Degradation of Membrane-bound Ganglioside GM1

STIMULATION BY BIS(MONOAICYLGLYCERO)PHOSPHATE AND THE ACTIVATOR PROTEINS SAP-B AND GM2-AP

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According to our hypothesis (Fürst, W., and Sandhoff, K. (1992) Biochem. Biophys. Acta 1126, 1–16) glycosphingolipids of the plasma membrane are digested after endocytosis as components of intraendosomal and intralysosomal vesicles and membrane structures. The lysosomal degradation of glycosphingolipids with short oligosaccharide chains by acid exohydrolases requires small, non-enzymatic cofactors, called sphingolipid activator proteins (SAPs). A total of five activator proteins have been identified as follows: namely the saposins SAP-A, -B, -C, and -D, which are derived from the single chain SAP-precursor protein (prosaposin), and the GM2 activator protein. A deficiency of prosaposin results in the storage of ceramide and sphingolipids with short oligosaccharide head groups. The loss of the GM2 activator protein blocks the degradation of the ganglioside GM2. The enzymatic hydrolysis of the ganglioside GM1 is catalyzed by β-galactosidase, a water-soluble acid exohydrolase. The lack of ganglioside GM1 accumulation in patients suffering from either prosaposin or GM2 activator protein deficiency has led to the hypothesis that SAPs are not needed for the hydrolysis of the ganglioside GM1 in vivo. In this study we demonstrate that an activator protein is required for the enzymatic degradation of membrane-bound ganglioside GM1 and that both SAP-B and the GM2 activator protein significantly enhance the degradation of the ganglioside GM1 by acid β-galactosidase in a liposomal, detergent-free assay system. These findings offer a possible explanation for the observation that no storage of the ganglioside GM1 has been observed in patients with either isolated prosaposin or isolated GM2 activator deficiency. We also demonstrate that anionic phospholipids such as bis(monoacylglycerol)phosphate and phosphatidylglycerol, which specifically occur in inner membranes of endosomes and in lysosomes, are essential for the activator-stimulated hydrolysis of the ganglioside GM1. Assays utilizing surface plasmon resonance spectroscopy showed that bis(monoacylglycerol)phosphate increases the binding of both β-galactosidase and activator proteins to substrate-carrying membranes.

The degradation of glycosphingolipids (GSLs)\(^1\) and of other components of the plasma membrane takes place in the acidic compartments of the cell. According to a new hypothesis, GSLs and other membrane-bound structures are digested after endocytosis as components of intraendosomal and intralysosomal vesicles and membrane structures (1, 2).

The in vivo degradation of GSLs with short oligosaccharide head groups requires water-soluble lysosomal exohydrolases and sphingolipid activator proteins (SAPs). A total of five SAPs is known, and they are encoded by only two genes. One gene codes for the GM2 activator protein (GM2-AP) (2, 3). The second gene codes for prosaposin, which is proteolytically processed to four highly homologous proteins (SAP-A, -B, -C, and -D) (1, 2, 4, 5).

The complete lack of all four SAPs in patients suffering from prosaposin deficiency (2, 5) and in prosaposin knockout mice (6) results in a lysosomal accumulation of GSLs with short oligosaccharide head groups and ceramide. An isolated defect of GM2-AP on the other hand leads to storage of the ganglioside GM2 (2, 5). An accumulation of GM1 is not observed in either case (2, 5).

These observations suggest that the lysosomal degradation of GM1 by acid β-galactosidase proceeds in the absence of activator proteins in vivo. On the other hand, in vitro studies have shown that the hydrolysis of micellar GM1 by acid β-galactosidase strictly depends on the presence of SAP-B or an acidic detergent (2, 7–10).

In order to solve this apparent contradiction, the enzymatic hydrolysis of GM1 was analyzed in an in vitro system, simulating the in vivo situation as closely as possible. GM1-carrying liposomes, doped with the lysosomal anionic phospholipid BMP, reported to be in the range of 4–17 mol % of the total phospholipids obtained from purified rat liver lysosomes (11–13), were prepared as a model of intralysosomal vesicles and incubated with water-soluble β-galactosidase in the presence of different activator proteins.

MATERIALS AND METHODS

Commercial Products—Phosphatidylcholine (egg yolk), phosphatidyl-inositol (bovine brain), dolichol phosphate, and cholesterol were purchased from Sigma. Ganglioside GM1 was from Fidia. Fine silica gel Lichroprep Si 60 and Lichroprep RP18 were obtained from Merck. All other chemicals were of analytical grade or the highest purity available.

