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S van Weely, ... , J M Tager, J M Aerts

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Research Article

The properties of control and 370Asn-->Ser glucocerebrosidase, the frequently encountered mutated form of the enzyme in type 1 Gaucher disease, were studied in vitro as well as in situ. The catalytic properties of purified 370Asn-->Ser glucocerebrosidase were highly dependent on the assay conditions. The enzyme was deficient in activity towards substrate and in reactivity with the irreversible inhibitor conduritol B-epoxide (CBE) when activated by the bile salt taurocholate. In the presence of more physiological activators, the lysosomal activator protein saposin C and phosphatidylserine, the 370Asn-->Ser enzyme was near normal in kinetic properties at pH values approximately 5, but not at higher pH. In intact fibroblasts, the enzymic activity of the 370Asn-->Ser glucocerebrosidase and its reactivity with CBE were found to be clearly deficient. However, in intact lymphoblasts from the same patients, the behavior of the mutant enzyme was near normal. The catalytic efficiency of 370Asn-->Ser glucocerebrosidase in situ was also found to be highly pH dependent. When intact lymphoblasts were cultured in the presence of permeant weak bases, which increase the pH of acidic intracellular compartments, the catalytic efficiency of the mutant enzyme, as assessed by its reactivity with CBE, became markedly impaired. Our findings indicate that the intralysosomal pH in the intact cell can be expected to have a critical influence on the activation state of 370Asn-->Ser glucocerebrosidase and [...]

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Role of pH in Determining the Cell-type-specific Residual Activity of Glucocerebrosidase in Type 1 Gaucher Disease

S. van Weely,* M. van den Berg,* J. A. Barranger,* M. C. Sa Miranda,* J. M. Tager,* and J. M. F. G. Aerts*

*E. C. Slater Institute for Biochemical Research, University of Amsterdam, Academic Medical Centre, 1105 AZ Amsterdam, The Netherlands; †Department of Human Genetics, University of Pittsburgh, Pittsburgh, Pennsylvania 15261; and ‡Instituto Genetica Medica Jacinto Magalhaes, 4000 Porto, Portugal

Abstract

The properties of control and $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase, the frequently encountered mutated form of the enzyme in type 1 Gaucher disease, were studied in vitro as well as in situ.

The catalytic properties of purified $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase were highly dependent on the assay conditions. The enzyme was deficient in activity towards substrate and in reactivity with the irreversible inhibitor conduritol B-epoxide (CBE) when activated by the bile salt taurocholate. In the presence of more physiological activators, the lysosomal activator protein saposin C and phosphatidylserine, the $^{370}\text{Asn} \rightarrow \text{Ser}$ enzyme was near normal in kinetic properties at pH values ~ 5 , but not at higher pH.

In intact fibroblasts, the enzymic activity of the $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase and its reactivity with CBE were found to be clearly deficient. However, in intact lymphoblasts from the same patients, the behavior of the mutant enzyme was near normal. The catalytic efficiency of $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase in situ was also found to be highly pH dependent. When intact lymphoblasts were cultured in the presence of permeant weak bases, which increase the pH of acidic intracellular compartments, the catalytic efficiency of the mutant enzyme, as assessed by its reactivity with CBE, became markedly impaired.

Our findings indicate that the intralysosomal pH in the intact cell can be expected to have a critical influence on the activation state of $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase and its ability to hydrolyse substrate. This phenomenon may partly underlie the marked heterogeneity in clinical manifestation of Gaucher disease among patients with this mutated form of glucocerebrosidase. (*J. Clin. Invest.* 1993. 91:1167–1175.) Key words: conduritol B-epoxide • glucosylceramide lipidosis • lysosomal storage disorder • saposin C

Introduction

Gaucher disease (glucosylceramidosis) is a recessively inherited lysosomal storage disorder in which the activity of glucocerebrosidase (EC 3.2.1.45) is deficient. The clinical mani-

festation of the disease is very heterogeneous with respect to age of onset and neurological involvement. In patients with the most frequently encountered form, type 1, the clinical presentation may vary from cases with severe splenomegaly and bone deterioration to cases without clear clinical complications (1). Type 2 and type 3 Gaucher disease patients develop neurological complications in infancy or childhood, respectively (1). Gaucher disease is genetically heterogeneous. Several mutations in glucocerebrosidase have been identified (2–6), and it has become clear that patients may have either two identical or, more generally, two different mutant glucocerebrosidase alleles (see e.g., references 7 and 8). Most type 1 Gaucher disease patients carry at least one copy of a mutant glucocerebrosidase allele in which a mutation at position 1,226 in the cDNA leads to substitution of G for A (cDNA nucleotides are numbered starting with the upstream ATG at number 1 [9]). This results in a substitution of serine for asparagine at amino acid position 370 in the mature protein (8, 10, 11). The $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase is present in normal amounts (12–15). In vitro, this enzyme is only poorly active under most conditions; however, when activated at acidic pH by negatively charged phospholipid and the natural lysosomal activator protein saposin C, formerly referred to as sphingolipid activator protein 2 (for a review, see reference 16), its activity is near normal (17). Another frequently encountered form of mutated glucocerebrosidase has a C for T substitution at nucleotide position 1,448, resulting in a proline for leucine substitution at position 444 in the mature protein. This mutation has been detected in various phenotypes of Gaucher disease. Most patients who are homozygous for this mutation develop a neuronopathic course of the disease (7, 11, 18–20). The $^{444}\text{Leu} \rightarrow \text{Pro}$ glucocerebrosidase results in reduced enzyme stability (15–21). In a third commonly encountered allele, a G insertion in the glucocerebrosidase gene at cDNA position 84 results in production of a truncated protein (22).

The consequences of the $^{370}\text{Asn} \rightarrow \text{Ser}$ substitution for catalytic efficiency of glucocerebrosidase in situ are not yet fully understood. It has previously been observed by us that the state of activation of $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase, and concomitantly its catalytic efficiency, differs in homogenates of fibroblasts and lymphoblasts obtained from the same type 1 Gaucher disease patients (23). We have now studied the properties of glucocerebrosidase in intact fibroblasts and lymphoblasts. First, the degradation of a fluorescent analogue of glucocerebroside by intact cells was measured. Second, the reactivity of glucocerebrosidase with the irreversible inhibitor conduritol B-epoxide (CBE)¹ was assessed. This inhibitor is analogous in

Address correspondence to J. M. F. G. Aerts, Publication Secretary, E. C. Slater Institute for Biochemical Research, University of Amsterdam, Academic Medical Centre, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands.

