ELSEVIER

Carbohydrate Research 307 (1998) 135–145

CARBOHYDRATE RESEARCH

Glycosphingolipids of skeletal muscle: I. Subcellular distribution of neutral glycosphingolipids and gangliosides in rabbit skeletal muscle

Johannes Müthing[a,*], Ulrich Maurer[a], Ulrich Neumann[b], Bernhard Kniep[c], Sabine Weber-Schürholz[d]

[a] *Universität Bielefeld, Technische Fakultät, Arbeitsgruppe Zellkulturtechnik, Postfach 100131, D33501 Bielefeld, Germany*

[b] *Clinic for Poultry of the Hannover School of Veterinary Medicine, 30559 Hannover, Germany*

[c] *Institute of Immunology, Technical University of Dresden, 01101 Dresden, Germany*

[d] *Institute for Developmental Biology, University of Bielefeld, 33501 Bielefeld, Germany*

Received 24 October 1997; accepted 16 January 1998

Abstract

Membrane vesicles were prepared from rabbit skeletal muscle, separated by sucrose density gradient centrifugation and characterized by their specific marker enzymes, ligand binding, and ion flux activities. The fractions obtained (in the order of increasing density) were sarcolemma (SL), T-tubules (TT), sarcoplasmic reticulum (SR1 and SR2) and triads/mitochondria (Tr/M). Their glycosphingolipid compositions were analyzed by biochemical and immunochemical methods with specific antibodies (TLC immunostaining) and characteristic patterns were obtained from respective membrane fractions, expressed on a protein basis. Glucosylceramide, the main neutral glycosphingolipid of rabbit muscle, was found in SL and TT fractions, whereas SR and Tr/M vesicles lack this compound. Lactosylceramide was selectively recovered in the SR1 fraction. G_{M3}(Neu5Ac), the main ganglioside in rabbit muscle, was found to account for 64% in the SL, 13% in the TT, 7% in the SR1, 3% in the SR2 and 13% in the Tr/M fractions. IV^3Neu5Ac–*n*LcOse$_4$Cer

Abbreviations: DAPI, 4',6-diamidine-2-phenylindole-dihydrochloride; DHP, dihydropyridines; DTAF, dichlorotriazinylamino-fluorescein; GSL(s), glycosphingolipid(s); HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; SL, sarcolemma; SR, sarcoplasmic reticulum; Tr/M, triads/mitochondria; TT, T-tubules. The nomenclature of sialic acids follows the suggestions of Reuter and Schauer [1], Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid. The designation of glycosphingolipids was performed according to the IUPAC–IUB recommendations [2] and the nomenclature of Svennerholm [3]. Glucosylceramide or Glcβ1–1Cer; lactosylceramide or LacCer, Ga1β1–4G1cβ1–1Cer; globotriaosylceramide or GbOse$_3$Cer, Ga1α1–4Ga1β1–4Glcβ1–1Cer; globoside or globotetraosylceramide or GbOse$_4$Cer, Ga1NAcβ1–3Galα1–4Galβ1–4Glcβ1–1Cer; gangliotetraosylceramide or GgOse$_4$Cer, Galβ1–3GalNAcβ1–4Galβ1–4Glcβ1–1Cer; lacto-*N*-*neo*tetraosylceramide or *n*LcOse$_4$Cer, Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer; lacto-*N*-*nor*hexaosylceramide or *n*LcOse$_6$Cer, Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer; G_{M3}, II^3Neu5Ac–LacCer; G_{M1}, II^3Neu5Ac–GgOse$_4$Cer; G_{D1a}, IV^3Neu5Ac, II^3Neu5Ac–GgOse$_4$Cer; G_{D1b}, II^3(Neu5Ac)$_2$–GgOse$_4$Cer; G_{T1b}, IV^3Neu5Ac, II^3(Neu5Ac)$_2$–GgOse$_4$Cer.

* Corresponding author. Tel: 49.521.1066320; Fax: 49.521.1066328; e-mail: jm@zellkult.techfak.uni-bielefeld.de

PII S0008-6215(98)00027-5

was mostly abundant in SL and decreased in the order SL > TT, Tr/M > SR1, SR2. IV^{6}Neu5Ac–*n*LcOse$_4$Cer was only detected in the SL and Tr/M fractions in noteworthy quantities. Ganglioseries gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} displayed homogeneous distribution patterns in each membrane preparation. They were expressed only in small amounts but mainly in SL, TT and Tr/M vesicles and to less extent in SR1 and SR2 fractions. The presence of G_{M3}(Neu5Ac) in the SL as well as on subcellular level was confirmed in transverse muscle cryosections by means of indirect immunofluorescence microscopy. The SL was brightly stained, but considerable intracellular fluorescence was observed as expected from the biochemical analyses. Thus, the neutral GSL and ganglioside expression of the SL and the intracellular membraneous network is different in skeletal muscle both in terms of quantitative and qualitative GSL composition as demonstrated in details by means of biochemical and immunochemical techniques. The modulatory functions of G_{M3} and gangliosides of the neolacto- and ganglio-series towards the voltage dependent Ca^{2+}-channel, largely preponderant in the triads-containing Tr/M fraction, is the subject of the accompanying paper (J. Müthing, U. Maurer, and S. Weber-Schürholz, *Carbohydr. Res.*, 307 (1998) 147–157).

Keywords: Antibodies; Immunohistochemistry; Membrane fractions; Subcellular localization; TLC immunostaining

1. Introduction

Structures, metabolism and functions of glycosphingolipids (GSLs) have been widely reviewed [4–10]. Neutral GSLs as well as gangliosides, which are characterized by the presence of one or more sialic acids [11,12], are located primarily in the external membrane of animal cells [13], but they are also found associated with intracellular organelles [14]. The predominant intracellular localization of lactosylceramide, the major human neutrophil neutral GSL, in granule-enriched rather than plasma membrane fractions has been reported [15]. Further examples are the association of globoside with the secretory granules of mouse bone marrow culture-derived mast cells [16] and the subcellular localization of the Forssman GSL in the Golgi complex, endosomes and lysosomes of canine epithelial cells [17]. Chromaffin granule membranes of bovine adrenal medulla were found to contain a relatively higher concentration of gangliosides than the membranes of microsomes and mitochondria [18]. As shown for the subcellular distribution and biosynthesis of rat liver gangliosides, gangliosides synthesized in the Golgi apparatus are transported not only to the plasma membrane, but to the endoplasmic reticulum and to other internal endomembranes as well [19]. Subcellular fractions of rat sciatic nerve [20], rat brain [21] and *Torpedo* electric organ [22] revealed clear differences in ganglioside patterns. Furthermore, formation of cytosolic ganglioside–protein complexes have been determined in human fibroblasts [23] and in guinea pig skeletal and cardiac muscle [24]. Finally, the association of GSLs with intermediate filaments of a variety of cell types has been well documented [25,26]. Intracellular pools of GSLs, while quantitatively less than the plasma membrane, may subserve important cellular functions (outlined in the accompanying paper in this issue [27]).

