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Analysis of glucocerebrosidase activity using *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-glucosylsphingosine demonstrates a correlation between levels of residual enzyme activity and the type of Gaucher disease

Irit MEIVAR-LEVY,* Mia HOROWITZ† and Anthony H. FUTERMAN*‡

*Department of Membrane Research and Biophysics, Weizmann Institute of Science, Rehovot 76100, Israel, and †Department of Cell Research and Immunology, Tel Aviv University, Ramat-Aviv 69978, Israel

Glucosylceramide, a degradation product of complex glycosphingolipids, is hydrolysed in lysosomes by glucocerebrosidase (GlcCerase). Mutations in the human GlcCerase gene cause a reduction in GlcCerase activity and accumulation of glucosylceramide, which results in the onset of Gaucher disease, the most common lysosomal storage disease. Significant clinical heterogeneity is observed in Gaucher disease, with three main types known, but no clear correlation has been reported between the different types and levels of residual GlcCerase activity. We now demonstrate that a correlation exists by using a radioactive, short-acyl chain substrate, *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-glucosylsphingosine ([^{14}C]hexanoyl-GlcCer). This substrate rapidly transferred into biological membranes in the absence of detergent [Futerman and Pagano (1991) Biochem. J. **280**, 295–302] and was hydrolyzed to *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-sphingosine ([^{14}C]hexanoyl-Cer) both *in vitro* and *in situ*, with an acid pH optimum. A strict correlation was observed between levels of [^{14}C]hexanoyl-GlcCer hydrolysis and Gaucher type in human skin fibroblasts. The mean residual activity measured *in vitro* for 3 h incubation in type 1 Gaucher fibroblasts (the mild form of the disease) was 46.3 ± 4.6 nmol of [^{14}C]hexanoyl-Cer formed per mg protein ($n = 9$), and in type 2 and 3 fibroblasts (the neuronopathic forms of the disease) was 19.6 ± 6.5 ($n = 9$). A similar correlation was observed when activity was measured *in situ*, suggesting that the clinical severity of a lysosomal storage disease is related to levels of residual enzyme activity.

INTRODUCTION

Lysosomes contain a variety of acid hydrolases responsible for the degradation of proteins, lipids and carbohydrates that are internalized via endocytosis (Kornfeld and Mellman, 1989). A large number of genetically determined defects in lysosomal hydrolases are known, manifested either by the absence of a particular hydrolase or by defects in its activity, resulting in accumulation of undegraded substrates, a progressive increase in size and number of lysosomes, and onset of a 'lysosomal storage disease' with its associated clinical manifestations (Neufeld, 1991).

Defects in the acid hydrolases that degrade sphingolipids are particularly common. The most prevalent lysosomal storage disease, Gaucher disease (Barranger and Ginns, 1989; Grabowski et al., 1990), results from deficiencies in the activity of glucocerebrosidase (D-glucosyl-*N*-acylsphingosine glucohydrolase, EC 3.2.1.45; GlcCerase), the enzyme that cleaves the β-glucosidic linkage of glucosylceramide (GlcCer) to produce ceramide and glucose. 36 mutations have so far been reported in the GlcCerase gene, including point mutations, insertional mutations, deletions and splicing mutations (Beutler, 1993; Mistry and Cox, 1993; Horowitz and Zimran, 1994). All of these mutations result in a large, but comparable, decrease in GlcCerase activity when measured *in vitro* using a variety of synthetic substrates (Hultberg and Ockerman, 1972; Dinur et al., 1984; Osiecki-Newman et al., 1987). Thus no correlation was observed *in vitro* between the $V_{max.}$ of GlcCerase towards 4-methylumbelliferyl β-D-glucopyranoside in different Gaucher alleles [see, for instance, mutations N370S (type 1) and L444P (type 3) in Horowitz and Zimran (1994)].

