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Degradation of Membrane-bound Ganglioside GM2 by β-Hexosaminidase A

STIMULATION BY GM2 ACTIVATOR PROTEIN AND LYSOSOMAL LIPIDS*

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According to a recent hypothesis, glycosphingolipids originating from the plasma membrane are degraded in the acidic compartments of the cell as components of intraendosomal and intralysosomal vesicles and structures. Since most previous *in vitro* investigations used micellar ganglioside GM2 as substrate, we studied the degradation of membrane-bound ganglioside GM2 by water-soluble β-hexosaminidase A in the presence of the GM2 activator protein in a detergent-free, liposomal assay system. Our results show that anionic lipids such as the lysosomal components bis(monoacylglycero)phosphate or phosphatidylinositol stimulate the degradation of GM2 by β-hexosaminidase A up to 180-fold in the presence of GM2 activator protein. In contrast, the degradation rate of GM2 incorporated into liposomes composed of neutral lysosomal lipids such as dolichol, cholesterol, or phosphatidylcholine was significantly lower than in negatively charged liposomes. This demonstrates that both, the GM2 activator protein and anionic lysosomal phospholipids, are needed to achieve a significant degradation of membrane-bound GM2 under physiological conditions. The interaction of GM2 activator protein with immobilized membranes was studied with surface plasmon resonance spectroscopy at an acidic pH value as it occurs in the lysosomes. Increasing the concentration of bis(monoacylglycero)phosphate in immobilized liposomes led to a significant drop of the resonance signal in the presence of GM2 activator protein. This suggests that in the presence of bis(monoacylglycero)phosphate, which has been shown to occur in inner membranes of the acidic compartment, GM2 activator protein is able to solubilize lipids from the surface of immobilized membrane structures.

The degradation of glycosphingolipids (GSLs)[1] endocytosed from the plasma membrane takes place in the acidic compartments of the cell. According to a recently proposed hypothesis of the topology of lysosomal digestion (1), GSLs reach the lysosomal compartments as membrane-bound components of intraendosomal and intralysosomal vesicles and structures. The degradation of GSLs with short oligosaccharide head groups and ceramide requires two proteins: a water-soluble lysosomal sphingolipid hydrolase and a sphingolipid activator protein (SAP) (2, 3). Recent studies suggest that SAPs mediate the interaction of water-soluble sphingolipid hydrolases with their respective membrane-bound substrates (4).

The enzymatic, lysosomal degradation of the ganglioside GM2 is catalyzed by β-hexosaminidase A (HexA) and requires the GM2 activator protein (GM2AP) as cofactor (5).

Human lysosomal β-hexosaminidases (EC 3.2.1.52) cleave off terminal β-glycosidically-bound *N*-acetylglucosamine and *N*-acetylgalactosamine residues from a number of glycoconjugates, including glycoproteins, oligosaccharides, and GSLs such as GM2, its asialo derivative GA2, and globoside (6). A total of three different β-hexosaminidase isoenzymes have been identified. These are composed of different combinations of two noncovalently linked, structurally related subunits, α and β (7–10). The β-homodimer is called the β-hexosaminidase B, the α-homodimer is called the β-hexosaminidase S, and the α,β-heterodimer is called the β-hexosaminidase A. Despite the fact that the active sites of the α and the β subunits are functionally very similar, the three isoenzymes have different substrate specificities (11). Only HexA is able to hydrolyze GM2 in the presence of the GM2AP at significant rates (12).

Defects or mutations in the genes of the subunits α and β or the gene encoding the GM2AP lead to the various forms of GM2 gangliosidosis. These storage diseases manifest themselves by a massive accumulation of GM2 and related glycolipids in neuronal lysosomes, leading to severe neurodegeneration. Depending on the defect and its severity, other tissues may also be affected by minor lipid and oligosaccharide accumulation (6).

GM2AP is one of five known SAPs. Saposin (SAP)-A, -B, -C, and -D are derived from a single precursor protein, prosaposin, through proteolytic processing (2). GM2AP is first synthesized as a 22-kDa precursor protein, which is subsequently processed in the lysosomes to the mature 20-kDa protein (13). Physicochemical measurements with lipid monolayers demonstrated the membrane-active properties of the GM2AP. GM2AP can immerse into monolayer membranes with a lateral pressure of up to 25 millinewtons/m. The lateral pressure of biological

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Dedicated to Prof. Saul Roseman on the occasion of his 80th birthday.

