Involvement of N-glycans in binding of Photorhabdus luminescens Tc toxin

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Abstract
Photorhabdus luminescens Tc toxins are large tripartite ABC-type toxin complexes, composed of TcA, TcB and TcC proteins. Tc toxins are widespread and have shown a tropism for a variety of targets including insect, mammalian and human cells. However, their receptors and the specific mechanisms of uptake into target cells remain unknown. Here, we show that the TcA protein TcdA1 interacts with N-glycans, particularly Lewis X/Y antigens. This is confirmed using N-acetylglucosamine transferase I (Mgat1 gene product)-deficient Chinese hamster ovary (CHO) Lec1 cells, which are highly resistant to intoxication by the Tc toxin complex most likely due to the absence of complex N-glycans. Restoring Mgat1 gene activity, and hence complex N-glycan biosynthesis, recapitulated the sensitivity of these cells to the toxin. Exogenous addition of Lewis X trisaccharide partially inhibits intoxication in wild-type cells. Additionally, sialic acid also largely reduced binding of the Tc toxin. Moreover, proteolytic activation of TcdA1 alters glycan-binding and uptake into target cells. The data suggest that TcdA1-binding is most likely multivalent, and carbohydrates probably work cooperatively to facilitate binding and intoxication.

KEYWORDS
bacterial toxin, glycan, receptor

1 | INTRODUCTION

The Photorhabdus toxin complex (PTC), also known as the Tc toxin, is a high-molecular-mass (1.7 MDa) insecticidal toxin complex, which is essential for Photorhabdus toxicity. It was first identified in the nematode-associated bacteria Photorhabdus luminescens and Xenorhabdus nematophila (Bowen & Ensign, 1998; Sergeant, Jarrett, Osley, & Morgan, 2003). However, Tc-like genes are widespread, and orthologues have been found in a variety of Gram-negative and Gram-positive bacteria, encompassing insect-, plant- and human-pathogens like Serratia entomaphila (Hurst, Glare, Jackson, & Ronson, 2000), Pseudomonas syringae (Guttman et al., 2002), Yersinia spp.
The Tc toxins are large tripartite ABC-toxin complexes (Ffrench-Constant & Waterfield, 2005; Sheets & Aktories, 2017; Sheets et al., 2011), composed of TcA, TcB and TcC components, which combine into a biologically active holotoxin complex (Ffrench-Constant & Waterfield, 2005; Lang et al., 2010; Roderer & Raunser, 2019; Sheets & Aktories, 2017). The TcA complex is a homopentamer and forms the binding and translocation component of the holotoxin (Gatsogiannis et al., 2013). The TcB–TcC complex forms a hollow coconu-like structure, that encases an active enzyme formed by the hypervariable C-terminal end of TcC (TcChvr) (Busby, Panjikar, Landsberg, Hurst, & Lott, 2013; Meusch et al., 2014). The toxins are also produced in a variety of isoforms likely to target a wide array of host species (Ffrench-Constant & Waterfield, 2005; Forst, Dowds, Boemare, & Stackebrandt, 1997). For instance, up to seven TcA- and TcB-type genes and three TcC-type genes have been identified in P. luminescens strain TTO1 (Ffrench-Constant & Waterfield, 2005; Roderer & Raunser, 2019).

The holotoxin binds to target cells through a yet unknown receptor and is probably taken up by receptor-mediated endocytosis. Acidification of the toxin-containing endosome induces conformational changes in TcA, which then perforates the endosomal membrane in a syringe-like mechanism, forming a pore through which the toxic TcChvr translocates into the host cell cytosol. Here, it re-folds with the help of cytosolic host chaperones into its active conformation (Lang et al., 2014). Two TcChvrs from P. luminescens have been characterized, TccC3 and TccC5. Both are ADP-ribosyltransferases, targeting actin and small GTPases of the Rho family, respectively (Lang et al., 2010; Lang, Schmidt, Sheets, & Aktories, 2011; Pfau mann, Lang, Schwan, & Stacke brandt, 2015). While TccC3 modifies actin at T148, resulting in increased actin polymerisation (Lang et al., 2011), ADP-ribosylation of Rho proteins (Q63 of RhoA and Q61 of Rac and Cdc42) locks the GTPases in a persistently active state. Thus, both toxins act in concert to induce actin polymerisation and clustering (Lang et al., 2010, 2011).

Recently, we showed that TcdA1 (one of the TcA proteins from P. luminescens) is processed by the Photorhabdus metalloprotease PrtA1 and by collagenase, leading to increased toxin activity (Ost, Ng’ang’a, Lang, & Aktories, 2019). This increased toxin activity was in part due to increased cell-surface binding of the cleaved TcdA1. Additionally, we and others showed that the Tc toxin could be used to deliver foreign peptides into target cells (Ng’ang, Ebner, Plessner, Aktories, & Schmidt, 2019; Roderer, Schubert, Sitsel, & Raunser, 2019). Here, we sought to identify cell-surface interactor of the cell binding component TcdA1 of P. luminescens Tc toxin. We report that TcdA1 interacts with N-glycans on target cells, particularly Lewis antigens as possible interactors. Moreover, we identified Chinese hamster ovary (CHO) cells devoid of complex N-glycans (Leε1 cells) as being completely resistant towards the P. luminescens holotoxin, whereas parental CHO cells are sensitive.

