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Biochemical and structural characterization of the novel sialic acid-binding site of *Escherichia coli* heat-labile enterotoxin LT-IIb

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ABSTRACT

The structurally related AB₅-type heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae* are classified into two major types. The type I group includes cholera toxin and *E. coli* LT-I, while the type II subfamily comprises LT-IIa, LT-IIb and LT-IIc. The carbohydrate binding specificities of LT-IIa, LT-IIb and LT-IIc are distinctive from those of cholera toxin and *E. coli* LT-I. While CT and LT-I bind primarily to the GM1 ganglioside, LT-IIa binds to gangliosides GD1a, GD1b, and GM1, LT-IIb binds only to the GD1a ganglioside, and LT-IIc binds to GM1, GM2, GM3 and GD1a. Previous studies of the binding properties of type II B-subunits have been focused on ganglio core chain gangliosides. To further define the carbohydrate binding specificity of LT-IIb B-subunits, we have herein investigated its binding to a collection of gangliosides and non-acid glycosphingolipids with different core chains. A high affinity binding of LT-IIb B-subunits to gangliosides with neolacto core chain, as *e.g.* NeuGc α 3- and NeuAc α 3-neolactohexaosylceramide, and NeuGc α 3- and NeuAc α 3-neolactooctaosylceramide was detected. An LT-IIb binding ganglioside was isolated from human small intestine and characterized as NeuAc α 3-neolactohexaosylceramide. The crystal structure of B-subunit of LT-IIb with the pentasaccharide moiety of NeuAc α 3-neolactotetraosylceramide (NeuAc-nLT: NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc) was determined providing the first information for sialic binding site in this sub-family, with clear differences with the one from CT and LT-I.

Summary statement (25 words)

LT-IIb, a toxin from diarrhea-causing *Escherichia coli* binds to sialylated glycoconjugates on target tissues, and present only partial similarity with the cholera toxin.

Short title

Novel carbohydrate-binding site of *E. coli* heat-labile enterotoxin LT-IIb (74 characters)

Keywords

Toxin B-subunit, carbohydrate-binding site, sialic acid binding.

Abbreviations

CT, cholera toxin; ETEC, enterotoxigenic *Escherichia coli*; LC-ESI/MS; liquid chromatography/electrospray ionization mass spectrometry; LTs, *E. coli* heat-labile enterotoxins; LT-I, *E. coli* heat-labile enterotoxin type I; LT-IIa, *E. coli* heat-labile enterotoxin type IIa; LT-IIb, *E. coli* heat-labile enterotoxin type IIb; LT-IIc, *E. coli* heat-labile enterotoxin type IIc; NeuAc-nLT: NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc; RT, room temperature.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is the cause of diarrheal diseases leading to several hundred million cases of diarrhea and several ten of thousand deaths annually [1]. The diarrhea is mainly due to the action of enterotoxins released from the bacteria. ETEC produces both heat-stable and heat-labile (LTs) enterotoxins. The LTs belong to AB₅ toxins, a family of oligomeric proteins consisting of an A-subunit with catalytic activity and a pentameric B-subunit responsible for the binding to host glycoconjugates [2, 3]. ETEC enterotoxins together with cholera toxin (CT) constitute one of the four subgroups of AB₅ toxins [3].

The family of ETEC enterotoxins is divided into type I and type II, where the type I B-subunits are most related to the CT B-subunits. There are three members of the ETEC type II heat-labile enterotoxins: LT-IIa, LT-IIb, and LT-IIc. The amino acid sequences of these A-subunits have only approximately 50-60% identity with the A-subunits of CT/LT type I, and there is no amino acid homology between the type II B-subunits and CT/LT type I B-subunits [4].

LTs and CT receptors are mainly gangliosides, a subclass of glycosphingolipids containing one or several sialic acid residues. Glycosphingolipids are classified on the basis of their carbohydrate core structures. In humans the lacto/type 1 (Gal β 3GlcNAc), neolacto/type 2 (Gal β 4GlcNAc), and globo/type 4 (Gal α 4Gal) core chains are the most common in non-acid glycosphingolipids, while gangliosides are mainly based on ganglio (Gal β 3GalNAc) or neolacto core chains. The primary receptor of B-subunits of CT and LT type I is the GM1 ganglioside [5], although LT type I also binds to glycoconjugates with terminal *N*-

acetyllactosamine [6, 7]. Binding of type II enterotoxin B-subunits have hitherto only been tested on gangliosides with ganglio core chain, demonstrating that LT-IIa binds to a range of such gangliosides, with highest affinity for the GD1b ganglioside, while LT-IIb interacts preferentially with gangliosides GD1a and GT1b [8, 9]. The gangliosides recognized by LT-IIc are GM1, GM2, GM3 and GD1a [10], with a preference for GM1 with long fatty acyl ceramide chain [9].

While a large amount of CT and LT type 1 structures bound to a variety of glycan receptors are available [11-14], the structural data on LT-IIb is limited [4, 15]. The overall structure of the LT-IIb holotoxin [4] closely resembles that of CT. Despite of the very limited sequence identity, the B-subunit of LT-IIb adopts a very similar fold as the B-subunits of CT and LT type 1. The putative ganglioside binding site of LT-IIb was proposed to be located in the same region as the GM1 binding sites of CT and LT type I [4].

In the present work, we further investigated the carbohydrate binding specificity of LT-IIb using our unique collection of gangliosides of various core chain series from different sources [16], and also a number of related non-acid glycosphingolipids. We also determined the first crystal structure of the B-subunit of LT-IIb with a ligand bound, namely with the pentasaccharide moiety of NeuAc α 3-neolactotetraosylceramide (NeuAc-nLT: NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc) establishing the molecular basis of the specificity and demonstrating the differences with the binding sites of LT-I/CT B-subunits.

EXPERIMENTAL PROCEDURES

Production of recombinant LT-IIb B-subunits

Recombinant LT-IIb B-subunits was produced from a synthetic gene purchased from ATG-Biosynthetics GmbH (Freiburg, Germany). This gene contained the entire coding sequence including the signal sequence. The signal peptide was removed by reverse PCR and the resulting plasmid was pML-LTB2b Δ s λ cI857. Induction of expression in *E. coli* strain BL21 gave rise to inclusion bodies containing the LT-IIb gene product.