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[1] The abbreviations used are: GSL, glycosphingolipid; BMP, bis(monoacylglycero)phosphate; Chol, cholesterol; DoP, dolichol phosphate; GD1a, ganglioside GD1a, NeuAcα.2→3Galβ1→3GalNAcβ1→ 4Gal(3←2αNeuAc)β1→4Glcβ1→1Cer; GM2, ganglioside GM2, GalNAcβ1→4Gal(3←2αNeuAc)β1→4Glcβ1→1Cer; GM2AP, GM2 activator protein; HexA, β-hexosaminidase A, 2-acetamido-2-desoxy-β-D-hexoside-acetamidodesoxyhexohydrolase A; LUV, large unilamellar vesicles; MUG, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside; SAP, sphingolipid activator protein; SAP-B, saposin B; SUV, small unilamellar vesicle; PC, phosphatidylcholine (egg yolk); PI, phosphatidylinositol (bovine brain); PA, phosphatidic acid (egg yolk); RU, relative unit(s).

membranes is believed to be significantly higher (14). It is conceivable, however, that the lateral pressure of the inner membranes of late endosomes and lysosomes could be less than 25 millinewtons/m due to their high degree of curvature and the enrichment of the unusual lysophospholipid bis(monoacylglycero)phosphate (BMP) (15).

Studies on the degradation of glucosylceramide show that glucocerebrosidase requires negatively charged detergents or phospholipids to reach its full enzymatic activity *in vitro* (16, 17). Negatively charged lipids such as dolichol phosphate (DoP) and BMP, which were identified as components of the membrane of late endosomes and lysosomes (15, 18), stimulated the degradation of membrane-bound glucosylceramide by glucocerebrosidase and SAP-C in a detergent-free, liposomal system up to 35-fold (4).

So far, *in vitro* studies on the degradation of GM2 were mostly performed using micellar ganglioside GM2 as substrate and have never been executed in the presence of anionic phospholipids. To analyze the influence of anionic lysosomal phospholipids on the hydrolysis of the membrane bound ganglioside, GM2 was incorporated into liposomes of varying composition, mimicking intralysosomal vesicles. The degradation rate of membrane-bound GM2 by HexA and GM2AP was studied as a function of the concentration of anionic lysosomal phospholipids in liposomes at acidic pH values, which prevail in the lysosomal compartment. We also investigated the influence of membrane curvature on the degradation rate using liposomes of varying size.

EXPERIMENTAL PROCEDURES

Commercial Products—Phosphatidylcholine (egg yolk) (PC), phosphatidylinositol (bovine liver) (PI), phosphatidic acid (egg yolk) (PA), dolichol (porcine liver), dolicholphosphate (porcine liver) (DoP), and cholesterol (Chol) were purchased from Sigma. Lichroprep RP18 was obtained from Merck. All other chemicals were of analytical grade or the highest purity available.

Chemical Synthesis—Ganglioside GM2 was tritiated by the galactose oxidase/[^{3}H]$NaBH_4$ method of Novak (1979) (19) to a specific activity of 104 Ci/mol. TLC followed by autoradiography did not reveal any contamination of the labeled GM2.

BMP was prepared as described previously (4).

Enzyme Preparation—HexA was purified from human liver to apparent homogeneity according to Schütte (20). Purity and identity were ascertained by SDS-polyacrylamide gel electrophoresis and Western blot. One unit of enzyme activity (U) is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MUG)/min at 37 °C.

Activator Preparation—Recombinant GM2AP was expressed in insect cells using the baculovirus expression system and purified as described previously (21).

SAP-B was isolated from the spleen of a patient with Gaucher disease (3). The purity and identity of the final preparation was ensured by SDS-polyacrylamide gel electrophoresis and Western blot analysis.

Vesicle Preparation—Large unilamellar vesicles (LUVs) were prepared by the following procedure: PC (50 mM, toluol/ethanol 2:1), BMP (5 mM, chloroform/methanol 2:1), PI (2.5 mM, chloroform), Chol (25.6 mM, chloroform/methanol 2:1), dolichol (2 mM, chloroform), DoP (0.75 mM, chloroform), PA (1.8 mM, chloroform/ethanol 1:1), and ^{3}H-labeled GM2 (0.5 mM, toluol/ethanol 1:1) were dissolved in organic solvents. Appropriate aliquots of the lipid solutions were mixed and dried under nitrogen. The lipid mixture was hydrated to a total lipid concentration of 2 mM in Tris/HCl buffer (1 mM, pH 7.4) and freeze-thawed ten times in liquid nitrogen to ensure solute equilibration between trapped and bulk solutions.