In the present study we examined the relationship between levels of residual GlcCerase activity and the type of Gaucher disease. As for all lysosomal storage diseases, Gaucher disease is characterized by marked clinical heterogeneity, varying from a chronic non-neuronopathic type (type 1) to infantile (type 2) and juvenile (type 3) neuronopathic types (Barranger and Ginns, 1989). However, no correlation has been reported between levels of residual GlcCerase activity measured *in vitro* and type or genotype (Barranger and Ginns, 1989; Beutler, 1993). We now use a radioactive short-acyl-chain analogue of glucosylceramide, *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-glucosylsphingosine ([^{14}C]-hexanoyl-GlcCer) (Futerman and Pagano, 1991), to examine this relationship. In contrast with other substrates used for measuring GlcCerase activity, such as 4-methylumbelliferyl β-D-glucopyranoside (Hultberg and Ockerman, 1972) (Figure 1), [^{14}C]hexanoyl-GlcCer rapidly and spontaneously transfers into biological membranes in the absence of detergent and consequently does not destroy membrane integrity, permitting analysis of enzyme activity *in situ* in live cells. Radioactive short-acyl-chain sphingolipid analogues have been used previously to measure the activity of various enzymes of sphingolipid synthesis and degradation both *in vitro* and *in vivo* (Futerman et al., 1990; Futerman and Pagano, 1991, 1992; Boudker and Futerman, 1993). Using cultured skin fibroblasts obtained from patients with Gaucher disease whose genotypes have been characterized

Abbreviations used: GlcCer, glucosylceramide; GlcCerase, glucocerebrosidase; [^{14}C]hexanoyl-Cer, *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-sphingosine; [^{14}C]hexanoyl-GlcCer, *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-glucosylsphingosine; C_6-NBD-GlcCer, *N*-{6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl}-D-*erythro*-glucosylsphingosine; [^{14}C]hexanoyl-SM, *N*-(1-[^{14}C]hexanoyl)-D-*erythro*-sphingosylphosphocholine.

‡ To whom correspondence should be addressed.

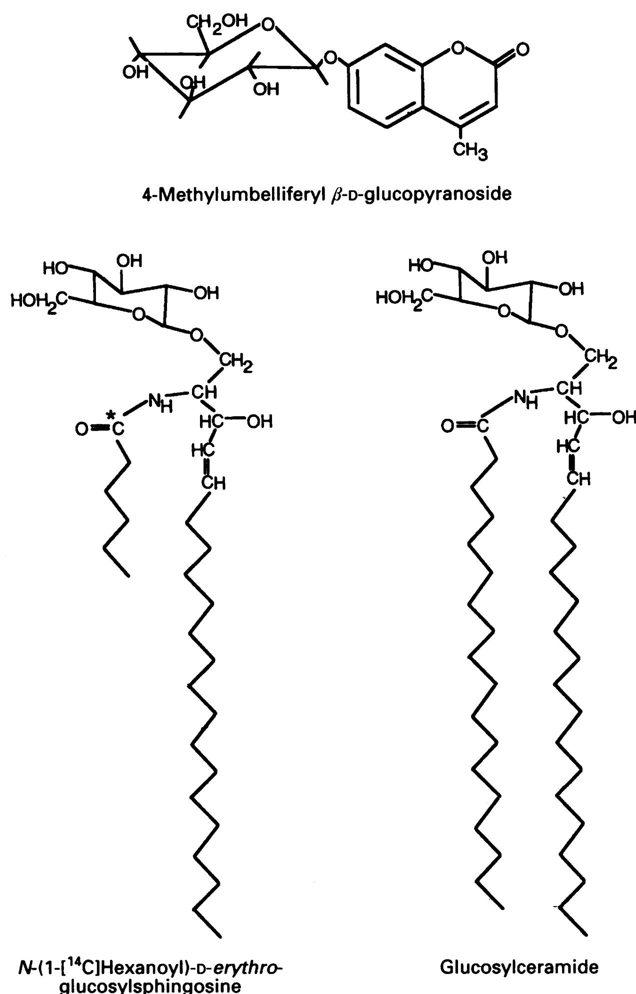

Figure 1 Molecular structure of substrates used to assay GlcCerase activity

The structure of [^{14}C]hexanoyl-GlcCer is compared with the natural substrate of GlcCerase, glucosylceramide, and a synthetic substrate, 4-methylumbelliferyl β-D-gluocopyranoside. The short acyl chain of [^{14}C]hexanoyl-GlcCer significantly increases the solubility of this substrate in aqueous solutions compared with the natural and synthetic analogues. The asterisk (*) denotes the radioactive carbon atom.

