TECHNICAL NOTES

Synthesis of a Fluorescent Ganglioside G_{M1} Derivative and Screening of a Synthetic Peptide Library for G_{M1} Binding Sequence Motifs

Niels Röckendorf,[†] Steffen Bade,[†] Timothy R. Hirst,[‡] Hans H. Gorris,[†] and Andreas Frey^{*,†}

Division of Mucosal Immunology, Department of Clinical Medicine, Research Center Borstel, Borstel, Germany, and John Curtin School of Medical Research, The Australian National University, Canberra, Australia. Received August 1, 2006; Revised Manuscript Received December 18, 2006

A ganglioside G_{M1} probe bearing a dark-red fluorescent dye at the sphingosine moiety of the molecule was prepared by a convenient one-pot synthesis. The labeled G_{M1} permitted the detection of the natural ganglioside G_{M1} ligand *Escherichia coli* heat-labile enterotoxin subunit B (EtxB) in picomole quantities on a solid support. When an epitope mapping of several ganglioside binding proteins and protein fragments was performed by screening a cellulose membrane-bound synthetic library of 64 16mer peptides with the new probe, several peptides displaying ganglioside G_{M1} affinity could be identified. We consider the labeled glycolipid described herein a versatile tool for manifold biochemical investigations.

INTRODUCTION

Carbohydrate structures are abundant on cell surfaces and occur in a wide variety, reflecting the diverse functions they exert in biological systems. Carbohydrate moieties have been shown to protect cells, to mediate cell attachment, and to participate in signal transduction pathways (1, 2). Among other possibilities, they can be attached to cell surfaces via linkage to sphingolipids (3), thereby forming glycosphingolipids, such as gangliosides.

Glycosphingolipids are composed of two functional units. Their lipid moiety, named ceramide, is responsible for anchoring the molecule in the outer leaflet of the lipid bilayer of the cell membrane. The glycosyl moiety of glycosphingolipids consists of a branched saccharide chain that extends from the membrane surface. The carbohydrate antenna acts as a receptor in intercellular communication processes but may also be exploited by microbial pathogens in order to infect the cells or to manipulate them (4). Bacterial toxins of the AB₅-type such as cholera toxin or heat-labile enterotoxin from *Escherichia coli* enter their target cells by binding to ganglioside G_{M1} followed by endocytosis of the complex formed. Once inside, the toxins induce a profound electrolyte and water release which in turn may improve the habitat of the bacteria or facilitate their spread (5, 6).

The multiple and important roles glycosphingolipids are recognized to play has sparked interest in both the development of traceable glycosphingolipid derivatives for studying their cell biology and the use of glycosphingolipid ligands for drug and vaccine targeting purposes. Several ganglioside derivatives carrying fluorescent labels either on their sphingosine or

saccharide moiety have been described and used for various applications (7–9). Their biological behavior seems to be strongly dependent on the site of fluorophore attachment as well as on the structure of the fluorophore itself (10), which limits their use in basic studies of glycosphingolipid function. Moreover, none of the fluorescent ganglioside derivatives described so far operates beyond the spectral range where autofluorescence of eukaryotic tissue or membrane supports is observed. Such autofluorescence not only hampers detection of the dye in live cells but may also interfere with fluorescence-based solid-phase assays. Consequently, assays with fluorophore-labeled glycolipids that involve the use of membrane supports or tissue material call for fluorescent dyes which emit light in the far-red to near-infrared region (650–1100 nm), since at these wavelengths, little background fluorescence is usually observed (11).

In order to exploit ganglioside G_{M1} as a gateway for drug or vaccine delivery, soluble as well as particulate ganglioside G_{M1} targeting systems have been developed (12–14). These systems are based on the B subunits of cholera toxin (CTB) or *Escherichia coli* heat-labile enterotoxin (EtxB) and were used, e.g., in mucosal immunizations to target antigens to epithelial cells of the mucosa-associated lymphoid tissue (13) or to investigate the accessibility of cell membranes of different intestinal cell types (15, 16). Importantly, the ganglioside binding capability of these proteinaceous ligands may be compromised when they are attached to particulate carriers for use in drug delivery systems (14). Thus, small peptides or peptoid structures with high binding affinity to gangliosides would be preferable for these purposes. The identification of such ganglioside ligands, however, requires traceable ganglioside G_{M1} derivatives that can be used without background problems for the screening of solid-phase bound substance libraries and thus must be superior to the currently available compounds.

In the present study, a new fluorescent ganglioside G_{M1} derivative is introduced which meets these requirements. The new probe is characterized regarding its EtxB binding behavior and utilized to identify short ganglioside G_{M1} binding peptides

* Corresponding author. Dr. Andreas Frey, Division of Mucosal Immunology, Research Center Borstel, Parkallee 22, D-23845 Borstel, Germany. Phone: +49-4537-188-562; Fax: +49-4537-188-693; E-mail: afrey@fz-borstel.de.