25 mL cultures of *E. coli* BL21 carrying pML-LTB2b Δ s λ cI857 were grown in 250 mL Erlenmeyer flasks in LB broth supplemented with 100 μ g/ml ampicillin overnight at 30 °C with shaking at 180 rpm. 5 mL aliquots of this culture were used to inoculate 500 mL fresh LB broth supplemented with 100 μ g/ml ampicillin in 2L Erlenmeyer flasks. The flasks were incubated for 2 h at 30 °C with shaking before induction of LT-IIb B-subunit expression by raising the temperature to 42 °C. The cultures were incubated for a further 3-4 h, and thereafter cells were harvested by centrifugation at 13,000 x g for 20 min, and the pellet was stored at -20 °C.

Cells were resuspended in Tris-HCl, 50 mM, pH 8, and lysed by treatment with lysozyme (100 mg/ml) and EDTA (1 mM), at room temperature (RT) for 30 min. MgCl₂ was added to a final concentration of 10 mM followed by a small amount of DNase (Roche). The mixture was incubated for 15 min at RT and a protease inhibitor cocktail was added at a concentration recommended by the manufacturer (Complete, Roche). Cells were disrupted by sonication on ice for 4-5 min with 2 s pulses at 2 s intervals at 60% amplitude, and the inclusion bodies harvested by centrifugation (4,000 x g, 7 min). The pellet was washed with ice cold phosphate-buffered saline, pH 7.3 (PBS), containing 0.1 % Triton X114 and re-suspended in

minimal volume of double deionized water. Urea solution (6.5 M) was added and left overnight with stirring at 4 °C. Undissolved debris was then removed by centrifugation at 48,000 x g for 30 min. The protein was refolded by dialysis overnight at 4 °C against 2 L of 50 mM Carbonate buffer (pH 9) containing 1 M Urea, followed by further extensive dialysis against the same buffer containing no urea. Insoluble debris was then removed by centrifugation (48,000 x g, 30 min). The supernatant was sterilized by filtration through a 0,22 µm filter. Assembled protein was purified by ion exchange followed size exclusion chromatography.

The protein preparations were analysed on 14 % Tris-Glycine gel (Invitrogen, Carlsbad, CA), according to the instructions of the manufacturer, and stained using Coomassie Brilliant Blue R-250.

Thermal shift assays

LT-IIb B-subunit samples were diluted to a final protein concentration of 0.1 mg/mL and mixed with SYPRO Orange (5x) in 25 µl. The samples were subjected to thermal denaturation using a Real Time PCR machine with a temperature gradient starting at from 25 and ascending to 100 °C at a heating rate of 1 °C/min. Protein unfolding was followed by the increase of the fluorescence values given by the SYPRO Orange probe. Datapoints are from the experimental values and all curves were integrated according to the previously described protocol [17].

Radiolabeling

Aliquots of 100 µg of B-subunits were labeled with ^{125}I , using Na^{125}I (100 µCi/ml; Amersham Pharmacia Biotech, Little Chalfont, U.K.), according to the IODO-GEN protocol of the manufacturer (Pierce, Rockford, IL), giving approximately 5×10^3 cpm/µg protein.

Reference glycosphingolipids

Total acid and neutral glycosphingolipid fractions were obtained by standard procedures [18]. The individual glycosphingolipids were isolated by repeated chromatography on silicic acid columns of the native glycosphingolipid fractions, or acetylated derivatives thereof. The identity of the purified glycosphingolipids was confirmed by mass spectrometry [19, 20] and proton NMR spectroscopy [21].

Thin-layer chromatography

Precoated silica gel 60 HPTLC plates with either a glass or aluminium support (Merck, Darmstadt, Germany) were used for thin-layer chromatography. The solvent system used for separation of glycosphingolipids was chloroform/methanol/water 60:35:8 (by volume). Glycosphingolipids were detected by the anisaldehyde reagent [22] or the resorcinol reagent [23].

Chromatogram binding assay

Binding of radiolabeled LT-IIb B-subunits to glycosphingolipids separated on thin-layer chromatograms was performed as described [7]. In short, 20 - 40 µg of mixtures of glycosphingolipids, or 20 ng - 4 µg of pure compounds, were separated on aluminium-backed thin-layer plates. Dried chromatograms were dipped for 1 min in diethylether/*n*-hexane (1:5, by volume) containing 0.5% (w/v) polyisobutylmethacrylate (Aldrich Chem. Comp. Inc., Milwaukee, WI). After drying, the chromatograms were soaked in PBS, containing 2%

bovine serum albumin and 0.1% NaN₃ (BSA/PBS), for 2 h at RT. Thereafter the plates were incubated with ¹²⁵I-labeled B-subunits diluted in BSA/PBS (1-2 x 10⁶ cpm/ml) for another 2 h at RT. After washing six times with PBS, and drying, the thin-layer plates were autoradiographed for 12 h using XAR-5 x-ray films (Eastman Kodak, Rochester, NY).

Isolation of human small intestinal gangliosides recognized by LT-IIb B-subunits

One total acid glycosphingolipid fraction from human small intestine (675 mg) was separated by repeated silicic acid chromatography, and followed by repeated chromatography on Iatrobeds (Iatrobeds 6RS-8060; Iatron Laboratories, Tokyo) columns, eluted with chloroform/methanol/water 60:35:8 (by volume), and finally chloroform/methanol/water 40:40:12 (by volume). Throughout the separation procedures, aliquots of the fractions obtained were analyzed by thin-layer chromatography, and fractions that were coloured green by anisaldehyde were tested for binding of LT-IIb using the chromatogram binding assay. The fractions were pooled according to the mobility on thin-layer chromatograms and their LT-IIb binding activity.

LC-ESI/MS of the LT-IIb binding acid glycosphingolipid fractions from human small intestine

Aliquots (20 µg) of ganglioside fractions A-1 and A-2 from human small intestine were dissolved in methanol:acetonitrile 75:25 (by volume), and separated on a 200x0.150 mm column, packed in-house with 5 µm polyamine II particles (YMC Europe GmbH, Dinslaken, Germany). An autosampler, HTC-PAL (CTC Analytics AG, Zwingen, Switzerland) equipped with a cheminert valve (0.25 mm bore) and a 2 µl loop was used for sample injection. An Agilent 1100 binary pump (Agilent technologies, Palo Alto, CA) delivered a flow of 250 µl/min, which was split down in an 1/16" microvolume-T (0.15 mm bore) (Vici AG

International, Schenk, Switzerland) by a 50 cm x 50 μ m i.d. fused silica capillary before the injector of the autosampler, allowing approximately 2-3 μ l/min through the column. Samples were eluted with an aqueous gradient (A:100% acetonitrile to B: 10 mM ammonium bicarbonate). The gradient (0-50% B) was eluted for 40 min, followed by a wash step with 100% B, and equilibration of the column for 20 min. The samples were analyzed in negative ion mode on a LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron, San José, CA), with an IonMax standard ESI source equipped with a stainless steel needle kept at -3.5 kV. Compressed air was used as nebulizer gas. The heated capillary was kept at 270 °C, and the capillary voltage was -50 kV. Full scan (m/z 500-1800, two microscan, maximum 100 ms, target value of 30,000) was performed, followed by data-dependent MS² scans (two microscans, maximum 100 ms, target value of 10,000) with normalized collision energy of 35%, isolation window of 2.5 units, activation $q=0.25$ and activation time 30 ms). The threshold for MS² was set to 500 counts.

