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Review

Labeled chemical biology tools for investigating sphingolipid metabolism, trafficking and interaction with lipids and proteins ☆

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ABSTRACT

The unraveling of sphingolipid metabolism and function in the last 40 years relied on the extensive study of inherited human disease and specifically-tailored mouse models. However, only few of the achievements made so far would have been possible without chemical biology tools, such as fluorescent and/or radio-labeled and other artificial substrates, (mechanism-based) enzyme inhibitors, cross-linking probes or artificial membrane models. In this review we provide an overview over chemical biology tools that have been used to gain more insight into the molecular basis of sphingolipid-related biology. Many of these tools are still of high relevance for the investigation of current sphingolipid-related questions, others may stimulate the tailoring of novel probes suitable to address recent and future issues in the field. This article is part of a Special Issue entitled Tools to study lipid functions.

1. Introduction

Sphingolipids have been defined for the first time by the German physician J. Ludwig Tudichum, who found this class of lipids to be miraculous, since it showed fractionation schemes when isolated from human brains that differed from the fractions found for other lipids. Since that time around 1884, the extraction and fractionation of sphingolipid-containing biomaterial together with the thin layer chromatographic separation of individual sphingolipids [1,2] and their visualization by either charring or autoradiography was the method of choice for many years. Molecular analyses of natural sphingolipid metabolism and the molecular basis of sphingolipid-related human disease were facilitated by the (semi-) synthesis of novel radiolabeled substrates. When thin layer chromatographic techniques were substituted by high performance liquid chromatography, radiolabeled substrates were replaced by fluorescently labeled intermediates and substrates, whose biochemical properties were sometimes slightly different from those of their natural counterparts. After the main questions of sphingolipid metabolism – the pathways and its topologies – had been largely clarified [3], chemical biology tools were developed to address questions like detailed enzyme mechanisms, topology and trafficking of individual lipids, lipid–lipid and lipid–protein interactions or the interplay of soluble enzymes with lipids at the lipid–water interface. Similar to other compound classes, biochemical mechanisms have been confirmed by specific enzyme inhibitors, which often hold promise for the development of novel drugs for fighting human disease. In this short review we will highlight several older and newer chemical biology tools, mainly labeled lipids, emphasizing those that have been developed in our research groups.

2. Sphingolipid biosynthesis

2.1. Radioactive labeling of sphingolipids

The reaction schemes, the topology and the enzymes involved in sphingolipid and glycosphingolipid biosynthesis are largely known today [4–6]. This has been mainly achieved by feeding of cells with different labeled substrates like serine, sphingosine, palmitic acid or galactose. The synthesis of sphingolipids usually starts with the condensation of serine and palmitoylCoA, as found by enzymatic conversion of radioactively labeled serine or palmitoylCoA with yeast extracts [7]. The resulting 3-ketosphinganine is then reduced to dihydrosphingosine

Abbreviations: 2-NBD-GM1, 2-(R/S)-nitrobenzodiazol-GM1; 2-NBD-GM2, 2-(R/S)-nitrobenzodiazol-GM2; BMP, bis(monoacylglycero)phosphate; BODIPY, 4,4′-difluoro-4-bora-(3a,4a)-diaza-s-indacene; CTB, cholera toxin subunit B; doxyl, 4,4-dimethyl-3-oxazolidinyloxy, free radical; DPH, diphenylhexatrienyl; FRET, Förster resonance energy transfer; GM2AP, ganglioside GM2 activator protein; HexA, β-hexosaminidase A; NBD, nitrobenzodiazol; NR, Nile Red; Sap, saposin; C3-TPD-GM2[^{14}C] C3-TPD-GM2, 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1–4)-α-D-neuraminyl-(2–3)-β-D-galactopyranosyl-(1–4)-β-D-glucopyranosyl-β-(1–1)-(2S,3R,4E)-2-[N-(p-(3-trifluoromethyl)diazirinyl)-phenylpropaneamido]-4-octadecen-1,3-diol; C3-TPD-GM2[^{14}C]C7-TPD-GM2, 2-acetamido-2-deoxy-β-D-galactopyranosyl-(1–4)-α-D-neuraminyl-(2–3)-β-D-galactopyranosyl-(1–4)-β-D-glucopyranosyl-β-(1–1)-(2S,3R,4E)-2-[N-(p-(3-trifluoromethyl)diazirinyl)-phenylheptaneamido]-4-octadecen-1,3-diol

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(sphinganine), which was shown using radiolabeled NADPH [8]. Metabolic studies with radiolabeled serine suggested that sphinganine is first acylated to form dihydroceramide, which is then finally desaturated yielding ceramide [9]. The question of at which step the double bond is introduced during sphingolipid synthesis was a matter of debate for some time, but finally was answered with the help of the acylation inhibitor Fuminosin B1 [10] and with feeding of cells with N-[1-^{14}C]-octanoyl-D-erythro-sphinganine, which was readily transformed to the corresponding ceramide [11,12]. Sphingosine as catabolic intermediate of the sphingolipid metabolism however can also be acylated by sphinganine N-acyl transferase, as shown by using different ^{14}C-labeled Acyl-CoAs [13]. Ceramide, which is barely abundant in plasma membranes, is the membrane anchor for glycosphingolipids (GSL) like the gangliosides and for the major membrane constituent sphingomyelin. For the synthesis of sphingomyelin, phosphoryl choline is transferred from phosphatidylcholine (lecithin) to ceramide, thereby also producing diacyl glycerol. A first topological study using ^{14}C-labeled N-hexanoylsphingosine (C6-ceramide) showed that the main portion of the sphingomyelin producing activity is localized to the lumen of the Golgi [14]. In order to study the biosynthesis of glycosphingolipids, early studies – besides using labeled serine – relied on feeding of cells with radioactive glucose and its incorporation into gangliosides [15]. However, glucose is rapidly degraded during glycolysis. The donation of labeled galactose is more straightforward because galactose is readily transformed into UDP-galactose which in turn can be converted into UDP-glucose and hence can label glucosylceramide as well as other glycosphingolipids [16].

GSL biosynthesis requires the abundance of the ceramide membrane-anchor [17] and involves a hierarchical addition of individual monosaccharides [4]. Notably, the involved glycosyl and sialyl transferases are not strictly substrate specific, which as a consequence results in a combinatorial biosynthesis of cellular ganglioside patterns [4,6]. Moreover, GSL biosynthesis is closely connected to intracellular transport of the biosynthetic intermediates through the ER/Golgi network to the plasma membrane [18]. The clarification of the GSL biosynthetic pathway thus involved the above-mentioned arsenal of radioactively labeled precursors in combination with Golgi-targeting agents [19] like Brefeldin A [10] or monensin [20]. Interestingly, the topology of the different intermediates strongly suggests the existence of a yet to be identified flippase, catalyzing the transition of GlcCer from the cytosolic to the luminal leaflet of the Golgi membranes. Two mechanisms for the translocation of GlcCer have been suggested. One involves multidrug transporters [21,22] whereas the other favors the participation of the cytosolic GlcCer transfer protein FAPP2 [23,24].

If labeled lipids are used for metabolic studies in living cells, the resulting pattern of labeled lipids is usually not only the consequence of anabolic but also of catabolic reactions. Catabolized probes can usually be recycled and transported to enter a different branch of biosynthesis, which can significantly complicate the interpretation of resulting lipid patterns. Thus, the timing is an important feature to be aware of when conducting metabolic assays within cellular environments. To more deeply investigate the bi-directional metabolism of sphingolipid probes, the ganglioside GM2, tritium-labeled in the sphingoid base, was metabolized in skin fibroblasts from normal and GM2 gangliosidosis subjects [25]. While in normal cells, but not in gangliosidosis cells the major part of the radioactivity was found in degradation products as well as in sphingomyelin, the amount of radioactively-labeled higher gangliosides like GM1 and GD1a was virtually the same in both cells, clearly indicating that the synthesis of the latter proceeds by direct glycosylation of GM2 [25].