Synthesis of [\(^{2}H\)]GMI—Ganglioside GM1 was tritiated by the galactose oxidase/[\(^{2}H\)]NaBH\(_{4}\) method to a specific activity of 60 Ci/mol (14).

\(^{1}\)The abbreviations used are: GSLs, glycosphingolipids; BMP, bis(monoacylglycerol)phosphate; GM1, ganglioside GM1, Galβ1–3Gal-1–4Glcβ1–4GlcNAcβ1–4Gal(3–2αNeuAcβ1–4Glcβ1–1Cer; GM2-AP, GM2 activator protein; LUV, large unilamellar vesicles; MuGal, 4-methylumbelliferyl-β-D-galactopyranoside; SAP-A, sphingolipid activator protein A or saposin A; SAP-B, sphingolipid activator protein B or saposin B; SAP-C, sphingolipid activator protein C or saposin C; SAP-D, sphingolipid activator protein D or saposin D; SUV, small unilamellar vesicle; PC, phosphatidylcholine; PI, phosphatidylinositol; AP, activator proteins; RU, response units.
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TLC followed by autoradiography did not indicate any contamination of the labeled GM1. 

Synthesis of GM1-Sugar (Monosialogangliotetraose)—The synthesis of the GM1-sugar moiety was performed according to a method developed by Wiegandt and Baschang (15). 

Enzyme Preparation—β-Galactosidase was purified to an apparent purity of 98% by ion exchange chromatography on hydroxyapatite (RP18, 1 ml), which was equilibrated with a solution of methanol/water (1:1, v/v) and sodium citrate buffer (50 mM, pH 4.2). 

Expression of the GM2 Activator Protein—The human GM2 activator protein (GM2-AP) was expressed in insect cells using the baculovirus expression system as described previously (19).

Vesicle Preparation—Unless stated otherwise large unilamellar vesicles (LUVs, for composition see below) were prepared by the following procedure (16). 

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The isolation of the [3H]galactose in the assays with GM1-sugar, which lacks the ceramide moiety, was achieved by small DEAE columns (8, 21). Therefore, SAP-B and GM2-AP were spiked with GM1, was varied in order to find out possible stimulatory effects of anionic lysosomal lipids such as BMP, which is specifically found in lysosomes (11–13) and intravesicular structures of late endosomes (22). LUVs with increasing concentrations of BMP were used as substrate-bearing liposomes. In the absence of BMP, the GM1 degradation rate was only moderately enhanced by the addition of SAP-B or GM2-AP. However, in the presence of 20 mol % of BMP, SAP-B increased the degradation rate by a factor of 200 (Fig. 1A) and GM2-AP by a factor of 110 (Fig. 1B).

Dilution Experiments—In order to find out whether the enzymatic degradation of liposome-bound GM1 takes place on the liposomal surface, in the surrounding aqueous solution or in both phases simultaneously, dilution experiments were performed. 

The Effect of SAP-A, -C, and -D and the Bilayer Curvature on the Enzymatic Degradation of GM1—To investigate further the specificity of the stimulating effect of SAP-B and GM2-AP on the conversion of GM1 to the ganglioside GM2, the effects of

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

Surface Plasmon Resonance (Biacore)—Plasmon resonance was measured at 25 °C using real time biomolecular interaction analysis in a Biacore instrument (BiaLite).

A sensorchip providing a preimmobilized surface with lipophilic anchors attached to a dextran matrix (Pioneer L1-Chip) was obtained from Biacore. LUVs, diluted in phosphate-buffered saline buffer (10 mM, 140 mM NaCl, 10 mM KCl, pH 7.4), were injected into the system to achieve a final signal increase of 8000 response units (RU). Sodium hydroxide (50 μl, 10 mM) was then injected at a high flow rate (100 μl/min) to remove multilamellar structures. This procedure leads to a stable baseline. 

SAP-B and β-galactosidase stock solutions were injected at concentrations of 5 μM (SAP-B) or 20 μM (β-galactosidase) in running buffer (50 mM sodium citrate buffer, pH 4.2), respectively. For all experiments the flow rate was set to 10 μl/min.

Determination of the Surface Charge of Liposomes—The surface charge of the vesicles was obtained by measuring the zeta potential. The zeta potential was determined utilizing the scattering microelectrophoresis implemented by the Malvern Zetasizer 3000HS (Malvern Instruments Ltd., Malvern). The experiments were performed at 25 °C in 20 mM sodium citrate buffer, pH 4.2.