Data acquisition and processing were conducted with Xcalibur software (Version 2.0.7). Manual assignment of glycosphingolipid sequences was done with the assistance of the Glycoworkbench tool (Version 2.1), and by comparison of retention times and MS² spectra of reference glycosphingolipids.

Crystallization and data collection

A solution of LT-IIb B-subunit (10 mg ml⁻¹) in sodium carbonate 50 mM pH 9 was sent to the High Throughput Crystallization laboratory (HTXlab, EMBL Grenoble, France) to screen for the best crystallization conditions. The protein was tested against the JCSG (Qiagen), Wizard I and II (Rigaku reagents), PACT (Qiagen), PEGS I (Qiagen), and plates 4 and 5 (Hampton) screens. The crystallization conditions that resulted in good quality crystals were reproduced

in house with the addition of 11 mM NeuAc-nLT (Sialyl-Lacto-N-tetraose from Elicityl, Crolles, France) to the crystallization buffer. After optimization, high quality crystals were obtained in 0.2 M KCl, 20% w/v PEG 3350 and 0.1 M sodium cacodylate pH 6. Diffraction data for the LT-IIb/NeuAc-nLT complex was collected to 1.72 Å on ID23-2 beamline using a Pilatus detector at the ESRF, Grenoble. Diffraction images were integrated using XDS program [24], and all further processing done using the CCP4 program suite [25].

Structure determination and refinement

The structure was solved by molecular replacement with PHASER [26] using the coordinates from the B-pentamer of the LT-IIb structure (PDB ID 1TII) as search model. The initial model was optimized using ARP/wARP [27] prior restrained maximum likelihood refinement with REFMAC 5.8 [28] iterated with manual rebuilding in Coot [29]. Five percent of the observations were set aside for cross-validation analysis, and hydrogen atoms were added in their riding positions and used for geometry and structure-factor calculations. Incorporation of the ligand was performed after inspection of the $2F_o - F_c$ weighted maps. Water molecules, introduced automatically using Coot, were inspected manually. The quality of the models was assessed using the PDB validation server (<http://wwpdb-validation.wwpdb.org/validservice/>) and coordinates were deposited in the Protein Data Bank under code 5G3L.

RESULTS

Recombinant expression and purification of LT-IIb B-subunits

The LT-IIb B-subunit was isolated in high yield, *i.e.* 30 mg of protein was obtained from 2 L culture medium. The purified protein migrated as a single band, and the apparent molecular weight of the monomeric protein was in agreement with the predicted molecular mass of

~11.8 kDa (Figure S1 in Suppl. Mat., lanes 3-5). The pentameric form of the LT-IIb B-subunit appears to run at a molecular weight of approximately 50 kDa in agreement with the predicted molecular mass (Figure S1 in Suppl. Mat., lanes 7-9). The protein was stable in several of the solvents tested, allowing us to store it in the refrigerator at 4 °C for weeks, without seeing a change in its activity. The protein thermostability and its buffer preference was determined using thermal shift assays, showing the highest T_m , of 77 °C in HEPES buffer pH 8.5 with 20 mM NaCl (Figure S2 in Suppl. Mat.).

Binding of LT-IIb B-subunits to mixtures of gangliosides

First the binding of radiolabeled LT-IIb B-subunits to mixtures of acid and non-acid glycosphingolipid fractions from various sources was tested. Thereby a distinct binding to some slow-migrating compounds in the acid fractions from rabbit thymus (Figure 1B, lane 1) and human neutrophils (Figure 1B, lane 2) was obtained. The binding-active gangliosides migrated at the level of sialyl-neolactotetraosylceramide (Figure 1, lane 7) and sialyl-neolactohexaosylceramide (Figure 1 lane 8), both of which were also recognized by the B-subunits. Taken together, this suggested a recognition of gangliosides with neolacto core chains, since this core chain is predominant among the gangliosides of both rabbit thymus and human neutrophils [30-33]. Binding of LT-IIb B-subunits to a minor slow-migrating compound in the acid fractions of human small intestine was also observed (Figure 1B, lanes 3-5).

Binding of LT-IIb B-subunits to reference gangliosides

Next, the binding of radiolabeled LT-IIb B-subunits to a collection of variant gangliosides [18] was examined (Figure 2 and Figures S3 and S4 in Suppl. Mat.). The results of the binding assays are summarized in Table 1). As reported previously [8, 9] the B-subunits

bound to the GD1a ganglioside, while no binding to the GD1b or GT1b gangliosides was obtained.

In addition, the B-subunits of LT-IIb bound to a number of gangliosides with neolacto core, as NeuAc α 3- and NeuGc α 3-neolactotetraosylceramide, NeuAc α 3- and NeuGc α 3-neolactohexaosylceramide, NeuAc α 3- and NeuGc α 3-neolactooctaosylceramide, the G-10 ganglioside and the G9-B ganglioside. Binding to the VIM-2 ganglioside was also occasionally obtained. However, several gangliosides with neolacto core were not recognized. Thus, a terminal disialyl unit, as in NeuAc α 8NeuAc α 3-neolactotetraosylceramide was not tolerated, and the gangliosides with terminal α 6-linked sialic acid (NeuAc α 6-neolactotetraosylceramide and NeuAc α 6-neolactohexaosylceramide) were not recognized. Furthermore, no binding to gangliosides with a sialyl-Le^x epitope occurred. Other non-binding compounds were the gangliosides GM1, GM3, GM2, GD3 and NeuAc α 3-globotetraosylceramide (not shown). No binding of LT-IIb to non-acid glycosphingolipids as *e.g.* neolactotetraosylceramide or gangliotetraosylceramide occurred, demonstrating that the terminal α 3-linked sialic acid of NeuAc α 3-/NeuGc α 3-neolactotetraosylceramide and the GD1a ganglioside, is necessary for for LT-IIb carbohydrate binding.

