Lysosomal Degradation on Vesicular Membrane Surfaces
ENHANCED GLUCOSYLKERAMIDE DEGRADATION BY LYSOSOMAL ANIONIC LIPIDS AND ACTIVATORS

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According to a recent hypothesis (Sandhoff, K., and Kolter, T. (1996) Trends Cell Biol. 6, 98–103), glycolipids, which originate from the plasma membrane, are exposed to lysosomal degradation on the surface of intralysosomal vesicles. Taking the interaction of membrane-bound lipid substrates and lysosomal hydrolases as an experimental model, we studied the degradation of glucosylceramides with different acyl chain lengths by purified glucocerebrosidase in a detergent-free liposomal assay system. Our investigation focused on the stimulating effect induced by lysosomal components such as sphingolipid activator protein C (SAP-C or saposin C), anionic lysosomal lipids, bis(monoacylglycerol)phosphate, and dolichol phosphate, as well as degradation products of lysosomal lipids, e.g. dolichols and free fatty acids. The size of the substrate-containing liposomal vesicles was varied in the study.

Enzymatic hydrolysis of glucoceramide carried by liposomes made of phosphatidylcholine and cholesterol was rather slow and only weakly accelerated by the addition of SAP-C. However, the incorporation of anionic lipids such as bis(monoacylglycerol)phosphate, dolichol phosphate, and phosphatidylinositol into the substrate carrying liposomes stimulated glucoceramide hydrolysis up to 30-fold. Dolichol was less effective. SAP-C activated glucoceramide hydrolysis under a variety of experimental conditions and was especially effective for the increase of enzyme activity when anionic lipids were inserted into the liposomes. Glucosylceramides with short acyl chains were found to be degraded much faster than the natural substrates. Dilution experiments indicated that the added enzyme molecules associate at least partially with the membranes and act there. Surface plasmon resonance experiments demonstrated binding of SAP-C at concentrations up to 1 μM to liposomes. At higher concentrations (2.5 μM of SAP-C), liposomal lipids were released from the liposome coated chip. A model for lysosomal glucoceramide hydrolysis is discussed.

The degradation of plasma membrane-derived glycolipids takes place in the acidic compartments of the cells. According to a recently proposed hypothesis on the topology of lysosomal digestion (1, 2), glycolipids of the plasma membrane reach the lysosomal compartment as components of vesicles through the endocytic route. In the case of glycolipids with short oligosaccharide head groups, two proteins are required for the physiological degradation of each glycolipid substrate, a water-soluble lysosomal exohydrolase and a sphingolipid activator protein (SAP). The latter facilitates the interaction between vesicle-bound substrate and water-soluble enzyme. The deficiency of activator proteins SAP-A, -B, -C, and -D in a multiple activator protein deficiency syndrome (2) or in SAP precursor knockout mice (3) results in an accumulation of glycolipids with a short oligosaccharide chain as well as of vesicle structures within the acidic compartment of the cells (4, 5). Endocytosis of the missing protein, the SAP precursor, by cultured mutant cells reverses both, the lipid and the vesicle accumulation (5).

For the lysosomal degradation of glucosylceramide, the enzyme glucocerebrosidase and the protein cofactor SAP-C (also called saposin C) are required (2, 6).

A series of in vitro studies has been performed to understand the interaction between the enzyme and the activator (7–15). Human acid β-glucosyl-N-acylphosphoglucohydrolase (glucocerebrosidase; EC 3.2.1.45) is a water-soluble lysosomal protein. It cleaves the linkage of its physiological substrate, glucosylceramide (GlcCer) as well as water-soluble synthetic β-glucosides, e.g., 4-methylumbelliferyl-β-D-glucoside (MuGlc) (7–16). Defective activity of the enzyme causes glucosylceramide accumulation in patients suffering from Gaucher disease, of which three clinical variants are known, i.e., types 1, 2, and 3 (17, 18).

A known activator of the enzyme is the small glycoprotein, SAP-C, which enhances hydrolysis rates of glucocerebrosidase in vitro (7–15) and in vivo (2, 6).