[†] Research Center Borstel.

[‡] The Australian National University.

by screening a solid-phase synthesized library of peptides derived from G_{M1} binding proteins and protein fragments.

EXPERIMENTAL PROCEDURES

Materials. Heat-labile enterotoxin subunit B (EtxB) from *Escherichia coli* was isolated according to a procedure described earlier (17). Casein (Hammarsten grade) was obtained from BDH (via VWR International, Darmstadt, Germany), Lyso-ganglioside G_{M1} was from Sigma-Aldrich (Taufkirchen, Germany), and DY650-NHS was from Dyomics (Jena, Germany). PVDF membranes (Westran clear signal membranes, 0.45 μ m pore size), nitrocellulose membranes (0.2 μ m pore size), cellulose membrane supports (Whatman 540), and a Minifold I 96-well blotting device were from Whatman International (Dassel, Germany). Fmoc amino acid building blocks with side chain protecting groups if required (*t*Bu (serine, threonine, and tyrosine); *Or*Bu (aspartic and glutamic acid); Boc (lysine and tryptophane); Acn (cysteine); Trt (histidine, asparagine, and glutamine), Pbf (arginine)) were obtained from Merck Biosciences (Bad Soden, Germany). Anhydrous hydroxybenzotriazole (HOBt) was from Dojindo Laboratories (Kumamoto, Japan) and dimethylformamide (DMF) from Promochem (Wesel, Germany). *N*-Methylpyrrolidone (NMP; Fluka, Taufkirchen, Germany). Fourier transform ion cyclotron resonance mass spectrometry was carried out on a Bruker Daltonics (Bremen, Germany) Apex II ESI-FT-MS instrument equipped with a 7 T activity-shielded magnet and an Apollo electrospray ion source in negative detection mode. Fluorescence intensities were quantitated with an Odyssey Infrared Imager running software version 1.2.15 (LI-COR Biosciences, Bad Homburg, Germany). Spatially addressable multiple peptide synthesis was carried out using a pipetting robot ASP 222 (Intavis Bioanalytical Instruments, Köln, Germany).

HPLC was performed using an Äkta Purifier (GE Healthcare, Freiburg, Germany). Purifications were carried out on a Jupiter (C18, 300 Å, 5 μ m, 4.6 $\times$ 250 mm) reversed-phase (RP) column (solvents ratio v/v reported) (Phenomenex, Aschaffenburg, Germany). Fourier transform ion cyclotron resonance mass spectrometry was carried out on a Bruker Daltonics (Bremen, Germany) Apex II ESI-FT-MS instrument equipped with a 7 T activity-shielded magnet and an Apollo electrospray ion source in negative detection mode. Fluorescence intensities were quantitated with an Odyssey Infrared Imager running software version 1.2.15 (LI-COR Biosciences, Bad Homburg, Germany). Spatially addressable multiple peptide synthesis was carried out using a pipetting robot ASP 222 (Intavis Bioanalytical Instruments, Köln, Germany).

Synthesis. For preparation of lyso G_{M1} -DY650 conjugates (1/2), lyso-ganglioside G_{M1} was dissolved in dry DMF at a concentration of 2 mg/mL (1.03 mM). 200 μ L of this solution was mixed with a solution of DY650-NHS in dry DMF (225 μ L, 2 mg/mL), diisopropylethylamine (DIPEA, 7 μ L) was added, and the mixture was incubated with shaking under an argon atmosphere at 30 °C. After 36 h, the reaction mixture was analyzed by ESI-MS, the solvent was removed in vacuo, and the residue was redissolved in acetonitrile/water (500 μ L, 20% (v/v)). The resulting solution was subjected to HPLC purification on RP-18 silica gel (A = water, B = acetonitrile, 20% B to 100% B, 1 mL/min, 90 mL, retention volume 67 mL), and product fractions were collected. ESI-MS (*m/z*): fraction 1 1947.95 [M^+] (1947.94 calcd for $C_{95}H_{145}N_5O_{35}S$); fraction 2 1975.98 [M^+] (1975.98 calcd for $C_{97}H_{149}N_5O_{35}S$) (see also Supporting Information).