2.2. Other substrates for metabolic labeling of sphingolipids

The problem of mixed anabolic and catabolic metabolisms of labeled substrates can also be circumvented by the usage of non-degradable substrates. Towards this end, a series of non-degradable labeled glycolipid analogs has been synthesized [26] in which the normal glycosidic

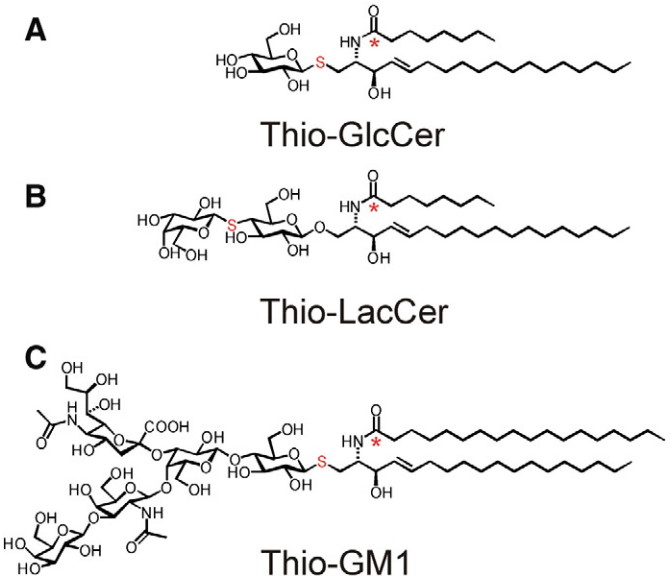

Fig. 1. Structures of some glycosphingolipids with thioglycosidic bonds. Glycosphingolipids with sulfur (shown in red) in place of oxygen in glycosidic bonds is shown for a non-degradable glucosylceramide Thio-GlcCer (A). The non-degradable lactosylceramide Thio-LacCer (B) has the thioglycosidic bond between the galactose and glucose residue, and the saccharide portion of ganglioside Thio-GM1 (C) is linked via sulfur to the ceramide moiety. The red asterisk denotes position of radiocarbon.

bonds were replaced by thioglycosidic bonds (Fig. 1). Interestingly, the thioglycosidic bonds are not cleavable by sphingolipid hydrolases. It was demonstrated that such analogs, which can have an additional fluorescent or radioactive label within the ceramide parts, are subject to direct glycosylation in cultured cells [27]. Such probes have been used to more efficiently track the biosynthetic metabolism of individual ganglioside analogs at a reduced background of labeled anabolic intermediates [28,29]. It is noteworthy, that even complex glycolipids like ganglioside GM1 containing a thioglycosidic bond to its labeled ceramide(s) are available by a facile semi-synthetic route starting from natural gangliosides (Fig. 1) [30]. With such derivatives like the thio-analog of β-D-glucosylceramide, it is possible to test whether biological responses to individual GSL species are likely attributable to their degradation products or not [31]. In addition to unwanted degradation of lipid probes, anabolic metabolism can also compromise the anticipated information of defined probes incubated with living cells. A well-known example is C6-NBD-ceramide, which readily stains the Golgi apparatus when incubated with living cells [32]. The fact that the same holds true for the 3-O-methyl ether, but not for the 1-O-methyl ether of C6-NBD-ceramide, which cannot be anabolized due to the methyl substituting the proton at the primary hydroxyl group, suggested that metabolism of ceramide is required for Golgi staining (Fig. 2) [33]. Indeed, a conversion of ceramide to sphingomyelin is the reason for this observation. Similar results were obtained in experiments to study the distribution and metabolism of fluorescent sphingosines and corresponding ceramides bearing the diphenylhexatrienyl (DPH) fluorophore in cultured human fibroblasts (Fig. 3) [34].

Classically, modified lipids are referred to as “labeled probes” when they contain modifications that give rise to detectable signals like UV-absorbance, fluorescence emission or ionizing radiation. However, even lipid analogs characterized by a simple modification in lipid chain length can be regarded as probes if they are used to test substrate specificity of enzymes or lipid transporters. As an example, artificial GSL substrates, truncated in their N-acyl chains helped to reveal significant differences in the substrate specificities of GM2 and GD3 synthase of Golgi vesicles [35]. Even more strikingly, such substrates provided a tremendous insight into the mode of action of sphingolipid activator proteins, which are essential for the lysosomal degradation of numerous

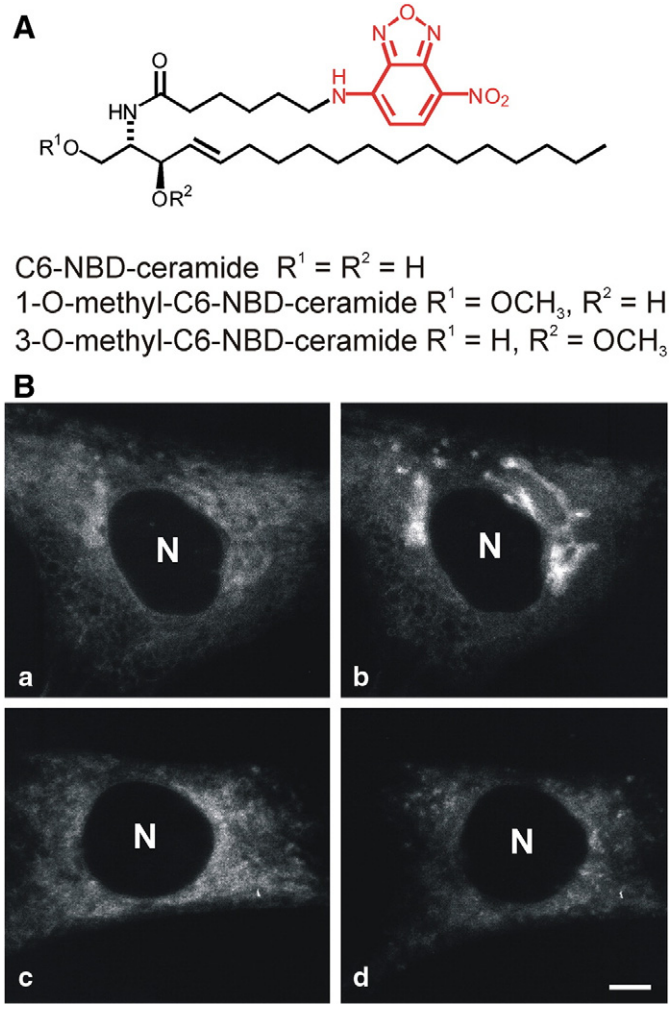

Fig. 2. NBD-ceramides for labeling cells in culture. (A) Structures of C6-NBD-ceramide and its O-methyl ethers. The NBD label is shown in red. (B) Shows labeling of human skin fibroblasts after 30 min incubation of cells at 7 °C with 3-O-methyl-C6-NBD-ceramide (a) or 1-O-methyl-C6-NBD-ceramide (c). After further incubation for 60 min at 7 °C prominent and perinuclear labeling was displayed only for the 3-O-methyl ether (b) but not for the 1-O-methyl ether (d) whose primary hydroxyl group is blocked for anabolism. Fig. 2(B) was modified from [33]. N = nucleus — Bar = 10 μm.

sphingolipids (see Section 3). Similarly, artificial long chain sphingoid bases incubated with murine primary cultured neurons were readily incorporated into glycosphingolipids, showing in this regard no explicit substrate selectivity of the involved biosynthetic enzymes [36].

3. Sphingolipid degradation

Sphingolipids are amphiphilic membrane components and hardly soluble in aqueous solutions. That's why sphingolipid catabolism mainly takes place at the membrane–water interface. For degradation sphingolipids occur at the luminal side of intraendosomal/-lysosomal vesicles and membranes following either endocytosis, phagocytosis or autophagy. The degradation requires the respective, mostly water-soluble, acid hydrolases. Many of the degradative steps depend on lysosomal lipid binding proteins, the so-called activator proteins GM2AP, Sap-A, Sap-B, Sap-C and Sap-D. In general a defect in either enzymes or activator proteins leads to severe storage diseases which are often-fatal. In particular the use of radioisotope-labeled exogenous sphingolipids in the investigation of sphingolipid catabolism offers some major advantages relative to the application of labeled simple biosynthetic precursors: (i) high sensitivity of detection of metabolites in cultured cells; (ii) specificity of metabolic processes of single sphingolipids; (iii) the possibility of gaining information on intracellular sphingolipid trafficking and on the intracellular sites of metabolic pathways; and last but not least (iv) information on inborn errors of glycosphingolipid metabolism.

3.1. Radioactive labeling of gangliosides and other sphingolipids

The application of labeled exogenous glycosphingolipids, especially gangliosides, in the study of ganglioside function, transport and metabolism has been fruitful over past decades [37]. Few procedures for labeling of sphingolipids have been described. The isotopes tritium and carbon-14 were mainly used. In earlier studies tritium could be incorporated into glycolipids, e.g. GM2, GM1, LacCer and GalCer that were prone to galactose oxidase via regeneration of a carbonyl group at C-6 of the terminal galactosamine or galactose residue, respectively, followed by reduction with sodium borotritiide [38,39]. Most tritium labeling methods use tritium gas, which, however, is difficult to handle and with platinum as catalyst produce unspecific-labeled compounds and many side products. A more ubiquitous simple approach and gas-free method introduced tritium into the sphingoid base of sphingolipids by saturation of the double bond using potassium or sodium borotritiide in the presence of palladium chloride or acetate [40,41]. In this procedure tritium and palladium, as catalyst, is generated in situ, hence avoiding the handling of free tritium gas. Alternatively, the allylic hydroxyl group of the sphingoid base can be oxidized to a keto function that upon reduction with borotritiide reverts to the allylic hydroxyl albeit in a configuration of 3R and 3S and with the proton at carbon 3 replaced by tritium [42]. The introduction of radiocarbon into glycosphingolipids via labeled fatty acids was made easy as soon as their respective lyso-compounds became available. In addition, double labeling of gangliosides became possible owing to at least two or more inherent amino functions generated by strong alkaline hydrolysis of some sphingolipids [41]. That's why e.g. a fluorescent label or a spin label (Fig. 4) could be incorporated into the ceramide portion and a radiolabel into the sugar residue of glycosphingolipids [43]. Alternately, the radiolabel could be introduced into the ceramide and a biotin (Fig. 5) or digoxigenine moiety into the neuraminic acid residue of ganglioside GM1 [44].

