The N370S (Asn³⁷⁰ → Ser) mutation affects the capacity of glucosylceramidase to interact with anionic phospholipid-containing membranes and saposin C

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The properties of the endolysosomal enzyme GCase (glucosylceramidase), carrying the most prevalent mutation observed in Gaucher patients, namely substitution of an asparagine residue with a serine at amino acid position 370 [N370S (Asn³⁷⁰ → Ser) GCase], were investigated in the present study. We previously demonstrated that Sap (saposin) C, the physiological GCase activator, promotes the association of GCase with anionic phospholipid-containing membranes, reconstituting in this way the enzyme activity. In the present study, we show that, in the presence of Sap C and membranes containing high levels of anionic phospholipids, both normal and N370S GCases are able to associate with the lipid surface and to express their activity. Conversely, when the amount of anionic phospholipids in the membrane is reduced (~20% of total lipids), Sap C is still able to promote binding and activation of the normal enzyme, but not of N370S GCase. The altered interaction of the mutated enzyme with anionic phospholipid-containing membranes and Sap C

was further demonstrated in Gaucher fibroblasts by confocal microscopy, which revealed poor co-localization of N370S GCase with Sap C and lysobisphosphatidic acid, the most abundant anionic phospholipid in endolysosomes. Moreover, we found that N370S Gaucher fibroblasts accumulate endolysosomal free cholesterol, a lipid that might further interfere with the interaction of the enzyme with Sap C and lysobisphosphatidic acid-containing membranes. In summary, our results show that the N370S mutation primarily affects the interaction of GCase with its physiological activators, namely Sap C and anionic phospholipid-containing membranes. We thus propose that the poor contact between N370S GCase and its activators may be responsible for the low activity of the mutant enzyme *in vivo*.

Key words: anionic phospholipid, Gaucher fibroblast, glucosylceramidase, large unilamellar vesicles, lysobisphosphatidic acid (LBPA), saposin C.

INTRODUCTION

Gaucher disease is a metabolic storage disorder characterized by the accumulation of GC (glucosylceramide) as a result of deficient GCase (glucosylceramidase) activity [1]. GCase is a 497 amino acid membrane-associated endolysosomal glycoprotein that requires anionic phospholipids and Sap (saposin) C to express its activity [2–6]. Sap C is a member of a family of four similar glycoproteins, Sap A, B, C and D, generated from a common precursor, prosaposin, in late endosomes/lysosomes [6]. The physiological significance of Sap C as GCase activator has been demonstrated by the description of cases of Gaucher disease caused by Sap C deficiency, where normal levels of GCase were unable to degrade GC [7,8].

In our past work, we have shown that Sap C reconstitutes enzyme activity by promoting the GCase association with lipid surfaces containing appropriate levels of anionic phospholipids [9,10]. At low pH values mimicking the acidic endolysosomal environment, Sap C strongly binds to anionic phospholipid-containing membranes [11]. The pH-dependent interaction of Sap C with phospholipid vesicles has been recently confirmed by NMR studies [12]. The insertion of Sap C dramatically perturbs the membrane, favouring the interaction of GCase with anionic phospholipids and stimulating the catalytic activity of the en-

zyme towards natural (GC) and artificial [MUGlc (4-methylumbelliferyl-β-D-glucopyranoside)] substrates [13].

Gaucher disease is highly heterogeneous and has been classified into three groups according to the clinical manifestations: type 1, the non-neuronopathic form; type 2, the acute neuronopathic form; and type 3, the subacute neuronopathic form [14,15]. A large number of GCase mutations that cause Gaucher disease have been identified [14,15]. It is to be expected that each enzyme mutation distinctly affects the mechanism by which GC is degraded by GCase with the assistance of Sap C. Two mutant alleles, N370S (Asn³⁷⁰ → Ser) and L444P account for the majority of the reported cases of Gaucher disease. The most prevalent GCase mutation observed in type 1 Gaucher disease is the N370S. Patients homozygous for this mutation can show moderate to severe phenotype. Screening studies on the prevalence of the N370S allele among Ashkenazi Jews suggest that only some of the N370S homozygotes are ascertained by clinical involvement, most of them having a normal or close to normal phenotype [16]. This observation raises the possibility that the *in situ* activity of N370S GCase might be modulated by specific factors and that the mutated enzyme, under favourable conditions, might function almost normally. The N370S GCase residual activity measured *in vitro* is highly variable; its value can range from 10 to 80% of the control, depending on the assay conditions and the

Abbreviations used: Chol, cholesterol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GC, glucosylceramide; GCase, glucosylceramidase; LBPA, lysobisphosphatidic acid; LUV, large unilamellar vesicle; MUGlc, 4-methylumbelliferyl-β-D-glucopyranoside; NPC, Niemann–Pick disease type C; PC, phosphatidylcholine; PS, phosphatidylserine; Sap, saposin.

