Published in final edited form as:

Nature. 2008 December 4; 456(7222): 648–652. doi:10.1038/nature07428.

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

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Abstract

AB₅ 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 AB₅ toxin secreted by Shiga toxigenic *Escherichia coli* (STEC)¹, which causes serious gastrointestinal disease in humans². SubAB causes haemolytic uraemic syndrome-like pathology in mice³ via SubA-mediated cleavage of BiP/GRP78, an essential endoplasmic reticulum chaperone⁴. Here we show that SubB has a strong preference for glycans terminating in the sialic acid *N*-glycolylneuraminic acid (Neu5Gc), a monosaccharide not synthesised 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 human lack of Neu5Gc biosynthesis, assimilation of dietary Neu5Gc creates high-affinity receptors on human gut epithelia and kidney vasculature. This, together

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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. synthesised oligosaccharides.

Supplementary Information is linked to the online version of this paper at www.nature.com/nature.

Author Information. The coordinates and structure factors for the SubB structures have been submitted to the Protein Data Bank under accession codes: 3DWA, 3DWP, 3DWQ. Raw glycan array data are available at www.functionalglycomics.org/glycomics/publicdata/selectedScreens.jsp.

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The authors declare that they have no competing financial interests.

with the human lack of Neu5Gc-containing body fluid competitors, 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 AB₅ toxins typically recognise cognate glycan receptors displayed on cell surface glycoconjugates^{5, 6}. 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 gain an understanding of 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 α 2–3-linked residues of the non-human sialic acid *N*-glycolylneuraminic acid (Neu5Gc) (Table 1). Much weaker binding was seen with those glycans that terminated in α 2–3-linked *N*-acetylneuraminic acid (Neu5Ac), which differs by one 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 SubAB_{A12} (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 sulfated-derivatives. Of all the glycans on the array, Neu5Gc α 23Gal β 1–4GlcNAc β – (#260) bound SubAB best. The binding of SubAB to this glycan is reduced 20-fold if the Neu5Gc is changed to Neu5Ac (#237); over 30-fold if the Neu5Gc linkage is changed from α 2–3 to α 2–6 (#263); and 100-fold if the sialic acid is removed (#152). The high binding of SubAB to structures #258, #260, and #261 indicate that it has a high affinity for terminal α 2–3-linked Neu5Gc with little discrimination for the penultimate moiety. Surface plasmon resonance analysis (Supplementary Fig. 2A) showed an approximately 10-fold higher SubAB binding response to Neu5Gc α 2–3Lac β than to Neu5Ac α 2–3Lac β . Competitive inhibition studies (Supplementary Fig. 2B) indicated that Neu5Gc α 2–3Lac β has a K_i of 2 mM, which is in the range reported for other monovalent sialic acid-protein interactions^{7, 8}. This high specificity of SubAB for Neu5Gc-terminating glycans is unique amongst bacterial toxins.

Next we determined the structure of the *apo*-form of the SubB pentamer (see Fig. 1 and Supplementary Table 1). As expected SubB protomer adopted the common OB (oligonucleotide/oligosaccharide-binding) fold⁹, typical of other AB₅ 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 glycoproteins¹⁰; SubB also contained a similar shallow pocket lined by similar residues. In all such previously studied AB₅ 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 the majority of free Neu5Gc (> 90%) 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 *via* multiple polar interactions with SubB (Supplementary Table 3). The predominant vdw interaction arose from Phe11, 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 a large number of direct and water-mediated hydrogen bonds, including interactions with the side chains of Asp8, Ser12, Glu36 and Tyr78. 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 Tyr78^{OH} and hydrogen bonds with the main chain of Met10. These key interactions could not occur with Neu5Ac, thus explaining the marked preference for Neu5Gc.

Given the glycan array data, we also established how a trisaccharide that terminated in Neu5Gc (Neu5Gc α 2–3Gal β 1–3GlcNAc; #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 Neu5Gc α 2–3Gal β 1–3GlcNAc is very common as a terminating structure of *N*-glycans in non-human animals.

Despite the commonality of OB-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 toxin^{11–13}. 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 on 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, Neu5Ac α 2–3Gal, 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 Tyr78 and Asp8 of SubB with the extra hydroxyl group of Neu5Gc have no such equivalents in Ptx. At the same position as Tyr78 in SubB is the small, non-polar Val167 side chain in Ptx S2/3 and there are no residues that overlay in the Ptx S2/3 region equivalent to Asp8 in SubB. The absence of these side chains in Ptx S2/3 provides a basis for understanding why Ptx, unlike SubB, is not specific for Neu5Gc.