2 RESULTS

2.1 TcdA1 interacts with N-glycans and Lewis antigens

To identify interactors of TcdA1, a glycan microarray screen was performed with the help of the Consortium for Functional Glycomics (CFG) (www.functionalglycomics.org). In line with the previously described varying binding capabilities of TcdA1 due to cleavage by metalloproteases like collagenase (Ost et al., 2019), we studied processed TcdA1 (TcdA1FPLC) and the respective unprocessed TcdA1 (TcdA1aff) as control. In addition, a TcdA1 preparation (TcdA1aff) was employed that was not pre-treated for processing (see Methods and Figure S1 for cleavage protocol and TcdA1 nomenclature). The screen data were further analysed using the online Glycan Array Dashboard (GLAD) software (https://glycotoolkit.com/GLAD/) (Mehta & Cummings, 2019). First, a force graph showing interaction of the protein samples with the top glycan hits was generated using default parameters (Figure 1a). All hits with an average relative fluorescence unit (RFU) above 1,000 were included in the analysis. The hits were then grouped according to their interaction with each sample. As shown in Table 1, 10 hits out of 585 tested met these criteria (Figure 1a). Two hits interacted with all samples (red oval), five hits interacted with three samples, that is, the cleaved toxins (5 and 50 μg) and 50 μg of TcdA1FPLC (blue oval). Finally, three hits interacted with two samples, the collagenase-cleaved samples only (green oval) (Figure 1a and Table 1). Binding profiles containing the top 20 hits of the 5 μg samples of TcdA1FPLC and TcdA1FPLC/Collag are shown in Figure 1b,c, respectively, while the 50 μg samples are shown in Figure S2a,b, respectively. Additionally, each glycan present in the top 10 hits (Figure 1a and Table 1) was labelled with the respective coloured circle in Figure 1b,c, as well as Figure S2a.b.

As shown in Table 1, Figures 1b and S2a, the overall top hits interacting with all toxin preparations (red circles) were N-glycans with terminal N-acetylgalactosamine (GlcNAc) or galactose residues (Figure 1d,e). A single sialylated-N-glycan shared by three samples (the two cleaved-TcdA1 samples and the 50 μg uncleaved-TcdA1 sample) was also observed in the top hits (Figure 1f). Highly represented in the top hits were fucosylated (Lewis X and Y) structures (Figure 1g–i), whose different variations interacted with at least two toxin samples (blue and green circles). See Table S1 for the list of all the glycans used in the screen.

The binding profiles also showed that cleavage of TcdA1 by collagenase (TcdA1FPLC/Collag) altered its pattern of interaction with the glycans (Figure S2c). Different glycans were enriched on cleaved and uncleaved proteins (compare Figures 1b,c and S2a,b). Additionally, two-fold higher RFU values were obtained from the cleaved TcdA1s compared to the uncleaved TcdA1 (TcdA1FPLC) component (Figures 1c & S2c), indicating higher glycan binding by the cleaved toxin. However, for the overall top glycan hit, glycan ID: 319, the cleaved samples and the 50 μg TcdA1FPLC sample showed similar RFUs (Figure S2c), suggesting saturation of glycan-binding sites.
Figure 1

Legend
- TcdA1\textsuperscript{PLC} _5 \mu g, TcdA1\textsuperscript{PLC/Collat} _50 \mu g, TcdA1\textsuperscript{PLC} _5 \mu g, TcdA1\textsuperscript{PLC/Collat} _5 \mu g
- Glycans included in the analysis
- Glycans shared by all four samples
- Glycans shared by three samples
- Glycans shared by two samples

(b) TcdA1\textsuperscript{PLC} (5 \mu g)

(c) TcdA1\textsuperscript{PLC/Collat} (5 \mu g)

(d) Complex-type N-glycan (ID 382)

(e) NeoLactosamine N-glycan (ID 319)

(f) Sialylated N-glycan (ID 475)

(g) Lewis type 1

(h) Lewis Y (ID 70)

(i) Lewis X (ID 154)

Lewis Y (ID 69)

Galactose, N-acetylglucosamine, Mannose, Fucose, Sialic acid

Legend on next page.
Possible saturation was also evident when comparing the two TcdA1FPLC/Collag samples (TcdA1C-5 μg and TcdA1C-50 μg) (Figure S2c), increasing the concentration of TcdA1FPLC/Collag from 5 to 50 μg minimally altered the respective binding profiles. The bound glycans remained largely the same (compare Figures 1b & S2a, Figures 1c & S2b) and the curves remained largely similar (Figure S2c). Taken together, these data suggest cleavage-related structure and glycan-binding changes on TcdA1.

To get a picture of the possible glycan-binding sites and domains on TcdA1, an in silico analysis was also performed using 3DLigandSite (Wass, Kelley, & Sternberg, 2010) and I-TASSER (Yang et al., 2015). 3DLigandSite is a web server for prediction of ligand-binding sites on full protein and/or domain structures. Similarly, I-TASSER is a software suite that models protein structure and function from sequences and structural similarity with characterized proteins in protein databases. For this analysis, TcdA1 was split into its various domains as described by Meusch et al., 2014 (Figure S3a), and then the different domains were submitted to 3DLigandSite and I-TASSER for prediction of potential ligands. Splitting of TcdA1 was necessitated by its large size, which could not be accommodated by the software. Ligands bound to similar structures were fitted onto the TcdA1 domains. Subsequently, all the predicted ligands on each domain were ranked according to the total number of hits found for each site, that is, the total number of ligand-binding proteins found for each ligand that showed structural similarity to the respective domain on TcdA1 (Tables S2 and S3).