Although muscles together make up approximately 40% of total body weight, there have been in the past only a few studies of the GSL expression in mammalian muscles. Several GSL structures from pig [28,29] and human skeletal muscle [30–32] have been identified, recently also confirmed by means of indirect immunofluorescence and TLC immunostaining techniques [33,34]. From early studies of the ganglioside composition of rabbit skeletal muscle [35,36] it became obvious that muscle represents the tissue with the lowest ganglioside content of about 28.4 nmol/g wet tissue compared to e.g. liver (293.3 nmol/g wet tissue) and brain (2015.6 nmol/g wet tissue) (data drawn from ref. [37]). G_{M3}(Neu5Ac) and glucosylceramide are the major GSLs of rabbit skeletal muscle, whereas gangliosides of the neolacto- and ganglio-series represent minor constituents [38,39]. However, only scarce data is available concerning the distribution and role of GSLs in the plasma and intracellular membranes of skeletal muscle [24,40]. Therefore, we investigated the detailed GSL composition of sarcolemma (SL), T-tubules (TT), sarcoplasmic reticulum (SR) and triads/mitochondria (Tr/M) enriched membrane preparations of rabbit

skeletal muscle. Profound qualitative as well as quantitative differences in the GSL expression could be demonstrated for the analyzed membrane preparations. The modulatory effects of muscle specific gangliosides towards voltage dependent Ca^{2+}-channels, which are enriched in the triad membrane fraction, will be presented in the accompanying paper [27].

2. Results

The subcellular distribution of neutral GSLs and gangliosides in skeletal muscle, i.e. their distribution in the SL, TT, SR1, SR2 and Tr/M membrane fractions has not been documented so far in detail. For this purpose, membrane vesicles were prepared from rabbit skeletal muscle, separated by sucrose density gradient centrifugation and characterized by their specific marker enzymes, ligand binding, and ion flux activities [41]. GSLs were extracted from whole muscle and from single membrane vesicle preparations. Since muscle represents the tissue with the lowest GSL content, GSL compositions of respective fractions were elucidated by means of the overlay technique [42].

Neutral GSLs and gangliosides from whole rabbit skeletal muscle.—The orcinol stained TLC of neutral GSLs from whole rabbit skeletal muscle is shown in Fig. 1A (lane b) in comparison to neutral GSLs from human erythrocytes (Fig. 1A, lane a; reference). Glucosylceramide represents the major neutral GSL in skeletal muscle, and only traces of lactosylceramide were detectable in the whole GSL extract. The resorcinol stained TLC of gangliosides from whole rabbit skeletal muscle is given in Fig. 1B (lane b) in comparison to gangliosides from human granulocytes (Fig. 1B, lane a) and human brain (Fig. 1B, lane c), which were used as references throughout this study (see below). Rabbit skeletal muscle is characterized by the expression of G_{M3}(Neu5Ac) and some minor gangliosides, which chromatograph on the level of reference neolacto-series type gangliosides from human granulocytes (Fig. 1B).

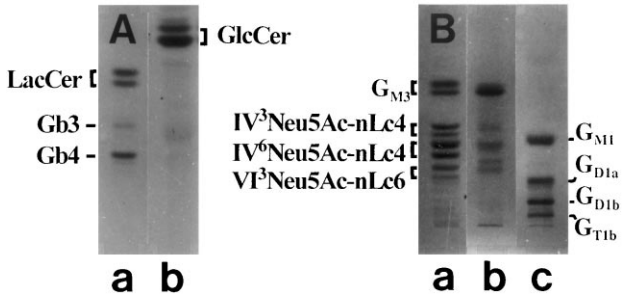

Fig. 1. (A) Orcinol stained TLC of neutral GSLs from rabbit skeletal muscle. Lane a: 2 μg of neutral GSLs from human red blood cells (reference); lane b: 5 μg of neutral GSLs from whole rabbit skeletal muscle. (B) Resorcinol stained TLC of gangliosides from rabbit skeletal muscle. Lane a: 10 μg of gang1iosides from human granulocytes (reference); lane b: 5 μg of gangliosides from whole rabbit skeletal muscle; lane c: 10 μg of human brain gangliosides (reference). Gb3 = globotriaosylceramide; Gb4 = globotetraosylceramide; GlcCer = glucosylceramide; *n*Lc4 = *n*LcOse$_4$Cer; *n*Lc6 = *n*LcOse$_6$Cer.

Membrane fractions.—Marker enzymes, ligand binding, and ion flux activities in membrane fractions from rabbit skeletal muscle are summarized in Table 1. The SL fraction was characterized by high Na^+,K^+-ATPase activity, enrichment of $C1^-$-channel, which was detected by specific binding of IAA-94, and enrichment of Na^+-channel, determined by specific saxitoxin ligand binding capacity, as reported by Weber-Schürholz et al. [41]. TT-rich fractions bound [^{3}H]PN200–110 (dihydropyridine receptors, Ca^{2+}-channels) with high specific activity. The Ca^{2+}-channel of muscle is predominantly localized in the triad fraction [43] and to lesser extent in the TT fraction [44], representing a minor underlying protein constituent within respective membrane fractions. The main compound of the SR1 and SR2 fractions is Ca^{2+}-ATPase and high specific activity of this enzyme identified both fractions as enriched for SR. The densest fraction ($\approx$36–40% sucrose) beared the mitochondrial marker succinate-cytochrome c reductase [41] and highest specific binding activity for the DHP receptor ligand [^{3}H]PN200–110 according to Imagawa et al. [43].

Neutral GSL expression in muscle membrane vesicles.—Glucosylceramide (GlcCer) which represents the dominant neutral GSL of rabbit muscle [39] was found in the SL and TT vesicles, whereas the SR2 and Tr/M fractions lack this GSL (Fig. 2). Lactosylceramide (LacCer) which had been detected only in trace amounts in whole homogenates of rabbit skeletal muscle (Fig. 1A, lane b), was selectively recovered in the SR1 fraction. Applying neutral GSLs that corresponded to 200 μg protein of each fraction, no relevant amounts of neutral GSLs were detected by orcinol stain in SR2 and Tr/M vesicles (Fig. 2).