3.2. Incorporation of labeled sphingolipids into cells in culture

Several methods have been applied to feed labeled exogenous sphingolipids to cells under study. These include complex formation of the lipid with bovine serum albumin or with low-density lipoprotein. Others used micelle formation as for gangliosides or integration of the labeled lipid into liposomes. Three modes of uptake by cells of ganglioside micelles have been observed [45] and reviewed [37]. Especially with micelle forming gangliosides the insertion of ganglioside molecules into the outer leaflet of plasma membranes could be achieved as demonstrated with electron spin resonance (ESR) spectroscopy [43]. The major portion of exogenous ganglioside micelles fed to cells in culture adhere to surface proteins of the plasma membrane and can be released by mild trypsinization. This portion still contains micelles as demonstrated for spin-labeled ganglioside analogs (gangliosides bearing a doxyl label in their acyl chain, Fig. 4). In micelles the spin-label are close to each other yielding a spin–spin broadening signal in ESR spectroscopy as was found for the ganglioside micelle-containing medium and the supernatant after trypsinization (Fig. 4B a, b). However, those ganglioside molecules truly inserted into the plasma membrane and diluted within the lipid layer gave rise to ESR spectra with the typical anisotropic axial pattern diagnostic of a fluid lipid membrane environment (Fig. 4B c). This true membrane insertion paved the way for the examination of ganglioside distribution and trafficking in cells.

Most of incorporated ganglioside molecules are conveyed to degradative compartments and hydrolyzed by acid glycohydrolases, starting from the non-reducing end, to yield free sugars and ceramide [46–48].

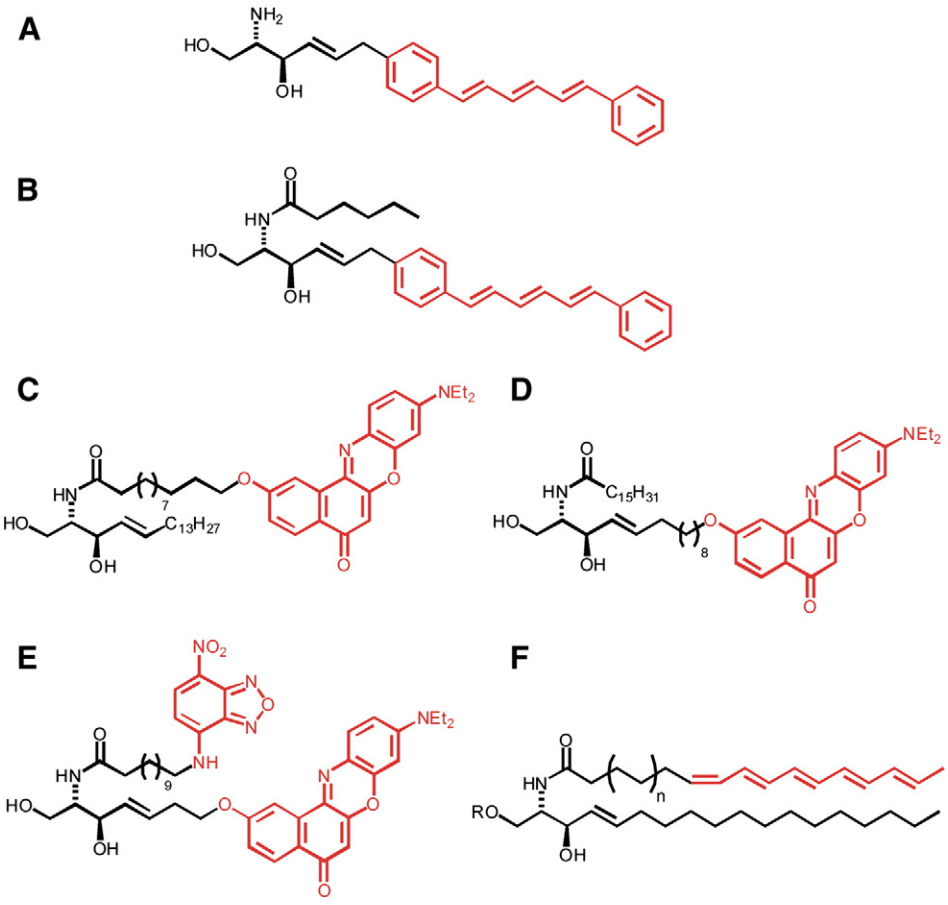

Fig. 3. Novel fluorescently-labeled ceramides. (A) DPH-sphingosine; (B) DPH-C6-ceramide; (C) acyl-NR ceramide; (D) sphingosyl-NR ceramide; (E) NR/NBD-ceramide used as FRET probe for ceramidases; (F) polyene ceramide (R = H) or sphingomyelin (R = phosphoryl choline). NR = Nile Red.

Ceramide, as such, cannot escape lysosomes in detectable amounts and is split by an acid ceramidase to fatty acid and sphingosine or labeled sphinganine. Small activator proteins assist most of these degradative steps [25,49,50]. All released building blocks exit lysosomes and are reused in a salvage pathway for de novo synthesis of lipids and glycoconjugates [29,51–56], or are further processed to small building blocks and energy production. Labeled sphingosine or sphinganine is also used for the synthesis of labeled sphingomyelin [47]. In a retroaldol reaction followed by oxidation of the generated palmitaldehyde sphingoid bases can be further processed to ethanolamine and palmitoylCoA, which in turn may enter the biosynthetic pathway of phosphoglycerolipids [47]. Besides catabolism direct glycosylation of a small percentage of administered exogenous GM2 and GM1 has also been observed in cultured human fibroblasts [25].

For the unambiguous diagnosis of inborn errors of glycolipid metabolism the administration of labeled exogenous gangliosides to cultured fibroblasts of afflicted patients is more reliable than enzyme assays employing water-soluble substrates which may be split by various enzymes; e.g. 4-methylumbelliferyl-β-galactoside is hydrolyzed not only by galactosylceramidase, the enzyme deficient in Morbus Krabbe, but also by GM1-β-galactosidase, the enzyme deficient in GM1 gangliosidosis, which later was called Morbus Landing [57]. In addition, a defect merely in the respective activator protein would not be detected by the use of water-soluble substrates [58]. That is why the uptake by and the metabolism of radiolabeled GM2 and GM1 in cultured fibroblasts of the respective patients have been examined [25,59,60]. Feeding of tritiated GM1 has also been successfully employed for the study of activator protein deficiencies [61].

3.3. Labeled glycosphingolipids in the study of catabolism on model membranes and in micelles

To gain further insight into sphingolipid degradation with regard to activator protein dependency, pH profile, kinetic constants and lipid requirements a series of experiments has been performed in our laboratories. Most of the studies were done in a detergent-free assay system on liposomes as model membranes designed to mimic intralysosomal vesicles and using mostly radioactive and fluorescent sphingolipids.

