# Membrane lipids regulate ganglioside GM2 catabolism and GM2 activator protein activity[S]

**Susi Anheuser, Bernadette Breiden, Günter Schwarzmann, and Konrad Sandhoff[1]**

LIMES Institute, Membrane Biology and Lipid Biochemistry Unit, Kekulé-Institut für Organische Chemie und Biochemie, Universität Bonn, D-53121 Bonn, Germany

Abstract   Ganglioside GM2 is the major lysosomal storage compound of Tay-Sachs disease. It also accumulates in Niemann-Pick disease types A and B with primary storage of SM and with cholesterol in type C. Reconstitution of GM2 catabolism with β-hexosaminidase A and GM2 activator protein (GM2AP) at uncharged liposomal surfaces carrying GM2 as substrate generated only a physiologically irrelevant catabolic rate, even at pH 4.2. However, incorporation of anionic phospholipids into the GM2 carrying liposomes stimulated GM2 hydrolysis more than 10-fold, while the incorporation of plasma membrane stabilizing lipids (SM and cholesterol) generated a strong inhibition of GM2 hydrolysis, even in the presence of anionic phospholipids. Mobilization of membrane lipids by GM2AP was also inhibited in the presence of cholesterol or SM, as revealed by surface plasmon resonance studies. These lipids also reduced the interliposomal transfer rate of 2-NBD-GM1 by GM2AP, as observed in assays using Förster resonance energy transfer.[S] Our data raise major concerns about the usage of recombinant His-tagged GM2AP compared with untagged protein. The former binds more strongly to anionic GM2-carrying liposomal surfaces, increases GM2 hydrolysis, and accelerates intermembrane transfer of 2-NBD-GM1, but does not mobilize membrane lipids.—Anheuser, S., B. Breiden, G. Schwarzmann, and K. Sandhoff. **Membrane lipids regulate ganglioside GM2 catabolism and GM2 activator protein activity.** *J. Lipid Res.* 2015. **56: 1747–1761.**

Components of eukaryotic plasma membranes reach the intraendolysosomal vesicles by endocytotic membrane flow (1). Those vesicles have been identified as platforms for lipid and membrane degradation (2). They differ from the limiting endosomal membrane by the absence of a glycocalix, a lower cholesterol content, and the cumulative occurrence of the anionic bis(monoacylglycero)phosphate (BMP) (3–5), which is of functional significance.

For the effective degradation of lysosomal sphingolipids with short oligosaccharide head groups, special lipid binding and transfer proteins (the sphingolipid activator proteins) are needed. The sphingolipid activator proteins consist of the GM2 activator protein (GM2AP) and the saposins (Saps) A–D. They all facilitate the interaction of the membrane-bound substrate with the water-soluble enzyme at the water-membrane interface and differ from sphingolipid transfer proteins of the cytosol (6). The anionic lysosomal phospholipid, BMP, is also known to dramatically enhance (glyco)sphingolipid hydrolysis by lysosomal hydrolases and sphingolipid activator proteins (7–9).

Even at low pH values (pH <5.0), BMP conveys negative surface charge to luminal lysosomal vesicles (10, 11). This favors an electrostatic binding of the positively charged activator proteins and the polycationic hydrolases, and enhances degradation of (glyco)sphingolipids substantially (7, 9, 10, 12–16).

The GM2AP, which has a hydrophobic binding cavity for lipids and a binding site for β-hexosaminidase A (HexA) (17–19), arranges the interaction of ganglioside GM2 with HexA and facilitates the formation of a Michaelis-Menten complex with access of the catalytic site to the carbohydrate head group (20, 21). The whole working mechanism,

however, is still a matter of debate. A scheme of the postulated mechanism and the liposomal activity assay can be seen in **Fig. 1**. Until now, it was not known exactly whether the degradation took place at the surface of the intralysosomal vesicles or in solution.

GM2AP is known to be a quite promiscuous lipid binding protein (22, 23). Its activity depends on numerous physiological parameters, including the lateral pressure of the lipid bilayer (24), the pH value (25), the vesicle curvature, and the presence of negatively charged lysosomal lipids, especially BMP (9). The isoelectric point of the GM2AP is situated at pH 4.8 (26). Due to the cumulated occurrence of lysine residues, it has positively charged surface domains, especially at the low pH values existent in the lysosomal environment. This contributes to the interaction of the activator protein with the anionic membrane-bound BMP.

Anionic membrane lipids have been identified as stimulating lipid modifiers of many membrane-associated reactions, e.g., phosphatidylglycerol (PG) stimulates steps of ganglioside biosynthesis at the Golgi compartment (27, 28). BMP, phosphatidic acid, phosphatidylserine, and phosphatidylinositol (PI) stimulate the transfer of cholesterol by Niemann-Pick protein type C2 (NPC2) (29–32). On the other hand, plasma membrane-stabilizing SM strongly inhibits cholesterol transfer by NPC2, even in the presence of anionic lipids (31). Therefore, SM has to be degraded first by the acid sphingomyelinase (ASM) to facilitate the release of cholesterol from inner membranes. Because of this, a defective ASM in Niemann-Pick disease types A and B not only leads to accumulation of SM, but also secondarily triggers the storage of cholesterol (33).

This work studies the function of membrane lipids in the regulation of (glyco)sphingolipid turnover. Therefore, we chose reconstitution experiments in a detergent-free liposomal in vitro approach. In all assays, liposomes were used as substrate-carrying membranes to mimic the intraendolysosomal vesicles. The lipid composition of the vesicles can be manipulated exactly, thus allowing analysis of the influence of the lipid composition, pH value, and ionic strength on GM2AP, affecting not only GM2 hydrolysis but also solubilization and transfer of membrane lipids.

## MATERIALS AND METHODS

### Materials

1,2-Dioleoyl-*sn*-glycerol-3-phosphocholine (DOPC), bis(monooleoylglycero)phosphate (BMP), multivalent cationic lipid 5 (MVL5), and 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (EPC) were purchased from Avanti Polar Lipids (Alabaster, AL). C18-SM and D-erythro-C18-ceramide (Cer) were purchased from Matreya (Pleasant Gap, PA). GM2 was available in our laboratory. Cholesterol, desipramine, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (MUG), 4-methylumbelliferone, PG, and PI were purchased from Sigma (Taufkirchen, Germany). Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (rhodamine-PE) triethylammonium salt was purchased from Life Technologies, Molecular Probes, Inc. (Eugene, OR).

Human embryonic kidney cells (HEK-293 cells) were from Sigma and TurboFectin 8.0 was from Origene Technologies, Inc. (Rockville, MD). MEM-medium and IPL41-medium were obtained from Gibco BRL (Bethesda, MD). Geneticin disulfate (G418) was obtained from Carl Roth GmbH (Karlsruhe, Germany). *Spodoptera frugiperda* (SF-21) cells were available in our laboratory. Sequencing of the GM2AP was done by Chromatec (Greifswald, Germany).

Ni-NTA-agarose Superflow was from Qiagen (Hilden, Germany), the cationic exchange material Poros HS 20 and Macro-Prep High S Support were obtained from Applied Biosystems/Applere (Freiburg, Germany) and Bio-Rad (Hercules, CA).
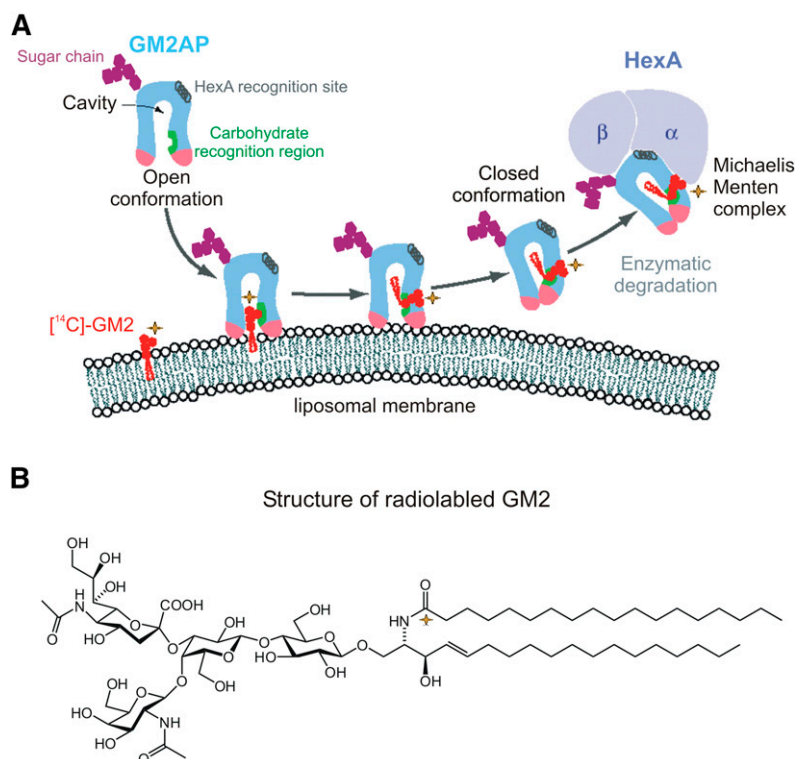


**Fig. 1.** Scheme of the in vitro assay for the degradation of liposomal bound ganglioside GM2 by HexA in the presence of GM2AP. A: Assay for enzymatic hydrolysis of liposomal GM2. The radiolabeled GM2 (red) is incorporated into liposomes (lipid composition variable), mimicking intralysosomal vesicles. The GM2AP recognizes membrane bound [14C]GM2 and presents it to the HexA (light blue) for degradation. The enzymatic digestion may occur either at the surface of the membrane or as shown in solution at the water-soluble Michaelis-Menten complex. The emerging [14C]GM3 was measured. GM2AP: purple, sugar chain; black, HexA recognition site; green, carbohydrate recognition region; pink, lipid binding side. B: Structure of the [14C]GM2 used in the assay shown in (A). Position of the radioactive label is marked by a yellow cross.

All other chemicals were obtained from Merck and Sigma, and were of analytical grade.

## Synthesis of 2-NBD-GM1

β-D-galactopyranosyl-(1,3)-2-acetamido-2-deoxy-β-D-galactopyranosyl-(1,4)-[α-D-N-acetyl-neuraminyl-(2,3)]-β-D-galactosylpyranosyl-(1,4)-β-D-glucopyranosyl-(1,1)-(2S,3R,4E)-2-[(2R/S-N-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)-amino)-octadecan-amido]-4-octadecen-1,3-diol (2-NBD-GM1) was synthesized in a multistep synthesis starting from the GM1 isolated from natural sources. The added functionality of 2-aminogangliosides allowed us to introduce the chromophore into the region between the polar head group and the hydrophobic anchor of the lipid (34). The chemical structure is shown in supplementary Fig. 2.

## Synthesis of [$^{14}$C]GM2

[$^{14}$C]GM2 was synthesized from its corresponding lyso-lipid, following previously reported procedures (35). The chemical structure can be seen in Fig. 1B.

## Protein preparation

Glycosylated recombinant GM2AP with hexahistidine-tag (His$_6$-tag) (g-rGM2AP-His$_6$) at the C terminus was expressed in the Baculovirus Expression Vector System using SF-21 cells, and was purified as reported previously (17). The molecular mass, determined by MALDI-MS, was 20.527 kDa.

Glycosylated recombinant GM2AP (g-rGM2AP) without any tag was expressed in HEK-293 cells and purified by cation exchange chromatography. The protein expression vector pCMV6-XL4 (supplementary Fig. 1), including *Homo sapiens* GM2 ganglioside activator transcript variant 1 (NM_00405), was bought from Origene Technologies, Inc. (Rockville, MD) as transfection-ready DNA (SC126904). The vector was transfected into competent *Escherichia coli* DH5α and grown overnight on lysogeny broth plates, containing ampicillin for selection of transfection. One single colony was grown overnight in liquid lysogeny broth medium with ampicillin. Contained vectors were purified using a QIAprep Spin Miniprep kit (Qiagen). Identity was proofed by DNA-sequencing (Seqlab, Göttingen, Germany).

The purified vector was transfected into HEK-293 cells (50–70% confluence) using TurboFectin 8.0 (Origene Technologies, Inc.) following the protocol. Selection of transfected cells was done for 2 weeks using G418.