### TABLE 1
Top 10 hits (out of 585 tested) of a glycan microarray screen that are shared by all tested samples of TcdA1 (TcdA1FPLC and TcdA1FPLC/Collag)

| Number | Glycan ID | IUPAC name | Common name |
|--------|-----------|------------|-------------|
| 1      | 382       | GlcNAcb1-2Manα1-6(GlcNAcb1-4[GlcnAcβ1-2]Manα1-3)Manβ1-4GlcNAcb1-4GlcNAcb-Sp21 | Complex-type N-glycan |
| 2      | 319       | Galβ1-3GlcNAcb1-2Manα1-6 (Galβ1-3GlcNAcb1-2Manα1-3) | Neo Lactosamine, N-glycan basic, Lewis type 1 |
| 3      | 363       | Galβ1-4GlcNAcb1-2Manα1-6(Galβ1-4GlcNAcb1-2)Manα1-3 Manβ1-4GlcNAcb1-4GlcNAcb-Sp19 | Lactosamine, N-glycan basic, Lewis type 2 |
| 4      | 70        | Fuca1-2Galβ1-4(Fuca1-3)GlcNAcb1-3Galβ1-4(Fuca1-3)GlcNAcb-Sp0 | Fucosylated, Lewis X, Lewis Y |
| 5      | 475       | Neu5Acα2-3Galβ1-4GlcNAcb1-2Manα1-6 (Neu5Acα2-3Galβ1-4GlcNAcb1-2Manα1-3) Manβ1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24 | Sialylated, fucosylated, N-glycan basic, Lewis type 2 |
| 6      | 465       | Fuca1-2Galβ1-4(Fuca1-3)GlcNAcb1-2Manα1-6 (Fuca1-2Galβ1-4(Fuca1-3)GlcNAcb1-2Manα1-3) Manβ1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp24 | Fucosylated, N-glycan basic, Lewis X, Lewis Y |
| 7      | 381       | Galβ1-4GlcNAcb1-6(Galβ1-4GlcNAcb1-2)Manα1-6 (Galβ1-4GlcNAcb1-4(Galβ1-4GlcNAcb1-2)Manα1-3) | Lactosamine, N-glycan basic, Lewis type 2 |
| 8      | 154       | Galβ1-4(Fuca1-3)GlcNAcb1-3Galβ1-4(Fuca1-3)GlcNAcb-Sp0 | Fucosylated, Lewis X |
| 9      | 348       | Galβ1-3GlcNAcb1-2Manα1-6 (Galβ1-3GlcNAcb1-2Manα1-3)Manβ1-4GlcNAcb1-4(Fuca1-6)GlcNAcb-Sp22 | Fucosylated, N-glycan basic, Lewis X, Lewis Y |
| 10     | 69        | Fuca1-2Galβ1-4(Fuca1-3)GlcNAcb1-3Galβ1-4(Fuca1-3)GlcNAcb-Sp0 | Fucosylated, Lewis X, Lewis Y |

Note: The hits are colour-coded according to the groups in Figure 1a.

**FIGURE 1**
Glycan microarray screen of TcdA1. (a) Force graph of the top hits interacting with the four TcdA1 samples (5 and 50 μg TcdA1FPLC and TcdA1FPLC/Collag). All hits with an average relative fluorescence unit (RFU) above 1,000 (grey circles) were linked to each TcdA1 sample to reveal shared interactions. The hits marked in red (red oval) were shared by all four samples, those in green (green oval) were shared by three samples and those in blue (blue oval) were shared by two samples. (b) Graph of the top 20 hits for 5 μg TcdA1FPLC (a.u., arbitrary units). Interaction of glycan 154 (labelled with one of the green circles) is missing for TcdA1FPLC, because it is not present in the top 20 hits for this sample. (c) Graph of the top 20 hits for 5 μg TcdA1FPLC/Collag. Red dots, overall top hits shared by all samples; blue dots, top hits shared by three samples; green dots, top hits shared by two samples. (d–i) Glycan structures appearing in the top hits; (d) complex-type N-glycan (ID: 382), (e) Lewis 1 neolactosamine N-glycan (ID: 319), (f) sialylated N-glycan (ID: 475), (g) Lewis Y (ID: 70), (h) Lewis X (ID: 154), (i) Lewis Y (ID: 69)
Interestingly, as shown in Table S2 and Figure S3, a variety of predicted glycan-binding sites were found on TcdA1, particularly on the putative receptor binding domains, which all had at least one predicted glycan-binding site. No sites were found on the α-helical shell domains, indicating that glycan-binding may be restricted to the putative receptor binding domains. Overall, the large variety of predicted glycan-binding sites mirrored the glycan screen, in that, many sites accommodated interactions with mostly N-acetylgalactosamine (GlcNAc), galactose (Gal), fucose (Fuc) and, to a lesser extent, mannose (Man) (Table S2). Expectedly, a single sialic acid–binding site was predicted on the neuraminidase-like domain of TcdA1. Furthermore, some domains had multiple glycans predicted on neighbouring sites, suggesting possible binding to composite glycan structures, rather than single monosaccharides. Table S3 lists all the neighbouring sites, suggesting possible binding to composite glycan sites. Taken together, the high density of putative glycan-binding sites on TcdA1 may explain the interaction with a large variety of N-glycans as observed from the glycan microarray screen.

To confirm the direct involvement of N-glycans, particularly Lewis X (LeX) antigens on cell intoxication by the tripartite holotoxin (PTC3), a LeX trisaccharide (Figure 2a) was synthesized (Biosynth, Basel, Switzerland). Subsequently, affinity-purified TcdA1 (TcdA1aff, 1.4 nM, Figure S1) was premixed with increasing concentrations of the sugar, incubated at room temperature (RT) for 2–5 min, then used to intoxicate HeLa cells together with the TcB-TcC-fusion (BC3) component (3.5 nM) (note, the complete tripartite toxin is called PTC3). As a negative control, Clostridioides difficile (formerly called Clostridium difficile) toxin B was used. As shown in Figure 2b,d, the LeX trisaccharides delayed cell intoxication by PTC3 in a concentration-dependent manner, while C. difficile toxin B was not affected by addition of the sugar (Figure 2c,e). Furthermore, exogenous addition of a mixture of the individual sugars constituting the LeX trisaccharide (Gal, GlcNAc and Fuc) at the same concentrations did not affect cell intoxication (Figure 2f). These results further supported the hypothesis of the involvement of LeX trisaccharides in the interaction of PTC3 with the cell surface. Additionally, the data suggested that the glycans must be in a defined complex, rather than single monosaccharides in order to interact with the toxin.