Binding of LT-IIb B-subunits to dilutions of gangliosides

Dilutions of gangliosides on thin-layer chromatograms were used for estimation of the relative binding affinities of LT-IIb for the binding active gangliosides. Here. NeuGc α 3-neolactohexaosylceramide was the preferred ligand with a detection limit at less than 20 ng (Figure S5B, lane 10), while the detection limits for NeuAc α 3-neolactotetraosylceramide and the GD1a ganglioside were approximately 800 ng (Figure S5A, lane 3).

Human small intestinal ganglioside recognized by LT-IIb B-subunits

The slow-migrating LT-IIb B-subunit binding ganglioside of human small intestine was isolated by repeated chromatographies on silicic acid and Iatrobeds columns, and the preparative procedure was monitored by binding of radiolabeled LT-IIb B-subunits on thin-layer chromatograms. Finally, two LT-IIb binding fractions were obtained and designated fractions A-1 and A-2 (Figure 3B, lanes 1 and 2), while the following non-binding fractions were designated A-3 and A-4 (Figure 3B, lanes 3 and 4).

LC-ESI/MS of the human small intestinal ganglioside recognized by LT-IIb B-subunits

The base peak chromatogram obtained by LC-ESI/MS of the LT-IIb binding ganglioside fraction A-2 from human small intestine had two $[M-2H^+]^{2-}$ ions at m/z 721 and m/z 941, respectively (not shown). The major $[M-2H^+]^{2-}$ ion at m/z 721 corresponded to a $[M-H^+]$ ion at m/z 1442, indicating a ganglioside with two NeuAc, two Hex, and d18:1-16:0 ceramide, and the mass spectrum obtained by MS² spectrum of the ion at m/z 721 was characteristic for the GD3 ganglioside (data not shown).

The $[M-2H^+]^{2-}$ ion at m/z 941 corresponded to a $[M-H^+]$ ion at m/z 1882, and indicated a ganglioside with one NeuAc, two HexNAc, four Hex, and d18:1-16:0 ceramide. MS² of the $[M-2H^+]^{2-}$ ion at m/z 941 (Figure 3C) gave a series of Y ions (Y_0 at m/z 536, Y_1 at m/z 698, Y_2 at m/z 860, Y_3 at m/z 1063, Y_4 at m/z 1225, Y_5 at m/z 1428, and Y_6 at m/z 1590), which along with the B- and C-type ions, demonstrated a ganglioside with NeuAc-Hex-HexNAc-Hex-HexNAc-Hex-Hex sequence and d18:1-16:0 ceramide. Thus, LC-ESI/MS allowed identification of NeuAc α 3-neolactohexaosylceramide in the LT-IIb binding ganglioside fraction isolated from human small intestine.

Crystal structure of LT-IIb in complex with Sialyl-lacto-N-tetraose

LT-IIb B-subunit gave crystals between one day to one week with either needle or parallelepipedic morphology, under different conditions (see Supplementary Table S1). In a second step, crystals of LT-IIb B-subunits were optimized manually using 20% peg 3350, 0.2M KCl and 0.1 M buffer pH 6.0-7.6 and the protein was co-crystallized with NeuAc-nLT. Data were collected at 1.72 Å resolution and indexed in the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 48.56$, $b = 68.02$, $c = 154.52$ Å. The Matthews coefficient [34] indicated the presence of five monomers in the asymmetric unit. The complex structure was solved by molecular replacement with a search for one pentamer using coordinates of PDB 1TII as model [4]. X-ray data collection statistics are shown in Supplementary Table S2. Monomers have been labelled D to H to follow classical AB_5 nomenclature. The monomers forming the B pentamer are almost identical, with root mean square (rmsd) values between 0.24 and 0.14 Å for the 98 equivalent C α . The pentamer is very similar to the one observed for the apo LT-IIb structure used as model with a rmsd of 0.35 Å for the 484 equivalent C α .

Electron density corresponding to carbohydrate ligands was observed in the protein crystal structure. Four of these ligands are positioned in the groove formed between loops of region 12 – 16 of one monomer and region 29 – 36 of an adjacent one (Figure 4). The Neu5Ac α 2-3Gal fragment of the NeuAc-nLT ligand could be modeled in the binding sites of monomers D, G, and H, whereas only the sialic acid moiety could be modelled in monomer E. The rest of the ligand was too disordered to be modelled since it was in the solvent area. No density was observed in the binding site of monomer F as a result of crystal contacts that would lead to steric conflict with the sialic acid moiety. Additional electron density could also identify a

sialic acid residue in a non-canonical binding site on the surface of monomer G, not far from the occupied binding site in the same monomer (Figure 4).

The groove between γ and q monomers corresponds to the expected binding site since proteins with OB-fold (a five-stranded closed β barrel) use the same face and regions to bind saccharide or nucleotides [35]. Nevertheless, the binding mode is completely different to the one observed for GM1 in LT-I or CT. Since the binding mode is very similar in the four occupied sites only monomer D is described in continuation. The terminal Neu5Ac residue of NeuAc-nLT establishes most contacts. The pyranose ring of NeuAc is sandwiched between the indole ring of Trp92_D and the side chain of Asn32_E, being stabilized by hydrophobic contacts (Figure 5A). Another hydrophobic residue, Ile30_E, faces the methyl of the *N*-acetylamino group. The oxygen atom at C4 and the *N*-acetyl group form direct and water-mediated H-bonds with the Asn31_E and Asn32_E residues. The carboxylate group of NeuAc receives hydrogen bonds from both the side chain hydroxyl group and the main chain amine of Thr14_D, whereas the deoxy carbon at position 2 of the ring makes an hydrophobic contact with the methyl group of Thr13_D. The galactose residue presents very limited contact with the protein surface, with only one water bridged hydrogen bond from O4 oxygen to Arg12 in monomers G and H and to neighboring Asn32 in monomer D. These contacts differ due to the two different conformations of the ligand (Supplementary Figure S6). The NeuAc-nLT ligand in positions G and H adopts similar conformations, with dihedral angles of ϕ (O-C₂-O₂-C₃') = 47 and 64°, and ψ (C₂-O₂-C₃'-C₄') = 112° and 103° at the NeuAc α 2-3Gal linkage, whereas a different conformation is seen for monomer D (ϕ = -69° and ψ = 110° [36]).

