Synthesis of novel NBD-GM1 and NBD-GM2 for the transfer activity of GM2-activator protein by a FRET-based assay system

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The ganglioside-activator protein is an essential cofactor for the lysosomal degradation of ganglioside GM2 (GM2) by β-hexosaminidase A. It mediates the interaction between the water-soluble exohydrolase and its membrane-embedded glycolipid substrate at the lipid-water interphase. Mutations in the gene encoding this glycoprotein result in a fatal neurological storage disorder, the AB variant of GM2-gangliosidosis. In order to efficiently and sensitively probe the glycolipid binding and membrane activity of this cofactor, we synthesized two new fluorescent glycosphingolipid (GSL) probes, 2-NBD-GM1 and 2-NBD-GM2. Both compounds were synthesized in a convergent and multistep synthesis starting from the respective gangliosides 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. Both fluorescent glycolipids exhibited an extremely low off-rate in model membranes and displayed very efficient resonance energy transfer to rhodamine-dioleoyl phosphoglycerol ethanolamine (rhodamine-PE) as acceptor. The binding to GM2-activator protein (GM2AP) and the degrading enzyme was shown to be unaltered compared to their natural analogues. A novel fluorescence-resonance energy transfer (FRET) assay was developed to monitor in real time the protein-mediated intervesicular transfer of these lipids from donor to acceptor liposomes. The data obtained indicate that this rapid and robust system presented here should serve as a valuable tool to probe quantitatively and comprehensively the membrane activity of GM2AP and other sphingolipid activator proteins and facilitate further structure-function studies aimed at delineating independently the lipid- and the enzyme-binding mode of these essential cofactors.

Key words: 2-amino-gangliosides/2-NBD-GM1/2-NBD-GM2/fluorescence resonance energy transfer/G2-activator protein

Introduction

The GM2-activator protein (GM2AP) is an essential cofactor for the degradation of ganglioside GM2 (GM2) by lysosomal β-hexosaminidase A (Conzelmann and Sandhoff, 1979). Its physiological significance is illustrated by the occurrence of a fatal neurological storage disorder, the AB variant of GM2-gangliosidosis, which results from its inherited deficiency (Conzelmann and Sandhoff, 1979). Similar in its function to the four other known sphingolipid activator proteins occurring in the lysosomal compartment, the saposins A, B, C, and D (Fürst and Sandhoff, 1992), GM2AP mediates the interaction between the water-soluble exohydrolase and its membrane-bound substrate at the lipid-water interphase. The mechanism by which the activator works is still a matter of conjecture. It is well known that GM2-activator binds avidly to a variety of anionic glycolipids in vitro (Hama et al., 1997), and that its membrane activity is critically influenced by several physiological parameters, including the lateral pressure of the lipid bilayer (Giehl et al., 1999), the pH (Bierfreund et al., 1999), the vesicle curvature, and the presence of negatively charged lysosomal lipids, especially bis(monoacylglycerol)phosphate (Werth et al., 2001). According to the liftase model of GM2AP function, this cofactor recognizes the ganglioside substrate within the membrane plane, complexes it, and lifts it out of the lipid bilayer, thereby giving the enzyme access to its carbohydrate headgroup (Meier et al., 1991; Sandhoff et al., 2001). The enzymatic degradation of the glycolipid substrate is then accompanied by a transient interaction between the enzyme and the activator protein (Yadao et al., 1997). It has also been suggested that GM2AP modifies the trisaccharide structure of GM2 by cleaving an intramolecular hydrogen bond between the terminal residues, thus facilitating the enzymatic attack on the N-acetyl-D-galactosamine (GalNAc) bond (Wu et al., 1994).

