Membrane assembly of Shiga toxin glycosphingolipid receptors and toxin refractiveness of MDCK II epithelial cells.

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Abstract  Shiga toxins (Stxs) are the major virulence factors of Stx-producing Escherichia coli (STEC), which cause hemorrhagic colitis and severe extraintestinal complications due to injury of renal endothelial cells, resulting in kidney failure. Since kidney epithelial cells are suggested additional targets for Stxs, we analyzed Madin-Darby canine kidney (MDCK) II epithelial cells for presence of Stx-binding glycosphingolipids (GSLs), determined their distribution to detergent-resistant membranes (DRMs), and ascertained the lipid composition of DRM and non-DRM preparations. Globotriaosylceramide and globotetraosylceramide, known as receptors for Stx1a, Stx2a, and Stx2e, and Forssman GSL as a specific receptor for Stx2e, were found to cooccur with SM and cholesterol in DRMs of MDCK II cells, which was shown using TLC overlay assay detection combined with mass spectrometry. The various lipoforms of GSLs were found to mainly harbor ceramide moieties composed of sphingosine (d18:1) and C24:1/C24:0 or C16:0 FA. The cells were highly refractory toward Stx1a, Stx2a, and Stx2e, most likely due to the absence of Stx-binding GSLs in the apical plasma membrane determined by immunofluorescence confocal laser scanning microscopy. The results suggest that the cellular content of Stx receptor GSLs and their biochemical detection in DRM preparations alone are inadequate to predict cellular sensitivity toward Stxs.—Legros, N., G. Pohlentz, D. Steil, I. U. Kouzel, I. Liashkovich, A. Meellmann, H. Karch, and J. Müthing. Membrane assembly of Shiga toxin glycosphingolipid receptors and toxin refractiveness of MDCK II epithelial cells. J. Lipid Res. 2018. 59: 1383–1401.

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Glycosphingolipids (GSLs) belong to the structurally very diverse group of sphingolipids (1–3) and are not just structural elements of cells, but also participate in intracellular and extracellular signaling (4–10). Besides the complex glycan headgroups, the ceramide lipid backbones have selective biochemical functions and involve the dynamic clustering of GSLs in microdomains of the plasma membrane referred to as lipid rafts (6, 11–14). Owing to their peculiar chemico-physical features, sphingolipids participate as a driving force in the formation of microdomains in biological membranes (15), in which cholesterol-rich lipid rafts are islands in the liquid-disordered membrane phase. Cholesterol, SM, and GSLs represent classical markers of lipid rafts, with GSLs as prime players in the development and spatial organization of such microdomains (16). Cholesterol renders lipid rafts relatively resistant to solubilization by nonionic detergents, allowing for the isolation of detergent-resistant membranes (DRMs) from the low-density fraction of a sucrose gradient after ultracentrifugation of detergent-spiked cell lysates (17). DRMs result from a fractionation procedure and exist by definition only in vitro in the presence of detergents. In as such, they provide information on how intrinsic properties of lipids (and proteins) that are found in DRM fractions influence their interaction among each other and with the corresponding

Abbreviations:  AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; CID, collision-induced dissociation; DRM, detergent-resistant membrane; Gb3Cer, globotriaosylceramide; Gb4Cer, globotetraosylceramide; GSL, glycosphingolipid; Le2Cer, lactosylceramide; MDCK II, Madin-Darby canine kidney II; MHC, monohexosylceramide; MS, mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; STEC, Stx-producing Escherichia coli; Stx, Shiga toxin.

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detergents. Novel available methods are single-molecule imaging for unraveling microdomain membrane structures (18–20), which are suitable for direct observation of the nanoscopic dynamics of membrane lipids in a living cell in contrast to a procedure based on detergent resistance (21).

GSLs are attachment sites for a huge number of bacteria and viruses, as well as targets for bacterial toxins (22–26). Plasma membrane segregation into microdomains is well recognized as a functional requirement for binding of pathogens and toxins to gain entry into cells (27–31). Shiga toxins (Stxs) from pathogenic Stx-producing Escherichia coli (STEC) bind to lipid raft-associated GSL receptors on the surface of target cells, followed by endocytosis and retrograde transport to intracellular targets (32–34). Stx is the primary virulence factor of STEC that provokes life-threatening systemic complications and makes this pathogen a public health problem of serious concern (35–38).

Stxs belong to the group of bacterial AB$_5$ toxins made up of a single 30 kDa A-subunit and five identical noncovalently linked 7 kDa B-subunits forming a doughnut-like structure with a central pore (39). The B pentamer binds to GSLs of the globo-series and is therefore dependent on these lipids for cellular uptake (34). Upon internalization, retrograding trafficking to the endoplasmic reticulum and transfer into the cytosol, the cleaved A$_1$ fragment exerts its ribotoxic effect, resulting in inhibition of protein biosynthesis and cell death (32, 33). The cytotoxic action of Stx is based on its N-glycosidase activity that depurinates a specific adenine residue in a conserved loop of the large rRNA. Stx also exhibits depurination activity toward nuclear DNA (40) and acts as a DNA repair inhibitor (41). Furthermore, Stxs are capable of activating multiple cell stress signaling pathways (38, 42). Protection of cells against Stxs can be provided either by inhibiting binding of the toxin to cells or by interfering with any of the subsequent steps required for its toxic effect (43). Analysis of the binding specificities of the Stx subtypes Stx1a, Stx2a, and Stx2e [for revised nomenclature of Stx subtypes, refer to Scheutz et al. 2012 (44)] revealed preferential binding of Stx1a to globohexaosylceramide (Gb3Cer, Galα4Galβ1Cer) and less intensive but clearly detectable interaction with globoheptaosylceramide (Gb4Cer; GalαNacβ3Galα4Galβ4Glcβ1Cer), whereas Stx2a favors Gb3Cer and exhibits only marginal adhesion toward Gb4Cer. In addition to Gb3Cer and priority for Gb4Cer, Stx2e represents the promiscuous Stx subtype that recognizes also two pentahexaosylceramides with Gb4Cer-elargened core structures: the Forssman GSL with GalαNacα3GalαNacβ3Galα4Galβ4Glcβ1Cer structure (45) and globohexaosylceramide (Gb3Cer) with Galβ3GalαNacβ3Galα4Galβ4Glcβ1Cer structure (46).

For the present, microvascular endothelial cells of human kidneys and the brain are considered the preferential direct cellular targets of Stxs (47–51). Stx-mediated injury of glomerular endothelial cells is deemed to be the key event in acute renal impairment, underlying the pathogenesis of hemolytic uremic syndrome that follows gastrointestinal infection and culminates as renal insufficiency and often fatal outcome (52–54). However, evidence has accumulated that Stx may attack, besides endothelial cells, also epithelial cells of the kidney. This has been shown for Stx-sensitive primary human renal epithelial cells (55–57) and various human renal epithelium-derived cell lines (56, 58–60). Direct effects of Stx2 on renal tubular epithelium and contribution to acute renal failure have been shown in mice, because they, like humans, do express the Stx receptor Gb3Cer in the tubular epithelium (60). Stxs are synonymously named as “verotoxins” or “verocytotoxins” (61, 62), owing to their cytotoxic capacity toward Vero cells derived from the kidney of an African green monkey. Vero cells are routinely used kidney epithelial cells to measure the cytotoxicity of Stx samples (63). Stx GSL receptors of Vero-B4 cells and their membrane microdomain lipid environment as well as Stx-mediated damage have been recently particularized (46). Similar to ubiquitously employed renal Vero cells, Madin-Darby canine kidney (MDCK) cells are widely used for studying biological issues of epithelial cells. Out of the MDCK variety pack, MDCK II cells are the most commonly used MDCK strain and are recommended for most studies (64). Previous reports have provided some data on the phospholipid and GSL composition of MDCK II cells, mostly based on TLC analysis (65–73). However, the exact structures of GSLs and phospholipids, their localization in membrane microdomains, especially of supposed Stx-binding GSLs, and susceptibility of MDCK II cells toward Stx1a, Stx2a, and Stx2e subtypes have not been investigated in detail so far.

Here, we report on the identification and structural characterization of globo-series Stx receptor GSLs of MDCK II cells for the three clinically important Stx1a, Stx2a, and Stx2e subtypes and their distribution to and lipid environment in DRMs, and we probed the three Stx subtypes for their cytotoxic effects toward MDCK II cells in cell culture assays. This study might be helpful to further our understanding of the functional role of Stx-binding GSLs in kidney epithelial cells.