Peptide libraries were synthesized on cellulose membrane supports following the procedure of Frank (18). The cellulose membranes were derivatized with Fmoc-protected β -alanine in DMF (2.5 mL (0.02 mL/cm² membrane), 0.2 M Fmoc- β -alanine, 0.2 M methylimidazol, 0.26 M diisopropylcarbodiimide (DIC), 24 h, room temperature (RT)). Excess hydroxyl groups were blocked (capped) with acetic anhydride in DMF (15 mL (0.13 mL/cm² membrane), 2% (v/v), 24 h, RT, rocking), and the membranes were washed 3 $\times$ with DMF (15 mL (0.13 mL/cm²

Table 1. Ganglioside G_{M1} -Binding Proteins and Peptides

set	protein/source	motif ^a [aa-aa]	peptide no. ^b	ref
I	cholera toxin B subunit	50–75	A1–A6	25
II	cholera toxin B subunit	50–75	A7–B4	26
III	<i>E. coli</i> enterotoxin B subunit	26–45	B5–B7	27
IV	amyloid β protein	1–40	B8–D4	28
V	phage display peptides	n.a. ^c	D5–D7	21
VI	saposin B	52–69	D8–E1	29
VII	saposin C	8–29	E2–E5	29
VIII	myelin basic protein	1–44	E6–G4	30
IX	<i>Staphylococcus aureus</i> leukocidin	270–287	G5–G6	31
X	cholera toxin B subunit	rational ^d	G7–H4	this study
XI	random peptides	n.a.	H5–H8	this study

^a Linear sequence motif proposed to be responsible for ganglioside G_{M1} binding. Numbering refers to the amino acid positions published in the respective reference. ^b Alphanumeric numbering of the 16mer peptides covering the motif indicated. The numbering refers to their position on the membrane (see Figure 2). ^c Not applicable. ^d Motifs designed rationally from crystal structure data ("linearized discontinuous motif").

membrane)). Fmoc cleavage was performed by incubating the membranes twice with piperidine in DMF (10 mL (0.09 mL/cm² membrane), 20% (v/v), 5 min each, RT, rocking). Presence of free amino functions on the cellulose membranes was confirmed with bromophenol blue. For that, membranes were washed 5 $\times$ with DMF (15 mL (0.13 mL/cm² membrane)), stained with bromophenol blue in DMF (15 mL (0.13 mL/cm² membrane), 0.01% (w/v), 10 min, RT, rocking), washed 3 $\times$ with 100% ethanol (15 mL (0.13 mL/cm² membrane)), and air-dried.

The above capping, washing, Fmoc-cleavage and staining steps were repeated between all synthesis cycles from the definition of the synthesis areas (spots) onward, but capping was reduced to 20 min after the third cycle.

For definition of the synthesis areas, 0.1 μ L of Fmoc- β -alanine-pentafluorophenyl (Pfp) ester (0.2 M Fmoc- β -alanine-Pfp-ester in NMP) was applied by a pipetting robot on defined membrane areas.

For subsequent synthesis cycles, the amino acids were converted into their corresponding HOBt esters immediately before use by adding 1.25 mol DIC per mole amino acid to a solution containing 0.4 M N- α -Fmoc-protected amino acid and 0.7 M HOBt in NMP (final concentration: 0.2 M amino acid, 0.35 M HOBt, 0.25 M DIC) and allowing the mixture to react for 30 min at RT. Precipitates were removed by a short centrifugation step, and 0.2 μ L of these N- α -Fmoc-protected amino acid active esters were applied to the respective synthesis areas. Coupling of each amino acid was repeated three times, and a minimum of 40 min reaction time was allowed in each synthesis cycle. After the last cycle, the peptides were N-terminally acetylated with acetic anhydride in DMF (15 mL (0.13 mL/cm² membrane) 2% (v/v), 20 min, RT, rocking).

Side chain protecting groups (except Acn) were removed by immersing the membranes twice in a cleavage cocktail (50% (v/v) trifluoroacetic acid, 3% (v/v) triisobutylsilane, and 2% (v/v) water in dichloromethane (DCM), 10 mL (0.09 mL/cm² membrane), 1 h each, RT, rocking). Subsequently, membranes were washed 4 $\times$ with DCM (15 mL (0.13 mL/cm² membrane)), 3 $\times$ with DMF (15 mL (0.13 mL/cm² membrane)), 4 $\times$ with 1 M acetic acid (15 mL (0.13 mL/cm² membrane)), and finally 3 $\times$ with 100% ethanol (15 mL (0.13 mL/cm² membrane)). Membranes were air-dried and desiccated overnight in vacuo and stored in the presence of desiccant at –20 °C. The sequences of the peptides synthesized are compiled in Table 2. The library was prepared in six replicas on a single cellulose membrane.