The crystal structure of unglycosylated, mature GM2AP expressed in Escherichia coli revealed a novel protein fold denoted β-cup, consisting of an eight-stranded antiparallel β-pleated sheet, which forms a central hydrophobic cavity. The dimensions of this pocket are such that it can accommodate the ceramide tail of GM2 (Wright et al., 2000). The structural analysis of lipid complexes of GM2AP (Wright et al., 2003) as well as of GM2AP in complex with platelet-activating factor (Wright et al., 2004), confirmed lipid binding within this cavity and revealed different lipid-binding modes within the apolar pocket. By photoaffinity labeling, we were able to identify a highly flexible surface loop of GM2AP as that part of the activator protein that directly interacts with the ganglioside substrate and to obtain further mechanistic insight into GM2 degradation (Wendeler et al., 2004).
All attempts to identify both the lipid- and the enzyme-binding region of GM2AP by site-directed mutagenesis and functional studies were so far hampered by the fact that the current assay system for GM2AP, which measures its stimulatory activity on the degradation rate of GM2 by β-hexosaminidase A (Klima et al., 1993), reflects simultaneously both the interaction of GM2AP with GM2, as well as the interaction of the GM2AP-GM2 complex with the enzyme. Therefore, the molecular basis underlying the GM2AP deficiency in patients with fatal AB variant of GM2 gangliosidosis remained elusive. Previously presented systems to evaluate the membrane activity of GM2AP include the transfer of radioactive GM2 between donor and acceptor vesicles (Conzelmann et al., 1982; Meier et al., 1991), and a fluorescence-quenching assay based on the self-quenching of the lipid-probe octadecylrhodamine (Smiljanic-Georgijev et al., 1997). Whereas the first method does not allow the continuous real-time monitoring of intervesicular lipid transfer and therefore impedes mechanistic studies, the latter system is not specific for ganglioside transfer activity and might not prove sensitive enough to reliably detect subtle differences in intervesicular transfer activity exhibited by GM2AP variants.

To investigate the glycolipid binding and transfer activity of GM2AP separated from its interaction with the enzyme, we developed a simple transfer assay that allowed rapid measurement of membrane activity of GM2AP and its variants. This assay is based on fluorescence-resonance energy transfer (FRET) from a fluorescent GM2 or ganglioside GM1 (GM1) (donor) to a quencher molecule (acceptor) within the same membrane (donor liposomes) and on an activator-mediated transfer of either fluorescent GM1 or GM2 from donor liposomes to acceptor liposomes that do not contain quencher molecules. It has been shown previously that both gangliosides are transferred almost equally well between membranes by GM2AP (Conzelmann et al., 1982). A prerequisite for the fluorescent gangliosides as well as for the quencher molecules to act as sensitive transfer probes is their low “off rate” (Brown et al., 1985) which impedes spontaneous transfer between membranes. As these fluorescent gangliosides are not commercially available, they had to be synthesized. The 7-nitrobenz-2-oxa-1,3-diazol (NBD) group was chosen as the donor fluorophore and the rhodamine group as a quenching fluorophore.

The structures of the two fluorescent ganglioside analogues 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1,4)-[α-D-neuraminyl-(2,3)]-β-D-galactopyranosyl-(1,4)-[β-D-glucopyranosyl(1,1)-(2S, 3R, 4E)-2-[(2-N-(7-nitrobenz-1,3-diazol-2-oxa-4-yl)-amino)-octadecanamido]-4-octadecen-1,3-diol (2-NBD-GM2) and 2-azido-octadecanoic acid as outlined in Schemes 1 and 2. In the first step, deacetyl-lyso-GM2 and -GM1 were obtained by alkaline hydrolysis of gangliosides GM2 and GM1 purified from bovine brain as described previously (Neuenhofer et al., 1985; Schwarzmann and Sandhoff, 1987). Briefly, the intermediate deacetylated gangliosides GM2 and GM1 (mono-deacetyl-lyso-GM2 and -GM1) containing two amino groups were obtained by vigorous alkaline hydrolysis of GM1 with potassium hydroxide in methanol at 100°C. By this procedure, the fatty acyl as well as the acetyl group of the sialic acid residue are completely removed with very little hydrolysis of the acetamido group of the N-acetylglactosamin moity of GM2 and GM1 (Schwarzmann and Sandhoff, 1987). The amino group of the sphingosine residue is more basic than those of the neuraminic acid moiety. Thus, selective acylation of the free amino group of the sphingosine moiety of deacetylated GM2 and deacetylated GM1 could be achieved with fluoromethoxycarbonyl chloride (Fmoc chloride). The Fmoc-protected compounds were subsequently re-acylated with acetic anhydride or [14C]acetic anhydride to yield, after removal of the protecting group, the unlabeled or radioactively labeled lysogangliosides. The protecting Fmoc group was removed by treating Fmoc-lysogangliosides with piperidine in N,N-dimethylformamide. At this step the synthesis converged.

