Incorporation of a non-human glycan mediates human susceptibility to a bacterial toxin

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AB₂ toxins comprise an A subunit that corrupts essential eukaryotic cell functions, and pentameric B subunits that direct target-cell uptake after binding surface glycans. Subtilase cytotoxin (SubAB) is an AB₂ toxin secreted by Shiga toxigenic Escherichia coli (STEC), which causes serious gastrointestinal disease in humans. SubAB causes haemolytic uremic syndrome-like pathology in mice through SubA-mediated cleavage of BIP/GRP78, an essential endoplasmic reticulum chaperone. Here we show that SubB has a strong preference for glycans terminating in the sialic acid N-glycolylneuraminic acid (Neu5Gc), a monosaccharide not synthesized in humans. Structures of SubB–Neu5Gc complexes revealed the basis for this specificity, and mutagenesis of key SubB residues abrogated in vitro glycan recognition, cell binding and cytotoxicity. SubAB specificity for Neu5Gc was confirmed using mouse tissues with a human-like deficiency of Neu5Gc and human cell lines fed with Neu5Gc. Despite lack of Neu5Gc biosynthesis in humans, assimilation of dietary Neu5Gc creates high-affinity receptors on human gut epithelia and kidney vasculature. This, and the lack of Neu5Gc-containing body fluid competitors in humans, confers susceptibility to the gastrointestinal and systemic toxicities of SubAB. Ironically, foods rich in Neu5Gc are the most common source of STEC contamination. Thus a bacterial toxin’s receptor is generated by metabolic incorporation of an exogenous factor derived from food.

The B subunits of AB₂ toxins typically recognize cognate glycan receptors displayed on cell-surface glycoconjugates. Receptor specificity is critical for the pathogenic process, as it determines host susceptibility, tissue tropism, and the nature and spectrum of the resultant pathology. Accordingly, we sought to understand the receptor specificity of SubAB. Glycan array analysis showed that Oregon-Green-labelled SubAB (OG–SubAB) had a high degree of binding specificity for glycans terminating with α2-3-linked residues of the non-human sialic acid Neu5Gc (Table 1). Much weaker binding was seen with those glycans that terminated in α(2-3)-linked N-acetyleneuraminic acid (Neu5Ac), which differs from Neu5Gc by the addition of a hydroxyl group from Neu5Gc (Fig. 1a). Table 1 is a list of glycans selected from the microarray analysis of SubAB toxin and a mutant derivative SubABA₁₂ (discussed later). This list represents the glycans on the array to which native SubAB has the highest apparent affinity and corresponding Neu5Ac derivatives, asialo- and sulphated-derivatives. Of all the glycans on the array, Neu5Gcα2-3Galβ1-4GlcNAcβ (#260) bound SubAB best. The binding of SubAB to this glycan is reduced 20-fold if the Neu5Gc is changed to Neu5Ac (#237); 30-fold if the Neu5Gc linkage is changed from α2-3 to α2-6 (#263); and 100-fold if the sialic acid is removed (#152). The high binding of SubAB to structures #258, #260 and #261 indicates that it has a high affinity for terminal α2-3-linked Neu5Gc, with little discrimination for the penultimate moiety. Surface plasmon resonance analysis (Supplementary Fig. 2a) showed an approximately tenfold higher SubAB binding response to Neu5Gcα2-3Lacβ than to Neu5Acα(2-3)-Lacβ. Competitive inhibition studies (Supplementary Fig. 2b) indicated that Neu5Gcα2-3Lacβ has an inhibition constant (Ki) of 2 mM, which is in the range reported for other monovalent sialic-acid–protein interactions. This high specificity of SubAB for Neu5Gc-terminating glycans is unique among bacterial toxins.