Fifteen cell culture flasks (175 cm$^2$) of stabile transfected HEK-293 cells grown to confluence were harvested, washed with PBS, dispersed in 15 ml water, and sonified (3 × 3 min, 120 W, Cuphorn, Branzon, MO). After centrifugation, the supernatant was adjusted to pH 4 and kept at 4°C for 12 h. After additional centrifugation, sodium phosphate was added to the supernatant to a final concentration of 50 mM.

Purification was done by cation exchange chromatography using a step gradient from 0 to 1 M NaCl in 50 mM phosphate buffer (pH 4). The column was washed with two column volumes 50 mM phosphate buffer (pH 4). Afterwards, the supernatant of the acid precipitation was applied to the column and washed with two column volumes 50 mM phosphate buffer (pH 4). Elution of proteins was done with 50 mM phosphate buffer (pH 4) adding steps of 0.2 M NaCl successively. The g-rGM2AP was found in high purity, concentrated in 15 ml eluate.

Purity of the protein preparations was better than 95%, as confirmed by SDS gel electrophoresis with silver staining (**Fig. 2**). Identity was confirmed by Western blotting, protein sequencing (Chromatec, Greifswald, Germany), and MALDI-MS (Autoflex II TOF/TOF; Bruker Daltonik, Bremen, Germany). The specified molecular mass was 18.007 kDa.
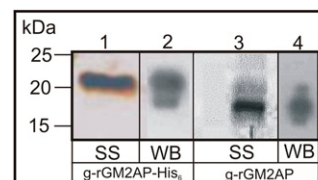


**Fig. 2.** SDS-PAGE with silver staining (SS) and Western blot (WB) of the two g-rGM2AP preparations. 1, g-rGM2AP-His6 (silver staining); 2, g-rGM2AP-His6 (Western blot); 3, g-rGM2AP (silver staining); 4, g-rGM2AP (Western blot).

The difference in molecular mass between g-rGM2AP and g-rGM2AP-His$_6$ is based in the additional six histidine-residues and a variant glycosylation resulting from the different expression systems.

HexA was purified from human liver to apparent homogeneity, as described previously (36). Purity and identity were verified by SDS gel electrophoresis with silver staining and Western blot. Activity was determined by a MUG/4-methylumbelliferone assay (37) with 0.8 µU/1 µg HexA. One unit of enzyme activity (1 U) is defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of MUG per minute at 37°C.

## Preparation of liposomes

Large unilamellar vesicles were prepared as previously described (38). 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 citrate buffer (pH 4.2) (unless stated otherwise). The dispersion was vortexed, sonified in a sonifier bath for 15 min, and subjected to eight freeze-thaw cycles to obtain a uniform distribution of buffer and solutes across the bilayers. Thereafter, the lipid suspension was passed through polycarbonate filters with a pore size of 100 nm in a mini-extruder (LiposoFast Avestin, Ottawa, Canada). Samples were subjected to 21 passes.

Unless stated otherwise, liposomes with no net charge contained 5 mol% cholesterol, the assay-specific marker lipids, and DOPC as a host lipid. Negatively charged liposomes contained 5 mol% cholesterol, 20 mol% BMP, the assay-specific marker lipids, and DOPC as a host lipid. When the composition of liposomes was varied, the amount of DOPC was adjusted appropriately.

## Determination of the lipid content of liposomes and final concentration of the liposomal suspension

Aliquots of the stock solution of liposomes, before and after passing through the membranes in the mini-extruder, were evaporated in a stream of nitrogen. The lipids were dissolved in 50 µl of chloroform/methanol (1/1, v/v) and separated by thin-layer chromatography with chloroform/methanol/water (60/25/4, v/v/v) as the mobile phase. After development, plates were air-dried, sprayed with a solution containing 8% (w/v) H$_3$PO$_4$ and 10% (w/v) copper (II) sulfate pentahydrate, and charred at 180°C for 10 min. The lipids were quantified using densitometry (Camag, Muttenz, Schweiz) at 595 nm. Determinations of lipid contents were done in triplicate. The indicated mole percent of lipid in the figures and in the text corresponds to the actual content following preparation of liposomes by extrusion.

## Activity assay with GM2AP and HexA

A scheme of the in vitro activity assay (9) for the enzymatic degradation of liposomal ganglioside GM2 is given in Fig. 1. Preparation of vesicles was done as shown above (see Preparation of liposomes). Lipid concentration of the negatively charged vesicles containing 2 mol% [$^{14}$C]GM2 was 0.5 mM.

Liposome dispersion (40 µl) was mixed with 10 µU HexA and 5 µg (0.277 pmol) g-rGM2AP or 5 µg (0.243 pmol) g-rGMAP-His$_6$, made up to 80 µl with buffer. The samples were kept on ice prior to addition of the proteins and afterwards incubated at 37°C for 1 h in 20 mM sodium citrate (pH 4.2) (unless stated otherwise). Afterwards the assay was put on ice and stopped by adding 20 µl methanol. Thereafter, the assays were dried under a stream of nitrogen, redissolved 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 of [$^{14}$C]GM3 and [$^{14}$C]GM2 were visualized with a Bio Imaging Analyzer 1000 (Fuji, Japan), and the quantification was performed with the image analysis software "Tina" (Raytest, Staubenhardt, Germany).

The mean error was less than 10%.

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

Biomolecular interaction analysis was carried out at 25°C with a Bialite instrument (Biacore, now GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Preparation of vesicles was done as shown above (see Preparation of liposomes). In the lipid mobilization assay, negatively charged liposomes with 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. One hundred microliter vesicles (0.5 mM total lipid concentration), diluted in 20 mM sodium citrate buffer (pH 4.2) (unless stated otherwise), were injected into the system at a flow rate of 5 ml/min. This resulted in a shift of approximately 2,000 resonance units (RUs), which corresponds to a lipid monolayer. GM2AP (0.2 mM) in running buffer [20 mM sodium citrate buffer( pH 4.2)] was injected into the flow cells at a rate of 20 µl/min for 3 min, followed by buffer alone.

## SPR study for analyzing the interaction of GM2AP with membrane lipids in the presence of desipramine

According to previous experiments with ASM (39), negatively charged liposomes containing 5 mol% cholesterol, 5 mol% BMP, and 10 mol% GM2 were immobilized on a Pioneer HPA chip. The running buffer was 20 mM sodium citrate buffer (pH 4.2). GM2AP (0.2 mM) in running buffer was injected into the flow cells at a rate of 20 µl/min. Afterwards, desipramine (30 mmol/l, dissolved in water) was injected for 3 min (flow rate 20 µl/min), followed by buffer alone.

## Lipid transfer assay by Förster resonance energy transfer

In the Förster resonance energy transfer (FRET) assay for intervesicular transfer of 2-NBD-GM1 (depicted in **Fig. 3**), donor and acceptor vesicles were prepared separately. Negatively charged liposomes (containing 5 mol% cholesterol and 20 mol% BMP) with 2 mol% 2-NBD-GM1 and 4 mol% rhodamine-PE in 20 mM sodium citrate (pH 4.2) were used as donor vesicles (unless stated otherwise). The acceptor vesicles were made of the same lipids without 2-NBD-GM1 and rhodamine-PE.

The final lipid concentrations in the assay were 4 and 24 nmol for donor and acceptor vesicles, respectively (donor-acceptor vesicle ratio is 1:6). The experiment was started by the addition of 0.083 pmol (1.5 µg) g-rGM2AP or 0.073 pmol (1.5 µg) g-rGM2AP-His$_6$. Increase in NBD-fluorescence in the acceptor vesicle was measured continuously, as described previously (34), with a spectrofluorophotometer (RF-5000; Shimadzu, Düsseldorf, Germany).

Fluorescence measurements were performed at 37°C using an excitation wavelength of 480 nm and an emission wavelength of 522 nm.

The value of 100% of accessible fluorescence (maximal dequench) ($F^{6/7(2\text{-}NBD)} - F^{D+A}$) was calculated as the measured fluorescence of liposomes containing 2 mol% 2-NBD-GM1 in the same dilution as the assay preparation divided by two and multiplied by 6/7 ($F^{6/7(2\text{-}NBD)}$). This was done because, in the FRET-assay, only the 2-NBD-GM1 of the outer leaflet can be transferred and 1/7 of the 2-NBD-GM1 stays back in the donor vesicle. The small amount of basis fluorescence of donor and acceptor vesicles at the beginning of the assay ($F^{D+A}$) was subtracted.

The percentage of the accessible fluorescence observed in the FRET assay was calculated accordingly as: $[F^{D+A+P} - F^{D+A}] / [(F^{6/7(2\text{-}NBD)} - F^{D+A})/100]$, where $F^{D+A+P}$ is the released fluorescence when GM2AP is added to donor and acceptor liposomes.

The "baseline of total fusion", shown in the figures, was experimentally determined: Former experiments indicated an enhanced fusion of liposomes by g-rGM2AP-His$_6$. Liposomes were prepared containing the combined lipid composition of all donor and acceptor vesicles of the FRET assay, representing the highest dequench of NBD-fluorescence that can be reached by vesicle fusion. The measured fluorescence value represents the baseline of total fusion shown in the figures. Fluorescent values above this line can be reached only by transfer of 2-NBD-GM1.

Because of its enhanced fusogenic properties, we did not use g-rGM2AP-His$_6$ in the FRET-assay.

The mean error was determined with less than 10%.

## RESULTS

Although GM2AP is known to play a key role in the lysosomal degradation of ganglioside GM2 (26), its exact function, mechanism, and regulation in the lysosomal environment are not well-defined. It was observed earlier by our group that the lipid composition of the GM2-carrying membranes is crucial for the interaction of GM2 with both GM2AP and HexA (9, 25). The lipid composition of the luminal endolysosomal vesicles changes drastically during endocytosis due to sorting out of cholesterol and digestion of other membrane lipids along the endocytotic pathway (4, 40–42). Cholesterol is sorted out by the transfer proteins NPC2 and Niemann-Pick protein type C1 (NPC1). NPC2, a soluble intralysosomal glycoprotein, transfers cholesterol to NPC1, a transmembrane protein of the endosomal membrane (5). For extraction of cholesterol by NPC2, the composition of the intraendosomal membrane is highly important. In former experiments we showed that BMP and Cer stimulate cholesterol transfer by NPC2 while SM inhibits it (31). Because SM is degraded to Cer by the ASM, this enzyme seems to play a major role in the efflux of cholesterol from the intraendosomal membrane.

To analyze those extensive coherences, liposomal reconstitution experiments using liposomes, which may mimic luminal vesicles of the late endosomal/lysosomal compartment, were carried out. The influence of various membrane lipids, such as BMP, cholesterol, Cer, and SM, on the ability of GM2AP to present GM2 to HexA for degradation, on its ability to mobilize membrane lipids, and its qualification to transfer a ganglioside analog, 2-NBD-GM1, between liposomal membranes were investigated.
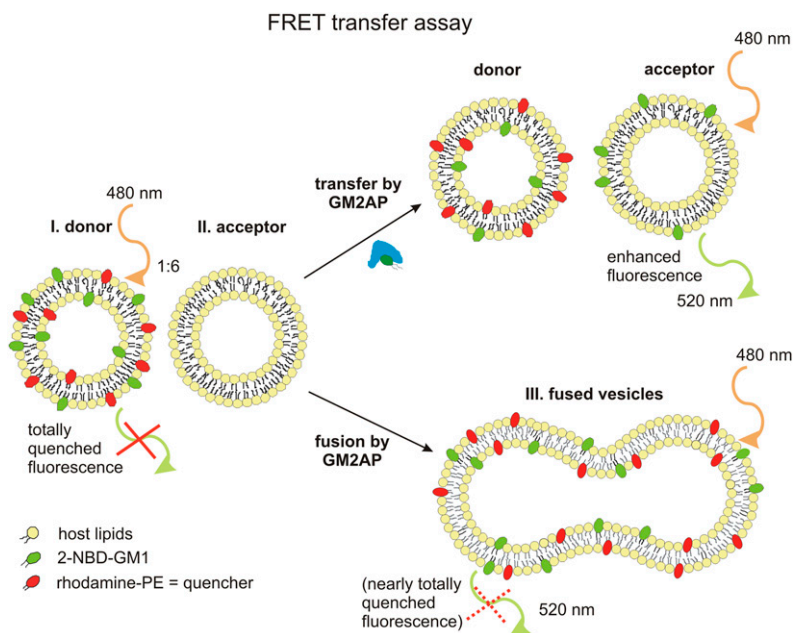
FRET transfer assay

**Fig. 3.** FRET assay for interliposomal lipid transfer. Transfer of fluorescent labeled lipid from donor to acceptor liposomes by GM2AP is monitored in real time (34). Fluorescence of the donor vesicles (I) containing the 2-NBD-GM1 is quenched by rhodamine-PE. The acceptor vesicles (II) do not contain any labeled lipid. The ratio of donor to acceptor vesicles is 1:6. Transfer of 2-NBD-GM1 is started by adding a catalytic amount (1.5 µg) of g-GM2AP or g-rGM2AP-His$_6$ to the assay volume of 400 µl. Incubation at 28°C differs between 15 and 90 min. The fluorescence of the NBD group is stimulated at a wavelength of 480 nm, and the increase of fluorescence in the acceptor vesicles is detected by 528 nm. 2-NBD-GM1 carries its NBD group between its hydrophobic lipid anchor and its sugar chain (supplementary Fig. 2) and has a "low off rate" comparable to that of GM1 in contrast to the commercially available NBD-derivatives (34). The commercially available NBD-derivatives carry their NBD group at the end of the acyl chain, which results in a "high off rate", caused by the amphiphilic character of the NBD group. Therefore these derivatives are not suitable analogs of ganglioside GM1 and not useable for our transfer studies. Fusion of donor and acceptor vesicles may also result in a slight increase of the fluorescence. The maximal achievable fluorescence by vesicle fusion has been determined by control experiments (see Materials and Methods) and is shown in the figures as "baseline total fusion." The basic composition of the donor vesicle is 2 mol% 2-NBD-GM1 and 4 mol% rhodamine-PE with PC as host lipid (BMP, cholesterol, etc. are varied as given in the figure). The acceptor vesicle composition is PC as host lipid (BMP, cholesterol, etc. are varied as given in the figures).