The lysogangliosides were then acylated with the N-succinimidyl ester of racemic 2-azido-octadecanoic acid to yield (2R/S)-azido-GM2 and -GM1. To avoid O-acylation of carbohydrate hydroxyl groups, the reaction was performed at low temperature in the presence of a large excess of methanol. The N-succinimidyl ester was obtained in good yield by treating 2-azido-octadecanoic acid with N-hydroxysuccinimide and equimolar amounts of dicyclohexylcarbodiimide in ethyl acetate. Racemic 2-azido-octadecanoic acid was prepared by treating racem 2-bromo-octadecanoic acid with sodium azide in aqueous dioxane.

The conversion of an azido into a functional amino group is usually and easily accomplished by catalytic hydrogenation. However, for the reduction of the azido group in 2-azido-GM2 and -GM1, this hydrogenation was inappropriate as it also reduced the double bond in the sphingoid moiety.
Therefore, dihydrogen sulfide in pyridine was the reducing agent of choice and led to (2R/S)-amino-GM2 and -GM1 in acceptable yields. At this stage it was also possible, by chromatography, to separate quite well (2S)-amino-GM2 from (2R)-amino-GM2. The separation of the two diastereomeric 2-amino-GM1 was hampered by the fact that this ganglioside analogue contained a sphingoid moiety of 18 as well as 20 C-atoms leading to a broad peak with the latter analogue eluting in the first part of the peak. Chromatographic separation of the respective azido compounds was not sufficient. Both chiral forms were expected as synthesis started from racemic 2-bromo-octadecanoic acid. For measurement of membrane activity of GM2AP and its variant forms with the analogue of GM1 it was, however, not necessary to separate the chiral forms as enzymatic studies showed that both (2S)-NBD-GM2 and (2R)-NBD-GM2 were degraded equally well by lysosomal β-hexosaminidase A in the presence of GM2AP (data not shown).

The fluorescent analogues NBD-GM2 and NBD-GM1 were obtained by treating the respective 2-amino-gangliosides with NBD fluoride in \( N,N \)-dimethylformamide in the presence of an aprotic acid scavenger. The products were purified by high performance liquid chromatography on prepacked silica gel columns Lobar either in an isocratic mode or using a two-step gradient of increasing polarity.

Product identity and purity were checked by thin-layer chromatography (TLC) and followed, if appropriate, by autoradiography and fluorescence. The structures of selected intermediates and of the final products were confirmed by ESI-TOF mass spectrometry.

To check whether the introduction of the NBD group altered the recognition and binding of the ganglioside by both the enzyme and the activator protein, the enzymatic degradation of both diastereomeric forms of 2-NBD-GM2 was tested in a micellar \textit{in vitro} system as described (Conzelmann and Sandhoff, 1979). Both stereoisomers of 2-NBD-GM2 were found to be enzymatically degraded like the normal GM2 in the presence of GM2AP (data not shown), indicating that the NBD-group does not impede binding of the glycolipid to both the enzyme and the activator protein.

\textit{Assay design}

Assay optimization involved assessment of the amount and ratio of donor and acceptor liposomes needed to provide a readily measurable fluorescence signal response over the time frame of the assay while minimizing the required quantities of the NBD-labeled glycolipid. Both FRET lipid probes were initially incorporated in equimolar amounts into large unilamellar donor vesicles whose composition

![Chemical structures of the two fluorescent glycosphingolipids (GSLs) 2-NBD-GM2 and 2-NBD-GM1.](image)
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Scheme 1. Synthesis of (2R/S)-NBD-GM1. *, position of label when radioactive acetic anhydride was used.
was chosen such as to mimic most closely the conditions on intralysosomal membranes. As the presence of anionic lipids like bis(monoacylglycerol)phosphate and phosphatidic acid was found to drastically stimulate the activity of GM2AP in surface plasmon resonance spectroscopy as well as the enzymatic degradation of GM2 (Werth et al., 2001), donor and acceptor liposomes contained 20 mol% phosphatidic acid. Initially, we also synthesized radioactively labeled NBD-GM1 and used this to quantitate most accurately the amount of fluorescent label incorporated into liposomes as well as the liposomal concentration in the assay mixture. To obtain a maximal possible increase in fluorescence, a five-fold excess of acceptor vesicles was employed in the assays.