MATERIALS AND METHODS

Cultivation of MDCK II cells

MDCK II cells were obtained from the European Collection of Animal Cell Cultures (Salisbury SP4 0JG, UK; catalog no. 00062107). The MDCK II cell line has been isolated from a high-passage parental cell line (NB-2) and represents the most commonly used strain of MDCK cell lines (64). MDCK II cells were cultivated in cell culture medium composed of MEM (PAA Laboratories GmbH, Pasching, Austria) supplemented with 2 mM L-glutamine and 10% FBS (PAA Laboratories GmbH) at 37°C in a humidified 5% CO$_2$/95% air atmosphere. The cells were grown in epithelial monolayers and were routinely passaged every 2–3 days using 0.25% Trypsin-EDTA (Lonza, Verviers, Belgium) before reaching the confluent state. Appropriate cell amounts for the isolation of lipids were produced in 175 cm$^2$ tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) as previously described for colon epithelial cell lines (74). The Vero-B4 kidney cell line was purchased from the Leibniz Institute DSMZ–German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany; DSMZ no. ACC 33). Cells were propagated and passaged...
in OptiPRO™ + 4 mM l-glutamine (Fisher Scientific GmbH, Schwerte, Germany). Stx-sensitive Vero-B4 cells served as positive control in cytotoxicity assays.

**Immunofluorescence microscopy**

MDCK II and Vero-B4 cells were seeded with 1 × 10⁵ cells/ml in 8-well polystyrene chamber slides (Thermo Fisher, Rochester, NY; catalog no. 177445) and propagated for 24 h until ~80% confluence. The medium was aspirated, and the cells were washed with PBS (Lonza), followed by fixation with 3.7% paraformaldehyde (Merck, Darmstadt, Germany) for 30 min. The fixed cells were washed with PBS and quenched with 0.2 M glycine, pH 7.2 (Roth, Karlsruhe, Germany) for 15 min. After washing with PBS, the cells were blocked with 5% (wt/vol) BSA (Serva Electrophoresis, Heidelberg, Germany) and unrelated goat anti-human transferrin polyclonal antibody in a concentration of 20 µg/ml (AHP858, Bio-Rad, Kidlington, UK) in PBS overnight at 4°C. After washing with PBS, the slides were incubated with Stx1a, Stx2a, and Stx2e for 1 h at a concentration of 0.5 µg/ml each. After one more washing step with PBS, the Stx1a-exposed slides were incubated with monoclonal mouse IgG anti-Stx1 antibody (clone VT109/4-E9b) and those exposed to Stx2a and Stx2e with monoclonal mouse IgG anti-Stx2 antibody (clone VT135/6-B9, both from Sifin GmbH, Berlin, Germany) in 1:500 dilution with 1% BSA at 4°C overnight. The slides were then washed three times with PBS and incubated for 1 h under light protection with BSA at 4°C overnight. The images were processed with Adobe Photoshop software (Adobe Systems Inc.).

**Cell cytotoxicity assay**

Cell viability was determined using the crystal violet assay as previously described (46, 74). In short, MDCK II cells were grown to confluence in tissue culture flasks (Greiner Bio-One), trypsinized, and seeded in 100 µl volumes in 96-well tissue culture plates (Corning Inc., Corning, NY) (initial cell seeding density of 2 × 10⁵ cells/well). One hundred microliters of purified Stx subtype solutions was applied in increasing toxin concentrations ranging from 10⁻⁶ ng/ml (1 fg/ml) up to 10⁵ ng/ml (1 µg/ml) diluted in cell culture medium to cell culture plate wells each in a total volume of 200 µl. Cells were exposed for 1 h to Stx at 37°C in a humidified atmosphere of 5% CO₂/95% air followed by incubation in 200 µl of cell culture medium without toxin for 48 h. Pure cell culture medium without toxin served as a control. The cultivation was completed by aspiration of the cell culture supernatant. Remaining adherent cells were fixed with formalin and stained with crystal violet, and cell survival was quantified photometrically as previously described (46, 74). Results represent the means ± SDs of 6-4 fold determinations and are depicted as percentage values of two untreated controls.

**Purification of neutral GSLs from MDCK II cells**

The isolation of neutral GSLs from lipid extracts of total cells was performed following established protocols that were previously published (46, 75, 76). Concisely, lipids were extracted from four independently produced biological replicates of MDCK II cells, each obtained from eight large (175 cm²) tissue culture flasks (Greiner Bio-One) of cells in the confluent state. Methanol was used as the first extraction solvent, followed by chloroform/methanol (1/2, vol/vol), chloroform/methanol (1/1, vol/vol), and chloroform/methanol (2/1, vol/vol) mixtures. The pooled extracts were evaporated under rotation, and coextracted phospholipids and triglycerides were saponified by mild alkaline treatment (46, 75, 76). Neutral GSLs were separated from negatively charged GSLs by anion-exchange column chromatography using a DEAE-Sepharose CL-6B column (GE Healthcare, Munich, Germany) according to standard procedures (77, 78).

**Reference GSLs, phospholipids, and cholesterol**

A preparation of reference-neutral GSLs isolated from human erythrocytes (R1) containing Gb3Cer (Galα4Galβ3Galβ1Cer) and Gb4Cer (GalNAcβ3Galα4Galβ3Galβ1Cer) (79, 80) was used as positive controls for antibody and Stx TLC overlay assays. A second neutral GSL reference mixture was prepared from mutton blood, which contained besides Gb3Cer and Gb4Cer the Forssman GSL (45). The nomenclature of the GSLs follows the IUPAC-IUB recommendations, 1997 (81). A phospholipid reference preparation (R3) comprising SM, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidylserine (PS) was used as described in several previous publications (46, 74, 75, 82). Cholesterol was purchased from Sigma-Aldrich (catalog no. C8667) and used as standard for quantification in DRM and non-DRM fractions. Purified GlcCer from human Gaucher’s spleen was purchased from Sigma-Aldrich (catalog no. G-9884), and a GalCer fraction was prepared from human brain according to standard procedures (77, 78, 83).

**Stx subtypes and antibodies**

Supernatants from bacterial cell cultures of *Escherichia coli* O145:H− (strain 2074/97, Stx1a), *E. coli* O111:H− (strain 03-06016, Stx2a) and from *E. coli* ONT:H− (strain 2777/91, Stx2e) were used for TLC overlay assay detection of Stx-binding GSLs (45) and for purification of Stx subtypes, which has been previously described for Stx2 from *E. coli* strain C600(933W) (84). Murine monoclonal IgG antibodies against Stx1 (clone VT 109/4-E9b, 3.9 mg/ml) and Stx2 (clone VT 135/6-B9, 2.75 mg/ml) were obtained from Sifin GmbH (Berlin, Germany). Polyclonal chicken IgY anti-lactosylceramide (anti-Le₂Cer), anti-Gb3Cer, and anti-Gb4Cer antibodies with previously described specificities (85–88) were used for TLC overlay assays. Monoclonal rat IgM (clone IIC2) anti-Forssmann GSL antibody was produced as previously described by Bethke et al. (89, 90). Secondary alkaline phosphatase (AP)-conjugated affinity-purified polyclonal rabbit anti-chicken IgY (code 303-055-033), goat anti-mouse IgG (code 115-055-003), and goat anti-rat IgG + IgM (code 112-055-044) antibodies were from Dianova.

**High-performance TLC and staining of lipids**

All lipid samples were applied onto silica gel 60 precoated glass plates (HPTLC plates, size 10 × 10 cm, thickness 0.2 mm; catalog no. 05633.0001, Merck) using an automatic sample applicator (Linomat 5, CAMAG, Muttenz, Switzerland). Neutral GSLs were chromatographed in chloroform/methanol/water (120/70/17, each by volume) (solvent 1) and stained with orcinol (91). The monohecosylceramides (MHCs) GlcCer and GalCer were separated as borate complexes in alkaline solvent 2 composed of chloroform/methanol/water/32% NH₄OH (65/25/4/0.5, each by volume) (87, 92). For this purpose, the plate was loaded with the
sample and exhaustively sprayed with 1.5% (wt/vol) aqueous Na₂B₄O₇ solution before chromatography, which was performed after careful drying. Phospholipids were separated in solvent 3 consisting of chloroform/methanol/isopropanol/triethylamine/0.25% aqueous KCl (30/9/25/18/6, each by volume) and stained with molybdenum blue Dittmer-Lester reagent (93, 94). Cholesterol was stained with manganese(II) chloride (75, 95) after TLC separation in solvent 4 comprising chloroform/acetone (96/4, vol/vol).