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presence of different activators [17,18]. A decreased sensitivity of N370S GCCase to inhibitors such as conduritol B epoxide, a compound which forms a covalent bond with aspartate residues of the enzyme, has also been reported [19,21].

Despite the many comparative studies performed to explain the differences in the response of control and N370S GCCase to activators and inhibitors [17–21], the consequences of this mutation for the mechanism of action of the enzyme are not fully understood. In order to identify factors that might affect the N370S GCCase activity in Gaucher cells, we analysed the properties of the mutated enzyme in more detail. Considering that impaired contact between the enzyme and its physiological activators might result in reduction of catalytic function, the capacity of N370S GCCase to interact with anionic phospholipids and Sap C was investigated. Since we have recently shown that the endolysosomal accumulation of Chol (cholesterol) in fibroblasts derived from patients with NPC (Niemann–Pick disease type C) interferes with the GCCase association with anionic phospholipids and Sap C-containing membranes [22], the intracellular distribution of Chol in N370S Gaucher fibroblasts was also assessed.

EXPERIMENTAL

Materials

DMEM (Dulbecco's modified Eagle's medium) was obtained from Euroclone (Wetherby, West Yorks., U.K.). Complete™ (protease inhibitor mixture) was obtained from Roche Diagnostics S.p.A., Applied Science (Monza, Italy). Methionine/cysteine-deficient DMEM was obtained from ICN Biomedicals (Irvine, CA, U.S.A.). PRO-MIX L-[³⁵S] (70 % L-[³⁵S]methionine and 30 % L-[³⁵S]cysteine; specific radioactivity, > 1000 Ci/mM), 1,2-di-oleoyl-L-3-phosphatidyl-L-[3-¹⁴C]serine (56 mCi/mmol), CNBr-activated Sepharose 4B beads and ECL® Western blotting reagents were from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Protein A–Sepharose CL-4B, filipin and Chol were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.). LysoTracker Red DND-99, ProLong anti-fade kit and Alexa Fluors were obtained from Molecular Probes (Eugene, OR, U.S.A.). SDS/PAGE reagents were from Bio-Rad (Hercules, CA, U.S.A.). PC (phosphatidylcholine) from egg yolk, PS (phosphatidylserine) from bovine brain and LBPA (lysobisphosphatidic acid) were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). L- α -Dipalmitoyl [2-palmitoyl-9,10-³H(N)] PC (82 Ci/mmol) and Kodak X-Omat Blue films were from PerkinElmer Life Sciences (Boston, MA, U.S.A.).

Cell cultures and GCCase extraction

Four human Gaucher fibroblast lines homozygous for the N370S mutation were utilized. Three of them, 8817 (370a), 8915 (370b) and 8945 (370c), were obtained from the National Institutes of Health. One cell line, 3699 (370d), was a gift from Dr M. Filocamo (Institute 'Giannina Gaslini', Genova, Italy). Control fibroblast cultures were established from skin explants of normal individuals. Normal and Gaucher fibroblasts were cultured in DMEM supplemented with 10 % (v/v) FBS (fetal bovine serum), 2 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Fibroblasts were harvested after reaching confluency. Cells were suspended in 50 mM phosphate buffer (pH 6.5), containing 0.5 % (v/v) Triton X-100 and one tablet of protease inhibitor mixture/50 ml (lysis buffer), and sonicated for 20 s. The homogenates were centrifuged for 30 min at 20 000 g and the supernatant used as crude fibroblast homogenate.

Antibodies

Mouse monoclonal anti-GCCase antibody (8E4) was kindly provided by Dr H. Aerts (E. C. Slater Institute for Biochemical Research, University of Amsterdam, the Netherlands). Rabbit polyclonal anti-GCCase antibody was kindly provided by Dr G. Murray of National Institutes of Health. Rabbit polyclonal anti-human Sap C antibody was prepared in our laboratory [11]. Mouse monoclonal anti-LBPA antibody (6C4) was a gift from J. Gruenberg (Department of Biochemistry, University of Geneva, Switzerland). Anti-actin monoclonal antibody was obtained from Oncogene Research Products (Boston, MA, U.S.A.).

GCCase purification

GCCase was purified from fibroblast homogenates by immunoaffinity chromatography following a previously described procedure [19,23]. Briefly, the homogenates were incubated overnight with CNBr-activated Sepharose 4B coupled with the anti-GCCase monoclonal antibody 8E4. The affinity resin was prepared according to the manufacturer's instructions. After binding of the enzyme, the resin was successively washed with 0.1 M citrate/0.5 M NaCl buffer (pH 6.0), with 30 % ethylene glycol in the same buffer, with 1 % Triton X-100 in the same buffer and with 50 % ethylene glycol in the same buffer. The enzyme was eluted with 90 % ethylene glycol in the same buffer. The final degree of purification was > 1000-fold as judged by SDS/PAGE.