(Firon et al., 1990), we now demonstrate that a correlation exists between levels of residual enzyme activity and the type of Gaucher disease.

EXPERIMENTAL

Materials

[1-^{14}C]Hexanoic acid (55.7 mCi/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Conduritol β-epoxide was from Matreya Inc. (Pleasant Gap, PA, U.S.A.). Silica-gel 60 plates were from Merck. Sodium dithionite was purchased from BDH Laboratories (Poole, Dorset, U.K.). Succinimidyl 6-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)aminohexanoate and sulforhodamine B were from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals, including D-*erythro*-sphingosine and D-*erythro*-glucosylsphingosine, were from Sigma. Solvents (analytical grade) were from Bio-Lab Laboratories (Jerusalem, Israel).

Preparation and analysis of lipids

[^{14}C]Hexanoyl-GlcCer and *N*-[6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-D-*erythro*-glucosylsphingosine (C_6-NBD-GlcCer) were synthesized by *N*-acylation of glucosylsphingosine using the *N*-hydroxysuccinimide ester of 1-[^{14}C]hexanoic acid or succinimidyl 6-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)aminohexanoate respectively as described by Futerman and Pagano (1991,1992) and Schwarzmann and Sandhoff (1987). *N*-(1-[^{14}C]Hexanoyl)-D-*erythro*-sphingosine ([^{14}C]hexanoyl-Cer) was prepared by the same procedure, except that sphingosine was used (Pagano, 1989; Futerman et al., 1990). [^{14}C]Hexanoyl-sphingolipids were prepared as a complex with defatted BSA (molar ratio 1:1) (Boudker and Futerman, 1993). Stock concentrations of radioactive lipids were determined by liquid-scintillation counting in a Packard 1500 Tri-Carb scintillation counter using Lumax/toluene (1:3, v/v) as scintillation fluid.

Cell culture

Human skin fibroblasts from normal donors and patients with Gaucher disease were obtained from the following sources (see Table 1 for cell codes): FS11 from Dr. Michel Revel, Weizmann Institute of Science, Rehovot, Israel; TJ, MAM, BRN, KW, BH, BM, RP and JP from the Eunice Kennedy Shriver Center, Waltham, MA, U.S.A.; 1607, 89.33, 89.25, 86.4, 84.72, 88.23, 89.62, 877 and 89.57 from the National Institutes of Health; NAP from Dr. H. Mozer, Johns Hopkins University, Baltimore, MD, U.S.A.; IS from Dr. K. Hanzer, University of Tübingen, Tübingen, Germany; S. S., MR and SN from Dr. Y. Zlotogora, Haddasah Medical School, Jerusalem, Israel. Fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 20% heat-inactivated fetal-calf serum (Kibbutz Beth Ha-Emek, Israel), 10 unit/ml penicillin, 10 μg/ml streptomycin and 20 mM glutamine. Cells were grown at 37 °C in an air/CO_2 (19:1) atmosphere.

Assay of GlcCerase activity

In vitro

Cells were grown until confluence and then washed three times with Dulbecco's modified Eagle's medium minus Phenol Red. Cells were harvested using a rubber policeman and homogenized in a hand-held Potter–Elvehjem homogenizer in 1 ml of Mes buffer (50 mM, pH 5.5). Homogenates were used either fresh or after storing at −80 °C. In one case, membranes were prepared by centrifugation at 80000 $g_{av.}$ for 10 min at 4 °C. Homogenates (10 μg) were incubated with 1 nmol of a [^{14}C]hexanoyl-GlcCer–defatted BSA complex in a final volume of 1 ml of Mes (50 mM, pH 5.5). No other components were added to the reaction mixture. Reactions were allowed to proceed for various times at 37 °C and stopped by addition of 2 ml of chloroform/methanol (1:2, v/v) prior to extraction and analysis of the radioactive lipids (see below).

In situ

[^{14}C]Hexanoyl-GlcCer–BSA complex (1 nmol) was added to the medium (Dulbecco's modified Eagle's medium minus phenol red) of a confluent monolayer of cells for 3 h at 37 °C. Cells were subsequently harvested and homogenized as described above. Aliquots were removed for protein assay (Bradford, 1976) prior to extraction of lipids (see below).