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1. Abbreviations used in this paper: CBE, conduritol B-epoxide; C₆-NBD glucosylceramide, 6-[N-7-nitrobenz-2-oxa-1,3 diazol-4-yl-aminocaproyl] sphingosyl β -D-glucoside; 4-MU- β -glucoside, 4-methylumbelliferyl- β -D-glucoside.

structure to the transition state of gluconolactone formed during hydrolysis of glucocerebroside. In contrast to the natural transition state compound, the epoxide is able to form a covalent bond with an aspartic acid group in the catalytic site, thus irreversibly inhibiting the enzyme (2, 24). It has elegantly been shown by Bieberich and Legler (25) that CBE rapidly enters lysosomes in intact cells and effectively inhibits the enzyme *in situ*.

Our investigation revealed differences between properties of glucocerebrosidase in intact fibroblasts and in intact lymphoblasts from type 1 Gaucher disease patients with $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase. The possible role of the natural activator protein saposin C and lysosomal pH in this connection was investigated. The results are reported here, and the implications are discussed.

Methods

Materials. CBE was purchased from Biomol Research Laboratories (Philadelphia, PA), sodium taurocholate grade A was purchased from Calbiochem (San Diego, CA); and phosphatidylserine, and 4-methylumbelliferyl- β -D-glucoside (4-MU- β -glucoside) was purchased from Sigma Immunochemicals (St. Louis, MO). C_6 -NBD glucosylceramide (6-[N-7-nitrobenz-2-oxa-1,3 diazol-4-yl-aminocaproyl] sphingosyl β -D-glucoside) was a kind gift from Dr. G. Schwarzmann (University of Bonn, Bonn, Germany). All other chemicals were of the purest grade commercially available.

Cell lines of Gaucher disease patients. Properties of glucocerebrosidase were studied in cultured skin fibroblasts and EBV transformed B lymphocytes (lymphoblasts) from Gaucher disease patients with a defined glucocerebrosidase genotype; i.e. genotypes $^{370}\text{Asn} \rightarrow \text{Ser}/^{370}\text{Asn} \rightarrow \text{Ser}$, $^{444}\text{Leu} \rightarrow \text{Pro}/^{444}\text{Leu} \rightarrow \text{Pro}$ and $^{370}\text{Asn} \rightarrow \text{Ser}/^{444}\text{Leu} \rightarrow \text{Pro}$. Fibroblasts were obtained by skin biopsies and lymphoblasts were obtained by transformation of peripheral blood B lymphocytes with EBV (23, 26). The cells were cultured in RPMI 1640 containing 10% (by volume) FCS and bicarbonate with 5% CO_2 in the gas phase. The glucocerebrosidase genotype of the patients was determined by allele-specific oligonucleotide hybridization (26). In the case of patient C (Table I), it has not as yet been excluded that the mutated $^{444}\text{Leu} \rightarrow \text{Pro}$ allele contains additional mutations (also referred to as complex pattern alleles). The cell biological consequences of the $^{444}\text{Leu} \rightarrow \text{Pro}$ mutation and one of the complex pattern alleles are known to be similar; i.e. the production of a rapidly degraded glucocerebrosidase (15).

The Gaucher disease patients homozygous or heterozygous for the $^{370}\text{Asn} \rightarrow \text{Ser}$ allele varied markedly in severity of nonneuronopathic manifestation of the disorder. The patients homozygous for the $^{444}\text{Leu} \rightarrow \text{Pro}$ allele had developed neurological complications already in infancy (type 2) or only in childhood (type 3).

Consistent with earlier findings, it was noted using PAGE in the presence of sodium dodecyl sulphate followed by Western blotting (27), that the amount of glucocerebrosidase antigen was normal in fibroblasts of patients homozygous for the $^{370}\text{Asn} \rightarrow \text{Ser}$ allele (i.e., type 1 patients) and very strongly reduced in fibroblasts of patients homozygous for the $^{444}\text{Leu} \rightarrow \text{Pro}$ allele (i.e., type 2 and 3 patients). In cells from patients who were genetic compounds carrying both alleles, intermediate amounts of cross-reactive glucocerebrosidase were detected.

Preparation of antisera. A monospecific rabbit antiserum to human placental glucocerebrosidase was prepared as described (28). A monospecific rabbit antiserum to saposin C was prepared as described (29).

Immunoaffinity chromatographic purification of glucocerebrosidase. Glucocerebrosidase was purified from various materials by immunoaffinity chromatography with immobilized antiglucocerebrosidase monoclonal antibodies 8E4 and 2C7 as immunosorbents exactly as described previously (30).

Table I. Relative Specific Activity of Glucocerebrosidase Immunopurified from Cultured Skin Fibroblasts of Gaucher Patients

Genotype	Patient	Relative specific activity measured in the presence of	
		Taurocholate/Triton	PS/saposin C
		%	%
370/370	A	16, 14	77, 88
	B	15, 8	79, 84
370/444	C	21, 20	92, 87
	D	22	89
444/444	E	81, 73	86, 82
	F	85	81
Control (n = 4)		88–115	92–107

Identical amounts of cross-reactive glucocerebrosidase in preparations from cultured skin fibroblasts of control subjects and Gaucher disease patients were bound in a microtiter plate in which monoclonal anti-human glucocerebrosidase antibody 8E4 was immobilised, as described in Methods. The relative specific activity is the enzymic activity per amount of cross-reactive glucocerebrosidase in a preparation from a cell line of a Gaucher disease patient as percentage of the mean value for preparations from four different control fibroblast cell lines. The activity of bound glucocerebrosidase was determined with two different assay mixtures, each well containing 150 μl assay mixture. The reaction was stopped by addition of 100 μl 1 M glycine-NaOH, pH 10.6. The taurocholate/Triton assay mixture contained 5 mM 4-MU- β -glucoside, 0.1% (by volume) Triton X-100, 0.5% (mass/vol) sodium taurocholate, 100:200 mM citric acid/sodium phosphate, pH 5.0; and the phosphatidylserine/saposin C assay mixture contained 5 mM 4-MU- β -glucoside, 10 $\mu\text{g}/\text{ml}$ phosphatidylserine, 2.4 $\mu\text{g}/\text{ml}$ saposin C preparation, 100:200 mM citric acid/sodium phosphate, pH 5.0. The specific activity of control glucocerebrosidase immobilized to monoclonal antibody 8E4 was 1.8 and 1.4 nmol/h per mg protein when measured in the presence of taurocholate/Triton and PS/saposin C, respectively. 370, $^{370}\text{Asn} \rightarrow \text{Ser}$ allele; 444, $^{444}\text{Leu} \rightarrow \text{Pro}$ allele.