Vesicle preparation

LUVs (large unilamellar vesicles) were prepared as previously reported [11]. Briefly, appropriate amounts of lipids dissolved in chloroform were mixed and the solvent evaporated under nitrogen. The dry lipids were dispersed by vortex-mixing in 2 mM L-histidine/2 mM Tes/150 mM NaCl/1 mM EDTA (pH 7.4), followed by ten cycles of freeze–thawing and finally passed 21 times through two stacked 0.1 µm-diameter-pore polycarbonate membranes in a Liposofast-Miniextruder (Avestin, Ottawa, ON, Canada). All vesicles were supplemented with trace amounts of labelled PC and/or PS and their concentration determined by radioactivity measurements.

GCCase assays

GCCase activity was measured utilizing either detergents or anionic phospholipid-containing LUVs and Sap C as enzyme activators [11]. In the first case, the assay mixture in the final volume (0.2 ml) contained citrate/phosphate buffer (50 mM in citrate, pH 5.6), 2.5 mM MUGlc, 0.1 % Triton X-100 and 0.25 % (w/v) sodium taurocholate. In the second case, the assay mixture in the final volume (0.2 ml) contained buffer A at pH 5.0 (10 mM acetate buffer, 150 mM NaCl and 1 mM EDTA), 2.5 mM MUGlc, various amounts of Sap C and 100 µg of LUVs.

The assay mixtures were incubated for 30 min at 37 °C. The extent of reaction was estimated fluorimetrically. One unit of GCCase is defined as the amount of enzyme that hydrolyses 1 nmol of MUGlc/h in the assay mixtures containing detergents.

Western blotting

SDS/PAGE was performed with 7.5 or 15 % (w/v) acrylamide gels [24]. After electrophoresis, the proteins were electroblotted on to PVDF membranes (Bio-Rad). GCCase was detected with the anti-GCCase monoclonal antibody 8E4 and Sap C with the anti-Sap C polyclonal antibody using an ECL® Western blotting kit, according to the manufacturer's instructions (Amersham Biosciences).

Metabolic labelling and immunoprecipitation of Sap C

Fibroblast cultures were grown until near confluency. Before being labelled, the cells were washed twice with ice-cold PBS supplemented with 1 mM MgCl₂ and 0.1 mM CaCl₂ and starved for 2 h in methionine- and cysteine-free medium containing 4 % dialysed FBS. This medium was replaced with the labelling medium (DMEM lacking methionine and cysteine and supplemented with PRO-MIX L-[³⁵S], 150 µCi/ml and 4 % dialysed FBS). After 1 h of incubation, the cells were washed three times with DMEM and non-radioactive chase medium was added (DMEM containing 4 % FBS). The cells were chased for the indicated periods and then harvested and disrupted in lysis buffer. The suspensions were subjected to a brief sonication and centrifuged at 20 000 *g* for 30 min. To remove DNA and histones, the supernatant solutions were incubated with 0.03 % protamine sulphate for 45 min at 4 °C and centrifuged as above. After the addition of 0.1 % BSA, the cell lysates were incubated with rabbit pre-immune serum overnight at 4 °C and non-specific complexes were precipitated with Protein A–Sepharose CL-4B. The clarified supernatants were then incubated with anti-Sap C antiserum. Cross-reacting material was precipitated with Protein A–Sepharose CL-4B. The immunocomplexes were washed four times with PBS containing 1 % BSA, 1 % Triton X-100, 1 % SDS, 0.4 % sodium deoxycholate and finally with PBS only. The washed precipitates were analysed by SDS/PAGE. Labelled proteins were detected by fluorography.

Binding of GCase to vesicles

For binding studies, similar amounts of purified control and N370S GCases, corresponding to 25 units of control and 2.5 units of N370S GCase respectively were incubated at 37 °C for 15 min with LUVs (100 µg of total lipids) and Sap C (10 µg) in 0.2 ml of buffer A, supplemented with 10 mM dithioerythritol and 20 µg of BSA. The mixtures were centrifuged with a 42.2 Ti rotor (Beckman, Fullerton, CA, U.S.A.), in polycarbonate centrifuge tubes (7 × 20 mm), at 120 000 *g* for 45 min. After separation of the supernatant solution, more than 90 % of the vesicles were found in the pellet, which was resuspended in 0.2 ml of buffer A containing 20 µg of BSA. The proteins of the supernatant and those arising from the pellet were precipitated by adding 10 vol. of cold acetone and keeping the samples on solid CO₂ for 1 h. After centrifugation at 41 000 *g* for 30 min at 4 °C, the acetone was removed by aspiration and evaporation under a stream of nitrogen. The proteins were dissolved in the electrophoresis loading buffer [50 mM Tris/HCl, pH 6.8, 100 mM dithiothreitol, 2 % (w/v) SDS and 10 % glycerol]. Free GCase in the supernatant and liposome-bound GCase in the pellet were identified by SDS/PAGE and Western blotting.