Unilamellar vesicles were prepared by passage through two polycarbonate filters (pore size, 100 nm; Avestin) mounted in tandem in a mini-extruder (Liposo-Fast; Avestin). Samples were subjected to 19 passes as recommended (22).

Small unilamellar vesicles (SUVs) were produced by sonification 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 minimize oxidation of lipids. Samples were centrifuged at 100,000 × *g* for 15 min. The liposomes used for the MUG assays were prepared as described above, but without the addition of GM2.

Liposomes used for the surface plasmon resonance spectroscopy were prepared as described above, but hydrated to a lipid concentration of 0.5 mM in PBS buffer (10 mM phosphate, 140 mM NaCl, 10 mM KCl, pH 7.4). Liposomes used for surface plasmon resonance spectroscopy contained unlabeled GM2.

Concentrations of liposomal lipid (LUVs and SUVs) were determined by measurement of the radioactively labeled GM2. For liposomes that did not contain radioactive GM2 but were prepared by the same protocol, identical concentrations were assumed.

Enzyme Assays—Enzyme assays using MUG as substrate contained the following components in a final volume of 200 μl: sodium citrate buffer (pH 4.2, 50 mM), MUG (2 mM), unilamellar liposomes (LUVs, 1 mM, amount of total liposomal lipids) composed of 20 mol % Chol and 80 mol % PC or 20 mol % Chol, 60 mol % PC, and 20 mol % PA. Enzyme assays were incubated for 15 min at 37 °C. Incubations were stopped by adding 1 ml of sodium carbonate/glycine buffer (pH 9.5, 200 mM each). After vigorous shaking the amount of liberated 4-methylumbelliferone was measured fluorometrically.

The standard incubation mixture using GM2 as substrate contained the following components in a final volume of 50 μl: bovine serum albumin (50 μg/ml), sodium citrate buffer (pH 4.2, 50 mM), unilamellar liposomes (LUVs or SUVs, total lipid concentration = 1 mM), GM2AP (0.08–2 μg), and β-hexosaminidase A (15 milliunits).

Liposomes had the following composition: [^{3}H]GM2 (10 mol %, 1.8 Ci/mol), Chol (20 mol %), PC (50–80 mol %), and BMP (0–25 mol %), dolichol (0–10 mol %), DoP (0–10 mol %), PI (0–30 mol %), or PA (0–25 mol %) as indicated in the legends to all of figures.

The standard incubation conditions were 37 °C for 30 min.

Enzyme assays were stopped by the addition of 50 μl of methanol. Terminated enzyme assays were loaded onto a *reverse phase* column (RP18, 1 ml) equilibrated with a solution of chloroform/methanol/0.1 M KCl (3:48:47, v/v/v). The column was eluted with 2 ml of the same solvent, and the radioactivity in the effluents was measured in a scintillation counter.

Surface Plasmon Resonance Spectroscopy (Biacore)—Biomolecular interaction analysis (surface plasmon resonance spectroscopy) was carried out at 25 °C with a Bialite instrument (Biacore).

Sensorchips providing a preimmobilized surface with lipophilic anchors attached to a dextran matrix (Pioneer L1 chip) were obtained from Biacore. LUVs (total lipid concentration = 0.5 mM), diluted in PBS buffer, were injected into the system at a flow rate of 5 μl/min in two steps (first injection, 60 μl; second injection, 40 μl). This resulted in a RU shift of 5000–7000 RU. 10 μl sodium hydroxide (25 mM) were then injected at 100 μl/min to remove multilamellar structures and to stabilize the base line, resulting in a RU shift of about 20–50 RU. GM2AP (0.2 μM) in running buffer (50 mM sodium citrate buffer, pH 4.2) was injected into the flow cells at a rate of 20 μl/min.

