have normal bile salt metabolism, despite the fact that *in vitro* GBA2 is capable of metabolizing glucosylated bile salt. The study from Yildiz and co-workers will undoubtedly excite interest in the role of glycosphingolipids in spermatogenesis and in particular the function of GBA2 in this process. A more detailed study of GBA2 and the GBA2 null mouse will provide additional information on the role of the ubiquitously expressed GBA2 in maintaining the homeostasis of glucosylceramide, ceramide, phosphatidylcholine, diacylglycerol, and sphingomyelin concentrations in specific membranes of multiple cell types. Such investigation may contribute to increased understanding of the role of this protein in the pathogenesis of Gaucher disease.

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Identification of the Non-lysosomal Glucosylceramidase as β-Glucosidase 2

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