## Expression and purification of recombinant glycosylated, tagged, and tag-free GM2AP

In this study, two different GM2AP preparations were used. The g-rGM2AP was expressed without any tag in HEK-293 cells and g-rGM2AP-His$_6$, by the Baculovirus Expression Vector System (17), in SF-21 cells. Both preparations were characterized for pureness and functionality (see the Materials and Methods).

Per 1 l expression medium, an amount of approximately 30 mg rGM2AP-His$_6$ was purified by cationic exchange chromatography and metal-ion affinity chromatography on Ni-NTA-agarose.

From 15 cell culture flasks (175 cm$^2$) with confluent stable transfected HEK-293 cells, 6 mg g-rGM2AP were achieved using acid precipitation and cationic exchange chromatography. The g-rGM2AP was found in high purity, concentrated in three fractions.

Previous experiments had shown that g-rGM2AP-His$_6$ has a highly enhanced membrane-fusion ability (43) (data not shown). Therefore, r-gGM2AP-His$_6$ was not used in our transfer studies.

## Transfer assay by FRET

The donor liposomes contained the fluorescent labeled lipid to be transferred and a quencher. Upon transfer of 2-NBD-lipid to quencher-free acceptor liposomes, the NBD-fluorescence rose according to the amount of lipid transferred. This transfer experiment could be impaired when donor and acceptor liposomes fuse (equal to dequench by dilution). Therefore, in the experiments, we used an enhanced amount of quencher molecules to ensure sufficient concentration in the fused vesicles to quench NBD-fluorescence. Thus, an increase of the NBD-fluorescence was due to the effect of protein-mediated transfer rather than protein-mediated fusion.

We used 2-NBD-GM1 in place of 2-NBD-GM2 in our transfer experiments, because it is known that GM1 and GM2 are equally bound to GM2AP (44), and the former was more available to us than the latter.

A scheme of the FRET assay is illustrated in Fig. 3.

To measure the fluorescence achieved by fusion of the liposomes contained in the assay preparations, the fluorescence value of liposomes with the various lipid compositions

of total fused liposomes were measured. These results served as the baseline of total fusion, which show the maximum fluorescence reachable by fusion of vesicles. Values going above this line can only be reached by transfer of 2-NBD-GM1 in the absence of fusion.

## His₆-tag has a high impact on the ability of GM2AP to present GM2 for degradation, to mobilize lipids, and to transfer 2-NBD-GM1 between membranes

**Figure 4** shows the strong influence of the six histidine residues at the C-terminal end of GM2AP on hydrolysis of
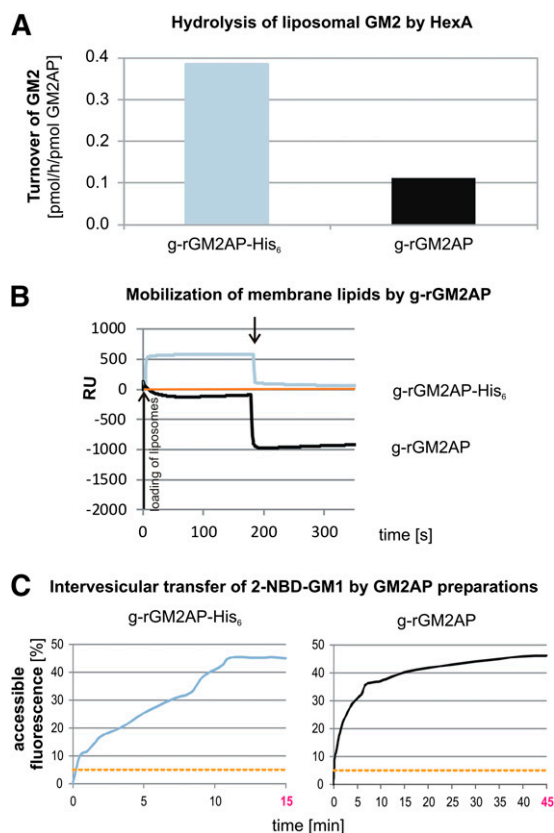


**Fig. 4.** The His₆-tag changes the abilities of GM2AP substantially. A: Liposomes containing 5 mol% cholesterol, 20 mol% BMP, and 2 mol% [¹⁴C]GM2, made up to 100 mol% by DOPC (called negatively charged liposomes in all experiments), were used in an in vitro liposomal activity assay with g-rGM2AP-His₆ (light blue) and g-rGM2AP (black). Mean error was determined with less than 10%. B: In contrast to g-rGM2AP-His₆ (light blue), g-rGM2AP (black) mobilized lipids from the HPA sensor chip. After immobilization of liposomes made of 5 mol% cholesterol, 20 mol% BMP, 10 mol% GM2, and DOPC as a host lipid (negatively charged liposomes), GM2AP 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 (orange) suggest mobilization of membrane lipids. C: Intervesicular transfer of 2-NBD-GM1 with negatively charged acceptor vesicles (liposome composition of 5 mol% cholesterol and 20 mol% BMP with DOPC as host lipid). Negatively charged donor vesicles contained additional 2 mol% 2-NBD-GM1 and 4 mol% rhodamine-PE. The transfer of 2-NBD-GM1 is substantively accelerated by a His₆-tag on the GM2AP (light blue lines) compared with g-rGM2AP (black lines). The "baseline total fusion" shown in the FRET measurements, shows the highest level of fluorescence reachable by fusion of vesicles. For definition of "accessible fluorescence" see the Materials and Methods.

GM2 by HexA, lipid mobilization, and lipid transfer by GM2AP. Using negatively charged liposomes (containing 20 mol% BMP and 5 mol% cholesterol beside the marker lipids and DOPC), hydrolysis of [¹⁴C]GM2 in assay preparations with g-rGM2AP-His₆ (light blue bar) is enhanced almost 4-fold compared with the hydrolysis of [¹⁴C]GM2 in the presence of the untagged g-rGM2AP (black bar) (Fig. 4A). This turnover-enhancing effect of the hexahistidine residue was measured at various liposomal lipid compositions.

It was further observed that the His₆-tag inhibits mobilization of membrane lipids by GM2AP (Fig. 4B). The RUs above the baseline (orange) 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.

Any increase of RU values indicates binding of additional material (e.g., GM2AP) to the lipid layer and any drop of RU values indicates loss of material, GM2AP or lipids, from the lipid layer. However, it remains unclear for all data above the baseline (RU = 0), whether the loss is due to removal of lipid or to removal of both, a mixture of protein and lipid.

If the RU value drops below the baseline, we can be certain that lipids from the lipid layer have been removed in amounts at least equal to the RU values given (8). Release and mobilization of liposomal lipids have been analyzed previously by using radiolabeled membrane lipids (8).

However, injection of His₆-tagged GM2AP resulted in an increase of RUs corresponding to adsorbed material at the liposome layer. After injection of buffer, the RUs dropped, but the signal did not go under the baseline, so that some material was released but no explicit mobilization of lipids could be detected (light blue line). On the other hand, injection of tag-free g-rGM2AP resulted first in an increase of RUs, and after injection of buffer, a decrease of the signal below the baseline was observed. This indicates that protein adsorbed to the liposome layer and material was released even before buffer was injected, thus triggering a massive mobilization of lipids (black line). So the His₆-tag seems to strongly inhibit the mobilization of membrane lipids by GM2AP (Fig. 4B).

The intervesicular transfer of 2-NBD-GM1 by both GM2APs was investigated by a FRET assay using liposomes with a negative or no net charge. Transfer of 2-NBD-GM1 was detected by a rise in fluorescence.

The increase of fluorescence was extremely accelerated by the His₆-tagged GM2AP (Fig. 4C). After 5 min, 17% of the transferrable 2-NBD-GM1 had been transferred by g-rGM2AP-His₆, while after 5 min almost no fluorescence was detectable in the assay with the untagged activator protein.

Earlier experiments suggested that g-rGM2AP-His₆ is capable of fusing liposomes (43). Therefore, g-rGM2AP-His₆ was not used in further FRET measurements.

## Hydrolysis of GM2, mobilization of membrane lipids, and transfer of 2-NBD-GM1 by GM2AP are dependent on a low pH value around 4.2

During maturation of lysosomes, the pH value is lowered to a pH value around 4. At this value, many lysosomal exohydrolases possess their pH optima. For hydrolysis of GM2 by HexA in the presence of GM2AP, a narrow pH profile has been observed at pH 4.2 ± 0.3 (25, 45, 46).

The effects on GM2 hydrolysis (**Fig. 5A**), mobilization of membrane lipids (Fig. 5B), and intervesicular transfer of 2-NBD-GM1 (Fig. 5C) were investigated at different pH values (3.0, 4.2, 5.0, and 6.0) using negatively charged liposomes. At pH 4.2, GM2 was degraded to GM3 in the presence of HexA and GM2AP (Fig. 5A). At pH values of 3.0, 5.0, and 6.0, no hydrolysis of liposomal GM2 could be observed in the detergent-free liposomal assay system.

In the lipid mobilization assay, the signal fell below the baseline at pH 4.2, indicating mobilization of membrane lipids by g-rGM2AP (Fig. 5B). At all other pH values, an increase in RUs could be detected, showing that the GM2AP attached to the lipid layer.

The highest intervesicular transfer rate of 2-NBD-GM1 (46%) was reached after 40 min at pH 4.2, while assay preparations with slightly lower pH values (3.0) and slightly higher pH values (5.0) resulted in greatly reduced transfer rates of about 30% (Fig. 5C). At pH values of 6.0 and 7.0, the intervesicular transfer rate was strongly reduced further to 8 and 2%, respectively.

## High ionic strength reduces hydrolysis of GM2, lipid mobilization, and transfer of 2-NDB-GM1

**Figure 6** shows the inhibiting effects of the high ionic strength on GM2AP functions (GM2 hydrolysis, mobilization of membrane lipids, and transfer of 2-NBD-GM1) using negatively charged liposomes. High ionic strength had an inhibitory effect on hydrolysis of GM2. In the case of g-rGM2AP-His$_6$, the hydrolysis of GM2 was nearly 5-fold higher without adding NaCl compared with the assays containing 113 or 226 mM NaCl. In the case of g-rGM2AP, the hydrolysis of GM2 was halved using buffer with NaCl (Fig. 6A).

Mobilization of lipids was observed only in the absence of NaCl addition (Fig. 6B). Transfer of 2-NBD-GM1 at the $t = 40$ min was reduced from 46 to 27% on the addition of 226 mM NaCl (Fig. 6C).

Therefore, the addition of sodium chloride was omitted in further studies.