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

RESULTS

Basic Experiments

To investigate the influence of acidic lipids on the rate of GM2 degradation under lysosome-like conditions, GM2 was incorporated into liposomes and incubated with HexA and GM2AP. In a series of preliminary experiments the influence of pH, time, and substrate concentration on GM2 degradation was investigated. Optimal degradation rates were obtained at pH 4.2 for ganglioside GM2 and at pH 4.5 for the artificial substrate MUG (data not shown). It was found that the rate of degradation was linear for up to 30 min using liposomes containing 10 mol % [^{3}H]GM2 and 20 mol % PA (data not shown). Experiments with increasing amounts of [^{3}H]GM2 (0–10 mol %) in liposomes containing 20 mol % PA resulted in an almost linear dependence of the degradation rate on substrate concentration (data not shown). Studies using the water-soluble synthetic substrate MUG showed no influence of GM2AP on the degradation rate. The hydrolysis was also not affected by the presence of liposomes containing 20 mol % PA or by the presence of both, GM2AP and the anionic lipid (data not shown).

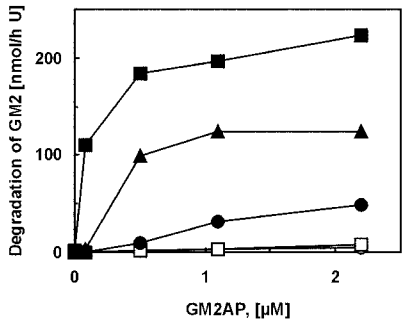

FIG. 1. **BMP and GM2AP stimulate the hydrolysis of LUV-bound ganglioside GM2.** The degradation of ganglioside GM2 inserted into LUVs doped with 0 mol % BMP (○), 5 mol % BMP (□), 10 mol % BMP (●), 20 mol % BMP (▲), or 25 mol % BMP (■) was measured in the presence of increasing concentrations of GM2AP (0–2.2 μM).

The Influence of Anionic Lysosomal Lipids on the Degradation of Liposomal GM2

The Effect of BMP on the Hydrolysis of Membrane-bound GM2—BMP is a unique, anionic lysophospholipid found specifically in the lysosomes (23–26) and in intravesicular structures of late endosomes (15). It may play a role in destabilizing these structures, thus facilitating the degradation of their components by lysosomal hydrolases. To determine the influence of BMP on the degradation of GM2, we tested how increasing concentrations of GM2AP affected the GM2 degradation rate in liposomes containing differing amounts of BMP (Fig. 1). This experiment demonstrated that GM2 hydrolysis requires the presence of GM2AP. Increasing amounts of BMP stimulated GM2 degradation in the presence of GM2AP, but not in its absence, whereas maximum stimulation of GM2 degradation was achieved by 0.5–1 μM GM2AP. We also tested how increasing amounts of BMP stimulate the GM2 degradation. LUVs containing 10 mol % [^{3}H]GM2 were spiked with increasing amounts of BMP and incubated with 15 milliunits of HexA in the presence of 0.5 μM GM2AP. Under these conditions, the incorporation of 25 mol % BMP into liposomes increased the GM2 degradation rate 180-fold compared with BMP-free liposomes (Fig. 2*A*).

To test the influence of the membrane curvature on the rate of degradation, SUVs were doped with increasing amounts of BMP and subjected to HexA and GM2AP. They displayed GM2 degradation rates that ranged about 40% above those measured for LUVs (data not shown).

In a study by Wu and co-workers (27), it was shown that the GM2AP-dependent degradation of micellar GM2 is increased by the additional presence of SAP-B in high concentrations (40-fold excess over GM2AP). The authors conclude that SAP-B might play a role in the degradation of GM2 *in vivo*. To test this hypothesis in our detergent-free assay system, we prepared LUVs containing 10 mol % [^{3}H]GM2 and either 70 mol % PC and 20 mol % Chol or 45 mol % PC, 20 mol % Chol, and 25 mol % BMP. GM2-containing LUVs were incubated with HexA in the presence of either SAP-B, GM2AP, or a mixture of the two activator proteins, using a 2-fold excess of SAP-B over GM2AP. In the absence of BMP, minimal GM2 degradation was detected in the presence of GM2AP and in the mixture of GM2AP and SAP-B, whereas no degradation took place in the presence of SAP-B alone, as expected (Fig. 3). Using BMP-containing liposomes, high degradation rates were detected in the presence of GM2AP. This, however, was not the case when SAP-B was used as activator protein (which, again, was expected), and no additional increase in the degradation rate was observed when SAP-B was added to GM2AP-containing incubation mixtures.