### 2.2 N-glycan-deficient cells are resistant to intoxication by PTC3

To gain more insight into N-glycan binding, Chinese hamster ovary (CHO) Lec1 cells, which do not produce complex and hybrid N-glycans, were used. These cells have a mutated Mgal gene, which encodes N-acetylgalactosaminyltransferase I (GlcNAc-TI) and hence lack N-glycans (Puthalakath, Burke, & Gleeson, 1996). However, they have short oligomannose trees and produce normal O-linked glycans (North et al., 2010). The cells were intoxicated with affinity-purified PTC3 (PTC3aff) (Figure S1) and, as a positive control, with Clostridium botulinum C2 toxin, which was shown to interact with N-glycans (Eckhardt, Barth, Blöcker, & Aktories, 2000). As a negative control, C. difficile toxin B was used. As shown in Figure 3a,b, Lec1 cells were resistant to PTC3 and C. botulinum C2 toxin, but not to C. difficile toxin B, indicating a dependence on complex N-glycans for PTC3 binding.

Comparatively, cells deficient in various monosaccharides that constitute N-glycans were sensitive to PTC3. Specifically, CHO Lec2 and Lec8 cells lacking sialic acid and galactose, respectively (North et al., 2010), as well as fucose-deficient leukocyte-adhesion deficiency II (LADII) cells (Wild, Luhn, Marquardt, & Vestweber, 2002) were all susceptible to PTC3. All cells were intoxicated with PTC3 for 5 hr, and the percentage of rounded cells was quantified. As shown in Figure S4a–c, none of the cell lines were resistant to intoxication by PTC3. Furthermore, neither Lec2 nor Lec8 cells showed decreased toxin binding compared to the wild-type CHO-K1 (Figure S4d). These data further supported the involvement of glycan complexes rather than single monosaccharides in toxin interaction with the target cells.

We sought to confirm that the gained resistance of Lec1 cells to PTC3 is due to deficiency in GlcNAc-TI activity. Accordingly, we transfected the Lec1 cells with Mgal gene, cloned in a pEGFP-N1 vector. This resulted in the transient expression of GlcNAc-TI-EGFP fusion protein (Figure 4a). As controls, cells were either left untreated, treated with Lipofectamine 2000 alone, or transfected with pEGFP empty vector (pEGFP-Control). Subsequently, the cells were intoxicated for 5 hours with PTC3FPLC (Figure 4b,c). Only cells transfected with Mgal showed significant cell rounding as a result of intoxication (Figure 4c). Non-transfected cells, as well as those treated with Lipofectamine alone, or carrying the empty vector, were still resistant to PTC3. Therefore, this result confirmed that the resistance of Lec1 cells to intoxication by PTC3 was due to the mutant Mgal gene.

Resistance towards PTC3 was confirmed through a post-intoxication ADP-ribosylation assay. Here, CHO-K1 wild-type and CHO-Lec1 mutant cells were either intoxicated for 3 hr with PTC3aff, or left untreated. Then, the cells were lysed and lysates incubated with the fused toxin components TcB-C3 (BC3, 0.7 μM), which possess actin-ADP-ribosylating activity, in the presence of biotin-NAD+ (50 μM). After the ADP-ribosylation reaction, samples were separated by SDS-PAGE and modification of actin was analysed by Western blotting using a horseradish peroxidase–avidin D. As depicted in Figure 5a,c, only the CHO-K1 cells were intoxicated and, subsequently, did not show any signal after in vitro ADP-ribosylation by BC3 (Figure 5b). The lack of signal in this lane confirmed the initial intoxication by PTC3, as all actin was already ADP-ribosylated and could not be modified any further by TccC3hvr (the enzyme contained in BC3). As a control, recombinant βγ-actin was used in the presence or absence of BC3. Only actin treated with both BC3 and biotin-NAD+ showed a signal (Figure 5b). Unmodified actin appeared to be degraded, when incubated at similar conditions (actin control).

To further confirm that resistance of Lec1 is due to deficient binding, interaction of the toxin with the cells was analysed by flow cytometry. Increasing concentrations of Dylight-488-labelled TcdA1FPLC/Colag were incubated with the cells for 15 min at 4°C,
FIGURE 2  Effects of exogenous addition of Lewis X trisaccharides to cell intoxication by the PTC. (a) Structure of the artificially synthesized fucosylated (Lewis X) trisaccharide. (b) Intoxication of HeLa cells for 4 hr with PTC3 consisting of TcdA1aff (1.4 nM) premixed with the indicated concentrations of sugars and the BC3 component (3.5 nM). As control, cells were left untreated. (c) Intoxication of HeLa cells for 4 hr with toxin B (4 pM) premixed with the indicated concentrations of sugars. (d) Quantification of the number of rounded cells in cells intoxicated in (b). (e) Quantification of the number of rounded cells in cells intoxicated in (c). (f) Intoxication of HeLa cells for 4 hr with PTC3 consisting of TcdA1aff (1.4 nM) premixed with monosaccharides constituting the Lewis X trisaccharide in (a), galactose, fucose and N-acetylglucosamine (GlcNAc). Unpaired, two-tailed t test (*p < .05, **p < .01, ns, not significant, n = 3, ±SEM). The scale bar is 80 μm.
before washing and analysis. Compared to wild-type CHO-K1 cells, Lec1 cells showed significantly reduced binding (Figure 6a). Furthermore, pre-treatment of TcdA1 with various monosaccharides, even at much higher concentrations (100 mM), before intoxication, neither affected interaction of PTC3 with Lec1 cells (Figure 6b) nor inhibited intoxication of CHO-wild-type cells (Figure 6c). Sialic acid is the only monosaccharide that showed an inhibitory effect on PTC3 action at lower concentrations (Figure 6d), indicating a more prominent role of this sugar in PTC binding and/or intoxication. This effect was studied in more detail with HeLa cells (see below). These data corroborate the dependence of PTC3 on cell-surface N-glycans for binding and intoxication of mammalian cells.