Next we determined the structure of the apo-form of the SubB pentamer (Fig. 1 and Supplementary Table 1). As expected the SubB protomer adopted the common oligonucleotide/oligosaccharide-binding fold, typical of other AB₂ toxins. The SubB structure most resembled the S2, S3 and S5 subunits of pertussis toxin (PTX; Supplementary Table 2), where the S2/3 subunits of PTX contained a shallow binding site for sialylated glycoproteins; SubB also contained a similar shallow pocket lined by similar residues. In all such previously studied AB₂ toxins, the sialic acid in question is Neu5Ac, a common sialic acid found in humans and other animals. We then determined the structure of SubB in complex with free Neu5Gc (Supplementary Table 1). Neu5Gc bound to SubB unambiguously (Fig. 1c), whereas identical experiments using Neu5Ac failed to show any binding. The shallow binding pocket of SubB bound Neu5Gc in the chair conformation (Fig. 1d). The Neu5Gc lay in the α-anomeric configuration, even though most free Neu5Gc (more than 90%) is in solution is in the β-anomeric form; SubB is therefore highly selective for the small fraction of α-anomeric Neu5Gc present in solution. Neu5Gc is coordinated mainly by multiple polar interactions with SubB (Supplementary Table 3). The predominant van der Waals interaction arose from Phe 11, which swivelled by 90° when Neu5Gc was bound, stacking parallel with the sugar ring and forming a cap over the binding site. In addition, the Neu5Gc was sequestered by many direct and water-mediated hydrogen bonds, including interactions with the side chains of Asp 8, Ser 12, Glu 36 and Tyr 78. Neu5Gc differs from Neu5Ac by the addition of a hydroxyl on the methyl group of the N-acetyl moiety of Neu5Ac. This extra hydroxyl present in Neu5Gc made crucial interactions with SubB: namely, the extra hydroxyl interacts with Tyr 78⁴⁹ and hydrogen bonds with the main chain of Met 10. These key interactions could not occur with Neu5Ac, thus explaining the marked preference for Neu5Gc.

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Given the glycan array data, we also established how a trisaccharide that terminated in Neu5Gc (Neu5Gcα2-3Galβ1-3GlcNAcβ; #258 in the array) bound SubB (Fig. 1e). The mode of binding of the Neu5Gc moiety in the monosaccharide and the trisaccharide complex was identical. The remaining two sugar moieties present in the trisaccharide extended to solvent, but were nevertheless able to contact SubB. The small number of additional interactions between SubB and the tertiary sugar in the trisaccharide (Fig. 1e) is consistent with the Neu5Gc moiety driving the specificity and affinity for the interaction with SubB. Of note, the sequence Neu5Gcα2-3Galβ1-3GlcNAcβ is very common as a terminating structure of N-glycans in non-human animals.

Despite the commonality of oligonucleotide/oligosaccharide-binding fold, neither the receptor specificity nor the location of the receptor-binding site is conserved throughout the AB5 toxin family. For Shiga and cholera toxins, whose receptors are glycolipids, the deep receptor-binding pockets are located on the membrane face of the toxin11–13. Like SubB14, PTX binds sialylated glycoproteins; moreover, the PTX S2/3 sialic-acid binding site is also shallow and in the same location, halfway down the sides of the pentamer (Fig. 1f–h). Examination of the SubB–Neu5Gc structure superposed onto the structure of PTX in complex with the disaccharide, Neu5Acα2-3Gal, reveals that both toxins bind sialic acid in the same orientation, with similar residues interacting with the sialic-acid head group (Fig. 1d, f). However, the interactions between Tyr 78 and Asp 8 of SubB with the extra hydroxyl group of Neu5Gc have no such equivalents in PTX. At the same position as Tyr 78 in SubB is the small, non-polar Val 167 side chain in PTX S2/3, and there are no residues that overlay in the PTX S2/3 region equivalent to Asp 8 in SubB. Of note, the sequence Neu5Gcα2-3Galβ1-3GlcNAcβ is very common as a terminating structure of N-glycans in non-human animals.

Table 1  Glycan array analysis of native SubAB and B subunit mutant SubABΔ12

<table>
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<tr>
<th>Glycan number</th>
<th>Structure</th>
<th>Mean relative fluorescence units</th>
<th>Coefficient of variation (%)</th>
<th>Mean relative fluorescence units</th>
<th>Coefficient of variation (%)</th>
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<tbody>
<tr>
<td>260</td>
<td>Neu5Gcα2-3Galβ1-4GlcNAcβ–Sp0*</td>
<td>37,606</td>
<td>4</td>
<td>590</td>
<td>10</td>
</tr>
<tr>
<td>261</td>
<td>Neu5Gcα2-3Galβ1-4Glcβ–Sp0</td>
<td>25,197</td>
<td>16</td>
<td>1,998</td>
<td>30</td>
</tr>
<tr>
<td>264</td>
<td>Neu5Gcαx–Sp8</td>
<td>25,114</td>
<td>19</td>
<td>172</td>
<td>87</td>
</tr>
<tr>
<td>258</td>
<td>Neu5Gcαx2-3Galβ1-3GlcNAcβ–Sp0</td>
<td>24,209</td>
<td>12</td>
<td>327</td>
<td>54</td>
</tr>
<tr>
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<td>Neu5Gcα2-3Galβ1-4GlcNAcβ–Sp0*</td>
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Data are presented for a selection of 40 of the 320 glycans present on the array. Complete data sets are available at: www.functionalglycomics.org/glycomics/publicdata/selectedScreens.jsp. Data are for quadruplicate array spots.