The degradation product of [^{14}C]hexanoyl-GlcCer, [^{14}C]-hexanoyl-Cer, can escape from lysosomes *in situ* and be subsequently metabolized in the endoplasmic reticulum–Golgi-apparatus complex to [^{14}C]hexanoylsphingomyelin (SM), [^{14}C]hexanoyl-GlcCer and other higher-order sphingolipids [see Figure 5 below and Koval and Pagano (1989)]. Thus, in order to determine the amount of [^{14}C]hexanoyl-Cer formed by the degradation of [^{14}C]hexanoyl-GlcCer *in situ*, the loss of [^{14}C]hexanoyl-Cer due to subsequent metabolism to [^{14}C]-hexanoyl-GlcCer and [^{14}C]hexanoyl-SM was determined. This was performed by incubating cells directly with [^{14}C]hexanoyl-Cer (see Figure 5 below) and calculating the ratio of [^{14}C]hexanoyl-GlcCer to [^{14}C]hexanoyl-SM formed. This ratio was then used to calculate the amount of [^{14}C]hexanoyl-Cer that was metabolized to [^{14}C]hexanoyl-GlcCer, exactly as described by Koval and Pagano (1989). GlcCerase activity *in situ* was calculated from the sum of the radioactivities in [^{14}C]hexanoyl-Cer, [^{14}C]hexanoyl-GlcCer, [^{14}C]hexanoyl-LacCer and [^{14}C]hexanoyl-SM.

Extraction and analysis of lipids

Lipids were extracted by the procedure of Bligh and Dyer (1959), dried under N_2, and resolved by t.l.c. using chloroform/methanol/15 mM $CaCl_2$ (60:35:8, by vol.) or chloroform/methanol/15 mM KCl (60:35:8, by vol.) as developing solvents. Radioactive lipids were identified after autoradiography by comparison with authentic [^{14}C]hexanoylsphingolipid standards (Futerman and Pagano, 1992). Lipids were recovered from the plates by scraping, and radioactivity was determined by liquid-scintillation counting.

Cell labelling with short-acyl-chain fluorescent lipid analogues

Fibroblasts were grown on 24 mm-diameter glass coverslips and pre-cooled at 7 °C for 5 min prior to addition of a C_6-NBD-GlcCer–defatted BSA complex (5 μM). Cells were incubated for 30 min at 7 °C, washed three times with Dulbecco's modified Eagle's medium minus Phenol Red, and incubated for a further 3 h at 37 °C in Dulbecco's modified Eagle's medium minus Phenol Red. For the first 1 h of the incubation period, sulforhodamine B (0.1 mg/ml) was present in the incubation medium. C_6-NBD-GlcCer-associated fluorescence was subsequently depleted from the plasma membrane by treatment with 80 mM sodium dithionite (Mcintyre and Sleight, 1991) for 5 min at 25 °C, and intracellular labelling was monitored by fluorescence microscopy using an Apochromat 63X/1.40 oil objective of an Axiovert 35 microscope, with appropriate filters for NBD or sulforhodamine fluorescence.

RESULTS

In vitro assay of GlcCerase using [^{14}C]hexanoyl-GlcCer

Initial studies were performed to characterize the enzyme activity that hydrolyses [^{14}C]hexanoyl-GlcCer in cell homogenates. [^{14}C]hexanoyl-GlcCer was hydrolysed by a membrane-associated enzyme with a pH optimum of 5.5 (not shown), and was completely inhibited by conduritol-β-epoxide, a specific inhibitor of lysosomal GlcCerase (Legler, 1977). Activity was dependent on both time (Figure 2a) and protein concentration (Figure 2b), and clear differences in enzyme activity were observed between normal and Gaucher fibroblasts. In addition, it was possible to distinguish between residual enzyme activities in cells taken from different Gaucher types, with higher activity observed for type 1 Gaucher fibroblasts (the non-neuronopathic type) than for types 2 and 3 (neuronopathic types) as a function of time or protein concentration (Figure 2). On the basis of these results, standard incubation conditions were chosen so that the rate of formation of [^{14}C]hexanoyl-Cer was linear with respect to time (60 min) and protein concentration (10 μg of protein) using 1 μM [^{14}C]hexanoyl-GlcCer.