Purification of saposin C. The activator protein was purified according to the procedure described (31).

Assessment of activator protein level. The level of activator protein was determined by a functional test as described (12).

The assay of glucocerebrosidase with 4-MU- β -glucoside as substrate. Fibroblasts were harvested by trypsinisation and lymphoblasts by centrifugation. The cells were washed in isotonic PBS and homogenized as indicated in the text. The contribution of lysosomal glucocerebrosidase to the total β -glucosidase activity was determined using CBE. Enzyme preparations were simultaneously preincubated for 5 min without CBE, or with 5 mM CBE to ensure that all glucocerebrosidase was inactivated by the irreversible inhibitor, and subsequently β -glucosidase activity was measured in the absence and presence of 1 mM CBE under the conditions indicated in the text. The CBE-inhibitable β -glucosidase activity is caused by the lysosomal glucocerebrosidase (31). Protein was determined as described by Lowry et al. (32) with BSA as standard.

Measurement of relative specific activity of glucocerebrosidase. Previous studies have shown that monoclonal anti-(human glucocerebrosidase) antibody 8E4 has the same binding affinity for control, $^{370}\text{Asn} \rightarrow \text{Ser}$ substituted and $^{444}\text{Leu} \rightarrow \text{Pro}$ substituted glucocerebrosidase (12, 13, 15). Thus, identical amounts of various types of glucocerebrosidase will be bound to a specific amount of immobilized monoclonal antibody 8E4 when the immunosorbent is saturated by incubation with an excess of an enzyme preparation. If the enzyme preparation

consists of a mixture of two forms of glucocerebrosidase, the bound glucocerebrosidase will be a comparable mixture. These binding characteristics of the monoclonal antibody 8E4 allow a simple analysis of the specific activity of mutated glucocerebrosidase per amount of enzyme protein as described previously (12). Briefly, identical amounts of antiglucocerebrosidase monoclonal antibody 8E4 were immobilized to wells of a microtiter plate. The wells were incubated with enzyme preparations containing an excess of glucocerebrosidase antigen. The activity of bound glucocerebrosidase was measured with the fluorogenic 4-MU- β -glucoside as substrate as described in the legend of Table I. Fluorescence in the wells of the microtiter plate was determined (Fluoroscan; Flow Laboratories Inc., McLean, VA) with excitation at 366 nm and emission at 450 nm.

Measurement of hydrolysis of C₆-NBD glucosylceramide in intact cells. C₆-NBD glucosylceramide was complexed with BSA as described (13). Cells were incubated for 24 h at 37°C with 5 μ M lipid-BSA complex in the absence or presence of excess CBE. The cells were harvested, and lipids were extracted and separated by thin layer chromatography. The fluorescent lipids were scraped off, extracted, and quantified fluorimetrically as described (13).

Immuno-electron microscopy. Cultured human skin fibroblasts and EBV-lymphoblasts were fixed and prepared for immunogold labeling and electron microscopy following the procedure described for fibroblasts (33). Frozen cell samples were cryosectioned and immunolabeled, and examined using a transmission electron microscope (model 300; Phillips Electronic Instruments Co., Mahwah, NJ) at an accelerating voltage of 60 kV.

Results

Enzymic properties of purified glucocerebrosidase

Glucocerebrosidase was purified by immunoaffinity chromatography from cultured skin fibroblasts. The recovery of enzyme in monomeric form (31) was always > 70% on the basis of enzymic activity as measured in the presence of taurocholate.

Relative specific activity. The enzymic activity per amount of cross-reactive glucocerebrosidase related to that of control enzyme (i.e., the relative specific activity) was determined for enzyme preparations from fibroblasts from various types of Gaucher disease patients (see Table I). When measured in the presence of taurocholate and Triton X-100, the relative specific activity of glucocerebrosidase in preparations of cells of patients homozygous for the ³⁷⁰Asn $\rightarrow$ Ser allele is abnormally low, and that of enzyme in preparations from cells of patients homozygous for the ⁴⁴⁴Leu $\rightarrow$ Pro allele is close to normal. The relative specific activity of glucocerebrosidase in preparations from cells of patients who are genetic compounds for the ³⁷⁰Asn $\rightarrow$ Ser and ⁴⁴⁴Leu $\rightarrow$ Pro alleles is also low, suggesting that they contain predominantly ³⁷⁰Asn $\rightarrow$ Ser glucocerebrosidase molecules.

When bound glucocerebrosidase is measured in the presence of phosphatidylserine and activator protein the relative specific activity of enzyme in the preparations from fibroblasts of all the patients is near normal (see Table I). In other words, under these conditions, the ³⁷⁰Asn $\rightarrow$ Ser glucocerebrosidase is normalized.

The amount of cross-reactive glucocerebrosidase can be estimated by comparing the data for relative specific activity with values for specific activity in the corresponding cell extracts. In this way, it was calculated that the concentration of glucocerebrosidase antigen is 80–100% in fibroblasts from patients with genotype ³⁷⁰Asn $\rightarrow$ Ser/³⁷⁰Asn $\rightarrow$ Ser, < 10% in cells from

patients with genotype ⁴⁴⁴Leu $\rightarrow$ Pro/⁴⁴⁴Leu $\rightarrow$ Pro and ~ 30% in cells from patients carrying both alleles.

Inactivation by CBE. The irreversible inhibition of glucocerebrosidase by CBE was determined for enzyme purified from fibroblasts of individuals with different glucocerebrosidase genotypes. Fig. 1 A shows the results when enzyme preparations were incubated with inhibitor in the presence of taurocholate and Triton X-100, and enzyme activity was subsequently determined under the same conditions. The glucocerebrosidase purified from fibroblasts of the patient homozygous for the ⁴⁴⁴Leu $\rightarrow$ Pro allele was inactivated in a manner similar to control enzyme, but the inactivation of enzyme purified from cells of the patient homozygous for the ³⁷⁰Asn $\rightarrow$ Ser allele was very slow. The inactivation of enzyme purified from cells of the genetic compound proceeded with intermediate kinetics. When the enzyme preparations were incubated with inhibitor in the presence of phosphatidylserine and activator protein at pH 5.0, and the enzyme activity was determined under the same condition a different picture was obtained (see Fig. 1 B). Under these conditions, the behavior of glucocerebrosidase purified from fibroblasts of all the Gaucher disease patients was not clearly different from that of control enzyme. This suggests that in the presence of phospholipid and activator protein the ³⁷⁰Asn $\rightarrow$ Ser glucocerebrosidase is not only 'normalized' in activity towards substrate, but also in reactivity with CBE.