The fifth ligand molecule is observed interacting with monomer G of LT-IIb B-subunit in an alternative position. Only the NeuAc residue could be modeled in the electron density. The

residue is lying flat in a very shallow domain near the canonical site of monomer G (Figure 5). The carboxylate groups establish hydrogen bonds with side chains of Arg51_G and Tyr55_F of the neighboring monomer helix, while the glycerol side chains interact with backbone nitrogen of Lys53_D and Asp54_D and with side chain of Tyr55_D. Water molecules establish several bridged hydrogen bonds, including with the neighboring oligosaccharide in the canonical binding site of monomer G.

Due to the divergent orientation of the anomeric oxygen residues, our attempt to model a single branched disialylated oligosaccharide, like the GD1a oligosaccharide (NeuAc α 3Gal β 3GalNAc β 4(NeuAc α 3)Gal β 4Glc), with one sialic acid in each site was not successful. However this hypothesis cannot be completely discarded.

DISCUSSION AND CONCLUSION

In this study, we have expressed a recombinant LT-IIb B-subunit and investigated the carbohydrate binding specificity by means of solid phase glycosphingolipid binding studies. By binding to a variety of glycosphingolipids separated on thin-layer chromatograms, we found that LT-IIb B-subunits bind with high affinity to gangliosides with neolacto core chain as *e.g.* NeuGc α 3- and NeuAc α 3-neolacto-hexa- and neolactooctaosylceramide. Previous studies of the carbohydrate recognition by LT-IIb were done using a limited number of gangliosides with ganglio core chain, which explains why the LT-IIb binding to neolacto based gangliosides was overlooked. Furthermore, by isolating LT-IIb binding gangliosides from human small intestine, we established the link between the NeuAc-neolacto gangliosides

presence on the target tissue and the LT-IIb infection of intestinal cells, where the protein recognition of these antigens promotes the initial steps of the disease.

Lymphocytes are also target cells of LT-IIb, since this protein is a potent adjuvant. Interestingly, the immune responses induced by LT-IIa and LT-IIb are distinctive from those induced by CT or LT-I [37]. While CT and LT-I induce Th2 responses to co-administered antigens, LT-IIa and LT-IIb give a more balanced Th1/Th2 response. These differences have been attributed to the binding of the B-subunits to different ganglioside receptors on lymphocytes [38]. The available data about human lymphocyte gangliosides structures is very limited. However, NeuAc-GM3 is the major ganglioside of both peripheral blood lymphocytes and monocytes, and these cells also have NeuAc-neolactotetraosylceramide [39, 40]. Thus, the identified NeuAc α 3-neolacto binding of LT-IIb may also have a role in the immunomodulatory activities of the protein.

The crystal structure of LT-IIb was solved in complex with the carbohydrate moiety of the newly identified ligand NeuAc-neolactotetraosylceramide. Due to the high flexibility of this linear pentasaccharide, only the terminal Neu5Ac α 2-3Gal disaccharide was observed in most binding sites. Sialic acid binds in the groove that was predicted to be the binding site, based on the *i*) overall fold similarity of OB proteins; *ii*) surface analysis of the crystal structure of the apo-protein; and *iii*) absence of ganglioside binding in mutants affecting the loop in region 13 and 14 lining the groove [15]. Indeed Thr13 and Thr14 were found critical of GD1a affinity [41], and we demonstrate here that the hydroxyl side chain of Thr14 gives a strong hydrogen bond to the sialic acid carboxylate group, with the methyl group of Thr13 making also hydrophobic contacts with the CH₂ at position 2 of NeuAc ring.

While the NeuAc binding site in LT-IIb is similarly positioned as those observed in CT and LT-I [3] the orientation of the glycan ligand (and therefore the network of contacts) is entirely different (Figure 6 and Figure S7 in Suppl. Mat.). The NeuAc in LT-IIb is rotated about 120° compared to the one in CT, accompanied with a sliding of the ring. The conformation of GM1 in CT could not be accommodated in LT-IIb binding site due to the conformation adopted by loop 30-35 (31-36 in CT). This loop conformation makes the binding site tighter in that position when compared with CT, efficiently tucking the NeuAc residue in that particular orientation. There are considerable differences - size and conformation wise - between loops 50-54 of LT-IIb and the corresponding (51-60) in CT. In CT, this loop is considerably bigger forming a second wall of a trench/gate, where the other end of the GM1 interacts.

A secondary binding site with affinity for blood group determinants has been identified on the side of the β -barrel of CT and LT-I B-subunits [14, 42]. However, a secondary binding site in the vicinity of the primary site, and accommodating the same ligand, has not previously been found in the B-subunits of *V. cholerae* or ETEC. In this respect LT-IIb resembles the Shiga-like toxins from *E. coli* where there are three carbohydrate binding sites in each B-subunit, providing an increased binding affinity to the globotriasylceramide receptor [43, 44]. However, at the present time, it is not clear if the secondary binding site could accommodate the second NeuAc of a divalent ligand such as GD1a or if it would bind to different sialylated glycolipids on cell surfaces. Our attempts to fit the two NeuAc residues of a single GD1a oligosaccharides in the two sites were not successful, so the second hypothesis seems to be more likely.

AUTHOR CONTRIBUTIONS

Dani Zalem, João P. Ribeiro and Annabelle Varrot carried out the experimental work. All authors contributed to the design of the experimental plans, and analyzed the data. Michael Lebens, João P. Ribeiro, Anne Imberty and Susann Teneberg wrote the manuscript.

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FIGURE LEGENDS

Figure 1. Binding of LT-IIb B-subunits to mixtures of gangliosides. Thin-layer chromatogram after detection with anisaldehyde (A), and autoradiogram obtained by binding of radiolabeled LT-IIb B-subunits (B). The chromatograms were eluted with chloroform/methanol/water 60:35:8 (by volume), and the binding assays were done as described under "Experimental procedures". The lanes were: Lane 1, Acid glycosphingolipids of rabbit thymus, 20 µg; Lane 2, Acid glycosphingolipids of human neutrophils, 40 µg; Lane 3, Acid glycosphingolipids of human small intestine (Individual I), 40 µg; Lane 4, Acid glycosphingolipids of human small intestine (Individual II), 40 µg; Lane 5, Acid glycosphingolipids of human small intestine (Individual III), 40 µg; Lane 6, Reference GM3 ganglioside (NeuAcα3Galβ4Glcβ1Cer), 4 µg; Lane 7, Reference NeuAcα3-neolactotetraosylceramide (NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 4 µg; Lane 8, Reference NeuGcα3-neolactohexaosylceramide (NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 2 µg.