Using these standard incubation conditions, a consistent difference was observed in GlcCerase activity between fibroblasts from different Gaucher types (Figure 3). Residual activity in type 1 fibroblasts varied from 12.0 to 33.1 nmol/mg of protein (Table 1) after 1 h incubation, with a mean value of 20.7 ± 6.9 ($n = 10$), and in type 2 and 3 fibroblasts varied from 3.9 to 12.4 nmol/mg of protein, with a mean value of 8.4 ± 2.8 ($n = 9$). Although some overlap in activity was observed after 1 h incubation between type 1 and types 2 and 3 fibroblasts (see for instance cell sources 86.4 and IS in Table 1), incubation with [^{14}C]hexanoyl-GlcCer for longer times (3 h) completely resolved any ambiguity in distinguishing residual enzyme activities between different Gaucher types (Table 1).

In situ assay of GlcCerase using [^{14}C]hexanoyl-GlcCer

Cultured cells were incubated with [^{14}C]hexanoyl-GlcCer *in situ*. Prior to these experiments we determined whether short-acyl-chain analogues of GlcCer were internalized via the endocytic pathway to endosomes and lysosomes in human skin fibroblasts, by using a short-acyl-chain fluorescent derivative of GlcCer, C_6-NBD-GlcCer. This fluorescent derivative has been used to study endocytosis in other cell types (Kok et al., 1989, 1991, 1992),

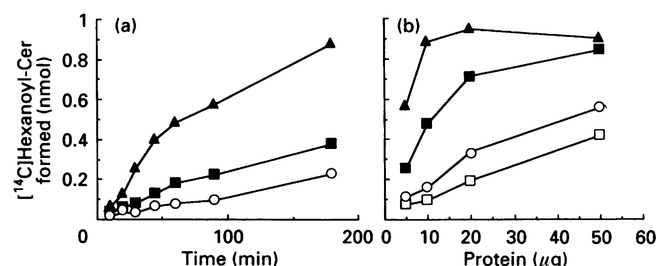

Figure 2 Characterization of [^{14}C]hexanoyl-GlcCer hydrolysis *in vitro*

[^{14}C]Hexanoyl-GlcCer (1 μM) was incubated with homogenates from normal fibroblasts (▲), and type 1 (■), 2 (□) and 3 (○) Gaucher fibroblasts. (**a**) [^{14}C]Hexanoyl-GlcCer hydrolysis as function of time using 10 μg of protein. (**b**) [^{14}C]Hexanoyl-GlcCer hydrolysis as a function of protein concentration after 180 min incubation. Each point represents the mean for at least two independent assays done in duplicate.

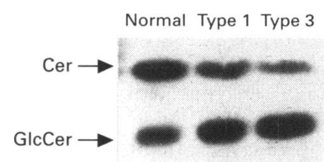

Figure 3 Chromatographic analysis of [^{14}C]hexanoyl-GlcCer hydrolysis *in vitro*

Homogenates from normal fibroblasts, and type 1 and type 3 Gaucher fibroblasts were incubated for 1 h with 1 μM [^{14}C]hexanoyl-GlcCer prior to extraction and separation by t.l.c.

Table 1 GlcCerase activity in normal and Gaucher fibroblasts *in vitro* and *in situ*

GlcCerase activity was assayed for either 1 or 3 h in *vitro* using standard incubation conditions (see the Results section); results represent the mean activity ± S.D for at least three independent assays. For the *in situ* assay, incubations were performed for 3 h with 80–120 μg of protein; results represent the mean activity ± S.D. for assays of six to ten individual culture plates. Activity is given as nmol of [^{14}C]hexanoyl-sphingolipids formed (see the Experimental section)/mg of protein. Data from type 2 (indicated by an asterisk, *) and 3 Gaucher types are pooled, owing to the limited number of subjects available with type 2 Gaucher.