*Sp0 and Sp08 designate CH2CH2NH2 and CH2CH2CH2NH2 linkers, respectively.
Figure 1 | Structural analysis of SubB–sialic acid interactions and comparison with other AB toxins. a, Structures of Neu5Gc and Neu5Ac, showing additional O at C11 of the former. b, SubB protomer (orange) superimposed upon PTX S2 (blue). Amino (N) and carboxy (C) termini of SubB and PTX S2 are designated by subscripts S and P, respectively. c, Representation of the pentameric SubB–Neu5Gc structure, with each protomer colour-coded. Cyan sticks represent the sugar, blue sticks represent nitrogen atoms, and red sticks represent oxygen atoms. d, Neu5Gc in sialic-acid receptor-binding site of SubB. The extra hydroxyl of Neu5Gc interacts with Tyr78 and Asp8. e, Trisaccharide Neu5Gc(2-3Gal[1-3]GlcNAcβ)ProN3 binding to SubB. ProN3 refers to the linker used in the synthesis. f, Neu5Ac(2-3Gal binding site of PTX S2/3, which shares similarity to the SubB binding site, namely: Ser12 in SubB, Ser104 in PTX S2/3; Gln36 in SubB, Arg125 in PTX S2/3; Phe11 in SubB, Tyr103 in PTX S2/3. PTX does not have the equivalent of Tyr78 and Asp8. g, Side-on view of SubB:Neu5Gc(2-3Gal[1-3]GlcNAcβ)ProN3. h, Side-on view of CtxB:GM1. In d–h, cyan sticks represent ligands, dark blue sticks represent nitrogen atoms, and red sticks represent oxygen atoms. Yellow sticks represent key residues in the protein backbone. Black dotted lines represent hydrogen bonds.

Figure 2 | Fluorescence microscopy and Neu5Gc-dependent cytotoxicity. a, Binding of SubAB mutants to Vero cells. Vero cells growing on coverslips were incubated with 1 μg ml⁻¹ OG–SubAB, OG–SubAB₁₇₁₂ or Texas Red–SubAB₁₇₁₂ for 60 min at 37 °C (scale bar, 25 μm). b, Binding of SubAB to kidney sections from wild-type and Cmah null mice. Frozen kidney sections were incubated with 1 μg ml⁻¹ OG–SubAB for 60 min at 37 °C, washed, and examined by epifluorescence microscopy (scale bar, 100 μm). c, Neu5Gc-dependent cytotoxicity. Cytotoxicity of SubAB for MDA-MB-231 cells or 293 cells after growth in medium supplemented with 3 mM Neu5Gc (Gc) or Neu5Ac (Ac) was determined as described in the Methods. Data shown are from a single experiment. Analysis of data pooled from triplicate experiments yielded CD₅₀ values of 3.92 ± 1.58 pg (mean ± standard error) and 13.17 ± 2.46 pg for MDA cells fed Neu5Gc or Neu5Ac (P = 0.034; two-tailed t-test). For 293 cells fed Neu5Gc or Neu5Ac the CD₅₀ values are 1.33 ± 0.30 pg and 11.58 ± 3.30 pg, respectively (P = 0.036). The mean increase in SubAB susceptibility (± standard error) for cells fed Neu5Gc versus Neu5Ac was 5.56 ± 2.71-fold for MDA cells and 8.35 ± 0.88-fold for 293 cells.

reduction of cytotoxicity by 88% (Supplementary Table 4), which is consistent with the importance of the interactions with the C8 and C9 hydroxyl groups present on both Neu5Gc and Neu5Ac.