Figure 2. Binding of LT-IIb B-subunits to reference gangliosides. Thin-layer chromatograms after detection with anisaldehyde (A), and autoradiogram obtained by binding of radiolabeled LT-IIb B-subunits (B). The chromatograms were eluted with chloroform/methanol/water 60:35:8 (by volume), and the binding assays were done as described under "Experimental procedures". The lanes were: Lane 1, NeuGc α 3-neolactoheptaosylceramide (NeuGc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 1 μ g; Lane 2, G9-B ganglioside (Gal α 3(Fuc α 2)Gal β 4GlcNAc β 6(NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 3, NeuAc-GD1a (NeuAc α 3Gal β 3GalNAc β 4(NeuAc α 3)Gal β 4Glc β 1Cer), 4 μ g; Lane 4, NeuAc-GD1b (Gal β 3GalNAc β 4(NeuAc α 8NeuAc α 3)Gal β 4Glc β 1Cer), 4 μ g; Lane 5, NeuAc-GT1b (NeuAc α 3Gal β 3GalNAc β 4(NeuAc α 8NeuAc α 3)Gal β 4Glc β 1Cer), 4 μ g.

Figure 3. Characterization of LT-IIb binding gangliosides from human small intestine (fraction A-2). (A and B) Binding of LT-IIb B-subunits to human small intestinal gangliosides. Thin-layer chromatogram after detection with anisaldehyde (A), and autoradiogram obtained by binding of radiolabeled LT-IIb B-subunits (B). The chromatograms were eluted with chloroform/methanol/water 60:35:8 (by volume), and the binding assays were done as described under "Experimental procedures". The lanes were: Lane 1, Fraction A-1 from human small intestine, 4 μ g; Lane 2, Fraction A-2 from human small intestine, 4 μ g; Lane 3, Fraction A-3 from human small intestine, 4 μ g; Lane 4, Fraction A-4 from human small intestine, 4 μ g; Lane 5, NeuGc α 3-neolactoheptaosylceramide (NeuGc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 1 μ g; Lane 6, NeuAc-GD1a (NeuAc α 3Gal β 3GalNAc β 4(NeuAc α 3)Gal β 4Glc β 1Cer), 4 μ g. (C) MS² of m/z 941 from LC-ESI/MS of fraction A-2. (D) Interpretation formula showing the deduced ganglioside sequence.

Figure 4. LT-IIb B pentamer with NeuAc-nLT bound. Overall representation of the pentamer of subunit B of LT-IIb with the monomers colored by chain. Display of $2F_o - F_c$ weighted electron density map electron density for the ligands at 1σ (0.41 eA^3)

Figure 5. Zoomed in view at the binding of NeuAc-nLT at monomer G. A : Cartoon representation of the primary and “secondary” binding sites of LT-IIb with the bound NeuAc-nLT, whose partial structure is shown in stick representation. The LT-IIb residues interacting with the ligand are displayed in green and the formed H-bonds as blue lines. Protein backbone is colored as a function of the monomer. Not all water molecules are shown for simplicity (*Note:* All highlighted residues belong to monomer G except for Tyr55_F). B: Surface representation of the surface highlighting the deep NeuAc primary binding site, and the shallow secondary one. Surfaces are colored according to protein monomer.

Figure 6. Comparison of fold and glycan binding site in CT and LT pentamers of B-subunit. Green : LT-IIb complexed with terminal NeuAc α 3-neolactoheptaosylceramide (this work), yellow LT-I and pink GM1 complexed with GM1 pentasaccharide (code 2XRQ and 3CHB respectively). All binding sites are displayed with same orientation of the protein beneath.

Fig. 1

A. Anisaldehyde detection

B. LT-IIb B-subunits

Fig. 2 A. Anisaldehyde
detection

B. LT-IIb
B-subunits

Fig. 3

A. Anisaldehyde detection

B. LT-IIb B-subunits

Fig. 4

Fig. 5

Fig. 6

Table 1. Glycosphingolipid binding of LT-IIb B-subunits

No. Trivial name	Structure	Binding	Source
<i>Simple compounds</i>			
1. NeuAc-GM3	NeuAc α 3Gal β 4Glc β 1Cer	- ^a	Human intestine
2. NeuAc-GD3	NeuAc α 8NeuAc α 3Gal β 4Glc β 1Cer	-	Bovine buttermilk
3. LacCer	Gal β 4Glc β 1Cer	-	Dog intestine
<i>Lacto/neolacto series</i>			
4. nLc ₄	Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Human neutrophils
5. nLc ₆	Gal β 4GlcNAc β 6(Gal β 4GlcNAc β 3)Gal β 4Glc β 1Cer	-	Bovine buttermilk
6. H type 2 penta	Fuc α 2Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Human erythrocytes
7. Le ^a penta	Gal β 3(Fuc α 4)GlcNAc β 3Gal β 4Glc β 1Cer	-	Human intestine
8. B type 2 hexa	Gal α 3(Fuc α 2)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Human erythrocytes
9. A type 1 hexa	GalNAc α 3(Fuc α 2)Gal β 3GlcNAc β 3Gal β 4Glc β 1Cer	-	Human intestine
10. Le ^b hexa	Fuc α 2Gal β 3(Fuc α 4)GlcNAc β 3Gal β 4Glc β 1Cer	-	Human meconium
11. B type 1 hepta	Gal α 3(Fuc α 2)Gal β 3(Fuc α 4)GlcNAc β 3Gal β 4Glc β 1Cer	-	Monkey intestine
12. NeuAc α 3nLc ₄	<u>NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer^b</u>	+	Human erythrocytes
13. NeuGc α 3nLc ₄	<u>NeuGcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer^b</u>	+	Piglet intestine
14. NeuAc α 6nLc ₄	NeuAc α 6Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Human meconium
15. NeuAc ₂ -nLc ₄	NeuAc α 8NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Human kidney
16. NeuAc α 3Lc ₄	NeuAc α 3Gal β 3GlcNAc β 3Gal β 4Glc β 1Cer	-	Human embryonic stem cells ^c
17. NeuAc α 3-Le ^x	<u>NeuAcα3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer</u>	-	Commercial
18. NeuAc-Sd ^a	GalNAc β 4(NeuAc α 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Moose intestine
19. NeuGc-Sd ^a	GalNAc β 4(<u>NeuGcα3</u>)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Moose intestine
20. NeuAc α 3-nLc ₆	<u>NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer</u>	+++	Human hepatoma
21. NeuGc α 3-nLc ₆	<u>NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer</u>	+++	Rabbit thymus
22. NeuAc α 3-nLc ₈	<u>NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer</u>	+++	Human erythrocytes
23. NeuGc α 3-nLc ₈	<u>NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer</u>	+++	Rabbit thymus
24. VIM-2	<u>NeuAcα3Galβ4GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer</u>	+	Human colon cancer
25. NeuAc α 3-Le ^x -nLc ₈	<u>NeuAcα3Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer</u>	-	Human pancreas cancer