TLC overlay assay detection of GSLs

TLC overlay assays using polyclonal chicken anti-Lc2Cer, anti-Gb3Cer, and anti-Gb4Cer antibodies, the monoclonal rat IgM anti-Forssman GSL antibody and bacterial supernatants containing Stx1a, Stx2a, and Stx2e subtypes were done as previously described (45, 75, 82, 88, 96). In short, after GSL separation, the silica gel layer requires fixation with polyisobutylmethacrylate (Plexigum P28, Röhm, Darmstadt, Germany) to prevent detachment from the glass plate. Polyclonal primary chicken anti-GSL antibodies were used at 1:2,000 dilutions and the supernatant from the anti-Forssman GSL producing hybridoma as 1:20 diluted solution in 1% (wt/vol) BSA in PBS. The Stx1a-, Stx2a-, and Stx2e-containing sterile-filtered bacterial supernatants were used undiluted; the anti-Stx1 and anti-Stx2 antibodies were applied in 1:1,000 dilution, and the secondary AP-conjugated antibodies were used at 1:2,000 dilutions (all in 1% BSA in PBS) as previously described (45, 76, 82, 88, 96, 97). Bound secondary antibodies were visualized with 0.05% (wt/vol) 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP; Roth) in glycine solution (pH 10.4), which generates a blue precipitate at sites of antibody binding on the TLC plate (91).

Densitometric quantification of stained phospholipids, cholesterol, and GSLs and immunodetected GSLs

The relative amounts of TLC-separated orcinol-stained and immunostained GSLs as well as molybdenum blue-colored and manganese(II) chloride-colored phospholipid and cholesterol bands, respectively, were determined by densitometry using a CD 60 scanner (Desaga, Heidelberg, Germany; software ProQuant®, version 1.06.0000). The scanner operated in reflectance mode at a wavelength of 544 nm (orcinol), 630 nm (BCIP), 700 nm (molybdenum blue), or 365 nm (cholesterol) with light beam slit dimensions of 0.02 × 4 mm.

Preparation of sucrose density gradient DRM and non-DRM fractions

Classical DRM and non-DRM fractions were prepared from sucrose density gradients according to the commonly used procedure described by Brown and Rose (67) with minor modifications as previously published (46, 74, 75, 82). Briefly, confluent grown MDCK II cells were dispersed in lysis buffer and the cell debris was removed by mild centrifugation (400 g). Next, membranes were separated from the supernatant by short ultracentrifugation (150,000 g). The membrane sediment was then solubilized in 1% Triton X-100 buffer and mixed in an equal ratio with 85% sucrose. The produced 42.5% sucrose solution was then overlayed on a discontinuous sucrose gradient of 30% and 5% sucrose and submitted to ultracentrifugation (200,000 g). From top to bottom of the gradient eight fractions each of 1.5 ml volumes were collected: upper three DRM-associated fractions (F1–F3) and lower five non-DRM fractions (F4–F8). The non-DRM fractions were further subgrouped into intermediate (F4–F6) and bottom fractions (F7–F8).

Isolation of phospholipids, cholesterol, and GSLs from sucrose density gradient fractions

Gradient fractions F1–F8 were dialyzed against deionized water at 4°C for 3 days to remove sucrose. A volume of 0.5 ml was taken from each fraction, freeze-dried, and resolved in chloroform/methanol (2/1, vol/vol) for TLC analysis of phospholipids. For GSL and cholesterol analysis, 0.5 ml freeze-dried aliquots of the gradient fractions were taken up in 0.5 ml of 1 N NaOH and kept for 1 h at 37°C for alkaline hydrolysis of phospholipids and triglycerides. Afterwards the samples were neutralized with HCl and desalted by dialysis (46, 74, 75, 82). After hyphosphilation, the extracts were adjusted to a concentration equivalent to 1 × 10⁷ cells/μl in chloroform/methanol (2/1, vol/vol).

ESI MS

MS¹ and MS² analyses of GSLs and phospholipids were performed on a SYNAPT G2-S mass spectrometer (Waters, Manchester, UK) equipped with a Z-spray source. The source settings were: temperature 80°C, capillary voltage 0.8 kV, sampling cone voltage 20 V, and offset voltage 50 V. Low energy collision-induced dissociation (CID) was used for GSL and phospholipid MS² analysis. To this end, the lipid precursor ions were selected in the quadrupole analyzer and ion mobility separation was employed (wave velocity 700–800 m/s, wave height 40 V, nitrogen gas flow rate 90 ml/min, helium gas flow rate 180 ml/min). Fragmentation was subsequently performed in the transfer cell using a collision gas (Ar) flow rate of 2.0 ml/min and collision energies up to 100 eV (Ekin). Individual GSLs and phospholipids were detected as singly charged monosodiated [M+Na]⁺ and/or protonated [M+H]⁺ species and structures were derived from CID spectra. Purified neutral GSLs from MDCKII cells, extracts from TLC-separated GalCer and GlcCer and immunostained Forssman GSL as well as lipid extracts from density gradient fractions F2 and F7 were dried under a stream of nitrogen, dissolved in methanol, and analyzed in the positive ion sensitivity mode.

Statistics

Nonparametric statistical analysis was performed in the R (Version 3.4.0) computing environment (98). The strength of association between phospholipids, cholesterol, and GSLs in DRM and non-DRM fractions obtained from MDCK II cells was determined using Spearman’s rank correlation coefficient r. Ranks were assigned to densitometrically determined values of the individual lipids, whereby rank 1 corresponds to the highest, rank 2 to the second highest, etc., and rank 8 to the lowest measured value. All tests were two-tailed, and the σr values were calculated with R software. σr values were adjusted for multiple comparisons using Bonferroni correction, and σr values were considered significant at adjusted P < 0.01.

RESULTS

Neutral GSLs at first glance

The initial analysis of isolated neutral GSLs from MDCK II cells using TLC followed by sugar staining with orcinol revealed a heterogeneous pattern comprising minor quantities of monohexosylceramides, dihexosylceramides, trihexosylceramides, and tetrahexosylceramides and a prevalent pentahexosylceramide (Fig. 1). From what we know of previous publications, the MHC fraction of MDCK cells should contain glucosylceramide (GlcCer) and galactosylceramide (GalCer) with variable relative content.
Relative and semiquantification of orcinol-positive bands was done by densitometric scanning as shown by the corresponding scan. Values of a representative biological replicate are given as percent of total neutral GSLs of MDCK II cells indicating major expression of proposed Forssman GSL.

### Fig. 1. Orcinol-stained thin-layer chromatogram of neutral GSLs from MDCK II cells.

Neutral GSLs corresponding to 1 x 10⁶ cells were cochromatographed with 20 µg of neutral GSLs from human erythrocytes (reference R1) and 20 µg of neutral GSLs from mutton blood (reference R2). Neutral GSLs were separated in solvent 1, and semiquantification of orcinol-positive bands was done by densitometric scanning as shown by the corresponding scan. Relative values of a representative biological replicate are given as percentages of total neutral GSLs of MDCK II cells indicating major expression of proposed Forssman GSL.

| GSL          | R1 % | R2 % | MDCK II % |
|--------------|------|------|-----------|
| MHC          |      | 20.6 |           |
| Lc2Cer       | 18.8 |      |            |
| Gb3Cer       | 10.6 |      |            |
| Gb4Cer       | 8.1  |      |            |
| Forssman     | 41.9 |      |            |

(65, 67–69), although an in-depth mass spectrometric investigation has not been performed so far. TLC separation of the dihexosylceramide suggests lactosylceramide (Lc2Cer) as the most likely structure. The trihexosylceramides and tetrahexosylceramides are expected to represent globo-series Gb3Cer and Gb4Cer, respectively, and the pentaehexosylceramide should correspond to Forssman GSL as the dominant GSL species of MDCK II cells, according to previous predictions (65–67, 69, 70, 73), although detailed structural data are lacking for the pentaehexosylceramide of MDCK II cells. The proposed globo-series GSLs of MDCK II cells cochromatograph with Gb3Cer and Gb4Cer of reference R1 (prepared from human erythrocytes) and reference R2 (prepared from mutton blood). The latter harbors Forssman GSL as the prevalent GSL with Gal\(\beta\)3Gal\(\beta\)3Gal\(\beta\)4Glc\(\beta\)1Cer pentaehexosylceramide structure (not to be confused with Gb5Cer with Gal\(\beta\)3Gal\(\alpha\)β3Gal\(\alpha\)4Gal\(\beta\)4Glc\(\beta\)1Cer structure), in addition to Gb4Cer, Gb3Cer, and Lc2Cer. As shown for a representative scan of MDCK II GSLs in Fig. 1, the MHC amount of hydroxylated GalCer (d18:1, 24:1-OH/24:0-OH) as the major GalCer species over the nonhydroxylated GalCer (d18:1, C24:1/C24:0) pendant (Fig. 2C and supplemental Table S1). MS analysis of the silica gel extract harboring the GalCer variants resulted in the identification of hydroxylated GalCer (d18:1, 24:1-OH/24:0-OH) as the major GalCer species over the nonhydroxylated GalCer (d18:1, C24:1/C24:0) pendant (Fig. 2C and supplemental Table S1). The situation was found to be inverse in the case of GalCer with short-chain FA showing highest abundance of GalCer (d18:1, C16:0) in comparison to minor GalCer (d18:1, C16:0-OH). The proposed structures of GlcCer and GalCer including observed ceramide heterogeneity and especially hydroxylation of respective FA were scrutinized by CID measurements as previously described for GlcCer and GalCer from human blood plasma (87) and are not further outlined here (data not shown). Collectively, GlcCer and GalCer differed in terms of the high degree of ceramide hydroxylation of GalCer, whereas GlcCer was not hydroxylated at all.