SubB residues that were considered critical for Neu5Gc binding were then mutated to determine their role in biologically-relevant toxin-receptor interactions. The most critical residue was Ser12, mutation of which abolishes interactions with the C1 carboxylate group of sialic acid; this reduced cytotoxicity for Vero cells by 99.98% (Supplementary Table 4). Also, binding of labelled mutated toxin (OG-SubAB_{A12}) to Vero cells could not be detected by fluorescence microscopy, confirming an effect on receptor recognition (Fig. 2a). Further glycan array analysis using OG-SubAB_{A12} indicated markedly reduced binding to the immobilized sialylated glycans (Table 1). The elimination of the hydroxyl of Tyr78 in the SubAB_{F78} mutant holotoxin also reduced Vero cell cytotoxicity by 96.9% (Supplementary Table 4), which highlights the importance of interactions with the hydroxyl group unique to Neu5Gc-containing glycans. The trace residual cytotoxicity of the SubAB_{F78} mutant is most likely attributable to SubB still being able to bind weakly to Neu5Ac-containing glycans, since Tyr78 would not be required for binding Neu5Ac. Labelled-SubAB_{F78} also exhibited reduced binding to Vero cells (Fig. 2a). The Gln36Ala mutation resulted in 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 CMP-*N*-acetylneuraminic acid hydroxylase (*Cmah*)-null mice¹⁵ that have 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 incorporate 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 to growth on 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 susceptibility to SubAB (CD₅₀ values decreased 5.56- and 8.35-fold, respectively), rendering them as sensitive as Vero cells (Fig 2c).

We have previously shown that some normal human tissues contain small quantities of Neu5Gc¹⁷. No tissues in *Cmah* null mice express Neu5Gc, as determined by a highly specific polyclonal chicken antibody, and by mass spectrometry¹⁵. Thus, human Neu5Gc must be derived from dietary sources, a mechanism confirmed by prior human volunteer studies¹⁷. We therefore studied human colon sections 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 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 since normal mouse serum contains high levels of Neu5Gc-containing glycoproteins, this 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 immobilised 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 competitors¹⁸.

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 α 2–3Gal β 1–3GlcNAc 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* mutation¹⁶. How then could the toxin mediate disease in humans? The answer most likely lies in the diet, as human ingestion of red meats and milk products results in Neu5Gc incorporation into human tissues¹⁷. 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 HUS caused by STEC (Fig. 3). This Neu5Gc can only have originated from the diet, as there is no known alternative pathway for Neu5Gc biosynthesis¹⁵. Ironically, red meat and dairy products (the richest dietary sources of Neu5Gc) are the very foods that are most commonly

contaminated with SubAB-producing STEC². Thus, through regular dietary intake of red meats and milk, humans may pre-sensitise 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.

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 previously^{19, 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 v3.0 of the glycan microarray of the Consortium for Functional Glycomics Core H (<http://www.functionalglycomics.org/static/consortium/resources/resourcecoreh8.shtml>). Further details are provided in the Methods.

DNA methods

Routine DNA manipulations were carried out essentially as described previously¹. DNA sequencing employed 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 described¹. 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 cells¹. Cell viability was assessed after a further 3 days incubation 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.

METHODS

Glycan array analysis

Labelled proteins were diluted to 0.1 mg/ml 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 cover slip for 60 min at room temperature. Cover slips were then gently removed and slides were washed by dipping 4 times in successive washes of TBS containing 0.05% Tween-20, TBS and deionized water. Slides were then spun for approximately 15 sec to dry and immediately

scanned in a PerkinElmer ProScanArray MicroArray Scanner using an excitation wavelength of 488 nm and ImaGene software (BioDiscovery, Inc., El Segundo, CA) to quantify fluorescence. The data are reported as average RFU of 4 of 6 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 carried out as detailed previously for the holotoxin⁴ 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-PAGE analysis and MALDI-TOF mass spectrometry (data not shown). SubB was concentrated to 2 mg/ml and 40 mM 3-(Decyldimethylammonio) propanesulfonate added before storing at 4 °C. Crystals of SubB grew in drops containing 1 μ l protein (2 mg/ml) and 1 μ l reservoir solution, with the reservoir solution consisting of 500 μ 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 Neu5Gc α 2-3Gal β 1-3GlcNAc β ProN₃ for 1 hour at 20 °C. Datasets from the soaked crystals were collected in-house using an R-axis IV⁺⁺ detector. X-ray data were autoindexed and processed with MOSFLM²¹, SCALA²² and the CCP4 suite of programs²³.