As further evidence of the biological significance of the high specificity of SubAB for Neu5Gc-terminating glycans, we showed that OG–SubAB bound to kidney tissue from wild-type mice, but not cytidine monophosphate (CMP)-N-acetylneuraminic acid hydroxylase (Cmah)-null mice¹⁵ that had a human-like genetic defect in the ability to convert CMP-Neu5Ac to CMP-Neu5Gc (Fig. 2b). Humans lack this enzyme owing to a mutation in the Cmah gene that occurred after evolutionary separation of the hominin lineage from the great apes¹⁶, suggesting the possibility of human genetic resistance to the toxin. However, human cells can metabolically assimilate Neu5Gc present in tissue culture media and incorporate it into cell-surface glycans¹⁷. We therefore manipulated the levels of Neu5Gc on the surface of human cell lines (MDA-MB-231 breast cancer cells and 293 embryonic kidney cells), which had been adapted for growth in human serum, resulting in undetectable amounts of Neu5Gc. These cells were then grown for 3 days in medium supplemented with 3 mM Neu5Gc or 3 mM Neu5Ac. In the former case, this increased Neu5Gc content to 50–75% of total sialic acid in membrane preparations (result not shown). Feeding MDA and 293 cells with Neu5Gc rather than Neu5Ac significantly increased their susceptibility to SubAB (50% cytotoxic dose (CD₅₀) values decreased 5.56- and
We have previously shown that some normal human tissues contain small quantities of Neu5Gc\(^1\). No tissues in Cmah-null mice express Neu5Gc, as determined by a highly specific polyclonal chicken antibody, and by mass spectrometry\(^5\). Thus, human Neu5Gc must be derived from dietary sources, a mechanism confirmed by previous studies of human volunteers\(^7\). We therefore studied human colon sections by using an IgY antibody with absolute specificity for Neu5Gc (unpublished improvements over ref. 15), and noted that Neu5Gc was present on the epithelial surfaces and in the crypts (Fig. 3a). Similar regions of human colon sections also bound native SubAB, but binding of SubAB\(_{A12}\) was markedly diminished (Fig. 3b). Importantly, pre-incubation with anti-Neu5Gc IgY, but not control IgY, substantially blocked binding of SubAB to human colon sections (Fig. 3c), demonstrating competition for the same epitope. Specific binding of SubAB to human kidney sections, particularly the glomerular endothelium, was also observed, but this was not seen for SubAB\(_{A12}\) (Fig. 3d). Human kidney vasculature also shows staining with the anti-Neu5Gc antibody (data not shown). Furthermore, binding to kidney tissue was not seen with SubAB\(_{F78}\), which is defective only in Neu5Gc-specific receptor interactions (Fig. 3e). Thus, binding of SubAB to human colon and kidney tissue is Neu5Gc dependent.

We also examined whether the inability of Cmah-null mice to express Neu5Gc affected in vivo susceptibility to injected SubAB. Surprisingly, the null mice had a slightly shorter median survival time (5 versus 6 days, \(P = 0.038\); Fig. 3f). We reasoned that because normal mouse serum contains high levels of Neu5Gc-containing glycoproteins, these would compete with receptors on the surface of tissues for SubAB, thereby providing partial protection against toxicity. In contrast, human and Cmah-null mouse serum would not have any such protective activity. Thus, in the null mouse, lack of protective serum glycoproteins could counteract any benefit derived from lack of expression of Neu5Gc on cell-surface glycoconjugates. Indeed, wild-type but not Cmah-null mouse serum competitively inhibited binding of SubAB to immobilized Neu5Gc-glycan (Fig. 3g). Similar results were obtained for chimpanzee serum (which, like wild-type mouse serum, is rich in Neu5Gc-glycoproteins) versus human serum (Fig. 3h). Furthermore, chimpanzee serum, but not human serum, inhibited binding of SubAB to human kidney sections (Fig. 3d). Given that chimpanzee serum glycoproteins otherwise share near identity with their human counterparts, the effects can be attributed to the presence or absence of Neu5Gc competitors\(^18\).