Together with three other small glycoproteins, more specifically the saposins A, B, and D, SAP-C is derived from a single precursor protein, the SAP precursor or prosaposin (2). The in vivo function of SAP-C was demonstrated by the identification of patients with an isolated deficiency of this glycoprotein (2). Its deficiency causes a juvenile type of Gaucher disease but not the severe infantile form (type 2). In addition, feeding of purified SAP-C to fibroblasts from a patient with a SAP precursor deficiency reduced the level of GlcCer storage, whereas SAP-A, -B, and -D were not effective (6).

Glucocerebrosidase also requires negatively charged detergents or phospholipids for its full enzymatic activity in vitro (7–16). Earlier kinetic studies with GlcCer were mainly per-

1 The abbreviations used are: SAP, sphingolipid activator protein (saposin); Biotin-X-DHPE, N-(4-(b-aminooxy)hexanoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; BMP, bis(monoacylglycerol)phosphate; GlcCer, glucosylceramide (glucosyl-N-erythro-2-N-acetylsphingosine); C2-GlcCer, [1-14C]glucosyl-N-erythro-2-N-acetylsphingosine; C8-GlcCer, [1-14C]glucosyl-N-erythro-2-N-hexanoylsphingosine; C12-GlcCer, [1-14C]glucosyl-N-erythro-2-N-decanoylsphingosine; C18-GlcCer, [1-14C]glucosyl-N-erythro-2-N-octadecanoylsphingosine; LUV, large unilamellar vesicles; MuGlc, 4-methylumbelliferyl-β-D-glucurononoside; PA, phosphatidic acid (egg yolk); PC, phosphatidylcholine (egg yolk); PI, phosphatidylinositol (bovine brain); SUV, small unilamellar vesicle; TES, N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid; glucocerebrosidase, β-glucosyl-N-acylphosphoglucohydrolase (EC 3.2.1.45).
formed in the presence of detergents in order to solubilize the lipophilic substrate or in the presence of phosphatidylserine, which is a minor component of the lysosomes (0–3% of the phospholipid composition) (19–21).

The acidic compartments of the cells contain negatively charged lipids such as bis(monoacylglycerol)phosphate (BMP), which has been localized in the intraorganellar vesicular structures of late endosomes (22), and dolichol phosphate (23). These molecular species as well as degradation products like dolichol and free fatty acids may influence the degradation of glucosylceramide. To verify the above mentioned model of the topology of lysosomal digestion, the experimental conditions should mimic the lipid composition and size of the vesicles as closely as possible. The convex curvature of the small vesicles (40–100 nm in diameter) (4) favors the spreading of the glycoconjugate head groups on their surface and makes them easy prey for exohydrolases in the presence of activator proteins. To mimic the in vivo situation, we studied the degradation of glucosylceramide-bearing liposomes by glucocerebrosidase in the presence of SAP-C. The liposomes with diameters ranging from 40–100 nm contained various lysosomal anionic lipids.

MATERIALS AND METHODS

Commercial Products—in [14C]Glucose (specific activity 230 Ci/mol) was from ICN. Phosphatidylcholine (egg yolk), phosphatidic acid (egg yolk), phosphatidylinositol (bovine liver), dolichol (porcine liver), dolichol phosphate (porcine liver), cholesterol, 1,2-dipalmitoyl-sn-glycero, sodium taurocholate, Triton X-100, phospholipase A2 (pig pancreas), and 4-methylumbelliferyl-β-D-glucopyranoside, were purchased from Sigma. Phenylphosphoryl dichloride was from Fluka, and Biotin-X-DHPE was from Molecular Probes, Inc. (Eugene, OR). SA™ and HPA™ sensor chips were purchased from Biacore. Fine silica gel Lichroprep Si 60 (1.2 cm × 100 cm column) with chloroform/methanol as eluent was served by Sarmientos et al. (16). The structures of the labeled and unlabeled products were analyzed by mass spectrometry (matrix-assisted laser desorption ionization mass spectrometry). The labeled compounds were diluted with the unlabeled products to reach a specific radioactivity of 2.4 Ci/mol.

Synthesis of [14C]Glucosylsphingosine—Glucosylsphingosine and [14C]Glucosylsphingosine (230 Ci/mol), as well as the glucosylceramides with different fatty acyl chains (C2-, C6-, C12-, and C18-GlcCer) were synthesized according to the method described by Sarmientos et al. (16).