2.3 | TcdA1 may interact with glycosaminoglycans (GAGs)

GAGs are linear, anionic polysaccharides that provide an ideal landing pad for proteins, macromolecules, bacteria, viruses and parasites (Kamhi, Joo, Dordick, & Linhardt, 2013; Tao et al., 2019). Therefore, to test whether they may be involved in TcdA1 binding, TcdA1off was premixed with heparin (HP), heparan sulfate (HS), the semi-synthetic GAG analogue dextran sulfate (DexS) and chondroitin sulfate (CS) and incubated for 2–5 min at RT. Then, together with BC3, it was used to intoxicate HeLa cells. HP, HS and DexS partially reduced cell intoxication (Figure S5a–c). The effect of CS was minimal (Figure S5d). Lower concentrations of HP and HS than of DexS were required to inhibit intoxication. Nevertheless, these results suggested some influence of GAGs on binding and intoxication by PTC3.

To study the influence of SA on PTC3, we first assessed its effect on HeLa cell intoxication. In this regard, TcdA1off was premixed with increasing concentrations of SA as previously described and then added to cells together with BC3. As shown in Figure 7a,b, SA inhibited intoxication of HeLa cells in a concentration-dependent manner, starting at 0.1 mM. Inhibition of intoxication was also observed on CHO-K1 cells (Figures 6d and 7c), indicating that this effect was not cell type-dependent. Next, we tested the effect of SA on the binding of TcdA1 on cells. Here, Dylight-488-labelled TcdA1FPLC/Collag premixed with increasing concentrations of SA was allowed to bind to HeLa cells. After washing, the amount of bound toxin was assessed by flow cytometry. Interestingly, while SA inhibited the binding of TcdA1FPLC/Collag to HeLa cells in a concentration-dependent manner (Figure 7d), toxin binding was observed at 0.1 mM. This result raised a discrepancy in the concentration dependency of effects of SA on toxin binding and intoxication.

To resolve the discrepancy between toxin binding and intoxication, we used a more sensitive assay to assess toxin binding. To this end, we analysed a BC3 complex where C3hvr (the enzyme contained in BC3) was replaced with MLuc7, a small luciferase from the marine copepod Metridia longa (Markova, Golz, Frank, Kalthof, & Vysotski, 2004). We had previously shown that MLuc7 was efficiently translocated through TcdA1, and that it subsequently provided a...
sensitive non-toxic read-out for cargo delivery into target cells (Ng’ang et al., 2019). Therefore, we used the direct pH 5-dependent membrane-translocation of Mluc7 by PTC3-Mluc7 at the plasma membrane as an indicator for toxin delivery into the cytosol (Ng’ang et al., 2019). TcdA1 pre-mixed with increasing concentrations of SA was added to HeLa cells, together with BC3-Mluc7 and allowed to bind for 1 hr at 4°C. Then, after washing, pore formation was induced by pH change to pH 5, to allow delivery of the luciferase into the cells. As shown in Figure 7e, a reduced but statistically significant luminescence was observed at 0.1 mM SA, thus confirming binding of

FIGURE 4  Re-expression of MgatI in CHO Lec1 cells. (a) Expression of EGFP-MgatI in Lec1 cells. Lysates (50 μg) from cells transfected with either pEGFP empty vector, or pEGFP-MgatI vector, were analysed by Western blot using an anti-EGFP antibody. Lane 1 = size marker, Lane 2 = lysate from cells carrying the pEGFP empty vector and Lane 3 = lysate from cells carrying the pEGFP-MgatI vector. GAPDH was used as a loading control. (b) Lec1 cells treated with Lipofectamine 2000 only, pEGFP empty vector, pEGFP-MgatI vector, or left untreated, respectively. Cells were transfected as described in methods and cultured overnight, then either intoxicated for 5 hr with PTC3FPLC/Collag (1.4 nM TcdA1FPLC/Collag + 3.5 nM BC3) (bottom panel), or left untreated (top panel). The scale bar is 80 μm. (c) Quantification of cells intoxicated in (b). Unpaired, two-tailed t test (**p < .001; ns, not significant, n = 3, ±SEM)
TcdA1 at this concentration, as was observed by flow cytometry (Figure 7d). As controls, we assessed the direct effect of SA on BC3-Mluc7 and PTC3-Mluc7 alone. As depicted in Figures 7f and S6, SA neither affected luciferase activity by PTC3-Mluc7 nor by BC3-Mluc7 alone, respectively.

In our experiments, SA has a significant effect if added in high concentrations (> 0.1 mM), but we did not detect any change in toxin binding or activity if SA was applied at lower concentrations, which are more physiological. This indicates that, at high concentrations, SA may mask important amino acids required for the toxin-receptor interaction due to its negative charge, rather than specific binding to the toxin.