### Neutral GSLs of MDCK II cells as receptors for Stx1a, Stx2a, and Stx2e

The preliminarily identified dihexosylceramide and globo-series neutral GSLs were confirmed by TLC immunostaining as shown in Fig. 3A and probed for their binding potential toward Stx subtypes Stx1a, Stx2a, and Stx2e as displayed in Fig. 3B. Polyclonal anti-Lc2Cer, anti-Gb3Cer, and anti-Gb4Cer antibodies bound to supposed Lc2Cer, Gb3Cer, and Gb4Cer, respectively, and the pentaehexosylceramide was recognized by a monoclonal anti-Forssman GSL antibody (Fig. 3A). The GSLs appeared as double bands showing some tendency to split into triple bands due to separation of respective upper band in two distinguishable bands, clearly visible in Fig. 3A for Gb3Cer and the Forssman GSL. In either overlay binding assays of Gb3Cer and Gb4Cer an additional immunopositive band appeared below at position of a putative pentaehexosylceramide and a hexaehexosylceramide, which might represent disaccharide-elongated GSLs with Gb3Cer and Gb4Cer core structures (not further analyzed). The Stx1a and Stx2a subtypes both primarily recognized Gb3Cer. In addition, Stx1a exhibited clearly visible adhesion to Gb4Cer and a not-further-characterized dihexosylceramide separating at the...
position of Le2Cer (Fig. 3B). Only trace positive bands were found for Stx2a regarding Gb4Cer and the putative dihexosylceramide. Importantly, Stx1a and Stx2a did not bind to Forssman GSL. This is in striking contrast to Stx2e subtype that bound to Forssman GSL, whereby Stx2e exhibited stronger interaction with Gb1Cer than with Gb3Cer (Fig. 3B). This promiscuous binding with distinct preference for Gb4Cer and singular binding toward Forssman GSL makes Stx2e unique among the various subtypes so far investigated, as previously reported (45). Detected heterogeneity of immunostained GSLs was proved by ESI MS, as shown below.

Structures of Le2Cer, Gb3Cer, Gb4Cer, and Forssman GSL from MDCK II cells

Individual GSL species were structurally characterized by ESI MS in the positive ion mode as monosodiated [M+Na]+ ions combined with CID.

Le2Cer. The collection of anti-Le2Cer immunopositive dihexosylceramide species was found to encompass Le2Cer variants with Cer (d18:1, C16:0) and Cer (d18:1, C24:1/C24:0) as the prevalent dihexosylceramide structures, with accompanying less abundant Le2Cer (d18:1, C24:1-OH/C24:0-OH) as the only hydroxylated Le2Cer species (Fig. 3A). In the order of decreasing signal intensities, we could identify further Le2Cer molecules with ceramides containing sphingosine (d18:1) substituted with C22:0, C20:0, C26:1/C26:0, and C18:0 FA (for synopsis of structures, refer to supplemental Table S1).

Gb3Cer. The overview mass spectrum shown in Fig. 4B gives evidence for Gb3Cer structures with Cer (d18:1, C16:0) and Cer (d18:1, C24:1/C24:0) as the principal lipiforms, the latter Gb3Cer variant with attendant but less abundant hydroxylated Cer (d18:1, C24:1-OH/C24:0-OH). Minor Gb3Cer species were those with Cer (d18:1, C22:0), Cer (d18:1, C26:1/C26:0), Cer (d18:1, C20:0), and Cer (d18:1, C18:0) as lipid anchors, in the order from higher to lower signal intensities (for synopsis of structures refer to supplemental Table S2). As an example for CID verification of proposed structures an MS2 spectrum of Gb3Cer (d18:1, C24:1/C24:0) together with the auxiliary fragmentation scheme is provided in supplemental Fig. S1A.

Gb4Cer. A very similar pattern of [M+Na]+ ions regarding lipid heterogeneity was obtained for Gb4Cer (Fig. 4C) when compared with the spectrum of Gb3Cer. Again, Cer (d18:1, C24:1/C24:0) [accompanied with low amounts of hydroxylated Gb4Cer (d18:1, C24:1-OH/C24:0-OH)] and Cer (d18:1, C16:0) were the prevalent lipid cores, followed by the presence of less abundant Gb4Cer with Cer (d18:1, C22:0) and Cer (d18:1, C26:1/C26:0) and minor Gb4Cer

extracts from an unstained parallel chromatogram using ESI MS in the positive ion mode. The dot (●) in the MS1 spectrum of GalCer (C) marks an ion, which could be attributed to a coeluted impurity from the silica gel of the TLC plate. MS data of GlcCer and GalCer are summarized in supplemental Table S1.
variants with Cer (d18:1, C20:0) and Cer (d18:1, C18:0) as lipid moieties (for synopsis of structures, refer to supplemental Table S2). As an example for CID verification of proposed structures, an MS$^2$ spectrum of Gb4Cer (d18:1, C22:0) together with the corresponding fragmentation scheme is provided in supplemental Fig. S1B.

Forssman GSL. Interestingly, GalNAcα3-elongated Gb4Cer, defined as the Forssman GSL, exhibited the same ceramide variability (Fig. 4D) as figured out for precursor Gb3Cer and Gb4Cer. Maximum ion abundancies were observed for Forssman GSLs with Cer (d18:1, C24:1/C24:0) and Cer (d18:1, C16:0), whereby the former ions were flanked with the corresponding hydroxylated Forssman GSLs with Cer (d18:1, C24:1-OH/C24:0-OH) ions. Further Forssman GSLs were minor variants with C22:0, C26:1/C26:0, C20:0, and C18:0 FAs in the respective ceramide portions as detected for Gb3Cer and Gb4Cer (for synopsis of structures refer to supplemental Table S2). As an example for CID verification of proposed structures MS$^2$ spectra of Forssman GSL with Cer (d18:1, C24:1/C24:0) and Cer (d18:1, C16:0) are shown, together with explanatory fragmentation schemes, in Fig. 5A and Fig. 5B, respectively.

Identification of cholesterol and phospholipids in DRM and non-DRM fractions

Supposed nonhomogenous lateral distribution of membrane lipids was investigated using DRM and non-DRMs prepared from sucrose density gradient fractions after ultracentrifugation. This principle allows us to roughly differentiate between components of the liquid-ordered phase corresponding to DRM fractions and the more fluid liquid-disordered phase (surrounding medium in which microdomains can freely float), corresponding to the non-DRM fractions. Gradient fractions from four independent cell approaches of MDCK II cells were prepared, and the occurrence of the key membrane lipids cholesterol, SM, and PC in sucrose gradient fractions F1–F8 (from top to bottom) was investigated and is portrayed for the average values in Fig. 6.

Cholesterol. Cholesterol was quantified as nanograms per 1 × 10$^6$ cells in the DRM fractions (F1–F3) and the non-DRM fractions (F4–F8), which were further subdivided into intermediate fractions (F4–F6) and bottom fractions (F7–F8), as demonstrated for four independent biological replicates in Fig. 6A. DRM fraction F2 exhibited on average, with 599 ng/10$^6$ cells, the highest cholesterol content, flanked by sizeable 200 ng/10$^6$ cells and minor 26 ng/10$^6$ cells in DRM-associated fraction F3 and F1, respectively. Notable quantities of cholesterol were found in bottom fractions F7 (308 ng/10$^6$ cells) and F8 (274 ng/10$^6$ cells), whereas intermediate fractions F4–F6 contained cholesterol amounts in the range between 22 and 55 ng/10$^6$ cells. Collectively, 53% of cholesterol was found in DRMs, preferentially in canonical DRM fraction F2 (38%), giving at least a hint for putative lipid raft association, and 47% distributed to the five non-DRM fractions (for synopsis of values, refer to supplemental Table S3).

SM. A highly significant enrichment in DRMs was detected for SM, which showed an exceptional high relative content of 69% in classical DRM fraction F2, accompanied by 18% and 6% in DRM-related fractions F3 and F1, respectively, amounting in total to 93% (Fig. 6B). In contrast, only marginal amounts of SM were detected in non-DRM fractions (for list of values, refer to supplemental Table S3). Because SM is known as a truly reliable lipid raft marker and according to its peak content in DRM fraction

![Fig. 3. TLC overlay assay detection of neutral GSLs from MDCK II cells with antibodies (A) and identification of Stx GSL receptors using Stx1a, Stx2a, and Stx2e subtypes (B). A: GSL amounts applied for Gb3Cer and Gb4Cer detection correspond to 1 × 10$^5$ cells, and those for Lc2Cer and Forssman GSL detection are equivalent to 1 × 10$^6$ and 5 × 10$^6$ cells, respectively. B: GSL amounts employed for the Stx1a, Stx2a, and Stx2e TLC overlay assays correspond to 1 × 10$^6$ cells, respectively. GSLs were separated in solvent 1 for neutral GSLs (A, B) and bound primary anti-GSL antibodies as well as anti-Stx1 or anti-Stx2 antibodies (B) were visualized with AP-conjugated secondary antibodies and BCIP as the substrate.](image-url)
F2, it is tempting to speculate about the association of SM with lipid rafts.