This study presents the first example of a bacterial toxin showing a marked preference for Neu5Gc-containing glycans and is potentially significant in the context of host susceptibility to toxin-mediated disease. Indeed, glycans terminating in Neu5Gc\(2\)Gal\(\beta\)-3GlcNAc sequences are widely expressed on the cells of many mammals including livestock, suggesting an evolutionary reason for the emergence of this selective binding preference. Neu5Gc is not produced by bacteria or plants, is low or absent in poultry and fish, but abundant in red meats (lamb, pork and beef) and in bovine milk. However, humans are the known exception among mammals, and Neu5Ac predominates because of the Cmah mutation\(^16\). How then could the toxin mediate disease in humans? The answer probably lies in the diet, as human ingestion of red meats and milk products results in Neu5Gc incorporation into human tissues\(^17\). We detected significant levels of Neu5Gc on human colonic epithelium, and Neu5Gc-specific binding of SubAB at this site, as well as in the kidney vasculature, the major target organ for haemolytic-uraemic syndrome caused by STEC (Fig. 3). This Neu5Gc can only have originated from the diet, as there is no known alternative pathway for Neu5Gc biosynthesis\(^15\). Ironically, red meat and dairy products (the richest dietary sources of Neu5Gc) are the very foods that are most commonly contaminated with SubAB-producing STEC\(^2\). Thus, through regular dietary intake of red meats and milk, humans may pre-sensitize their tissues to a key virulence factor of a major pathogen that occurs sporadically in the same foods. Furthermore, because of the absence of protective Neu5Gc-bearing glycoproteins in their serum and other body fluids, humans who have consumed foods with high Neu5Gc content may actually be hyper-susceptible to the toxin, as illustrated in Supplementary Fig. 1.

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**Figure 3** | Neu5Gc-dependent binding of SubAB to human tissues and toxicity of SubAB in wild-type and Cmah-null mice. A. Frozen sections of human colon were stained with chicken anti-Neu5Gc or control IgY at 5 \(\mu\)g ml\(^{-1}\), followed by anti-chicken IgY–HRP conjugate, and examined by immunohistochemistry\(^7\) (scale bar, 100 \(\mu\)m). B. Similar human colon sections were overlaid with or without 1 \(\mu\)g ml\(^{-1}\) SubAB or SubAB\(_{A12}\) and bound toxin was detected using rabbit anti-SubAB and Cy3-labelled goat anti-rabbit IgG and examined by epifluorescence microscopy (see Methods) (scale bar, 50 \(\mu\)m). C. Human colon sections were overlaid first with anti-Neu5Gc or control IgY at 5 \(\mu\)g ml\(^{-1}\), followed by 1 \(\mu\)g ml\(^{-1}\) SubAB, and bound toxin was detected as for B. Background control sections received only rabbit anti-SubAB, followed by Cy3-labelled anti-rabbit IgG (scale bar, 100 \(\mu\)m). D. Human kidney sections were overlaid 1 \(\mu\)g ml\(^{-1}\) SubAB, SubAB\(_{A12}\) or SubAB\(_{F78}\), and in the presence or absence of 10% human or chimpanzee serum, as indicated. Bound toxin was detected as for B (scale bar, 50 \(\mu\)m). E. Wild-type and Cmah-null mice (\(n = 8\) each) were injected intraperitoneally with 200 ng \(\text{g}^{-1}\) purified SubAB in a total of 100 \(\mu\)l PBS, and survival time was recorded. Kaplan–Meier survival curves were plotted and statistical analysis (Wilcoxon–Gehan test) was performed in GraphPad Prism. G. h. Inhibition of SubAB binding to immobilized Neu5Gc-(2-3)-Lac-HSA by wild-type versus Cmah-null mouse serum (f), or human versus chimpanzee serum (g) was assayed by enzyme-linked immunosorbent assay (ELISA) as described in the Methods. Data are expressed as the percentage of a control with no serum and are the mean ± standard error of triplicate wells and representative of three independent experiments.
METHODS SUMMARY

Purification and labelling of SubAB. SubAB holotoxin, and derivatives with B-subunit mutations, were purified by Ni-NTA chromatography and labelled with Oregon Green, as described previously16–20.

Structure determination. SubB was crystallized, and the Se-Met-labelled SubB structure was solved using the multiple anomalous dispersion technique. Crystal soaks and difference Fourier analysis was used to locate the Neu5Gc-containing binding sites. Further details are provided in the Methods.

Glycan array analysis. Binding of OG-labelled SubAB and mutant derivatives thereof to immobilized glycans was investigated using a printed array of 320 glycan targets on version 3.0 of the glycan microarray of the Consortium for Functional Glycomics Core H (http://www.functionalglycomics.org/static/consortium/resources/resourceroch8.shtml). Further details are provided in the Methods.

DNA methods. Routine DNA manipulations were performed essentially as described previously16. DNA sequencing used dye-terminator chemistry and an ABI 3700 sequencer.