Fluorescence microscopy

Cells were grown on Labteck chamber slides (Nunc, Naperville, IL, U.S.A.) and fixed with 4 % (w/v) paraformaldehyde in PBS for 30 min. Cells were then rinsed with PBS, permeabilized for 7 min with 0.05 % saponin and incubated for 2 h with 3 % (w/v) BSA.

For intracellular free unesterified Chol staining, fixed cells were incubated for 30 min with filipin solution (0.05 % in PBS). The cells were observed with a UV 330–380 filter.

For labelling of acidic organelles, living cells were incubated for 30 min with 100 nM LysoTracker Red DND-99 in culture medium at 37 °C, before fixation.

For double immunostaining, the cells were incubated for 1 h with a specific rabbit polyclonal primary antibody (anti-Sap C or

polyclonal anti-GCase), rinsed twice with PBS and incubated for 1 h with the secondary anti-rabbit antibody conjugated with Alexa Fluor 594. The cells were then rinsed twice with PBS, incubated for 1 h with a specific mouse monoclonal primary antibody (anti-GCase 8E4 or anti-LBPA), rinsed twice with PBS and incubated with the secondary anti-mouse antibody conjugated with Alexa Fluor 488.

Finally, the cells were mounted with ProLong anti-fade reagent and the fluorescence was viewed by fluorescence microscopy or by confocal laser-scanning microscopy as previously described [22].

For cellular double staining of Chol and anti-LBPA, the cells were observed with an Olympus BX52 fluorescence microscope equipped with an excitation filter at 490 nm and an emission filter at 525 nm for Alexa Fluor 488 (green) and with a UV 330–380 filter for filipin (blue). The images were acquired with the IAS 2000 software and elaborated with Adobe Photoshop.

The other apparatus utilized was a Leica TCS SP2, equipped with an argon–krypton laser, double-dichroic splitters (488/568 nm), 520 nm barrier filter for Alexa Fluor-488 (green), and 590 nm barrier filter for Alexa Fluor-594 (red) observations. Image acquisition and processing were conducted by using the SCANware and Multicolor Analysis (Leica Lasertechnik, Heidelberg, Germany) and Adobe Photoshop software programs. Signals from different fluorescent probes were taken in parallel and co-localization was detected in yellow.

Primary antibodies were used at the following dilutions: anti-Sap C (1:300), monoclonal anti-GCase (1:300), polyclonal anti-GCase (1:100) and anti-LBPA (1:80).

RESULTS

N370S GCase activity and mass in Gaucher fibroblasts

In order to characterize the N370S GCase, four Gaucher cell lines homozygous for this mutation were analysed for the residual enzyme activity. In the Gaucher fibroblast homogenates, GCase activity, measured in the presence of taurocholate and Triton X-100 as enzyme activators, was approx. 10 % of the normal value (Figure 1). When a combination of Sap C and LUVs composed of an anionic phospholipid such as PS and Chol, a major constituent of biological membranes, was added to the assay mixture instead of detergents, the N370S GCase activity in all four Gaucher homogenates dramatically increased, reaching 50–70 % of the control value. These observations are in agreement with previous findings, which showed that a PS dispersion together with Sap C was able to restore the catalytic function of N370S GCase [18].

The effect of the level of anionic phospholipids in the LUVs was also evaluated. When the PS content was reduced, the activity of the mutated but not of the control enzyme dramatically decreased. As shown in Figure 1, the N370S GCase activity, measured in the presence of Sap C and 20 % PS LUVs, was approx. 15 % of the control value. It is thus evident that the evaluation of the residual enzyme activity in fibroblast extracts of Gaucher patients carrying the N370S mutation is highly dependent on the composition of the assay mixture and can be modulated by the percentage of anionic phospholipids in the activating vesicles.

We then estimated the amount of N370S GCase protein and the pattern of its molecular mass forms in the four Gaucher cell lines. Figure 2 shows the Western-blot analysis of the homogenates, indicating that the amount of the mutated enzyme protein cross-reacting with the monoclonal anti-GCase antibody 8E4, which has the same affinity for control and N370S GCase [19,25], is very similar to that of the control fibroblasts. Moreover, the pattern of the various enzyme forms revealed by immunoblotting

Figure 1 Effect of different activators on the GCase activity in control and N370S Gaucher fibroblast extracts

The GCase activity in normal and N370S Gaucher fibroblast extracts (cell lines 370a, 370b, 370c, and 370d) was determined in at least three separate preparations for each cell line utilizing assay mixtures containing either detergents (Triton X-100 and taurocholate) or Sap C and LUVs as activators (see the Experimental section). No enzyme activity was detected in both the normal and N370S Gaucher fibroblasts in the absence of activators. An identical amount of protein (10 μ g) was used in each assay. The concentration of Sap C was 5 μ M. LUVs were composed of one among PS/Chol, 75:25 (75% PS); PS/PC/Chol, 60:15:25 (60% PS); and PS/PC/Chol, 20:55:25 (20% PS). Data represent the means $\pm$ S.D.

