Membrane lipids and their degradation compounds control GM2 catabolism at intralysosomal luminal vesicles

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Running title: GM2 catabolism is regulated by lysosomal storage compounds

Abbreviations: BMP: bis(monoacylglycerol)phosphate, Cer: ceramide, Chol: cholesterol, DAG: diacylglycerol, DOPC: dioleoyl-L-α-phosphatidylcholine, GM2AP: GM2 activator protein, g-rGM2AP: glycosylated recombinant GM2 activator protein, g-rGM2AP-His6: glycosylated recombinant GM2 activator protein with His6-tag, Hex A: β-hexosaminidase A, His6-tag: hexahistidine-tag, ILV: intralysosomal luminal vesicle, lyso-PC: lysophosphatidylcholine, MUF: 4-methylumbelliferone, MUGS: 4-methylumbelliferyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside, RU: resonance units, Sa: sphinganine, So: sphingosine, SPR: surface plasmon resonance
Abstract

The catabolism of ganglioside GM2 is dependent on three gene products. Mutations in any of these genes result in a different type of GM2 gangliosidosis (Tay-Sachs disease, B1 variant, Sandhoff disease and the AB-variant), with GM2 as major lysosomal storage compound. GM2 is also a secondary storage compound in lysosomal storage diseases like Niemann-Pick disease type A, B and C with primary storage of SM and cholesterol, respectively. Reconstitution of GM2 catabolism at liposomal surfaces carrying GM2 revealed that incorporation of lipids into the GM2 carrying membrane like cholesterol, SM, sphingosine and sphinganine inhibit GM2 hydrolysis by β-hexosaminidase A assisted by GM2 activator protein, while anionic lipids, ceramide, fatty acids, lyso-phosphatidylcholine and diacylglycerol stimulate GM2 catabolism.

In contrast, the hydrolysis of the synthetic, water soluble substrate 4-methylumbelliferyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside was hardly affected by membrane lipids such as ceramide or SM, nor was it stimulated by anionic lipids like bis(monoacylglycerol)phosphate, either added as liposomes, detergent micelles or lipid aggregates.

Moreover, we could show that hydrolysis inhibiting lipids had also an inhibiting effect on the solubilization and mobilization of membrane-bound lipids by GM2 activator protein, while the stimulating lipids enhanced lipid mobilization.

Keywords: lipid transfer protein, sphingolipids, gangliosides, cholesterol, sphingomyelin, bis(monoacylglycerol)phosphate, sphingosine, GM2 activator protein, solubilization of lipids, enzymology of membrane lipids
Introduction:

Amphiphilic lipids and proteins are ubiquitous building blocks of biological membranes, preserving specific pattern in subcellular membranes. Intralysosomal luminal vesicles (ILVs) are the platform for the catabolism of membrane lipids (1). Their lipid composition varies from the limiting endosomal and lysosomal perimeter membranes by a much lower cholesterol (Chol) content, an enhanced occurrence of bis(monoacylglycero)phosphate (BMP) (2-4) and the absence of a glycocalix. A disturbance of the physiological lipid composition in the lysosomal system may well contribute to the molecular and clinical pathology of a multitude of lysosomal storage disorders (5, 6).

To enable a physiological turnover of membrane lipids, they have to reach the ILVs of the endolysosomal system e.g. by endocytotic membrane flow. Along the endocytotic pathway the lipid composition of the intraendolysosomal vesicles to the ILVs changes radically. Plasma membrane stabilizing lipids are sorted out, e.g. Chol is secreted by Niemann-Pick protein type 1 and 2 (7-9), the anionic BMP accumulates as an intermediate of phosphatidylglycerol catabolism (10) whereas a multiplicity of membrane lipids is depleted, e.g. SM by degradation (11, 12). Proper catabolism of sphingolipids with short oligosaccharide head groups requires special lipid binding and transfer proteins, so called sphingolipid activator proteins, which mediate the interaction of the membrane-bound lipid substrate and the water-soluble enzyme (13-16). They consist of the saposins A-D and the GM2 activator protein (GM2AP).

It is known that (glyco)sphingolipid hydrolysis is enhanced by BMP (17-25), an anionic lipid of the ILVs, which is of functional significance and acts as a stimulating lipid modifier (23). It places negative surface charge to the luminal vesicles (18, 22, 23), even
at the low pH range in the lysosome (pH 4-5) (26, 27). The GM2AP possesses an isoelectric point around pH 4.8 (15), caused by the stacked occurrence of lysine residues, what results in positively charged surface domains at low lysosomal pH values and makes an allowance to the interaction with the ILVs covered by a negative zeta potential which becomes more negative by the anionic membrane-bound BMP and other anionic lipids (22).

The GM2AP, which is known to be a quite promiscuous lipid binding protein (28, 29), forms a stoichiometric complex with membrane-bound ganglioside GM2, which is recognized by β-hexosaminidase A (Hex A), forming a soluble Michaelis-Menten complex (14, 30). Until now, it is not exactly known whether the catabolism of GM2 takes place at the surface of the ILVs, in solution or at both ways simultaneously. Mutations that affect the function of Hex A or GM2AP can inhibit the crucial catabolic pathway, resulting in lysosomal storage diseases such as Tay-Sachs disease, Sandhoff disease, AB variant or B1 variant of GM2 gangliosidosis (31). Furthermore GM2 accumulates as secondary storage compound in different lysosomal storage disorders, for example in Niemann-Pick disease (32, 33) and in different mucopolysaccharidoses (34).

SM or Chol are the primary storage compounds in Niemann-Pick disease type A, B and C, respectively. The hydrolysis of GM2 is inhibited by both lipids (23). We now analyzed the influence of other membrane lipids and their degradation intermediates occurring in the lysosomal compartment, ceramide (Cer), fatty acids, lysophosphatidylcholine (lysophosphatidylcholine (lyso-PC), diacylglycerol (DAG), sphingosine (So) and sphinganine (Sa). All tested lipids influenced the catabolism of membrane bound GM2 by Hex A assisted by GM2AP substantially, while the turnover of the synthetic, water soluble substrate 4-
methylumbelliferyl-6-sulfo-2-acetamido-2-deoxy-β-D-glycopyranoside (MUGS) by Hex A was not affected by membrane lipids.
Materials and Methods

Materials

1,2-Dioleoyl-sn-glycerol-3-phosphocholine (DOPC), So and C18:1-BMP were purchased from Avanti Polar Lipids (Alabaster, AL). D-erythro-stearoyl-ceramide, and GM2 were available in our laboratory. Chol, 1,2-dipalmitoylglycerol (DAG), Sa, lyso-palmitoyl-PC, stearic acid, 4-methylumbelliferone (MUF), and MUGS were purchased from Sigma (Taufkirchen, Germany). Stearoyl-SM was from Matreya (Pennsylvania, PA) All other chemicals were obtained from Merck and Sigma. All other chemicals and solutions were of analytical grade.