		Assay conditions		
Type and cell source	Genotype	*In situ*	*In vitro* (1 h)	*In vitro* (3 h)
Normal				
FS11	+/+	5.7 ± 0.1	79.8 ± 3.4	
TJ	+/+		72.6 ± 12.8	
MAM	+/+		48.0 ± 5.6	
Average ...			66.8 ± 16.7	
Type 1				
1607	N370S/RecTL	4.5 ± 1.3	28.4 ± 4.3	45.7 ± 9.8
BRN	N370S/RecTL	4.4 ± 0.7	13.7 ± 3.0	50.3 ± 4.5
KW	N370S/N370S	3.3 ± 1.1	16.8 ± 3.2	34.0 ± 9.1
NAP	N370S/N370S	3.4 ± 0.6	25.7 ± 5.4	44.7 ± 5.3
89.33	N370S/IVS2 + 1		19.5 ± 6.3	51.6 ± 14.6
BH	N370S/RecNcil (444)	3.1 ± 0.4	18.7 ± 4.0	44.2 ± 8.2
89.25	N370S/?	3.6 ± 0.4	33.1 ± 2.7	
BM	N370S/?	3.2 ± 0.3	14.2 ± 2.3	41.9 ± 2.6
86.4	R463C/?	3.4 ± 1.0	12.0 ± 1.9	44.6 ± 0.5
R.P.†	K157Q/D140H;E326K	2.9 ± 0.5	23.5 ± 5.2	53.5 ± 8.7
Average ...		3.5 ± 0.6	20.7 ± 6.9	46.3 ± 4.6
Types 2/3				
IS	?/?	2.3 ± 0.5	12.4 ± 1.3	28.7 ± 4.3
84.72	L444P/L444P	2.4 ± 0.4	11.3 ± 2.3	26.2 ± 5.4
88.23	L444P/L444P	2.2 ± 0.3	11.8 ± 3.6	17.5 ± 2.7
S.S	L444P/L444P	2.1 ± 0.3		
MR	D409H/D409H	1.7 ± 0.3	6.7 ± 1.1	21.3 ± 5.8
89.62	L444P/L444P	2.0 ± 0.6	9.6 ± 2.2	16.2 ± 2.2
877*	L444P/RecNcil	1.3 ± 0.2	9.5 ± 2.5	25.7 ± 5.6
SN*	L444P/IVS2 + 1	2.1 ± 0.5	3.9 ± 1.3	8.6 ± 1.6
89.57*	D409H/IVS2 + 1	1.7 ± 0.8	4.7 ± 1.4	13.4 ± 4.1
J.P.†	K157Q/D140H;E326K	0.9 ± 0.4	7.7 ± 1.7	19.1 ± 6.2
Average ...		1.8 ± 0.5	8.4 ± 2.8	19.6 ± 6.5

† Even though R.P. and J.P. are brothers with the same mutation, they were diagnosed clinically as different Gaucher phenotypes (Firon et al., 1990); the difference in clinical expression is not due to the fact that the same mutation leads to differing severity of disease, but due to other inherited differences between the brothers (M. Horowitz, unpublished work).

although some ambiguity has been reported concerning its mode of internalization. In human skin fibroblasts, C_6-NBD-GlcCer was internalized to a population of small vesicles (Figure 4, panel A) that were co-labelled by a fluid-phase marker, sulforhodamine B (Figure 4, panel B), indicating that C_6-NBD-GlcCer can be transported via endosomes to lysosomes, where it is degraded by lysosomal GlcCerase. This was confirmed by biochemical analysis *in situ* (Figure 5, lane A), in which [^{14}C]hexanoyl-GlcCer was degraded to [^{14}C]hexanoyl-Cer, which was subsequently metabolized in the endoplasmic reticulum/Golgi-apparatus complex (Futerman and Pagano, 1991; Futerman et al., 1990; Koval and Pagano, 1989) to [^{14}C]hexanoyl-SM, [^{14}C]hexanoyl-GlcCer (Figure 5, lane B) and [^{14}C]hexanoyl-LacCer.