Lyso G_{M1} –DY650 Binding to EtxB. EtxB was dissolved in physiological phosphate buffer (10 mM sodium phosphate buffer, 150 mM NaCl, pH 7.5) at a concentration of 5 mg/mL

Table 2. Peptide Sequences Synthesized on Cellulose Membrane Supports for Screening of G_{M1}-Binding Capacity^a

No.	Sequence	No.	Sequence
A1	VEVPGQHIDSQKK	E1	YSEIAIQMMMHMQ
A2	VPGQHIDSQKKAI	E2	CEFLVKEVTKLID
A3	GQHIDSQKKAIER	E3	FLVKEVTKLIDNN
A4	HIDSQKKAIERMK	E4	VKEVTKLIDNNKT
A5	DSQKKAIERMKNT	E5	EVTCLIDNNKTEK
A6	SQKKAIERMKNTL	E6	ASQKRPSQRHGSK
A7	VEVPGSQHIDSQK	E7	QKRPSQRHGSKYL
A8	VPGSQHIDSQKKA	E8	RPSQRHGSKYLAT
B1	GSQHIDSQKKAI	F1	SQRHGSKYLATAS
B2	QHIDSQKKAIERM	F2	RHGSKYLATASTM
B3	IDSQKKAIERMKD	F3	GSKYLATASTMDH
B4	SQKKAIERMKDTL	F4	KYLATASTMDHARHGF
B5	SYTESMAGKREMV	F5	LATASTMDHARHG
B6	TESMAGKREMVII	F6	TASTMDHARHGFL
B7	SMAGKREMVITF	F7	STMDHARHGFLPR
B8	LEVPTDGNAGLLA	F8	MDHARHGFLPRHR
C1	VPTDGNAGLLAEP	G1	HARHGFLPRHRDT
C2	TDGNAGLLAEPQI	G2	RHGFLPRHRDTGI
C3	GNAGLLAEPQIAM	G3	GFLPRHRDTGILD
C4	AGLLAEPQIAMFC	G4	LPRHRDTGILDSI
C5	LLAEPQIAMFCGR	G5	KYEVNWKTHEIKV
C6	AEPQIAMFCGRLN	G6	YEVNWKTHEIKVK
C7	PQIAMFCGRNLNH	G7	AEYHNTQIHTLND
C8	IAMFCGRNLNMHN	G8	ATFQVEVPGSQHI
D1	MFCGRNLNMHNQ	H1	AKVEKLCVWNNKT
D2	CGRLNMHMNVQNG	H2	GALSETYSFIKEY
D3	RLNMHMNVQNGKW	H3	ESLAGKREQSDIH
D4	NMNMNVQNGKWDS	H4	ASDWYDEMLTWN
D5	DFRRLPGAFWQLR	H5	DGTVSTHECRLEY
D6	GWYKGRARPVSA	H6	FSHSGEGDMIWER
D7	VWRLAPFFSNRL	H7	HHCSILKEVWHVK
D8	SQYSEIAIQMMMH	H8	PDQDGVVTAMHGF

^a No. indicates the alphanumeric numbering of the respective peptide and refers to its position on the membrane (see Figure 2).

(86 μ M), twofold serial dilutions were prepared (concentration range: 5 mg/mL to 80 μ g/mL), and 1 μ L of each dilution was mixed with 100 μ L Dulbecco's phosphate buffered saline (D-PBS; 2.7 mM KCl, 1.5 mM KH₂PO₄, 136 mM NaCl, 8.1 mM Na₂HPO₄, pH 7.4). PVDF membranes were soaked in ethanol for 10 min, washed with water and PBS, and transferred to a 96-well blotting device in the wet state. The EtxB solutions diluted in D-PBS were applied to the membranes under gentle vacuum. Then, the membranes were incubated for 4 h at RT in blocking solution (0.1% (w/v) Tween 20 in D-PBS (PBST)) followed by overnight incubation at RT in a solution of **2** (approximately 10 ng/mL (approximately 5 nM), in 10 mL PBST). They were washed 6 $\times$ for 10 min with PBST and 2 $\times$ for 10 min with D-PBS. Fluorescence was measured with an Odyssey infrared imager at 700 nm. Measurements were performed in triplicate; signals are reported as mean fluorescence intensities $\pm$ SEM above cutoff. The cutoff was determined according to a method described earlier (19).

LysoG_{M1}-DY650 Binding to Peptide Libraries. The cellulose membrane-bound peptide libraries were blocked with casein/PBS (1% casein in D-PBS) for 3 h at RT and incubated

Figure 1. Detection of heat-labile enterotoxin B subunit (EtxB) from *Escherichia coli* by lysoG_{M1}-DY650. Decreasing amounts of EtxB were immobilized on a PVDF membrane and detected with lysoG_{M1}-DY650. Fluorescence emission intensities of bound lysoG_{M1}-DY650 at 700 nm are given in arbitrary units (mean $\pm$ SEM of triplicate experiments).

overnight at RT with a solution of **2** (approximately 10 ng/mL (approximately 5 nM) in 10 mL casein/PBS). They were washed 6 $\times$ for 10 min with 10 mL PBST and 2 $\times$ for 10 min with 10 mL D-PBS, and the bound lysoG_{M1}-DY650 was quantitated on the wet membranes in the Odyssey infrared imager at 700 nm. The experiment was performed in quintuplicate using five identical libraries that had been synthesized in parallel. Signals are reported as mean fluorescence intensities $\pm$ SEM above average background outside the spot areas. For all spots, an "average signal" was calculated as the mean of the fluorescence intensities of all spots. From this average signal, the upper and lower cutoff values to classify true binders (signals above upper cutoff) and nonbinders (signals below lower cutoff) was determined using *t*-statistics according to a method described earlier (19).

