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Structural basis for promiscuity and specificity during Candida glabrata invasion of host epithelia

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The human pathogenic yeast Candida glabrata harbors more than 20 surface-exposed, epithelial adhesins (Epa) for host cell adhesion. The Epa family recognizes host glycans and discriminates between target tissues by their adhesin (A) domains, but a detailed structural basis for ligand-binding specificity of Epa proteins has been lacking so far. In this study, we provide high-resolution crystal structures of the Epa1A domain in complex with different carbohydrate ligands that reveal how host cell mucin-type O-glycans are recognized and allow a structure-guided classification of the Epa family into specific subtypes. Further detailed structural and functional characterization of subtype-switched Epa1 variants shows that specificity is governed by two inner loops, CBL1 and CBL2, involved in calcium binding as well as by three outer loops, L1, L2, and L3. In summary, our study provides the structural basis for promiscuity and specificity of Epa adhesins, which might further contribute to developing anti-adhesive antimycotics and combating Candida colonization.
preference for glycans containing galactose at nonreducing ends (Fig. 1A and B). Both α- and β-linked galactoses are bound by Ep1A, but the binding capability for Galβ1-terminated glycans is significantly higher than for those with Galα-terminal residues. Previous binding studies with Ep1A presented at the surface of S. cerevisiae indicated that this adhesin does not discriminate between mucin-relevant Galβ1-3 and Galβ1-4 disaccharides (15). Here, also no obvious preference was found when comparing Galβ1-3 and Galβ1-4-containing glycans, but all of the best binders within the Galβ1-4 group contain terminal Galβ1-3 branches (Fig. 1B). To further discriminate between stringent primary and nonstringent secondary specificities, binding at lower protein concentrations was conducted. At 50 μg/mL, Ep1A, a moderate decrease in the group of Galβ1-4-containing glycans was observed that sharply drops at 1 μg/mL. Likewise, oligosaccharides with terminal α-linked galactose moieties show diminished binding at restricting Ep1A concentrations, further pointing to a preference for β-linkage. Best binders are Galβ1-3 group members, especially those with a Galβ1-3GalNAc motif including the T-antigen (glycans 137 and 140, Fig. 1A). In contrast, β4-linked lactosides, which are known to inhibit Candida adherence to host cells in the millimolar range (7), show only weak (lactose, glycans 169 and 170) or very moderate (α-acetyltactosamine, glycans 167 and 168) binding to Ep1A. In summary, our data provide a refined specificity profile for Ep1A and offer compelling evidence that this adhesin prefers Galβ1-3 disaccharides, but lacks high affinity for Galβ1-4 disaccharides. This conclusion is further supported by our fluorescence titration analysis (Fig. S1), which demonstrates that Ep1A has at least a 16-fold higher binding affinity for the T-antigen (Kd = 2.1 ± 0.3 μM, Galβ1-3GalNAc) than for milk-derived lactose (34.6 ± 6.1 μM), which is likely to contain significant amounts of a Galβ1-3Glc contaminant as delineated by our structural analysis below.

Overall Ep1A Structure. To determine the structure of Ep1A, crystals were obtained at low pH, but only in the presence of high concentrations of lactose. The Ep1A crystal structure was solved by molecular replacement, using a truncated homology model based on the S. cerevisiae Flo5A structure (12). Despite a pairwise sequence identity of less than 25%, the resulting structure (Tables S1 and S2) reveals a high similarity with Flo5A, as indicated by an rmsd of 1.4 Å over 121 common Cα atoms (Fig. 2A). The barrel-shaped Ep1A domain has an overall dimension of 57 × 42 × 36 Å3, consists of 15 β-strands, and is complexed via a calcium ion to its respective disaccharide ligand (Fig. 2A). Eleven of the strands form an antiparallel β-sandwich motif with PA14/Flo5-like topology (16). An L-shaped stretch, composed of the remaining four β-strands and the N and C termini, wraps around the β-sandwich and protects its bottom end from solvent access (12). This end of Ep1A is adjacent to the repetitive stalk of the Ep1 B domain. The two disulfide bridges C50–C179 and C180–C263, respectively, which covalently tether the N and C termini to the β-sandwich domain (Fig. 2A), are preserved in Flo5, suggesting that this rigid linkage between A and B domains is a general feature of GPI-CWP adhesins.