Figure 2 Level and molecular mass forms of N370S GCase in N370S Gaucher fibroblasts

The level and the pattern of molecular mass forms of GCase were determined by Western-blot analysis of GCase antigen from control and N370S Gaucher fibroblast homogenates (cell lines 370a, 370b, 370c and 370d). Identical amounts of protein (5 μ g) were loaded in each lane. The number on the left refers to the molecular mass (kDa) of albumin standard. The blots were reprobed for β -actin as a control of protein content. Each experiment was repeated more than three times and gave similar results.

was also similar in control and Gaucher fibroblasts (Figure 2). These findings are consistent with literature reports describing the presence of intermediate and mature enzyme species ranging in molecular mass from 56 to 66 kDa in fibroblasts from both normal individuals and patients with type 1 Gaucher disease [26,27].

Sap C level, biosynthesis and maturation in N370S Gaucher fibroblasts

Since Sap C is the physiological activator of GCase, variation in its mass or its processing might affect the N370S GCase activity.

Figure 3 Level and processing of Sap C in N370S Gaucher fibroblasts

(A) Sap C level was determined by Western-blot analysis of control and N370S Gaucher fibroblast homogenates (cell lines 370a, 370b, 370c and 370d). Identical amounts of protein (5 μ g) were loaded in each lane. The blots were reprobed for β -actin as a control of protein content. (B) Control and N370S Gaucher fibroblasts were pulsed for 1 h and then chased as indicated. Immunoprecipitation, SDS/PAGE and fluorography were performed as described in the Experimental section. The numbers on the left refer to the molecular-mass standards (kDa). Each experiment was repeated more than two times and gave similar results.

We therefore investigated the level of Sap C in the four N370S Gaucher cell lines. As shown in Figure 3(A), the Western-blot analysis indicates that the amount of Sap C in the Gaucher fibroblasts was similar to or higher than that present in the control fibroblasts.

The biosynthesis and processing of Sap C were also examined by pulse-chase experiments (Figure 3B). After 1 h pulse, two prosaposin precursor forms ($\sim$ 65–70 kDa) were observed. After 6 h, prosaposin was converted almost completely to Saps both in the control and in the N370S Gaucher fibroblasts. Thus the maturation of Sap C was essentially normal in the Gaucher fibroblasts.

Effect of Sap C and anionic phospholipids on the activity of N370S GCase

As shown in Figure 1, in the presence of appropriate amounts of physiological activators, N370S GCase can express an almost normal activity. To evaluate better the N370S GCase activation, the effects of Sap C and anionic phospholipids such as PS and LBPA were analysed in detail. Sap C-free control and N370S GCases were obtained after purification of the enzymes from fibroblast extracts using immunoaffinity chromatography [19,23]. The absence of Sap C was verified by immunoblotting (results not shown). The enzyme preparations were examined by Western-blot analysis, loading on to gels increasing amounts of GCase. The results of the analysis show that bands of approximately the same intensity were observed when 10 units of normal enzyme and 1 unit of N370S GCase were loaded on to the gel (Figure 4). Thus the specific activity relative to the amount of cross-reacting immunological material of the control enzyme is ten times higher than that of the mutated enzyme.

The stimulation by Sap C of purified control and N370S GCases was evaluated in the presence of LUVs of different composition. The LUVs contained increasing amounts of anionic phospholipids

Figure 4 Immunoblots of normal and N370S GCases purified from fibroblast homogenates

Aliquots of the purified enzyme preparations containing predetermined enzyme units were analysed by Western blotting. Lanes were loaded with 0.5, 2 and 3 units (U) of control enzyme or with 0.1, 0.2 and 0.3 unit of N370S GCase as indicated. Each experiment was repeated more than two times and gave similar results.