RESULTS AND DISCUSSION

Preparation of a LysoG_{M1}-DY650 Derivative. Since the carbohydrate part of ganglioside G_{M1} is essential for ligand binding, one runs the risk of inactivating the molecule when a bulky group, such as a fluorophore, is attached. The only locale that may be permissive to modification or replacement seems to be the generic fatty acid moiety residing on the amino group of sphingosine. We thus decided to substitute the fatty acid with a dark red fluorescent dye in order to obtain a traceable probe. A G_{M1} derivative that already lacks the fatty acid moiety and contains a free amino function instead is lysoganglioside G_{M1} (lysoG_{M1}) (20). Consequently, lysoG_{M1} was chosen as starting material.

As a fluorophore, we selected DY650, a member of the cyanine dye family that carries a lipophilic C-6 spacer and fluoresces at 674 nm after excitation at 653 nm. Due to its emission in the dark red, DY650 should be especially useful in all experiments where autofluorescence is to be expected. Autofluorescence of crude biological samples or organic membrane materials peaks around 550 nm and thus coincides with the emission spectra of popular fluorophores such as Bodipy-FL, which is often used as a label for glycosphingolipids (10). In comparison to Bodipy-FL, the DY650 fluorophore shows a twofold higher absorption as well as a doubled Stokes shift, and it is suitable for readout with a variety of fluorescence imagers using red to near-infrared excitation sources. Besides its favorable optical properties, DY650 offers chemical advan-

Scheme 1. Synthesis of Fluorescent Probes 1 and 2

tages, too. The molecular structure of this dye contains no net charge, since the dye's negatively charged sulfo group is compensated by its positively charged aromatic oxonium substructure. Coulomb interactions and hence unspecific binding of DY650 conjugates to charged residues in sample materials should therefore be reduced in comparison to other dyes, which often carry multiple ionic groups in order to increase their solubility in aqueous media.

The preparation of the lyso G_{M1} –DY650 conjugate was designed as a one-pot synthesis in which lyso G_{M1} was deprotonated by the sterically hindered base diisopropylethylamine (DIPEA) and reacted with DY650–NHS in dimethylformamide (DMF) in the dark at 30 °C. The reaction was monitored by ESI-MS. It revealed that product formation was slow and came to completion after 36 h only. We attribute this rather low reactivity of the NHS-ester to the fact that the reacting amino function is located at a sterically hindered secondary carbon. Purification of the crude lyso G_{M1} –DY650 conjugate was achieved by RP-HPLC, and two product fractions containing the fluorescent glycoconjugates 1 and 2 with molecular weights of 1948 and 1976 were collected (Scheme 1). The occurrence of two different product molecules is due to the fact that the lyso G_{M1} , which we used as starting material in the synthesis, is a mixture of two compounds which differ solely in the length of their alkyl chain. The fluorescent G_{M1} derivative 1 carries a C_{15} alkyl chain in its sphingosine moiety, while derivative 2 has a C_{13} alkyl chain in this position. Since fluorescent probe 2 could be enriched to higher purity than 1, derivative 2 was used in all further experiments.

Binding of Lyso G_{M1} –DY650 to a G_{M1} Ligand. We first investigated the probe's binding behavior toward a natural G_{M1} binding partner, heat-labile enterotoxin subunit B (EtxB) from *Escherichia coli*, in a dot blot-type assay. Using this setup, a detection limit of 10 pmol EtxB per dot was reached (Figure 1), despite the fact that the probe was excited at a suboptimal wavelength (680 nm) because no laser source for the dye's excitation maximum was available. Even higher sensitivities may be achievable if excitation of the probe is performed at 653 nm, the excitation maximum of DY650. Thus, lyso G_{M1} –DY650 was able to bind with high affinity to the natural G_{M1} ligand EtxB, demonstrating that the binding properties of the pentasaccharide moiety were retained in the labeled molecule.

Binding of Lyso G_{M1} –DY650 to Putative G_{M1} Binding Peptides. We finally wanted to validate the newly developed lyso G_{M1} –DY650 derivative as a tool for the detection of molecules which display a high affinity to ganglioside G_{M1} . Our ultimate goal is the identification of small ganglioside G_{M1} binding peptides that might be used for drug targeting.