26.	Gal β 4GlcNAc β 6(<u>NeuAcα6Galβ4GlcNAcβ3</u>)Gal β 4Glc β 1Cer	-	Bovine buttermilk
27.	Gal β 4GlcNAc β 6(<u>NeuAcα6Galβ4GlcNAcβ3</u>)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	-	Human meconium
28. NeuAc-G-10	<u>NeuAcα3Galβ4GlcNAcβ6</u> (<u>NeuAcα3Galβ4GlcNAcβ3</u>)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	+	Human erythrocytes
29. G9-B	Gal α 3(<u>Fucα2</u>)Gal β 4GlcNAc β 6(<u>NeuAcα3Galβ4GlcNAcβ3</u>)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer	+	
<hr/>			
Human erythrocytes			
<i>Ganglio series</i>			
30. GgO ₃	GalNAc β 4Gal β 4Glc β 1Cer	-	Guinea pig erythrocytes
31. GgO ₄	Gal β 3GalNAc β 4Gal β 4Glc β 1Cer	-	Mouse intestine
32. GM2	GalNAc β 4(<u>NeuAcα3</u>)Gal β 4Glc β 1Cer	-	Human brain
33. GM1	Gal β 3GalNAc β 4(<u>NeuAcα3</u>)Gal β 4Glc β 1Cer	-	Human brain
34. GD1a	<u>NeuAcα3Galβ3GalNAcβ4</u> (<u>NeuAcα3</u>)Gal β 4Glc β 1Cer	+++	Human brain
35. GD1b	Gal β 3GalNAc β 4(<u>NeuAcα8NeuAcα3</u>)Gal β 4Glc β 1Cer	-	Human brain
36. GT1b	<u>NeuAcα3Galβ3GalNAcβ4</u> (<u>NeuAcα8NeuAcα3</u>)Gal β 4Glc β 1Cer	-	Human brain
<hr/>			
<i>Globo series</i>			
37. Gb3	Gal α 4Gal β 4Glc β 1Cer	-	Human erythrocytes
38. Gb4	GalNAc β 3Gal α 4Gal β 4Glc β 1Cer	-	Human erythrocytes
39. Forssman	GalNAc α 3GalNAc β 3Gal α 4Gal β 4Glc β 1Cer	-	Dog intestine
40. NeuAc α 3-Gb4	NeuAc α 3GalNAc β 3Gal α 4Gal β 4Glc β 1Cer	-	Human embryonic stem cells ^c

a) Binding is defined as follows: +++ denotes an intense and highly reproducible staining when 4 μ g of the glycosphingolipid was applied on the thin-layer chromatogram, + denotes an occasional staining, while - denotes no binding even at 4 μ g.

b) The NeuAc/NeuGc α 3Gal β 4GlcNAc and NeuAc α 3Gal β 3GalNAc sequences are underlined.

c) Binding of LT-IIb to compounds 16 and 40 was evaluated using the slow-migrating ganglioside fraction from human embryonic stem cells (fraction 181C) [45].

SUPPLEMENTARY MATERIAL

Biochemical and structural characterization of the novel sialic acid-binding site of *Escherichia coli* heat-labile enterotoxin LT-IIb

Table S1. Successful crystallization conditions from assays at High Throughput Crystallization laboratory (HTXlab, Grenoble, France) using LT-IIb B-subunit (10 mg ml⁻¹) in sodium carbonate 50 mM pH 9.

Salt	pH, buffer	Precipitant (w/v)	Morphology
LiCl 1 M	Trisodium acetate 0.1M, pH 4	20% PEG 6000	Needle-like
LiCl 1 M	Citric acid 0.1M, pH 4	30% PEG 6000	
LiCl 1 M	Citric acid 0.1M, pH 5	30% PEG 6000	
MgCl ₂ 0.2M	Sodium cacodylate/ HCl 0.1M, pH 6.5	20% PEG 1000	Single crystal
Na ₂ HPO ₄ 0.2M	-	20% PEG 3350	
Na ₂ SO ₄ 0.2M	Bis-tris propane 0.1M	20% PEG 3350	
KCl 0.2M	-	20% PEG 3350	
KI 0.2M	-	20% PEG 3350	
KSCN 0.2M	-	20% PEG 3350	
K/Na tartare 0.2M	-	20% PEG 3350	
Trisodium citrate 0.2M	-	20% PEG 3350	
-	Citric acid 0.1M, pH 4	(NH ₄) ₂ SO ₄ 1.6M	

Table S2. X-ray data collection and refinement statistics

Data Collection					
Space group	P 2 ₁ 2 ₁ 2 ₁				
Cell dimensions a, b, c (Å), α, β, γ (deg)	48.56, 68.02, 154.51, 90.0, 90.0, 90.0				
Nb. reflections	271309				
Nb. Unique reflections	54271				
resolution (Å)	48.56 – 1.72 (1.75-1.72)				
R _{merge} (within I+/I-)	0.07 (0.47)				
I/σ(I) (within I+/I-)	2.89 (3.00)				
Completeness (%)	98.4 (96.4)				
redundancy	5.0 (4.5)				
Refinement					
Resolution (Å)	46.33 – 1.72				
Nb. Reflections	51509				
Nb free reflections	2713				
R _{work}	17.1				
R _{free}	19.6				
rms deviation bond lengths (Å) bond angles (deg)	0.018 1.714				
Chain	D	E	F	G	H
Nb Atoms protein	768	750	749	747	750
ligand	32	21	NA	53	32
water	143	117	100	119	110
B factor protein	17.6	17.1	16.9	16.7	15.5
ligand	31.6	34.9		30.6	21.0
water	27.8	27.4	26.2	28.3	29.1
Ramachandran Allowed	99				
Favored	100				
Outliers	0				
PDB Code	5G3L				