The product was purified by column chromatography (Lichroprep Si 60 (1.2 cm × 100 cm column) with chloroform as eluent. Fractions containing bis-1-palmitoyl-sn-glycero-3-phosphoric acid (Synthetic BMP)—BMP was prepared as described previously (24) with the following modifications. A solution of 250 mg of an-1,2-dipalmitoylglycerol (0.44 mmol) and 42 mg of 0.22 mmol) of phenylphosphoryl dichloride was stirred in 2-ml anhydrous pyridine under argon in a screw-capped vial at 37 °C for 24 h. Two volumes of water were added, and the mixture was centrifuged at 3000 rpm. The precipitate was washed three times with water and dried under reduced pressure. The crude solid product was dissolved in a minimum of chloroform and chromatographed on Lichroprep Si 60 (1.2 cm × 100-cm column) with chloroform as eluent. Fractions containing bis-1,2-dipalmitoyl-sn-glycero-3-(phenylphosphoryl) (compound I) were collected and lyophilized. Bis-1,2-dipalmitoylglycerolphosphate (compound II) was obtained by hydrogenolysis of compound I in the presence of platinum oxide. The product was applied to a column and eluted at first with chloroform to remove residues of dipalmitoylglycerol and subsequently with chloroform/methanol (2:1, v/v). The pure product compound II was lyophilized from benzene.

Bis-1-palmitoyl-sn-glycero-3-phosphate was derived by enzymatic hydrolysis of compound II by phospholipase A2 from porcine pancreas in a mixture of diethylether and sodium borate buffer, pH 8.3, as described before (24).

The product was purified by column chromatography (Lichroprep Si 60, 1.2 cm × 100 cm) in a chloroform/methanol/water (80:20:1) system. The yield of the lyso compound was 20 mg. Its structure was confirmed by mass spectrometry (fast atom bombardment mass spectrometry, matrix-assisted laser desorption ionization mass spectrometry).

Enzyme Source—The modified lysosomal glucocerebrosidase (Ceredase™), manufactured by Genzyme (Boston) from human placenta, was a gift from Dr. Hans Aerts (Amsterdam).

The concentrated enzyme preparation was stored in 1% human serum albumin in order to stabilize the glucocerebrosidase activity and was diluted with deionized water immediately before use.

One unit of enzyme activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of MuGlc/min in a detergent-containing assay. The final volume of 0.2 ml contained the following substances: 0.1 x citrate, 0.1 phosphate buffer, pH 5.5, 1 mM MuGlc, 0.4% (w/v) Triton X-100, 0.02% (w/v) sodium taurocholate.

Purification of SAP-C—SAP-C was isolated from the spleen of a patient with Gaucher disease as described previously (6). The purity of the SAP-C preparation was ensured by polyacrylamide gel electrophoresis, Western blotting, and matrix-assisted laser desorption ionization mass spectrometry (data not shown).

 Vesicle Preparation—Large unilamellar vesicles (LUVs) were prepared by the following procedure (25). Phosphatidylcholine, cholesterol, [14C]Glucosylceramide, and the other lipids (see Figs. 1–7) were dissolved in organic solvents (ethanol, chloroform, methanol). Appropriate aliquots of lipid solutions were mixed, dried under nitrogen, and kept under vacuum for at least 1 h. The lipid mixture was hydrated at a concentration of 6.25 mM in deionized water and freeze-thawed 10 times in liquid nitrogen to ensure solute equilibration between trapped and bulk solutions.

The multilamellar vesicles were pressed through polycarbonate filters (pore size, 100 nm; Nucleopore) mounted in a minietruder (Liposofast; Avestin). We subjected samples to 19 passes through two filters in tandem as recommended (25).

Small unilamellar vesicles (SUVs) were produced by sonication of LUVs with a Microtip sonicator (Branson, Danbury, CT) at 0 °C for 40 min (intervals of 15-s sonification, 30-s pause) under a stream of argon to avoid a degradation of the lipids resulting from the high temperatures. Subsequently, they were centrifuged at 100,000 × g for 15 min. The concentration of the liposomes (SUVs and LUVs) was proved by measuring their radioactivity.

In order to determine the proportion of the accessible substrate, glucosylceramide-containing SUVs and LUVs were exhaustively treated with glucocerebrosidase (500 microunits) for 2 h. Under these conditions, about 50% of glucosylceramide in SUVs and LUVs was degraded (16).