Synthesis of [14C] GM2

Synthesis of [14C] GM2 was synthesized from its corresponding lyso-lipid, following published procedures (35).

Protein preparation

Preparation of glycosylated recombinant GM2AP (g-rGM2AP) and glycosylated recombinant GM2AP with hexahistidine-tag (g-rGM2AP-His6) was done as described before (23). Hex A was purified from human placenta to apparent homogeneity as described before (36).

Preparation of liposomes

Large unilamellar vesicles were prepared as previously described (23, 37). Liposomes without BMP contained 5 mol% Chol, 2 mol% assay specific lipids as [14C] GM2, the lipid of interest (0-40 mol%) and DOPC as a host lipid in 20 mM sodium citrate buffer,
pH 4.2. BMP containing liposomes, carrying additional negative net charge, contained additive 20 mol% BMP. The amount of DOPC was adjusted to the varied lipid composition. Lipid concentration was 50 mM.

**Hex A activity assay with soluble substrate MUGS**

In this assay the turnover of MUGS to MUF was measured.

2 mU Hex A and 0.25 mM MUGS were incubated in 20 mM sodium citrate, pH 4.2 (final volume of 80 µl) at 37°C for 30 min. After that the assay was stopped with 400 µl stop-solution (0.2 M Na₂CO₃ + 0.2 M glycine, pH 9.8). Fluorescence of the formed MUF was measured with a spectrofluorophotometer (RF-5000, Shimadzu, Düsseldorf, Germany). Fluorescence measurements were performed using an excitation wavelength of 365 nm and an emission wavelength of 440 nm.

Beside this basic assay composition liposomes, Triton-X 100 micelles or lipid aggregates were added. Lipid composition of all three formulations was 5 mol% Chol, 20 mol% BMP, 0-40 mol% Cer or SM made up to 100 mol% by DOPC, lipid concentration was 50 mM.

The added liposomes were prepared as shown above.

For forming Triton-X 100 micelles appropriate amounts of lipids from stock solutions were mixed and dried under a stream of nitrogen. The lipid mixture was then dispersed in 1 ml of 20 mM sodium citrate buffer pH 4.2 containing 625 µg Triton-X 100. The dispersion was vortexed and sonified in a sonifier bath for 15 min.
To build lipid aggregates the dried lipid mixture was dispersed in 1 ml of 20 mM sodium citrate buffer pH 4.2. The dispersion was vortexed and sonified for 3x3 min (Brandson Sonifier 250, BRANSON Ultrasonics Corporation, Danbury, U.S.A.).

In case of addition of g-rGM2AP the added amount was 0.06 pmol.

The amount of generated MUF was determined by a straight calibration line of 0, 1, 2, 4, 6, 8, 10 nmol MUF in a volume of 80 µl mixed with 400 µl stop-solution.

**Liposomal activity assay with GM2AP and Hex A**

Preparation of vesicles was done as shown above. Lipid concentration of the vesicles containing 2 mol% [14C] GM2 was 0.5 mM. The assay was done following the published procedure (23).

The 40 µl liposome dispersion was mixed with 2 mU Hex A and for BMP containing vesicles with 0.06 pmol g-rGM2AP or g-rGMAP-His6, for vesicles free of BMP with 0.3 pmol g-rGM2AP or g-rGMAP-His6, made up to 80 µl with 20 mM sodium citrate buffer pH 4.2. Different amounts of GM2AP were chosen, because the GM2 turnover at membranes free of BMP is quite small (23). The samples were incubated at 37°C for 30 min. Afterwards the assay was put on ice and stopped by adding 20 µl chloroform/methanol (1:1, v:v).

Quantification of the generated [14C] GM3 from [14C] GM2 was done by thin layer chromatography. Therefore the preparations were dried under a stream of nitrogen, re-dissolved with 20 µl chloroform/methanol (1/1, v/v), vortexed and sonified for 15 min. After that, the solution of lipids was applied to a high-performance thin-layer chromatography plate (Merck, Darmstadt, Germany). Lipids were separated in
chloroform/methanol/0.22% CaCl$_2$ (55/45/10, v/v/v). Radioactive bands were visualized with Typhoon Fla 7000 and the quantification was performed with the image analysis software Image Quant TL (both GE healthcare, Buckinghamshire, UK).

The mean error was less than 10%.

**Lipid mobilization assay by surface plasmon resonance spectroscopy (SPR)**

The lipid mobilization assay was done following the published procedure (23).

SPR, a biomolecular interaction analysis, was carried out at 25°C with a Bialite instrument (Biacore, now GE healthcare, Buckinghamshire, UK).

In the lipid mobilization assay negatively charged liposomes with 5 mol% Chol, 20 mol% BMP and 10 mol% GM2 in 20 mM sodium citrate buffer (pH 4.2) were used (unless stated otherwise).

Sensorchips providing an immobilized surface with lipophilic anchors attached to a dextran matrix (Pioneer HPA chip) were obtained from Biacore. 100 µl vesicles (0.5 mM total lipid concentration) in 20 mM sodium citrate buffer, pH 4.2, were injected into the system at a flow rate of 5 ml/min. This resulted in a shift of approximately 2000 resonance units (RU), which corresponds to a lipid monolayer (see manual of Pioneer HPA chip). The lipid layer loaded chip was set as “baseline” (RU=0) and corresponds to 100% of loaded lipid material.

0.2 mM GM2AP in running buffer (20 mM sodium citrate buffer, pH 4.2) was injected into the flow cells at a rate of 20 ml/min for 3 min, followed by buffer alone.

The RUs above the baseline (yellow) represent material bound to the lipid layer during the experiment, whereas negative RUs below the baseline represent material loss,
which not only includes GM2AP added during the experiment, but also lipid material mobilized and released from the lipid layer. However, it remains unclear for all data above the baseline (RU=0), if the loss is due to removal of lipid or to a removal of both, a mixture of protein and lipid.

Only if the RU value drops below the baseline it is certain, that lipids from the lipid layer have been removed, in amounts equal to the RU values given or more, in case some unknown amount of protein still stays bound to the chip. Release and mobilization of liposomal lipids have been analyzed before by using radiolabeled membrane lipids (38).