We accomplished an epitope mapping by designing a peptide library consisting of candidate sequences that were taken from known or alleged G_{M1} binding proteins or protein fragments (Table 1) by breaking down the respective sequence motifs into overlapping 16mer peptides (12 to 15 amino acids overlap). We also included three 15mer peptides which had been identified previously as potential G_{M1} ligands in phage display experiments (21) and thus could serve as controls. For the sake of comparability, they were extended to 16mers by addition of a C-terminal alanine.

Last, we completed our peptide library with peptides that we deduced from the crystal structure of cholera toxin B-pentamer cocrystallized with bound G_{M1} (22). Amino acids that are part of the G_{M1} binding pocket and are involved in G_{M1} binding were selected and assembled sequentially by linear arrangement.

This way, 64 different 16mer peptide sequences were obtained (Table 2) and synthesized on a cellulose membrane support using the SPOT technique (18, 23). With this method, such a high peptide density in each spot of the membrane can be achieved that even weak ligand–peptide interactions are detectable due to multivalency effects. This advantage of SPOT-synthesized peptide libraries is of particular value in our case, as we aimed to detect a carbohydrate–peptide interaction, and this type of interaction is notoriously weak.

Identification of short ganglioside G_{M1} binding peptides was performed by incubating the cellulose membrane-bound library with lyso G_{M1} –DY650 and quantitating the fluorescence intensities of the single peptide spots after excitation at 680 nm (Figure 2). Background fluorescence of the membrane support was very low due to the long excitation wavelength used and the blocking of the cellulose membrane with 1% (w/v) casein/PBS. The entire procedure proved highly reproducible, as indicated by the low coefficient of variation that 5 independent screening experiments yielded for each single peptide (mean coefficient of variation for 64 peptides: 11.6%).

The peptides were classified according to their fluorescence intensities into two groups, nonbinders and binders. A peptide was defined as nonbinder if the fluorescence intensity of the respective spot was significantly lower than the “average signal” calculated from the mean fluorescence of all 64 spots (one-tailed *t* test, $P < 0.05$). Likewise, an upper cutoff value as threshold for significantly higher than average signals was determined. Peptide spots with a fluorescence intensity above this cutoff display better than “average” binding with a probability of $\geq 99.5\%$ and were hence defined as true binders.

With these discrimination criteria, 25 peptides were classified as nonbinders (Figure 2B, open bars), 15 peptides could not be assigned to either group (Figure 2B, hatched bars), and 24

Figure 2. Detection of peptides capable of binding lysoG_{M1}-DY650. A library of 64 different peptides was synthesized onto a cellulose membrane support. After blocking, the membrane was incubated with lysoG_{M1}-DY650 and fluorescence was measured at 700 nm. (A) Image of the peptide library as generated by the infrared imager depicting the fluorescence signals obtained after incubation of the membrane with lysoG_{M1}-DY650. (B) Quantitation of G_{M1} binding to single peptide spots. Filled bars represent signal intensities of peptides assigned to the group of binders; open bars represent nonbinders; hatched bars represent peptides which could not be assigned to either group. Roman numerals indicate the respective set to which the peptides belong (see Table 1). Individual peptides are numbered alphanumerically according to their position on the membrane (for sequences, see Table 2). Fluorescence emission intensities determined for each single spot at 700 nm are shown (mean $\pm$ SEM of five experiments). The horizontal line indicates the upper cutoff value used to define true G_{M1} binders.

peptides were found to be binders (Figure 2B, filled bars). All three phage-display-derived, alanine-extended peptides that had been shown to interact with G_{M1} in previous experiments (24) (peptide spots D5, D6, and D7 in Figure 2A) belong to the latter group, a finding that we consider a promising preliminary result pertaining to the validation of our screening system. In our assay setup, lysoG_{M1}-DY650 showed a stronger interaction with peptide D5 than with peptides D6 and D7 (Figure 2B). This is in conflict with the original report (21) where peptide D7 showed the highest inhibition of CTB binding to G_{M1}. We reason that these discrepancies are due to the different assay formats used. Originally, the G_{M1} binding performance of the three peptides had been determined in solution, whereas our screening system measures the binding behavior of peptides attached to a solid phase.

The highest fluorescence intensity achieved in our assay resulted from binding of lysoG_{M1}-DY650 to peptide G4, which encompasses the C-terminal part of the myelin basic protein fragment. For peptides B8–D4 derived from amyloid β protein, signal intensity peaks at candidate C7, which indicates that the sequence motif of C7 is mainly responsible for the interaction between ganglioside G_{M1} and amyloid β protein (Table 1).