The liposomes for the assays with MuGlc were prepared as described above, but without the addition of GlcCer.

For the Biacore measurements with the SA™ chip, 0.1 mol % Biotin-X-DHPE was added to the lipid solution before drying it. The liposomes were diluted in TES buffer (final concentration 10 mM TES, 300 mM NaCl, 2 mM CaCl2, pH 7.0) to a lipid concentration of 0.05 mM.

The size of the liposomes was controlled by electron microscopy. The SUVs had a diameter of 90–120 nm, and the SUVs were about 40 nm in diameter (data not shown).

Enzyme Assays—The standard incubation mixtures contained the following components in a final volume of 40 μl: human serum albumin (20 ng), sodium citrate buffer (50 mM, pH 4.5), unilamellar liposomes (LUVs or SUVs), SAP-C as indicated, and glucocerebrosidase (25–50 microunits, 1–3 ng). Ceredase™ was used as a glucocerebrosidase source for all assays. Comparative experiments of Ceredase™ and human serum albumin free native glucocerebrosidase showed that small amounts of human serum albumin did not effect the activation of glucocerebrosidase by SAP-C and acidic lipids as previously reported by another working group (15). The standard incubation conditions were 37 °C for 20 min. Incubation time and the amount of enzyme were chosen in such a way that less than 5% of C18-GlcCer and less than 20% of C2-GlcCer were degraded.

Unless stated otherwise, the lipid concentration was kept constant at 3.125 mM, and the liposomes had the following composition: [14C]Glucosylceramide (3 mol %, 2.4 Ci/mol), cholesterol (23 mol %), acidic lipids (0–40 mol %), and phosphatidylcholine (34–74 mol %). In addition, the liposomes contained dolichol or dolichol phosphate when indicated.

The termination of the enzyme assay and the phase distribution to separate the glucose were conducted as described previously (16). The whole upper phase was loaded onto a reverse-phase column (RP18, 1 ml), which was equilibrated with a 100 mM glucose/200 mM KCl solution. The column was eluted with 3 ml of the same solvent, and the radioactivity in the effluents was measured in a scintillation counter.

The enzyme assays using MuGlc (2 mM) as substrate were conducted in a total volume of 200 μl at 37 °C for 20 min and contained liposomal glucocerebrosidase (12.5 microunits) in sodium citrate buffer (50 mM, pH 5.0). Incubations were stopped by adding a solution of 0.2 M Na2CO3 and 0.2 M glycine, pH 9.5 (1 ml). After vigorous shaking, the amount of 4-methylumbelliferone was measured fluorometrically.

Plasmon Resonance (Biacore)—Plasmon resonance was measured at 25 °C using real-time biomolecular interaction analysis in a Biacore instrument (Biaanalytical).
A sensor chip providing a preimmobilized surface with streptavidin attached to a dextran matrix (SA™ chip) was obtained from Biacore. Biotinylated LUVs, diluted in TES buffer (10 mM, 300 mM NaCl, 1 mM CaCl₂, pH 7.0), were injected into the system to achieve a final signal increase of 2500 response units (RU). SAP-C stock solution was injected at different concentrations (0.625–2.5 μM) in running buffer (50 mM sodium citrate buffer, pH 4.5). For all experiments, the flow rate was set to 20 μl/min.

The interaction of SAP-C and a lipid monolayer was measured with an HPA™ chip. A solution (10 mM TES, 300 mM NaCl, 2 mM CaCl₂, pH 7.0) of SUVs (0.5 mM) was loaded on a chip at a low flow rate (2 μl/min) over a period of 3 h and 15 min (six injections of 30 min each with intervals for a short buffer wash (100 μl/min) of 2–3 min) until no further increase of the response signal was observed. Sodium hydroxide (10 mM) was then injected at a high flow rate (100 μl/min) to remove multilamellar structures, which resulted in a stable baseline. The complete coverage of the chip surface with lipid was confirmed by the lack of nonspecific binding of bovine serum albumin (1 mg/ml) (20).

After this procedure, a dilution of SAP-C (2.5 μM) was exposed to the lipid monolayer at a flow rate of 20 μl/min in sodium citrate buffer (50 mM, pH 4.5).