3 | DISCUSSION

Tc toxins are widespread and have shown a tropism for a variety of targets ranging from insect, to mammalian and human cells (Ffrench-Constant & Waterfield, 2005; Hinchliffe, Hares, Dowling, & Ffrench-Constant, 2010; Sheets & Aktories, 2017). However, their receptors and specific mechanisms of uptake into the target cells remain unknown. The glycan screen performed in this study revealed interactions with a range of N-glycan structures independent of the terminal sugar residues. The top hit in the array was a bi-antennary N-glycan (ID 319) probably mediating higher affinity interaction due to an enhanced number of binding sites; however, monovalent N-glycans chains, including fucosylated oligo-N-acetyllactosamine chains (Lewis X and Lewis Y epitopes), also appeared among the top hits. Furthermore, in silico prediction of ligand-binding sites on TcdA1 revealed a variety of glycan-binding sites spread throughout TcdA1’s putative receptor binding domains. The strongest support for the crucial role of N-glycans was obtained by the total resistance of the CHO-Lec1 cells, which lack complex type N-glycans, towards PTC3. Indeed, restoring Mgat1 gene activity, and hence complex N-glycan biosynthesis, recapitulated the sensitivity of these cells to the toxin. Importantly, resistance to intoxication was primarily due to lack of PTC3 binding onto the cells and not to inhibition of enzyme activity. The ADP-ribosylating C3hvr enzyme was able to modify actin in lysates of non-intoxicated CHO-K1 control cells, but not in previously intoxicated CHO-K1 cells, whose actin had already been modified during intoxication. In contrast, C3hvr was able to modify actin in lysates of intoxicated and non-intoxicated Lec1 cells, indicating that the enzyme was still active.

Complex and hybrid N-glycan structures have been identified in insects (Aoki et al., 2007; Vandenborre et al., 2011; Walski, De Schutter, Van Damme, & Smagghe, 2017), which are the primary targets of these toxins and also in vertebrates (North et al., 2010; Schiller, Hykollari, Yan, Paschinger, & Wilson, 2012), to which the toxin has shown activity (Lang et al., 2011). Notably, Le⁴-trisaccharide-dependent inhibition of intoxication was relatively weak, in the micromolar to the millimolar range, indicating medium-to-low affinity. This suggests that they may be co-receptors, acting co-ordinately or synergistically with other receptors to facilitate binding. The large size of the toxin supports this argument, as it would probably require multiple interactions to gain and maintain stability on the surface for injection. Although fucose-, galactose- and sialic acid-terminated structures were among the top hits in the glycan array, neither Fuc- nor Gal-deficient (as a result of that lacking most sialic acid) cells showed resistance to intoxication. Furthermore, none of the monosaccharides added in physiological concentrations affected cell intoxication, both when applied singly or in a mix. Exclusively, sialic acid reduced
intoxication in high concentrations, which most likely is due to its negative charge and not due to specific interference with the toxin-receptor interaction. The saccharides, however, reduced toxin activity as part of the Le^x^-trisaccharide, suggesting that they probably need to be incorporated into glycan structures to have an effect.

Lewis antigens are typical constituents on cell membranes of human red blood cells (Marcus & Cass, 1969) and are targets for norovirus (Kubota et al., 2012; Rydell, Svensson, Larson, Johannes, & Römer, 2013) or cholera toxin (Cervin et al., 2018; Heim, Hodnik, Heggeland, Anderluh, & Krengel, 2019; Wands et al., 2015). Similarly,
Fucosylated glycans are present in insects (Stanton et al., 2017); however, unlike in vertebrates, fucosylated structures as part of Lewis-type antigens are less abundant than core α-1,6- and difucosylated (α-1,6 and α-1,3) structures (Rendic et al., 2006, 2010). This, therefore, suggests that these structures might not be universal targets of TcdA1 on insects, but only on some, like Lepidopterans, which do possess α-1,3-fucosylated Lewis-like structures on glycoproteins (Stanton et al., 2017).

Very recently, while our manuscript was in preparation, the group of Stefan Raunser reported on the interaction of glycans with TcdA1 (Roderer et al., 2020). They also identified Lewis antigens as interaction sites of TcdA1. Moreover, using molecular docking, they could demonstrate the interaction of BSA-Lewis X with the so-called receptor binding domain D. This interaction was of low affinity and supports the view of a co-receptor function. In contrast, our findings with
CHO-Lec1 cells, which are only marginally sensitive towards the Tc toxin, suggest that N-glycans play a leading and essential role in the interaction of the toxin with host cells. This was also supported by increased glycan binding by the collagenase-cleaved toxin (TcdA1FPLC/Collag), which has also shown increased cell binding and intoxication kinetics (Ost et al., 2019). CHO-Lec1 cells were also resistant to TcdA1FPLC/Collag. Importantly, glycan structures on Lec1 cells are much reduced, leaving only Man5GlcNAc2 core structures, but does not affect O-glycans (North et al., 2010) (Figure S7a). Therefore, binding to N-glycans may require, at least, the next level of GlcNAc addition, which is catalysed by the missing Manat1 gene. After the action of Mgat1 and mannosidase II, additional GlcNAc-transferases create more terminal GlcNAc residues, which are not further extended by galactoses in Lec8 cells (Kawar et al., 2005) (Figure S7b). As these Lec8 cells are sensitive to PTC3, the critical step that makes cells sensitive to the toxin seems the addition of GlcNAc residues to N-glycans. Exactly an N-glycan terminating with three GlcNAc residues was the top hit in the glycan array, followed by several other N-glycans further extended. This is also the last step that is common between mammals and insect.

We and Roderer and coworkers (Roderer et al., 2020) showed the binding of heparins and fucosylated structures of Lewis-type antigens onto various types of TcA toxin components. In each case, we observed a relatively moderate inhibition of the toxin effect by the addition of these compounds, with heparin showing the strongest effects. However, CHO mutant (pgsA-745) cells, which lack or have reduced CS and HS content (Esko, Stewart, & Taylor, 1985), exhibited no reduced intoxication, suggesting cell-type-dependent variation in the effect of GAGs.

Roderer et al. (Roderer et al., 2020) suggested that because GAGs form long negatively charged polymers on the cell surface, extending 20–150 nm from the plasma membrane, they could act as primary low-affinity receptors that provide a landing pad for the toxin. This initial binding on GAGs could then be followed by binding to N-glycans, such as the Lewis antigens, which themselves are also not essential, as they are absent in sensitive Lec8 and LADII cells. Notably, of all the TcA proteins they studied, only TcdA1 from *P. luminescens* showed significant binding to N-glycan fragments. For none of the other TcA proteins, a significant binding to the glycan microarray was evident, even at higher protein concentrations.