Analogous findings to the results presented in Table I and Fig. 1 were made for glucocerebrosidase purified from urine samples, spleens, and lymphoblasts from type 1 Gaucher disease patients (not shown). No significant differences were ob-

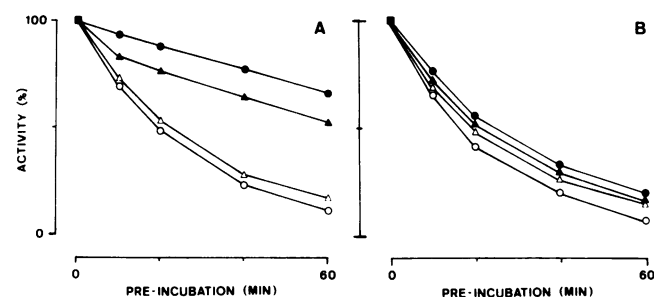


Figure 1. Inactivation of purified glucocerebrosidase by conduritol B-epoxide. Glucocerebrosidase was purified from fibroblasts of individuals with known glucocerebrosidase genotype by immunoaffinity chromatography as described previously (30). The purified enzyme was preincubated at room temperature for the times indicated with 25 μ M CBE in 100:200 mM citric acid/sodium phosphate buffer, pH 5.0, containing 0.1% (mass/vol) BSA and 10% (by volume) ethylene glycol. The preincubation mixture furthermore contained either 0.1% (by volume) Triton X-100 and 0.5% (mass/vol) sodium taurocholate (A) or 10 μ g/ml phosphatidylserine and 2.4 μ g/ml saposin C preparation (B). Subsequently, the enzyme preparation was diluted 10-fold in preincubation mixture from which CBE was omitted and to which 5 mM 4-MU- β -glucoside was added. The activity is expressed as a percentage of that found without preincubation. The results of a typical experiment are shown. Similar results were obtained in three to five other experiments and with different batches of CBE. The values in parentheses indicate the specific activity of immunopurified glucocerebrosidase (nanomoles per hour per milligram of protein) in the presence of taurocholate/Triton X-100. $\circ$, Control (2.8); $\bullet$, ³⁷⁰Asn $\rightarrow$ Ser/³⁷⁰Asn $\rightarrow$ Ser (0.21); $\blacktriangle$, ³⁷⁰Asn $\rightarrow$ Ser/⁴⁴⁴Leu $\rightarrow$ Pro (0.31); $\triangle$, ⁴⁴⁴Leu $\rightarrow$ Pro/⁴⁴⁴Leu $\rightarrow$ Pro (2.0).

served between enzyme preparations from the various sources examined.

It is of importance to note that the reactivity of glucocerebrosidase with CBE is to some extent a good reflection of the activity of the enzyme towards substrate. This was also observed when membrane suspensions were tested instead of pure enzyme preparations.

Enzymic properties of glucocerebrosidase in intact fibroblasts and lymphoblasts

Activity towards substrate. The glucocerebrosidase activity in cultured fibroblasts and lymphoblasts from type 1 Gaucher disease patients and control subjects was determined by loading cells with C₆-NBD glucosylceramide as described in Methods. Recently we established the existence of a nonlysosomal glucocerebrosidase activity in cells that is insensitive to CBE. The properties of the nonlysosomal glucocerebrosidase, which is not deficient in Gaucher disease materials, are to be described in a separate publication. To allow discrimination between the lysosomal (CBE-sensitive) and nonlysosomal (CBE-insensitive) glucocerebrosidase cells were pretreated with and without an excess of CBE. The lysosomal glucocerebrosidase activity was determined as the CBE-inhibitable hydrolysis of C₆-NBD glucosylceramide (Table II). The residual activity in fibroblasts of patients with the ³⁷⁰Asn → Ser allele was always less than that in lymphoblasts from the same patients.

Inactivation by CBE. The kinetics of the irreversible inhibition of glucocerebrosidase upon incubation of intact cells with CBE was examined (Fig. 2). Glucocerebrosidase in control fibroblasts and in ⁴⁴⁴Leu → Pro/⁴⁴⁴Leu → Pro fibroblasts was clearly inactivated more rapidly than the enzyme in ³⁷⁰Asn → Ser/³⁷⁰Asn → Ser fibroblasts when enzyme activity was subsequently measured either in the presence of taurocholate and Triton X-100 (Fig. 2 A) or in the presence of phosphatidylserine and activator protein at acid pH (Fig. 2 B). In ³⁷⁰Asn → Ser/⁴⁴⁴Leu → Pro fibroblasts, the inactivation of glucocerebrosidase appeared to be more rapid when the activity was subsequently measured in the presence of taurocholate and Triton

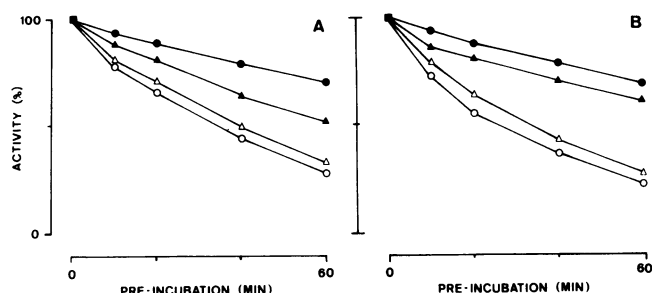


Figure 2. Inactivation of glucocerebrosidase in intact fibroblasts by conduritol B-epoxide. Cells were preincubated in culture medium for different periods of time with 100 μ M CBE. After harvesting and washing with PBS, the fibroblasts were homogenized in 50 mM potassium phosphate, pH 6.5, containing 0.25% (by volume) Triton X-100. β -Glucosidase activity was determined with 5 mM 4-MU- β -glucoside as substrate either in 100:200 mM citric acid/sodium phosphate, pH 5.2, 0.1% (by volume) Triton X-100 and 0.2% (mass/vol) sodium taurocholate (A) or in 100:200 mM citric acid/sodium phosphate, pH 5.0, 10 μ g/ml phosphatidylserine, and 2.4 μ g/ml saposin C preparation (B). The results of a typical experiment are indicated. Similar data were obtained in at least two independent experiments. $\circ$, Control; $\bullet$, ³⁷⁰Asn → Ser/³⁷⁰Asn → Ser; $\blacktriangle$, ³⁷⁰Asn → Ser/⁴⁴⁴Leu → Pro; $\triangle$, ⁴⁴⁴Leu → Pro/⁴⁴⁴Leu → Pro.