Presentation of Data — All data are means of at least triplicate.
Results

Whereas soluble macromolecules can be degraded directly by soluble hydrolases, special mechanisms are necessary for the turnover of membrane bound (glyco)sphingolipids. The lipid binding and transfer protein, GM2AP, is known to play an essential role in the catabolism of GM2 by Hex A at the ILVs (15). As shown before, the catabolism of GM2 by the water-soluble Hex A occurs in cooperation with GM2AP and is strongly modified by the lipid composition of the GM2 carrying membranes (17, 23, 39). To investigate this coherence more precisely we reconstituted GM2 catabolism at ILVs of the lysosomal compartment in a liposomal system. Further on we used a lipid mobilization assay to analyze the g-rGM2AP mediated solubilization of membrane lipids and their degradation products occurring in the lysosomes, such as SM, Chol, DAG, Cer, lyso-PC, free fatty acids, So and Sa.

For comparison we also analyzed the influence of membrane lipids on the turnover of the synthetic, water soluble substrate MUGS by Hex A.

The cleavage of soluble MUGS is almost independent of the presence of GM2AP and membrane lipids

The soluble, synthetic substrate MUGS is usually used to assay Hex A activity in research for lysosomal storage disorders (40, 41). It is known that Hex A shows a broad pH profile (3.0-7.6) against soluble, synthetic substrates (42), but an extremely sharp one at pH 3.5-4.6 for the hydrolysis of membrane bound GM2 in the presence of its cofactor GM2AP (17, 23, 39, 43).

As a control for the strong dependence of GM2 hydrolysis by Hex A in presence of GM2AP on the lipids of the GM2 carrying membranes, such as BMP, Cer and SM (17,
23) we assayed the influence of membrane lipids on the cleavage of the synthetic soluble substrate MUGS by Hex A. The MUGS-turnover by Hex A was analyzed in the absence and presence of membrane lipids, liposomes, Triton-X 100 micelles, “lipid aggregates” and/or g-rGM2AP. Liposomes were used to mimic ILVs, Triton-X micelles were chosen because Hex A is able to cleave GM2 bound to detergent micelles (44-47). “Lipid aggregates” were free of detergent to suspend severe side effects eventually caused by the use of detergent in Triton-X 100 micelles.

The cleavage of the soluble synthetic substrate MUGS is almost completely independent of GM2AP, the presence of membrane liposomes (Fig. 1A), lipid aggregates or Triton-X 100 micelles (data not shown), while the hydrolysis of membrane-bound, liposomal GM2, strongly depends on all of these factors (Fig. 1B).

A physiological relevant hydrolysis of membrane-bound [14C] GM2 by Hex A was only detectable in the presence both GM2AP and anionic lipids like BMP (Fig. 1B, left chart). Addition of 40 mol% Cer to BMP containing liposomes resulted in a 4-fold stimulation of GM2 catabolism by Hex A in presence of GM2AP, while the addition of 40 mol% SM to BMP containing liposomes reduced GM2 hydrolysis to 40% (Fig. 1B, right chart). The GM2AP independent turnover of the synthetic, soluble substrate by Hex A was independent of the addition of liposomes in every composition (Fig. 1A) and also independent of addition of Triton-X 100 micelles or lipid aggregates in similar compositions (data not shown).
Storage compounds of Niemann-Pick diseases, SM and Chol inhibit GM2 turnover and lipid mobilization

SM is degraded by acid sphingomyelinase to Cer in the late endosomal compartment but accumulates in ASM deficient Niemann-Pick disease type A and B. It is known that in Niemann-Pick disease type A and B the storage of SM provokes the secondary accumulation of Chol. However, the storage of Chol in Niemann-Pick disease type C triggers the secondary accumulation of SM (48, 49). In previous studies we could show, that both lipids are of great significance for the catabolism of liposomal bound GM2 (23). Using liposomal reconstitution experiments, consisting of liposomes carrying \(^{14}\text{C}\) GM2 which is not able to leave the membrane spontaneously because of its hydrophobic ceramide tail (containing two long hydrophobic chains), we now analyzed those coherences more precisely (Fig.2, 3). On the other hand we did lipid mobilization assays to analyze the lipid solubilization properties of GM2AP (Fig. 4). The resonance units (RU) above the baseline (yellow) represent material bound to the lipid layer during the experiment, whereas negative RUs below the baseline represent loss of lipid material mobilized and released from the lipid layer.

Any increase of RU values indicates binding of additional material (e.g. g-rGM2AP) to the lipid layer and any drop of RU values indicates loss of material, GM2AP or lipids, from the sensor chip. For detailed information see material and methods. Release and mobilization of liposomal lipids have been analyzed before by using radiolabeled membrane lipids (38).

A dose dependent inhibitory effect of SM on GM2 hydrolysis by Hex A was obtained in presence of GM2AP using BMP-containing, negatively charged liposomes (Fig. 2 A). It
was also observed in BMP-free liposomes (Fig. 3 A), while the level of GM2-turnover reached only 12-17% of that observed in case of the much more negatively charged, BMP-containing, liposomes. SM also retarded the lipid mobilization by g-rGM2AP strongly (Fig. 4 A) in a dose dependent manner. At 30-40 mol% SM in the liposomal preparation, g-rGM2AP bound to the lipid layer, but hardly released any material even when buffer was injected.

In the late endosomal compartment Chol is sorted out from the inner endosomal membranes by NPC2 and NPC1 proteins, avoiding the inhibition of GM2 catabolism (4, 10). High Chol levels inhibited both GM2 hydrolysis and lipid mobilization by GM2AP (Fig. 2 B, Fig. 3 B and Fig. 4 B), the inhibiting effect was tightened and enhanced by rising Chol concentrations. Using BMP-containing liposomes with an enhanced negative net charge and 40 mol% Chol, the turnover of GM2 was reduced down to 30% compared to liposomes containing only 5 mol% Chol (Fig. 2 B). A similar effect was also observed using liposomes free of BMP (Fig. 3 B) but the amount of hydrolyzed GM2 was only 12-20% compared with the GM2 turnover using BMP-containing liposomes.

Enhanced Chol concentration caused a drastically reduced mobilization of lipids. Introducing 30-40 mol% Chol into the liposomal preparation resulted in an enhanced binding of g-rGM2AP to the lipid layer but prohibited any lipid mobilization (Fig. 4 B).

**Lysosomal degradation products, Cer, DAG, lyso-PC and stearic acid stimulate GM2 hydrolysis and lipid mobilization by GM2AP**

Lysosomal degradation of phospho- and glycolipids generates lipid products such as lyso-PC, DAG, Cer and fatty acids in the lysosomal compartment. Their influence on
GM2 hydrolysis was examined. The stimulating effects of DAG, Cer, stearic acid and lyso-PC on GM2 hydrolysis in negatively charged, BMP containing, GM2 carrying liposomes are shown in Fig. 2 C-F.