Interestingly, for a related Yersinia entomophaga ABC toxin, GlcNAc-containing glycans were the main binding partners (Piper et al., 2019).

Like the LeX antigens, the marginal effects observed indicate that GAGs and sialic acids are also likely not high-affinity targets, further supporting their role as an initial landing pad for the toxin. Notably, Lec1 cells expressed more than the double amount of GAG compared to wild-type cells (Fujitani et al., 2013), yet they were resistant to TcdA1.

The appearing picture of our experiments and of related studies by others as described above is that Tc toxins might bind with low affinity to a variety of different glycans, possibly by different binding sites, but none of these structures alone are essential for binding. We, as well as Roderer et al., established that the formation of complex glycans is, however, essential for toxin binding. We could also show that further extension is not required. Whether the GlcNAc residues attached in this essential step are directly involved in toxin binding, remains to be determined.

### 4 | EXPERIMENTAL PROCEDURES

#### 4.1 Materials

Cell culture medium, Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 and foetal calf serum (FCS) were purchased from Biochrom (Berlin, Germany). Cell culture materials were obtained from Greiner (Frickenhausen, Germany), Ni-IDA resin was from Macherey-Nagel (Düren, Germany), Bafilomycin A1 was purchased from Enzo Life Sciences (Lörrach, Germany). Dynasore was purchased from Sigma (now Merck, Darmstadt, Germany). DyLight 488 was purchased from Thermofisher (Schwerte, Germany). *Clostridium histolyticum* collagenase type IA was purchased from Sigma (now Merck, Darmstadt, Germany). Lewis X trisaccharide was synthesized by Biosynth (Basel, Switzerland). All glycosaminoglycans were purchased from Sigma (now Merck, Darmstadt, Germany).

#### 4.2 Protein expression and purification of *Photorhabdus* toxins

Proteins were expressed and purified as previously described (Gatsogiannis et al., 2013; Lang et al., 2017). In short, *E. coli* BL21-CodonPlus cells were transformed with *Photorhabdus luminescens* TcdA1 and TcdB2-TccC3 (BC3) genes in pET28a vector, and protein expression was induced with 75 μM and 25 μM IPTG, respectively. After 24 hr, all protein-expressing cells were harvested and resuspended in lysis buffer (300 mM NaCl, 20 mM Tris−HCl, pH 8.0, 1 mM DTT, 500 μM EDTA, and 10% glycerol) supplemented with DNase (5 μg/mL), lysozyme (1 mg/ ml), and 1 mM PMSF. After sonication, the cell lysate was incubated with Ni-IDA resin and loaded onto empty PD-10 columns and purified by affinity chromatography. The His6-tagged proteins were eluted with 500 mM NaCl, 20 mM Tris−HCl, pH 8.0, 0.05% Tween-20, 500 mM imidazole, and 5% glycerol. The protein-containing fractions were pooled and dialysed in a buffer containing 100 mM NaCl, 50 mM Tris, pH 8.0, 0.05% Tween-20 and 5% glycerol. Subsequently, the proteins were stored in storage buffer (100 mM NaCl, 50 mM Tris, pH 8.0, 0.05% Tween-20 and 30% glycerol).

#### 4.3 Cleavage of TcdA1

In some instances, affinity purified TcdA1 (TcdA1aff) was further processed by cleavage using collagenase as described by (Ost et al., 2019). For this, 3 μg of TcdA1 was first incubated for 1 hr with
programs. ImageJ/Fiji software, followed by subsequent analysis using statistics periods, the cells were visualised and pictures taken. Toxin effects cells together with the BC3 complex. After the indicated incubation 

split into its various domains as described by (Meusch et al., 2014). To predict potential glycan binding sites on TcdA1, the protein was pre-incubated for 2 min at room temperature, then, added to the 

| 4.4 | Glycan microarray screen |

To identify interactors of TcdA1, a glycan microarray screen was performed with the help of the Consortium for Functional Glycomics (CFG) (www.functionalglycomics.org). Version 5.4 of the printed array which consists of 585 glycans in replicates of six was used. In line with the previously described varying binding capabilities of TcdA1 due to cleavage by metalloproteases, we studied processed TcdA1 (TcdA1 FPLC/Collag) and the respective unprocessed TcdA1 (TcdA1 FPLC) as control. The toxins were sent to the CFG as either fluorescently labelled with Dylight488 or unlabelled. In the latter case, a TcdA1-specific mouse monoclonal antibody, followed by a fluorescently labelled secondary antibody were used to probe the toxin bound onto the microarray. Subsequently, the screen data were first tabulated and analysed in Excel, followed by visualisation and analysis using the online Glycan Array Dashboard (GLAD) software (https://glycotoolkit.com/GLAD/) (Mehta & Cummings, 2019) provided by the CFG.

| 4.5 | Cell cytotoxicity assays |

For cytotoxicity experiments, cells were seeded in culture dishes and incubated in starvation medium (0.5% FCS) together with the respective toxins. Where pharmacological inhibitors were used, the cells were pre-incubated with the inhibitors for the stated duration before intoxication. For intoxication in the presence of glycans, TcdA1 was premixed with each sugar at the indicated concentrations, pre-incubated for 2–5 min at room temperature, then, added to the cells together with the BC3 complex. After the indicated incubation periods, the cells were visualised and pictures taken. Toxin effects were quantified by counting the number of rounded cells using the ImageJ/Fiji software, followed by subsequent analysis using statistics programs.