X-100 (Fig. 2 A) than when it was measured with phosphatidylserine and activator protein (Fig. 2 B). This apparent discrepancy results from the fact that with activator protein and phosphatidylserine, one measures predominantly ³⁷⁰Asn → Ser enzyme, which is slowly inactivated, whereas in the presence of taurocholate and Triton X-100, ⁴⁴⁴Leu → Pro enzyme contributes significantly to the total activity.

Comparison of different cell types. Cultured fibroblasts and lymphoblasts were incubated with CBE for different periods of time, and subsequently, enzymic activity of glucocerebrosidase was measured in the presence of taurocholate and Triton X-100.

Table III shows again that lysosomal glucocerebrosidase in intact fibroblasts from patients with the ³⁷⁰Asn → Ser allele was clearly less sensitive to inhibition by CBE compared to enzyme in corresponding cells of a control subject and a patient homozygous for the ⁴⁴⁴Leu → Pro glucocerebrosidase allele. However, glucocerebrosidase in cultured lymphoblasts from a control subject, a patient homozygous for the codon 370 substitution, and a patient with both the codon 370 and codon 444 substitutions was comparably inactivated by CBE. This cell-type-specific difference between fibroblasts and lymphoblasts was also observed in three type 1 Gaucher disease patients with one allele with the codon 370 mutation and an as yet unidentified mutated allele (not shown).

Immunocytochemical analysis of glucocerebrosidase and activator protein saposin C in cultured cells

The apparent difference in glucocerebrosidase activity in intact lymphoblasts and fibroblasts could, in principle, be caused by a relatively higher concentration of activator protein in lysosomes of lymphoblasts. To test this possibility, the relative intralysosomal concentrations of glucocerebrosidase and activator protein in fibroblasts and lymphoblasts were studied by immunoelectron microscopy using specific antisera. Fig. 3 shows some examples of double-labeling of glucocerebrosidase and saposin in lysosomes in fibroblasts and lymphoblasts from

Table II. Glucocerebrosidase Activity In Situ towards C₆-NBD Glucosylceramide in Fibroblasts and Lymphoblasts from a Control Subject and a Type 1 Gaucher Disease Patient

Cells from	Percent conversion of C ₆ -NBD glucosylceramide to ceramide + sphingomyelin in					
	Fibroblasts			Lymphoblasts		
	-CBE	+CBE	CBE-Inhibitable	-CBE	+CBE	CBE-Inhibitable
Control subject	60	21	39	26	13	13
Gaucher patient	35	29	6	21	12	9

Cells grown in RPMI 1640 culture medium with 5% CO₂ in the gas phase were preincubated with or without 100 μ M CBE for 24 h at 37°C. Next, C₆-NBD glucosylceramide/BSA (5 μ M:5 μ M) was added. After 24 h at 37°C, the cells were extensively washed and the NBD-lipids were extracted and analysed using thin layer chromatography as described in Methods. Glucocerebrosidase activity is expressed as the percent conversion of C₆-NBD glucosylceramide to C₆-NBD ceramide and C₆-NBD sphingomyelin. Fluorescence in other lipids was always <4%. Results of one experiment are shown; similar results were obtained in two independent experiments.

Table III. Inactivation of Glucocerebrosidase in Intact Cells by Conduritol B-Epoxyde

Cell type	Activity nmol/h per mg protein in nonpreincubated cells		CBE-inhibitable activity (percent of that in nonpreincubated cells) after preincubation with CBE for			
	-CBE	+CBE	10	20	40	60
			min			
Fibroblasts						
Control	384.2	1.0	80	66	45	30
370/370	37.8	0.7	95	87	79	68
370/444	20.7	0.7	90	81	64	52
444/444	17.2	0.4	82	69	48	32
Lymphoblasts						
Control	17.1	0.4	75	55	29	16
370/370	1.5	0.3	80	63	32	21
370/444	1.2	0.3	78	59	33	17

Cells grown in RPMI 1640 culture medium containing 10% (by volume) FCS with 5% CO₂ in the gas phase were incubated at 37°C with 100 µM CBE for different periods of time, washed extensively at 4°C with PBS to remove excess inhibitor and harvested. Next, the cells were homogenized in 50 mM potassium phosphate, pH 6.5, containing 0.25% (by volume) Triton X-100. β-Glucosidase activity was measured with 5 mM 4-MU-β-glucoside as substrate in 100:200 mM citric acid/sodium phosphate (pH 5.2) containing 0.1% (by volume) Triton X-100 and 0.2% (mass/vol) sodium taurocholate. At each preincubation time point, enzyme activity was measured in the presence of 1 mM CBE in one sample and in the absence of CBE in a parallel sample. Enzyme activity was related to total cellular protein. The CBE-inhibitable enzymic activity at each time point is expressed as percentage of that in cells not preincubated with inhibitor. The results of a typical experiment are shown. Analogous results were obtained in at least three independent experiments. 370, ³⁷⁰Asn → Ser allele; 444, ⁴⁴⁴Leu → Pro allele.

a control subject and a patient with the genotype ³⁷⁰Asn → Ser/⁴⁴⁴Leu → Pro. Quantification of the differently sized gold particles in lysosomes shows that the ratio of labeled activator protein to labeled glucocerebrosidase is not significantly different in fibroblasts and lymphoblasts of the control subject and the Gaucher disease patient (Table IV). The amount of activator protein was also quantified by means of a functional test in which the ability of denatured protein preparations from different cell types to activate purified glucocerebrosidase was measured. As shown in Table V, the amount of activator protein was greater in fibroblasts than in lymphoblasts, but there was no difference between control and Gaucher fibroblasts, or between control and Gaucher lymphoblasts. Thus, the apparent difference in properties of glucocerebrosidase in lymphoblasts and fibroblasts from patients with ³⁷⁰Asn → Ser glucocerebrosidase cannot simply be attributed to a higher amount of intralysosomal activator protein in the lysosomes of the former cells.