For the donor vesicles containing either 2-NBD-GM2 or 2-NBD-GM1 as well as equimolar amounts of rhodamine-dioleoyl phosphoglycerol ethanolamine (rhodamine-PE), the NBD-fluorescence was quenched, showing the efficient resonance energy transfer between the 2-NBD-gangliosides and the rhodamine-PE in the model bilayer membrane. Upon

**Scheme 1.** continued
addition of a five-fold excess of acceptor vesicles consisting of phosphatidylethanolamine, cholesterol, and phosphatidic acid, no change in the emission spectra was observed. This remained unaltered when emission scans were obtained after 90 min. Therefore, over the time course of the experiment, no spontaneous transfer of NBD-glycolipid between donor and acceptor vesicles could be detected in the absence of GM2AP, consistent with a negligible off-rate of the glycolipids from the donor membranes.

The addition of 3% Triton X-100 to the vesicle mixture resulted in an immediate increase in NBD-fluorescence to a value half of that maximal value observed for donor vesicles containing no quencher. This is in agreement with the complete solubilization of the liposomes by the detergent and the previously reported observation that Triton X-100 quenches NBD fluorescence in micellar systems by about 50% (Pagano et al., 1981).

**FRET-based transfer assay for GM2AP**

The addition of catalytic amounts of GM2AP (25–62.5 nM) to the vesicle mixture containing donor and acceptor liposomes produced an immediate increase in NBD fluorescence. As shown in Figure 2, the observed signal response in the initial phase of the reaction was dependent on the amount of GM2AP added. In contrast, the maximum value observed for the fluorescence intensity remained unchanged irrespective of the amount of GM2AP added. All curves showed saturation kinetics. The observed response in NBD fluorescence is consistent with the resonance transfer changes accompanying the transfer of NBD-GM1 or NBD-GM2 from the donor to the acceptor vesicles. Whereas the initial glycolipid transfer rate is dependent on the amount of GM2AP added, the net transfer equilibrium is apparently independent of the GM2AP concentration.

**Discussion**

Fluorescence resonance energy transfer is well established as a convenient and nondestructive method providing valuable information about the structure and dynamics of macromolecules (Wu and Brandt, 1994). Applied to the study of lipid trafficking and transport, it offers the advantage of continuous real-time monitoring of the transfer process (Mattjus et al., 1999; Corsico et al., 2004; Rao et al., 2004).

Here, we present a synthetic approach to two new fluorescent ganglioside probes and their application in a novel
assay system, which permits the investigation of the lipid transfer activity of GM2 activator protein.

As the NBD group attached to the methyl end of the acyl chain can significantly alter the orientation of the modified lipid within the membrane bilayer and might considerably change the interfacial properties of the membrane surface as well as the “off rate” of the modified lipid (Chattopadhyay and London, 1988; Chattopadhyay, 1990; Tremblay et al., 1998; Schwarzmann, 2001), we decided to introduce the NBD group close to the polar head region of the glycolipids. This proved possible through the synthesis of aminogangliosides and the exploitation of the added functionality to anchor the NBD-group. The chosen position of the fluorophore offers the advantage of neither perturbing the hydrophobic lipid bilayer core nor the polar oligosaccharides of gangliosides that are to be recognized by GM2AP. Also, this position of the NBD group was found to leave the binding of the modified lipid to both the activator protein and the degrading enzyme unaffected.

Recently, a FRET assay for a galactosylceramide transfer protein was described (Rao et al., 2004). In their assay, the authors used an anthryl vinyl group attached to the end of the acyl chain of galactosylceramide as the fluorescent donor. For the assay of GM2AP, this approach seemed not desirable as the bulky anthryl vinyl group may affect the lipid accommodation inside the hydrophobic protein pocket, thereby altering substrate-binding affinity and specificity. Furthermore, the precursor of NBD-gangliosides, that is, aminogangliosides, presented here have the potential of being immobilized on appropriate surfaces, which might facilitate the rapid screening of various parameters influencing the membrane transfer activity in microtiter-plate format. This class of compounds also offers the unique possibility of incorporating a third acyl chain into the lipid backbone, thereby drastically changing the hydrophobic properties of the gangliosides. This would allow to investigate whether the then impaired liftase function of GM2AP reduces the enzymatic ganglioside degradation, and to further delineate the mechanistic details of this reaction at the lipid-water interphase.