Which structural motifs define the ligand-binding site in Ep1A? In contrast to S. cerevisiae flocculin, Ep1A lacks the Flo5-like subdomain, which in budding yeast confers specific interactions with terminal α1,2-mannoside ligands (12). Surprisingly, the difference electron density map within the binding pocket of the Ep1A/lactose cocryystals clearly ruled out lactose, i.e., Galβ1-4Glc, as bound ligand. At 1.5 Å resolution only a Galβ1-3Glc disaccharide, a contaminant from commercially available lactose, was consistent with the electron density observed (Fig. 2B). This serendipitous finding corroborates the strong preference of Ep1A for Galβ1-3 over excess of Galβ1-4-linked disaccharides as indicated by our glycan profiling and

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**Fig. 1.** Glycan binding by the Ep1A domain. (A) Binding profiles of Ep1A at different protein concentrations (1, 50, and 200 μg/mL), using the CFG array V4.1 harboring 451 different glycan structures. Relative fluorescence units as monitored reflect relative affinities toward the corresponding glycan. Glycans bound by Ep1A are indicated by their CFG array numbers. Overall, Ep1A recognizes exclusively terminal galactosides. Green, Galα group; red, Galβ1-3 group; blue, Galβ1-4 group. (B) Groups that are recognized by Ep1A are shown; their locations within the CFG array are given in parentheses. Structural formulas are described according to the CFG nomenclature. At 200 μg/mL, Ep1A recognizes both Galβ1-3 (red) and Galβ1-4 (blue) glycans. However, the latter can harbor terminal β1-3 ramifications (red dotted lines). At low concentrations, Ep1A has a much narrower specificity profile.
fluorescence titration analyses. The binding site of the Galβ1–3 disaccharide is formed of five loops: two inner, short ones involved in calcium binding and ligand recognition (CBL1 and CBL2) and three outer, longer loops (L1, L2, and L3), which shield CBL1 and CBL2 from solvent access (Fig. 2D). CBL1 links β-strand 8 with 9 and is hallmarked by an unusual cis-peptide between D165 and D166, the DcisD motif. This motif is crucial for the C-type lectin function of Flo5A (12) and is conserved throughout the entire Epa family (Fig. S2). CBL2 links β-strands 12 and 13 and directly contributes to specific glycan binding by four of its side chains, R226, E227, Y228, and D229 (Fig. 2D and E), which we further refer to as CBL2 positions I–IV (Fig. 3). Finally, the Ca2+-binding site comprises the side chain of N225, the carbonyl groups of peptide bonds within CBL2, and the carboxylic side chains of D165 and D166, the DcisD motif of CBL1. Moreover, the Ca2+ ion directly interacts with the nonreducing galactose moiety over the C-3 and 4-hydroxyl groups (Fig. 2B) and is essential for glycan binding (Fig. S1).

Disaccharide Recognition by the Inner and Outer Subsites of Epa1A. What is the structural basis for disaccharide discrimination by Epa1A? The Epa1A-Galβ1–3Glc complex indicates that at least two subsites are crucial for glycan recognition: (i) the inner subsite, which is formed of CBL1, the Ca2+ ion, R226 from CBL2, and W198 from loop L3 and confers specific binding to the terminal galactose, and (ii) the outer subsite, which consists of the side chains of CBL2 together with loops L1, L2, and L3 and interacts with the peripheral hexose moiety (Fig. 2A and C). Moreover, loop L1 is not part of the core PA14/Flo5A-like domain, but connects β-strands 2 and 3. Also, a disulfide bridge formed by C78 and C119 cross-links L1 and L2 to arrest their conformation for shielding the binding site from bulk solvent.

The Epa1A-Galβ1–3Glc complex further reveals that the galactose moiety in the inner subsite adopts a flipped orientation, which differs from the orientation of mannose in Flo5A (12). This flipped binding mode is crucial for coaligating the pyranose moiety with residue W198 from loop L3 as well as for hydrogen bonding between the R226 side chain and the 2- and 3-hydroxyls of galactose (Fig. 2D). This suggests that the indole side chain of W198 together with R226 is crucial for selecting the terminal galactose (Figs. S3 and S4), a conclusion that is supported by our finding that an Epa1A variant shows only marginal binding activity (Fig. S5). We also performed molecular dynamics studies for ligand binding by Epa1A (Movies S1, S2, and S3), which indicate that W198 may exert an organizing effect on loop L3, because they show that the stretch W198–T202 of loop L3 adopts the observed degree of order only upon complexation of galactose.