RESULTS

In order to mimic the in vitro situation of ganglioside catabolism in the acidic compartments of the cell as closely as possible, unilamellar liposomes, carrying membrane-bound ganglioside GM1 as substrate, were used and incubated with β-galactosidase in the presence of various sphingolipid activator proteins.

An Acidic Environment Is Required for the Degradation of GM1 and Artificial Substrates in the Liposomal System—Optimal degradation rates were obtained for the GM1, the GM1-sugar, and the artificial substrate MuGal at an acidic pH value of 4.2 (data not shown).

The Hydrolisis of Membrane-bound GM1 Is Stimulated by Both SAP-B and GM2-AP—Several in vitro studies showed that SAP-B enhances the hydrolysis of micellar GM1 by β-galactosidase (2, 7, 8, 10). Transfer experiments also demonstrated that the GM2-AP did not only bind the ganglioside GM2 but also GM1 (8, 21). Therefore, SAP-B and GM2-AP were tested as possible activators of the enzymatic hydrolysis of membrane-bound GM1. The lipid composition of liposomes, spiked with GM1, was varied in order to find out possible stimulatory effects of anionic lysosomal lipids such as BMP, which is specifically found in lysosomes (11–13) and intravesicular structures of late endosomes (22). LUVs with increasing concentrations of BMP were used as substrate-bearing liposomes. In the absence of BMP, the GM1 degradation rate was only moderately enhanced by the addition of SAP-B or GM2-AP. However, in the presence of 20 mol % of BMP, SAP-B increased the degradation rate by a factor of 200 (Fig. 1A) and GM2-AP by a factor of 110 (Fig. 1B).

In the standard incubation mixture, containing particles of pure BMP (lipid concentration 200 μM), which was equilibrated with a solution of methanol/water (1:1, v/v) and sodium citrate buffer (50 mM, pH 4.2) and freeze-thawed 10 times to ensure solute equilibration between trapped and bulk solutions.

The multilamellar vesicles were pressured through poly carbonate filters, mounted in mini-extruder (LiposoFast; Avestin). The samples were subjected to 19 passes through two filters in tandem as recommended (20).

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 in order to minimize degradation of lipids as a result of the high temperatures that occur during the sonification process. The concentration of liposomes was determined by measuring their radioactivity. The liposomes used for the assays with the GM1-sugar moiety were prepared as described above but without the addition of GM1.

Enzyme Preparation—The enzyme preparation was purified to an apparent purity of 98% by ion exchange chromatography on hydroxyapatite (RP18, 1 ml), which was equilibrated with a solution of methanol/water (1:1, v/v) and sodium citrate buffer (50 mM, pH 4.2, 50 μl), respectively. For all experiments the flow rate was set to 10 μl/min.
these two activators were compared with those of SAP-A, -C, and -D. The enzyme assays were performed with LUVs and SUVs, composed of 10 mol % [3H]GM1, 10 mol % BMP, 20 mol % cholesterol, and 60 mol % PC or with particle structures consisting of 10 mol % [3H]GM1 and 90 mol % BMP. The latter structures were prepared by the same procedure as described for SUVs and LUVs.

Besides SAP-B, under the chosen experimental conditions, only SAP-A is able to stimulate the GM1 degradation to a minor degree, whereas SAP-C and SAP-D have no significant influence on the rate of GM1 hydrolysis (Fig. 3, A and B).

On the basis of our proposed model for the digestion of GSLs as components of intralysosomal vesicles (1, 2), it was anticipated that the size of liposomes might have an influence on the degradation rates of GSLs in general. The effect of the membrane curvature on the rate of hydrolysis was recently demonstrated for the degradation of glucosylceramide by acid glucocerebrosidase (16). As seen in Fig. 3A, hydrolysis rates were higher when GM1 was incorporated into SUVs rather than in LUVs. This effect was mainly observed in the presence of SAP-B and GM2-AP. In the absence of these activators the degradation rates were very low in the LUV as well as in the SUV system.