Similar to [^{14}C]hexanoyl-GlcCer hydrolysis in *vitro*, clear differences in residual enzyme activity were observed *in situ* between fibroblasts obtained from different Gaucher types. Activity in type 1 fibroblasts varied from 2.9 to 4.5 nmol/mg of protein with a mean value of 3.5 ± 0.6 (n = 9), and for type 2 and 3 varied from 0.9 to 2.4 nmol/mg of protein with a mean value of 1.8 ± 0.5 (n = 10) (Table 1). There was no overlap in residual enzyme activities between type 1 or types 2 and 3 fibroblasts in any of the cells. Moreover, cells with similar mutations in GlcCerase had similar specific activities *in situ* (see for instance mutation L444P/L444P in type 3 fibroblasts, and mutation N370S/RecTL in type 1 fibroblasts), indicating that a correlation may exist not only between the type but also between the genotype and levels of residual enzyme activity.

DISCUSSION

Short-acyl-chain sphingolipid analogues such as [^{14}C]hexanoyl-GlcCer rapidly and spontaneously transfer into biological membranes in the absence of detergent (Pagano and Sleight, 1985; Futerman et al., 1990; Futerman and Pagano, 1992). This property makes them particularly useful substrates for measuring the activity of membrane-associated enzymes under conditions in which membrane integrity is not destroyed. For most synthetic substrates of GlcCerase, such as 4-methylumbelliferyl β-D-glucopyranoside, mixtures of detergents and phospholipids are required to solubilize either the enzyme or substrate and obtain maximal activity (Hultberg and Ockerman, 1972; Van Weely et al., 1991). In such mixtures, the physical state under which the enzymic reaction proceeds is far removed from conditions existing *in situ*, inasmuch as both substrate and enzyme are present in aqueous solutions as complexes with detergents. In contrast, since short-acyl-chain lipids rapidly transfer into biological

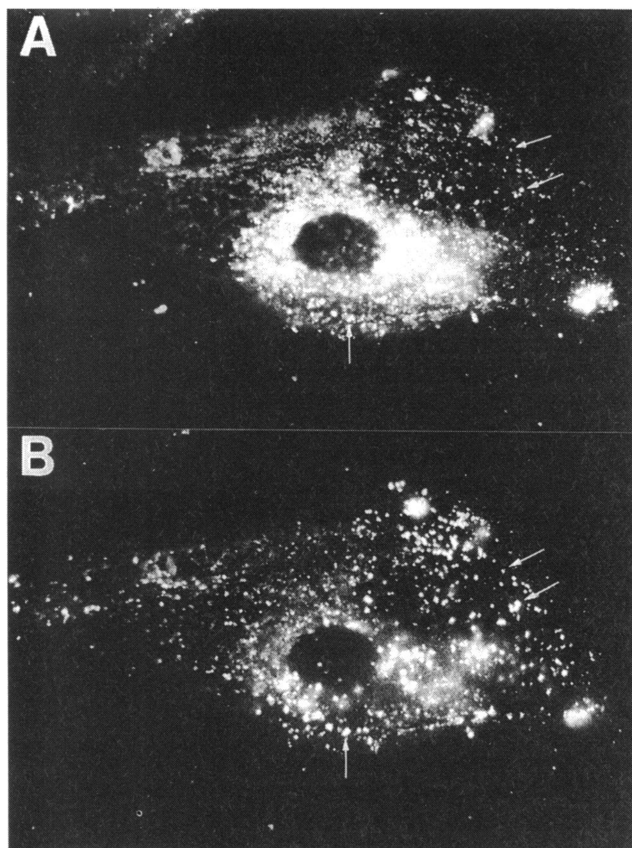

Figure 4 C_6-NBD-GlcCer labels the endocytic pathway in cultured human skin fibroblasts

Gaucher fibroblasts (type 3) were labelled with C_6-NBD-GlcCer (panel A) and sulforhodamine B (panel B). Some areas of overlap of NBD- and rhodamine-fluorescence are indicated by arrows.