The ganglioside GM2 activator protein (GM2AP) is an essential cofactor for the lysosomal degradation of ganglioside GM2 by β-hexosaminidase A (HexA). It mediates the interaction between the water-soluble exohydrolase and its membrane-embedded glycolipid substrate at the lipid–water interface [62]. In order to efficiently and sensitively probe the glycolipid binding and membrane activity of this cofactor by a simple FRET assay, we synthesized two new fluorescent glycosphingolipid probes, 2-NBD-GM1 and 2-NBD-GM2 (Fig. 6). The chromophore nitrobenzoxadiazol (NBD) was introduced into the region between the polar head group and the hydrophobic anchor of the lipid ensuring that their NBD group is positioned at the lipid–water interface when the ganglioside is in lipid bilayers. Both compounds were synthesized in a convergent and multistep synthesis starting from the respective gangliosides isolated from natural sources [63]. Both fluorescent glycolipids exhibited an extremely low off-rate in model membranes and displayed very efficient resonance energy transfer to rhodamine-dioleoyl phosphoglycerol ethanolamine as acceptor. The NBD group did not interfere with the binding of 2-NBD-GM2 to GM2AP and the exohydrolase, for the degradation of 2-NBD-

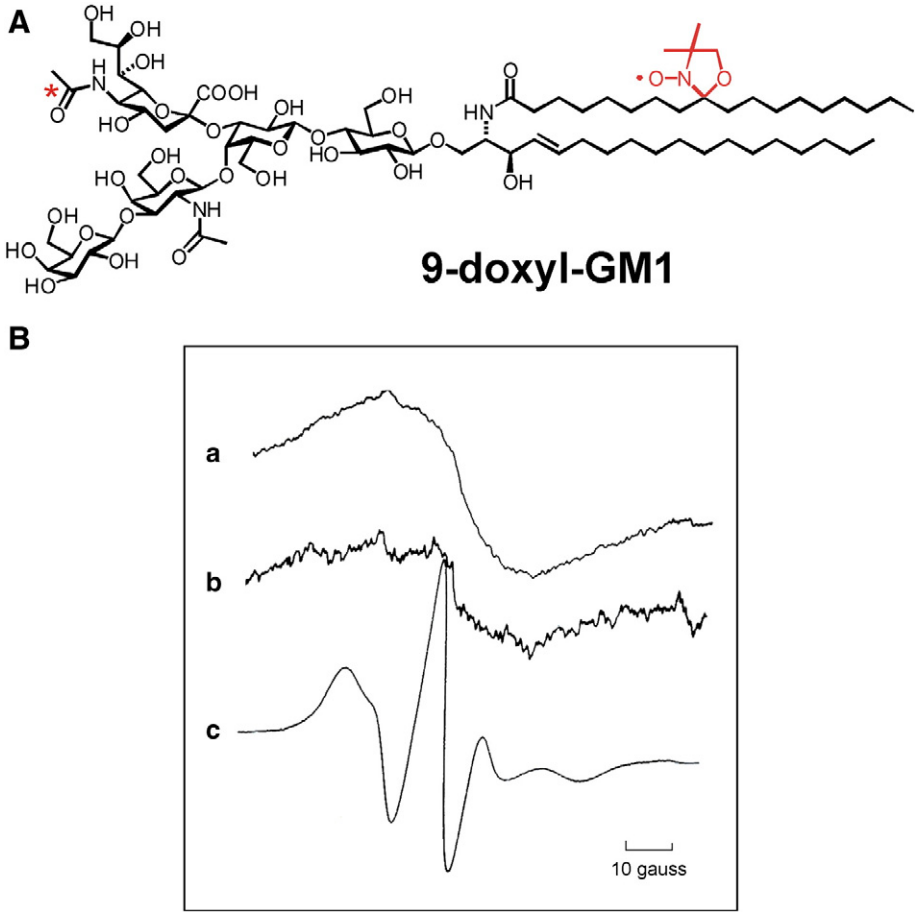

Fig. 4. Use of spin-labeled gangliosides for the demonstration of ganglioside insertion into cellular membranes. (A) Presents as an example the structure of ganglioside GM1 with a spin-probe (red) in position 9 of its acyl chain (9-doxyl-GM1). (B) Shows the ESR spectra obtained for the recovered incubation medium (a), of the supernatant of the mild trypsinization (b), and of the cell pellet following this treatment (c). Spin-spin broadening is obtained for the medium (a) and supernatant (b) demonstrating the presence of ganglioside micelles. The characteristic anisotropic spectrum (c) demonstrates ganglioside insertion into cellular membranes. The red asterisk denotes position of radiocarbon. Fig. 4(B) was modified from [43].

GM2 was shown to be unaltered compared to natural GM2. Thus, a novel Förster resonance energy transfer assay was developed to monitor in real time the protein-mediated intervesicular transfer of these lipids from donor to acceptor liposomes. Hence this rapid and robust system 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.

Recently, we synthesized two ceramide derivatives modified either within the sphingoid part or the fatty acid part with Nile Red (NR) (Fig. 3) [64]. Nile Red is a dye, which in its free form readily inserts into lipid phases, where its fluorescence is strongly enhanced compared to polar environments. Both NR-modified ceramides were readily degraded by the acid and neutral ceramidases, with lower Michaelis-constants but also lower V_{max} values compared to their NBD-modified counterparts. A second highly interesting feature of the NR-dye is the fact that its excitation maximum almost perfectly overlaps with the emission spectra of NBD, which anticipates the construction of lipid FRET probes [65]. Based on our previous studies, we constructed a doubly labeled ceramide, displaying NBD in the fatty acid part and NR in the sphingoid portion. Either with acid ceramidase or with recombinant neutral ceramidase in vitro, the probe was used to quantitatively monitor enzymatic cleavage in real time [66]. The probe showed almost 100% FRET in living cells, as indicated by photo-bleaching experiments. When applied to cells in form of a BSA complex the probe was, however, for unknown reason exclusively located to the Golgi without cleavage. Future experiments will concentrate on liposomal formulations which might direct the probe into cellular compartments with higher ceramidase activities.

The specificity of glucosylceramide β-glucosidase (glucosylceramidase) from human placenta [67] was investigated in the absence of the activator protein Sap-C with labeled glucosylceramides that differed in acyl chain length as well as in the configuration of the ceramide residue [68]. For these experiments structurally modified glucosylceramides were part of unilamellar liposomes. The reaction between the water-soluble enzyme and the liposomal substrates was significantly dependent on the structure of the lipophilic aglycon moiety. Especially the length of the acyl chain and thus the off-rate of the substrate from membranes turned out to be very significant. The off-rate describes the ease with which membrane-embedded lipid molecules may be loosened from its hydrophobic environment. It is thus obvious that lipids with long acyl chains have a lower off-rate than lipids with short acyl chains. Hence glucosyl-N-acetyl-sphingosines (D-erythro and L-threo) were better substrates for the enzyme than the corresponding glucosylceramides with longer acyl chains. The L-threo derivatives were poorer substrates with higher apparent Km values than the corresponding D-erythro derivatives. The short acyl chain glucosyl-N-acetyl-sphingosine and the cationic glucosylsphingosine were the most effective competitive inhibitors of the hydrolysis of long acyl chain glucosylceramide. This is of no surprise because these lipids are less tightly bound within the lipid bilayer and hence more easily accessible to a hydrophobic pocket of the membrane associated enzyme [69]. The affinity of sphingolipids such as sphingosine,

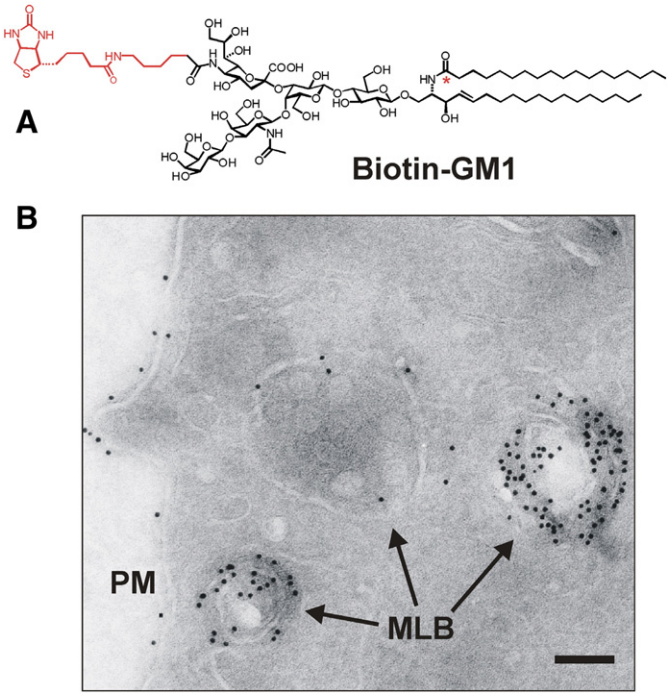

Fig. 5. Use of radioactive Biotin-GM1 for tracking cell-incorporated gangliosides in endocytosis. (A) Shows the structure of ganglioside GM1 with a spacer-linked biotin residue (marked red) in the sialic acid moiety. The red asterisk denotes position of radiocarbon. (B) Shows immunolabeling of a cryosection of fibroblasts that were incubated with Biotin-GM1 for 3 days. Cryosections were labeled with goat anti-biotin antibodies conjugated to gold particles (10 nm). Biotin-GM1 is distributed over the plasma membrane (PM) and the luminal membranes of multilamellar bodies (MLB) of late endosomes or lysosomes. Bar 100 nm. Fig. 5(B) was modified from [98].

D-erythro-ceramide and D-galactosyl-N-acetyl-D-erythro-sphingosine for a hydrophobic site of the membrane bound enzyme may also explain their non-competitive inhibition of the hydrolysis of membrane-bound glucosylceramide. Dilution experiments confirmed that at least a fraction, if not most, of the enzyme associates with the liposomes. Acidic phospholipids in liposomes caused a higher stimulation (30–40-fold) of the glucosylceramide β-glucosidase than acidic sphingolipids (sulfatide, gangliosides GM1 and GD1a, 3–10-fold) [68]. The importance of the off-rate of substrates from liposomal membranes became obvious also in the degradation of lactosylceramides and gangliosides GM1 by lysosomal β-galactosidases (galactosylceramidase and GM1-β-galactosidase) and sulfatides by arylsulfatase A [70,71].