Figure S1. Purified recombinant LT-IIb B-subunits. LT-IIb B-subunit preparation separated on a 14 % Tris-Glycine gel and stained using Coomassie Brilliant Blue R-250. Lane 1, molecular mass standards; lane 2, boiled CTB; lane 3, boiled LTB-IIb; lane 4, boiled LTB-IIb; lane 5, boiled LTB-IIb; lane 6, unboiled CTB; lane 7, unboiled LTB-IIb; lane 8, unboiled LTB-IIb; lane 9, unboiled LTB-IIb. When the pentamer is boiled, it denatures into monomers, with a predicted molecular mass of ~11.8 kDa

Figure S2. Thermal shift assay (TSA) data for LT-IIb B-subunit (derived curved). Two inflections are seen. The first regards the pentamer dissociation (around 46 °C) while the second represents the monomer unfolding, which takes place at higher temperatures. A small T_m variation (72 – 77 °C) is seen in these tested conditions. The data for the protein in water pH 7.3 solution is shown in a light blue color; water + 20mM NaCl, in dark blue; HEPES pH 8.5, in pink; and HEPES + 20mM NaCl, in grey.

Figure S3. Binding of LT-IIb B-subunits to reference gangliosides . Thin-layer chromatograms after detection with resorcinol (A), and autoradiogram obtained by binding of radiolabeled LT-IIb B-subunits (B). The chromatograms were eluted with chloroform/methanol/water 60:35:8 (by volume), and the binding assays were done as described under "Experimental procedures". The lanes were: Lane 1, NeuAc-GD1a (NeuAc α 3Gal β 3GalNAc β 4(NeuAc α 3)Gal β 4Glc β 1Cer), 4 μ g; Lane 2, NeuGc α 3-neolactohexaosylceramide (NeuGc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 3, NeuAc-G-10 ganglioside (NeuAc α 3Gal β 4GlcNAc β 6(NeuAc α 3Gal β 4GlcNAc β 3)Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 4, NeuGc α 3-neolactohexaosylceramide (NeuGc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), and NeuGc α 3-neolactooctaosylceramide (NeuGc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 5, NeuAc α 3-neolactooctaosylceramide (NeuAc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 6, VIM-2 ganglioside (NeuAc α 3Gal β 4GlcNAc β 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 7, NeuAc α 3-Le^x-neolactooctaosylceramide (NeuAc α 3Gal β 4(Fuc α 3)GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 8, NeuAc α 6-neolactohexaosylceramide (Gal β 4GlcNAc β 6(NeuAc α 6Gal β 4GlcNAc β 3)Gal β 4Glc β 1Cer), 4 μ g.

A. Resorcinol detection

1 2 3 4 5 6 7 8

B. LT-IIb B-subunits

1 2 3 4 5 6 7 8

Figure S4. Binding of LT-IIb B-subunits to reference gangliosides. Thin-layer chromatograms after detection with resorcinol (A), and autoradiogram obtained by binding of radiolabeled LT-IIb B-subunits (B). The chromatograms were eluted with chloroform/methanol/water 60:35:8 (by volume), and the binding assays were done as described under "Experimental procedures". The lanes were: Lane 1, NeuAc α 3-neolactotetraosylceramide (NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 2, NeuGc α 3-neolactotetraosylceramide (NeuGc α 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 3, NeuAc α 6-neolactotetraosylceramide (NeuAc α 6Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 4, GM1 ganglioside (Gal β 3GalNAc β 4(NeuAc α 3)Gal β 4Glc β 1Cer), 4 μ g, and NeuAc α 8NeuAc α 3-neolactotetraosylceramide (NeuAc α 8NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 5, NeuAc α 3-Le^x (NeuAc α 3Gal β 4(Fuca α 3)GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; lane 6, NeuGc α 3-neolactoheptaosylceramide (NeuGc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g.

A. Resorcinol detection

B. LT-IIb B-subunits

Figure S5. Binding of LT-IIb B-subunits to dilutions of gangliosides. The chromatograms were eluted with chloroform/methanol/water 60:35:8 (by volume), and the binding assay was done as described under "Experimental procedures". The lanes on (A) were: Lane 1, NeuAc α 3-neolactotetraosylceramide (NeuAc α 3-nLc4, NeuAc α 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), and GD1a ganglioside (NeuAc α 3Gal β 3GalNAc β 4(NeuAc α 3)Gal β 4Glc β 1Cer), 4 μ g each; Lane 2, NeuAc α 3-nLc4 and GD1a, 2 μ g each; Lane 3, NeuAc α 3-nLc4 and GD1a, 800 ng each; Lane 4, NeuAc α 3-nLc4 and GD1a, 400 ng each; Lane 5, NeuAc α 3-nLc4 and GD1a, 200 ng each; Lane 6, NeuGc α 3-neolactohexaosylceramide (NeuGc α 3-nLc6, NeuGc α 3Gal β 4GlcNAc β 3Gal β 4GlcNAc β 3Gal β 4Glc β 1Cer), 4 μ g; Lane 7, NeuGc α 3-nLc6, 2 μ g; Lane 8, NeuGc α 3-nLc6, 800 ng; Lane 9, NeuGc α 3-nLc6, 400 ng; Lane 10, NeuGc α 3-nLc6, 200 ng. The lanes on (B) were: Lane 1, NeuAc α 3-nLc4 and GD1a 800 ng each; Lane 2, NeuAc α 3-nLc4 and GD1a, 400 ng each; Lane 3, NeuAc α 3-nLc4 and GD1a, 80 ng each; Lane 4, NeuAc α 3-nLc4 and GD1a, 40 ng each; Lane 5, NeuAc α 3-nLc4 and GD1a, 20 ng each; Lane 6, NeuGc α 3-nLc6, 800 ng; Lane 7, NeuGc α 3-nLc6, 400 ng; Lane 8, NeuGc α 3-nLc6, 80 ng; Lane 9, NeuGc α 3-nLc6, 40 ng; Lane 10, NeuGc α 3-nLc6, 20 ng.

Figure S6. Overlay of the NeuAc-nLT conformations in LT-IIb B-subunit main binding site (D and E monomers are shown with yellow and orange carbons, A monomer is displayed with green carbons). No water molecules are shown for clarity.

Figure S7. Overlay of binding site of LT-IIb (green) and CT (gray) illustrating the different orientation of NeuAc .