## BMP stimulates enzymatic digestion of GM2, the mobilization of membrane lipids, and the intervesicular transfer of 2-NBD-GM1 by GM2AP

BMP is a unique lysophospholipid basically found in lysosomes and late endosomes, especially in their intravesicular membranes (4, 29). Formerly it has been shown that the degradation of (glyco)sphingolipids by lysosomal hydrolases in the presence of sphingolipid activator proteins is enhanced by adding BMP to the lipid composition of artificial vesicles (9, 10, 14).
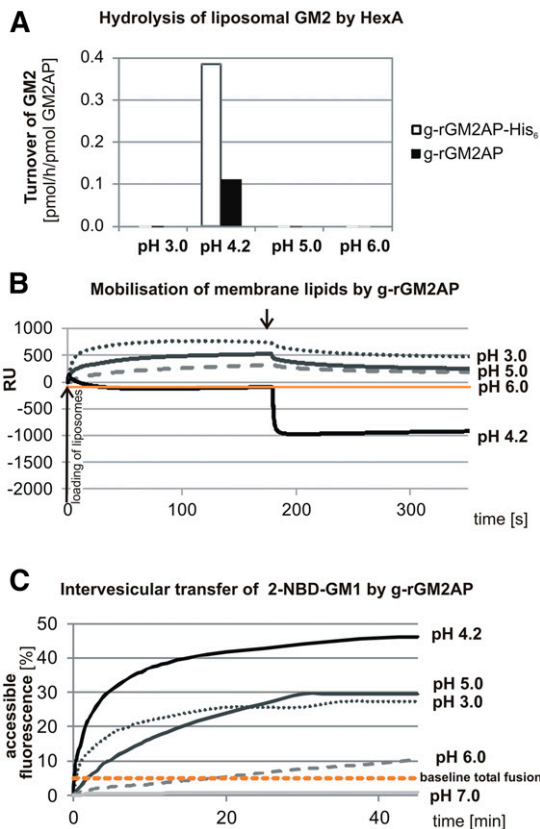
**Fig. 5.** Turnover of liposomal GM2 by HexA in presence of GM2AP, mobilization of membrane lipids, and transfer of 2-NBD-GM1 by GM2AP are sharply dependent on acidic pH values. A: Turnover of [$^{14}$C]GM2 in the liposomal activity assay depends strongly on a pH value around 4.2. The influence of the pH value on the conversion of GM2 to GM3 by HexA in the presence of g-rGM2AP (black/gray) and g-rGM2AP-His$_6$ (white) was investigated in an in vitro liposomal assay using negatively charged liposomes. Significant hydrolysis of GM2 to GM3 was only observed at pH 4.2. Mean error was determined with less than 10%. B: Using negatively charged liposomes, binding and mobilization of membrane lipids by g-rGM2AP was extremely dependent on the pH value. At a pH of 4.2, the injected g-rGM2AP adsorbed to the liposome layer; material was released, in part, before injection of buffer. After injection of buffer, material was released up to 50% (black line). However, at pH 3.0, 5.0, and 6.0 (gray lines, dashes and dots), no substantial mobilization of lipids was detected. C: Negatively charged liposomes contained 2-NBD-GM1, rhodamine PE, 5 mol% cholesterol, 20 mol% BMP, and as host lipid DOPC in 20 mM sodium citrate (pH 4.2) (black line), pH 3.0 (gray dots), pH 5.0 (gray line), pH 6.0 (gray dashes), and pH 7.0 (light gray line). The intervesicular transfer of 2-NBD-GM1 from donor to acceptor liposomes depends on the pH value. The highest transfer activity was observed at the pH value of 4.2, and (black line). At pH 5.0 and pH 3.0 the transfer of 2-NBD-GM1 was reduced (dark gray line and dots), while it was nearly negligible at pH 6.0 and pH 7.0 (gray dashes and light gray line). For a definition of "accessible fluorescence," see the Materials and Methods.

We could demonstrate that BMP (20–35 mol%) and other anionic lipids like PI and PG (20 mol%) strongly stimulate the GM2AP-mediated hydrolysis of GM2 by HexA (**Fig. 7A**, supplementary Fig. 3). In the case of g-rGM2AP-His$_6$, the hydrolysis rate was enhanced 5-fold (from 0.07 pmol/h/pmol GM2AP without BMP to 0.38 pmol/h/pmol GM2AP using 20 mol% BMP). In the case of g-rGM2AP,
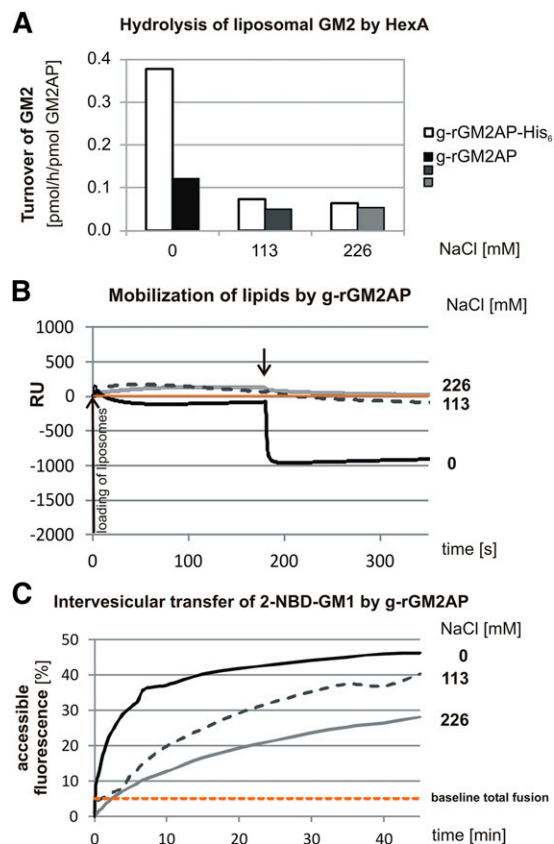
**Fig. 6.** Influence of ionic strength on the turnover of liposomal GM2, mobilization of membrane lipids, and transfer of 2-NBD-GM1. A: High ionic strength reduced hydrolysis of GM2 by HexA in presence of g-rGM2AP (black/gray) or g-rGM2AP-His₆ (white). The influence of ionic strength was investigated in an in vitro liposomal activity assay using negatively charged liposomes. The highest hydrolysis rate was measured at pH 4.2. Mean error was determined with less than 10%. B: Binding, as well as mobilization of membrane lipids by g-rGM2AP, depends on ionic strength, as shown by SPR studies, using immobilized negatively charged liposomes. Without adding NaCl, g-rGM2AP mobilized approximately half of the chip bound lipids (black line). Higher ionic strength had an inhibiting effect on the lipid mobilizing abilities of g-rGM2AP (gray dashes and gray line). Orange line, baseline after loading of liposomes. C: FRET measurements showed a reduced intervesicular transfer of 2-NBD-GM1 by g-rGM2AP using a citrate buffer (pH 4.2) with high ionic strength (113 mM NaCl, gray dashes; 226 mM NaCl, gray line). The negatively charged donor liposomes contained 2 mol% NBD-GM1 and 4 mol% rhodamine-PE, respectively. The orange baseline of total fusion indicates the fluorescence obtainable by fusion of vesicles. For definition of accessible fluorescence, see the Materials and Methods.

a 10-fold enhancement from 0.01 pmol/h/pmol GM2AP to more than 0.1 pmol/h/pmol GM2AP was reached by using 20 mol% BMP.

As shown in Fig. 7B, the mobilization of membrane lipids by GM2AP was only observed in the presence of BMP (10–35 mol%). Using uncharged liposomes or slightly negatively charged liposomes with 5 mol% BMP, the signal did not fall below the baseline, indicating no detectable mobilization of chip bound material. In the presence of liposomes with 10 mol% BMP, an increase of RUs was observed, indicating that the injected g-rGM2AP was
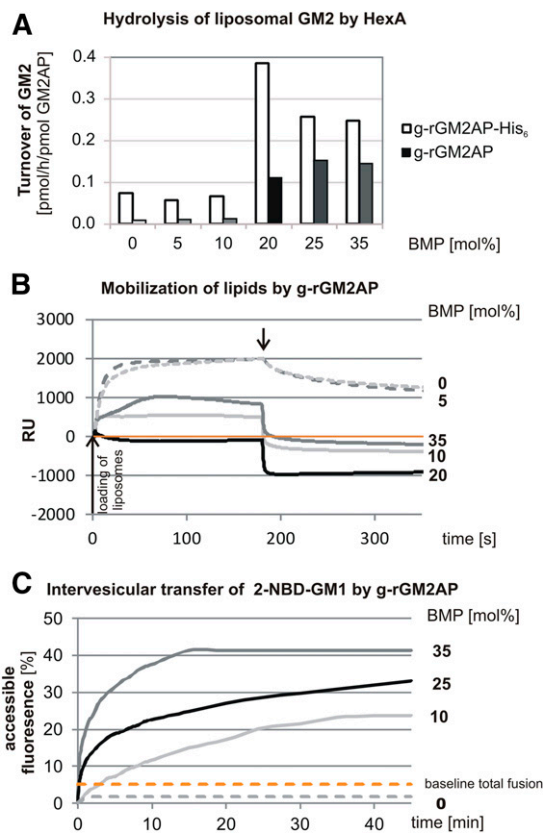


**Fig. 7.** BMP enhances hydrolysis of liposomal GM2, mobilization of membrane lipids, and transfer of 2-NBD-GM1 by GM2AP. A: Turnover of GM2 was investigated in an in vitro liposomal activity assay using negatively charged liposomes with 5–35 mol% BMP and uncharged liposomes (0 mol% BMP). Enhancement of BMP stimulated the hydrolysis of [¹⁴C]GM2 using g-rGM2AP (black/gray) and g-rGM2AP-His₆ (white). Mean error was determined with less than 10%. B: The SPR binding and mobilization studies were done with uncharged liposomes and negatively charged liposomes containing 5–35 mol% BMP. Using liposomes with 0–5 mol% BMP, the injected g-rGM2AP adsorbed to the lipid layer (light gray dashes); using 10 mol% BMP or 35 mol% BMP, little mobilization of lipids was observed (light gray line and gray line). Substantial lipid mobilization could be detected (black line) only with liposomes containing 20 mol% BMP. C: Intervesicular transfer of 2-NBD-GM1 was measured by a FRET assay using negatively charged liposomes with varying BMP content and uncharged liposomes. Without BMP in the liposomes, no transfer of 2-NBD-GM1 could be measured (light gray dashes). Enhancement of BMP content up to 35 mol% (gray line) led to a successive enhancement of 2-NBD-GM1 transfer by g-rGM2AP. For a definition of accessible fluorescence, see the Materials and Methods.

adsorbed to the lipid layer. The signal dropped by washing with buffer, giving evidence that material was mobilized and released. Afterwards, the signal fell slightly below the baseline, indicating that only a small amount of chip bound lipids was released (light gray line). A similar result was obtained using 35 mol% BMP (gray line). In liposomes containing 20 mol% BMP, the injection of g-rGM2AP resulted in a signal going far below the baseline, indicating that up to 50% of total chip bound lipids were mobilized (black line).

The intervesicular transfer of 2-NBD-GM1 took place only in the presence of BMP and was nearly doubled in the

presence of 35 mol% BMP compared with 10 mol% BMP in the liposomal membranes (Fig. 7C).