Protein, cholesterol and lipid bound sialic acid contents of muscle membrane fractions.—The protein, cholesterol and ganglioside derived sialic acid

Table 1
Summary of marker enzymes, ligand binding, and ion flux activities in membrane fractions from rabbit skeletal muscle [a]

Sucrose (%)	Membrane vesicles	Major enrichment [b]	Enzyme activities	Ligand binding
0–14	SL	SL	Na^+, K^+–ATPase	IAA-94, saxitoxin
14–25	SL/TT/SR	TT	—	(PN200-110)
25–28	SR	SR1	Ca^{2+}–ATPase	—
28–35	TT/SR	SR2	Ca^{2+}–ATPase	—
36–40	Tr/SR/M	Tr/M [c]	Succinate-cytochrome c reductase	PN200-110

[a] Data drawn from Weber-Schürholz et al. [41].
[b] Abbreviations used throughout the text.
[c] Abundant triad structures, identified by electron microscopy.

contents of muscle membrane fractions are summarized in Table 2. The absolute amounts as well as concentrations are given to stress the different composition of each membrane fraction. The lowest protein concentration was assayed in the SL fraction (1.8 mg/mL) and highest concentrations were found in SR2 and Tr/M fractions (17.6 and 15.9 mg/mL, respectively) according to increasing densities of respective fractions. Cholesterol concentrations varied from 0.3 mg/mL (SL) up to 0.9 mg/mL (Tr/M). Maximum sialic acid concentration was detected in the Tr/M (7.7 μg/mL) and lowest in the TT fraction (1.8 μg/mL). To gain more insights into the membrane architecture and specific membrane assembly, the cholesterol-to-protein ratios and lipid bound, i.e. ganglioside derived sialic acid-to-protein ratios were calculated for each membrane fraction. These data, estimated on a protein basis, are shown in Fig. 3. The SL and TT fractions are characterized by high cholesterol values compared to ca. fourfold lower contents in

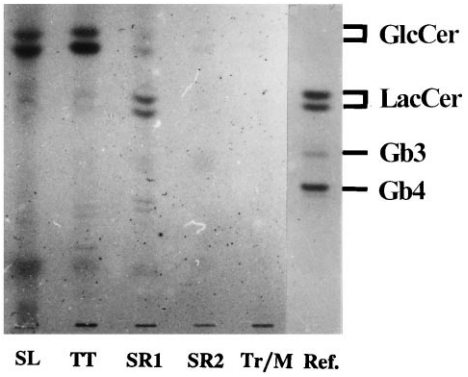

Fig. 2. Distribution of neutral glycosphingolipids in SL, TT, SR and Tr/M membranes from rabbit skeletal muscle. Neutral GSLs corresponding to 200 μg protein of each membrane fraction were chromatographed and stained with orcinol; 2 μg of neutral GSLs from human red blood cells were used as reference (Ref.). Gb3 = globotriaosylceramide; Gb4 = globotetraosylceramide; GlcCer = glucosylceramide; LacCer = lactosylceramide.

both SR fractions and Tr/M vesicles (Fig. 3A). The ganglioside content, determined via sialic acid quantification by HPLC and also calculated on a protein basis, was enriched in SL compared to all the other membrane fractions (Fig. 3B). The sialic acid profile revealed Neu5Ac quantities of 2.5 μg in SL, 0.5 μg in TT and Tr/M each, 0.35 μg in SR1 and 0.15 μg in SRT2, corresponding to 1 mg protein of each fraction. Thus, analyses of TT and Tr/M probes gave ca. fivefold lowered amounts, followed by sevenfold and 17-fold decreases in SR1 and SR2, respectively, compared to SL. Neu5Gc was not detected in either membrane fraction according to previous results from whole rabbit skeletal muscle [39].

G_{M3} expression in muscle membrane vesicles.—G_{M3}(Neu5Ac) is well known to be the predominant ganglioside in rabbit skeletal muscle [36] [38] [39]. Quantification of resorcinol stained bands by TLC scanning resulted in G_{M3}(Neu5Ac) distribution of about 64% in the SL, 13% in the TT, 7% in the SR1, 3% in the SR2 and 13% in the Tr/M fractions. These results corresponded exactly to the HPLC analysis of ganglioside derived Neu5Ac shown in Fig. 3B. Overall approximately two thirds of G_{M3} were found in the SL whereas the considerable amount of about one third was detected on subcellular level.

Distributions of neolacto-series gangliosides in muscle membrane fractions.—The procedure for selective detection of terminally α2–6 and α2–3 sialylated neolacto-series gangliosides involves immunostaining of separated gangliosides with an anti-*n*$LcOse_4Cer$ antibody without *V. cholerae* neuraminidase treatment and after enzyme incubation, respectively [45]. G_{M3}(Neu5Ac) and the neolacto-series gangliosides IV^3Neu5Ac-*n*$LcOse_4Cer$, IV^6Neu5Ac-*n*$LcOse_4Cer$, and VI^3Neu5Ac-*n*$LcOse_6$Cer from human granulocytes (for structures see Table 1 in the accompanying paper by Müthing

Table 2
Protein, cholesterol and sialic acid contents of membrane fractions from rabbit skeletal muscle

Membrane fraction	Volume	Protein		Cholesterol		Sialic acid	
	(mL)	(mg)	(mg/mL)	(mg)	(mg/mL)	(μg)	(μg/mL)
SL	2.3	4.2	1.8	0.7	0.3	10.4	4.5
TT	2.1	7.4	3.5	1.3	0.6	3.7	1.8
SR1	2.8	34.9	12.4	1.2	0.4	10.0	3.6
SR2	3.1	54.7	17.6	1.9	0.6	8.8	2.8
Tr/M	12.3	195.3	15.9	10.6	0.9	95.1	7.7

et al. [27] served as references [46,47]. Each ganglioside migrates as a double band due to substitution with C_{24}-fatty acid (upper band) and a C_{16}-fatty acid (lower band). After incubation of human granulocyte gangliosides with *V. cholerae* neuraminidase on a plasticized silica gel HPTLC plate, IV^{3}Neu5Ac–*n*LcOse$_4$Cer and VI^{3}Neu5Ac–*n*LcOse$_6$Cer as well as IV^{6}Neu5Ac–*n*LcOse$_4$Cer were detected by immunostaining with the anti-*n*LcOse$_4$Cer antibody (Fig. 4A, reference). On the other hand, the overlay assay without neuraminidase treatment showed two positive IV^{6}Neu5Ac–*n*LcOse$_4$-Cer bands (Fig. 4B, reference). The steric hindrance of sialic acid bound at position 3 to the terminal galactose prevents binding of the antibody to the masked Ga1β1–4GlcNAc-sequence, whereas sialylation at position 6 to the terminal galactose does not impair recognition [45]. The two immunostained bands above G_{M3} (Fig. 4) represent contaminations of *n*LcOse$_4$Cer within the ganglioside fraction as explained in a previous paper [39].