In donor vesicles containing equal amounts of NBD-ganglioside and rhodamine-PE, the NBD fluorescence was almost completely quenched, indicating efficient resonance energy transfer between the two fluorophores in model membranes. In the absence of GM2AP, no transfer of NBD-glycolipid between donor and acceptor vesicles could be detected.

GM2AP was shown to transfer 2-NBD-GM1 and -GM2 from the donor to acceptor vesicles. Whereas the initial glycolipid transfer rate was found to be dependent on the amount of GM2AP added, the net transfer equilibrium was independent of the GM2AP concentration.

The GM2 activator is an intensively investigated protein, however, many aspects of its mode of action are still unknown. In particular, no quantitative data are available describing the kinetic and thermodynamic characteristics of its lipid transfer properties. In addition, the protein regions responsible for the at least three distinct functions of GM2AP, the oligosaccharide recognition site, the lipid-binding site, and the contact region to the enzyme, remain elusive.

The assay presented here allows for the first time to investigate accurately the lipid-binding and -transfer activity of GM2AP separated from its interaction with the lysosomal enzyme β-hexosaminidase A. It can be anticipated that this greatly facilitates structure-function studies aimed at delineating the lipid-binding and the enzyme-binding site. In addition, the membrane parameters that influence the kinetics of the transfer mediated by GM2AP can be probed in detail, and the continuous nature of FRET provides the opportunity for comprehensive quantitative mechanistic studies.

In conclusion, we describe a synthetic approach to new fluorescent glycosphingolipids (GSL) and their application as valuable tools in FRET-based assay systems for intervesicular transfer that can easily be adapted to the study of other sphingolipid activator proteins.

**Materials and methods**

**Materials**

GM2 from postmortem human brain of a Tay-Sachs patient and GM1 from bovine brain were available in our laboratory. [1–14C]Acetic anhydride (spec. activity 4.22 TBq/mol) was obtained from Amersham-Buchler (Braunschweig, Germany), N,N′-dicyclohexylcarbodiimide, Fmoc chloride, N-hydroxysuccinimide, 7-nitrobenz-1,3-diazol-2-oxa-4-yl fluoride (NBDF), N,N-diisopropylethylamine and piperidine were obtained from Fluka (Buchs, Switzerland). LiChroprep RP-18, silica gel Si 60 (15–40 μm), prepacked silica gel chromatography columns Lobar, TLC plates with fluorescence indicator (silica gel Si 60 F254) were from E. Merck (Darmstadt, Germany). Phosphatidylcholine and phosphatidic acid (both dioleoyl) were from Sigma (Taufkirchen, Germany). All other reagents and chemicals used in this study were of the highest purity available.

β-Hexosaminidases A was purified from postmortem human liver to apparent homogeneity as described (Schuette, 1999). Recombinant GM2AP was expressed in the baculovirus expression vector system and purified as reported previously (Wendeler et al., 2003).

**Synthesis of NBD-GM1 and NBD-GM2**

As the synthesis of lysogangliosides has been described in detail elsewhere (Schwarzmann and Sandhoff, 1987), this section will focus on the synthesis of the final product starting from lyso-GM2 except for the synthesis of radiocarbon labeled lyso-GM1.

**Synthesis of (2R/S)-NBD-[14C]GM1**

N-Fmoc-lyso-[14C]GM1. N-Fmoc-deacetyl-lyso-GM1 (10.3 mg, about 7 μmol) (prepared as described previously, Schwarzmann and Sandhoff, 1987) was dissolved in dimethylformamide (300 μL) and N,N-diisopropylethylamine (7 μL). [1–14C]Acetic anhydride (4.38 μmol, 18.5 MBq, spec. activity 4.22 TBq/mol) in benzene (50 μL) was added and the mixture kept at 20°C overnight. As the reaction was not complete, further 18.5 MBq of acetic anhydride was added. After 10 h, the reaction was monitored by TLC with chloroform/methanol/15 mM calcium chloride (60:35:8, by
vol.) as the mobile phase on TLC plates with fluorescence indicator. A little remaining educt ($R_t = 0.13$) and mainly product ($R_t = 0.20$) were detected by fluorescence quench and the product also by phosphoimaging of its radioactivity. $N$-Fmoc-lyso-$[^{14}C]$GM1 was purified by chromatography on a prepacked silica gel column Lobar A with chloroform/methanol/water (60:35:8, by vol.) as eluant. The elution profile was monitored by TLC as above. All fractions containing the pure product were pooled and dried in a stream of nitrogen. The yield was 5.8 mg (55%).