| 4.6 | In silico prediction of glycan binding sites on TcdA1 |

To predict potential glycan binding sites on TcdA1, the protein was split into its various domains as described by (Meusch et al., 2014). Then, the different domains were submitted to 3DLigandSuite (Wass et al., 2010) and I-TASSER (Yang et al., 2015) for the prediction of potential ligands and ligand-binding sites. Both Phyre2 and I-TASSER were used for structure modelling cases of the different protein domains. The resultant structures were then employed in I-TASSER and 3DLigandsite to predict ligand binding sites. For this, TcdA1 domains were superimposed to similar structures in protein structure databases and their subsequent known ligands fitted onto the TcdA1 domains. Subsequently, all the predicted glycan ligands were quantified according to the total number of predicted sites per domain.

| 4.7 | Trans-epithelial electrical resistance assays |

For Trans-epithelial electrical resistance (TEER) assays, the Electrical Cell-substrate Impedance Sensing (ECIS) system (Applied biophysics) was used. Caco-2 cells were seeded onto 8-well 8W10E+ ECIS arrays (Ibidi GmbH) and cultured for 2 days. When TEER values remained constant at ~1,000–1,600 Ω (100% confluence), assays were performed. The ECIS arrays were first pre-cooled on ice for 15 min before intoxication. The toxins were allowed to bind for 1 hr on ice, and then the arrays were transferred to 37°C where TEER was measured. Subsequently, the resistance readings were tabulated and analysed.

| 4.8 | Fluorescence-activated cell sorting analysis |

For flow cytometry-based analysis of toxin binding to target cells, proteins labelled with DyLight488 (Thermo Scientific) according to the manufacturer’s recommendations were used. First, cells cultured on 10 cm dishes were detached using 10 mM EDTA in PBS, washed twice with PBS and adjusted to 500,000 cells/ml. Second, the labelled proteins were added to cells and incubated in the dark at 4°C on a rotary shaker. Third, after the indicated incubation time, the cells were washed twice in PBS and subjected to Fluorescence-activated cell sorting (FACS) analysis. Cell surface-bound fluorescence was detected with an argon ion laser (488 nm) and 530 nm band-pass filter.

| 4.9 | Post-intoxication ADP-ribosylation of Actin |

CHO-K1 and Lec1 cells intoxicated for 3 hr by PTC3 were collected and lysed (lysis buffer; 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM PMSF and 2.5 mM DTT). Then, after lysis, in vitro ADP-ribosylation of actin was performed as previously described (Lang et al., 2014). Briefly, BC3 (0.7 μM) and biotin-labelled NAD+ (70 μM) were added to the lysates and incubated for 20 min at 37°C. As a control, recombinant βγ-actin (1.9 μM) was used. The reactions were stopped by addition of Laemmli buffer. Afterwards, the samples were subjected to SDS-PAGE, blotted and the biotin-labelled, that is, ADP-ribosylated actin, was detected with horseradish
peroxidase–avidin and the ECL system. The amount of total actin is shown as loading control.

4.10 | Transfection of CHO Lec1 cells

Cells were seeded on 12-well plates and allowed to grow overnight. The transfection mix consisting of Opti-MEM containing Lipofectamine 2000 (6 μl) and plasmid DNA (3 μg of the empty pEGFP-N1 vector and 10 μg of MgalI) was prepared according to the manufacturer’s instructions. Then after a medium change, the transfection mix was added. The cells were then incubated overnight. Subsequently, after a medium change to starvation medium (0.5% FCS), the cells were intoxicated for 5 hr with PTC3FPLC/Collag (1.4 nM TcdA1FPLC/Collag + 3.5 nM BC3) and pictures taken hourly. Additionally, some transfected cells were harvested and lysed (lysis buffer; 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1 mM PMSF, and 2.5 mM DTT). The lysates were then separated on an SDS-PAGE gel, transferred on to nitrocellulose membranes and blotted with an antibody against EGFP (cell signalling).

4.11 | pH-dependent bioluminescence assays

For pH-dependent bioluminescence assays, HeLa cells were first incubated with 100 nM of Bafilomycin A1 (Enzo life sciences) for 30 min at 37°C. Then, they were transferred to ice, precooled to 4°C. Afterwards, BC3-Mluc7 only or PTC3-Mluc7, treated with, or without sialic acid, was added and incubated for 1 hr in 1x Hank’s balanced salt solution (HBSS) buffer supplemented with 20 mM Hepes, pH 7.5, to allow for toxin binding. This experimental setup ensured toxin binding at the cell surface without uptake into the cells. Then, to induce injection of Mluc7 into the cells by the surface-bound toxin complex, the pH of the surrounding medium was changed to acidic pH 5 medium (DMEM medium with 0.5% FCS and 20 mM MES, pH 5). This pH change mimicked the endosomal pH required to induce pore formation and translocation of the payload into the cytosol. These cells in pH medium were incubated for 1 min at 37°C before the medium was shifted back to pH 7.5. Afterwards, the cells were washed and treated with trypsin/EDTA for 2 min at 37°C to degrade extracellular protein and to simultaneously detach them from the plate. Finally, the cells were harvested and lysed in passive lysis buffer (Promega) and transferred into an infinite M200 microplate reader (Tecan) for detecting luciferase activity in the presence of coelenterazine H (Biotium).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTIONS

Peter Njenga Ng’ang’a, Lina Slukstaitė, and Gudula Schmidt performed experiments and prepared figures. Peter Njenga Ng’ang’a, Klaus Aktories, Hans Bakker, and Gudula Schmidt wrote the manuscript. Klaus Aktories, Gudula Schmidt, Alexander E. Lang, and Winfried Römer supervised the project. All authors reviewed the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study (Figure 1) are openly available under (www.functionalglycomics.org), reference number [cfg_rRequest_3630] or available in the supplementary material of this article.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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