Effect of pH on activity of ³⁷⁰Asn → Ser glucocerebrosidase
Pure enzyme. The influence of pH on enzymic activity of purified splenic ³⁷⁰Asn → Ser glucocerebrosidase and control enzyme was studied using acetate and citric acid/sodium phosphate buffers in the pH range 4.0–6.0. Fig. 4 A shows that the activity of ³⁷⁰Asn → Ser glucocerebrosidase in the presence of

activator protein and phospholipid has a very sharp pH optimum. At pH values > 5.4 in acetate buffer and > 5.2 in citric acid/sodium phosphate buffer, the mutated enzyme is markedly reduced in specific activity. It was noted that in the presence of higher amounts of activator protein, the specific activity of ³⁷⁰Asn → Ser glucocerebrosidase was also close to normal at pH values between 4.3 and 5.0, but not at pH values > 5.0 (not shown; see also [17]). It appears that the ³⁷⁰Asn → Ser substitution in glucocerebrosidase leads to an increased pH dependency of the enzyme with respect to activation by activator protein. Particularly at higher pH values, the mutated enzyme is poorly activated by saposin. Fig. 4 B shows that also the reactivity of ³⁷⁰Asn → Ser glucocerebrosidase with CBE is extraordinarily pH dependent in the presence of activator protein and phospholipid. At higher pH values, the mutated enzyme is dramatically less sensitive for CBE-induced inactivation compared to control enzyme.

Similar results to those presented in Fig. 4 were obtained with enzyme isolated from fibroblasts and lymphoblasts from patients homozygous for the ³⁷⁰Asn → Ser allele. Furthermore, ³⁷⁰Asn → Ser glucocerebrosidase isolated from 3T3 fibroblasts transfected with site-directed mutagenized human glucocerebrosidase cDNA (15) showed a comparable pH dependency (not shown).

Manipulation of intralysosomal pH with weak bases. Intact fibroblasts and lymphoblasts were exposed to NH₄Cl, a weak base, to increase the intralysosomal pH, as described by Ohkuma and Poole (34). These authors have shown that in mouse macrophages addition of increasing concentrations of NH₄Cl leads to a concomitant increase in pH of intracellular acidic compartments (including lysosomes), as monitored by the extent of quenching of the fluorescence of endocytosed dextran-fluorescein isothiocyanate. For instance, on addition of 10 mM NH₄Cl, the pH of such compartments increases from ~ 4.8 to ~ 6.2. Fig. 5 shows that with increasing amounts of NH₄Cl, glucocerebrosidase in cells becomes less sensitive to inactivation by CBE, as would be expected from the results of Fig. 4 B. This effect of NH₄Cl is reversible (not shown). It should be noted that in the absence of NH₄Cl glucocerebrosidase is much more effectively inactivated by CBE in Gaucher disease lymphoblasts than in Gaucher disease fibroblasts. However, at increasing concentrations of NH₄Cl, the rate of inactivation of glucocerebrosidase in lymphoblasts resembles that in fibroblasts from the same Gaucher disease patient. Similar observations were made using methylamine (not shown).

The findings presented in Figs. 4 and 5 suggest that the intralysosomal pH to which glucocerebrosidase is exposed might on the average be lower in cultured lymphoblasts than in fibroblasts. As an approximate measure of the pH in lysosomes (and other acidic compartments), we measured the accumulation of radioactively labeled weak base chloroquine (35). We observed that about seven times more radioactively labeled chloroquine was trapped by lymphoblasts than by fibroblasts when related to the amount of glucocerebrosidase present in the two cell types, whereas the immunogold labeling studies suggest that the concentration of glucocerebrosidase is about twice as high in lysosomes of fibroblasts than of lymphoblasts (see Table IV). This suggests that the average intralysosomal pH is higher in fibroblasts than in lymphoblasts.

Assessment of activation state of glucocerebrosidase. When control cells are homogenized in detergent-free buffer, a pro-

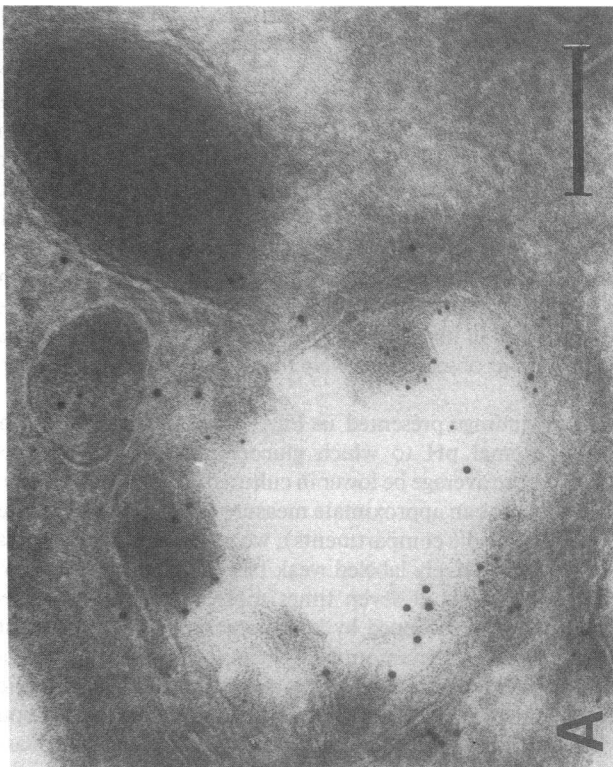
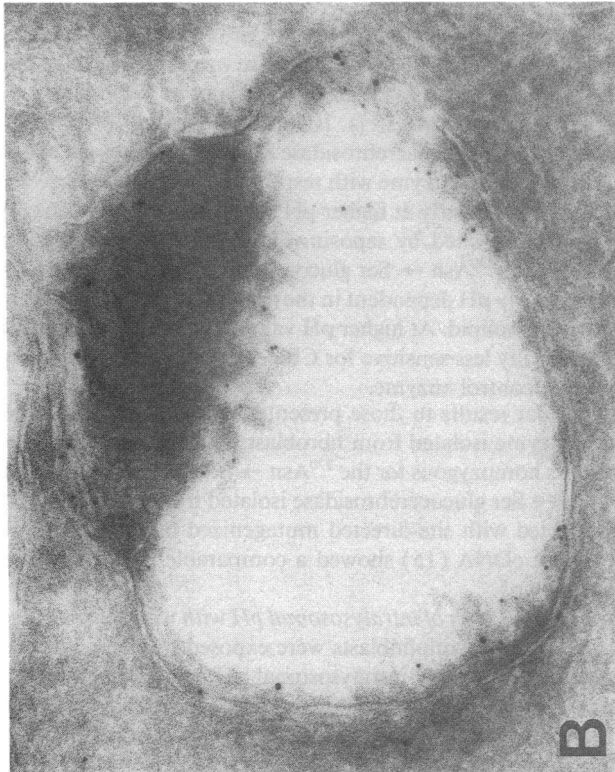