When analyses were performed with both galactosidases using lactosylceramides with varying acyl chain lengths as substrates in unilamellar liposomes and naturally occurring sphingolipid activator proteins Sap-B and Sap-C to stimulate the reaction it turned out that Sap-B was a better activator than Sap-C for the reaction catalyzed by GM1-β-galactosidase. On the other hand Sap-C stimulated preferentially lactosylceramide hydrolysis by galactosylceramidase. The enzymic hydrolysis of liposome-integrated lactosylceramides was significantly dependent on the structure of the lipophilic aglycon moiety of the lactosylceramide increasing with decreasing length of its fatty acyl chain (C2 > C4 > C6 > C8 > C10 > C18), thus strengthening the notion that a reduced hydrophobicity leads to a higher off-rate of lipids from membranes and to a better availability for the enzyme. This is corroborated by the observation that in the presence of detergents the degradation rates were independent of the acyl chain length. Kinetic and dilution experiments indicated that Sap-B forms water-soluble complexes with both lactosylceramide and GM1. These complexes were recognized by GM1-β-galactosidase as optimal substrates in the same mode, as postulated for the hydrolysis of sulfatides by arylsulfatase A [72]. In the presence of Sap-B GM1-β-galactosidase was more active

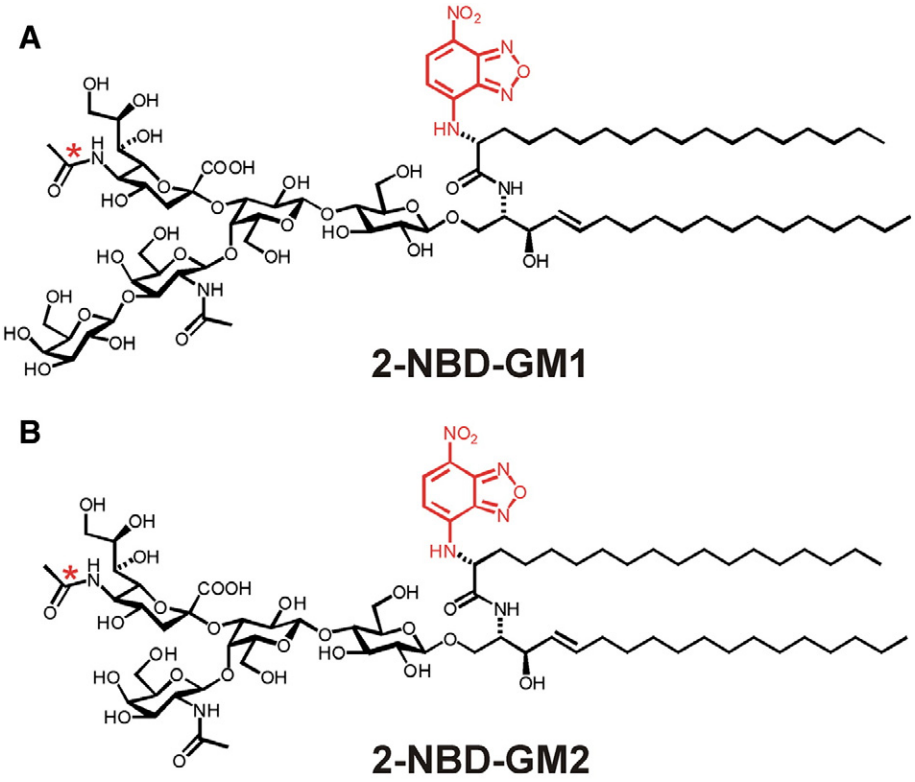

Fig. 6. Structure of NBD-gangliosides used for FRET assays. The NBD-label (shown in red) is attached to the alpha position of the acyl chain of GM1 (2-NBD-GM1) (A) and the respective GM2 (2-NBD-GM2) (B). The R configuration with respect to the orientation of the label is shown. Both R and S configured analogs were obtained in the synthesis and can be separated chromatographically. The red asterisk denotes position of radiocarbon.

than without on hydrolyzing liposomal GM1 and lactosylceramides. On the other hand, dilution experiments indicated that degradation of galactosylceramide and lactosylceramide by galactosylceramidase proceeds almost exclusively on liposomal surfaces.

It has been shown that the degradation of ganglioside GM2 requires a so-called GM2 activator protein GM2AP. A lack of this protein as in GM2 gangliosidosis variant AB is fatal for the afflicted person and may cause death at a very early age [73]. In membranes natural ganglioside GM2 is embedded within its neighboring lipids in a strong hydrophobic fashion and thus has a low off-rate (see above). The terminal GalNAc residue is consequently not extended far enough from the lipophilic surface to be recognized by the active center of the water-soluble HexA. Two models are described in the literature to explain the mode of action of GM2AP as to GM2 degradation. In one model this activator protein may act as a membrane or micelle disturbing small protein with the result that GM2 is loosened within the lipophilic neighborhood presumably forming a soluble GM2–GM2AP complex whose binding to HexA renders the oligosaccharide of GM2 accessible to the active center of the enzyme [62,74]. If this holds true then analogs of ganglioside GM2 with short acyl chains or no acyl chain at all and hence with an increased critical micellar concentration and with a concomitant higher off-rate from membranes and micelles should become degradable by HexA even in the absence of GM2AP. We studied various labeled derivatives of GM2 differing mainly in the hydrophobicity of their acyl chain [75]. When in liposomal membranes, the analogs with an acyl chain of medium length still required the activator protein for degradation by HexA. However, the GM2 analogs with an acetyl rather than a stearoyl residue or with no acyl chain at all were degraded by HexA in the absence of the activator protein. In micelles all analogs with short or no acyl chains were split by HexA without the need of the activator protein. This shows that the oligosaccharide group per se is the substrate of the enzyme and that GM2AP is not necessary to induce a special conformation of the sugar portion for recognition by HexA. Our findings are in contrast to the second model in which a conformational change in the oligosaccharide portion of GM2 is postulated, which has to be evoked by the activator protein and that is believed to be necessary for degradation of GM2 [76]. Our model is corroborated by the observation that the terminal GalNAc residue of the ganglioside GalNAcGD1a, when embedded at a low molar percentage in liposomal membranes, is split by HexA even in the absence of GM2AP. Although tightly embedded in the membrane via the ceramide residue of GalNAcGD1a its terminal GalNAc residue seems to extend far enough from the membrane surface to reach the active site of HexA. However, in micelles solely composed of GalNAcGD1a no degradation takes place. In this case the oligosaccharide groups may be too crowded and tightly packed to allow access of the active site of the enzyme. As soon as the density of the oligosaccharide chains is attenuated by the addition of taurodeoxycholate (TDC), i.e. when in mixed micelles of GalNAcGD1a und TDC, the degradation by HexA of GalNAcGD1a to GD1a was almost complete [75].

3.4. Inhibitors of acid sphingomyelinase

Among the different kinds of probes for unraveling sphingolipid metabolism, inhibitors of the involved enzymes play a very important role. Conventional probes can induce an increase or decrease of a specific enzyme activity that may coincide with a certain biological event. A causative connection however can only be drawn by a targeted blockage of individual enzymes, be it by knock-out or knock-down technologies or by the introduction of specific enzyme inhibitors. There are several excellent reviews on inhibitors of sphingolipid degradation available [77,78]. In the present review we want to focus on inhibitors of acid sphingomyelinase that have been developed in our laboratories [79]. During the last years, the acid sphingomyelinase has been implicated in a variety of different diseases like acute lung injury [80], cystic fibrosis [81], major depression [82,83], and cancer [84]. Unfortunately, there is no generally accepted inhibitor for this enzyme available. The tricyclic antidepressants like desipramine and amitriptyline enrich in the lysosomes and induce the proteolytic degradation of this enzyme [85] and most likely of several others, including the acid ceramidase [17]. Due to their indirect mode of inhibition, desipramine and other molecules with similar mode of action have been termed "functional inhibitors of acid sphingomyelinase" (FIASMAs) [86]. Despite their unspecific and indirect mode of action, it cannot be neglected that this compound class has been proven to be a tremendously useful tool in sphingolipid research, not at least due to the fact that most of them are clinically approved drugs. We have developed a group of directly-acting phosphoinositide inhibitors (Fig. 7) [87] and a carbohydrate-based version (Fig. 7) [88], both of which are derived from the naturally occurring, but biologically unstable lipid phosphatidylinositol-3,5-bisphosphate that was earlier shown to be a potent inhibitor of the acid sphingomyelinase [89]. Inspired by the phosphoinositide analogs, we furthermore developed a series of bisphosphonate inhibitors, with the α-aminobisphosphonate termed ARC39 being the most potent (IC_{50} = 20 nM) inhibitor of purified acid sphingomyelinase known