The *apo*-form of SubB was phased by the SAD method using SeMet inflection point data and the PHENIX Autosol suite²⁴. An initial model of 460 out of 630 residues was built by RESOLVE²⁵, and this was used as input for ARP/wARP²⁶. The model was completed by rounds of manual building in COOT²⁷ followed by refinement in REFMAC5²⁸ with 5-fold NCS 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 115–120 residues). The apo-SubB structure was used, via PHASER²⁹, to solve the sugar-bound structures, with residues rebuilt and refined as described for the *apo* structure. Ligands were constructed using the PRODRG2 server³⁰. Clear density was observed for the various sugars in the SubB binding sites and these were further confirmed by constructing F_o-F_c 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, Q36A, or Y78F substitutions in the B subunit were constructed using overlap extension PCR mutagenesis. For the S12A mutant, this involved high fidelity PCR amplification (Expand® High Fidelity PCR kit; Roche Molecular Diagnostics, Germany) of *subAB*-positive O113:H21 STEC DNA using primer pairs pETsubAF / SubBA12R and SubBA12F / pETsubBR (Supplementary Table 5). This generated two fragments with the necessary mutation in the S12 codon of *subB* incorporated into the overlapping region by the SubBA12R and SubBA12F primers. The two separate PCR products were purified, mixed together and the complete *subAB* region reamplified using primer pair pETsubAF / pETsubAR. These primers incorporate *Bam*HI and *Xho*I restriction sites, enabling direct cloning into pET-23(+) (Novagen, Madison, WI), followed by transformation into *E.*

coli BL21(DE3) (Novagen). The other mutations were constructed in an analogous manner, using first round primer pairs pETsubAF / SubBA36R and SubBA36F / pETsubBR for Q36A, and pETsubAF / SubBF78R and SubBF78F / pETsubBR 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 SubAB, SubAB_{A12}, or SubAB_{F78}, with or without 10% human or chimpanzee serum, as indicated, for one hour at room temperature. Slides were then washed and fixed in 10% buffered formalin for 15 minutes and washed again. The sections were overlaid with rabbit anti-SubA at 1:5000 in 1% BSA/PBS and incubated for 1 hour at room temperature, washed again and overlaid with Cy3-labeled goat anti-rabbit IgG (Jackson ImmunoResearch) for 1 hour at room temperature. Slides were washed, mounted using aqueous mounting medium and viewed using epifluorescence using a Zeiss Axiophot microscope with appropriate excitation and barrier filters. Digital photomicrographs were taken using a Sony CCD camera and NIH image software and photo panels constructed using Adobe Photoshop and Illustrator.

SubAB binding inhibition ELISA

Inhibition of SubAB binding to immobilized Neu5Gc α 2–3Lac-HSA by wild type versus *Cmah* 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 Neu5Gc α 2–3Lac-HSA at 4 °C overnight, and then blocked at 4 °C overnight. Serum was added at the indicated concentrations, followed by 15 ng/ml SubAB. After two hours incubation at room temperature, the plates were washed 4 times in PBS. Rabbit anti-SubAB serum diluted 1:5,000 was added. The plates were incubated for two hours at room temperature, followed by another washing step, and then horse-radish peroxidase-conjugated to goat anti-rabbit IgG, 1:20,000, for two hours at room temperature. After washing, 3,3',5,5'-Tetramethylbenzidine (Sigma) was added to the plates; the reaction was stopped after 15 minutes by 2 M 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 RU of Neu5Ac- α 2–3Lac β -biotin and Neu5Gc- α 2–3Lac β -biotin was immobilized onto Streptavidin-coupled sensor chips (BIAcore). SubAB was passed over all flow cells at 20 µl/min 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 Neu5Gc- α 2–3Lac β -biotin (3 µM to 4 mM) for 1h at room temperature before being passed over the chip at 5 µl/min for 3 min. After each injection the surface was regenerated with 3 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acknowledgements