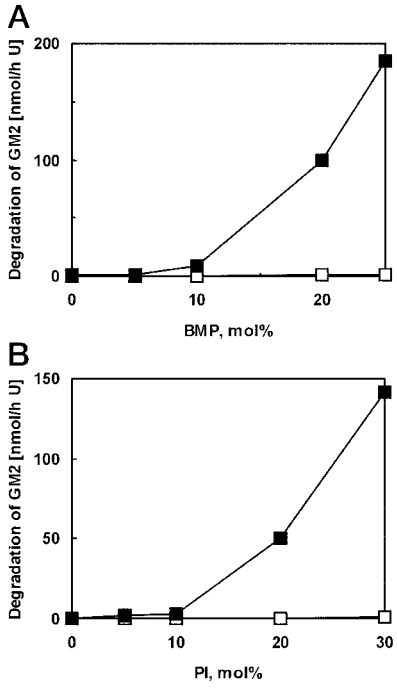

FIG. 2. **Lysosomal lipids in LUVs stimulate the enzymatic hydrolysis of ganglioside GM2 in the presence of GM2AP.** All assays were conducted with ganglioside GM2-carrying LUVs as substrates in the absence (□) and presence (■) of GM2AP (0.5 μM) as described under "Experimental Procedures," using LUVs with various proportions of synthetic BMP (0–25 mol %) (*A*) or PI (0–30 mol %) (*B*).

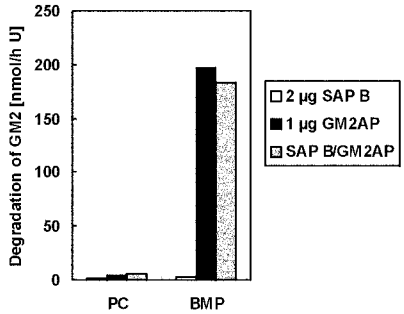

FIG. 3. **SAP-B does not stimulate the hydrolysis of membrane-bound ganglioside GM2 by β-hexosaminidase** *A*, degradation was measured with ganglioside GM2 carrying LUVs containing 80 mol % PC and 20 mol % Chol (*PC*) or 55 mol % PC, 20 mol % Chol, and 25 mol % BMP (*BMP*) in the presence of 2 μg of SAP-B, 1 μg of GM2-AP, or both activator proteins.

Since we do not observe an effect of SAP-B on the GM2AP-dependent degradation of GM2 in our liposomal system using similar amounts of SAP-B and GM2AP, we conclude that the effect observed in the micellar system by Wu and co-workers (27) does probably not play a role under physiological conditions.

Influence of Other Lysosomal Lipids on the Degradation of GM2 in Liposomes—Not only BMP, but also the negatively charged phospholipid PI, has been found in lysosomes in relatively high concentrations (15, 23–25). Dolichol is a polyisoprenoide and one of the largest lipids occurring in mammalian cells. It consists of an α-saturated isoprene unit and occurs either as a free alcohol or in its phosphorylated form as DoP. Both, dolichol and DoP, were found to occur in lysosomes in high concentrations compared with other organelles (18). GD1a is a negatively charged glycosphingolipid, the degradation of which takes place in the lysosomes. To investigate whether the stimulation of GM2 degradation by BMP is an unspecific effect of negatively charged lipids or of lipids that destabilize mem-

branes such as dolichol (18), the effect of these lipids on the degradation of liposomal GM2 was determined.

Liposomes containing 10 mol % [^{3}H]GM2 and increasing amounts of one of these four lipids were incubated with HexA either in the presence or in the absence of GM2AP. Neither dolichol nor GD1a or dolichol phosphate showed a significant stimulatory effect on GM2 degradation. Degradation rates were in the range of the measuring accuracy for all concentrations measured (0–2 nmol/h U) (data not shown). However, a pronounced stimulation was detected in the presence of PI and GM2AP (Fig. 2*B*). A concentration of 30 mol % PI in the liposomes stimulated the degradation of membrane-bound GM2 150-fold.

Interaction of GM2AP with Liposomes of Different Compositions

To analyze how GM2AP interacts with lipid bilayers, surface plasmon resonance measurements were performed. The effect of the presence of GM2 and acidic phospholipids in the lipid bilayers was studied in detail. In the first set of experiments, liposomes composed of neutral lipids and rising concentrations of GM2 were immobilized on a Pioneer L1 chip. The strongest binding of GM2AP to the immobilized lipid bilayer was observed when the membrane structures did not contain GM2 (Fig. 4*A*). Unexpectedly, the binding signal progressively decreased with rising amounts of GM2.