The presence of lactosamine-containing gangliosides in muscle membrane vesicles was revealed by this method and considerable differences in the distribution of neolacto-series gangliosides to the single membrane fractions could be observed: IV^{3}Neu5Ac–*n*LcOse$_4$Cer was mainly found in the SL and its distribution to the other membranes

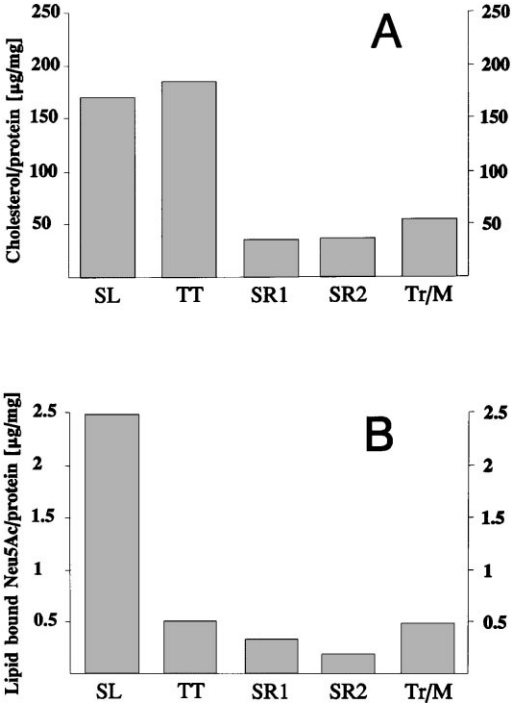

Fig. 3. Cholesterol-to-protein (A) and lipid bound sialic acid-to-protein ratios (B) for SL, TT, SR and Tr/M membranes from rabbit skeletal muscle.

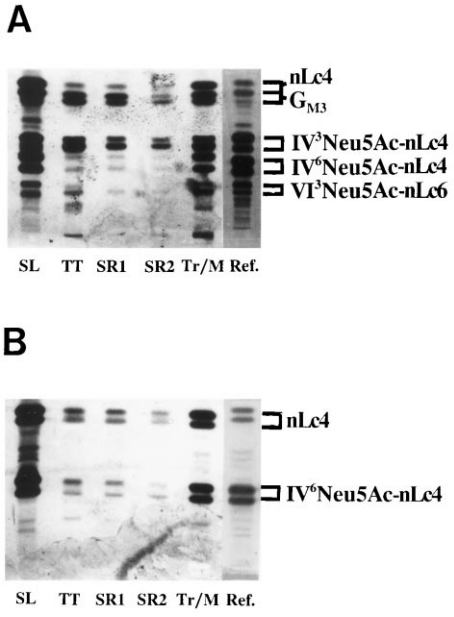

Fig. 4. Distribution of neolacto-series gangliosides in SL, TT, SR and Tr/M membranes from rabbit skeletal muscle. Chromatographed gangliosides were immunostained on TLC plates with anti-*n*LcOse$_4$Cer polyclonal antibody after (A) and without (B) preceding *V. cholerae* neuraminidase treatment. Gangliosides corresponding to 40 μg (A) and 80 μg (B) protein of each membrane fraction were applied. 5 μg (A) and 2 μg (B) of neolacto-series gangliosides from human granulocytes were used as references (Ref.). *n*Lc$_4$ = *n*LcOse4Cer; *n*Lc6 = *n*LcOse$_6$-Cer.

decreased in the order SL > TT, Tr/M > SR1, SR2 (Fig. 4A). IV^{6}Neu5Ac–*n*LcOse$_4$Cer was found in the SL and Tr/M vesicles in considerable quantities, whereas only trace amounts were detectable in TT and SR fractions, also proved by TLC immunostaining without preceding neuraminidase treatment (Fig. 4B).

Distributions of ganglio-series gangliosides in muscle membrane fractions.—G_{M1} can be visualized on silica gel fixed TLC plate with choleragenoid (=cholera toxin B subunit). Gangliosides G_{D1a}, G_{D1b} and G_{T1b} can be detected by conversion with *V. cholerae* neuraminidase to G_{M1} prior to treatment with choleragenoid according to Wu and Ledeen [48]. The suitability of this specific overlay assay is demonstrated in Fig. 5, using a reference ganglioside mixture from human brain (for structures see Table 2 in the accompanying paper by Müthing et al. [27]. G_{M1} of human brain was detected by choleragenoid without previous neuraminidase treatment (Fig. 5B, reference), whereas the higher sialylated gangliosides with a G_{M1}-backbone were detected with it (Fig. 5A, reference).

Ganglio-series gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} which had been found in trace amounts with this highly sensitive assay in whole rabbit skeletal muscle [39], were detected in different quantities in respective membrane fractions. Homogeneous distribution patterns were revealed among the membrane fractions indicating major expression of ganglio-series gangliosides G_{M1} and G_{D1a} compared to minor G_{T1b} and G_{D1b} (Fig. 5A). Within the five membrane samples, these compounds were most abundant in SL and TT vesicles and less abundant in Tr/M fractions, followed by SR1 and SR2.

Immunofluorescence microscopy.—The presence of GSLs in the SL as well as on subcellular level was proved in muscle cryosections using indirect immunofluorescence microscopy. For example, the immunofluorescence staining of a transverse cryosection of rabbit skeletal muscle with anti-G_{M3}(Neu5Ac) antibody is shown in Fig. 6A in parallel with the DAPI staining of nuclear DNA in the same field (Fig. 6B). Anti-G_{M3}(Neu5Ac) reacted strongly with the SL of muscle fibers as expected from the biochemical analyses. The SL was brightly stained expressing regular and even distribution of immunostained G_{M3}(Neu5Ac) along the membrane, but intracellular fluorescence was observed as well.

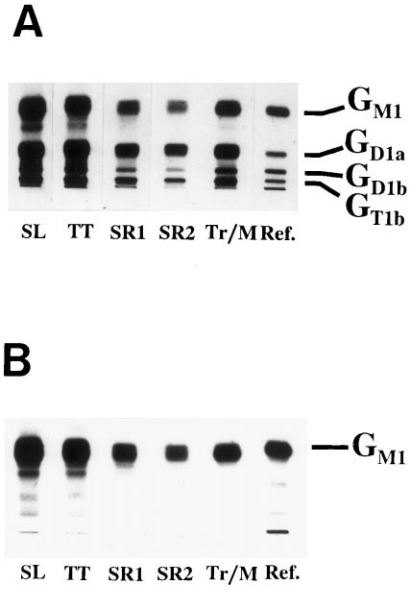

Fig. 5. Distribution of ganglio-series gangliosides in SL, TT, SR and Tr/M membranes from rabbit skeletal muscle. Chromatographed gangliosides were stained on TLC plates with choleragenoid after (A) and without (B) preceding *V. cholerae* neuraminidase treatment. Gangliosides corresponding to 40 μg (A) and 80 μg (B) protein of each membrane fraction were applied; 1 μg (A) and 5 μg (B) of human brain gangliosides were used as references (Ref.).