Cer arises in the late endosome as a degradation product of SM and glycosphingolipids (19, 22). Whereas SM inhibits GM2 catabolism (Fig. 2 A, Fig. 3 A), Cer stimulates it. (Fig. 2 D, Fig. 3 D) (23). The hydrolysis of GM2 was enhanced with heightened concentrations of the lipid of interest. The highest increase of GM2 hydrolysis in presence of g-rGM2AP was observed in the presence of 40 mol% lyso-PC (enhancement to nearly 6 fold) (Fig. 2 F) or stearic acid (enhancement 5.5 fold) while Cer at 40 mol% was slightly less effective (Fig. 2 D).

Though the rate of GM2 hydrolysis using liposomes free of BMP (Fig. 3) was about an order of magnitude lower than in BMP containing liposomes (Fig. 2), the stimulating effects of DAG, Cer, stearic acid and lyso-PC could still be detected (Fig. 3 C-F). 40 mol% DAG nearly doubled GM2 hydrolysis by Hex A in presence of r-gGM2AP (Fig. 3 C) while Cer at 40 mol% enhanced GM2 turnover more than 2-fold (Fig. 3 D) and lyso-PC at 40 mol% led to an 2.6 fold stimulation of GM2 turnover (Fig. 3 F).

The catabolism of GM2 by Hex A was always enhanced more effectively when the more protonated g-rGM2AP-His₆ with hexahistidine-tag (His₆-tag) was used instead of the natural occurring g-rGM2AP.

It was also observed, that the stimulating lipids DAG, Cer, stearic acid and lyso-PC facilitate the solubilization and mobilization of membrane lipids by GM2AP (Fig. 4 C-F). In control experiments without lipid modifiers (red lines in Fig. 4) injection of g-rGM2AP in the early beginning resulted in a little increase of RUs, corresponding to an absorption
of GM2AP at the lipid layer. The RU signal than dropped below the baseline during the injection of g-rGM2AP, and proceeded descending after injection of buffer, indicating release of membrane lipids (and g-rGM2AP). The addition of DAG (10-40 mol%) resulted in a concentration dependent acceleration of lipid release but did not enhance the overall amount of lipids released (Fig. 4 C). 70% of lipid material bound to the chip were released from the chip in the control experiment. The addition of 30-40 mol% DAG to the liposomal preparation resulted only in a small increase of lipid mobilization up to 75% (Fig. 4 C). In case of 10 mol% Cer the total amount of mobilized material was extremely reduced to $\frac{1}{3}$ of the control (0 mol% Cer, red line), whereas 20-30 mol% of Cer were less inhibitory (Fig. 4 D). 30 mol% Cer resulted in 70% mobilization of lipid material, comparable to liposomes free of Cer. Using 40 mol% Cer lipid mobilization was slightly enhanced, resulting in 75 mol% (Fig. 4 D).

The addition of stearic acid (10-40 mol%) led to a reduced and concentration dependent binding of g-rGM2AP to the lipid layer and an accelerated and more complete mobilization of lipids up to 85% of the loaded lipids (Fig. 4 E).

Low levels of lyso-PC (10-20 mol%) in the liposomal preparation enhanced the binding of g-rGM2AP to the lipid layer but the signal did not undergo the baseline until buffer was injected. After injection of buffer the signal dropped slightly beyond the baseline, indicating mobilization of lipids, but did not reach the level which was achieved using liposomes without lyso-PC. A lyso-PC concentration of 20 mol% resulted in a small amount of released lipids even before injection of buffer. Enhancing the lyso-PC concentration to 30 or 40 mol% led to an accelerated mobilization of membrane lipids, indicated by undergoing the control line (red), already before buffer was injected. The
final amount of lost material (lipids and protein) was not enhanced compared to measurements without lyso-PC. So lyso-PC levels (10-20 mol%) reduced lipid mobilization, while higher concentrations (30-40 mol%) mobilized lipids faster compared to the control experiment (red line) (Fig. 4 F).

Whereas g-rGM2AP steadily mobilized lipids from BMP containing liposomes (Fig. 4) it did not mobilize any lipids from BMP-free, less negatively charged lipid layers (Fig. 5). Even the incorporation of Cer (0-40 mol%) or stearic acid (0-40 mol%) did not lead to lipid mobilization by g-rGM2AP.

g-rGM2AP bound to the BMP-free lipid layer containing Cer concentrations of 0-40 mol% at an assay pH of 4.2. With injection of buffer some amount of the protein was released but the signal did not reach or undergo the baseline, indicating no release of lipids (Fig. 5 A). By the use of BMP-free liposomes containing stearic acid (0-40 mol%) binding of g-rGM2AP to the lipid layer was slightly reduced by enhanced concentrations of the fatty acid. Injection of buffer resulted in the release of a part of g-rGM2AP. The signal dropped but did not reach the baseline, producing no evidence for the removal of lipids.

Cationic sphingoid bases, stored in NPC, inhibit GM2 hydrolysis and lipid mobilization by GM2AP

The cationic sphingoid bases So and Sa arise in the lysosomal compartment by the degradation of Cer. Their levels are increased in some lysosomal storage disorders like NPC, where they are thought to increase the pH-value (50-53). The cationic amphiphiles, So and Sa turned out to inhibit the catabolism of GM2 by Hex A in presence of GM2AP strongly, using negatively charged liposomes with BMP (20 mol%)
Adding 20-40 mol% So or Sa to the liposomal membrane blocked the catabolism of GM2 completely, an inhibition is also observed with liposomes free of BMP (Fig. 3 G, H), where the rate of GM2 hydrolysis was reduced to 6-17% compared to BMP-containing ones.

Similar effects were seen with g-rGM2AP-His, carrying a His6-tag, though the GM2 turnover was always increased compared to experiments with natural occurring, untagged g-rGM2AP.

Also the lipid mobilization was inhibited by So and Sa in a dose dependent manner. Enhancing the liposomal level of the sphingoid bases So and Sa reduced the mobilization of membrane lipids by g-rGM2AP strongly. Adding 30-40 mol% So to the liposomal formulation g-rGM2AP bound strongly to the lipid layer. After injection of buffer the protein dissolved, slightly undergoing the baseline, suggesting a minor mobilization of membrane lipids (Fig. 4 G). When 30-40 mol% Sa were used, g-rGM2AP bound to the membrane layer, dissolved when buffer was added but did not undergo the baseline (Fig. 4 H).

The six histidine residues at the C-terminal end of g-rGM2AP-His6 do not only have strong influence on hydrolysis of GM2 by Hex A using various lipid compositions. The His6-tag also inhibited mobilization of membrane lipids by GM2AP as observed before (23). Because of the unnatural behavior of the His6-tagged protein we did not use it for SPR studies.
Discussion

Membrane lipids play a central role as regulators and modifiers of cellular functions mediated by the plasma membrane and organellar membranes. The specific composition of subcellular membranes, including their lipid pattern, is significant for the function of membrane properties (54), e.g. high concentration of Chol in the plasma membrane for the regulation of Na+, K+-ATPase (55).