**PC.** PC shows a balanced appearance in DRM fraction F2 (35% relative amount) and bottom fraction F7 (31% relative amount) along a scattered distribution to intermediate F4–F6, bottom F8, and the F2 adjacent fractions F1 and F3 (Fig. 6C). Although relevant concentrations were detectable in DRM fraction F2, its similar distribution pattern to fractions corresponding to the liquid-ordered and the liquid-disordered membrane phase may exclude PC as a marker for cellular microdomains of MDCK II cells.

**Structural fine characterization of DRM and non-DRM phospholipids**

The phospholipids of canonical DRM fraction F2 and fraction F7 as a typical non-DRM fraction from the bottom zone of the sucrose gradient were subjected to a detailed compositional analysis by ESI MS. TLC separation of phospholipids revealed PC and SM as major compounds of fraction F2, whereby the latter is marked with twin arrowheads (due to its appearance as double band), indicating SM as a DRM-specific phospholipid (Fig. 7A). Truncated (single-tailed) lyso-PC, which separates far below PC and slightly below SM, represents a characteristic compound of the bottom fractions and is marked with an arrowhead in the non-DRM fraction F7 indicating lyso-PC as a non-DRM-specific phospholipid (Fig. 7A).

Individual SM and PC molecules appeared exclusively as protonated $[M+H]^+$ ions in the MS 1 spectrum of the F2 fraction (Fig. 7B). Precisely, $[M+H]^+$ ions at m/z 703.57 and 813.68/815.69, highlighted in gray, represent SM (d18:1, C16:0) and SM (d18:1, C24:1/C24:0), respectively. Ions of PC molecules at m/z 760.58 could be assigned to lipoform PC (34:1), which dominated the spectrum, followed by PC (36:2/36:1) at m/z 786.59/788.61 and PC (32:1) with m/z 732.55 in the order of detected intensities. PE was found to be a less abundant phospholipid species, with PE (36:1) and PE (38:2/38:1) lipoforms at m/z 746.58 and 772.61/774.61, respectively.

The most obvious difference for the F7 bottom fraction was the presence of lyso-PC (highlighted by grayed boxes in the spectrum) and complete lack of SM ions in this non-DRM fraction (Fig. 7C). Highly intense $[M+H]^+$ ions at m/z 522.35, accompanied by minor sodiated $[M+Na]^+$ ions with m/z 544.33, could be assigned to lyso-PC (18:1). The second detected lysophospholipid was lyso-PC (16:0) with a characteristic m/z value of 496.33, which was also undetectable in the DRM F2 fraction. Thus, the two lyso-PC variants could be identified as definite non-DRM markers, which were solely present in the liquid-disordered, but not in the liquid-ordered, phase of cell membranes of MDCK II cells.

An additional difference observed in fraction F7, when compared with F2, was the increase in relative intensity of selective GSL species. MS1 data of Lc2Cer are summarized in supplemental Table S1. MS1 data of Gb3Cer, Gb4Cer, and Forssman GSL are compiled in supplemental Table S2.
Stx GSL receptors and toxin refractiveness of MDCK II cells

PC (36:2) [accompanied by loss of PC (36:1)] and concomitant relative decrease of PC (34:1), indicating opposing presence of these two phospholipids in the DRM and the non-DRM fraction and increase of saturation in PC species in the DRM fraction over the non-DRM fraction.

Identification of Stx GSL receptors in DRM and non-DRM fractions

Although being membrane compounds with rather low abundance among cholesterol and phospholipids in the outward-facing part of the bilayer of the plasma membrane, GSLs can be unequivocally determined in DRM and non-DRM fractions, as shown in Fig. 8.

Gb3Cer. Antibody-mediated detection of the Stx receptor GSL Gb3Cer in gradient fractions exhibited clear DRM preference as exemplarily shown for one out of four biological replicates (Fig. 8A). The averaged values of relative quantities of Gb3Cer obtained from four biological replicates are displayed in a bar chart (Fig. 9A) and are summarized in supplemental Table S4. On an average, 86% of Gb3Cer distribute to grouped DRM fractions (F1–F3), 6% to intermediate fractions (F4–F6), and 8% to the bottom fractions (F7 and F8).

Gb4Cer. An example out of four biological replicates of TLC immunostains of Gb4Cer in gradient fractions is shown in Fig. 8B, giving evidence for preferred presence of Gb4Cer in DRM fractions. The calculation of the average Gb4Cer distribution revealed 79% of Gb4Cer in DRM fractions F1–F3, 7% in intermediate fractions F4–F6, and 14% in the bottom fractions F7 and F8, indicating its preponderant occurrence in DRMs (supplemental Table S4).

Forssman GSL. The antibody-mediated Forssman GSL detection in an exemplarily shown gradient distribution pattern demonstrates strict preference of this GSL to DRM fractions (Fig. 8C). Based on the calculation of the average Forssman GSL distribution revealed 79% of Forssman GSL in DRM fractions F1–F3, 7% in intermediate fractions F4–F6, and 14% in the bottom fractions F7 and F8, indicating its preponderant occurrence in DRMs (supplemental Table S4).

Statistics of DRM and non-DRM distribution of membrane lipids

The distribution of cholesterol, SM, PC, and GSLs to DRMs and non-DRMs was assessed more precisely by non-parametric statistical analysis using Spearman’s rank correlation coefficient \( r_S \). To find out relationships between the analyzed lipids, we assigned ranks to percentage values of the gradient fractions of cholesterol, SM, and PC (see supplemental Table S3) and of the GSLs Gb3Cer, Gb4Cer, and Forssman GSL (see supplemental Table S4), starting with rank number 1 for the highest value, followed by rank number 2 of the second highest value until rank number 8 that corresponds to the lowest value. By doing so, Gb3Cer, Gb4Cer, and Forssman GSL were found to correlate with the canonical lipid raft marker SM, as expected from their distribution profiles obtained from the sucrose gradient.

Fig. 5. MS\(^2\) spectra and corresponding fragmentation schemes of antibody-detected Forssman GSL (d18:1, C24:1/C24:0) (A) and Forssman GSL (d18:1, C16:0) (B) from MDCK II cells. The singly charged [M+Na]\(^+\) precursor ions at \( m/z \) 1562.95/1564.93 of the Cer (d18:1, C24:1/C24:0) lipiforms (A) and those at \( m/z \) 1452.82 of the Cer (d18:1/C16:0) lipiform of the Forssman GSL (B) were selected from an MS\(^1\) spectrum of the neutral GSL fraction of MDCK II cells, which contains immunopositive Forssman GSL (see Fig. 3A). The positions of Forssman GSL species with C24:1/C24:0 FA (A) and C16:0 FA (B) are marked with arrowheads in the respective inserted TLC immunostain. Internal glycan fragments were assigned with numbers from 1 to 5: 1, \( Y_2/B_3 \) and \( Y_5/B_5 \) (m/z 347.08 for the C24 species and m/z 347.09 for the C16 species); 2, \( Y_2/C_5 \) and \( Y_3/C_4 \) (m/z 365.10 for both species); 3, \( Y_5/B_5 \) (m/z 388.11 for both species); 4, \( Y_5/B_5 \) (m/z 509.14 for both species); and 5, \( Y_5/C_5 \) (m/z 527.15 for both species).
fractions for SM (Fig. 6B) and the three GSLs (Fig. 9). The calculated rank correlation coefficients of $r_S = 0.755\ (P = 6.985 \times 10^{-6})$ for Gb3Cer, $r_S = 0.609\ (P=2.564 \times 10^{-3})$ for Gb4Cer, and $r_S = 0.724\ (P = 3.463 \times 10^{-5})$ for Forssman GSL versus SM suggests preferential colocalization of GSLs with SM tested at the 1% significance level. Similar but less pronounced, correlation coefficients of $r_S = 0.671\ (P = 3.118 \times 10^{-4})$ for Gb3Cer and $r_S = 0.644\ (P = 8.392 \times 10^{-4})$ for Forssman GSL versus cholesterol gave evidence for relationship between the membrane distribution of these two GSLs and cholesterol at the 1% significance level. Spearman $r_S = 0.714\ (P = 5.462 \times 10^{-5})$ for Gb4Cer and cholesterol was nearly the same as in comparison to SM. These data are not surprising from the distribution patterns of cholesterol (Fig. 6A) and GSLs (Fig. 9), which exhibited obvious relation in terms of high content of cholesterol and GSLs in the DRM fractions. On the other hand, a correlation coefficient of $r_S = 0.321\ (P = 8.881 \times 10^{-1})$ for cholesterol and SM and of $r_S = 0.397\ (P = 2.953 \times 10^{-1})$ for PC and SM exclude a correlation between these lipids. Thus, based on the cooccurrence of GSLs with SM and cholesterol in DRMs a lipid raft association can be statistically hypothesized for the Stx-binding GSLs Gb3Cer, Gb4Cer, and Forssman GSL based on calculated correlation coefficients.