Table IV. Quantification of Gold Labeling of Glucocerebrosidase and Saposin C in Lysosomal Structures in Fibroblasts and Lymphoblasts from a Control Subject and a Type 1 Gaucher Patient

Cell line	Antigen	Number of gold particles/lysosome in fibroblasts			Number of gold particles/lysosome in lymphoblasts		
		Mean±SEM	n	Range	Mean±SEM	n	Range
Control	β-Glu	8.1±1.2	44	0–34	3.9±0.5	83	0–31
	Saposin C	11.6±2.1	43	0–51	5.4±0.7	84	0–30
	Saposin C/β-Glu	1.61±0.28	38	0–9	2.00±0.37	69	0–18
Gaucher	β-Glu	6.0±0.9	20	0–15	2.5±0.7	33	0–16
	Saposin C	9.1±1.4	20	1–22	5.8±1.0	33	0–29
	Saposin C/β-Glu	2.04±0.43	19	0–7	2.98±1.10	25	0–29

For experimental details, see the legend to Fig. 3. Lysosomal structures present in electron micrographs of a typical immunolabeling experiment were quantified. The number of lysosomal structures analysed is indicated in the table. β-Glu, glucocerebrosidase.

portion of glucocerebrosidase remains in activated form, as reflected by a high ratio of enzymic activity measured in the absence of additional activators compared to that measured in the presence of taurocholate (see [23]). The bile salt abolishes any original activation of glucocerebrosidase and activates fully all enzymes present (23). Table VI shows that the activity in the absence of activator is relatively higher in a homogenate of lymphoblasts than that in a homogenate of fibroblasts. When the cells are pretreated with NH₄Cl to increase the pH of intracellular acidic compartments (see above), this difference disappears and glucocerebrosidase is only poorly active in the absence of additional activator in homogenates from both cell types. This finding supports the assumption that on the average, glucocerebrosidase is present in a more activated state in intact lymphoblasts than in fibroblasts because of an intralysosomal pH difference.

Discussion

The properties of the ³⁷⁰Asn → Ser glucocerebrosidase in vitro are intriguing: Under most assay conditions, this mutated enzyme is impaired in activity towards substrate. However, in the presence of the natural activator protein saposin C and phosphatidylserine, the enzyme functions in a near normal fashion at an appropriate acidic pH. To obtain information on the properties of this mutated enzyme in situ, the irreversible inhibitor CBE has been used, an approach that was developed and used previously by other investigators (25, 36, 37). Although it is obvious that the reaction of glucocerebrosidase with CBE involves only part of the total reaction mechanism of substrate hydrolysis (discussed in more detail in [2]), analysis of CBE-induced inactivation of glucocerebrosidase is informative because the reactivity of (mutant) glucocerebrosidase with CBE appears to reflect to a large extent the ability of enzyme to be active towards substrate.

Some of our findings are consistent with those of earlier studies. First, it has been reported by Grabowski and co-workers (36, 37) that the mutant form of glucocerebrosidase in Ashkenazi Jewish type 1 Gaucher disease patients referred to as group B mutant enzyme is less efficiently inactivated by CBE than control enzyme either in fibroblast extracts or in partially purified splenic preparations. It is now almost certain that group B mutant enzyme is identical to the ³⁷⁰Asn → Ser glucocerebrosidase (2). Second, it has been reported that glucocerebrosidase in intact fibroblasts from Ashkenazi Jewish Gaucher disease type 1 patients was less efficiently inactivated by CBE compared to enzyme in cells from control subjects and from other Gaucher disease patients (36). Thus, in the studies cited above, particular stress was laid upon the ethnic background of type 1 Gaucher disease patients. We have found that the rate of inactivation of glucocerebrosidase by CBE in fibroblasts from

Table V. Level of Activator Protein (Saposin C) in Fibroblasts and Lymphoblasts from a Control Subject and a Type 1 Gaucher Patient

Cell type	Cell line	Activator U/mg protein
Fibroblasts	Control	0.58
	Type 1 Gaucher	0.53
Lymphoblasts	Control	0.21
	Type 1 Gaucher	0.26

Cell extracts were boiled for 5 min, centrifuged, and the supernatants were tested for their capacity to stimulate the enzymic activity of purified placental glucocerebrosidase as described (12). 1 U of activation is defined as the amount of activator protein that stimulates the activity of glucocerebrosidase by a factor of 100.

Figure 3. Immunocytochemical visualisation of glucocerebrosidase and saposin C. Fibroblasts and lymphoblasts of a control subject and a Gaucher disease type 1 patient heterozygous for the ³⁷⁰Asn → Ser and ⁴⁴⁴Leu → Pro glucocerebrosidase substitutions were washed in PBS, fixed, cryosectioned and processed for immunogold double labeling as described (33). The immunoglobulins were visualized with protein A conjugated with 10-nm gold particles (antiglucocerebrosidase) and with protein A conjugated with 5-nm gold particles (anti-saposin C). Identical results were obtained when glucocerebrosidase was visualized with a 5-nm gold probe and the activator protein was visualized with a 10-nm gold probe. (A) Fibroblasts of the control subject; (B) fibroblasts of the Gaucher patient; (C) lymphoblasts of the control subject; and (D) lymphoblasts of the Gaucher patient. Bar, 0.2 μm.