Whereas Chol and SM are stabilizing the plasma membrane, they are inhibitory for some key reactions in lysosomal glycosphingolipid catabolism (6). On the other hand, the catabolism of (glyco)sphingolipids such as GM2 (17, 23), GM1 (18), sulfatide (21), glycosylceramide (25), Cer (19) and SM (22) and phospholipids (56) is extremely enhanced by anionic lipids as BMP at lysosomal pH values, mainly due to an electrostatic binding of protonated cationic lysosomal proteins to the negatively charged membranes of the liposomes and the ILVs carrying the (glyco)sphingolipid to be digested. The ILVs are enriched with BMP and may contain other anionic phospholipids which carry a negative charge at low pH-values, resulting in a negative zeta potential of the ILV membranes (22). A cartoon of the conditions at the ILVs in the lysosome can be seen in Fig. 6.

Disturbed lysosomal sphingolipid degradation, often caused by an inherited hydrolase deficiency, results in lysosomal storage disorders, a group of at least 40 hereditary diseases. In most of those disorders several lipids accumulate, indigestible primary storage compounds, and others, as secondary storage material (57).

In this study we examined the regulatory and modifying influence of some primary storage compounds and a multitude of membrane lipids and their degradation products on the catabolism of membrane bound GM2 by Hex A assisted by GM2AP in
comparison to their influence on the turnover of the synthetic, water soluble substrate MUGS by Hex A, often used to assay Hex A activity in patients samples with lysosomal storage disorders.

Addition of increasing concentrations of bilayer destroying lipids to the liposomal membranes, fatty acids, sphingoid bases (Sa, So) and DAG, however, may well generate other lipid aggregates than lipid bilayer liposomes, e.g. lipid layers, aggregates or micelles. However this would also apply to ILV in the living cell that incorporate those lipids released as intermediates of lipid catabolism at higher concentrations.

Membrane lipids and their degradation compounds regulate GM2 hydrolysis and the lipid mobilization by GM2AP, but do not affect Hex A catalyzed hydrolysis of soluble synthetic substrates

The GM2-cleaving Hex A is a promiscuous glycosidase, hydrolyzing a multitude of soluble β-N-acetyl-glucosaminides and -galactosaminides, e.g. oligosaccharides, mucopolysaccharides, glycolipids and synthetic, soluble substrates like MUGS (44, 58). Though Hex A can attack soluble substrates like MUGS directly, soluble Hex A does not recognize amphiphilic, membrane bound substrates like GM2 in absence of the cofactor GM2AP (42, 59, 60) but it interacts with the stoichiometric GM2-GM2AP-complex as substrate, forming a Michaelis-Menten complex (61) (Fig. 6 A). The inherited absence of GM2AP causes GM2 storage in AB-Variant of GM2-gangliosidoses (62, 63).

Hex A shows a broad pH profile (3.0-7.6) against soluble, synthetic substrates (42), but a sharp one at pH 3.8-4.6 for the cleavage of membrane bound amphiphilic GM2 in the presence of its cofactor GM2AP (15, 17, 23, 64). In contrast to MUGS hydrolysis, the cleavage of membrane bound liposomal GM2 depends completely on the cofactor
protein GM2AP and the lipid composition of the liposomal membrane (Fig. 2, 3) (17, 23).

In contrast to the catabolism of membrane bound GM2, the main storage compound of GM2-gangliosidoses (Tay-Sachs disease, Sandhoff disease, AB variant or B1 variant), the cleavage of soluble synthetic substrates like MUGS is hardly affected by the presence of GM2AP and membrane lipids either present in form of liposomes, lipid aggregates or detergent micelles (Fig. 1).

Also stabilizing lipids of the plasma membrane, Chol and SM, and its final lysosomal degradation products, Sa and So, strongly inhibit catabolism of liposomal bound GM2 (Fig. 2 A-B, G-H, 3 A-B, G H), but not that of soluble MUGS (data not shown). Whereas several intermediates of lysosomal lipid degradation DAG, Cer, fatty acids and lyso-PC, stimulate enzymatic hydrolysis of liposomal bound GM2 up to five-fold (Fig. 2 C-F, 3 C-F), they do not affect that of soluble MUGS (Fig. 1).

Summarized, lipid modifiers mainly affect the functions of the GM2AP at the membrane surface, the electrostatic binding of proteins to the surface of the GM2 containing membranes, the mobilization of membrane lipids by GM2AP (Fig. 4) and the GM2AP dependent Hex A catalyzed GM2 hydrolysis (Fig. 2, 3). They may also affect the Michaelis-Menten complex formation (GM2-GM2AP-Hex A) directly, but they do not affect the Hex A catalyzed hydrolysis of synthetic, water-soluble substrates like MUGS.

**Electrostatic interactions between protonated lysosomal proteins and negatively charged ILVs**

At low pH values, e.g. 4-4.8, most lysosomal proteins, hydrolases and sphingolipid activator proteins, are positively charged and concentrate on the surface of lipid
substrate carrying, negatively charged ILVs to speed up lipid hydrolysis at the vesicle surface (22, 23).

In reconstitution experiments liposomes containing 20 mol% BMP or other anionic phospholipids (phosphatidylglycerol, phosphatidylinositol, phosphatic acid) carry a negative zeta potential at their surface (22). This negative surface charge of ILVs should attract and concentrate positively charged proteins like acid sphingomyelinase (22, 65), glucocerebrosidase (25) and other hydrolases and sphingolipid activator proteins like GM2AP (23, 38, 66) at their surface. Due to the additional protonation of the His6-tag at low pH values, the electrostatic attraction of His6-tagged sphingolipid activator proteins like g-rGM2AP-His6 is further enhanced to the GM2-carrying vesicular surface and leads to a stronger concentration at the vesicular surface and an increase of the catabolic reaction rate (Fig. 2, 3). On the other hand cationic amphiphilic drugs added to the assay (or fed to cultured fibroblasts) (65, 67) can compensate the negative surface charge and trigger the release of vesicle bound proteins, thereby reducing sphingolipid and glycosphingolipid catabolism (65, 67). The release of ILVs bound lysosomal hydrolases can also facilitate their proteolytic digestion in the lysosomes and trigger a drug induced lipidosis (65, 67).