In the second set of experiments, 20 mol % of BMP were incorporated into the bilayers together with rising amounts of GM2 (Fig. 4*B*). Binding curves followed a similar pattern as observed for lipid bilayers without BMP, although the binding signals were somewhat reduced. The most notable effect of the presence of BMP was a dramatic drop of the binding signal shortly after GM2AP injection. This drop was most pronounced for membrane structures containing high amounts of GM2. In all bilayers containing GM2 and BMP, the signal dropped below the initial base-line value after about 360 s, reaching a minimum of −800 RU in the presence of 20 mol % GM2 (Fig. 4*B*).

To exclude the possibility that the effect observed for GM2AP is nonspecific, we studied the binding of bovine serum albumin and SAP-B to lipid bilayers containing BMP and GM2. Bovine serum albumin tethers very strongly (11,300 RU), whereas SAP-B binds weaker than GM2AP (250 RU). The binding curves follow a similar pattern as observed for lipid bilayers composed of neutral lipids and GM2 and show no dramatic drop of the binding signal as observed in the presence of BMP and GM2AP (data not shown).

Since the binding of GM2AP to gangliosides had been reported not to be completely specific for GM2 (5, 28), we decided to analyze whether a similar effect as that observed for GM2 was also observed for other glycolipids. To do this, we investigated the binding of GM2AP to lipid bilayers containing 10 mol % GM1, glucosylceramide, or GM3, all spiked with 10 mol % BMP, by surface plasmon resonance. The binding curve for GM3 showed a similar pattern as that observed for lipid bilayers doped with 10 mol % BMP only (Fig. 5). The same was true for glucosylceramide, although the total binding signal was reduced (data not shown). The binding curve of GM2AP to GM1, however, is comparable with the one observed for GM2, including the strong drop in the signal upon injection of the activator protein (Fig. 5). These results indicate that GM2AP differentiates between different glycolipids but is not completely specific for GM2, which is in accordance with earlier results (5, 28).

The observed drop in the plasmon resonance signal suggests that GM2AP can either extract lipids from immobilized lipid bilayers containing BMP and GM2 or GM1 or lead to a destabilization of the bilayers with concomitant loss of lipids.

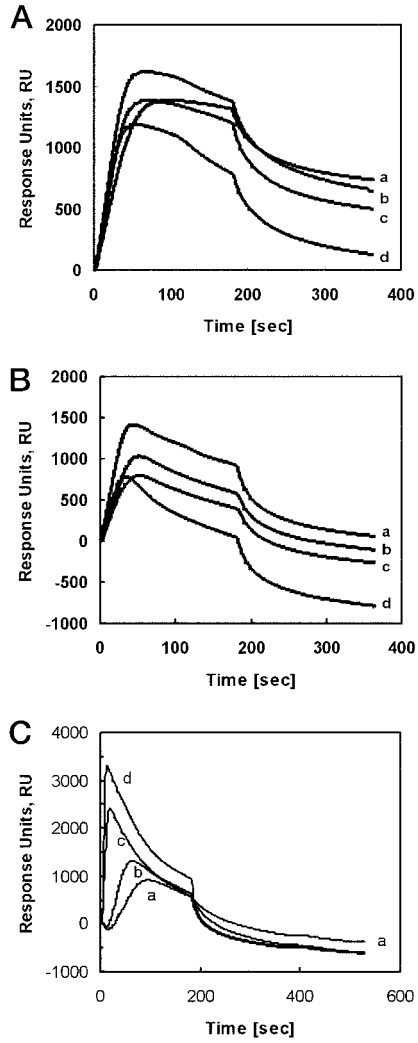

FIG. 4. **Interaction of GM2 activator protein with immobilized vesicles.** LUVs were immobilized on a Pioneer L.1 sensorchip. GM2AP (0.2 μM) was injected at a flow rate of 20 μl/min in 50 mM sodium citrate buffer (pH 4.2) for 180 s. In the next step, protein-free buffer was injected for 180 s. The measurement started with the injection of GM2AP. *A*, the binding curves were obtained with LUVs bearing Chol (20 mol %)/PC (various mol %) and 0 mol % GM2 (*trace a*), 5 mol % GM2 (*trace b*), 10 mol % GM2 (*trace c*), or 20 mol % GM2 (*trace d*). *B*, the binding curves were obtained with LUVs bearing Chol (20 mol %)/BMP (20 mol %)/PC (various mol %) and 0 mol % GM2 (*trace a*), 5 mol % GM2 (*trace b*), 10 mol % GM2 (*trace c*), or 20 mol % GM2 (*trace d*). *C*, the binding curves were obtained with LUVs bearing Chol (20 mol %)/PC (50 mol %)/BMP (20 mol %) and GM2 (10 mol %). GM2AP was injected with a concentration of 0.2 μM (*trace a*), 0.4 μM (*trace b*), 1 μM (*trace c*), or 2 μM (*trace d*).