We thank the staff at GMCA, Advanced Photon Source, Chicago for assistance with data collection. This research was supported by a Program Grant from the National Health and Medical Research Council of Australia (NHMRC) (to A.W.P. & J.C.P.), a NHMRC Project Grant (to T.B. & A.W.P.), a grant from the National Institute of General Medical Sciences to the Consortium for Functional Glycomics, RO1 grants from the National Institutes of Health (to A.W.P, J.C.P. & J.R. X.C. and A.V.) and from the ARC Centre of Excellence in Structural and Functional Microbial Genomics (J.R.). J.R. is supported by an Australian Research Council Federation Fellowship; T.B. by a NHMRC Career Development Award; J.C.P. by a NHMRC Australia Fellowship. We also thank Christopher J. Gregg for assistance with *in vivo* experiments and with collection of data and Lisa Wiggleton for technical assistance with tissue sectioning and staining.

Figure 1. Structural analysis of SubB-sialic acid interactions and comparison with other AB₅ toxins
a, Structures of Neu5Gc and Neu5Ac, showing additional O at C11 of the former. **b**, SubB protomer (orange) superimposed upon Ptx S2 (blue). N and C termini of SubB and Ptx S2 are designated with subscript S and P, respectively. **c**, Cartoon representation of the pentameric SubB-Neu5Gc structure, with each protomer colour-coded. Cyan sticks represent the sugar, with blue sticks representing nitrogen atoms and red sticks representing oxygen atoms. **d**, Neu5Gc in sialic acid receptor binding site of SubB. The extra hydroxyl of Neu5Gc points interacts with Tyr78^{OH} and also hydrogen bonds with the main chain of Met10. **e**, Trisaccharide Neu5Gc α 2-3Gal β 1-3GlcNAc β ProN₃ binding to SubB. ProN₃ refers to the linker used in the synthesis. **f**, Neu5Ac α 2-3-Gal 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 α 2–3Gal β 1-GlcNAc β ProN₃. **h**, Side-on view of CtxB:GM₁. In panels d-h, cyan sticks represent ligands, with dark blue sticks representing nitrogen atoms and red sticks 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 $\mu\text{g/ml}$ OG-SubAB, OG-SubAB_{A12} or Texas Red-SubAB_{F78} 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 $\mu\text{g/ml}$ 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 Summary. Data shown are from a single experiment. Analysis of data pooled from triplicate experiments yielded CD_{50} values of 3.92 ± 1.58 pg (mean $\pm$ standard error) and

13.17 $\pm$ 2.46 pg for MDA cells fed Neu5Gc or Neu5Ac, ($P = 0.034$; 2-tailed t -test). For 293 cells fed Neu5Gc or Neu5Ac, the CD₅₀ values are 1.33 $\pm$ 0.30 pg and 11.58 $\pm$ 3.30 pg, respectively ($P = 0.036$). The mean fold increase in SubAB susceptibility ($\pm$ SE) for cells fed Neu5Gc vs Neu5Ac is 5.56 $\pm$ 2.71 for MDA cells and 8.35 $\pm$ 0.88 for 293 cells.

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 μ g/ml, followed by anti-chicken IgY-HRP conjugate, and examined by immunohistochemistry¹⁷ (scale bar, 100 μ m). **b**, Similar human colon sections were overlaid with or without 1 μ g/ml SubAB or SubAB_{A12} and bound toxin was detected using rabbit anti-SubA and Cy3-labeled goat anti-rabbit IgG and examined by epifluorescence microscopy (see Methods) (scale bar, 50 μ m). **c**, Human colon sections were overlaid first with anti-Neu5Gc or control IgY at 5 μ g/ml, followed by 1 μ g/ml SubAB, and bound toxin was detected as for panel b. Background control sections received only rabbit anti-SubAB, followed by Cy3-

labeled anti-rabbit IgG (scale bar, 100 μ m). **d & e**, Human kidney sections were overlaid 1 μ g/ml 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 panel b (scale bar, 50 μ m). **f**, Wild type and *Cmah* null mice (n = 8 each) were injected intraperitoneally with 200 ng/g purified SubAB in a total of 100 μ 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 α 2–3Lac-HSA by wild type versus *Cmah* null mouse serum (f), or human versus chimpanzee serum (g) was assayed by ELISA as described in the Methods. Data are expressed as % of a control with no serum and are the mean $\pm$ SE of triplicate wells and representative of three independent experiments.