Cytotoxicity of Stx1a, Stx2a, and Stx2e toward MDCK II cells

MDCK II epithelial cells were exposed to increasing concentrations of Stxs from 1 fg/ml (10$^{-6}$ ng/ml) up to 1 µg/ml (10$^3$ ng/ml) and compared concerning Stx-mediated cytotoxicity with Vero-B4 cells known as the Stx-sensitive epithelial cell line. MDCK II cells did not show any response upon exposure to the three tested Stx subtypes as shown in Fig. 10. No viability decrease could be determined after Stx1a treatment. By contrast, Stx1a-caused reduction in viability of Vero-B4 cells started at a toxin concentration of 0.1 ng/ml and dropped further down to 20% cell viability after treatment with 1 µg/ml, the highest toxin concentration applied (Fig. 10A). Stx2a did not exert any reduction in survival of MDCK II cells, but caused a significant decrease in viability toward Vero-B4 cells in the range between 10 ng/ml and 1 µg/ml, with 60% cell viability at the uppermost concentration used (Fig. 10B). MDCK II cells were also found being resistant toward Stx2e, whereas Vero-B4 cell survival decreased to 68% after treatment with 1 µg/ml toxin (Fig. 10C). In summary, MDCK II cells were resistant toward Stx1a, Stx2a, and Stx2e, even at high concentration of 1 µg/ml applied in cell culture assays.

![Fig. 6. Distribution of cholesterol (Chol) (A), SM (B), and PC (C) in sucrose density gradient fractions of MDCK II cells analyzed by TLC separation. Ultracentrifugation gradient fractions F1–F8 were numbered according to increasing sucrose content (5, 30, and 42.5%) from top (F1) to bottom (F8) and further grouped into top (F1–F3), intermediate (F4–F6), and bottom (F7 and F8) fractions. F1–F3 represent DRM, and F4–F8 non-DRM fractions. Phospholipids were quantified after TLC separation in solvent 3 and cholesterol in solvent 4 by densitometric scanning of separated bands. A: Cholesterol amounts are depicted as nanograms per 1 × 10$^6$ cells and as rounded percentages normalized to 100%. B, C: Values for SM and PC were obtained from lipid preparations corresponding to 5 × 10$^6$ cells, respectively, and are displayed as rounded percentage values normalized to 100%. The synopsis of cholesterol and phospholipid measurements from four independent biological replicates is provided in supplemental Table S3. Phospholipids of gradient fractions F2 (DRMs) and F7 (non-DRMs, bottom fraction) were further analyzed by MS (see Fig. 7).](1392-fig6.png)
Immunofluorescence microscopic detection of Stx-binding GSLs

Subcellular localization of the Stx-binding receptor GSLs in MDCK II and Vero-B4 cells was performed by immunolabeling and subsequent imaging with confocal laser scanning microscopy. The ability of the microscope to excite and detect fluorophores within a thin section of the sample has allowed us to assess the distribution of the Stx-binding GSLs within the apical and basal plasma membrane as well as intracellularly as shown for representative areas of MDCK II and Vero-B4 cell cultures in Fig. 11A, B, respectively. The exposure of MDCK II cells to the three Stx subtypes revealed an extremely sparse distribution of hardly detectable Stx1a-, Stx2a-, or Stx2e-immunopositive foci on the apical cell surface in the vicinity of the cell nucleus (Fig. 11A, apical plane). Clearly clustered Stx-positive spots were visible on subcellular level enriched in the perinuclear area of MDCK II cells ranked with Stx2e > Stx1a > Stx2a (Fig. 11A, midplane). An almost homogeneous distribution of strong immunopositive clusters was detected on the basal cell surface in the order of Stx2e > Stx1a > Stx2a according to the observed decline in fluorescence intensity (Fig. 11A, basal plane). These findings suggest the prevalent occurrence of Stx-binding GSLs, which have been detected in the DRM fractions of MDCK II cells (see Figs. 8 and 9), in the basal plasma membrane. Moreover, absence of Stx-binding GSLs on the cell surface demonstrated by immunofluorescence microscopy could explain the refractiveness of MDCK II cells to the three Stx subtypes.

A weakly fluorochrome-speckled apical cell surface of Vero-B4 was observed upon application of the three Stx subtypes (Fig. 11B, apical plane), indicating a low toxin accessibility being a little bit more pronounced for Stx1a when compared with Stx2a and Stx2e. Subcellular immunostaining with Stx2a revealed a significantly lower fluorescence intensity compared with higher intensity upon application of Stx1a and Stx2e, indicating also a more compact fluorescence in the perinuclear area in case of Stx1a and a more regular distribution in the cytosol in case of Stx2a (Fig. 11B, midplane). Among the three Stx subtypes, Stx1a exhibited the strongest interaction with the surface of the basal membrane of Vero-B4 cells, followed by Stx2a and Stx2e, which gave a moderate immunofluorescence stain (Fig. 11B, basal plane). In general, clusters of Stx-positive spots were more pronounced in Vero-B4 cells when compared with MDCK II cells. Whether the slightly higher cell-surface binding capacity of Stx1a among the applied three Stx subtypes toward its receptor GSLs on the apical plasma membrane could be the reason of the greater susceptibility of Vero-B4 cells toward Stx1a (see Fig. 10) remains questionable.

Fig. 7. Distribution of phospholipids to sucrose density gradient fractions of MDCK II cells (A) and mass spectra of phospholipids from DRM fraction F2 (B) and non-DRM fraction F7 (C). Gradient fractions (F1–F8) were prepared, and phospholipids were separated in solvent 3 as described in Fig. 6. The phospholipids PC, SM, and Lyso-PC (A) were detected after TLC separation with molybdenum blue reagent in lipid extracts corresponding to 5 × 10^6 cells and matched to a phospholipid reference mixture (R3). The arrowheads point to SM (F2) and Lyso-PC (F7 and F8) as specific markers of DRMs and non-DRMs, respectively. The ESI MS spectra (B, C) were recorded in the positive-ion mode, and phospholipids were preferentially detected as singly charged protonated [M+H]^+ species and to less extent as sodium [M+Na]^+ adducts. SM distributes preferably to DRM fraction F2 (B, highlighted in gray) and Lyso-PC to non-DRM fractions as shown for bottom fraction F7 (C, highlighted in gray). PC distributes almost equally to DRM fraction F2 and non-DRM fraction F7.
not yet been fully elucidated for MDCK II cells in previous studies (65–70, 73). We filled this gap of knowledge by a comprehensive compositional analysis of GSLs and phospholipids of MDCK II cells (as further discussed below).

The functional importance of GlcCer and GalCer for shaping the polarity of epithelial cells has been shown for MDCK I and II cells, indicating that the observed different ratios of GlcCer and GalCer synthesis in epithelial tissues may regulate the intracellular transport and localization of GSLs (101, 102). We could show for the MDCK II cells analyzed by us striking differences in the ceramide moieties of GlcCer and GalCer, which have not been described before. GlcCer and GalCer differed in terms of the marked hydroxylation of GalCer, whereas GlcCer did not show this modification. Hydroxylated GalCer (d18:1, 24:1-OH/24:0-OH) as the major GalCer species and GalCer (d18:1, C22:0-OH) dominated over the nonhydroxylated GalCer (d18:1, C24:1/C24:0) and GalCer (d18:1, C22:0) species, respectively. However, the question of whether this pronounced ceramide heterogeneity might have an influence on lipid sorting in the Golgi apparatus and subcellular localization of GSLs remains open and might be an aspect for future investigations. Regarding the ratios of GlcCer (50.2%) and GalCer (49.8%) determined by us for MDCK II cells, similar relative amounts were recently detected for mouse kidney with 58% GlcCer and 42% GalCer (103). For analytical reasons, it is noteworthy to mention the elegant novel hydrophilic interaction chromatography-based LC/MS² method, which allowed for separation, identification, and quantification of endogenous GlcCer and GalCer stereoisomers (103). This technique might be extremely helpful for unequivocal differentiation between GlcCer and largely coseparating GalCer of cells like invariant natural killer T cells, which were found to express GlcCer as the sole neutral GSL (104).