Two linear CTB-derived motifs could be assigned to the group of binders. These peptides, A4 and A5, vary by only one amino acid, an asparagine instead of an aspartic acid, from peptides B3 and B4, but exhibit a much higher fluorescence intensity than the latter. This point mutation originates from a discrepancy in the reported parent sequences (25, 26) and illustrates nicely that the exchange of a single amino acid alone is able to alter the affinity of a peptide ligand to its target. Apparently, a negative charge is highly unfavorable at the respective position. This assumption is supported by the fact that peptide B1 which shares a major part of its sequence with its “neighboring” peptides, B2–B4, but lacks their aspartic acid—and hence the negative charge—displays a similar signal intensity as the other “uncharged” candidate peptides A3–A6. Regardless of the underlying reason for this charge effect, the example emphasizes the value of our assay, which is evidently capable of accurately reflecting the differences in the G_{M1} binding affinities of these closely related peptides.

The peptides derived from the other G_{M1} binding protein fragments exhibited little or no binding to lysoG_{M1}-DY650, which suggests that the G_{M1} binding properties of those proteins mainly reside in their three-dimensional structure.

Intriguingly, some of the candidate sequences which we designed on the basis of the structural data of the CTB–G_{M1} complex show rather high fluorescence signals, rendering them true binders (peptides H1, H2, and H4). This demonstrates that rational design may constitute a worthwhile route toward the identification of peptidic ganglioside G_{M1} ligands.

ACKNOWLEDGMENT

We thank Dr. Buko Lindner for performing the mass spectrometrical analyses. This work was funded by the German Federal Ministry of Education and Research (BMBF, grants 13N8473 and 01KO113) and by the German Research Foundation (DFG, grant FR 958/4-1).

Supporting Information Available: ESI-MS spectra and HPLC profile of ganglioside–dye conjugates. This material is available free of charge via the Internet at <http://pubs.acs.org/BC>.

LITERATURE CITED

- (1) Murrell, M. P., Yarema, K. J., and Levchenko, A. (2004) The systems biology of glycosylation. *ChemBiochem* 5, 1311–1313.
- (2) Hakomori, S. I., and Igarashi, Y. (1995) Functional role of glycosphingolipids in cell recognition and signaling. *J. Biochem.* 118, 1091–1103.
- (3) Futerman, A. H., and Hannun, Y. A. (2004) The complex life of simple sphingolipids. *EMBO Rep.* 5, 777–782.
- (4) Yates, A. J., and Rampersaud, A. (1998) Sphingolipids as receptor modulators. An overview. *Ann. N. Y. Acad. Sci.* 845, 57–71.
- (5) Spangler, B. D. (1992) Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol. Rev.* 56, 622–647.
- (6) De Haan, L., and Hirst, T. R. (2004) Cholera toxin: a paradigm for multi-functional engagement of cellular mechanisms. *Mol. Membr. Biol.* 21, 77–92.
- (7) Nakagawa, T., Tani, M., Kita, K., and Ito, M. (1999) Preparation of fluorescence-labeled G_{M1} and sphingomyelin by the reverse