Figure 5 Effect of anionic phospholipids and Sap C on the activation of control and N370S GCases

The activities of similar amounts of purified control (A) and N370S (B) GCases, corresponding to 1 unit of control and 0.1 unit of N370S GCase respectively were measured in the presence of LUVs and increasing amounts of Sap C as described in the Experimental section. LUVs were composed of one among PS/Chol, 75:25 (75% PS, ○); PS/PC/Chol, 60:15:25 (60% PS, ▲); PS/PC/Chol, 20:55:25 (20% PS, □); LBPA/PC/Chol, 20:55:25 (20% LBPA, ●); and PC/Chol, 75:25 (75% PC, ■). Each point represents the mean of at least three replicates. The deviation was within $\pm 5\%$ of each data point.

and 25% Chol, which was included in the liposomes to mimic better the biological membranes. As shown in Figure 5(A), Sap C efficiently promoted the activity of control GCase in a concentration-dependent manner in the presence of LUVs containing 75, 60 or 20% anionic phospholipids. Also purified N370S GCase was stimulated by Sap C in combination with LUVs containing 75 or 60% PS, the activity increasing more than 15 times when measured in the presence of 5 μ M Sap C, but the activation significantly decreased when LUVs contained 20% PS or LBPA (Figure 5B). As already observed with crude fibroblast extracts (see Figure 1), these results indicate that control and N370S GCases respond differently to the Sap C-mediated activation in the presence of membranes containing only 20% anionic phospholipids. In the absence of anionic phospholipids (75% PC LUVs), neither the control nor the mutated enzyme was efficiently stimulated by Sap C (Figure 5).

Effect of Sap C and anionic phospholipids on the membrane binding properties of N370S GCase

GCase can express its activity only when bound to the Sap C–anionic phospholipid complex [9,10]. To investigate whether the

Figure 6 Comparison of the binding of control and N370S GCases to membranes containing anionic phospholipids and Sap C

For binding experiments, similar amounts of purified control and N370S GCases were incubated with Sap C and LUVs as reported in the Experimental section. The vesicles were composed of one among LBPA/PC/Chol, 20:55:25 (20% LBPA); PS/PC/Chol, 20:55:25 (20% PS); and PS/PC/Chol, 60:15:25 (60% PS). After centrifugation, the free and liposome-bound enzymes were evaluated by SDS/PAGE and Western blotting. Lanes 1, free enzyme; lanes 2, membrane-bound enzyme. Each experiment was repeated at least three times and gave similar results.

decreased activation of N370S corresponds to an impaired binding, purified control and mutated enzyme preparations containing equivalent amounts of enzyme protein were incubated with Sap C and LUVs as model of biological membranes. After centrifugation, the free and vesicle-bound enzymes were qualitatively evaluated by Western-blot analysis. As shown in Figure 6, most of the control GCase was bound to LUVs containing 20% PS or LBPA. In contrast, N370S GCase did not associate with these membranes and remained free in the supernatant. When LUVs contained an increased amount of anionic phospholipids (60% PS LUVs), both control and N370S GCases bound to the vesicles. A comparison of Figures 5 and 6 shows that conditions promoting enzyme activation similarly favour the interaction of the enzyme with LUVs. These results show that normal GCase is able to bind to membranes containing 20% anionic phospholipids and Sap C much better than the mutant enzyme. This difference disappears when the percentage of anionic phospholipids on the lipid surface is very high.

Chol accumulates in the late endosomes/lysosomes of N370S Gaucher fibroblasts

We have previously shown that, in NPC1 cells, the interaction of GCase with Sap C and LBPA-containing membranes is impaired by the storage of free Chol [22]. Chol accumulation in late endosomes/lysosomes has been found in several sphingolipid storage diseases [34]. This observation prompted us to examine the intracellular distribution of Chol in the four Gaucher cell lines from patients homozygous for the N370S mutation. As shown in Figure 7, increased filipin fluorescence relative to control cells was observed in each cell line. The intensity of the fluorescence was slightly weaker than that previously observed in NPC1 cells [22], but the Chol distribution in vesicular structures was identical. To investigate the nature of these structures, the co-localization between Chol and LBPA was evaluated by immunofluorescence. Figure 7(B) shows that, in the Gaucher fibroblasts, Chol accumulated in vesicles containing LBPA, a marker of late endosomal–lysosomal organelles.

Subcellular localization of N370S GCase, Sap C and LBPA in Gaucher fibroblasts

In view of the altered membrane binding properties of N370S GCase and of the storage of Chol, which might affect the organization of endolysosomal membranes, immunostaining experiments were performed to compare the intracellular distribution of N370S GCase, Sap C and LBPA in the normal and Gaucher fibroblasts by confocal laser-scanning microscopy. Figure 8(A)

Figure 7 Chol accumulates in endosomal/lysosomal vesicles in N370S Gaucher fibroblasts

(A) Control and Gaucher fibroblasts (N370S a, b and c) were cytochemically stained with filipin for Chol as reported in the Experimental section. Scale bars, 10 μ m. (B) The Gaucher cell line N370S d was cytochemically stained with filipin for Chol (left panel) and immunostained for LBPA, an endosomal/lysosomal marker (middle panel). The right panels show the merged image. Scale bars, 10 μ m.

shows that, in control fibroblasts, GCase, Sap C and LBPA completely co-localize, confirming previous data [22]. In contrast, Figure 8(B) shows that most of the mutated enzyme is present in vesicles negative for Sap C and LBPA, indicating that N370S GCase resides in structures distinct from that containing its physiological activators.