**Enhancement of GM2 turnover by removal of inhibiting membrane lipids from ILVs and generation of modifying lipid intermediates**

Plasma membrane stabilizing lipids, Chol and SM, inhibit several key steps in sphingolipid catabolism (7) (Fig. 2 A-B, 3 A-B). The conversion of inhibiting SM to stimulating Cer at ILVs of late endosomes, facilitates the removal of Chol by NPC2 from the ILVs and stimulates the catabolism of several sphingolipids (22).
The action of the promiscuous phospholipase C, the acid sphingomyelinase, and of the lysosomal phospholipase A2 will generate a series of degradation products, DAG, Cer, fatty acids and lyso-PC (68-70). Also those early degradation products of phospholipids and sphingolipids like DAG, Cer, fatty acids and lyso-PC will disturb the bilayer structures of ILVs and create lipid micelles and aggregates in the lumen of the lysosomes, which can be digested much faster by lysosomal lipases and glycosidases than the lipids in the vesicular membranes.

DAGs can accumulate transiently in membranes, and cause changes in the physical properties of the bilayer. They tend to form inverted micellar structures since their polar head group is small. This leads to the formation of small areas of unstable negative curvature in membranes that facilitate membrane fission and fusion (71, 72).

It is known that Cer induces transbilayer flip-flop (73), induces membrane efflux from LUVs, and creates Cer rich domains in plasma membranes (74, 75). Also its strong stimulation of GM2 turnover may be based on its bilayer labilizing abilities.

The micelle forming lipid, lyso-PC, can be generated in the lysosome as an intermediate of phosphatidylcholine catabolism. Formed in ILVs it may well labilize the stability of ILV membranes, thereby stimulating GM2 and lipid mobilization. Stearic acid induces formation of micelles and due to its negative surface charge at slightly acidic conditions facilitates lipid degradation by soluble and protonated lysosomal enzymes.

The bilayer disturbing lipids DAG, Cer, stearic acid and lyso-PC elevated the GM2 turnover in a concentration dependent manner, using BMP containing liposomes with an enhanced negative surface charge (Fig. 2 C-F). This influence was also observed in BMP-free liposomes, exhibiting, however, rather low metabolic rates due to an extremely reduced negative surface charge (Fig. 3 C-F). Similar effects had been observed before
concerning the turnover of glycosylceramide (25) and other glycosphingolipids. Moreover DAG has been described to act as a stimulator for acid sphingomyelinase (22, 76). Its stimulatory effects on sphingomyelin and GM2 hydrolysis may be based by its bilayer perturbing properties. They may be also responsible for the slightly stimulating effects on lipid mobilization as observed in SPR studies (Fig. 4 C).

The potent stimulatory impact of the micelle forming stearic acid on GM2 hydrolysis and lipid mobilization may be based on its cone-shaped form, having a voluminous hydrated head group and a single hydrophobic chain. With a pK value of about 4.8, its stimulatory impact maybe also increased by a small percentage of still unprotonated and negatively charged stearic acid molecules at the pH range of GM2 hydrolysis at 3.8 to 4.6 (39).

Further on, of all lipids mentioned above, only micelle forming stearic acid had a strong stimulating effect on lipid mobilization by g-rGM2AP in SPR studies (Fig. 4 C-F). This observation may be very important also for other lipid bilayer disturbing and micelle forming fatty acids, released in large amounts by triacylglycerol and phospholipid catabolism in the lysosomal system.

SM is reported to stabilize Chol in the membrane by shielding its OH-Group (77). During maturation of ILVs SM is degraded to Cer (78, 79). As in former experiments (23), we could show, that the zwitterionic and bilayer stabilizing SM inhibits the GM2AP dependent GM2 hydrolysis, even in presence of the stimulating lysosomal lipid BMP (2, 17, 38) (Fig. 2 A). This is supported by the strong inhibition of g-rGM2AP mediated lipid mobilization by SM containing membranes (Fig. 4 A).

Chol is the major steroid constituent of mammalian membranes. It has membrane stabilizing properties, enables the lipid-ordered phase of lipid bilayer structures and reduces the membrane permeability for ions (55, 80, 81). During endocytosis the Chol
content decreases in the intraendolysosomal vesicles. It is sorted out by the sterol binding proteins NPC1 and NPC2 (4, 7, 22, 82, 83). As shown before (23) Chol is a strong inhibitor for hydrolysis of GM2 (Fig. 2 B, 3 B). It triggers GM2 accumulation in NPC disease and inhibits lipid mobilization by g-rGM2AP (Fig. 4 B) even in presence of the stimulating lysosomal lipid BMP (2, 17, 38).

So and Sa are two free sphingoid bases whose pathomechanisms have been reviewed previously (84). They are highly cytotoxic and closely associated to the primary defects in Krabbe disease, Fabry disease and Niemann-Pick disease (53, 85-87). They are strong inhibitors of glycosylceramide hydrolysis (25, 88) what is based in their positive net charge. As components of the lipid substrate carrying membrane they reduce the electrostatic interaction between the cationic enzyme or protein and the negatively charged membrane surface (88). It is postulated, that they act in a similar way as CADs inducing a lipidosis. Like desipramine (65) they may trigger a dissociation of acid spingomyelinase and other hydrolases from the lipid layers, thereby reducing the catabolic rates. Even low concentrations of amphiphilic amines e.g. So and the CAD imipramine can provoke a “NPC phenotype” (89). Indeed So and Sa have strong inhibitory influence on GM2 turnover (Fig. 2 G, H) as seen before for glycosylceramide catabolism (25). They also inhibit lipid mobilization by GM2AP strongly as seen in SPR studies (Fig. 4 G, H).

Levels of sphingoid bases in the lysosomal compartment are mostly unknown, but they may well increase in lysosomes especially when their egress is impaired by storage material.
Severe side effects of the His₆-tag

The His₆-tag was added to recombinant GM2AP to enable its simple purification. At lysosomal pH values the increased cationic charge of the His₆-tagged GM2AP, however, may enhance its electrostatic binding to the negatively charged surface of BMP containing liposomes and ILVs. This may well increase the GM2AP concentration at the substrate carrying vesicle surfaces and thereby enhance the Hex A mediated GM2 hydrolysis in the *in vitro* activity assay as observed in Fig. 3, 4. The GM2 turnover enhancing effect of the His₆-tag is independent of the vesicle formulation in the activity assays (Fig. 2 A-H, 3 A-H) and seems to be based on the additional positive charge of the 6 additional histidine residues protonated at low pH-values what should result in a stronger electrostatic binding to the negatively charged liposomal membrane. Based on these findings we postulate that the hydrolysis of GM2 by Hex A in presence of g-rGM2AP-His₆ takes place at the membrane surface and not in solution, leading to the conclusion that His₆-tagged membrane active proteins should not be used in reconstitution assays.