Electrospray ionization time of flight mass spectrometry (ESI-TOF MS) in the negative mode gave two peaks of double negatively charged species $[M–2H]^{2–}$ at $m/z$ 750.8 and 764.8 with C18 and C20 sphingosine, respectively.

**Lyso-$[^{14}C]$GM1.** Fmoc-lyso-$[^{14}C]$GM1 (5.8 mg, 3.85 μmol) was kept for 20 h at 20°C in a mixture of methanol (500 μL), dimethylformamide (200 μL) and piperidine (300 μL). TLC with chloroform/methanol/15 mM calcium chloride (60:35:8, by vol.) as the mobile phase demonstrated that the educt ($R_t = 0.20$) had completely disappeared. Instead a double band ($R_t = 0.12$ and 0.14) showed the formation of radioactive lyso-GM1 with C18 and C20 sphingosine moieties, respectively. This was expected as the parent GM1 from bovine brain contained both C18 and C20 sphingosine in almost equal amounts. Solvents were removed azeotropically in a nitrogen jet with several additions of benzene, and the residue (about 90% yield) was used without further purification in the next synthetic step.

**Lyso-$[^{14}C]$GM1.** Lyso-$[^{14}C]$GM1 (about 3.5 μmol) was N-acylated with the N-succinimidyld derivative of (2R/S)-azido-octadecanoic acid (a total of 9 μmol) in a mixture of dimethylformamide (100 μL), N,N-diisopropylpropylethylamine (10 μL), and methanol (10 μL). The addition of the azido ester was at 0°C, and the reaction monitored by TLC with chloroform/methanol/15 mM calcium chloride (60:35:8, by vol.) as the mobile phase. When a new product ($R_t = 0.21$) had appeared on the expense of lyso-GM1 solvents were evaporated and the residue purified by chromatography on a prepacked silica gel column Lobar A with chloroform/methanol/water (65:25:4, by vol.; 66 mL) followed by chloroform/methanol/water (60:35:8, by vol.; 90 mL) as eluant. Radioactive fractions containing the pure product ($R_t = 0.21$) were collected and dried to yield 2.1 μmol 2-Azido-$[^{14}C]$GM1 (3.3 mg, 60%).

**2(R/S)-Azido-$[^{14}C]$GM1.** 2(R/S)-Azido-$[^{14}C]$GM1 (3.3 mg, 2.1 μmol) was treated for 12 h with hydrogen sulfide (0.5 M) in pyridine. The reaction mixture was dried azeotropically in a nitrogen jet with several additions of benzene, and the residue (about 65% yield as determined by radioactivity) was purified by chromatography on a prepacked silica gel column Lobar A with chloroform/methanol/2.5 M ammonia (60:35:8, by vol.) as eluant. The elution profile was monitored by TLC with chloroform/methanol/water (60:35:8, by vol.) as the mobile phase. All fractions containing the pure radioactive product ($R_t = 0.16$) were pooled and dried in a stream of nitrogen. After purification the yield was 1.8 mg (54%).

**2(R/S)-NBD-$[^{14}C]$GM1.** To (2R/S)-amino-$[^{14}C]$GM1 (1.8 mg, 1.1 μmol) dissolved in a mixture of DMF (100 μL), methanol (100 μL) and N,N-diisopropylethylamine (15 μL) NBD fluoride (4.1 mg, 22 μmol) was added at 0°C. The mixture was kept for 30 min and then for 12 h at 20°C. After drying in a nitrogen jet, the residue was purified by chromatography on a prepacked silica gel column Lobar A with 45 mL of chloroform/methanol/water (65:25:4, by vol.) followed by 160 mL of chloroform/methanol/water (60:35:8, by vol.) as eluant. The elution profile was monitored by TLC with chloroform/methanol/water (60:35:8, by vol.) as the mobile phase. All fluorescent and radioactive fractions containing the pure product ($R_t = 0.22$ to $R_t = 0.19$) were collected separately, dried in a stream of nitrogen and then dissolved in methanol for analysis. Total yield as determined by radioactivity was 0.5 μmol (45%). ESI-TOF MS in the negative mode of early fractions ($R_t = 0.23$) of (2R/S)-NBD-$[^{14}C]$GM1 gave one peak of double negatively charged species $[M–2H]^{2–}$ at $m/z$ 875.93 (calculated 875.96) representing the molecule with C20 sphingosine moiety, whereas a later fraction ($R_t = 0.21$) of (2R/S)-NBD-$[^{14}C]$GM1 with mainly C18 sphingosine residue yielded one peak of double negatively charged species $[M–2H]^{2–}$ at $m/z$ 861.95 (calculated 861.93). A clear-cut separation of the two chiral forms was not observed.