DISCUSSION

GSLs are amphiphilic components of the plasma membrane of eucaryotic cells. After endocytosis, GSLs reach the lysosomal compartments of the cells where they are catabolized by a series of lysosomal glycosidases. According to our recent model of endocytosis and lysosomal digestion (1, 2), GSLs are degraded as components of intraendosomal and intralysosomal membrane structures, rather than as components of the limiting lysosomal membrane. Such intralysosomal vesicles with a diameter in the range of 50–100 nm were detected in the biopsy tissue of a patient with prosaposin deficiency (29). Several factors such as membrane curvature, membrane pressure, and lipid composition may make such vesicles more accessible for the lysosomal hydrolases than the limiting membrane. To test this hypothesis, we studied the enzymatic hydrolysis of GM2, which requires lysosomal HexA and GM2AP as a cofactor (30–

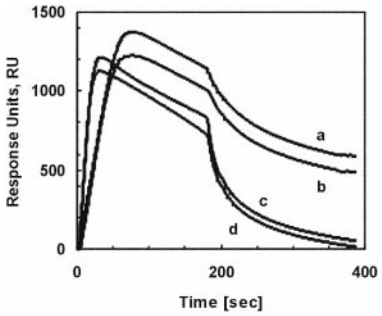

FIG. 5. **Interaction of GM2 activator protein with immobilized vesicles.** LUVs were immobilized on a Pioneer L.1 sensorchip. GM2AP (0.2 μM) was injected at a flow rate of 20 μl/min in 50 mM sodium citrate buffer (pH 4.2) for 180 s. In the next step, protein-free buffer was injected for 180 s. The measurement started with the injection of GM2AP. The binding curves were obtained with LUVs bearing Chol (20 mol %)/PC (60 mol %)/BMP (10 mol %) and GM3 (10 mol %) (*trace a*), PC (10 mol %) (*trace b*), GM2 (10 mol %) (*trace c*), or GM1 (10 mol %) (*trace d*).

32) in a detergent-free assay system mimicking the intralysosomal conditions. The studies were performed at pH 4.2, which is optimal for the enzymatic reaction and typical for the lysosomal content (33). The substrate was presented to the HexA as a component of liposomal membranes, either of LUVs with an average diameter of 100 nm or of SUVs with an average diameter of 40 nm. Liposomes were composed of lipids that had been detected in the acidic compartment of the cell or had been specifically localized to its inner membranes as BMP. In contrast to the presentation of the substrate as micelles, this is believed to be the most appropriate model for the lysosomal digestion of membrane-bound GSL substrates.

The incorporation of acidic lipids occurring in lysosomes such as BMP or PI into the liposomes led to a strong stimulation of the degradation of GM2 in the presence of GM2AP (up to 180-fold at 25 mol % BMP). A significant enzymatic degradation of GM2 was only observed in the presence of both, an anionic lipid and the GM2AP.

Although the concentrations of acidic lipids used in this study are quite high, they can still be considered to be in a realistic physiological range. The determination of lipid concentrations in cellular organelles is difficult, and results vary considerably with the cells and the method of organelle isolation used. Nevertheless, several investigations found increased amounts of BMP and PI in lysosomes. The relative amounts of BMP in lysosomes that were reported vary between 7 mol % (23) and 28 mol % (24), but most reports state values of more than 10 mol % of total lysosomal lipid. The corresponding values determined for PI are in the order of 5–10 mol % (25). The determination of lipid concentrations on intraendosomal and intralysosomal membrane structures was not possible to date, so that a further enrichment of the lipids in these structures has to be considered. For BMP it has been demonstrated immunologically that it is localized specifically on intraendosomal membranes (15). Anionic lipids like PA, sulfatide, etc., which also stimulated GM2 hydrolysis *in vitro*, have not been detected in lysosomes and are therefore of no physiological significance.