As mentioned before (23), the His₆-tag has more than one side-effect on the overall properties of GM2AP. It enhances turnover of GM2 by Hex A, assisted by GM2AP, speeds up the intermembrane lipid transfer of 2-NBD-GM1 in Förster resonance energy transfer studies and inhibits lipid mobilization by GM2AP in SPR studies (23).
Conclusion and Outlook

In this study we characterized the impact of various membrane lipids on the GM2 catabolism at and in the ILVs. Primary GM2 storage is known to be based on mutations in the alpha and beta Hex A subunits (Tay Sachs disease and Sandhoff disease), and in the GM2 activator (AB variant). Here, the influence of changes in ILV lipid composition on GM2 catabolism as regulator of GM2 turnover, possibly also affecting lysosomal storage, has been less illuminated. We present clear evidence that Chol and SM as well as the lysosomal degradation compounds So and Sa could inhibit GM2AP assisted GM2 hydrolysis by Hex A. Furthermore, incorporation of Cer, DAG, fatty acids and lyso-PC into the GM2-carrying membrane could stimulate it. In contrast to the altered activities of GM2AP assisted GM2 catabolism the Hex A catalyzed hydrolysis of the synthetic water soluble substrate MUGS was not affected by all lipid alterations. These findings may provide an explanation for secondary GM2 storage as a consequence of lipid alterations induced by lysosomal storage disorders.

Posttranscriptional and posttranslational generated compounds and other cofactors in the micro-environment of the substrate-carrying membranes regulate and modify the lipid-cleaving activity of catabolic lysosomal hydrolases effectively, in contrast to the hydrolysis of soluble synthetic substrates like MUGS. Besides known factors like pH, nature of the substrate, (15, 23, 39), ionic strength (23) etc., the GM2AP assisted hydrolysis of GM2 is influenced by the membrane lipids (23) comprised in the ILVs and by storage compounds of lysosomal storage diseases (Anheuser et al., submitted), that can trigger a secondary lipid accumulation and affect pathogeneses in patients. Further on we found a clinical phenotype correlation between patients with GM2 gangliosidosis and metachromatic leukodystrophy and the sphingolipid-degrading activity of their
cultured fibroblasts. (90, 91). Lipid cleaving activity of hydrolases appears to be strongly modified by the molecular micro-environment of the reaction (22). Also membrane properties might be important for the rate of sphingolipid catabolism in the lysosomes. Lateral pressure (as assigned for GM2AP action (92)) and curvature (as demonstrated for Cer (93)) of the vesicle membranes are important, but will be only of transient influence. The fast generation of lipid bilayer destroying catabolic products, like the massive release of micelle-forming free fatty acids by the degradation of tri-and diglycerides, sphingolipids and phospholipids and the generation of lyso-sphingolipids and lyso-phospholipids will rapidly destroy the membrane bilayer structure of the ILVs and generate a multitude of micelles and other lipid aggregates within the lysosomes. They will be a much better prey for enzymatic catabolism than lipid components of intact membrane structures. This became obvious when comparing in vitro studies of micellar with membrane-bound sulfatides and ganglioside GM2, respectively, (94) as substrates of catabolic hydrolases. Also the inhibiting action of CADs (Anheuser et al. submitted) and sphingoid bases will change with the disappearance of intact membrane structures. Lysosomal storage of ILV membranes is not only massive in inherited defects of lysosomal hydrolases and sphingolipid activator proteins, but also becomes prominent in healthy animals by feeding massive amounts of CADs (95, 96) or excessive amounts of lipids and other nutrients. Under normal conditions healthy tissues hardly show any ILV structures. Those are apparently readily degraded (97-99).

Therefore, we postulate that membrane lipids of organellar membranes modify the activity of many organellar proteins. Pathological alterations of their lipid composition may interfere with cellular metabolism (for instance with the lipid and membrane metabolism in obesity, Alzheimer's disease and Parkinson's disease). Therefore, the
lipid pattern of organelles should be analyzed *in situ* as soon as an appropriate technology will be available with sufficiently high spatial and temporal resolution. Lipidomics of tissues and cells is of only limited value. Though the overall cholesterol content in the brain appears to be normal, a slight cholesterol increase in the endosomes and lysosomes is fatal for the patients with Niemann-Pick disease type C (84, 100, 101).
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Fig. 1: Hydrolysis of the synthetic, water soluble substrate MUGS by Hex A (A) and turnover of liposome-bound [\(^{14}\)C] GM2 by Hex A in presence of GM2AP (B)

Usually the activity of Hex A is determined by an assay using the water soluble, synthetic substrate MUGS. Further on it is known that the hydrolysis of the natural membrane bound GM2 by Hex A occurs only in presence of GM2AP (17, 23) and is strongly depended on the presence of membrane lipids such as BMP, Cer and SM (23) (B). The highest GM2 turnover was reached in the presence of GM2AP, ceramide and anionic BMP in the liposomal membranes.
To investigate the influence of membrane lipids on the digestion of MUGS by Hex A, liposomes containing 5 mol% Chol, 0 or 20 mol% BMP, 0 or 40 mol% Cer and SM, respectively, and DOPC as a host lipid (0.5 mM in 20 mM citrate buffer, pH 4.2), were added to the assay mixture. However, addition of different liposomes, containing additional Cer or SM, to the assay mixture had no significant effect on the turnover of MUGS (A, right chart). Further, neither GM2AP nor anionic membrane lipids (BMP) had influence on the cleavage of the synthetic soluble substrate MUGS by Hex A (A, left chart).

To mimic the conditions at the intralysosomal vesicles the turnover of membrane-bound GM2 by Hex A in presence of GM2AP was measured in a liposomal assay system (B). Liposome composition was as given above, containing additional [14C] GM2 (2 mol%) and 0 or 20 mol% BMP.

GM2 turnover was inhibited by SM, even in presence of BMP, and strongly enhanced by Cer (B, right chart). SEM, (n=3).

BMP: bis(monoacylglycero)phosphate, Cer: ceramide, Chol: cholesterol, DOPC: dioleoyl-phosphatidylcholine, g-rGM2AP: glycosylated recombinant GM2 activator protein, Hex A: β-hexosaminidase A, MUGS: 4-methylumbelliferyl-6-sulfo-2-acetamido-2-deoxy-β-D-glucopyranoside
Fig. 2: GM2 hydrolysis is strongly modified by membrane lipids in BMP containing liposomes

The influence of increasing liposomal concentrations of SM (A), Chol (B), DAG (C), Cer (D), stearic acid (E), lyso-PC (F), So (G), Sa (H) on the conversion of GM2 to GM3 by Hex A in the absence of GM2AP (red), and the presence of g-rGM2AP (green) and g-rGM2AP-His$_6$ (blue) was investigated in an *in vitro* liposomal assay using negatively charged, BMP-containing liposomes. Liposomal composition was Chol (5 mol%), BMP (20 mol%), lipid of interest (0-40 mol%) and $[^{14}C]$ GM2 (2 mol%), made up to 100 mol% by DOPC.