Preliminary studies of several research groups working with MDCK II cells have provided evidence for the presence of globo-series neutral GSLs and Forssman GSL in kidney epithelial MDCK II cells (65–70, 73), but their structures have not been elucidated so far. Our comprehensive structural characterization revealed Gb3Cer and Gb4Cer lipoforms with ceramide moieties mainly composed of C24:1/C24:0 or C16:0 FA and sphingosine (d18:1) in MDCK II cells being comparable to those previously found in monkey Vero-B4 kidney (46) and human Caco-2 and HCT-8 colon epithelial cell lines (74). Furthermore, the most prominent Gb3Cer and Gb4Cer lipoforms of canine kidney (MDCK II), monkey kidney (Vero-B4), and human colon epithelial cell lines (Caco-2 and HCT-8) resemble those of human brain and glomerular microvascular endothelial cell lines (75, 105, 106), as well as primary human cerebral (107) and primary human renal endothelial cells (108), whereby the latter may represent the major targets in human STEC infections (47, 51). In the MS² spectra of all neutral GSLs of MDCK II cells analyzed by us, inspected signals at m/z 292.30 with abundances ranging from 3% to 24% relative to the peaks of the W⁺⁺ ions of sphingosine (d18:1) at m/z 264.27 were detected. These signals might derive from secondary fragment ions of sphingosine.

Fig. 8. Distribution of Stx receptors Gb3Cer (A), Gb4Cer (B), and Forssman GSL (C) in sucrose density gradient fractions of MDCK II cells analyzed by TLC immunostaining. Gradient fractions (F1–F8) were prepared and assigned as described in Fig. 6. GSLs were separated in solvent 1 and TLC immunostains of separated GSL extracts of fractions F1–F8 were performed with anti-Gb3Cer (A), anti-Gb4Cer (B), and anti-Forssman GSL antibody (C). Amounts of 2 and 0.2 µg of neutral GSLs from human erythrocytes (A), anti-Gb4Cer (B), and anti-Forssman GSL antibody (C). GSL extracts of fractions F1–F8 were performed with anti-Gb3Cer (A) and the anti-Gb4Cer (B) TLC overlay assay, respectively. An amount of 0.2 µg of the neutral GSLs from human erythrocytes (reference R1) were used as positive control in the anti-Gb3Cer (A) and subcellular localization of GSLs remains open.

DISCUSSION

Two strains of MDCK cells, namely, MDCK I and II, have been found to exhibit strikingly different GSL compositions (65). While the MDCK I strain was characterized by main expression of MHCs besides dihexosylceramide and trihexosylceramide, the MDCK II expressed in addition neutral GSLs of MDCK II cells analyzed by us, indicating that the observed differences in the ceramide moieties of GlcCer and GalCer in epithelial tissues may regulate the intracellular transport and localization of GSLs (101, 102). We could show for the MDCK II cells analyzed by us striking differences in the ceramide moieties of GlcCer and GalCer, which have not been described before. GlcCer and GalCer differed in terms of the marked hydroxylation of GalCer, whereas GlcCer did not show this modification. Hydroxylated GalCer (d18:1, 24:1-OH/24:0-OH) as the major GalCer species and GalCer (d18:1, C22:0-OH) dominated over the nonhydroxylated GalCer (d18:1, C24:1/C24:0) and GalCer (d18:1, C22:0) species, respectively. However, the question of whether this pronounced ceramide heterogeneity might have an influence on lipid sorting in the Golgi apparatus and subcellular localization of GSLs remains open and might be an aspect for future investigations. Regarding the ratios of GlcCer (50.2%) and GalCer (49.8%) determined by us for MDCK II cells, similar relative amounts were recently detected for mouse kidney with 58% GlcCer and 42% GalCer (103). For analytical reasons, it is noteworthy to mention the elegant novel hydrophilic interaction chromatography-based LC/MS² method, which allowed for separation, identification, and quantification of endogenous GlcCer and GalCer stereoisomers (103). This technique might be extremely helpful for unequivocal differentiation between GlcCer and largely coseparating GalCer of cells like invariant natural killer T cells, which were found to express GlcCer as the sole neutral GSL (104).

Preliminary studies of several research groups working with MDCK II cells have provided evidence for the presence of globo-series neutral GSLs and Forssman GSL in kidney epithelial MDCK II cells (65–70, 73), but their structures have not been elucidated so far. Our comprehensive structural characterization revealed Gb3Cer and Gb4Cer lipoforms with ceramide moieties mainly composed of C24:1/C24:0 or C16:0 FA and sphingosine (d18:1) in MDCK II cells being comparable to those previously found in monkey Vero-B4 kidney (46) and human Caco-2 and HCT-8 colon epithelial cell lines (74). Furthermore, the most prominent Gb3Cer and Gb4Cer lipoforms of canine kidney (MDCK II), monkey kidney (Vero-B4), and human colon epithelial cell lines (Caco-2 and HCT-8) resemble those of human brain and glomerular microvascular endothelial cell lines (75, 105, 106), as well as primary human cerebral (107) and primary human renal endothelial cells (108), whereby the latter may represent the major targets in human STEC infections (47, 51). In the MS² spectra of all neutral GSLs of MDCK II cells analyzed by us, inspected signals at m/z 292.30 with abundances ranging from 3% to 24% relative to the peaks of the W⁺⁺ ions of sphingosine (d18:1) at m/z 264.27 were detected. These signals might derive from secondary fragment ions of sphingosine.
However, if, e.g., m/z 1,452.84 (around 12% m/z 292.30 in MS²) corresponds to a mixture of Forssman GSL (d18:1, C16:0) and Forssman GSL (d20:1, C14:0), an additional peak at m/z 1,424.81 for Forssman GSL (d18:1, C14:0) would have been expected, which was not at all observed. In addition, corresponding species neither of Lc2Cer, Gb3Cer, nor Gb4Cer were detected, which makes the assignment of m/z 292.30 to W″ of sphingosine (d20:1) at least debatable. Because this point is not yet clarified and is beyond the scope of this investigation, the main compounds bearing sphingosine (d18:1) are presented in the figures and listed in the tables. Anyway, the biological function of this ceramide variability is largely unknown, although differential binding of Stxs to the various Gb3Cer lipoforms may indicate a significant role in the eventual pathogenic outcome of renal glomeruli restricted hemolytic uremic syndrome caused by STEC infections (109, 110). Importantly, the unsaturation level of Gb3Cer acyl chains has been shown to have a drastic impact on lipid bilayer properties and phase behavior. For instance, binding of the Stx B-subunit to a bilayer harboring Gb3Cer with saturated acyl chains did not lead to the formation of tubular invaginations, whereas invagination occurs for unsaturated Gb3Cer (111–113). Furthermore, Gb3Cer with long acyl chains from one leaflet can interdigitate into the opposing leaflet and lead to significant bilayer rigidification and immobilization of the lipid tails (114). Moreover, the lipid environment and Gb3Cer acyl chain structure impact carbohydrate conformation suggesting broad implications for GSL macromolecule recognition and ligand binding (115). The major difference when compared with the various endothelial and epithelial cells analyzed so far by us was the uncommon high rate of specific FA hydroxylation of Lc2Cer, Gb3Cer, Gb4Cer, and Forssman GSL of MDCK II cells, which was found to be restricted to the long-chain C24:1/C24:0 FAs. Interestingly, this constraint was not observed for GalCer, where C16:0-OH and C22:0-OH were detected in addition to C24:1-OH- and C24:0-OH-carrying GalCer species. It is tempting to speculate that hydroxylation of the Stx receptors Gb5Cer, Gb4Cer, and Forssman GSL might influence the toxin–cell interaction, because increased binding of verotoxins to hydroxylated C22 FA-containing GSL was reported by Binnington et al. (116).

Forssman GSL, the prevalent neutral GSL of MDCK II cells, and Gb5Cer, which has been previously detected in Vero-B4 cells (46), represent structures not expressed by human endothelial and human epithelial cells. Both GSLs do not interact with human pathogenic Stx1a and Stx2a, whereas animal pathogenic Stx2e, the causative virulence factor of pig edema disease released by swine pathogenic STEC, has been shown to promiscuously bind to Gb5Cer of Vero-B4 cells (46) and to Forssman GSL (45). The binding to Forssman GSL, in addition to Gb3Cer and Gb4Cer, is shown in this study for MDCK II cells. The biological impact of binding of the swine-pathogenic Stx2e to these two pentahexosylceramides is unknown. Functionally, Forssman GSL plays a key role in apical and basal membrane sorting of MDCK II cells (66, 117), and the interaction of

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**Fig. 9.** Distribution of Stx receptors Gb3Cer (A), Gb4Cer (B), and Forssman GSL (C) in sucrose density gradient fractions of MDCK II cells analyzed by TLC immunostaining. GSLs of the gradient fractions (F1–F8) were separated in solvent 1 and immunostained as described in preceding Fig. 8. GSLs were quantified by densitometric scanning of separated bands. Values for Gb3Cer and Gb4Cer were obtained from GSL preparations corresponding to 2.5 × 10⁶ cells and those for Forssman GSL, being equivalent to 2 × 10⁵ cells and are displayed as rounded percentage values normalized to 100%. The synopsis of Gb3Cer, Gb4Cer, and Forssman GSL from four independent biological replicates is provided in supplemental Table S4.
Forssman GSL with galectin-9 is important for polarizing MDCK cells (118).