Presentation of Data—All data presented are means of at least duplicate determinations. Most single values are the mean of three determinations. All individuals values are in the range of ±5% up to ±15% of the mean.

RESULTS

Basic Experiments

To mimic the in vivo situation, GlcCer was presented to the water-soluble enzyme as a component of a liposomal lipid bilayer in the absence of detergents. With this system, the following basic experiments were performed.

pH Dependence—For the enzymatic hydrolysis of GlcCer and the truncated GlcCer derivatives, optimal rates were obtained at pH 4.5 in the presence and absence of SAP-C. Assays with the artificial, water-soluble substrate, MuGlc, were performed at pH 5.0, which was optimal for its enzymatic hydrolysis. These data were consistent with previous data obtained by other investigators using a detergent-free system (7, 16).

SAP-C Dependence—LUVs were used as substrate-bearing liposomes, spiked with different concentrations of PA. In the absence of PA, the addition of SAP-C generated only a rather low rate of GlcCer hydrolysis (Fig. 1). The increasing amount of PA up to concentrations of 20 mol % stimulated the enzymatic rate dramatically up to 30-fold in the absence and up to 20-fold in the presence of SAP-C. An optimal stimulation by SAP-C was reached at about 2.5–3 μM (Fig. 1).

Dilution Experiments—In order to clarify whether the enzymatic degradation of liposomal-bound GlcCer presented as a component of liposomes takes place 1) on the liposomal surfaces, 2) in the surrounding aqueous solution, or 3) in both phases simultaneously, dilution experiments were performed under various conditions (Fig. 2, A and B). In the absence of SAP-C, a dilution of the standard incubation assay up to 5-fold with buffer reduced the reaction rate only slightly (Fig. 2A), much less than similar dilutions of the water-soluble substrate, MuGlc (Fig. 2B). On the other hand, in the presence of SAP-C, whether at a constant concentration throughout the dilution experiment or at a constant amount, an intermediate decrease of GlcCer hydrolysis rates was observed (Fig. 2A).

The Role of Lysosomal Lipids in the Degradation of Liposomal Glucosylceramide

BMP—BMP is a unique, anionic lysosphospholipid specifically found in lysosomes (19–21) and intravesicular structures of late endosomes (22). The biosynthesis of this lipid is also localized in these acidic compartments of the cell (28, 29). BMP reaches its highest concentrations (15% of the total phospholipid) in the pulmonary alveolar macrophages (27). Its part of the total phospholipid composition in purified rat liver lysosomes is about 4–17 mol % (19–21).

Increasing concentrations of BMP in LUVs stimulated the hydrolysis of GlcCer by glucocerebrosidase up to ~30-fold at 40 mol % BMP (from 0.3–0.4 to ~10.5 μmol/unit-h), (Fig. 3A). The reaction rate was further increased by the addition of SAP-C.

Phosphatidylidylinositol—Phosphatidylidylinositol (PI) is another acidic lipid found in lysosomes. Its concentration in rat liver lysosomes is about 5–8 mol % of all phospholipids (19–21). PI is also a strong stimulator of the hydrolysis of glucosylceramide, reaching a factor of 24 at 40 mol % PI (Fig. 3B).

Dolichol and Dolichol Phosphate—Dolichol is a polyisoprenoid and one of the largest lipids occurring in cells. This polyunsaturated compound has an α-saturated isoprene unit that may exist as the free alcohol or may be phosphorylated or esterified with a fatty acid (23). High concentrations of dolichol and dolichol phosphate were found in lysosomes (4.5 and 0.4 μg/mg of protein) (23). Both seem to increase the fluidity of phospholipid bilayers and to destabilize lipid membranes (23). As shown in Fig. 3C, dolichol (10 mol %) embedded in LUVs that were spiked with 10 mol % PA stimulated the degradation of GlcCer by glucocerebrosidase in the presence of SAP-C up to 2-fold and in the absence of SAP-C up to 4-fold. On the other hand, dolichol phosphate appears to be the strongest stimulator of the enzymatic hydrolysis of GlcCer in LUVs (Fig. 3D). It was more effective than any of the other anionic phospholipids tested. In the absence of SAP-C, 10 mol % of dolichol phosphate induced a 35-fold (from 0.3–0.4 to ~13 μmol/unit-h) stimulation (Fig. 3D).