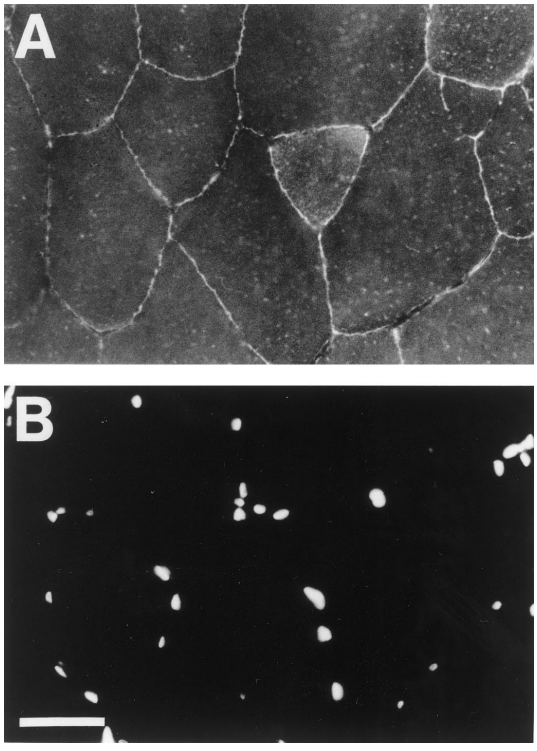

Fig. 6. Immunofluorescence staining of a transverse cryosection of rabbit skeletal muscle with anti-G_{M3}(Neu5Ac) antibody. Fluorescence micrograph (A) and DAPI staining (B) of the same field. Original magnification ×400. Bar = 50 μm.

3. Discussion

Most of our current knowledge on GSL biosynthesis and degradation has been emerged from metabolic studies in liver and neuronal tissue [7,49]. Serine palmitoyltransferase, 3-dehydrosphinganine reductase and sphinganine *N*-acetyltransferase are involved in the first steps of sphingolipid biosynthesis [50]. The localization of these enzymes was confirmed in mouse and rat liver, facing the cytosolic side of the endoplasmic reticulum [51,52]. The biosynthesis of GlcCer has been localized at the cytosolic surface of Golgi subfractions of various cell types [53]. The sequence of biosynthetic steps of sphingolipid and GSL biosynthesis is presumed to be accompanied by intracellular movement of the stepwise-growing molecules most likely by vesicle-bound membrane flow from the endoplasmic reticulum through the Golgi cisternae to the plasma membrane. Some evidence was provided from rat liver investigations that glucosyltransferase and galactosyltransferase-2, leading to glucosyl- and lactosylceramide, respectively, face the cytoplasmic side of the Golgi apparatus, whereas sialyltransferase-1, responsible for G_{M3}-biosynthesis, and the other late acting enzymes face the luminal side [54]. On the other hand, transbilayer movement of monohexosyl sphingolipids has been proposed [55] to be required for higher GSL biosynthesis, i.e. glucosylceramide translocates from the cytosolic to the luminal leaflet of the Golgi membrane [56] where the conversion of glucosyl- into lactosylceramide by galactosyltransferase-2 takes place [55].

Concerning these early steps in sphingolipid and GSL biosynthesis, very little is known about the glycosyl- and sialyltransferases in mammalian skeletal muscle. The GSL biosynthesis has been studied with rat and mouse myoblast cell lines, which form multinucleated myotubes in vitro, resembling normal muscle syncytia [57,58]. Their results implicated, that the biosynthesis of GSLs is tightly regulated during myogenesis in vitro and suggests a role for membrane gangliosides in muscle cell differentiation. With respect to our data of the subcellular distribution of neutral GSLs, the expression of GlcCer in the SL and TT fractions and its absence in the SR1, SR2 and Tr/M vesicles as well as the specific enrichment of LacCer in the SR1 preparation suggest a compartmentation of glucosyltransferase and galactosyltransferase-2, membrane traffic or a specific sorting mechanism in rabbit skeletal muscle, however, speculative at this stage of research. Moreover, the selective enrichment of lactosylceramide, the substrate of sialyltransferase-1 generating the predominant ganglioside G_{M3}, supports the hypothesis of membraneous movement along the biosynthesis of GSLs. These data also indicate different subcellular localization of glucosyl-, galactosyl- and sialyltransferase. Studies on the glycoconjugate metabolism in developing rabbit skeletal muscle membranes [59] revealed a strong decrease in sialyltransferase-1 activity during development from neonatal to adult stage in sarcoplasmic reticulum in comparison to sarcolemma, being in good agreement with our findings of preponderance of G_{M3} in the SL fraction. Thus, major biosynthesis of G_{M3} in skeletal muscle is presumably located in the sarcolemma, i.e. in the plasma membrane of myotubes.

G_{M3} represents one of the major GSLs in extraneuronal tissues. Much effort has been spent to the biochemical characterization of sialyltransferase-1 focussed on rat liver tissue [60–63] and the glycosyltransferases, which are involved in the biosynthesis of the asialo-, a-, b- and c-series gangliosides [64,65]. The gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}, characteristic for mammalian neuronal tissue, were identified as very minor constituents in rabbit skeletal muscle and showed rather quantitative than qualitative differences in their subcellular distribution patterns. On the other hand, quantitative as well as qualitative alterations of α2–3- and α2–6-sialylated neolacto-series gangliosides were observed in the muscle vesicle preparations. IV^{3}Neu5Ac–*n*LcOse$_4$Cer dominated in the SL and Tr/M fractions, accompanied by high levels of IV^{6}Neu5Ac–*n*LcOse$_4$Cer, the latter being almost completely absent in the TT, SR1 and SR2 vesicles. Extremely low abundancy of IV^{3}Neu5Ac–*n*LcOse$_4$Cer was found to be characteristic for both SR1 and SR2 preparations. From this data it can be concluded, that α2–3- and α2–6-sialyltransferases may act at different sites in the muscle or that specific sorting mechanisms are responsible for the subcellular restricted expression of IV^{6}Neu5Ac–*n*LcOse$_4$Cer in the SL and Tr/M vesicles. Gangliosides of the paragloboside series isolated from chicken muscle have been described [66] and the corresponding sialyltransferase, which transfers sialic acid to the non-reducing terminus of *n*LcOse$_4$Cer and *n*LcOse$_6$Cer, has been partially purified from the same tissue [67]. However,

nothing is known about its subcellular localization in muscle so far.

A large body of accumulated evidence points to the plasma membrane as the principal subcellular locus of gangliosides (and GSLs generally). There are, however, several indications of intracellular pools as well, which, while quantitatively less than the plasma membrane, may subserve important subcellular functions. The modulatory functions of G_{M3}, neolacto- and ganglio-series gangliosides has been proved in reconstituted Ca^{2+}-channel containing vesicles from the Tr/M fraction. This data is presented and discussed in the accompanying paper [27].