**2(R)-NBD-GM2 and (2S)-NBD-GM2.**

**2(R)-Azido-GM2.** Lyso-GM2 containing exclusively C18 sphingosine (5.6 mg, 5 μmol) was N-acylated with the N-succinimidyld derivative of (2R/S)-azido-octadecanoic acid (a total of 12.5 μmol) in a mixture of dimethylformamide (100 μL), N,N-diisopropylethylamine (10 μL) and methanol (10 μL). The addition of the azido ester in ethyl acetate (100 μL) was at 0°C and the reaction monitored by TLC with chloroform/methanol/15 mM calcium chloride (60:35:8, by vol.) as the mobile phase. After 12 h, at 4°C a new product ($R_t = 0.25$) had appeared on the expense of lyso-GM2 (2$R_t = 0.14$). Then solvents were evaporated and the residue purified by chromatography on a prepacked silica gel column Lobar A with chloroform/methanol/water (65:25:4, by vol.; 45 mL) followed by chloroform/methanol/water (60:35:8, by vol.; 105 mL) as eluant. Fractions containing the pure product ($R_t = 0.25$) were collected and dried to yield 3.5 μmol 2-azido-GM2 (5.0 mg, 70%). Characterization was by ESI-TOF MS in the positive mode and yielded the sodium salt of $[M+Na]^+$ at $m/z$ 1469.90 (calculated 1469.82) and double charged sodium salt of $[M+2Na]^{2+}$ at $m/z$ 746.39 (calculated 746.41).

**2(R/S)-Amino-GM2.** 2-Azido-GM2 (5.0 mg, 3.5 μmol) was treated for 12 h with hydrogen sulphide (0.5 M) in pyridine. When an aliquot showed the disappearance of the azido compound and the appearance of two new products ($R_t = 0.23$ and 0.21), the reaction mixture was dried azeotropically in a nitrogen jet with several additions of benzene, and the residue was purified by chromatography on a prepacked silica gel column Lobar A with chloroform/methanol/2.5 M ammonia (60:35:8, by vol.) as eluant. The elution profile was monitored by TLC with chloroform/methanol/water (60:35:8, by vol.) as the mobile phase. All
amine-reactive fractions containing product ($R_f = 0.23$) and ($R_f = 0.21$), respectively, were pooled separately and dried in a stream of nitrogen. The yield was about 25% (1.2 mg to 1.3 mg, 0.88 μmol) for each pool. Characterization was by ESI-TOF MS in the positive mode and yielded [M+Na]$^+$ at $m/z$ 1421.79 (calculated 1421.83), [M+H]$^+$ at $m/z$ 1399.80 (calculated 1399.83) and double charged [M+2Na]$^{2+}$ at $m/z$ 722.41 (calculated 722.40).