Fatty Acids—The influence of oleic acid and hexanoic acid was studied in the presence and absence of SAP-C using LUVs with and without PA (10 mol %). Oleic acid induced a noticeable increase of the GlcCer degradation only in the presence of PA, while the degradation was hardly affected by the addition of short chain n-hexanoic acid (Fig. 4). With oleic acid, an optimum stimulation was reached at a concentration of 7 mM (data not shown) and thus about twice as high as the lipid concentration of the liposomes (3.125 mM) added.

The degradation rate was further increased by the addition of SAP-C (up to 1.5-fold) in the presence of oleic acid (7 mM) and 10 mol % PA.

Curvature

On the basis of our recently proposed model (1, 2) for digestion of glycosphingolipids on intralysosomal vesicles, the size of the liposomes should affect the degradation rates. As can be
seen from Fig. 5A, enzymatic hydrolysis rates were in each case higher when glucosylceramide was presented as a component of SUVs than of LUVs. This was observed in the presence and absence of SAP-C regardless of the PA concentration used. The most remarkable differences occurred at low PA concentrations (0–10 mol %). Here, depending on the PA concentrations used, glucosylceramide of SUVs was hydrolyzed about 4–7 times faster in the absence of SAP-C and up to 4 times faster in the...
Glucosylceramide and other glycosphingolipids are amphipathic components of the plasma membrane. GlCer has a poor solubility in aqueous solution and aggregates. After endocytosis, glycosphingolipids of the plasma membrane reach the lysosomal compartments of the cell by a vesicular membrane flow. According to our recent model of endocytosis and lysosomal digestion (1, 2), glycosphingolipids and also GlCer are degraded within the lumen of the acidic compartments of the cell as components of intraendosomal and intralysosomal vesicles. Hydrolysis of GlCer is facilitated by lysosomal glucocerebrosidase and SAP-C, which activates the enzyme (2, 9, 11, 14) and supports the interaction of water-soluble glucocerebrosidase and vesicle-bound GlCer (7, 8).

In order to mimic the topology of the intralysosomal situation as closely as possible, we studied the enzymatic GlCer hydrolysis at the acidic pH of 4.5 in a detergent-free assay system, in which the glycolipid substrate was presented to a water-soluble glucocerebrosidase as a component of liposomal membranes.

Since the diameter of the intralysosomal vesicles seen in the biopsy tissue of a patient with pSAP deficiency was in the range of 50–100 nm (4), LUVs with an average diameter of 100 nm and small, usually less stable SUVs with an average diameter of 40 nm were used.

Enzymatic hydrolysis of synthetic, water-soluble MuGlc proceeds mainly in the aqueous space of the incubation mixture, even if liposomes are added, as indicated by the dilution experiment in Fig. 2. The water-soluble substrate was readily hydrolyzed at an appreciable rate in the absence of any stimulators. The addition of LUVs or SUVs containing the anionic phospholipid PA and the addition of SAP-C both stimulated enzymatic MuGlc hydrolysis in a similar mode and to a similar extent as that of liposomal GlCer. This indicates that anionic lipids (more effective and probably more accessible to the enzyme when presented on SUVs rather than on LUVs) and SAP-C activate glucocerebrosidase directly as suggested by previous studies (9, 11, 14). These observations are also in agreement with in vitro studies that have shown that anionic detergents, anionic lipids like phosphatidylserine, and oleic acid stimulate glucocerebrosidase, even in the absence of SAP-C (9–16).

Since synthetic detergents, however, do not occur in vivo and since phosphatidylserine and oleic acid as used in previous studies (7, 8, 9, 11–15, 30) are only minor components of the lysosomal compartment (19–22), we also used acidic lysosomal lipids such as BMP, PI, dolichol phosphate inserted into the liposomal membranes, and dolichol and fatty acids as possible stimulators for the degradation of liposomal GlCer in the presence of SAP-C.

Dilution of the liposome-containing incubation mixture with buffer in the absence of SAP-C resulted only in a marginal reduction of GlCer hydrolysis (Fig. 2A) but in a drastic decline of the hydrolysis rate of the water-soluble substrate MuGlc (Fig. 2B). This indicates that GlCer hydrolysis occurred predominantly on the substrate-carrying membrane surfaces. However, a significant drop in GlCer hydrolysis was observed after dilution in the presence of SAP-C (Fig. 2A). This suggests that SAP-C solubilizes GlCer from liposomal surfaces so that part of the enzymatic reaction can occur in the aqueous volume surrounding the vesicles.