4. Experimental

Preparation of membrane vesicles.—Fractions of membrane vesicles were preparaed from 380 g fresh skeletal muscle tissue (back and hindlegs) of a specific pathogen free inbred rabbit (Hoechst, Frankfurt, Germany) according to the method of Mitchell et al. [68], modified in some parts by Weber-Schürholz et al. [41]. The fractions obtained after sucrose density gradient centrifugation contained vesicles, enriched in the following membrane systems (in the order of increasing density): sarcolemma (SL), T-tubules (TT), sarcoplasmic reticulum (SR), subdivided into SR1 and SR2 (containing smaller and larger vesicles, respectively), and triads and mitochondria (Tr/M). Membrane fractions were monitored by the distribution of marker enzymes, ligand binding, and flux activities as reported [41]. All the membrane characteristics are summarized in Table 1. [^{3}H]IAA-94 was used as the ligand for the Cl^--channel and [^{3}H]saxitoxin for the Na^+-channel. [^{3}H]PN200–110 binds with high specificity to Ca^{2+}-channels (=DHP-receptors). Na^+,K^+-ATPase is the characteristic enzyme for SL, Ca^{2+}-ATPase for SR and succinate–cytochrome c reductase for mitochondria. Triads were identified by use of electron microscopy according to Mitchell et al. [68].

Determination of protein, cholesterol and sialic acids.—Protein concentration was determined with the bicinchoninic acid method (Pierce Chemical Company, Rockford, IL, USA) according to Smith et al. [69]. This protein assay combines the well known reduction of Cu^{2+} by protein to Cu^+ in alkaline medium with the cuprous (Cu^+) ion detecting property of bicinchoninic acid. Bovine serum albumin was used as protein standard.

Total cholesterol was determined using an enzyme/colorimetry assay according to Allain et al. [70]. Cholesterol esterase, cholesterol oxidase and peroxidase enzyme cascade finally generate a quinoneimine dye, which is photometrically quantified at 500 nm.

Lipid bound sialic acids (Neu5Ac and Neu5Gc) were determined as their fluorescent derivatives by HPLC, essentially as described by Hara et al. [71]. Sialic acids were released from gangliosides with 25 mM H_2SO_4 (2.5 h, 80 °C) and then converted with 1,2-diamino-4,5-methylenedioxybenzene (DMB, Sigma, Deisenhofen, Germany) into their fluorescent derivatives. Reference Neu5Ac was from Biomol (Hamburg, Germany) and Neu5Gc from Sigma. Isocratic HPLC was performed using an RP_{18} column (250×4.6 mm, ODS 5 μm; Beckman, München, Germany) as the stationary phase and acetonitrile/methanol/water (7:9:110) as the mobile phase with a flow rate of 1.2 mL/min. The derivatives were detected with a fluorometer, operating at an excitation wavelength of 373 nm and an emission wavelength of 448 nm.

Isolation of GSLs from whole rabbit skeletal muscle and muscle membrane preparations.—Whole skeletal muscles were homogenized with an Ultraturrax (TP 18/10; Janke & Kunkel, Staufen, Germany) after grinding with a mincing machine and 100 g were used for GSL extraction. GSLs were extracted from whole skeletal muscle and the single membrane fractions with chloroform/methanol (2:1), (1:2) and chloroform/methanol/water (30:60:8), each by vol. Chloroform and methanol of analytical grade (Merck, Darmstadt, Germany) were distilled before use. Gangliosides and neutral GSLs were separated according to standard procedures [72] by anion exchange chromatography on DEAE–Sepharose (Pharmacia Fine Chemicals, Freiburg, Germany) and further purified by adsorption chromatography on Iatrobeads 6RS-8060 (Macherey & Nagel, Düren, Germany) according to Ueno et al. [73]. Whole gangliosides were eluted with chloroform/methanol (1:2, by vol.). The neutral GSL-containing fraction was applied to a silica gel 60 column (Merck) and whole neutral GSLs were eluted with chloroform/methanol (40:60). The final purification of GSLs was performed by Florisil chromatography as their peracetylated derivatives [74].

Reference GSLs.—A reference neutral GSL fraction with LacCer, $GbOse_3Cer$ and $GbOse_4Cer$ as main components was prepared from human

erythrocytes according to standard procedures as described above. A ganglioside mixture composed of G_{M3}(Neu5Ac), IV^{3}Neu5Ac–*n*LcOse$_4$Cer (=sialylparagloboside), IV^{6}Neu5Ac–*n*LcOse$_4$Cer, and VI^{3}Neu5Ac–*n*LcOse$_6$Cer was isolated from human granulocytes [46]. Human brain gangliosides were purchased from Supelco Inc. (Bellefonte, PA, USA).

Thin layer chromatography.—Glass-backed silica gel 60 precoated high-performance thin-layer chromatography plates (HPTLC plates, size 10×10 cm, thickness 0.2 mm, Art. 5633, Merck, Darmstadt, Germany) were used [42]. GSLs were applied to the plates with the automatic TLC applicator AS 30 (Desaga, Heidelberg, Germany). Neutral GSLs were separated in chloroform/methanol/water (120:70:17), and gangliosides were chromatographed in chloroform/methanol/water (120:85:20) containing 2 mM $CaCl_2$. Neutral GSLs were visualized with orcinol [75] and gangliosides with resorcinol [76].

G_{M3}(Neu5Ac) was quantified by densitometry with a CD 60 scanner (Desaga) equipped with an IBM compatible personal computer and densitometric software. Intensities of bands were measured in reflectance mode at 580 nm with a light beam slit of 0.1×2 mm.

TLC immunostaining (overlay technique).—The immunostaining procedure was carried out according to Magnani et al. [77] with some modifications [78] and as described recently in detail [42,79]. Affinity chromatography purified rabbit anti-chicken IgG antibody labeled with alkaline phosphatase was purchased from Dianova (Hamburg, Germany). Bound antibodies were visualized with 5-bromo-4-chloro-3-indolylphosphate.

Detection of neolacto-series gangliosides.—Neuraminidase treatment of neolacto-series gangliosides with α2–3 substituted sialic acid is necessary prior to immunostaining with the anti-*n*LcOse$_4$Cer antibody on HPTLC plates (see above), whereas α2–6 sialylated neolacto-type gangliosides can be detected without enzyme treatment, since sialylation at position 6 of the terminal galactose does not hinder recognition [45]. Briefly, gangliosides were chromatographed and the silica gel fixed plate was incubated with 2.5 mU/mL *V. cholerae* neuraminidase (EC 3.2.1.18, Behring, Marburg, Germany) for 2 h at room temperature. For detection of α2–6 sialylated neolacto-type gangliosides, the plates were incubated with buffer only without enzyme. The plates were then immunostained with the chicken anti-*n*LcOse$_4$Cer antibody, followed by incubation with the alkaline phosphatase labeled second antibody.

Detection of G_{M1}-type gangliosides.—The HPTLC binding assay using choleragenoid (cholera toxin B subunit) for specific detection of G_{M1} was developed by Magnani et al. [80], and was used according to the modifications described by Pörtner et al. [81]. Briefly, gangliosides were chromatographed and the silica gel fixed HPTLC plate was incubated with 250 ng/mL choleragenoid (Sigma, No. C-7771). Goat anti-choleragenoid antiserum (Calbiochem, No. 227040, Frankfurt a.M., Germany) and alkaline phosphatase conjugated rabbit anti-goat IgG antiserum (Dianova) were used for immunostaining. To reveal the presence of G_{M1}-core gangliosides G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b}, the plates were incubated with 50 mU/mL *V. cholerae* neuraminidase (18 h, 37 °C) prior to combined choleragenoid-immunostaining. This technique was originally developed by Wu and Ledeen [48].