Beside the detailed structures of the Stx receptors Gb3-Cer, Gb4Cer, and Forssman GSL of MDCK II cells, we provide in this study data on their occurrence in DRMs, which are commonly used as model membranes to explore hypothetical association of membrane constituents with lipid rafts (17). This has been previously shown for certain lipids and proteins using MDCK II cells. In particular, MDCK II cells were the study object for elucidation of sorting of glycosylphosphatidyl inositol-anchored proteins to GSL-enriched membrane subdomains (67–69) with focus on the characterization of proteins in DRM complexes (70, 119) or to follow sorting of newly synthesized galactosphingolipids to the cell surface (102). Preliminary compositional analysis of neutral GSLs from MDCK II cell-derived DRMs revealed the presence of MHCs (GlcCer and GalCer, collectively named as cerebrosides), dihexosylceramide (Lc2Cer), trihexosylceramide and tetrahexosylceramide (suggested Gb3Cer and Gb4Cer), as well as proposed Forssman GSL (67–70, 72). Focusing on the Stx-binding GSLs Gb3Cer and Gb4Cer (holds for Stx1a, Stx2a, and Stx2e) and Forssman GSL (holds for Stx2e), we could show their preferred distribution to DRM fractions F1–F3 and concomitant codistribution with cholesterol and SM, which are commonly considered as classical lipid raft markers along with hypothetical association of the Stx receptor GSLs with microdomains. Such association of Gb3Cer, Gb4Cer, and Forssman GSL might be of functional importance to Stx-mediated cytotoxicity, because the organization of GSLs, including Gb3Cer, into lipid rafts has been reported to be central to the pathology (kidney failure) of STEC infection (109, 110).

Clustering of extracellular proteins on the plasma membrane is required to perform specific cellular functions such as signaling and endocytosis (120). Membrane-mediated clustering originates from the membrane’s physical properties such as line tension, lipid depletion, and membrane curvature as well as additional attractive forces that arise from protein-induced perturbation of a membrane’s fluctuation (120). In its initial formulation (121), the lipid raft concept suggested that mixtures of certain amphipathic molecules phase-separate into nanodomains, which coexist in thermodynamic equilibrium with the bulk membrane (120). Multicomponent lipid membranes have the capacity to form domains associated with the liquid-ordered (Lσ)–liquid-disordered (Ld) coexistence (120), and many cell membranes have lipid compositions that are close to Lσ–Ld coexistence in equilibrium driven by lipid chain melting and cholesterol (120, 122, 123). Differences in molecular packing of the raft and bulk membrane lipids drive the phase separation, whereby proteins directly influence the distribution of lipid rafts (124) and contribute to the stabilization of supramolecular protein–lipid raft assemblies that accomplish certain biological functions (12, 31, 125). Proteins can induce disk-like lipid nanodomains, and Stx represents an example for macroscopic phase separation and most likely also nanoscopic domain formation. The study of Stx has led to the proposal of the GlycoLipid–Lectin

Fig. 10. Refractiveness of MDCK II cells toward Stxs. MDCK II cells were exposed to Stx1a (A), Stx2a (B), or Stx2e (C) with increasing toxin concentrations ranging from lowest concentration of $10^{-6}$ ng/ml to the highest concentration of $10^{-5}$ ng/ml. Stx susceptibility was determined in comparison to reference Vero-B4 cells known as Stx-sensitive cells. Viability measurements of six biological replicates are depicted as mean percentage values related to two untreated controls.
hypothesis including the following principal steps: receptor binding and toxin clustering at the plasma membrane, the formation of membrane invaginations and tubular endocytic pits, scission of these invaginations to form endocytic carriers, and their intracellular trafficking to endosomes and the retrograde route (126). Stx clusters readily suggesting a membrane-mediated mechanism that drives toxin molecules together as shown for interaction on model membranes (111) and on cells (127). Binding of Stx to Gb3Cer results in a small increment of local curvature, whereby several Stx molecules create a tubular membrane invagination that drives toxin entry into the cell (128), which then leads to the formation of tubular endocytic pits according to the GlycoLipid–Lectin hypothesis (126). Thus, the functional importance of clustering of GSLs in lipid rafts regarding Stx-mediated effects has been impressively shown in these shortly discussed studies. Data obtained from DRM investigations, although being aware that DRMs and lipid rafts are different things (129, 130), can provide some information on how intrinsic properties of lipids (and proteins) that are found in DRM fractions influence their interaction among each other and with the used detergent. It seems possible therefore (or is even likely) that properties, which lead to the association of certain classes of lipids with DRMs, are also relevant for their biological functions as raft lipids (129, 131).

In our study, additional emphasis was placed on analyzing the phospholipids of the DRM and non-DRM fractions. Mass-spectrometric analysis of MDCK II cell-derived gradient fractions revealed prevalence of SM [more precisely SM (d18:1, C16:0) and SM (d18:1, C24:1/C24:0)] in DRM fraction F2 and complete lack of this sphingolipid in the non-DRM fractions. In contrast to this, lyso-PC [more precisely lyso-PC (16:0) and lyso-PC (18:1)] was identified as a marker of the non-DRM bottom fractions F7 and F8. The reverse distribution of SM and lyso-PC is not a unique feature of MDCK II cells, because a very similar phospholipid assembly of DRM and non-DRM fractions has been described previously by us for human leukocyte-derived Raji (B cell) and THP-1 (monocyte) cell lines (82), human endothelial (107, 108) as well as monkey Vero-B4 cells (46). The degree of saturation in PC species was enhanced in the DRM fraction over the non-DRM fraction, i.e., the relative ion intensities of PC (34:1) and PC (36:1) were significantly higher in the mass spectrum of DRM fraction F2 than in F7 and concomitantly accompanied by an increase of ion intensities of PC (34:2) and PC (36:2) in the F7 fraction.

The susceptibility toward Stx1a, Stx2a, and Stx2e subtypes has been, to the best of our knowledge, hitherto unknown for MDCK II cells. The MDCK II strain used in our study was resistant to the treatment with human-pathogenic Stx1a and Stx2a, as well as swine-pathogenic Stx2e when compared with sensitive Vero-B4 cells. The results suggest that the cellular content of Stx receptor GSLs and their biochemical detection in DRM preparations are insufficient to predict cellular sensitivity toward the various Stx.

Fig. 11. Immunofluorescence microscopic detection of Stx-binding GSLs in MDCK II (A) and Vero-B4 cells (B). The epithelial cell lines were grown in chamber slides and incubated with Stx1a, Stx2a, or Stx2e as indicated. Bound Stx1a and Stx2 subtypes were detected with anti-Stx1 and anti-Stx2 antibody, respectively, in conjunction with Alexa Fluor 488-labeled secondary antibodies. Fluorescence imaging was performed for the apical cell surface (apical plane), on intracellular level (midplane) and at the basal cell surface (basal plane). The immunofluorescence micrographs merged with the DAPI stains of the nuclear DNA are shown.
subtypes. Importantly, Stx-binding GSLs were virtually undetectable on the apical cell surface of MDCK II cells using immunofluorescence confocal laser scanning microscopy. This could be the most plausible reason for resistance of immunofluorescence confocal laser scanning microscopy. Other membrane compounds that might hamper access of Stxs cannot be excluded. A further hypothetical explanation could be that cholesterol may mask cell membrane GSLs (132) or induce a tilt in the GSL headgroups (133), rendering Stx receptor GSL-positive cells refractory to the cytotoxic action of Stxs. Another explanation could be different endocytosis rates and/or intracellular trafficking routes of the three Stx subtypes, which may occur in MDCK II and Vero-B4 cells. However, these interpretations are highly speculative at this stage of research, and the reason for the phenomenon of Stx-resistance despite presence of Stx-binding GSLs in the basal plasma membrane and the perinuclear space of MDCK II cells, remains to be determined in future research projects. Actually, to the best of our knowledge, it has never been tested whether Stxs can inhibit protein biosynthesis in MDCK II cells and it remains unknown, whether cells survive despite an inhibition of protein biosynthesis or whether the toxins fail to be targeted to the cytosol and thereby to modify ribosomes. Interestingly, Stx from Shigella dysenteriae has been shown in previous studies to inhibit protein biosynthesis in human epithelial cell lines and MDCK-I cells (134).

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