## High cholesterol levels strongly inhibit hydrolysis of GM2, mobilization of membrane lipids, and intervesicular transfer of 2-NBD-GM1

In the late endosomal compartment, cholesterol is sorted out from the inner membranes by NPC2 (47). The inhibitory effects of high cholesterol levels (20–40 mol%) in negatively charged liposomes on GM2 hydrolysis are shown in **Fig. 8A**, while the mobilization of membrane lipids and transfer of 2-NBD-GM1 are shown in Fig. 8B and Fig. 8C,
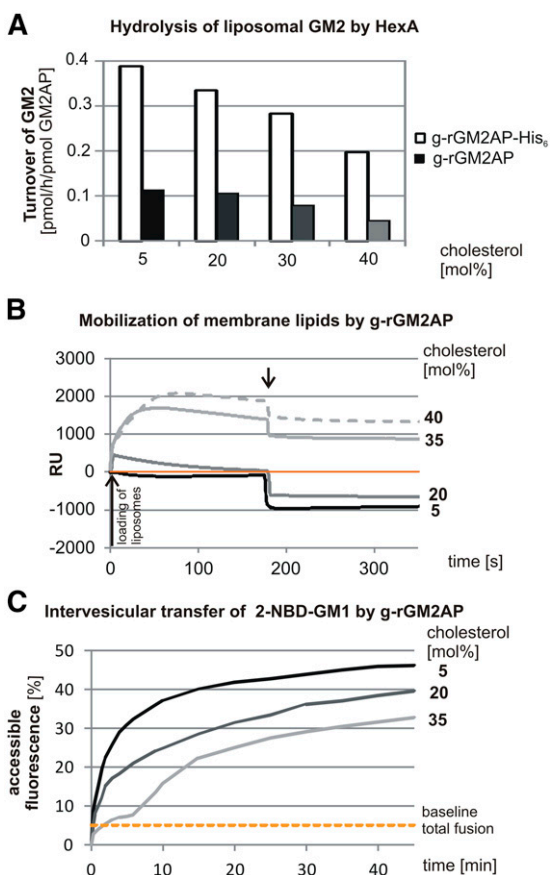


**Fig. 8.** High cholesterol levels inhibit hydrolysis of liposomal GM2, mobilization of membrane lipids, and transfer of 2-NBD-GM1 by GM2AP. A: A high cholesterol content of 40 mol% strongly inhibited the hydrolysis of [$^{14}$C]GM2 by HexA in the presence of GM2AP [g-rGM2AP (light gray) and g-rGM2AP-His$_6$ (white)] using negatively charged liposomes. However, a cholesterol content below 20 mol% had negligible influence on the turnover of [$^{14}$C]GM2 by g-rGM2AP (dark gray) and g-rGM2AP-His$_6$ (white). Mean error was determined with less than 10%. B: The injected g-rGM2AP adsorbed to the lipid layer but almost no material was released by injection of protein free buffer (light gray line and dashes) only when using negatively charged liposomes with high cholesterol levels of 35–40 mol%. Using cholesterol ≤20 mol%, g-rGM2AP adsorbed to the lipid layer and lipids were mobilized, especially when GM2AP free buffer was injected (gray and black lines). C: High cholesterol levels reduced the rate of intervesicular transfer of 2-NBD-GM1 by g-rGM2AP in the FRET assay (gray and light gray lines) compared with liposomes with 5 mol% cholesterol (black) using negatively charged liposomes. For a definition of accessible fluorescence, see the Materials and Methods.

respectively. The results are compared with the normally used negatively charged liposomes with 5 mol% cholesterol and 20 mol% BMP. Increasing cholesterol concentration up to 40 mol% inhibited the GM2AP-mediated hydrolysis of GM2 by HexA (Fig. 8A). The GM2 degradation was reduced by about 60% in the presence of g-rGM2AP and 40 mol% cholesterol, whereas 20 mol% cholesterol led to a decrease of only 10%. His-tagged g-rGM2AP-His$_6$ also reduced the hydrolysis by about 15% at 20 mol% cholesterol and to around 50% at 40 mol% cholesterol.

Transfer of 2-NBD-GM1 is reduced by the inclusion of 20–35 mol% cholesterol to anionic vesicles (Fig. 8C). With liposomes containing 5 mol% cholesterol, 47% transfer of 2-NBD-GM1 was observed after 40 min, compared with 32% observed on the addition of 35 mol% cholesterol.

## SM inhibited and Cer enhanced hydrolysis of GM2, while mobilization of membrane lipids and intervesicular transfer of 2-NBD-GM1 by GM2AP were inhibited by both lipids

In the late endosomes, SM is degraded to Cer by ASM (14). Furthermore, it has been shown that Cer functions as a cholesterol competitor and promotes the exit of cholesterol from membranes (48). Therefore, both SM and Cer may play important roles in lysosomal lipid degradation.

The inhibiting effects of SM (20 mol%) and the stimulating effects of Cer (20 mol%) in negatively charged liposomes on GM2 hydrolysis are shown in **Fig. 9A**. Further on, the inhibiting effects on mobilization of membrane lipids (Fig. 9B) and transfer of 2-NBD-GM1 (Fig. 9C) compared with negatively charged liposomes with 5 mol% cholesterol and 20 mol% BMP were observed.

SM (20 mol%) inhibited the GM2AP-mediated hydrolysis of GM2 by HexA from 0.11 pmol/h/pmol GM2AP to 0.08 pmol/h/pmol GM2AP, representing 27% inhibition; while 20 mol% Cer enhanced turnover of GM2 by almost 100% (to 0.21 pmol/h/pmol GM2AP) (Fig. 9A). The inhibiting effect of SM was reversed by the inclusion of an equal amount (20 mol%) of Cer into the vesicles.

A mixture of both SM and Cer at medium concentrations (10 mol% each) showed the best results for hydrolysis of GM2 by enhancing it effectively to 0.3 pmol/h/pmol (**Fig. 10A**).

Cer strongly reduced the mobilization of membrane lipids from negatively charged vesicles, while SM inhibited it almost completely (Fig. 9B). In the presence of Cer (20 mol%), the signal fell slightly below the baseline, indicating that g-rGM2AP was able to mobilize lipids under these conditions (green line). In the presence of SM (20 mol%), g-rGM2AP was adsorbed to the lipid layer and material was released by injection of buffer without going below the baseline (red line). The highest mobilization of membrane lipids was observed in the absence of both SM and Cer (black line).

A mixture of both SM and Cer, at medium concentration, resulted in nearly the same findings as measurements with Cer alone (Fig. 10B).

SM (20 mol%) also extremely reduced intervesicular transfer of 2-NBD-GM1 at negatively charged vesicles, from
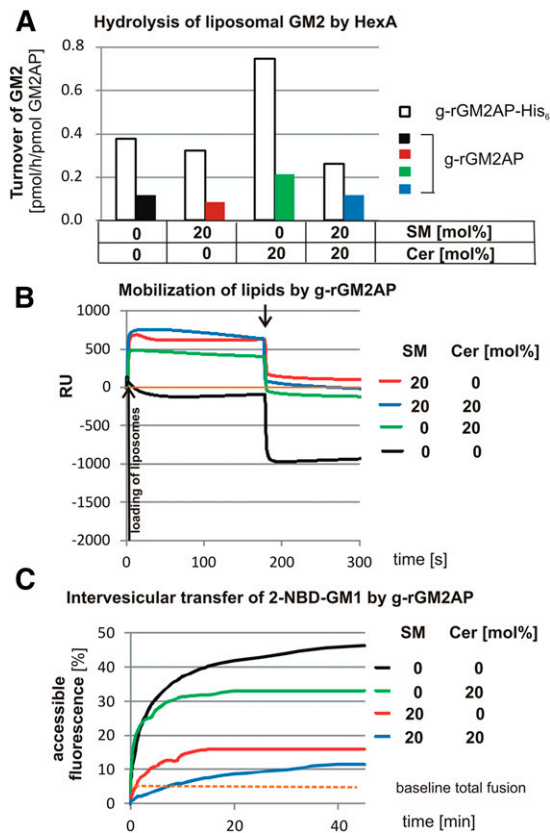
**Fig. 9.** Influence of SM and Cer on hydrolysis of liposomal GM2, mobilization of membrane lipids, and intervesicular transfer of 2-NBD-GM1 by GM2AP. A: SM reduces, while Cer enhances. hydrolysis of GM2 by HexA in the presence of g-rGM2AP (black, red, green, blue) and g-rGM2AP-His₆ (white) in the in vitro liposomal activity assay using negatively charged liposomes containing SM (0 or 20 mol%) and Cer (0 or 20 mol%). Mean error was determined with less than 10%. B: After immobilization of negatively charged liposomes with 0 or 20 mol% Cer and 0 or 20 mol% SM, g-rGM2AP was injected to the flow cell. The highest mobilization of lipids was measured (black line) in the absence of both lipids. In the presence of liposomes with Cer (20 mol%), g-rGM2AP was able to mobilize lipids (green line); while in presence of SM (20 mol%, red line) and a mixture of both lipids (blue line), the signal did not fall below the baseline (orange line). C: The 2-NBD-GM1 fluorescence was measured continuously in a FRET assay using negatively charged liposomes with 0 or 20 mol% Cer and 0 or 20 mol% SM. The highest transfer of 2-NBD-GM1 was achieved with liposomes without SM and Cer (black line). SM and Cer alone had inhibiting effects (red and green lines), while the lowest transfer was achieved with the combination of Cer and SM (blue line). For a definition of accessible fluorescence, see the Materials and Methods.

47 to 16% (*t* = 40 min), while the inhibition caused by Cer was less pronounced (Fig. 9C). A mixture of SM and Cer (10 mol% each) resulted in a mean transfer of 2-NBD-GM1 (12%) (Fig. 10C).

## DISCUSSION

Membrane lipids are essential building blocks of biological membranes. Therefore a genetic block (knockout) of their biosynthesis is detrimental to life. In addition, many membrane lipids are precursors for the biosynthesis
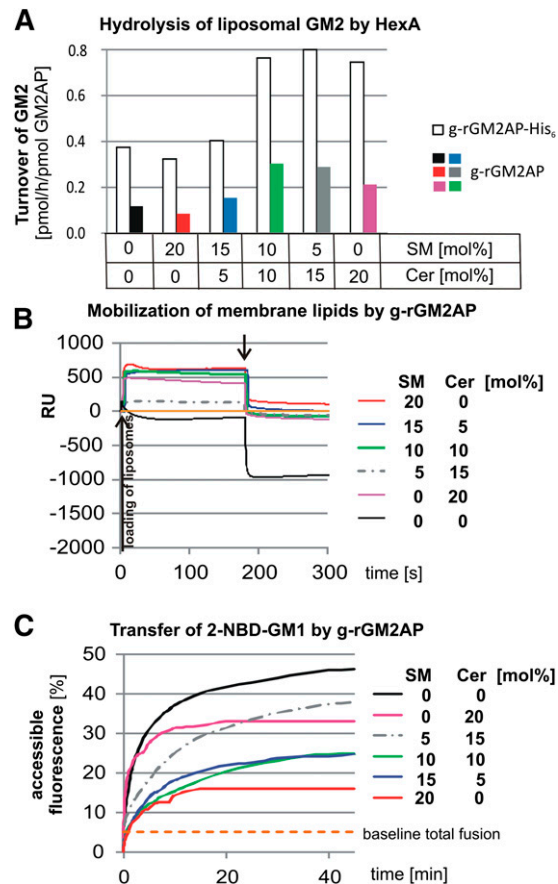


**Fig. 10.** Increasing Cer levels enhanced hydrolysis of liposomal GM2 by HexA in presence of GM2AP, but not mobilization of lipids and intervesicular transfer of 2-NBD-GM1 by r-gGM2AP; whereas increasing SM levels are always inhibitory in all three assay systems. A: A reduction of SM level and an increase of Cer leads to an enhanced hydrolysis of liposomal GM2 by HexA in the presence of g-rGM2AP (filled columns) and g-rGM2AP-His₆ (white). An equimolar presence of both SM (10 mol%) and Cer (10 mol%) resulted in the highest turnover rates for GM2. Mean error was determined with less than 10%. B: The addition of SM and Cer leads to reduced lipid mobilization (colored lines) compared with the studies without both lipids (black line). C: SM and Cer slow down intervesicular transfer of 2-NBD-GM1 by g-rGM2AP in the FRET-assay. SM (10 or 20 mol%) or Cer (20 mol%) reduced transfer of 2-NBD-GM1 (colored lines). The highest transfer of 2-NBD-GM1 was achieved with liposomes without SM and Cer (black line). For a definition of accessible fluorescence, see the Materials and Methods.

of other membrane lipids. For example, Cer is needed as a precursor of other (glyco)sphingolipids, such as glucosylceramide, SM, and gangliosides such as GM1 and GM2. Thus a knockout affecting the biosynthesis of one of these lipids would fatally interfere with proper formation of membrane lipids. Therefore, it would not allow the elucidation of its regulatory functions with regard to the metabolism of other membrane lipids.

Therefore, we used three different in vitro approaches, based on liposomes with well-defined and modifiable lipid compositions, to investigate the regulatory influence of individual membrane lipids on the capability of GM2AP, a lipid binding and transfer protein, to transfer lipids such as 2-NBD-GM1 between membranes, to mobilize membrane

bound lipids, and on the GM2AP-mediated hydrolysis of liposomal GM2 by HexA.

## The His$_6$-tag changes the overall properties of GM2AP

The His$_6$-tag has been used for facilitated isolation and purification of recombinant proteins for a couple of years. The g-rGM2AP-His$_6$, expressed and purified in our lab, showed higher activity in liposomal assays for hydrolysis of GM2 by HexA than g-rGM2AP (Fig. 4A), but a loss of its ability to mobilize membrane lipids was observed (Fig. 4B). This was independent of its concentration, the lipid composition of the liposomes, the buffer system, and the pH value used (data not shown). The g-rGM2AP-His$_6$ bound tightly to the negatively charged vesicles, presumably mediated by extra positive charges carried by the His$_6$-tag at low pH values.