This notion is supported by surface plasmon resonance studies.
Enzymatic Degradation of Glucosylceramide

Fig. 5. Curvature of vesicles stimulates the enzymatic hydrolysis of GlcCer and MuGlc in the presence of PA and SAP-C. A, LUVs and SUVs, doped with different proportions of PA and 3 mol % \(^{14}\text{C}\)glucosylceramide, were incubated with 50 microunits of glucocerebrosidase, were incubated with 50 microunits of glucocerebrosidase in the absence and in the presence of SAP-C (2.5 \(\mu\text{M}\)) and assayed as described under "Experimental Procedures" as follows: LUVs (●), LUVs with SAP-C (●), SUVs (○), and SUVs with SAP-C (▲). B, assay mixtures contained 2 mM MuGlc, unilamellar liposomes (3.125 \(\mu\text{M}\) lipid) of different size and varying amounts of PA without and with SAP-C (2.5 \(\mu\text{M}\)) as follows: LUVs (●), LUVs with SAP-C (●), SUVs (○), and SUVs with SAP-C (▲). The enzyme assays were carried out as described under "Experimental Procedures."

Fig. 6. Hydrolysis of LUV-bound GlcCer as a function of the fatty acyl chain length. The degradation of the following LUV-bound substrates (3 mol %) were measured: C2-GlcCer, C6-GlcCer, C12-GlcCer, and C18-GlcCer. Assays were conducted in the absence of SAP-C (○), in the presence of 2.5 \(\mu\text{M}\) SAP-C (●), in the presence of 20 mol % PA (○), and in the presence of 20 mol % PA and 2.5 \(\mu\text{M}\) SAP-C (▲) as described under "Experimental Procedures."

(Fig. 7, A–D). First of all, these studies demonstrate the binding of SAP-C presented in the running buffer to liposomes composed of phosphatidylcholine and cholesterol. This association is increased by the incorporation of BMP. Furthermore, if BMP (20 mol %) is inserted in the vesicles, an increased SAP-C concentration of 2.5 \(\mu\text{M}\), as used in the enzyme assays, resulted in a dramatic drop of the resonance signal, suggesting a solubilization of the liposomal lipids from the liposomal membranes.

Incorporation of BMP into the GlcCer-bearing vesicles resulted not only in a labilization of the membranes by SAP-C but also in a strong stimulation of GlcCer hydrolysis in the absence and in the presence of SAP-C. Therefore, it can be assumed that the solubilization of liposomal lipids by SAP-C may well facilitate the interaction between GlcCer and glucocerebrosidase.

On the other hand, SAP-C binds only weakly to planar PC monolayers, suggesting the importance of curved membrane structures for the SAP-C-stimulated GlcCer hydrolysis.

Although the BMP used in this study was a synthetic product and contained only saturated fatty acids, it turned out to be one of the best stimulators of GlcCer hydrolysis in vitro (25-fold stimulation at 30 mol %). In contrast to the synthetic BMP, the physiological product is composed of a mixture of numerous fatty acids that are believed to be acyl chains mainly esterified at the secondary position of the glycerol backbone (28, 29).

This compound has been identified in intracellular vesicles (22) and may therefore be directly involved in the lysosomal degradation of the vesicles. A comparable acceleration of GlcCer hydrolysis was also obtained with another lysosomal lipid, namely dolichol phosphate (35-fold stimulation at 10 mol %). This compound is supposed to destabilize membranes (23). Even very small quantities of dolichol phosphate (1.25 mol %) induced a stimulation of GlcCer degradation by the hydrolase in the presence and in the absence of SAP-C. At a concentration of 10 mol %, dolichol phosphate triggers the highest degradation rates of all acidic lipids in LUVs. These high rates may be caused by a combined effect of membrane destabilization and enzyme stimulation. The presence of a negatively charged group (phosphate) seems to be essential for the activation of the enzyme, because dolichol itself only gives a considerable stimulation of glucocerebrosidase in association with an anionic lipid like PA, whereas cationic amphiphiles such as glucosylsphingosine and sphingosine inhibit (16). As an overall result, anionic lysosomal lipids like BMP, PI, dolichol phosphate, and PA display a general stimulatory effect in the liposomal assay system.