Table 1
Glycan array analysis of native SubAB and B subunit mutant SubAB_{A12}.[#]

Glycan #	Structure	SubAB		SubAB _{A12}	
		Avg. RFU	%CV	Avg. RFU	%CV
260	Neu5Gca2-3Galβ1-4GlcNAcβ-Sp0*	37606	4	590	10
261	Neu5Gca2-3Galβ1-4Glcβ-Sp0	25197	16	1998	30
264	Neu5Gca-Sp8*	25114	19	172	87
258	Neu5Gca2-3Galβ1-3GlcNAcβ-Sp0	24209	12	327	54
15	α-Neu5Ac-Sp11	8836	30	1242	46
31	[3OS03]Galβ1-3(Fuca1-4)GlcNAcβ-Sp8	3094	10	1375	41
33	[3OS03]Galβ1-3GlcNAcβ-Sp8	3006	15	3808	45
231	Neu5Aca2-3Galβ1-4(Fuca1-3)GlcNAcβ-Sp8	2401	8	1587	72
46	Neu5Aca2-3[6OSO3]Galβ1-4GlcNAcβ-Sp8	2254	16	2997	11
36	[3OS03]Galβ1-4GlcNAcβ-Sp0	1923	22	3426	4
34	[3OS03]Galβ1-4(Fuca1-3)GlcNAcβ-Sp8	1878	36	2398	32
237	Neu5Aca2-3Galβ1-4GlcNAcβ-Sp8	1862	31	4247	8
240	Neu5Aca2-3Galβ1-4Glcβ-Sp8	1799	10	1127	25
243	Neu5Aca2-6GalNAcβ1-4GlcNAcβ-Sp0	1730	18	1871	67
117	Galβ1-3(Fuca1-4)GlcNAc-Sp0	1549	42	1395	55
68	Fuca1-2Galβ1-4(Fuca1-3)GlcNAcβ-Sp8	1385	30	1204	53
225	Neu5Aca2-3Galβ1-3GlcNAcβ-Sp0	1315	75	1563	16
257	Neu5Gca2-3Galβ1-3(Fuca1-4)GlcNAcβ-Sp0	1244	36	2250	14
259	Neu5Gca2-3Galβ1-4(Fuca1-3)GlcNAcβ-Sp0	1163	37	1719	11
263	Neu5Gca2-6Galβ1-4GlcNAcβ-Sp0	1154	34	859	16
245	Neu5Aca2-6Galβ1-4GlcNAcβ-Sp0	1048	34	1989	38
262	Neu5Gca2-6GalNAcα-Sp0	1003	10	125	23
37	[3OS03]Galβ1-4GlcNAcβ-Sp8	989	21	1362	63
217	Neu5Aca2-3Galβ1-3(Fuca1-4)GlcNAcβ-Sp8	922	14	335	42
10	GalNAcα-Sp8	857	39	938	43
230	Neu5Aca2-3Galβ1-4(Fuca1-3)GlcNAcβ-Sp0	798	26	344	24
134	Galβ1-3GlcNAcβ-Sp8	678	121	197	33

Glycan #	Structure	SubAB		SubAB _{AI2}	
		Avg. RFU	%CV	Avg. RFU	%CV
246	Neu5Acα2-6Galβ1-4GlcNAcβ-Sp8	658	30	755	26
154	Galβ1-4Glcβ-Sp0	644	41	789	48
118	Galβ1-3(Fucα1-4)GlcNAc-Sp8	604	31	411	59
242	Neu5Acα2-6GalNAcα-Sp8	564	16	434	24
133	Galβ1-3GlcNAcβ-Sp0	539	25	604	24
239	Neu5Acα2-3Galβ1-4Glcβ-Sp0	484	46	318	23
49	9-O-AcNeu5Acα2-6Galβ1-4GlcNAcβ-Sp8	478	42	204	59
4	Neu5Gcβ2-6Galβ1-4GlcNAc-Sp8	471	24	176	45
92	GalNAcβ1-4GlcNAcβ-Sp0	419	15	235	24
152	Galβ1-4GlcNAcβ-Sp0	374	40	755	27
67	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ-Sp0	326	52	273	103
155	Galβ1-4Glcβ-Sp8	299	32	164	73
153	Galβ1-4GlcNAcβ-Sp8	298	20	123	44

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 mean relative fluorescence units (RFU) for quadruplicate array spots and % coefficient of variation.

* Sp0 and Sp8 designate CH₂ CH₂ NH₂ and CH₂ CH₂ CH₂ NH₂ linkers, respectively.