Fluorescence microscopy.—The preparation of sections from skeletal muscle, the immunostaining procedure and microscopic evaluation were performed essentially as described by Müthing et al. [39] and Cacić et al. [33].

Acknowledgements

We express our warmest thanks to Professor Dr.-Ing. J. Lehmann for his generous support of this work. The expert technical assistance of Mrs. H. Doedens and Mrs. C. Wiegandt (Developmental Biology Unit, University of Bielefeld) is also gratefully acknowledged. Furthermore we thank Dr. H. Brandt for HPLC analysis of sialic acids and Mrs. K. Priljević for immunofluorescence microscopic work. This work was financed by the Deutsche Forschungsgemeinschaft SFB 223 Pathomechanisms of Cellular Interactions, Project C06.

References

[1] G. Reuter and R. Schauer, *Glycoconjugate J.*, 5 (1988) 133–135.
[2] IUPAC–IUB Commission on Biochemical Nomenclature, *Eur. J. Biochem.*, 79 (1977) 11–21.
[3] L. Svennerholm, *J. Neurochem.*, 10 (1963) 613–623.
[4] R.K. Yu and M. Saito, *Structures and Localization of Gangliosides*, in R.U. Margolis and R.K.

Margolis (Eds.), *Neurobiology of Glycoconjugates*, Plenum Press, New York, 1989, pp 1–42.
[5] R.W. Ledeen, *Biosynthesis, Metabolism and Biological Effects of Gangliosides*, in R.U. Margolis and R.K. Margolis (Eds.), *Neurobiology of Gycoconjugates*, Plenum Press, New York, 1989, pp 43–83.
[6] S.-I. Hakomori, *J. Biol. Chem.*, 265 (1990) 18713–18716.
[7] G. Schwarzmann and K. Sandhoff, *Biochemistry*, 29 (1990) 10865–10871.
[8] R.L. Schnaar, *Glycobiology*, 1 (1991) 477–485.
[9] C.B. Zeller and R.B. Marchase, *Am. J. Physiol.*, 262 (1992) C1341–C1355.
[10] G. van Echten and K. Sandhoff, *J. Biol. Chem.*, 268 (1993) 5341–5344.
[11] A. Varki, *Glycobiology*, 2 (1992) 25–40.
[12] R. Schauer, S. Kelm, G. Reuter, P. Roggentin, and L. Shaw, *Biochemistry and Role of Sialic Acids*, in A. Rosenberg (Ed.), *Biology of the Sialic Acids*, Plenum Press, New York, 1995, pp 7–67.
[13] T.E. Thompson and T.W. Tillack, *Ann. Rev. Biophys. Biophys. Chem.*, 14 (1985) 361–386.
[14] B.K. Gillard, L.T. Thurmon, and D.M. Marcus, *Glycobiology*, 3 (1993) 57–67.
[15] F.W. Symington, W.A. Murray, S.I. Bearman, and S.-I. Hakomori, *J. Biol. Chem.*, 262 (1987) 11356–11363.
[16] H.R. Katz and K.F. Austen, *J. Immunol.*, 136 (1986) 3819–3824.
[17] I.L. van Genderen, G. van Meer, J.W. Slot, H.J Geuze, and W.F. Voorhout, *J. Cell Biol.*, 115 (1991) 1009–1019.
[18] M. Sekine, T. Ariga, T. Miyatake, Y. Kuroda, A. Suzuki, and T. Yamakawa, *J. Biochem.*, 95 (1984) 155–160.
[19] G.R. Matyas and D.J. Morré, *Biochim. Biophys. Acta*, 921 (1987) 599–614.
[20] K.H. Chou, C.E. Nolan, and F.B. Jungalwala, *J. Neurochem.*, 44 (1985) 1898–1912.
[21] P. Palestini, M. Masserini, A. Fiorilli, E. Calappi, and G. Tettamanti, *J. Neurochem.*, 61 (1993) 955–960.
[22] R.W. Ledeen, M.-F. Diebler, G. Wu, Z.-H. Lu, and H. Varoqui, *Neurochem. Res.*, 18 (1993) 1151–1155.
[23] V. Chigorno, M. Valsecchi, D. Acquotti, S. Sonnino, and G. Tettamanti, *FEBS Lett.*, 263 (1990) 329–331.
[24] K.-F.J. Chan and Y. Liu, *Glycobiology*, 1 (1991) 193–203.
[25] B.K. Gillard, J.P. Heath, L.T. Thurmon, and D.M. Marcus, *Exp. Cell. Res.*, 192 (1991) 433–444.
[26] B.K. Gillard, L.T. Thurmon, and D.M. Marcus, *Cell Motil. Cytoskel.*, 21 (1992) 255–271.
[27] J. Müthing, U. Maurer, and S. Weber-Schürholz, *Carbohydr. Res.*, 307 (1998) 147–157.
[28] K. Nakamura, M. Nagashima, M. Sekine, M. Igarashi, T. Ariga, T. Atsumi, T. Miyatake, A. Suzuki, and T. Yamakawa, *Biochim. Biophys. Acta*, 752 (1983) 291–300.
[29] T. Ariga, M. Sekine, K. Nakamura, M. Igarashi, M. Nagashima, T. Miyatake, A. Suzuki, and T. Yamakawa, *J. Biochem.*, 93 (1983) 889–893.
[30] L. Svennerholm, Å. Bruce, J.E. Månsson, B.-M. Rynmark, and M.-T. Vanier, *Biochim. Biophys. Acta*, 280 (1972) 626–636.
[31] Y.-T. Li, J.-E. Månsson, M.-T. Vanier, and L. Svennerholm, *J. Biol. Chem.*, 248 (1973) 2634–2636.
[32] K.C. Leskawa, P.E. Buse, E.L. Hogan, and A.J. Garvin, *Neurochem. Pathol.*, 2 (1994) 19–29.
[33] M. Cacić, J. Müthing, I. Kracun, U. Neumann, and S. Weber-Schürholz, *Glycoconjugate J.*, 11 (1994) 477–485.
[34] J. Müthing and M. Cacić, *Glycoconjugate J.*, 14 (1997) 19–28.
[35] F.E. Lassaga, I. Albarracin de Lassaga, and R. Caputto, *J. Lipid Res.*, 13 (1972) 810–815.
[36] M. Iwamori and Y. Nagai, *J. Biochem.*, 84 (1978) 1609–1615.
[37] M. Iwamori and Y. Nagai, *Biochim. Biophys. Acta*, 665 (1981) 214–220.
[38] K. Nakamura, T. Ariga, T. Yahagi, T. Miyatake, A. Suzuki, and T. Yamakawa, *J. Biochem.*, 94 (1983) 1359–1365.
[39] J. Müthing, U. Maurer, K. Sostarić, U. Neumann, H. Brandt, S. Duvar, J. Peter-Katalinić, and S. Weber-Schürholz, *J. Biochem.*, 115 (1994) 248–256.
[40] L.H. Wang, Y.P. Tu, X.Y. Yang, Z.C. Tsui, and F.Y. Yang, *FEBS Lett.*, 388 (1996) 128–130.
[41] S. Weber-Schürholz, E. Wischmeyer, M. Laurien, H. Jockusch, T. Schürholz, D.W. Landry, and Q. Al-Awqati, *J. Biol. Chem.*, 268 (1993) 547–551.
[42] J. Müthing, *J. Chromatogr. A*, 720 (1996) 3–25.
[43] T. Imagawa, A.T. Leung, and K.P. Campbell, *J. Biol. Chem.*, 62 (1987) 8333–8339.
[44] V. Flockerzi, H.-J. Oeken, F. Hofmann, D. Pelzer, A. Cavalié, and W. Trautwein, *Nature*, 323 (1986) 66–68.
[45] J. Müthing and U. Neumann, *Biomed. Chromatogr.*, 7 (1993) 158–161.
[46] J. Müthing, F. Unland, D. Heitmann, M. Orlich, F.-G. Hanisch, J. Peter-Katalinić, V. Knäuper, H. Tschesche, S. Kelm, R. Schauer, and J. Lehmann, *Glycoconjugate J.*, 10 (1993) 120–126.
[47] M.N. Fukuda, A. Dell, J.E. Oates, P. Wu, J.C. Klock, and M. Fukuda, *J. Biol. Chem.*, 260 (1985) 1067–1082.
[48] G. Wu and R. Ledeen, *Anal. Biochem.*, 173 (1988) 368–375.
[49] K. Sandhoff and T. Kolter, *Trends Cell. Biol.*, 6 (1996) 98–103.