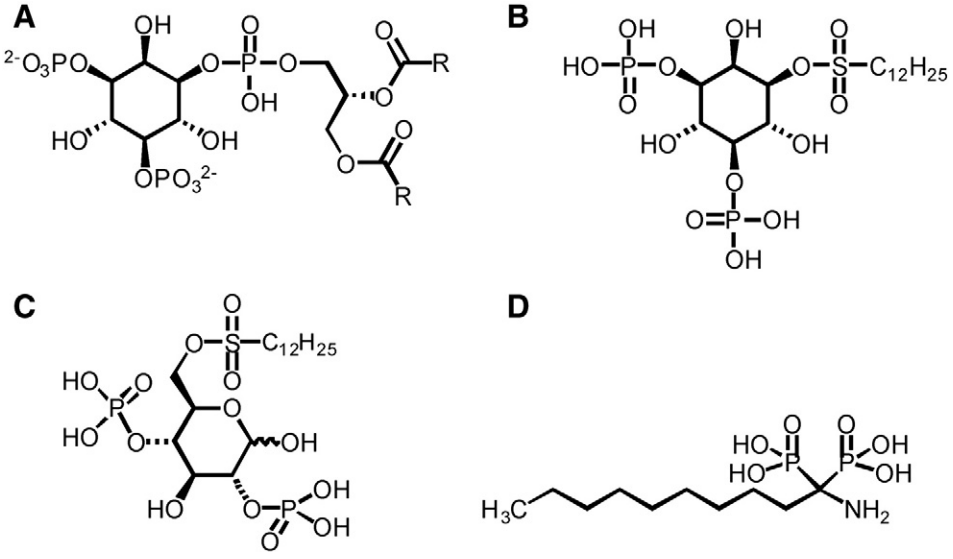

Fig. 7. Inhibitors of the acid sphingomyelinase. (A) Phosphatidyl-3,5-bisphosphate (PtdIns-3,5-P_2); (B) sulfonic ester analog "C-16"; (C) glucose-based analog of PtdIns-3,5-P_2; (D) aminobisphosphonate "ARC-39".

today [90]. Besides its activity in cell culture, it also inhibits edema formation in excised rat-lungs at lower concentrations than reported for desipramine [90,91]. Despite these encouraging features, it must be mentioned that its specificity and mode of action in cellular environment still await further clarification [78].

4. Sorting of membrane lipids

The lipid composition of biological membranes like the plasma membrane is tightly regulated and only few of the underlying mechanisms have been elucidated [92]. In fact, this regulation appears to have many levels beyond the mere differential regulation of biosynthetic and catabolic enzymes. The regulation of a membrane's lipid composition also involves transport proteins, ion gradients across the membrane, including pH gradients, and last but not least the lipids themselves [13].

Many biosynthetic enzymes and transporters like the sphingomyelin synthases [93] or the ceramide transport protein CERT [14] have been cloned and characterized only recently. Classical studies with the goal to follow sphingolipid trafficking in biosynthesis involve NBD- or BODIPY-labeled ceramide [32], sphingomyelin [94] and glucosylceramide [18]. Since these lipid species are modulated within the lipid chains (usually N-acyl part) the fluorescent labels obviously do not severely interfere with the enzymatic modification to higher functionalized lipids. In some cases, however, these modified lipids do not faithfully mimic their natural counterpart in all aspects of metabolism and intracellular trafficking. For instance a BODIPY-labeled GlcCer when fed to cultured fibroblasts labeled the Golgi compartment whereas a non-degradable NBD-labeled GlcCer did not, but instead labeled lysosomal membranes as one would expect [37,95]. Since the fluorescent tag does not only indicate the lipid's subcellular position, but also its metabolic origin, such probes are invaluable tools for matching the biosynthesis of complex sphingolipids with their individual subcellular transport [18]. Since several, if not most of the labeled sphingolipids fed to cells are subject to degradation rather than anabolic modifications [25], the use of non-cleavable lipids that are on the other hand subject to further modification to more complex lipids, is highly advisable (see Section 2) [27].

The lipid sorting that is connected to the lysosomal degradation of membranes is much better understood, not least due to a set of lysosomal storage disorders caused by the dysfunction of lysosomal hydrolases and lipid transporters. The changes in lysosomal lipid composition in the context of sphingolipidoses have been a matter of investigation for decades and led to detailed hypotheses of the principles underlying endocytosis and degradation of membrane lipids [96]. The main pathway starts within the plasma membrane by invagination of caveolae, which get internalized by endocytosis. While the surrounding membranes of endosomes and lysosomes are protected against lipolytic degradation, probably by a dense network of glycocalix, there are separated vesicles within endosomes and lysosomes, where membrane digestion takes place. To gain insight into structural aspects of GSL degradation, feeding studies were performed in cell culture using radioactively labeled ganglioside GM1 carrying either a biotin or a digoxigenin label at its sialic acid part [44]. The biotinylated ganglioside GM1 was then used to track this molecule from the plasma membrane to intralysosomal membrane vesicles by immuno-electron microscopy, thereby providing insight into intraendosomal and intralysosomal membrane morphology (Fig. 5) [97,98]. Noteworthy, metabolic studies revealed that the modified GM1 was degraded to GM2 and GM3, respectively, while the modification at the sialic acid prevented further degradation by mammalian sialidases [99].

As pointed out in Section 3, complex glycolipids like gangliosides are stepwise processed from their non-reducing ends of their carbohydrate parts. Moreover, we and other groups have collected significant evidence that the constitutive lysosomal degradation of sphingolipids progresses in a strictly consecutive fashion, which is controlled by pH value on the one hand and the lipid composition of the intraendosomal and intralysosomal vesicles on the other hand. Since the sphingolipid hydrolases differ in their pH optima, pH value is a very important factor in determining hierarchical sphingolipid degradation and thus lipid sorting. As an example, the acid sphingomyelinase has its pH optimum around pH 5.5 and thus will be active in early to late endosomes, while the acid ceramidase is active only at pH values < 5 and thus strictly active in the lysosomes. Based on the observations from lysosomal storage disorders, we have established several liposomal in vitro assays that make use of purified hydrolases and sphingolipid activator proteins. Such assays allow for testing the influence of individual lipids on the activity of the respective hydrolases or transporters. Early studies already revealed a stimulation of acid sphingomyelinase by negatively charged lysosomal lipids like bis(monoacylglycero)phosphate (BMP) [100]. Later, we established surface plasmon resonance experiments, which are in full agreement with the enzymatic assay [68] and underline the importance of the anionic lysosomal lipid BMP, which appears to be a key activator for membrane digestion [101]. Since sphingolipid degradation essentially takes place at the lipid–water interface, the ability of enzymes and supporting proteins to perturb the lipid surface seems to be decisive for an effective turnover. Recent research on lipid microdomains that segregate into liquid ordered and liquid disordered phases has made clear, that also the affinity (or rather packing features) among different lipid species has major impact on membrane properties. As an example, it was shown that cholesterol displays high affinity to sphingomyelin containing domains, while it can be replaced by ceramide, which is even a stronger binder to its metabolic precursor [102]. The hint that such principles can also hold true for intralysosomal membranes comes from nature itself. The Niemann–Pick Type C diseases, like types A and B, are characterized by a partial loss of acid sphingomyelinase activity and by a massive accumulation of sphingomyelin. Today it is known that the primary origin for the cell's inability to degrade sphingomyelin is a lack in cholesterol sorting that is caused by a defect in either of the cholesterol transporter proteins NPC1 and NPC2. This is likely accompanied by a concomitant attenuation of sphingomyelinase activity, which leads to a reduced degradation of sphingomyelin. To more thoroughly follow lipid transfer between different membranes, we developed a novel liposomal assay for the intermembrane transfer of cholesterol by Niemann–Pick C2 protein, which contains differentially labeled types of lysosomal lipids [103]. Using this assay, we were able to gain insight into the role of endosomal membrane lipids and NPC2 in cholesterol transfer and membrane fusion [104]. Indeed, all data obtained from such assays suggest that besides the pH, the lipid composition of the inner lysosomal membranes determines which enzymes or transporters will be active at the water–lipid interface and which ones will be not. As an example the cholesterol transfer by NPC2 is inhibited by sphingomyelin and stimulated by anionic phospholipids like BMP [104]. As a result, the degradation and thus the concentration of individual lipids underlie strict temporal control during the maturation from early endosomes to lysosomes.