Turnover of $[^{14}C]$ GM2 in the liposomal activity assay was strongly stimulated by DAG (C), Cer (D), stearic acid (E) and lyso-PC (F) and inhibited by SM (A), Chol (B), So (G) or Sa (H). In absence of GM2AP no hydrolysis of GM2 was detectable (A-H). SEM, (n=3).

BMP: bis(monoacylglycero)phosphate, Cer: ceramide, Chol: cholesterol, DAG: diacylglycerol, GM2AP: GM2 activator protein, g-rGM2AP: glycosylated recombinant GM2 activator protein, g-rGM2AP-His$_6$: glycosylated recombinant GM2 activator protein with hexahistidine-tag, Hex A: β-hexosaminidase A, His$_6$-tag: hexahistidine-tag, lyso-PC: lysophosphatidylcholine, Sa: sphinganine, So: sphingosine
Fig. 3: GM2 digestion is also affected by membrane lipids in BMP-free liposomes

The influence of the liposomal lipids SM (A), Chol (B), DAG (C), Cer (D), stearic acid (E), lyso-PC (F), So (G) and Sa (H) on the conversion of GM2 to GM3 by Hex A in the presence of g-rGM2AP (green) and g-rGM2AP-His$_6$ (blue) were investigated using,
BMP-free liposomes: Turnover of GM2 was fundamentally reduced compared to liposomes containing 20 mol% BMP, carrying a stronger negative net charge (see Fig. 2).

Enhanced liposomal DAG (C), Cer (D), stearic acid (E) or lyso-PC (F) concentrations led to an enhanced hydrolysis of GM2 by Hex A in presence of g-rGM2AP (green) and g-rGM2AP-His6 (blue) in the in vitro liposomal activity assay (C-F) while increasing SM (A), Chol (B), So (G) and Sa (H) concentrations reduced it (A, B, H, G). In absence of GM2AP no hydrolysis of GM2 was detectable (A-H). SEM, (n=3).

BMP: bis(monoacylglycero)phosphate, Cer: ceramide, Chol: cholesterol, DAG: diacylglycerol, GM2AP: GM2 activator protein, g-rGM2AP: glycosylated recombinant GM2 activator protein, g-rGM2AP-His6: glycosylated recombinant GM2 activator protein with hexahistidine-tag, Hex A: β-hexosaminidase A, lyso-PC: lysophosphatidylcholine, Sa: sphinganine, So: sphingosine
Fig. 4: Mobilization of membrane lipids from BMP-containing, negatively charged lipid layers by GM2AP is strongly modified by the liposomal lipid composition.
Binding and mobilization of membrane lipids by g-rGM2AP was investigated in SPR studies. After immobilization of negatively charged liposomes, containing 0-40 mol% lipid of interest, 20 mol% BMP, 5 mol% Chol, 10 mol% GM2 and DOPC as a host lipid, g-rGM2AP was injected into the flow cell, (2.5 µM in 20 mM sodium citrate, pH 4.2) at a flow rate of 20 µl/min for 180 s. This was followed by injection of protein free buffer (220 s, 20 µl/min) indicated by an arrow. Curves falling below the baseline (yellow) suggest solubilization and mobilization of membrane lipids.

Loading of lipids resulted in a shift of approximately 2000 Resonance units (RU), which corresponds to a lipid monolayer and was set as “baseline” (RU=0). The Baseline corresponds to 100% of loaded lipid material. The RUs above the baseline (yellow) represent material bound to the lipid layer during the experiment, whereas negative RUs below the baseline represent material loss, which not only includes protein (GM2AP) added during the experiment, but also lipid material mobilized and released from the lipid layer. Only if the RU value drops below the baseline we can be certain, that lipids from the lipid layer have been removed, in amounts equal to the RU values given or more, in case some unknown amount of protein still stays bound to the chip.

Mobilization of lipids from lipid layers consisting of 0 mol% lipid of interest, 20 mol% BMP and 5 mol% cholesterol by g-rGM2AP was set as control for all experiments (red line). All lines shown are one representative out of three.

BMP, DAG, Cer, stearic acid and lyso-PC stimulated lipid mobilization by GM2AP, while SM, Chol, So and Sa inhibited it.
Fig. 5: GM2AP could not mobilize lipids from BMP-free lipid layers

Mobilization of membrane lipids by g-rGM2AP was investigated in SPR studies. Conditions were equal to that of Fig. 4. Liposomes did not contain BMP.

BMP: bis(monoacylglycero)phosphate, Cer: ceramide, Chol: cholesterol, DAG: diacylglycerol, g-rGM2AP: glycosylated recombinant GM2 activator protein, lyso-PC: lysophosphatidylcholine, RU: resonance units, Sa: sphinganine, So: sphingosine
Fig. 6: Cartoon for enzymatic hydrolysis of membrane bound ganglioside GM2 by Hex A in the presence of GM2AP (A) and turnover of the soluble substrate MUGS by Hex A (B)
A: Model for the hydrolysis of membrane bound GM2 by Hex A, assisted by GM2AP, at the surface of luminal lysosomal vesicles. Assisted by the hydrophobic loops (bluish grey), the GM2AP can bind to the membrane and penetrates into the hydrophobic region of the bilayer. Its hydrophobic cavity can include the ceramide tail of GM2 and other lipids (61) and stay at the membrane (I). The conformation of the lipid GM2AP complex can also change to a more closed conformation thus becoming more water soluble and detach from the lipid layer as a water-soluble Michaelis-Menten-complex (II), exposing the GM2 to the water-soluble Hex A for degradation.

Hydrolysis of GM2 by Hex A assisted by GM2AP is based on a pH level from 3.8-4.6 combined with a low ionic strength (23, 39). It is enhanced by the membrane curvature of the intralysosomal luminal vesicles (ILVs) and a negative charge due to anionic lipids like BMP. However, high lateral pressure, cationic amphiphilic drugs (CADs), SM, Chol, and sphingoid bases reduce GM2 hydrolysis.

Modified after (5, 6)

B: \textit{In vitro} Hex A is able to cleave the synthetic substrate MUF without assistance of auxiliary proteins. The turnover of MUGS to MUF is unaffected by the regulatory system involved in the lysosomal turnover of GM2 by Hex A and GM2AP shown in A.

BMP: bis(monoacylglycerophosphate), CADs: cationic amphiphilic drugs, GM2AP: GM2 activator protein, Hex A: \(\beta\)-hexosaminidase A, ILV: intralysosomal luminal vesicle, MUF: 4-methylumbelliferone, MUGS: 4-methylumbelliferyl-6-sulfo-2-acetamido-2-desoxy-\(\beta\)-D-glucopyranoside, PC: phosphatidylcholine,