[50] A.H. Futerman, *Ceramide Metabolism—Compartmentalized in the Endoplasmic Reticulum and Golgi Apparatus*, in D. Hoekstra (Ed.), *Current Topics in Membranes*, Vol. 40, Academic Press, San Diego, CA, 1994, pp 93–110.
[51] E.C. Mandon, I. Ehses, J. Rother, G. van Echten, and K. Sandhoff, *J. Biol. Chem.*, 267 (1992) 11144–11148.
[52] K. Hirschberg, J. Rodger, and A.H. Futerman, *Biochem. J.*, 290 (1993) 751–757.
[53] D. Jeckel, A. Karrenbauer, K.N.J. Burger, G. van Meer, and F. Wieland, *J. Cell Biol.*, 7 (1992) 259–267.
[54] M. Trinchera, M. Fabbri, and R. Ghidoni, *J. Biol. Chem.*, 266 (1991) 20907–20912.
[55] H. Lannert, C. Bünning, D. Jeckel, and F. Wieland, *FEBS Lett.*, 342 (1994) 91–96.
[56] K.N.J. Burger, P. van der Bijl, and G. van Meer, *J. Cell Biol.*, 133 (1996) 15–28.
[57] K.C. Leskawa, R.E. Erwin, P.E. Buse, and E.L. Hogan, *Mol. Cell. Biochem.*, 83 (1988) 47–54.
[58] K.C. Leskawa and E.L. Hogan, *Mol. Cell. Biochem.*, 96 (1990) 163–173.
[59] F.C. Clark and P.B. Smith, *Biochim. Biophys. Acta*, 755 (1983) 56–64.
[60] H. Kadowaki, L.A. Symanski, K.E. Rys-Sikora, and R.S. Koff, *J. Lipid Res.*, 30 (1989) 1789–1797.
[61] L.J. Melkerson-Watson and C.C. Sweeley, *J. Biol. Chem.*, 266 (1991) 4448–4457.
[62] H. Kadowaki and M.A. Grant, *J. Biol. Chem.*, 269 (1994) 14931–14938.
[63] H. Kadowaki and M.A. Grant, *J. Lipid Res.*, 36 (1995) 1274–1282.
[64] G. Pohlentz, D. Klein, G. Schwarzmann, D. Schmitz, and K. Sandhoff, *Proc. Natl. Acad. Sci. USA*, 85 (1988) 7044–7048.
[65] H. Iber, C. Zacharias, and K. Sandhoff, *Glycobiology*, 2 (1992) 137–142.
[66] J.-L. Chien and E.L. Hogan, *Biochim. Biophys. Acta*, 620 (1980) 454–461.
[67] S. Dasgupta, J.-L. Chien, and E.L. Hogan, *Biochim. Biophys. Acta*, 1036 (1990) 11–17.
[68] R.D. Mitchell, P. Palade, and S. Fleischer, *J. Cell Biol.*, 96 (1983) 1008–1016.
[69] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, and D.C. Klenk, *Anal. Biochem.*, 150 (1985) 76–85.
[70] C.C. Allain, L.S. Poon, C.S.G. Chan, W. Richmond, and P.C. Fu, *Clin. Chem.*, 20 (1974) 470–475.
[71] S. Hara, Y. Takemori, M. Yamaguchi, M. Nakamura, and Y. Ohkura, *Anal. Biochem.*, 164 (1987) 138–145.
[72] R.W. Ledeen and R.K. Yu, *Methods Enzymol.*, 83 (1982) 139–191.
[73] K. Ueno, S. Ando, and R.K. Yu, *J. Lipid Res.*, 19 (1978) 863–871.
[74] T. Saito and S.-I. Hakomori, *J. Lipid Res.*, 12 (1971) 257–259.
[75] V.P. Skipsky, *Methods Enzymol.*, 35 (1975) 396–425.
[76] L. Svennerholm, *Biochim. Biophys. Acta*, 24 (1957) 604–611.
[77] J.L. Magnani, B. Nilsson, M. Brockhaus, D. Zopf, Z. Steplewski, H. Koprowski, and V. Ginsburg, *J. Biol. Chem.*, 257 (1982) 14365–14369.
[78] J. Müthing and P.F. Mühlradt, *Anal. Biochem.*, 173 (1988) 10–17.
[79] J. Müthing, *TLC in Structure and Recognition Studies of Glycosphingolipids*, in E.F. Hounsell (Ed.), *Glycoanalysis Protocols*, Humana Press, Totowa, NJ, in press.
[80] J.L. Magnani, D.F. Smith, and V. Ginsburg, *Anal. Biochem.*, 109 (1980) 399–402.
[81] A. Pörtner, J. Peter-Katalinić, H. Brade, F. Unland, H. Büntemeyer, and J. Müthing, *Biochemistry*, 32 (1993) 12685–12693.