5. Probing lipid–lipid and lipid–protein interactions

In recent years enormous strides have been made in the development of methods for the study of lipid–lipid and lipid–protein interactions. Mostly fluorescent sphingolipids have been described that bear NBD, BODIPY or pyrene groups in their lipid portion (Fig. **8**). Each of these dyes has specific advantages and disadvantages [105]. The relatively polar NBD dye for instance, locates itself on the lipid–water interface [106]. The BODIPY-modified sphingolipids much more resemble their natural counterparts, due to the more hydrophobic nature of BODIPY. Nonetheless, BODIPY-modified sphingolipids have been shown not to insert into ordered lipid domains (also called lipid rafts) [107], which is regarded as a highly important characteristic of sphingolipids [108]. These drawbacks have been circumvented by the development of the pentaene-sphingolipids by the Thiele group, who replaced the natural mono-unsaturated part of the sphingoid base by

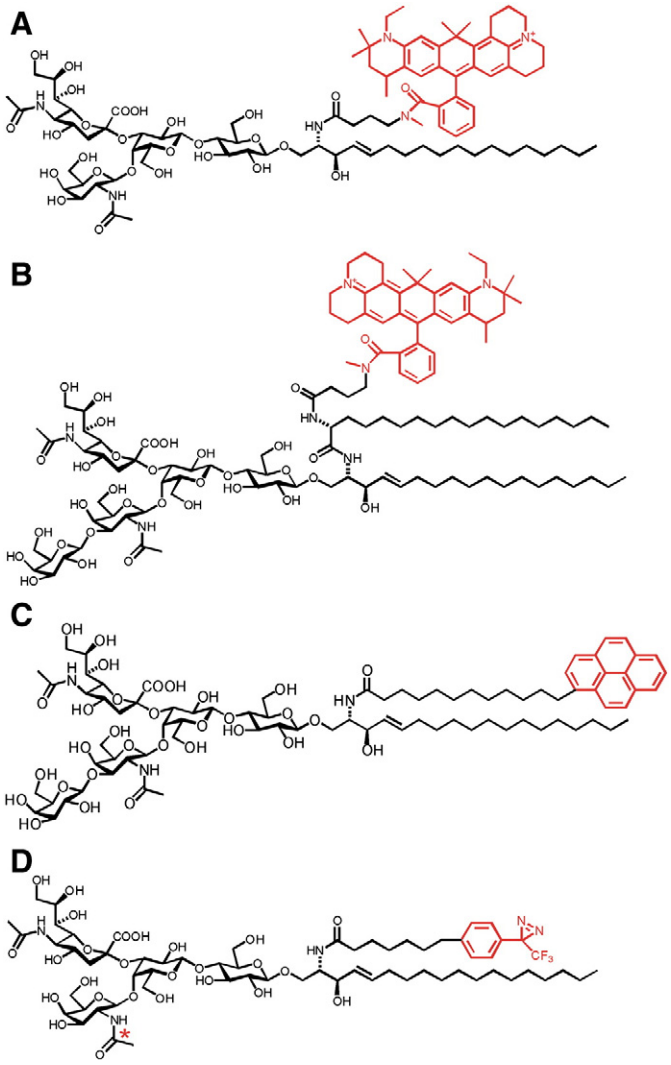

Fig. 8. Examples of labeled gangliosides for use in STED nanoscopy, excimer technique and photo-coupling. (A) ATTO647N-lyso-GM2; (B) ATTO647N-GM1; (C) Py-C12-GM1; (D) C7-TPD-GM2, red asterisk denotes position of radiocarbon.

a conjugated pentaene (Fig. 3) [109]. A further approach for studying the biosynthetic pathway of sphingolipids was the use of a non-disturbing alkyne-sphinganine, (2S,3R)-2-aminooctadec-17-yn-1,3-diol, which upon coupling by click chemistry to an azido-fluorophore became fluorescent also in its conjugates [110].

For our studies of lipid–lipid and lipid–protein interactions we used both spin-labeled and fluorescent gangliosides. In addition, lipid–protein interactions were also performed with sphingolipid photoaffinity labels (Fig. **8**).

5.1. Lipid–protein interactions

The function of membrane associated and integrated proteins depend on their interactions with membrane lipids. The activation of various peripheral proteins is critically dependent on the lipid composition of the membrane. Some integral proteins require specific lipids for optimal function and their activity is commonly sensitive to the dynamics of the lipid bilayer. For an optimal activity of membrane-bound cardiolipin synthase and β-hydroxybutyrate dehydrogenase phosphatidylethanolamine and phosphatidylcholine, respectively, are essential [111,112]. The use of spin-labeled membrane lipids for the study of membrane dynamics by electron spin resonance (ESR) spectroscopy has proved to be particularly fruitful. Using this technique lipids interacting directly with the membrane protein can be distinguished from the bulk lipids. For some studies we have synthesized various authentic spin-labeled gangliosides that carry a spin probe (doxyl group) within their acyl chains. Starting from the respective lysogangliosides spin-labeled gangliosides GM3, GM2, GM1, and GD1b were synthesized by acylation with activated esters of stearic acids that carried a doxyl group at the carbon 5 or 9 or 14 [41]. Gangliosides spin-labeled at the carbon in position 14 in the acyl chain were used to study their interaction with the (Na^+, K^+)-ATPase. This enzyme usually requires phosphatidylcholine and phosphatidylethanolamine as boundary lipids for activity in a cholesterol-rich lipid bilayer [113]. When studied in membranes from the shark *Squalus acanthias* or in dispersions of the extracted membrane lipids the association of the protein to GD1b was modest yet stronger than to the monosialogangliosides. The latter display little selectivity in the lipid–protein interaction relative to spin-labeled phosphatidylcholine. However, the degree of motional restriction of the ganglioside's acyl chain is smaller than for the doxyl-labeled acyl chain of phospholipids on interaction with the ATPase consistent with a location of the ganglioside's doxyl group closer to the polar interface of the membrane [114]. This is likely due to the polar sialooligosaccharide head group, which may pull out the ceramide residue from the bilayer by one to two carbons, compared to the diacyl glycerol backbone of the phospholipids [43]. Similar results were obtained for the ganglioside interactions with the acetylcholine receptor in membranes from *Torpedo marmorata* electric organ [115]. Though GM1 is one of the major gangliosides in these membranes other gangliosides such as GM3, GM2, and GD1b are also found as constituents and are not excluded from the first shell of lipids surrounding the protein and thus may potentially affect the acetylcholine receptor function via a direct ganglioside–protein interaction.

Another aspect of lipid–protein interaction is the association of lysosomal lipid binding proteins to certain sphingolipids and is best studied by either fluorescent sphingolipids or sphingolipids carrying a photoactivatable group (Fig. **8**D) [63,116]. The photoreactive probe should preferably be a carbene rather than a nitrene precursor since the nitrene group is prone to easy rearrangement and thus to inactivation. Two radiocarbon-labeled analogs of ganglioside GM2 that contained either a p-(3-trifluoromethyl-diazirinyl)-phenylpropionic (C3-TPD-GM2) or a p-(3-trifluoromethyl-diazirinyl)-phenylheptanonic (C7-TPD-GM2) acid residue as photoreactive probe were used to study the binding region of the GM2 activator protein GM2AP, an essential cofactor for the degradation of GM2 by lysosomal HexA. These photolabile short acyl chain fatty acids replaced the normally present acyl chain of GM2. Upon photolysis these GM2 analogs covalently inserted into the protein GM2AP and the binding region could be studied by ESI-Q-TOFF mass spectrometry of radiolabeled peptides generated by trypsinization. Both labels specifically attached into a part of the surface loop that was previously identified as the most flexible and hydrophobic region in the crystal structure of the activator [117]. These results provide strong evidence that this loop constitutes the part of the activator protein that directly interacts with the ganglioside substrate, suggesting that the hydrophobicity and the great structural mobility of this element are crucial for the loosening of the membrane-embedded GM2, its stabilization inside the spacious cavity of GM2AP, and its orientation to the enzyme's active site [116].

The ability of GM2AP to facilitate intermembranous transfer of gangliosides from donor to acceptor membranes was demonstrated by Förster resonance energy transfer (FRET) using novel NBD-labeled analogs of ganglioside GM2 and GM1 that bear the nitrobenzoxadiazol group at the alpha position of the acyl chain (Fig. 6). Hence, if incorporated into membranes their NBD group is located at the interface between the polar and hydrophobic region of membranes. The usefulness of these analogs has been described already in Section 3.3.

The carbene precursor 3-azi-1-[(2-acetamido-2-deoxy-1-β-D-galactopyranosyl)thio]butane [118] behaved as an active site-directed inhibitor of the human lysosomal β-hexosaminidase B and was applied to study its active site. Upon irradiation the generated carbene attached

covalently to the enzyme and caused a 15% inactivation of the enzyme's activity. Following trypsinization of the photo-coupled enzyme one prominently labeled peptide was isolated and sequenced. However, no modified peptide could be detected suggesting that the label had been bound in a labile fashion, presumably in an ester bond. Indeed, when the labeled peptide was treated by ammonia a former glutamate residue was transformed into a glutamine residue [119].