These results corroborate earlier findings that an elongation of GM2AP by a peptide of 10 histidine residues not only affected membrane binding properties of GM2AP, but also affected its lipid extraction properties, as observed with N-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (dansyl-DHPE) (49).

The isoelectric point of the tag-free GM2AP was found to be 4.8 [by measurements (26) and calculation (ExPASY ProtParam)], whereas that of g-rGM2AP-His$_6$ was calculated to be 6.1. This higher value resulted from positive charges of the hexahistidine residue.

We assume that the disturbing influence of the His$_6$-tag is based on its positive charge at low pH values. In the case of the GM2AP, the C-terminal-tag places positive charges at the opposite side of the positively charged domain of the protein (43). This may influence lipid binding properties and the ability of GM2AP to escape from negatively charged membranes (11, 16).

The observation that the tightly membrane bound His$_6$-tagged g-rGM2AP-His$_6$ strongly stimulated hydrolysis of liposomal bound GM2 by HexA (Figs. 4–10A) suggests that the g-rGM2AP-His$_6$-mediated hydrolysis by HexA occurred directly at the liposomal surface and not after forming a soluble complex, as postulated earlier for the untagged GM2AP (26, 50, 51).

The g-rGM2AP-His$_6$, or a number of g-r-GM2AP-His$_6$ molecules, may also bind two vesicles simultaneously. Bringing two vesicles (donor and acceptor) together could facilitate the intervesicular transfer of 2-NBD-GM1 by r-gGM2AP-His$_6$, even if r-gGM2AP-His$_6$ would not leave the vesicular membrane.

Preliminary experiments suggested that the His-tagged g-rGM2AP-His$_6$ may also fuse liposomes (43). Because fusion would compromise the transfer assay, we did not use the recombinant g-rGM2AP-His$_6$ for transfer assays, but used, instead, the naturally occurring tag-free GM2AP which is hardly fusogenic (Schwarzmann G, Breiden B, Sandhoff K, unpublished observations). In all intervesicular transfer experiments, we used 4 mol% quencher (rhodamine-PE) in the donor liposomes, twice as high as the level of the fluorophore (2 mol%). Under these conditions, the highest level of dequench of NBD-fluorescence reached by complete fusion of donor and acceptor vesicles was much lower than the fluorescence levels obtained by intervesicular transfer of the 2-NBD-GM1. The NBD-fluorescence measured above the level reached by complete fusion was considered to be generated by transfer of 2-NBD-GM1. Therefore, a baseline of total fusion was included in the results of the FRET measurements as a control.

## Lysosomal degradation of ganglioside GM2 is dependent on a pH value around 4.2 and is inhibited by high ionic strength

In lysosomes, the catabolism of GM2 occurs at luminal vesicular surfaces, mainly by the water-soluble HexA (and to a small part by the labile HexS) with the essential help of a lipid transfer protein, GM2AP. GM2AP interacts with HexA (50) and seems to lift membrane bound GM2 and present it to the water-soluble hydrolase. The optimal hydrolysis of liposomal GM2 by HexA in the presence of GM2AP takes place in a narrow pH range around 4.2 (25, 26); whereas on the contrary, HexA has a broad pH profile of pH 3–7.4 with synthetic water-soluble substrates (45).

In reconstitution experiments with liposomes containing GM2, but no other negatively charged lipids, as models of the luminal intralysosomal vesicles, the degradation of GM2 by HexA resulted in a physiologically irrelevant low catabolic rate, even in the presence of GM2AP. This low catabolic rate could be increased by several stimulating modifiers, such as low ionic strength and anionic lipids, as reported earlier (8, 9, 25). In addition to the stimulating lipid modifiers, we now report also on inhibitory lipid modifiers, cholesterol, and SM, which even inhibit hydrolysis of GM2 in the presence of stimulatory BMP.

During endocytosis, the lipid content of the intralysosomal membranes is changed dramatically. While the content of BMP is enhanced (4, 29, 52), cholesterol levels are reduced simultaneously and the lysosomes are acidified. The pH value changes from 7.4 at the plasma membrane to 4.2–5.5 in the lysosome (53, 54). Accordingly, the pH optima of many lysosomal exohydrolases are found in this range [e.g., pH 4.7 for GM1-β-galactosidase (55), pH 4.5 for arylsulftase A (56), and pH 3.8–4.3 for acid ceramidase (57)].

Whereas HexA has a rather broad pH profile in the range of 3–7.4 with water-soluble synthetic substrates (45, 46), hydrolysis of GM2 by HexA in the presence of GM2AP is confined to a rather narrow pH value around 4.2 (Fig. 5A), as observed before (25, 45). Accordingly, we found that the mobilization of membrane lipids by g-rGM2AP was also extremely dependent on a low pH value around 4.2 (Fig. 5B), whereas the transfer of 2-NBD-GM1 by g-rGM2AP showed a broader pH profile, with an optimum at pH 4.2 (Fig. 5C).

The catabolism of ganglioside GM2 is not only strongly affected by the frequently changing lipid composition of intraendolysosomal membranes, but also by ionic strength. Addition of 113 mM or 226 mM NaCl to 20 mM sodium citrate buffer reduced hydrolysis of GM2 by HexA in the presence of g-rGM2AP or g-rGM2AP-His$_6$ (Fig. 6A). Also, the intervesicular transfer of 2-NBD-GM1 and mobilization of membrane bound lipids by GM2AP were reduced

by increasing ionic strength (Fig. 6B, C). This corroborates former data on the hydrolysis of GM2 (25) and the mobilization of lipids by Sap D (8).

## Membrane lipids regulate the functionality of membrane-associated proteins

It is known that the lipid composition is essential for membrane-based cellular functions. Lipids act as modifiers, for example, as first and second messengers in signal transduction and by regulating membrane-associated enzymes and receptors. Also the specific lipid composition of subcellular membranes is crucial for specific lipid-protein interactions, such as of COP I protein function modulated by SM (58) and inhibition of phospholipase D by Cer (59).

Anionic phospholipids, such as BMP, strongly stimulate the lysosomal catabolism of a multitude of membrane bound (glyco)sphingolipids, such as that of GM1 (10), GM2 (9), sulfatide (60), sulfated gangliotriaosylceramide (12), glucosylceramide (16), CER (13), and SM (11). Malfunction in the degradation of lysosomal sphingolipids results in lipid accumulation causing fatal lipid storage diseases (61), while the accumulating material affects many other lysosomal proteins leading to a lysosomal traffic jam (33).

Incorporation of the anionic BMP into the GM2-carrying liposomal membranes stimulates GM2 hydrolysis more than 10-fold in the presence of HexA and GM2AP. This may be caused by an increased electrostatic interaction between negatively charged GM2 carrying liposomes and positively charged protonated catabolic proteins at the acidic pH values of the incubation mixture at pH 4.2, rather than by an additional and independent effect of BMP on the membrane structure.

Indeed, incorporation of PG or PI, instead of BMP, caused an even stronger stimulation of GM2 hydrolysis (supplementary Fig. 3). This is also in agreement with the observation that anionic lipids, found to occur in lysosomes in substantial quantity like PI and PG (62-65), stimulate GM1 hydrolysis by β-galactosidase in the presence of either Sap B or GM2AP even better than BMP (10).

However, the incorporation of a cationic lipid (EPC or MVL5) led to a low hydrolysis rate of GM2 in the range of that observed with uncharged vesicles (supplementary Fig. 3). Furthermore, the addition of a cationic amphiphilic drug into the assay mixture (desipramine 30 mmol/l) reduced GM2 hydrolysis drastically, presumably by reduced electrostatic interaction or even repulsion (supplementary Fig. 4B). In SPR studies, the cationic amphiphilic drug, desipramine, also disturbed the binding of GM2AP to the membrane lipids so that the protein was released (supplementary Fig. 4A). A similar effect had been observed before using membrane bound ASM (39).

As an essential and ubiquitous integral part of mammalian membranes, cholesterol assumes crucial structural and regulatory functions (66–68). It is a major steroid constituent of animal tissues, which stabilizes plasma membranes, reduces membrane permeability for ions, and enables the liquid-ordered phase of lipid bilayers (66, 68). However, the cholesterol content decreases in the intraendolysosomal vesicles during endocytosis (4, 69). Cholesterol is released and secreted from these luminal vesicles mainly by two sterol binding proteins, NPC2 and NPC1 (11, 31, 47, 69, 70). Those intraendolysosomal vesicles have been identified as platforms for lysosomal glycosphingolipid, sphingolipid, and membrane degradation (71–73). The drop in cholesterol content is as typical for the maturation of the luminal vesicles as is the increase in BMP content and acidification of the lysosomes. The sorting out of cholesterol and the degradation of lipids in the endocytotic pathway should heavily affect the composition and function of the luminal endolysosomal vesicles. Therefore, the question is whether cholesterol might be inhibitory to lysosomal catabolism of glycosphingolipids and has to be secreted first from the luminal vesicles, before glycosphingolipids can be digested properly by the lysosomal juice (5, 73)

Our experiments indeed show that a high cholesterol content (40 mol%), as found in neuronal plasma membranes (68), strongly inhibits turnover of GM2, intervesicular transfer of 2-NBD-GM1, and the mobilization of lipids by GM2AP, even in presence of BMP (Fig. 8). These findings confirm earlier observations on the essential functions of Sap A and Sap B, for the glycosphingolipid metabolism, that are impaired by high cholesterol levels (7, 8). Furthermore, it was shown that a defect of the protein NPC2 in Niemann-Pick disease type C not only caused an accumulation of cholesterol in the liver and brain, but also, as a secondary effect, caused increased storage of gangliosides such as GM2 (74, 75).

It is known that anionic lipids like BMP stimulate GSL degradation. BMP is generated in the late endosomes and lysosomes and is considered as a marker lipid for those organelles. Its long half-life is based on its unusual configuration and may possibly explain the extreme influence of this lipid on membrane degradation (3). Other anionic phospholipids are quickly degraded by the lysosomal phospholipase A2, reducing their influence. BMP has been reported to enhance degradation of sphingolipids and glycosphingolipids by lysosomal exohydrolases in the presence of sphingolipid activator proteins (9, 10, 13, 14). However, as shown here, it enhances not only hydrolysis of GM2, but also the intervesicular transfer of 2-NBD-GM1 and mobilization of membrane lipids by GM2AP (Fig. 7). BMP is formed within luminal vesicles presumably originating from PG (76, 77), and is a secondary storage compound in gangliosidoses (78) and SM storage diseases (79).

BMP carries a negative charge at endolysosomal pH values (pH 4–5) (11), generating an electrostatic interaction between cationic proteins (such as GM2AP and HexA) and negatively charged vesicular surfaces. This seems to be very important for mobilization of membrane bound GM2 by GM2AP and proper catabolic rates of vesicle bound ganglioside GM2. Though the physiological function of intervesicular lipid transfer and lipid mobilization by GM2AP and other SAPs is not clear, one may speculate that they might facilitate together with micelle-forming compounds (such as fatty acids or lysophospholipids released within the lysosome) the disintegration of luminal vesicles in the endolysosomal compartment and thereby enhance membrane and lipid digestion.

SM has an affinity to cholesterol and is reported to shield the OH-group of cholesterol like an umbrella, stabilizing it in the membrane (80). During maturation from endosomes, SM of the intraendosomal and intralysosomal vesicles is degraded to Cer by ASM, with a pH optima of 5.0 (81, 82). So SM, which blocks cholesterol transfer by NPC2 in liposomal assays (31), is replaced by the activating Cer, which competes with cholesterol for niches in the membrane, facilitating cholesterol efflux from the membrane (83).

Our experiments also show that zwitterionic SM, in contrast to the anionic BMP, has an inhibitory influence on the intervesicular transfer of 2-NBD-GM1, the hydrolysis of GM2, and the mobilization of membrane bound lipids by GM2AP. Furthermore, its degradation product, Cer, had a stimulatory effect on the hydrolysis of GM2 by HexA in the presence of g-rGM2AP and g-rGM2AP-His$_6$ (Fig. 9A) (green, white), but an inhibitory impact on lipid mobilization (Fig. 9B) (green line) and transfer of 2-NBD-GM1 (Fig. 9C) (green line).