The outer subsite apparently selects the type of glycosidic bondage and nature of the hexose moiety linked to the terminal galactose. The Epa1A-Galβ1–3Glc structure shows that the site for recognition of Galβ1–3-linked hexoses not only comprises E227 and Y228 of CBL2 (Fig. 2B and D), but also loops L1 and L2 lining the outer site. The glutamic acid side chain projected from position II forms hydrogen bonds with the 2-hydroxyls of both hexoses of Galβ1–3Glc as well as with loop L2 via the peptide group of G118–C119. Likewise, the L2 loop wraps with A115–G118 around Y228 and thereby stabilizes its packing with the pyranose ring of the glucose within the Galβ1–3Glc ligand.

To obtain further insights into disaccharide binding specificity, the structure of the cognate Epa1A-T-antigen complex was solved at 1.24 Å resolution. This structure reveals the same mode of glycan recognition at its inner subsite as found for the Epa1A-Galβ1–3Glc complex, but major differences at the outer subsite (Fig. 2C and E). Whereas in the Epa1A-Galβ1–3Glc complex the glucose moiety is coplanar to galactose, the N-acetylgalactosamine moiety of the T-antigen adopts an orthogonal conformation when complexed to Epa1A. This diverging conformation is apparently caused by the hydrogen bond between the axial 4-hydroxyl group and E227 of CBL2. As shown above, the Epa1A binding site is highly specific for disfavoring Galβ1–4-linked glycans. Molecular modeling of a lactose complex and subsequent molecular dynamics studies show that a β-4 linkage fails to position the second hexose in a defined conformation within the outer subsite, e.g., by packing with Y228 (Fig. S4 and Movies S3 and S4). Nevertheless, the outer subsite harbors sufficient flexibility to allow different binding modi, as shown for the T-antigen. This suggests that EpaA domains permit a certain degree of promiscuity for disaccharide binding in their outer, but not in their inner ligand-binding subsite.

Differential Promiscuity of Epa Subtypes Is Dominated by CBL2. We next generated a sequence alignment of 19 EpaA domains that is based on the structures of Epa1A and Flo5A (Fig. S2) and provides a structure-guided phylogenetic tree of the Epa family (Fig. 3A). Four prominent branches appear in this tree, which cluster 11 EpaA domains into four subtypes, which were named Epa1 (Epa1A, Epa6A, Epa7A), Epa2 (Epa2A, Epa4A, Epa5A, Epa19A), Epa3 (Epa3A, Epa22A), and Epa9 (Epa9A, Epa10A), respectively. Remarkably, the CBL2 positions I–IV are mostly sufficient to discriminate between the different subtypes (Fig. 3B). For Epa1 subtype members, this motif consists of REYD or RDNN and, and the Epa2 and Epa9 subtype adhesins contain related RDNN or RDYH motifs. Only Epa3 subtype proteins show a highly diverging IGKD motif. In addition, the Epa9 subtype differs from others by a prominent elongation within the L1 loop.

To explore how CBL2 affects the specificity of different Epa subtype proteins, the CBL2 motif of Epa1A was exchanged for the motif of Epa2A and Epa3A (Epa1→2A and Epa1→3A variants). Because the Epa1 subtype includes two different CBL2 signatures, an Epa1→6A variant was also generated. The Epa9 subtype was not further investigated due to its diverging L1 loop. In the next step, fluorescence titration analysis of the subtype-switched Epa1A variants was performed. This analysis revealed that the binding affinities of the Epa1→6A variant for lactose and the T-antigen resemble those of Epa1A.
(K_D = 33.5 ± 6.6 μM and 1.7 ± 0.4 μM). In contrast, the Epa1→2A variant has a fourfold lower affinity for lactose (K_D = 128 ± 16.3 μM). Unfortunately, this variant rapidly quantifies of binding despite its obvious interaction with this glycan. For the Epa1→3A variant, we detected only extremely low affinity toward lactose (K_D = 273 ± 5.7 mM) and a 15-fold less efficient binding of the T-antigen (K_D = 30.0 ± 6.1 μM) compared to Epa1. To further explore the role of the CBL2 motif, the subtype-switched Epa1A variants were subjected to CFG glycan arrays, revealing further differences in their ligand-binding specificity (Fig. S6). In contrast to Epa1, the Epa1→6A variant was found to recognize glycans within the Galβ3–4Gal β1–3-linked galactose moieties within the Galβ1–4 group, with glycans 164 and 165 found as best binders, as well as Galβ1–4 terminated glycans like N-acetyllactosamine (glycan 168). As found in the fluorescence titration analysis, lactose is bound with much weaker affinity by this variant, similar to Epa1 (Fig. 4). For Epa1→2A and Epa1→3A, we found strongly diminished binding compared to Epa1A and Epa1→6A, which were highly biased toward glycans with terminal α-linked galactose moieties. In summary, all variants analyzed by glycan array profiling are highly specific for galactose in the inner subsite, whereas the outer subsite is promiscuous, being occupied by glucose, N-acetylgalactosamine, galactose, or N-acetylgalactosamine (Figs. S6 and S7), a finding that explains previous observations (15).