Cell culture and cytotoxicity assays. SubAB and mutant derivatives were assayed for cytotoxicity on Vero cells as previously described17. Cytotoxicity was also assayed on MDA-MB-231 (human breast cancer) and 293 (human embryonic kidney) cells, which had been adapted for growth in human serum to eliminate presence of Neu5Gc. Cells were then cultured for 3 days in 96-well plates in medium (RPMI and DMEM, respectively) supplemented with 3 mM Neu5Gc or Neu5Ac, and then exposed to serial dilutions of SubAB, as described for Vero cells17. Cell viability was assessed after incubation for a further 3 days by staining with 10% Alamar blue (Serotec), according to the manufacturer's instructions.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions E.B. and A.W.P. contributed equally. E.B. T.B. and M.C.J.W. crystallized SubB, solved the structure and contributed to manuscript preparation. A.W.P. constructed mutants and contributed to design and interpretation of experiments, project management and writing of the manuscript. J.C.P. and J.R. contributed to design and interpretation of experiments, project management and writing of the manuscript. D.C.C. and U.M.T. performed experiments. D.F.S. performed and interpreted glycan array experiments. J.C.L., N.M.V. and A.V. designed, performed and interpreted experiments relating to Neu5Gc on cells and tissues and to cytotoxicity in vivo, and contributed to manuscript preparation. H.Y., S.H. and X.C. synthesized oligosaccharides. A.V., T.B. and J.R. are joint senior and corresponding authors.

Author Information The coordinates and structure factors for the SubB structures are deposited in Protein Data Bank under accession numbers 3DWA, 3DPW and 3DWQ. Raw glycan array data are available at www.functionalglycomics.org/glycomics/publicdata/selectedScreens.jsp. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to A.V. (avarki@ucsd.edu), J.R. (jamie.rossjohn@med.monash.edu.au) or T.B. (travis.beddow@med.monash.edu.au).
**METHODS**

**Glycan array analysis.** Labelled proteins were diluted to 0.1 mg ml⁻¹ in Tris-buffered saline (TBS: 20 mM Tris, 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, pH 7.4) containing 1% BSA and 0.05% Tween-20, and an aliquot (70 μl) was applied to separate microarray slides and incubated under a coverslip for 60 min at room temperature. Coverslips were then removed and slides were washed by dipping four times in successive washes of TBS containing 0.05% Tween-20, TBS and deionized water. Slides were then spun for approximately 15 s to dry and immediately scanned in a PerkinElmer ProScanArray MicroArray Scanner using an excitation wavelength of 488 nm and Imagen software (BioDiscovery) to quantify fluorescence. The data are reported as average relative fluorescence units of four of six replicates (after removal of the highest and lowest values) for each glycan represented on the array.

**Purification and crystallization of SubB.** Purification of SubB was performed as described previously for the holotoxin with the following modifications. SubB was eluted from an Ni-NTA column using wash buffer containing 500 mM imidazole and further purified by gel filtration using an S200 16/60 column (GE Healthcare) pre-equilibrated in 20 mM Tris HCl, 150 mM NaCl, 2 mM EDTA, pH 7.0. Fractions containing SubB were pooled and purity was assessed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry (data not shown). SubB was concentrated to 2 mg ml⁻¹ and 40 mM 3-(decyldimethylammonio) propane sulphonate was added before storing at 4 °C. Crystals of SubB grew in drops containing 1 μl protein (2 mg ml⁻¹) and 1 μl reservoir solution, with the reservoir solution consisting of 500 g/l of 16% (w/v) PEG 3350, 100 mM sodium cacodylate, pH 6.2, and 200 mM ammonium fluoride. Selenomethionine-substituted SubB was purified and crystallized under the same conditions.

**X-ray data collection, structure determination and refinement.** X-ray data were collected for SeMet-labelled SubB at the GMCA-CAT beamline at the Advanced Photon Source, Chicago. Data were collected from a single crystal at three wavelengths: inflection point, peak and high energy remote. Native SubB crystals were soaked by the addition of 1 mM of Neu5Gc, Neu5Ac or the trisaccharide Neu5Gc2-3Galβ1-3GlcnAcβProN for 1 h at 20 °C. Datasets from the soaked crystals were collected in-house using an R-axis IV Autosol suite (CCLRC, 1997).