The highest rate of GM2 hydrolysis was measured using model membranes containing 10 mol% SM and 10 mol% Cer (Fig. 10A) (green, blue). These observations are in agreement with the assumption that in intralysosomal vesicles, both lipids exist in a mixture and that their concentration is of significant importance.

Substantial mobilization of lipids and optimal intervesicular transfer of 2-NBD-GM1 by GM2AP was achieved only with liposomes free of Cer and SM. On the other hand, a high rate of GM2 hydrolysis was achieved in negatively charged vesicles at pH 4.2, in the combined presence of Cer and SM, both at medium levels. Using negatively charged vesicles with 20 mol% SM, the transfer of 2-NBD-GM1 was reduced by more than 60%. Also, mobilization of lipids was massively inhibited by adding SM to the vesicle preparation. Apparently at least a portion of SM has to be degraded first in the late endosomes by ASM, to annul its inhibitory action on cholesterol secretion by NPC2 (31) and on the mobilization and degradation of GM2.

## CONCLUSION

The regulatory roles of some late endosomal lipids (cholesterol, SM, Cer, and BMP) for sphingolipid digestion in the liposomes have been elucidated.

BMP, generated in luminal endolysosomal vesicles, stimulates intervesicular transfer of 2-NBD-GM1, mobilization of membrane lipids as mediated by GM2AP, and hydrolysis of liposomal GM2 by HexA in the presence of GM2AP. At neutral liposomes without negatively charged lipids like BMP, only little, nearly negligible GM2 degrading activity, little transfer of 2-NBD-GM1, and no lipid mobilization by GM2AP, were observed. With BMP in the liposomes, hydrolysis of GM2, transfer of 2-NBD-GM1, and lipid mobilization by GM2AP were enhanced strongly, thereby showing that an anionic environment is essential for all characteristic features of GM2AP. Also, Cer has a stimulating effect on lipid mobilization by GM2AP and the hydrolysis of GM2 by HexA in the presence of GM2AP.

SM and cholesterol, essential plasma membrane stabilizing lipids, inhibited GM2 turnover and mobilization of lipids by GM2AP. The combined presence of Cer and SM (both medium levels, as postulated in the intralysosomal membranes), however, greatly enhanced the turnover of membrane bound GM2 in negatively charged vesicles (containing 20 mol% BMP) at pH 4.2.

It has been observed that transfer of 2-NBD-GM1, turnover of GM2 by HexA in the presence of GM2AP, and the mobilization of membrane lipids by GM2AP are extremely enhanced by BMP and inhibited by SM and high cholesterol levels. Hydrolysis of GM2 was also stimulated by high Cer levels and by rather low pH values at about 4.2.

The His$_6$-tag, routinely added to recombinant proteins to facilitate their purification, however, had a high impact on the overall properties of GM2AP in liposomal assay systems. The hexahistidine residue on g-rGM2AP-His$_6$ changed the abilities of GM2AP substantially, leading to enhanced hydrolysis of GM2, accelerated intervesicular transfer of 2-NBD-GM1, and drastic inhibition of the mobilization of membrane bound lipids.

Furthermore, though the His$_6$-tagged g-rGM2AP-His$_6$ enhanced GM2 turnover by HexA, it blocked mobilization of membrane lipids by GM2AP, indicating that the GM2 degradation, as mediated by HexA in presence of the His-tagged g-rGM2AP-His$_6$, can proceed mainly at the liposomal membrane surface.

## REFERENCES

1. Kolter, T., and K. Sandhoff. 1999. Sphingolipids—their metabolic pathways and the pathobiochemistry of neurodegenerative diseases. *Angew. Chem. Int. Ed. Engl.* **38:** 1532–1568.
2. Sandhoff, K., and T. Kolter. 1996. Topology of glycosphingolipid degradation. *Trends Cell Biol.* **6:** 98–103.
3. Matsuzawa, Y., and K. Y. Hostetler. 1979. Degradation of bis (monoacylglycero)phosphate by an acid phosphodiesterase in rat liver lysosomes. *J. Biol. Chem.* **254:** 5997–6001.
4. Möbius, W., E. van Donselaar, Y. Ohno-Iwashita, Y. Shimada, H. F. Heijnen, J. W. Slot, and H. J. Geuze. 2003. Recycling compartments and the internal vesicles of multivesicular bodies harbor most of the cholesterol found in the endocytic pathway. *Traffic.* **4:** 222–231.
5. Wang, M. L., M. Motamed, R. E. Infante, L. Abi-Mosleh, M. S. Brown, and J. L. Goldstein. 2010. Identification of surface residues on Niemann-Pick C2 essential for hydrophobic handoff of cholesterol to NPC1 in lysosomes. *Cell Metab.* **12:** 166–173.
6. Malinina, L., D. K. Simanshu, X. Zhai, V. R. Samygina, R. Kamlekar, R. Kenoth, B. Ochoa-Lizarralde, M. L. Malakhova, J. G. Molotkovsky, D. J. Patel, et al. Sphingolipid transfer proteins defined by the GLTP-fold. *Q. Rev. Biophys.* Epub ahead of print March 23, 2015; doi:10.1017/S003358351400016X.
7. Locatelli-Hoops, S., N. Remmel, R. Klingenstein, B. Breiden, M. Rossocha, M. Schoeniger, C. Koenigs, W. Saenger, and K. Sandhoff. 2006. Saposin A mobilizes lipids from low cholesterol and high bis(monoacylglycerol)phosphate-containing membranes: patient variant saposin A lacks lipid extraction capacity. *J. Biol. Chem.* **281:** 32451–32460.
8. Remmel, N., S. Locatelli-Hoops, B. Breiden, G. Schwarzmann, and K. Sandhoff. 2007. Saposin B mobilizes lipids from cholesterol-poor and bis(monoacylglycero)phosphate-rich membranes at acidic pH.

Unglycosylated patient variant saposin B lacks lipid-extraction capacity. *FEBS J.* **274:** 3405–3420.

9. Werth, N., C. G. Schuette, G. Wilkening, T. Lemm, and K. Sandhoff. 2001. Degradation of membrane-bound ganglioside GM2 by beta-hexosaminidase A. Stimulation by GM2 activator protein and lysosomal lipids. *J. Biol. Chem.* **276:** 12685–12690.

10. Wilkening, G., T. Linke, G. Uhlhorn-Dierks, and K. Sandhoff. 2000. Degradation of membrane-bound ganglioside GM1. Stimulation by bis(monoacylglycero)phosphate and the activator proteins SAP-B and GM2- AP. *J. Biol. Chem.* **275:** 35814–35819.

11. Oninla, V. O., B. Breiden, J. O. Babalola, and K. Sandhoff. 2014. Acid sphingomyelinase activity is regulated by membrane lipids and facilitates cholesterol transfer by NPC2. *J. Lipid Res.* **55:** 2606–2619.

12. Hepbildikler, S. T., R. Sandhoff, M. Kölzer, R. L. Proia, and K. Sandhoff. 2002. Physiological substrates for human lysosomal beta-hexosaminidase S. *J. Biol. Chem.* **277:** 2562–2572.

13. Linke, T., G. Wilkening, F. Sadeghlar, H. Mozcall, K. Bernardo, E. Schuchman, and K. Sandhoff. 2001. Interfacial regulation of acid ceramidase activity. Stimulation of ceramide degradation by lysosomal lipids and sphingolipid activator proteins. *J. Biol. Chem.* **276:** 5760–5768.

14. Linke, T., G. Wilkening, S. Lansmann, H. Moczall, O. Bartelsen, J. Weisgerber, and K. Sandhoff. 2001. Stimulation of acid sphingomyelinase activity by lysosomal lipids and sphingolipid activator proteins. *Biol. Chem.* **382:** 283–290.

15. Salvioli, R., M. Tatti, F. Ciaffoni, and A. M. Vaccaro. 2000. Further studies on the reconstitution of glucosylceramidase activity by Sap C and anionic phospholipids. *FEBS Lett.* **472:** 17–21.

16. Wilkening, G., T. Linke, and K. Sandhoff. 1998. Lysosomal degradation on vesicular membrane surfaces. Enhanced glucosylceramide degradation by lysosomal anionic lipids and activators. *J. Biol. Chem.* **273:** 30271–30278.

17. Wendeler, M., T. Lemm, J. Weisgerber, J. Hoernschemeyer, O. Bartelsen, U. Schepers, and K. Sandhoff. 2003. Expression of recombinant human GM2-activator protein in insect cells: purification and characterization by mass spectrometry. *Protein Expr. Purif.* **27:** 259–266.

18. Wright, C. S., L. Z. Mi, S. Lee, and F. Rastinejad. 2005. Crystal structure analysis of phosphatidylcholine-GM2-activator product complexes: evidence for hydrolase activity. *Biochemistry.* **44:** 13510–13521.

19. Wendeler, M., J. Hoernschemeyer, D. Hoffmann, T. Kolter, G. Schwarzmann, and K. Sandhoff. 2004. Photoaffinity labelling of the human GM2-activator protein. Mechanistic insight into ganglioside GM2 degradation. *Eur. J. Biochem.* **271:** 614–627.

20. Meier, E. M., G. Schwarzmann, W. Fürst, and K. Sandhoff. 1991. The human GM2 activator protein. A substrate specific cofactor of beta-hexosaminidase A. *J. Biol. Chem.* **266:** 1879–1887.

21. Sandhoff, K., T. Kolter, and K. Harzer. 2001. Sphingolipid activator proteins. *In* The Metabolic and Molecular Basis of Inherited Disease. C. R. Scriver, A. L. Beaudet, W. S. Sly, et al., editors. McGraw-Hill, New York. 3371–3388.

22. Hama, Y., Y. T. Li, and S. C. Li. 1997. Interaction of GM2 activator protein with glycosphingolipids. *J. Biol. Chem.* **272:** 2828–2833.

23. Wendeler, M., J. Hoernschemeyer, M. John, N. Werth, M. Schoeniger, T. Lemm, R. Hartmann, H. Kessler, and K. Sandhoff. 2004. Expression of the GM2-activator protein in the methylotrophic yeast Pichia pastoris, purification, isotopic labeling, and biophysical characterization. *Protein Expr. Purif.* **34:** 147–157.

24. Giehl, A., T. Lemm, O. Bartelsen, K. Sandhoff, and A. Blume. 1999. Interaction of the GM2-activator protein with phospholipid-ganglioside bilayer membranes and with monolayers at the air-water interface. *Eur. J. Biochem.* **261:** 650–658.

25. Bierfreund, U., T. Lemm, A. Hoffmann, G. Uhlhorn-Dierks, R. A. Childs, C. T. Yuen, T. Feizi, and K. Sandhoff. 1999. Recombinant GM2-activator protein stimulates in vivo degradation of GA2 in GM2 gangliosidosis AB variant fibroblasts but exhibits no detectable binding of GA2 in an in vitro assay. *Neurochem. Res.* **24:** 295–300.

26. Conzelmann, E., and K. Sandhoff. 1979. Purification and characterization of an activator protein for the degradation of glycolipids GM2 and GA2 by hexosaminidase A. *Hoppe Seylers Z. Physiol. Chem.* **360:** 1837–1849.

27. Sandhoff, K., and K. Harzer. 2013. Gangliosides and gangliosidoses: principles of molecular and metabolic pathogenesis. *J. Neurosci.* **33:** 10195–10208.

28. Yusuf, H. K., G. Pohlentz, and K. Sandhoff. 1984. Ganglioside biosynthesis in Golgi apparatus: new perspectives on its mechanism. *J. Neurosci. Res.* **12:** 161–178.

29. Kobayashi, T., M. H. Beuchat, M. Lindsay, S. Frias, R. D. Palmiter, H. Sakuraba, R. G. Parton, and J. Gruenberg. 1999. Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport. *Nat. Cell Biol.* **1:** 113–118.

30. Babalola, J. O., M. Wendeler, B. Breiden, C. Arenz, G. Schwarzmann, S. Locatelli-Hoops, and K. Sandhoff. 2007. Development of an assay for the intermembrane transfer of cholesterol by Niemann-Pick C2 protein. *Biol. Chem.* **388:** 617–626.

31. Abdul-Hammed, M., B. Breiden, M. A. Adebayo, J. O. Babalola, G. Schwarzmann, and K. Sandhoff. 2010. Role of endosomal membrane lipids and NPC2 in cholesterol transfer and membrane fusion. *J. Lipid Res.* **51:** 1747–1760.