In order to characterize better the intracellular structures containing N370S GCase and Sap C, Gaucher fibroblasts were incubated with LysoTracker Red, a probe that accumulates in acidic organelles such as lysosomes. As shown in Figure 9, Sap C almost completely co-localizes with LysoTracker Red, while N370S GCase only partially, indicating that the enzyme resides in less acidic vesicles than Sap C.

DISCUSSION

In the present study, we have characterized the properties of the lysosomal enzyme GCase carrying the mutation most commonly found in Gaucher patients, N370S. In particular, we have investigated which specific alteration of the GCase mechanism of action may impair the activity of the mutated allele. In the past, we have shown that GCase can fully express its activity only after the Sap C-mediated interaction with anionic phospholipids [9,10]. The notion that the interaction between GCase and the Sap C–anionic phospholipid complex is required for the enzyme activation is

supported by the fact that neither anionic phospholipids alone nor Sap C in combination with membrane devoid of anionic phospholipids can efficiently stimulate the enzyme activity [9,10]. Our present results show that the capacity of N370S GCase to interact with its physiological activators, anionic phospholipids and Sap C, to form an activated complex is markedly altered in Gaucher fibroblasts.

At low pH values similar to those of the endolysosomal environment, Sap C strongly binds to and perturbs anionic phospholipid-containing lipid surfaces promoting in turn the association and activation of GCase [10]. Beside the presence of Sap C, the amount of anionic phospholipids in the membrane is a key factor for the binding of GCase. An extensive enzyme association with lipid surfaces occurs when the anionic phospholipid level exceeds a threshold of 10% [10]. In contrast, in the present study, we have observed a poor association of N370S GCase with membranes containing up to 20% anionic phospholipids. The mutated enzyme was able to bind and express its activity only in the presence of membranes highly enriched in anionic phospholipids.

Otherwise, the properties of N370S GCase are similar to those of the control enzyme. Actually, we have found an almost normal level of enzyme protein in the four Gaucher cell lines coming from patients homozygous for the N370S allele, an observation consistent with the intrinsic stability of the enzyme previously reported [19]. Moreover, the N370S mutation does not significantly affect the maturation of the enzyme. Final maturation of GCase occurs in late endosomes/lysosomes where the precursor and the intermediate forms are transported and modified by the sequential action of lysosomal exoglycosidases [28]. The occurrence of mature forms of N370S GCase is thus an indication that the mutated protein reaches the late endosomal/lysosomal compartment. Also, the level and processing of Sap C were normal in the Gaucher fibroblasts.

The recent elucidation of the crystal structure of GCase has allowed the localization of N370, which is distant from that of the amino acid residues directly involved in catalysis [29]. This is consistent with the notion that the N370S mutation results in the synthesis of a protein that might be potentially active, but has altered membrane binding properties. In order to understand how the N370S mutation affects the enzyme interaction with membranes, it is necessary to ascertain how this mutation affects the three-dimensional structure of the enzyme. At the moment, only the structure of the normal GCase has been clarified [29].

Since, in the presence of LUVs containing high levels of anionic phospholipids, Sap C can stimulate N370S GCase *in vitro*, it can be envisaged that also *in vivo* Sap C, interacting with membrane domains enriched in anionic phospholipids, has the potential to promote the activity of N370S GCase. High concentrations of anionic phospholipids have been observed in the endolysosomal compartment, where the membranes are organized as a mosaic of specialized domains. Some membranes are enriched in an anionic phospholipid such as LBPA up to 70–80% of total phospholipids, while others are enriched in the neutral phospholipid PC [30,31]. Variations in the anionic phospholipid level and/or organization might locally modulate the residual activity of N370S GCase, explaining at least in part the great phenotypic variability observed in the Gaucher patients homozygous for the N370S mutation.

In the past, the subcellular localization of GCase and Sap C has been determined by immunocytochemistry [32,33]. In control fibroblasts, the enzyme and the Sap have been visualized in endolysosomes, predominantly bound to the endolysosomal membranes. Consistent with this, we have observed by immunofluorescence microscopy that in normal fibroblasts GCase completely co-localizes with Sap C and LBPA ([22] and the results of