The apo-form of SubB was phased by the single-wavelength anomalous dispersion (SAD) method using SeMet inflection point data and the PHENIX Autosol suite (26). An initial model of 460 out of 630 residues was built by RESOLVE (27), and this was used as input for ARP/WARP. The model was completed by rounds of manual building in COOT (28) followed by refinement in REFMAC5 (ref. 28) with fivefold non-crystallographic symmetry maintained until the final stages of refinement. Waters and PEG 400 molecules were added in COOT to give a final SubB model consisting of a homopentamer of 588 residues (with each B subunit between 115 and 120 residues). The apo-SubB structure was used, through PHASER (29), to solve the sugar-bound structures, with residues rebuilt and refined as described for the apo structure. Ligands were constructed using the PRODRG2 server (30). Clear density was observed for the various sugars in the SubB binding sites and these were further confirmed by constructing Fₐ-Fᵢ omit maps. Data collection and refinement statistics are shown in Supplementary Table 1. The stereochemistry and overall quality of each of the structures was confirmed by CCP4 programs.

**Site-directed mutagenesis of subB.** Derivatives of SubAB with either S12A, SubBA36F or SubBA36F/pETsubAB for Q36A, and pETsubAB/SubB78R and SubB78F/pETsubAB for Y78F. Mutations were confirmed by sequencing, and expression of intact SubAB protein was confirmed by SDS–PAGE and western blotting. Holotoxins carrying confirmed B-subunit mutations were purified as for native toxin.

**Tissue immunohistochemical studies.** Human colon or kidney sections were overlaid with or without 1 μg ml⁻¹ SubAB, SubABα₁₂ or SubABβ₉₈, with or without 10% human or chimpanzee serum, as indicated, for 1 h at room temperature. Slides were then washed and fixed in 10% buffered formalin for 15 min and washed again. The sections were overlaid with rabbit anti-SubA at 1:5,000 in 1% BSA/PBS and incubated for 1 h at room temperature, washed again and overlaid with Cy3-labelled goat anti-rabbit IgG (Jackson Immunoresearch) for 1 h at room temperature. Slides were washed, mounted using aqueous mounting medium and viewed using epifluorescence with a Zeiss Axiopt microscope with appropriate excitation and barrier filters. Digital photomicrographs were taken using a Sony CCD (charge-coupled device) camera and National Institutes of Health image software and photo panels constructed using Adobe Photoshop and Illustrator.

**SubAB binding inhibition ELISA.** Inhibition of SubAB binding to immobilized Neu5Gc2-3LacHSA by wild-type versus S12A-null mouse serum, or human versus chimpanzee serum, was assayed as follows. All reagents were diluted and blocking was done in 1% fish gelatin. Costar 96-well ELISA plates were coated with saturating amounts of Neu5Gc2-3LacHSA at 4 °C overnight, and then blocked at 4 °C overnight. Serum was added at the indicated concentrations, followed by 15 ng ml⁻¹ SubAB. After 2 h incubation at room temperature, the plates were washed four times in PBS. Rabbit anti-SubA serum diluted 1:5,000 was added. The plates were incubated for 2 h at room temperature, followed by another washing step, and then horseradish peroxidase conjugated to goat anti-rabbit IgG (1:20,000, for 2 h at room temperature. After washing, 3,3',5,5'-tetramethylbenzidine (Sigma) was added to the plates; the reaction was stopped after 15 min by 2 mM sulphuric acid and absorbance was read at 450 nm.

**Surface plasmon resonance.** Experiments were conducted at 25 °C on a Biacore 3000 instrument using HBS buffer (10 mM HEPES–HCl, pH 7.4, 800 mM NaCl and 0.005% surfactant P20; supplied by the manufacturer). Approximately 200 resonance units of Neu5Ac2-3Lacβ1-biotin and Neu5Gc2-3Lacβ1-biotin was immobilized onto streptavidin-coupled sensor chips (Biacore). SubAB was passed over all flow cells at 20 μl min⁻¹ for 1 min. The final response was calculated by subtracting the response of the control surface from the glycan surface. For inhibition studies, SubAB (100 nM) was incubated with increasing concentrations of Neu5Gc2-3Lacβ1-biotin (3 μM to 4 mM) for 1 h at room temperature before being passed over the chip at 5 μl min⁻¹ for 3 min. After each injection, the surface was regenerated with three injections of 10 mM glycine, pH 2.0. The experiments were performed in duplicate. The amount of SubAB bound at equilibrium was used to generate the inhibition curve that was analysed by nonlinear regression using PRISM software (version 3.0).

22. Evans, P. R. in Proc. CCP4 Study Weekend on Recent Advances in Phasing 97–102 (CCLRC, 1997).