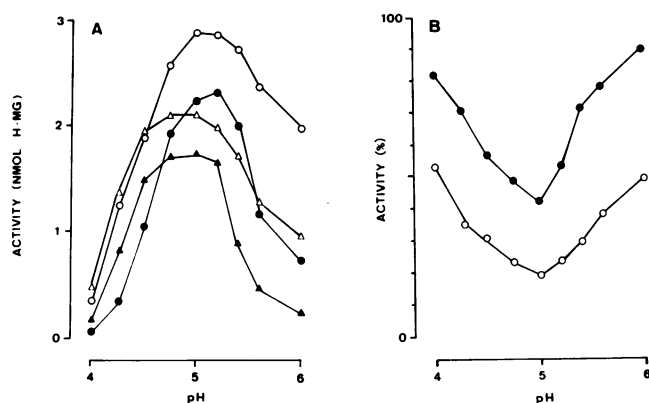


Figure 4. Influence of pH on the properties of $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase, purified from spleen. The effect of pH on the properties of immunoaffinity purified control and $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase in the presence of $10 \mu\text{g/ml}$ phosphatidylserine and $2.4 \mu\text{g/ml}$ saposin C was determined. (A) The activity of glucocerebrosidase was determined with 5 mM 4-MU- β -glucoside as substrate in the presence of 50 mM acetate buffer ($\circ$, $\bullet$) or $50:100 \text{ mM}$ citric acid/sodium phosphate buffer (Δ , $\blacktriangle$) varying in pH from 4.0 to 6.0. $\circ$ and Δ , control; $\bullet$ and $\blacktriangle$, $^{370}\text{Asn} \rightarrow \text{Ser}/^{370}\text{Asn} \rightarrow \text{Ser}$. (B) The inactivation of glucocerebrosidase by CBE was determined in $50:100 \text{ mM}$ citric acid/sodium phosphate buffer (pH range 4.0–6.0). Enzyme activity was determined as described in the legend of Fig. 1. $\circ$, Control; $\bullet$, $^{370}\text{Asn} \rightarrow \text{Ser}/^{370}\text{Asn} \rightarrow \text{Ser}$.

$^{370}\text{Asn} \rightarrow \text{Ser}/^{370}\text{Asn} \rightarrow \text{Ser}$ patients and, to a lesser extent, in fibroblasts from the more frequently encountered patients heterozygous for this mutation, is slow. However, we were not able to distinguish type 1 Gaucher disease patients with Ashkenazi Jewish background from patients with non-Jewish background on the basis of CBE-induced inactivation.

During this study, the important observation was made that the activation state of glucocerebrosidase in the lysosomes as measured *in situ* differs in fibroblasts and lymphoblasts. This finding substantiates earlier studies carried out with homogenates from the two cell types (23). The cell-type-specific difference is particularly evident in the case of $^{370}\text{Asn} \rightarrow \text{Ser}$

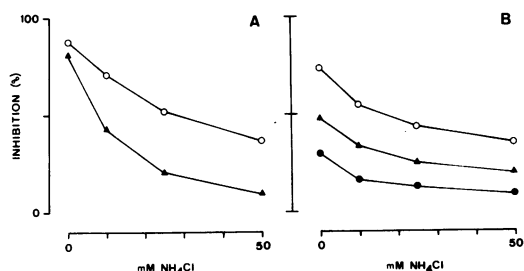


Figure 5. Influence of ammonia on inactivation of glucocerebrosidase in intact cells by conduritol B-epoxide. Cells were preincubated for 30 min at 37°C in culture medium containing different concentrations of NH_4Cl . Next, $100 \mu\text{M}$ CBE was added to the medium and the cells were harvested immediately or 1 h later. Glucocerebrosidase activity per milligram total cellular protein was determined as described in the legend to Table III. The results of a typical experiment are shown. Similar results were obtained in at least two independent experiments. (A) Lymphoblasts; (B) fibroblasts. $\circ$, Control; $\bullet$, $^{370}\text{Asn} \rightarrow \text{Ser}/^{370}\text{Asn} \rightarrow \text{Ser}$; Δ , $^{370}\text{Asn} \rightarrow \text{Ser}/^{444}\text{Leu} \rightarrow \text{Pro}$.

Table VI. Activation State of Glucocerebrosidase in Homogenates of Control Cells

Cell type	Cells preincubated for 30 min with	Glucocerebrosidase activity in homogenate with		
		No additions (A)	Taurocholate/Triton X-100 (B)	A/B
		nmol/h		
Fibroblasts	No additions	79.6	153.2	0.52
	25 mM NH ₄ Cl	57.2	159.1	0.36
Lymphoblasts	No additions	10.0	14.1	0.71
	25 mM NH ₄ Cl	5.3	13.9	0.38

Cells were harvested, washed in PBS and homogenized in $10:20 \text{ mM}$ citric acid/sodium phosphate buffer, pH 4.0, by brief sonication. Enzyme activity was measured in two reaction mixtures. The reaction mixture without an added activator contained $100:200 \text{ mM}$ citric acid/sodium phosphate, pH 4.0, and 5 mM 4-MU- β -glucoside. The other reaction mixture contained $100:200 \text{ mM}$ citric acid/sodium phosphate, pH 5.2, 0.1% (by volume) Triton X-100 and 0.2% (mass/vol) sodium taurocholate as activators, and 5 mM 4-MU- β -glucoside. Assays were performed in the absence and presence of 1 mM CBE to determine the contribution of glucocerebrosidase to the total β -glucosidase activity. The results of a typical experiment are given. Similar results were obtained in two other independent experiments.

glucocerebrosidase, the catalytic properties of which are so strongly determined by the state of activation of the enzyme.

Our studies suggest that the intralysosomal pH determines the state of activation of glucocerebrosidase. Again, this is particularly relevant for $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase: the activation of this mutant enzyme by activator protein is extraordinarily pH sensitive. Thus, a cell-type-specific difference in intralysosomal pH could explain the difference in properties of $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase in intact fibroblasts and lymphoblasts. Moreover, variability between individuals in intralysosomal conditions may underlie the heterogeneity in severity of the clinical expression of Gaucher disease in individuals with the same glucocerebrosidase genotype. In this connection, it is of interest to mention that some homozygotes for the $^{370}\text{Asn} \rightarrow \text{Ser}$ glucocerebrosidase mutation are not or only very mildly affected clinically (8, 38), and that a strict correlation between glucocerebrosidase genotype and Gaucher disease clinical phenotype has not always been noted; e.g., see reference 39. In Gaucher disease, glucocerebroside accumulation is restricted to macrophage-like cells. It will, therefore, be of interest to establish the intralysosomal pH and the state of activation of glucocerebrosidase in macrophages from patients with different degrees of severity of the clinical manifestation. Such studies are underway.

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