Figure 8 N370S GCCase poorly co-localizes with Sap C and LBPA

(A) Normal fibroblasts were double-immunostained for GCCase and Sap C (top panels) or GCCase and LBPA (bottom panels) as reported in the Experimental section. Note that the enzyme was visualized with the monoclonal antibody 8E4 (green, top-left panel) or with a polyclonal anti-GCCase antibody (red, bottom-left panel). The right panels show an enlargement of the regions outlined by the boxes in the merged panels. Scale bars, 10 μ m. (B) N370S GCCase fibroblasts were double-immunostained for the mutated enzyme and Sap C (top panels) or for the enzyme and LBPA (bottom panels). The right panels show an enlargement of the regions outlined by the boxes in the merged panels. Scale bars, 10 μ m. A comparison of the merged images in (A) and (B) shows that, in normal fibroblasts, all the intracellular vesicles are yellow, indicating that each vesicle contains GCCase, Sap C and LBPA, while in Gaucher cells, most of the vesicles are either green or red, indicating that the enzyme often resides in vesicles devoid of Sap C and LBPA.

the present study). In contrast, immunofluorescence microscopy has presently revealed that most of the N370S GCCase in Gaucher fibroblasts resides in vesicles devoid of Sap C and LBPA. Ap-

parently, the mutated enzyme and its activators partition into distinct subsets of endolysosomal vesicles. These results corroborate those of the binding experiments (see Figure 6). The

Figure 9 N370S GCase resides in less-acidic organelles than Sap C

N370S GCase fibroblasts were incubated with LysoTracker Red as reported in the Experimental section. The cells were then immunostained for the enzyme (green, upper-middle panel) or Sap C (green, bottom-middle panel). A comparison of the merged images shows that the vesicles containing Sap C also contain LysoTracker Red (all the vesicles are yellow), while several vesicles containing the enzyme are green indicating that they are devoid of LysoTracker Red. Scale bars, 10 μ m.

poor co-localization with LBPA, an anionic phospholipid present only in the internal membranes of late endosomes/lysosomes [30], indicates an alteration in the interaction of N370S GCase with these membranes.

Moreover, we have found that the N370S Gaucher fibroblasts accumulate free Chol in LBPA-positive structures. The storage of Chol in endolysosomal organelles has been observed in several sphingolipid storage diseases [34], but the mechanism by which intracellular Chol is elevated is unknown. It has been hypothesized that the endolysosomal accumulation of sphingolipids, due to the primary defect in their catabolism, leads to the sequestration of Chol, which is bound and immobilized as it circulates through the cell [35]. Chol accumulation in turn interferes with the function(s) exerted by LBPA on endolysosomes [36–38]. Actually, it has been shown that the LBPA membranes play a key role in the sorting, targeting and distribution of specific proteins and that Chol storage results in the redistribution of these proteins from their normal localization. Since anionic phospholipids also regulate the Sap C and GCase partition into lipid surfaces [9–11,39], it is likely that the presently observed Chol storage in the LBPA-containing structures of Gaucher fibroblasts contributes to alter the distribution of these two proteins in the endolysosomes.

The reduced contact among N370S GCase, Sap C and LBPA probably affects the activity of the enzyme *in situ* by preventing the formation of the complex among N370S GCase and its activating factors. A similar situation was recently observed in NPC1 cells

where the endolysosomal accumulation of Chol impaired the co-localization of GCase with Sap C, the two proteins distributing into distinct vesicular structures [22]. It is worth noting that a marked accumulation of GC has been observed not only in Gaucher cells, but also in several tissues of patients affected by NPC [40,41].

Although in Gaucher fibroblasts N370S GCase and Sap C do not often reside in the same structures, both proteins are present in the endolysosomal compartment as indicated by their normal maturation. The endolysosomal system comprises a series of intracellular acidic vesicles such as late endosomes, lysosome/late endosomes hybrid organelles and mature lysosomes. The extent of acidification varies within these organelles, the pH gradients being both dynamically and functionally interconnected [42,43]. Immunofluorescence microscopy has presently revealed that, in N370S Gaucher fibroblasts, the extent of co-localization of Sap C and N370S GCase with LysoTracker Red, a probe that selectively accumulates in acidic organelles, is different. LysoTracker completely co-localizes with Sap C, but only partially with N370S GCase. Apparently, the two proteins distribute in vesicles of different acidity, Sap C being present in more acidic structures than the mutated enzyme. The pH of late endosomes is typically less than 5.5 and that of lysosomes can be as low as 4.5 [44]. Most likely, during the intracellular transport Sap C reaches the lysosomes, while N370S GCase is mostly present in less acidic endosomes.

In conclusion, we have found for the first time that the N370S mutation alters the capacity of the enzyme to interact with anionic phospholipids and Sap C-containing membranes. A further limitation of the contact between the enzyme and its physiological activators might result from the intracellular Chol accumulation presently observed in N370S Gaucher fibroblasts. Our results suggest that the impaired function of N370S GCase cannot be simply attributed to altered catalytic properties of the enzyme, but can also depend on the physical separation among N370S GCase, Sap C and anionic phospholipids.

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