Then the degradation rates of particle structures, composed only of 90 mol % BMP and 10 mol % GM1, were tested. In contrast to the above mentioned results, these systems showed high hydrolysis rates already in the absence of the sphingolipid activator proteins GM2-AP and SAP-B. Therefore, the stimulatory effects caused by SAP-B and GM2-AP were diminished significantly (Fig. 3B).
LysoSomal Anionic Lipids Activate the Degradation of GM1 in Combination with SAP-B or GM2-AP—Although the degradation of GM1 was very low in liposomes consisting of PC and cholesterol, a marked stimulation of the enzyme-catalyzed reaction rates was obtained by the introduction of acidic lipids into substrate-containing liposomes. Fig. 4 shows the GM1 degradation rate in the presence of different lysoSomal anionic lipids at a concentration of 10 mol %. These anionic phospholipids exhibited a strong stimulatory effect in the presence of GM2-AP or SAP-B. The highest degradation rates could be achieved with PI-bearing vesicles in the presence of SAP-B.

Hydrolysis of Water-soluble Substrates, GM1-Sugar, and MuGal—Previous studies suggest that SAP-B and GM2-AP facilitate the interaction of membrane-bound sphingolipid substrates and water-soluble enzymes (2, 7, 8, 21). In order to rule out a possible direct stimulation of the enzyme β-galactosidase by these activators, the hydrolysis of the water-soluble GM1-sugar moiety was examined in the presence and absence of activator proteins and liposomes, spiked with different concentrations of BMP.

Neither the addition of vesicles, composed of neutral lipids and up to 20 mol % BMP, nor the addition of activator proteins affected the degradation rates of the water-soluble substrate (data not shown). However, particles composed of pure BMP inhibited the hydrolysis of the GM1-sugar to 20–50% (data not shown). This effect was more pronounced in presence of SUV-like particles. The surface charge of the liposomes. The surface charge of LUVs (100 mM, 0.5 mM) were immobilized on a Pioneer L1 sensorchip to give a response signal of 8,000 RU. SAP-B (5 μM) (A) and β-galactosidase (20 nM) (B) were injected at a flow rate of 10 μl/min in 50 mM sodium citrate buffer, pH 4.5, for 180 s. Then, as indicated by an upward arrow, protein-free buffer was injected. The binding curves were obtained with membrane structures, bearing PC (80 mol %)/cholesterol (20 mol %) (a), PC (70 mol %)/cholesterol (30 mol %)/GM1 (10 mol %) (b), PC (60 mol %)/cholesterol (20 mol %)/BMP (20 mol %) (c), or PC (50 mol %)/cholesterol (20 mol %)/BMP (20 mol %)/GM1 (10 mol %) (d).

Comparable results were obtained when MuGal was used as a water-soluble substrate instead of the GM1-sugar. Neither mixed vesicles nor activator proteins nor a combination of both influenced the hydrolysis of MuGal (data not shown).

However, addition of pure BMP prepared as LUV- or SUV-like particles caused a strong inhibition of MuGal hydrolysis (data not shown).

Interaction of SAP-B and β-Galactosidase with Liposomes Is Triggered by BMP—By assuming that the association of SAP-B and β-galactosidase on the membrane surface might play a decisive role for the degradation of GM1, we analyzed the binding behavior of SAP-B and β-galactosidase toward four different populations of neutral and anionic membrane structures using surface plasmon spectroscopy (Biacore) (Fig. 5). The injection of a 5 μM solution of SAP-B resulted in a strong binding signal of 760 RU toward membrane structures that were spiked with 20 mol % BMP, whereas the association with membrane structures consisting of PC and cholesterol proved to be less strong (470 RU). The incorporation of 10 mol % GM1 to the lipid bilayers increased the binding of SAP-B only slightly, whereas the incorporation of BMP into these bilayers increased the binding of β-galactosidase to the membranes more strongly (from 135 to 240 RU at 20 mol % BMP).

SAP-B, however, was not able to potentiate the capacity of BMP to mediate the binding of β-galactosidase to the membrane structures (Fig. 6).

ζ potential measurements were carried out to ascertain the surface charge of the liposomes. The surface charge of LUVs containing 20% BMP was found to be −54 mV in a 20 mM citrate buffer, pH 4.2, indicating that BMP was still negatively charged at that acidic value.

DISCUSSION

In higher eukaryotes, GSLs are catabolized by the sequential release of sugar units by a series of lysosomal glycosidases. It has been well documented that the lysosomal degradation of GSLs with short oligosaccharide structures requires the support of protein cofactors.