Other lipids that occur in the lysosomes such as dolichol, phosphatidylcholine, or dolichol phosphate did not have a similar stimulatory effect on the degradation of GM2 as it was observed for BMP and PI. Acidic gangliosides such as GD1a, which are degraded in the lysosomes and have been shown to disintegrate phospholipid bilayers at concentrations above 20 mol % (34), also showed no significant stimulatory effect at concentrations between 2.5 and 20 mol %. This demonstrates that the effect is not due to an unspecific destabilization of the membranes by anionic lipids. The fact that dolichol or its phosphate do not stimulate the reaction, although they are known to strongly destabilize membranes (18), implicates that a more complex mechanism than the simple perturbation of the membrane is the basis for the stimulatory effect of BMP and PI.

A direct interaction of the acidic lipids with the degrading enzyme, as it has been proposed for the degradation of glucosylceramide by glucocerebrosidase and SAP-C (4), has to be ruled out though, since the degradation of the water-soluble synthetic substrate MUG is not affected by the presence of either GM2AP or liposomes containing acidic lipids.

The specificity of the stimulatory effect of GM2AP on the reaction is high. An additional stimulation of the reaction by SAP-B, which was reported by Wu and co-workers for micellar GM2 (27) was not reproduced in our liposomal system using similar concentrations of both activators. We therefore conclude that this effect is not physiologically significant.

To find out more about the mechanism by which BMP stimulates the lysosomal degradation of GM2, the binding of GM2AP to immobilized lipid bilayers was studied by surface plasmon resonance spectroscopy. Our Biacore studies demonstrate that at pH 4.2 GM2AP binds to neutral lipid bilayers composed of PC and Chol as well as to anionic bilayers, which in addition contain GM2 and/or BMP. At pH 7, the binding of GM2AP to all liposomes tested was much weaker. However, the presence of BMP in the membrane structures did not lead to an increase of GM2AP binding (at pH 4.2) but to a significant drop of the resonance signal even below the base line (Fig. 4*B*). This effect was not observed in the absence of BMP and indicates a solubilization of lipids from the membranes in the presence of both, BMP and GM2AP, which increases with increasing ganglioside GM2 concentrations. This solubilization is also dependent on concentrations of GM2AP (Fig. 4*C*). Other proteins (bovine serum albumin, SAP-B) also bind to the membrane, but do not lead to a significant drop of the resonance signal, which indicates that the effect observed is specific for GM2AP and is not caused by an unspecific replacement of lipid structures by protein. These observations suggest an interaction of GM2AP with BMP, which leads to destabilization of the membrane.

To investigate the specificity of the observed effect for the ganglioside GM2, we used bilayers containing GM1, GM3, or glucosylceramide in the plasmon resonance measurements. Lipid bilayers containing GM3 or glucosylceramide showed a similar GM2AP binding curve as membranes devoid of ganglioside, whereas the GM2AP binding behavior is comparable for bilayers containing either GM1 or GM2, including the strong drop of the signal upon injection of GM2AP. This demonstrates that GM2AP shows some specificity but is not completely specific for GM2, which is in agreement with earlier glycolipid transfer studies using GM2AP (5, 28). An interesting point is that studies on the enzymatic degradation of GM1 led to the conclusion that the GM2AP, similar to SAP-B, stimulates this degradation step in the presence of BMP (35). Whether, therefore, the strong drop in the plasmon resonance signal, which was observed for GM1 and GM2, is due to the same effect that causes the activator function of GM2AP is still speculative, but an interesting point to consider.

The BMP-induced stimulation of glycolipid degradation is a phenomenon that is not limited to GM2 and the GM2AP. Similar results were obtained for the degradation of glucosylceramide by glucocerebrosidase and SAP-C (4) and of GM1 by β-galactosidase and SAP-B or GM2AP (35). These results together with the data presented here strongly support our hypothesis that the lysosomal degradation of glycosphingolipids takes place on intralysosomal membrane structures rather than in the limiting lysosomal membrane. BMP and presumably PI as components of these structures may contribute to a

selective destabilization of such membrane structures, whereas the BMP-free limiting lysosomal membrane (15) is not affected. The specific lipid composition of different membrane structures may therefore serve to differentiate between them, protecting the limiting lysosomal membrane from digestion on one hand and marking the components of intralysosomal membranes for degradation on the other.

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