Next, the adhesion behavior of different Epa subtypes was analyzed in vivo, using a heterologous S. cerevisiae system to express EpaA domains on the cell surface (Fig. S8) and the human colorectal, epithelial cell line Caco-2. The T-antigen is a typical mammalian surface glycian, which is present at high densities on Caco-2 cell surfaces, but not on fungal cells. As expected, S. cerevisiae strains that lack an EpaA domain failed to adhere to epithelial cells (Fig. 5A). In contrast, strains presenting Epa1A or subtype-switched Epa1→6A strongly adhere to Caco-2 cell layers. Obviously, T-antigen–presenting epithelial cells are well recognized by the Epa1 subtype independently of the used sequence of its CBL2 motif (Fig. S4). Furthermore, the 15-fold lower affinity of Epa1→3A to recognize the T-antigen in vitro is mirrored in vivo, because strains presenting either Epa1→3A or native Epa3 only inefficiently bind to Caco-2 cells (Fig. 5A). Epa1→2A that clearly mediates binding to Caco-2 cells presents a different scenario. Given an only fourfold reduced in vitro binding affinity to lactose compared to Epa1A and Epa1→6A (Fig. S1), one may expect that the affinity or at least k_D of Epa1→2A against the T-antigen is similar to that of the Epa1A subtype. However, the native Epa2A domain fails to confer significant epithelial cell binding, suggesting that in this case switching the specificity and binding characteristics between Epa subtypes seem to be more than exchanging the CBL2 motif. Additional structural features of the outer subsite like the L1 and L2 loops may hence contribute to binding and specificity. Nevertheless, our results indicate that the CBL2 loop plays a prominent role in conferring specificity and promiscuity to Epa family adhesins and thereby significantly affects efficient host cell recognition.

**Subtle Structural Changes Cause Promiscuity.** To obtain further detailed insights into Epa protein functionality, the structures of the subtype-switched Epa1A variants were determined by using the orthorhombic crystal form obtained from cocystalization with lactose. This analysis revealed structural alterations of the Epa1A variants, which were restricted to their glycan-binding sites when compared to Epa1 (Fig. 4). Similar to Epa1, the Epa1→2A and Epa1→6A variants were found to harbor the Galβ1–3-linked di-saccharide with a well-defined positioning of the galactose moiety in the inner subsite. In contrast to Epa1, however, the terminal glucose residue was present in two alternative conformations within the outer subsite, similar to Epa1→6A (Fig. 4). This suggests that the changes at positions II and III in CBL2 (E227D, Y228N) cause strongly diminished binding to Caco-2 cells (Fig. 5A). As a consequence, the reducing end of the di-saccharide can almost freely rotate due to increased entropic contribution to binding. Thus, sterically demanding glycans appear to be more easily accommodated in Epa6-like binding sites, because a conclusion that is supported by our glycan profiling. In addition, the changes at positions II and III in CBL2 (E227D, Y228N) reduce the negative charge next to the galactose ligand, which shows increased promiscuity for Epa1→6A, because it efficiently binds to Galβ3–4Galf as well as to Galectin joined glycans (Fig. 5B and S6C). Interestingly, Epa6 not only confers epithelial cell adhesion, but also is involved in the formation of biofilms, a prerequisite for C. glabrata to colonize inert surfaces such as clinical catheters (17, 18). The reduced Epa6 specificity and tendency to biofilm formation may thus promote host persistence due to improved resistance to antifungal agents. Accordingly, Epa6 has been assigned a role in urinary tract infections (19).