Aside from these experiments, in a model system, we studied protein mediated ganglioside clustering by means of FRET. For these experiments derivatives of acidic glycosphingolipids such as sulfatide and GM1 as well as uncharged glycosphingolipids such as GA1 and GalCer were synthesized that were labeled in their ceramide moiety with either DPH-propionic acid or NBD-aminohexanoic acid. When exited with light of the appropriate wavelength DPH emission is quenched by NBD if both labels are in close proximity, i.e. in the range from 1.5 to 7.5 nm. At low concentrations of both DPH- and NBD-labeled GM1 in model membranes (0.125 mol% of total lipid) there was little if any quench of DPH emission, for the energy transfer is inversely proportional to the sixth power of label's distance [120]. However, the addition of pentavalent cholera toxin subunit B (CTB) concentrated these ganglioside analogs in the plane of the membrane to yield a maximum quench of DPH emission when the ratio of CTB to GM1 reached 1 to 5. In this case the labels of bound GM1 molecules are apart by only 2 to 3 nm [121].

Another example for protein–lipid interaction stresses the importance of the hydrophobicity of lipids with respect to the physiological consequences of such an interaction. The simian virus (SV40) binds with pentavalent protein subunits to its natural receptor GM1 before it is internalized by cells via a tubular invagination process within the plasma membrane. GM1 molecules with shorter than their natural acyl chains or short-acyl chain PE analogs, bearing in their head group the GM1 sialooligosaccharide, and thus are less tightly incorporated into the exoplasmic leaflet of the plasma membrane do not support tubular invagination and infection of the cell whereas all analogs with long acyl chains do [122].

5.2. Lipid–lipid interactions

The interaction of lipid molecules with each other or with other lipid molecules in membranes or micelles has been studied using fluorescent and radioactive as well as spin-labeled lipid analogs. For many years cholesterol-mediated lipid interactions giving rise to ordered lipid microdomains in cellular membranes have been proposed for important membrane-associated processes such as transmembrane signaling events. Particularly sphingolipids like sphingomyelin and gangliosides have been suggested to be components of such domains [108]. A controversial discussion has come up as to the existence and size of lipid microdomains in living cells. Rafts could not be found in plasma membranes of living cells even by stimulated emission depletion (STED) far-field fluorescence nanoscopy, a suitable detection technique by far extending the resolution of light microscopy [123]. In this study various sphingolipids, i.e. sphingomyelin and gangliosides with a fluorophore either in the lipophilic ceramide (Fig. **8**) or in the polar head group were used to detect single diffusing lipid molecules in nanosized areas in the plasma membrane of cells. When the probed area in membranes came down to about 20 nm or smaller, the labeled sphingolipids unlike the appropriately labeled phosphoglycerolipids were transiently trapped in cholesterol-rich lipid complexes in spots smaller than 20 nm diameter and with a transit time from 10 to 20 ms.

Pyrene-labeled gangliosides are well suited to study, by use of the excimer technique, the formation of ganglioside micelles as well as their distribution and lateral diffusion in phosphatidylcholine membranes. For these studies 12-(1-pyrenyl)dodecanoic acid was covalently attached to lysogangliosides GM1, GM2, GM3, GD1a, and GD1b (Fig. **8**) [41]. The 12-(1-pyrenyl)dodecanoic acid substitute of phosphatidylcholine was used for comparison. All pyrene-labeled gangliosides (Py-gangliosides) were present in aqueous solution in a predominantly micellar form down to 2×10^{-8} M, which is the technical limit of this method. This concentration is above the critical micellar concentration of gangliosides by almost one to two orders of magnitude [124]. The tendency to aggregate was highest for PyGD1a and PyGD1b. In fluid dipalmitoylphosphatidylcholine bilayers the excimer-to-monomer fluorescence intensity ratio of pyrene-labeled gangliosides PyGM1, PyGM2, PyGM3, PyGD1a, and PyGD1b increased linearly with ganglioside concentration. The calculated diffusion coefficients for gangliosides are comparable to 1.6×10^{-7} cm^2/s, which is the diffusion coefficient of pyrene-labeled phosphatidylcholine [125]. In comparison to phosphatidylcholine, the diffusion of monosialogangliosides was slightly increased, while that of disialogangliosides PyGD1a and PyGD1b was slightly decreased. In dimyristoylphosphatidylcholine bilayers the pyrene-labeled disialogangliosides displayed ideal mixing whereas the mixing of pyrene-labeled monosialogangliosides was less ideal with the host lipids. Ca^{2+} ions up to 200 mM had no significant effect on ganglioside diffusion. An increase in bulk viscosity of the medium that surrounded the phosphatidylcholine bilayer had only a minor effect, if at all, on the diffusion of pyrene-labeled gangliosides that were intercalated in the bilayer [126,127].

Synaptic plasma membranes isolated from calf brain contain among other proteins a sphingomyelinase and a sialidase as lipid-metabolizing enzymes. These enzymes seem to be responsible for the homeostasis of both sphingolipids in the plasma membrane. To study their enzymic parameters exogenous substrates, i.e. labeled sphingomyelin and ganglioside GD1a, respectively, were used. In these investigations it is of utmost importance that the exogenous substrate has the correct orientation to be recognized by the enzyme's active site. Therefore, a crucial question in such studies is whether the exogenous lipid has been fully integrated into the membrane rather than being simply adsorbed on the surface or trapped within membrane vesicles. The spin-labeled method has been shown to be useful in determining the degree of incorporation of exogenously added lipids into cell membranes. The ESR spectra discriminate between labeled lipid which is incorporated and dispersed within the lipid phase of the membrane and that which co-sediments with the membranes but remains undispersed either because it is simply adsorbed on the membranes or is occluded within vesicles or segregated membrane patches. We have therefore used synthetic doxyl-labeled sphingomyelin and ganglioside GD1a for the study of their incorporation into microsomal membranes. Whereas GD1a could be transferred and incorporated from their micellar state sphingomyelin needed powerful sonication for its integration into the membrane [128,129].

6. Outlook

Sphingolipids are expressed in a cell-type, differentiation- and age-dependent manner. As lipophilic and amphiphilic molecules they are either components of membranes or protein-bound, but hardly soluble in aqueous solutions. Their general functions have been investigated in vivo by the analysis of inborn errors of sphingolipid metabolism in man and model animals, especially in knock-out mice or by gene silencing techniques in culture cells. As discussed above, appropriately labeled sphingolipids and sphingolipid analogs are useful chemical biology tools to investigate sphingolipid metabolism, trafficking and their interaction with lipids and proteins. The combined use of in vivo and in vitro tools available has allowed a first glance at sphingolipid functions.

However, the significance of the multitude of individual glycosphingolipid and sphingolipid structures, as identified by advanced mass spectrometry, is still poorly understood. E.g. what are the functions of several hundred different ceramides that are generated by six promiscuous ceramide synthases? The ceramides differ in their sphingoid bases and acyl chains, with different chain lengths, degrees of hydroxylation and unsaturation. What do they contribute as membrane anchors to the functions of sphingolipids and glycosphingolipids and as components of

membranes with respect to the overall collective properties of the self-assembling membranes?

Evidence that diversity of ceramide structure is important comes from model mice generated by silencing a single ceramide synthase. These display an altered ceramide expression and often fatal pathological phenotypes [130–133].

How do sphingolipid compositions change under such pathological conditions or during cellular differentiations at subcellular locations and how do their properties and functions, intracellular metabolism, membrane trafficking and flow change?

We even do not know much about the distribution of individual ceramide (or other sphingolipid) species in different subcellular organelles including the nuclei and how they affect their functional performance.

The complexity of these questions is furthermore increased by the low and often broad specificity of enzymes and binding proteins involved in sphingolipid metabolism. Not only are lysosomal hydrolases and multifunctional sphingolipid binding proteins of the lysosomal compartment promiscuous [134], but many glycosyltransferases involved in GSL biosynthesis are as well [4,6]. Furthermore, variant patient proteins may have changed substrate specificities. E.g. HexA hydrolyzes a variety of glycolipids, glycoproteins, and poly- and oligosaccharides containing terminal β-N-acetylgalactosamine or β-N-acetylglucosamine residues. Variant HexA molecules as found in patient tissues can have a changed substrate specificity. Whereas HexA from Tay–Sachs patients has lost its activity against both anionic and neutral substrates, allelic mutations in variant B1 patients generate variant HexA molecules which are still active on neutral but not on anionic substrates, including the main storage compound ganglioside GM2 [134].

In view of this complexity, further molecular, genetic and chemical biology tools [110] have to be developed for the analysis of basic sphingolipid functions at the crucial organellar and subcellular level.

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