(2R)-NBD-GM2 and (2S)-NBD-GM2. To each isolated pool of the above 2R- and 2S-amino-GM2 (1.2 mg, 0.88 μmol) dissolved in a mixture of dimethylformamide (100 μL), methanol (50 μL) and N,N-disopropylethylamine (5 μL) solid NBD fluoride (3.0 mg, 16 μmol) was added at 0°C. The mixture was kept at 0°C for 40 min and then for 12 h at 20°C. After drying in a nitrogen jet, the respective residue was purified by preparative chromatography on thin-layer plates (20 by 20 cm) with chloroform/methanol/15 mM calcium chloride (60:35:8, by vol.) as the mobile phase. Fluorescent bands containing the pure product with $R_f = 0.28$ and $R_f = 0.25$, respectively, were scraped off the plates and extracted from silica gel with mixtures of chloroform and methanol. After drying, each sample was freed of salt and silicic acid by reversed phase chromatography on LiChroprep RP-18 as described (Williams and McCluer, 1980). Elution was performed with chloroform/methanol (1:1, by vol.). After drying in a stream of nitrogen, the samples were then dissolved in methanol for analysis. Total yield as determined by fluorescence with reference to known amounts of NBD-C6-ceramide was about 0.35 μmol (0.55 mg, 40%). ESI-TOF MS in negative ion mode of one known amounts of NBD-C6-ceramide was about 0.35 μg (0.55 mg, 40%). ESI-TOF MS in negative ion mode of one known amounts of NBD-C6-ceramide was about 0.35 μg (0.55 mg, 40%). ESI-TOF MS in negative ion mode of one known amounts of NBD-C6-ceramide was about 0.35 μg (0.55 mg, 40%). ESI-TOF MS in negative ion mode of one known amounts of NBD-C6-ceramide was about 0.35 μg (0.55 mg, 40%). ESI-TOF MS in negative ion mode of one known amounts of NBD-C6-ceramide was about 0.35 μg (0.55 mg, 40%). ESI-TOF MS in negative ion mode of one known amounts of NBD-C6-ceramide was about 0.35 μg (0.55 mg, 40%). ESI-TOF MS in negative ion mode of one known amounts of NBD-C6-ceramide was about 0.35 μg (0.55 mg, 40%). ESI-TOF MS in negative ion mode of one known amounts of NBD-C6-ceramide was about 0.35 μg (0.55 mg, 40%). ESI-TOF MS in negative ion mode of one known amounts of NBD-C6-ceramide was about 0.35 μg (0.55 mg, 40%). ESI-TOF MS in negative ion mode of one known amounts of NBD-C6-ceramide was about 0.35 μg (0.55 mg, 40%).

Vesicle preparation

Donor vesicles were prepared with the following composition: Phosphatidylcholine (56 mol%), cholesterol (20 mol%), phosphatidic acid (20 mol%), either NBD-GM2 or NBD-GM1 (2 mol%), and rhodamine-PE (2 mol%). Acceptor vesicles consisted of phosphatidylcholine (60 mol%), cholesterol (20 mol%), and phosphatidic acid (20 mol%). Appropriate amounts of lipids from stock solutions were mixed and dried under nitrogen. The lipid mixture was then hydrated in citrate buffer (40 mM, pH 4.2) and this dispersion subjected to 10 freeze-thaw cycles. Large unilamellar vesicles were then prepared by extrusion 21 times through two polycarbonate filters with a pore size of 100 nm mounted in tandem in a mini-extruder (Liposofast, Avestin, Mannheim, Germany). This preparation contained in addition to unilamellar also some b- and trilamellar vesicles as revealed by electron microscopy of negatively stained preparations (data not shown).

FRET-based transfer assay

The final donor vesicle concentration in the assay mixture was 20 nmol lipid per mL, the concentration of acceptor vesicles 100 nmol lipid per mL in a total volume of 320 μL in 50 mM citrate buffer, pH 4.2.

The transfer of NBD-GM1 or NBD-GM2 was started by the addition of catalytic amounts of GM2AP (25–62.5 nM) to the vesicle mixture. Fluorescence measurements in a quartz cuvette were performed in a Shimadzu RF 5000 instrument (Kyoto, Japan) with an excitation wavelength of 480 nm. NBD emission was measured at 522 nm. Transfer was monitored at 28°C. Under our experimental conditions, photobleaching was negligible as for each time point (Figure 2) the shutter was opened for 3–4 s only resulting in a total of 39–52 s of illumination. With continuous illumination photobleaching over 1 h was linear with a loss of fluorescence of less than two relative fluorescence units per minute.

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Abbreviations

ESI-TOF MS, electrospray ionization time of flight mass spectrometry; Fmoc, fluorenylmethyloxycarbonyl; FRET, fluorescence resonance energy transfer; GM2AP, GM2-activator protein; GM1, ganglioside GM1; GM2, ganglioside GM2; GSL, glycosphingolipid; NBD, 7-nitrobenz-2-oxa-1,3-diazol; PE, dioleoyl phosphoglycerol ethanolamine; TLC, thin-layer chromatography.

References