Oleic acid was also an effective stimulator in our assay system as described for another assay system (30). However, concentrations as high as 7 mm of oleic acid were needed to achieve maximal effects on GlcCer hydrolysis. An optimal stimulation was only observed in the presence of PA (10 mol %).

Additional components and parameters stimulating the enzymatic hydrolysis of the liposome-bound substrate GlcCer effectively (see also Fig. 8) are 1) the addition of SAP-C, 2) an increasing curvature of the liposomes, and 3) truncation of the acyl chain length of the GlcCer substrate as described below.

1) We obtained an optimal stimulation by SAP-C at a concentration of about 2.5 \(\mu\text{M}\), which apparently already solubilized liposomal lipids (Fig. 7) and was about 2000 times higher than that of glucocerebrosidase. High stimulatory factors of up to 6-fold were found with SAP-C only in the presence of low concentrations of anionic phospholipids (10 mol % in the case of PA). In the presence of high anionic phospholipid concentrations, SAP-C stimulation was diminished to about 1.4-fold (30 mol % PA).

2) Increasing curvature of the liposomes also increased the GlcCer hydrolysis in the absence and presence of SAP-C (Fig. 5, A and B). A detectable stimulation was already observed at
lower concentrations of PA when SUVs were used. A remote possibility is that the conversion of LUVs to SUVs may redistribute GlcCer significantly from the inner to the outer membrane of the liposomes and increase the proportion of the available substrate. However, the difference of the distribution of lipids between LUVs (100 nm) and SUVs (40 nm) is less than 15% (16, 31). Even if we assume that the whole amount of GlcCer would reside in the outer leaflet of the SUVs, the increase of the degradation cannot surpass the factor 2 and explain the exceeding hydrolysis rates of up to 700% in SUVs (at 10 mol % PA). Furthermore, SUVs free of GlcCer stimulate the degradation of the water-soluble substrate MuGlc more effectively than LUVs, which is independent of the lipid substrate distribution.

3) Furthermore, hydrolysis was accelerated by reducing the acyl chain length of the lipid substrate (Fig. 6) and thereby increasing its solubility in aqueous solutions and possibly its accessibility for glucocerebrosidase. Relatively high degradation rates were achieved by C2- and C6-GlcCer in the absence of SAP-C or anionic lipids. The stimulating effect of SAP-C was decreased in comparison with the physiological substrate (C18-GlcCer). The short chain analogues, C2- and C6-GlcCer, being less tightly bound to the lipid bilayer than the long chain analogues (C12- and C18-GlcCer), are presumably lifted more easily from the lipid bilayer, and therefore they are more readily available for the active site of the enzyme without the requirement of SAP-C or anionic lipids (Fig. 8).

From these facts it can be concluded that SAP-C is important for the glucocerebrosidase to reach the natural bound substrate. The stimulation of glucocerebrosidase by SAP-C and acidic lipids seems to be a synergistic effect, because smaller amounts of acidic lipid are apparently needed for a notable stimulation by SAP-C.

Model for the Lysosomal Degradation of GlcCer—Deficiency of the SAP precursor (pSAP, prosaposin) in humans and in SAP precursor knockout mice results not only in a storage of GlcCer and other glycosphingolipids with short oligosaccharide chains but also in the accumulation of intralysosomal vesicle structures with a diameter of 40–100 nm (4). Both accumulations are reversed by feeding the missing protein, pSAP, to mutant fibroblasts in cell culture (5).

Based on the foregoing and also other observations (1, 2), a new model of the topology of lysosomal digestion has been developed, which is supported by the in vitro studies presented here. SAP-C and anionic lysosomal phospholipids, especially BMP, which has been localized in such vesicles (22), labilize the GlcCer-carrying vesicles and facilitate the interaction of GlcCer with water-soluble glucocerebrosidase. Apparently, SAP-C not only activates the enzyme but also binds to the vesicular surface (Fig. 8).

It remains an open question to what extent the stimulation of glucosylceramide hydrolysis as catalyzed by glucocerebrosidase in vivo is caused by acidic lipids and to what extent by SAP-C. Unfortunately, the lipid composition of intralysosomal membranes or vesicles is not known at present.

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