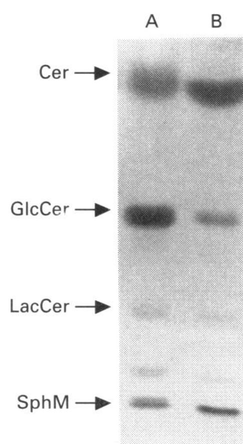

Figure 5 Chromatographic analysis of [^{14}C]hexanoyl-GlcCer hydrolysis and [^{14}C]hexanoyl-Cer metabolism *in situ*

Type 1 Gaucher fibroblasts were incubated with 1 nmol of [^{14}C]hexanoyl-GlcCer (lane A) or 1 nmol [^{14}C]hexanoyl-Cer (lane B) for 3 h prior to extraction and separation by t.l.c.; incubation with [^{14}C]hexanoyl-Cer was performed to calculate the amount of [^{14}C]hexanoyl-Cer that was metabolized to [^{14}C]hexanoyl-GlcCer (see the Experimental section). Lipids were identified by comparison with authentic short-acyl-chain sphingolipid standards as indicated. LacCer, lactosylceramide; SphM, sphingomyelin.

membranes from donors such as liposomes or protein (i.e. defatted albumin) complexes in the absence of detergent, the enzymic reaction proceeds in the natural environment of the membrane. Radioactive short-acyl-chain analogues of GlcCer and SM have been used previously to measure both GlcCerase (Futerman and Pagano, 1991) and sphingomyelinase activity (Futerman et al., 1990). In liver fractions, the majority of GlcCerase activity was recovered in a fraction that contained most of a lysosomal marker enzyme (Futerman and Pagano, 1991). We have now confirmed the specificity of [^{14}C]hexanoyl-GlcCer as a substrate for acid GlcCerase in human skin fibroblasts and also showed that a fluorescent derivative of GlcCer, C_6-NBD-GlcCer, is internalized along the endocytic pathway in these cells. Thus [^{14}C]hexanoyl-GlcCer can be used as a substrate to measure GlcCerase activity in human skin fibroblasts both *in vitro* and *in situ*.

We have now analysed GlcCerase activity in cell lines derived from Gaucher patients with different mutations in the GlcCerase gene [for details about the mutations, see Horowitz and Zimran (1994)]. The major finding is the demonstration that a direct correlation exists between levels of residual lysosomal GlcCerase activity and the type of Gaucher disease, using [^{14}C]hexanoyl-GlcCer as substrate. Theoretical considerations previously suggested that such a correlation exists (Conzelmann and Sandhoff, 1983/84; Leinekugel et al., 1992). According to this model, small changes in residual enzyme activity below a critical threshold value would significantly affect the rate of degradation of lysosomal substrates, but changes in levels of enzyme activity above the threshold would have no effect, since the enzyme is present at higher levels than that required to hydrolyse all the substrate. Direct proof of this proposal for Gaucher disease was lacking since no assay was available which permitted accurate analysis of low levels of residual GlcCerase activity. Two recent studies examined whether a correlation exists between residual GlcCerase activity and Gaucher types. In the first (Van Weely et al., 1991), C_6-NBD-GlcCer was used as substrate for *in situ* experiments and no correlation was observed, although incubation times (24 h) were much longer than those used in the current study (3 h), and the amount of C_6-NBD-Cer that was metabolized back to C_6-NBD-GlcCer was not calculated. In the second study (Agmon et al., 1993), lissamine rhodamine sulphonylamidosphingosyl derivatives were used to assay GlcCerase activity in cell extracts and in intact skin fibroblasts. In intact cells, long incubations revealed differences in the ratio of fluorescent ceramide to fluorescent GlcCer between type 1 and type 2 or 3 Gaucher fibroblasts, supporting the suggestion that there is a correlation between levels of residual enzyme activity and the type of Gaucher disease.

Although we have observed a consistent correlation between residual enzyme activity and Gaucher type, it should be emphasized that reticuloendothelial cells are affected to a greater extent in Gaucher disease than the skin fibroblasts used in this study. Analyses of GlcCerase using [^{14}C]hexanoyl-GlcCer might therefore demonstrate larger changes in residual enzyme activity between different types in reticuloendothelial cells. In addition to its potential diagnostic role, [^{14}C]hexanoyl-GlcCer could also be a useful substrate in attempting to understand the kinetic and molecular properties of GlcCerase in its natural membrane environment, and in helping to understand the role of the sphingolipid activator proteins (Furst and Sandhoff, 1992) that are necessary for the normal catabolism of natural sphingolipid substrates.

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