In the Epa1→2A-Galβ1–3Glc complex, the glucose adopts an orthogonal conformation similar to that of the Epa1→6A-Galβ1–3Glc complex (Fig. 4). Having the same exchanges at CBL2 positions II and III, the Epa1→2A variant recognizes Galectin joined glycans as well. In addition, the change in position IV (D229N) reduces the negative charge next to the galactose ligand. As a consequence, Epa1→2A exhibits a marked preference for terminal galactosides that are sulfated at the 6-hydroxyl position (Fig. 5B). These glycan structures are prevalent in sulfomucins, which are in turn predominant components of...
intestinal mucosa (20)). The structure of the Epa1→2A-Galβ1–3Glc complex offers sufficient room in the inner subsite to position the 6-sulfate of these terminal sulfo-galactosides close to N229. In contrast, the Epa1 subtype is hindered to recognize sulfo-galactosides due to possible electrostatic repulsion by N229. In contrast, the Epa1 subtype is hindered to recognize sulfo-galactosides due to possible electrostatic repulsion by N229. Finally, the Epa1→3A variant that is strongly impaired for in vitro and in vivo ligand binding harbors a glycerol molecule in its binding pocket that stems from the cryo buffer (Fig. 4). The glycerol is coordinated to Ca$^{2+}$ via two vicinal hydroxyl groups similar to the galactose moiety in Epa1. The Epa3 subtype is characterized by a highly divergent CBL2 region with positions I–III being replaced by IGK. Apparently, a change at position I from arginine to isoleucine (R226I) is sufficient to weaken the interaction with galactose in the inner subsite and to cause impaired discrimination of glycerol from hexoses (Fig. S6B). Nevertheless, Epa1→3A still has residual affinity toward the T-antigen, i.e., 15-fold lower than that of other Epa1A variants (Fig. 4). Finally, Epa1→3A binds more efficiently to 6-sulfated galactosides than Epa1. This indicates that the lysine residue at position III of the Epa3 subtype CBL2 is suitably positioned to form a salt bridge with the 6-sulfate of the sulfo-galactoside bound to the inner subsite. This may overcome the electrostatic interference by aspartate at position IV that is observed for the Epa1 subtype.

**Conclusions**

Our detailed structural and functional characterization of Epa1A and subtype-switched variants demonstrates that the specificity of *C. glabrata* Epa adhesins for different glycan structures is exerted by a distinct region of their A domain. This region comprises the calcium-coordinating loops CBL1 and CBL2 as well as parts of loop L3 and bears modular characteristics to generate Epa subtype variability. The specificity profiles of Epa1A and its variants strengthen and refine the link between these *C. glabrata* epithelial adhesins and the cores of mucin-type O-glycans (15). Importantly, we found that the T-antigen, which represents the core 1 of mucin-type O-glycans (21) and is part of most mucin-derived glycans (22), is one of the best ligands for all EpaA variants investigated in this study. Other glycans that we found to be recognized by Epa1A and variants are also related to mucins and

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**Fig. 4.** Specificity and binding pockets of subtype-switched Epa1A variants. (Upper) CFG glycan array profiles for Epa1A variants at 200 μg/mL protein concentration. Strong binders are indicated by their CFG array number. Unlike Epa1A, Epa1→6A lacks the strict Galβ1–3 specificity and instead binds galactose-comprising glycans within the Galα, Galβ1–4, and Galβ1–3 groups (green, blue, and red regions, respectively), indicating increased promiscuity. Epa1→2A and Epa1→3A have narrowed specificity profiles with Epa1→3A preferring terminal α-linked galactose-comprising glycans. The lower fluorescence signals of Epa1→2A and Epa1→3A indicate weaker binding to glycans than that of Epa1A and Epa1→6A. (Lower) Top view of subtype-switched Epa1A-binding sites with SIGMAA-weighted omit electron densities for ligands complexed to Epa1→6 (anhydride gray, contouring level: 0.8e, 0.29electrons/A$^3$), Epa1→3A (blue, 0.8e, 0.28electrons/A$^3$) and Epa1→2A (green, 0.6e, 0.19electrons/A$^3$). The orientation of the disaccharide within the binding pocket is shown (Upper Right Inset). CBL loops of the inner subsite are marked in cyan and L loops of the outer subsite in orange.