32. Cheruku, S. R., Z. Xu, R. Dutia, P. Lobel, and J. Storch. 2006. Mechanism of cholesterol transfer from the Niemann-Pick type C2 protein to model membranes supports a role in lysosomal cholesterol transport. *J. Biol. Chem.* **281:** 31594–31604.

33. Walkley, S. U., and M. T. Vanier. 2009. Secondary lipid accumulation in lysosomal disease. *Biochim. Biophys. Acta.* **1793:** 726–736.

34. Schwarzmann, G., M. Wendeler, and K. Sandhoff. 2005. Synthesis of novel NBD-GM1 and NBD-GM2 for the transfer activity of GM2-activator protein by a FRET-based assay system. *Glycobiology.* **15:** 1302–1311.

35. Schwarzmann, G., and K. Sandhoff. 1987. Lysogangliosides: synthesis and use in preparing labeled gangliosides. *Methods Enzymol.* **138:** 319–341.

36. Schütte, C. 1999. Strukturuntersuchungen an β-Hexosaminidase B und GM2-Aktivatorprotein durch proteinchemische Methoden und MALDI-Massenspektrometrie. Thesis: University of Bonn, Bonn, Germany.

37. Sandhoff, K., E. Conzelmann, and H. Nehrkorn. 1977. Specificity of human liver hexosaminidases A and B against glycosphingolipids GM2 and GA2. Purification of the enzymes by affinity chromatography employing specific elution. *Hoppe Seylers Z. Physiol. Chem.* **358:** 779–787.

38. MacDonald, R. C., R. I. MacDonald, B. P. Menco, K. Takeshita, N. K. Subbarao, and L. R. Hu. 1991. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta.* **1061:** 297–303.

39. Kölzer, M., N. Werth, and K. Sandhoff. 2004. Interactions of acid sphingomyelinase and lipid bilayers in the presence of the tricyclic antidepressant desipramine. *FEBS Lett.* **559:** 96–98.

40. Lange, Y., M. H. Swaisgood, B. V. Ramos, and T. L. Steck. 1989. Plasma membranes contain half the phospholipid and 90% of the cholesterol and sphingomyelin in cultured human fibroblasts. *J. Biol. Chem.* **264:** 3786–3793.

41. Schoer, J. K., A. M. Gallegos, A. L. McIntosh, O. Starodub, A. B. Kier, J. T. Billheimer, and F. Schroeder. 2000. Lysosomal membrane cholesterol dynamics. *Biochemistry.* **39:** 7662–7677.

42. Hao, M., S. X. Lin, O. J. Karylowski, D. Wustner, T. E. McGraw, and F. R. Maxfield. 2002. Vesicular and non-vesicular sterol transport in living cells. The endocytic recyclingcompartment is a major sterol storage organelle. *J. Biol. Chem.* **277:** 609–617.

43. Anheuser, S. 2011. Entwicklung eines kombinierten Lipidtransfer-und Membranfusions-Assays zur Untersuchung membranaktiver Proteine. Thesis: University of Bonn, Bonn, Germany. urn:nbn: de:hbz:5N-27013

44. Conzelmann, E., J. Burg, G. Stephan, and K. Sandhoff. 1982. Complexing of glycolipids and their transfer between membranes by the activator protein for degradation of lysosomal ganglioside GM2. *Eur. J. Biochem.* **123:** 455–464.

45. Sandhoff, K., and W. Wassle. 1971. Enrichment and characterization of 2 forms of human N-acetyl-beta-D-hexosaminidase. *Hoppe Seylers Z. Physiol. Chem.* **352:** 1119–1133.

46. Sandhoff, K., K. Harzer, W. Waessle, and H. Jatzkewitz. 1971. Enzyme alterations and lipid storage in three variants of Tay-Sachs disease. *J. Neurochem.* **18:** 2469–2489.

47. Gallala, H. D., and K. Sandhoff. 2011. Biological function of the cellular lipid BMP-BMP as a key activator for cholesterol sorting and membrane digestion. *Neurochem. Res.* **36:** 1594–1600.

48. Lange, Y., J. Ye, and T. L. Steck. 2005. Activation of membrane cholesterol by displacement from phospholipids. *J. Biol. Chem.* **280:** 36126–36131.

49. Ran, Y., and G. E. Fanucci. 2009. Ligand extraction properties of the GM2 activator protein and its interactions with lipid vesicles. *Biophys. J.* **97:** 257–266.

50. Wendeler, M., N. Werth, T. Maier, G. Schwarzmann, T. Kolter, M. Schoeniger, D. Hoffmann, T. Lemm, W. Saenger, and K. Sandhoff.

2006. The enzyme-binding region of human GM2-activator protein. *FEBS J.* **273:** 982–991.

51. Fürst, W., and K. Sandhoff. 1992. Activator proteins and topology of lysosomal sphingolipid catabolism. *Biochim. Biophys. Acta.* **1126:** 1–16.

52. Schwarzmann, G., A. von Coburg, and W. Möbius. 2000. Using biotinylated gangliosides to study their distribution and traffic in cells by immunoelectron microscopy. *Methods Enzymol.* **312:** 534–562.

53. Coffey, J. W., and C. De Duve. 1968. Digestive activity of lysosomes. I. The digestion of proteins by extracts of rat liver lysosomes. *J. Biol. Chem.* **243:** 3255–3263.

54. Mellman, I., R. Fuchs, and A. Helenius. 1986. Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* **55:** 663–700.

55. Hanada, E., and K. Suzuki. 1979. Activation of human brain galactosylceramidase by phosphatidylserine. *Biochim. Biophys. Acta.* **575:** 410–420.

56. Stinshoff, K., and H. Jatzkewitz. 1975. Comparison of the cerebroside sulphatase and the arylsulphatase activity of human sulphatase A in the absence of activators. *Biochim. Biophys. Acta.* **377:** 126–138.

57. Bernardo, K., R. Hurwitz, T. Zenk, R. J. Desnick, K. Ferlinz, E. H. Schuchman, and K. Sandhoff. 1995. Purification, characterization, and biosynthesis of human acid ceramidase. *J. Biol. Chem.* **270:** 11098–11102.

58. Contreras, F. X., A. M. Ernst, P. Haberkant, P. Bjorkholm, E. Lindahl, B. Gonen, C. Tischer, A. Elofsson, G. von Heijne, C. Thiele, et al. 2012. Molecular recognition of a single sphingolipid species by a protein's transmembrane domain. *Nature.* **481:** 525–529.

59. Mansfield, P. J., S. S. Carey, V. Hinkovska-Galcheva, J. A. Shayman, and L. A. Boxer. 2004. Ceramide inhibition of phospholipase D and its relationship to RhoA and ARF1 translocation in GTP gamma S-stimulated polymorphonuclear leukocytes. *Blood.* **103:** 2363–2368.

60. Matzner, U., B. Breiden, G. Schwarzmann, A. Yaghootfam, A. L. Fluharty, A. Hasilik, K. Sandhoff, and V. Gieselmann. 2009. Saposin B-dependent reconstitution of Arylsulfatase A activity in vitro and in cell culture models of metachromatic leukodystrophy. *J. Biol. Chem.* **284:** 9372–9381.

61. Schulze, H., and K. Sandhoff. 2011. Lysosomal lipid storage diseases. *Cold Spring Harb. Perspect. Biol.* **3:** pii:a004804.

62. Kobayashi, T., E. Stang, K. S. Fang, P. de Moerloose, R. G. Parton, and J. Gruenberg. 1998. A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. *Nature.* **392:** 193–197.

63. Wherrett, J. R., and S. Huterer. 1972. Enrichment of bis-(monoacylglyceryl) phosphate in lysosomes from rat liver. *J. Biol. Chem.* **247:** 4114–4120.

64. Stremmel, W., and H. Debuch. 1976. Bis(monoacylglycero)phosphate–a marker lipid of secondary lysosomes? *Hoppe Seylers Z. Physiol. Chem.* **357:** 803–810.

65. Bleistein, J., H. G. Heidrich, and H. Debuch. 1980. The phospholipids of liver lysosomes from untreated rats. *Hoppe Seylers Z. Physiol. Chem.* **361:** 595–597.

66. Vance, D. E., and H. Van den Bosch. 2000. Cholesterol in the year 2000. *Biochim. Biophys. Acta.* **1529:** 1–8.

67. Tabas, I. 2002. Cholesterol in health and disease. *J. Clin. Invest.* **110:** 583–590.

68. Mouritsen, O. G., and M. J. Zuckermann. 2004. What's so special about cholesterol? *Lipids.* **39:** 1101–1113.

69. Friedland, N., H. L. Liou, P. Lobel, and A. M. Stock. 2003. Structure of a cholesterol-binding protein deficient in Niemann-Pick type C2 disease. *Proc. Natl. Acad. Sci. USA.* **100:** 2512–2517.

70. Xie, X., M. S. Brown, J. M. Shelton, J. A. Richardson, J. L. Goldstein, and G. Liang. 2011. Amino acid substitution in NPC1 that abolishes cholesterol binding reproduces phenotype of complete NPC1 deficiency in mice. *Proc. Natl. Acad. Sci. USA.* **108:** 15330–15335.

71. Möbius, W., V. Herzog, K. Sandhoff, and G. Schwarzmann. 1999. Gangliosides are transported from the plasma membrane to intralysosomal membranes as revealed by immuno-electron microscopy. *Biosci. Rep.* **19:** 307–316.

72. Möbius, W., V. Herzog, K. Sandhoff, and G. Schwarzmann. 1999. Intracellular distribution of a biotin-labeled ganglioside, GM1, by immunoelectron microscopy after endocytosis in fibroblasts. *J. Histochem. Cytochem.* **47:** 1005–1014.

73. Burkhardt, J. K., S. Hüttler, A. Klein, W. Möbius, A. Habermann, G. Griffiths, and K. Sandhoff. 1997. Accumulation of sphingolipids in SAP-precursor (prosaposin)-deficient fibroblasts occurs as intralysosomal membrane structures and can be completely reversed by treatment with human SAP-precursor. *Eur. J. Cell Biol.* **73:** 10–18.

74. Kaye, E. M. 2011. Niemann-Pick C disease: not your average lysosomal storage disease. *Neurology.* **76:** 316–317.

75. Zervas, M., K. Dobrenis, and S. U. Walkley. 2001. Neurons in Niemann-Pick disease type C accumulate gangliosides as well as unesterified cholesterol and undergo dendritic and axonal alterations. *J. Neuropathol. Exp. Neurol.* **60:** 49–64.

76. Frentzen-Bertrams, M., and H. Debuch. 1981. Production of bis(monoacylglycero)phosphate from phosphatidylglycerol in isolated liver lysosomes of chloroquine-pretreated rats. *Hoppe Seylers Z. Physiol. Chem.* **362:** 1229–1236.

77. Hullin-Matsuda, F., K. Kawasaki, I. Delton-Vandenbroucke, Y. Xu, M. Nishijima, M. Lagarde, M. Schlame, and T. Kobayashi. 2007. De novo biosynthesis of the late endosome lipid, bis(monoacylglycero) phosphate. *J. Lipid Res.* **48:** 1997–2008.

78. Akgoc, Z., M. Sena-Esteves, D. R. Martin, X. Han, A. d'Azzo, and T. N. Seyfried. 2015. Bis(monoacylglycero)phosphate: a secondary storage lipid in the gangliosidoses. *J. Lipid Res.* **56:** 1006–1013.

79. Vanier, M. T. 1983. Biochemical studies in Niemann-Pick disease. I. Major sphingolipids of liver and spleen. *Biochim. Biophys. Acta.* **750:** 178–184.

80. Fantini, J., and F. J. Barrantes. 2013. How cholesterol interacts with membrane proteins: an exploration of cholesterol-binding sites including CRAC, CARC, and tilted domains. *Front. Physiol.* **4:** 31.

81. Barnholz, Y., A. Roitman, and S. Gatt. 1966. Enzymatic hydrolysis of sphingolipids. II. Hydrolysis of sphingomyelin by an enzyme from rat brain. *J. Biol. Chem.* **241:** 3731–3737.

82. Quintern, L. E., T. S. Zenk, and K. Sandhoff. 1989. The urine from patients with peritonitis as a rich source for purifying human acid sphingomyelinase and other lysosomal enzymes. *Biochim. Biophys. Acta.* **1003:** 121–124.

83. Megha, and E. London. 2004. Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function. *J. Biol. Chem.* **279:** 9997–10004.