- hydrolysis reaction of sphingolipid ceramide N-deacylase as substrates for assay of sphingolipid-degrading enzymes and for detection of sphingolipid-binding proteins. *J. Biochem.* 126, 604–611.
- (8) Schwarzmann, G., Wendeler, M., and Sandhoff, K. (2005) Synthesis of novel NBD- G_{M1} and NBD- G_{M2} for the transfer activity of G_{M2} -activator protein by a FRET-based assay system. *Glycobiology* 15, 1302–1311.
- (9) Song, X., and Swanson, B. I. (1999) Direct, ultrasensitive, and selective optical detection of protein toxins using multivalent interactions. *Anal. Chem.* 71, 2097–2107.
- (10) Burns, A. R., Frankel, D. J., and Buranda, T. (2005) Local mobility in lipid domains of supported bilayers characterized by atomic force microscopy and fluorescence correlation spectroscopy. *Biophys. J.* 89, 1081–1093.
- (11) Williams, R. J., Narayanan, N., Casay, G. A., Lipowska, M., Strekowski, L., Patonay, G., Peralta, J. M., and Tsang, V. C. W. (1994) Instrument to detect near-infrared fluorescence in solid-phase immunoassay. *Anal. Chem.* 66, 3102–3107.
- (12) Asou, H. (1990) Localisation of ganglioside G_{M1} with biotinylated cholera toxin. *Methods Enzymol.* 184, 405–408.
- (13) O'Dowd, A. M., Botting, C. H., Precious, B., Shawcross, R., and Randall, R. E. (1999) Novel modification to the C-terminus of LTB that facilitate site-directed chemical coupling of antigens and the development of LTB as a carrier for mucosal vaccines. *Vaccine* 17, 1442–1453.
- (14) Olivier, V., Meisen, I., Meckelein, B., Hirst, T. R., Peter-Katalinic, J., Schmidt, M. A., and Frey, A. (2003) Influence of targeting ligand flexibility on receptor binding of particulate drug delivery systems. *Bioconjugate Chem.* 14, 1203–1208.
- (15) Frey, A., Giannasca, K. T., Weltzin, R., Giannasca, P. J., Reggio, H., Lencer, W. I., and Neutra, M. R. (1996) Role of the glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting. *J. Exp. Med.* 184, 1045–1059.
- (16) Mantis, N. J., Frey, A., and Neutra, M. R. (2000) Accessibility of glycolipid and oligosaccharide epitopes on rabbit villus and follicle-associated epithelium. *Am. J. Physiol.* 278, G915–G923.
- (17) Amin, T., and Hirst, T. R. (1994) Purification of the B-subunit oligomer of *Escherichia coli* heat-labile enterotoxin by heterologous expression and secretion in a marine vibrio. *Protein Expression Purif.* 5, 198–204.
- (18) Frank, R. (1992) Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron* 48, 9217–9232.
- (19) Frey, A., Di Canzio, J., and Zurakovski, D. (1998) A statistically defined endpoint titer determination method for immunoassays. *J. Immunol. Methods* 221, 35–41.
- (20) Sonnino, S., Acquotti, D., Kirschner, G., Uguaglianza, A., Zecca, L., Rubino, F., and Tettamanti, G. (1992) Preparation of lyso- G_{M1} ($II_3Neu_5AcGgOse_4$ long chain bases) by a one-pot reaction. *J. Lipid Res.* 33, 1221–1226.
- (21) Matsubara, T., Ishikawa, D., Taki, T., Okahata, Y., and Sato, T. (1999) Selection of ganglioside G_{M1} -binding peptides by using a phage library. *FEBS Lett.* 456, 253–256.
- (22) Merritt, E. A., Sarfaty, S., van den Akker, F., L'Hoir, C., Martial, J. R., and Hol, W. G. J. (1994) Crystal structure of cholera toxin B-pentamer bound to receptor G_{M1} pentasaccharide. *Protein Sci.* 3, 166–175.
- (23) Wenschuh, H., Gausepohl, H., Germeroth, L., Ulbricht, M., Matuschewski, H., Kramer, A., Volkmer-Engert, R., Heine, N., Ast, T., Scharn, D., and Schneider-Mergener, J. (2000) Positionally addressable parallel synthesis on continuous membranes. In *Combinatorial Chemistry: A Practical Approach* (Ferrini, H., Ed.) pp 95–116, Oxford University Press, Oxford, U.K.
- (24) Siebert, H. C., Born, K., Andre, S., Frank, M., Kaltner, H., von der Lieth, C. W., Heck, A. J. R., Jimenez-Barbero, J. J., Kopitz, J., and Gabius, H. J. (2005) Carbohydrate chain of ganglioside G_{M1} as a ligand: identification of the binding strategies of three 15 mer peptides and their divergence from the binding modes of growth-regulatory galectin-1 and cholera toxin. *Chem.—Eur. J.* 12, 388–402.
- (25) Delmas, A., and Partidos, C. D. (1996) The binding of chimeric peptides to G_{M1} ganglioside enables induction of antibody responses after intranasal immunization. *Vaccine* 14, 1077–1082.
- (26) Kurosky, A., Markel, D. E., and Peterson, J. W. (1977) Covalent structure of the β chain of cholera enterotoxin. *J. Biol. Chem.* 252, 7257–7264.
- (27) Matkovic-Calogovic, D., Loregian, A., D'Acunto, M. R., Battistutta, R., Tossi, A., Palu, G., and Zanotti, G. (1999) Crystal structure of the B subunit of *Escherichia coli* heat labile enterotoxin carrying peptides with anti-Herpes simplex virus type 1 activity. *J. Biol. Chem.* 274, 8764–8769.
- (28) Kakio, A., Nishimoto, S., Yanagisawa, K., Kozutsumi, Y., and Matuzaki, K. (2002) Interaction of amyloid β -protein with various gangliosides in raft-like membranes: importance of G_{M1} ganglioside-bound form as an endogenous seed for alzheimer amyloid. *Biochemistry* 41, 7385–7390.
- (29) Champagne, M. J., Lamontagne, S., and Potier, M. (1994) Binding of G_{M1} -ganglioside to a synthetic peptide derived from the lysosomal sphingolipid-activator-protein saposin B. *FEBS Lett.* 347, 265–267.
- (30) Tseng, S. F., Deibler, G. E., and DeVries, G. H. (1999) Myelin basic protein and myelin basic protein peptides induce the proliferation of schwann cells via ganglioside G_{M1} and the FGF receptor. *Neurochem. Res.* 24, 255–260.
- (31) Nariya, H., Izaki, K., and Kamio, Y. (1993) The C-terminal region of the S component of staphylococcal leukocidin is essential for the biological activity of the toxin. *FEBS Lett.* 329, 219–222.

BC0602376