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**Fig. 5.** In vivo binding of EpaA domains and the distribution of mucin-derived glycans. (A) Relative adhesion to epithelial cells as conferred by different EpaA domains was determined by using a heterologous *S. cerevisiae* expression system. *S. cerevisiae* strains presenting different EpaA domains on Flo11 BC stalks were incubated with a monolayer of Caco-2 cells and adhesion was determined after 2 h. Colors correspond to the different EpaA subtypes as shown in Fig. 3A. (B) Pie charts show glycan types of the CFG array for which binding signals exceed either 20% or 50% of the signal of the best binder. For each glycan type (color as above), the core structures are shown below in CFG standard notation. Nonassigned glycans are grouped as “other sugars” (yellow).
include other mucin-type O-glycan cores or N-glycans, e.g., sulfogalactose-containing glycans.

Our study further reveals that the structural basis for the specificity and promiscuity in Epa–glycan interactions is significantly affected by the properties of CBL2. This finding is consistent with previous domain-swapping experiments between Epa6 and Epa1/7, which identified a larger, CBL2-covering region for controlling specificity (15). Specificity of the inner subsite for galactose is dictated by the recognition of the cis-diol form by the 3- and 4-hydroxyls. Furthermore, position I of CBL2 is occupied by an arginine in all Epa subtypes except Epa3. This crucial residue forms two hydrogen bonds with the 2-hydroxyl of the bound galactose and packing to loop L1 via W79 and Y83 stabilizes its conformation. In the outer subsite, position III of CBL2 presents either large aromatic residues (Y, Epa1, Epa9, Epa10, Epa12, Epa15, and Epa20; F, Epa23) or polar amino acids like asparagine (Epa2, Epa4, Epa5, Epa6, and Epa19). Whereas the former promote packing with planar glucose/galactose derivatives, resulting in specific interactions with either terminal Galβ1-3Glc(NAc) or Galβ1-3Gal(NAc), the latter can increase promiscuity (Epa6) or enable additional binding. E.g., sulfated galactoses (Lys: Epa3, Epa22). Position IV appears to mainly control the degree of allowed modification in the terminal galactose moiety. Together, CBL2 positions I–IV may be proposed to form a simple structural code for Epa specificity. However, the differences found for binding to host cell monolayers by Epa1→2A and Epa2A as well as the high-resolution structure of the Epa1A-T-antigen complex with its orthogonal binding mode for the GalNAc moiety challenge this notion and imply that the periphery formed by the loops L1 and L2 adds to the specificity of Epa adhesins.

In summary, our study suggests that variable presentation of Epa family members with diversified outer subunits at the cell surface of C. glabrata is one of the key elements for efficient and tissue-specific host invasion and could explain the clinical behavior of this human pathogenic fungus (23). Our structure-based insights that Candida–host interaction crucially depends on the conserved nature of an inner galactose-binding site of Epa adhesins may further contribute to the development of tailored antymycotics to combat this emerging pathogen.

**Methods**

**Protein Production and Purification.** Recombinant Epa1A and variants were generated in a thioredoxin and glutathione reductase-deficient *Escherichia coli* strain. Epa1A was purified by NiNTA-affinity and size-exclusion chromatography.

**Phylogenetic Analysis.** Phylogenetic analysis was performed by the neighboring-joining method, using Clustal X2.0 (24), COBALT (25), or a local copy of t-coffee (26) implemented with 3DCoffee (27) for structure-based alignments. Preliminary targets were selected with the help of BLAST.

**Crystallization and Structure Determination.** Epa1A/lactose cocrystals belonging to space group P2221 were obtained from ammonium sulfate-containing conditions. Epa1A-T-antigen cocrystals were obtained by soaking Ca2+-stripped Epa1A/lactose cocrystals. The structure of Epa1A was then solved by molecular replacement and refined using REFMACS or PHENIX.

**High-Throughput Glycan-Binding Assays.** The CFG glycan array consists of different groups of oligosaccharides that are presented by mammalian cells. Recombinant, fluorescently labeled Epa1A domains were applied to the CFG array V4.1 chips at concentrations ranging from 1 μg/mL to 200 μg/mL. Chip surfaces were repeatedly washed; remaining fluorescence was measured and quantified.

**Fluorescence Titrations.** Fluorescence titrations of Epa1A and variants were performed against lactose and the T-antigen. Binding was followed at an emission wavelength of 346 nm by excitation of intrinsic tryptophan fluorescence at 295 nm. Fluorescence quench was recorded during titration and fitted using a one-site plus unspecific-binding model.

**In Vivo Adhesion Assays.** In vivo adhesion of Epa proteins to epithelial cells was determined using a nonadhesive *S. cerevisiae* strain presenting the different EpaA domains at the cell surface (Tables S3–S5). Adhesion assays were performed as previously described (28).

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