A number of in vivo studies suggest, however, that the digestion of GM1 by lysosomal β-galactosidase does not require the presence of an activator protein (2, 5, 6), whereas the
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hydrolysis of micellar GM1 in vitro strictly depends on the addition of SAP-B (2, 7, 8, 10). To solve this apparent contradiction, we tested the stimulation of the activators SAP-B and GM2-AP in a detergent-free, liposomal system at pH 4.2, which is believed to mimic the in vivo situation as closely as possible. Based on our recently proposed model for the degradation of GSLs as components of intralysosomal vesicles, the natural substrate GM1 was presented to the water-soluble enzyme as a component of liposomal membranes.

Since the diameter of the intralysosomal vesicles found in the biopsy tissue of a patient with pSAP deficiency was in the range of 50–100 nm (23), LUVs with an average diameter of 100 nm and SUVs with an average diameter of 40 nm were used.

Membrane-bound GM1, as part of heterogeneously composed liposomes, was hydrolyzed to a significant extent only in the presence of both an anionic lysosomal or endosomal lipid such as BMP and an activator protein, SAP-B or GM2-AP. Even high proportions of BMP (up to 20 mol %) could not noticeably stimulate the degradation of GM1 by β-galactosidase without the addition of one of these activator proteins. Fig. 1 shows that SAP-B as well as GM2-AP stimulates the hydrolysis most effectively at high proportions of BMP (20 mol %). On the other hand, activator proteins were not needed as stimulators for GM1 hydrolysis if lipid structures were solely composed of BMP (90 mol %) and GM1 (10 mol %).

A comparable acceleration of GM1 hydrolysis was also obtained with another lysosomal acidic lipid, namely dolichol phosphate. This component is supposed to destabilize membranes and is known to be a strong stimulator of the glucocerebroside (16). Indeed, dolichol phosphate was more efficient than BMP to activate GM1 hydrolysis in the presence of SAP-B or GM2-AP. In the liposomal assay system, anionic lysosomal lipids like BMP, dolichol phosphate, and PI, also stimulate the enzymatic hydrolysis of other sphingolipids effectively, such as ganglioside GM2,2 glucosylceramide (16), sphingomyelin (24), and ceramide.7

In order to gain a greater insight into the mechanism of GM1 degradation, the effects of vesicles and activator proteins on the hydrolysis of the water-soluble substrates GM1-sugar and MuGal were examined.

Neither the addition of SAPs nor the addition of LUVs or SUVs, spiked with different proportions of BMP (up to 20 mol %), or a combination of both had an appreciable influence on the rate of hydrolysis of the water-soluble substrates. Therefore, neither BMP nor activator proteins can be considered as direct activators of β-galactosidase, a matter that has been proposed to be relevant for glucocerebrosidase (2, 16).

Moreover, the addition of increasing concentrations of particles, composed of pure BMP, inhibited the hydrolysis of GM1-sugar and MuGal drastically, suggesting that β-galactosidase becomes bound to the negatively charged membrane and therefore cannot interact effectively with the water-soluble substrates in the free solution.

This notion is supported by surface plasmon resonance studies. They show that both SAP-B and β-galactosidase can bind to membrane structures composed of PC and cholesterol only. Binding of both proteins is increased by the incorporation of BMP (20 mol %) and GM1 (10 mol %) into the lipid membrane. Binding of SAP-B to the liposomal surface, however, does not facilitate the binding of β-galactosidase. SAP-B, being a polycation at pH 4.2, presumably binds to the negatively charged lipid head groups of the BMP incorporated into liposomes and thereby decreases the surface charges. This could reduce its affinity for the polycation β-galactosidase. The binding of the activator proteins and/or the β-galactosidase to the membrane surface appears to be weak since the dilution of the liposome-containing incubation mixture with buffer resulted in a drastic decline of the hydrolysis rate of GM1.

The hydrolysis of membrane-bound GM1 by water-soluble β-galactosidase starts obviously at the water-lipid interface of the liposomes and is strictly dependent on the presence of either SAP-B or GM2-AP (see the model in Fig. 7). Since β-galactosidase binds to the surface of lipid bilayers already in the absence of activator proteins, and activator proteins apparently do not promote the binding of β-galactosidase to the liposomal surface, the activators must have a different function. They are membrane-active proteins, and GM2-AP has been shown to

2 N. Werth, C. Schutte, G. Wilkening, T. Lemm, and K. Sandhoff, submitted for publication.

3 T. Linke, G. Wilkening, F. Sadeghlar, E. Schuchman, and K. Sandhoff, submitted for publication.
insert into lipid monolayers (25). Therefore, we assume that they disturb the lipid organization of the adjacent lipid layer, bind GM1, and thereby lift it to the active site of the water-soluble enzyme.

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