Structural Hot Spots Determine Functional Diversity of the Candida glabrata Epithelial Adhesin Family*

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Background: The pathogenic yeast Candida glabrata harbors more than 20 epithelial adhesins (Epas).

Results: Epas are lectins with binding pockets that contain conserved and variable structural features determining ligand binding affinity and specificity.

Conclusion: The functionally diverse Epa family evolved in C. glabrata for efficient host infection.

Significance: Epa-mediated host-ligand binding is a therapeutic target to combat C. glabrata infections.

For host colonization, the human fungal pathogen Candida glabrata is known to utilize a large family of highly related surface-exposed cell wall proteins, the lectin-like epithelial adhesins (Epas). To reveal the structure-function relationships within the entire Epa family, we have performed a large scale functional analysis of the adhesion (A) domains of 17 Epa paralogs in combination with three-dimensional structural studies of selected members with cognate ligands. Our study shows that most EpaA domains exert lectin-like functions and together recognize a wide variety of glycans with terminal galactosides for conferring epithelial cell adhesion. We further identify several conserved and variable structural features within the diverse Epa ligand binding pockets, which affect affinity and specificity. These features rationalize why mere phylogenetic relationships within the Epa family are weak indicators for functional classification and explain how Epa-like adhesins have evolved in C. glabrata and related fungal species.

Adhesion of pathogens to host surfaces is a crucial first step for successful tissue invasion and infection. For this purpose, human pathogenic fungi often dispose of large repertoires of cell wall-associated adhesins, as exemplified by Candida albicans and Candida glabrata representing two of the major agents of human fungal infections (1–5). Most known fungal adhesins have a common modular architecture that is thought to contribute to a high variability of the cell surface properties (6). Typically, fungal adhesins are secreted proteins that consist of an N-terminal region for adhesion (A domain)4, a large central segment contained a variable number of highly glycosylated serine- and threonine-rich repeats (B domain), and a C-terminal region carried a glycosylphosphatidylinositol (GPI) anchor required for attachment to the cell wall (7). Therefore, these proteins are also referred to as GPI-anchored cell wall-associated adhesins (8). Detailed analysis of a few selected adhesins from C. albicans and C. glabrata has shown that A domains function by recognizing either host peptide (9, 10) or glycan structures (11, 12) and provided first resolution insights into the structural features required for ligand binding and discrimination (13, 14). With the exception of these first examples, however, the precise structures and functions of most fungal adhesins are unknown.

The genome of C. glabrata contains an extraordinarily large number of more than 60 sequences that encode typical GPI-anchored cell wall-associated adhesins (5, 15, 16). The largest group comprises the EPA (epithelial adhesion) family that, depending on the strain, contains between 17 (CBS138) and 23 (BG2) members (17, 18). EPA-like adhesins are also present in other species of the Nakaseomyces genus, a group of six yeasts that include C. glabrata and whose genomes have recently been sequenced (19). Remarkably, the two other human pathogenic Nakaseomyces, Candida bracarensis and Candida nivariensis, contain 12 and 9 EPA-like genes, respectively, whereas only a single copy can be found in the nonpathogenic Nakaseomyces delphensis. The other nonpathogenic Nakaseomyces, Nakaseomyces bacillisporus and Candida castellii, contain no EPA-like adhesins that are only distantly related to the EPA family. The best characterized EPA adhesins

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The abbreviations used are: A domain, adhesion domain; Epa, epithelial adhesin; CBL, calcium binding loop; Gal, galactose linked via a α-glycosidic bond; Galβ, galactose linked via a β-glycosidic bond; Galα1–3Gal, α1–3-galactobiose; Galβ1–3Gal, β1–3-galactobiose; Galβ1–3GalNAc, T-antigen; Galβ1–3GlcNAc, lacto-N-biose; Galβ1–4GlcNAc, N-acetyl-d-lactosamine; CFG, Consortium for Functional Glycomics.
are *C. glabrata* Epa1 (CAGL0E06644), Epa6 (CAGL0C00110g), and Epa7 (CAGL0C05643g), which confer adhesion to human epithelial cells (11, 12, 20).

Chip-based glycan array screening in combination with *Saccharomyces cerevisiae* cells presenting EpaA domains or with proteins purified from *Escherichia coli* has revealed that Epa1, Epa6, and Epa7 act as lectins with related ligand binding specificities (12, 14). This is in agreement with the idea that Epad adhesins enable *C. glabrata* to bind to host cells by recognition of mucin-type O-glycans (1, 21). Moreover, Epa1, Epa6, and Epa7 prefer carbohydrate structures with a terminal galactose unit linked via 1–3- or 1–4-glycosidic bonds to glucose, galactose, or their *N*-acetylated derivatives. However, Epa1 and Epa7 prefer β1–3- and β1–4-linked galactosides, whereas Epa6 is not able to discriminate between α- and β-glycosidic linkages. High resolution crystal structures of the Epa1 A domain (Epa1A) in complex with cognate disaccharide ligands have recently provided the structural basis for ligand binding specificity (14).

These structures show that Epa1A is related to the A domain of *S. cerevisiae* flocculins (Flo adhesins), e.g. Flo5A (gene ID, 856618), and in its core contains a PA14 domain initially characterized in the anthrax-protective antigen (22). For efficient carbohydrate binding, Epa1A utilizes a unique DcisD Ca\(^{2+}\)-binding motif that is also found in Flo5A, but not in other C-type lectins (23, 24). In contrast to Flo5A, substrate specificity in Epa1A is not mediated by a flocculin-specific subdomain but by two calcium-binding loops, CBL1 and CBL2, present in the inner part of the ligand binding pocket and by three outer loops. The A domains of all other Epa family members are also PA14 proteins and contain a DcisD motif, suggesting that they act as lectins. However, their ligand-binding patterns and/or precise binding pocket structures are largely unknown.

To determine the diversity of ligand-binding patterns within the Epa family, we here performed a comprehensive functional characterization of the adhesion domains of 17 Epa family members present in the *C. glabrata* strain CBS138 using large scale glycan array profiling and proteins purified from *E. coli*. Efficiency of individual A domains to bind to epithelial cells was assessed *in vivo* by an *S. cerevisiae*-based expression system, whereas the binding activity of selected A domains was further characterized *in vitro* by titration with different disaccharide ligands. To obtain new insights into the structural features conferring substrate binding and specificity in the Epa adhesin family, we compared the crystal structures of Epa1A and Epa6A in complex with diverse disaccharide ligands comprised of galactose linked via α- or β-glycosidic bonds to a secondary sugar. Our study shows that the Epa adhesin family recognizes a wide variety of α- and β-linked galactosides as well as nongalactosidic terminal glycans and provides novel insights into the structural motifs that enable ligand discrimination.

**Experimental Procedures**

**Yeast Strains**—For isolation of the EPAA domains, the *C. glabrata* strain CBS138 (American Type Culture Collection ATCC2001) was used. *In vivo* adhesion assays were performed with the nonadhesive *S. cerevisiae* strain BY4741 (European *Saccharomyces cerevisiae* Archive for Functional Analysis) carrying appropriate plasmids (Table 1) and with a triple-auxotrophic derivative of CBS138. Standard methods for yeast culture medium and transformation were used as described previously (25).

**Plasmids**—All plasmids used in this study are listed in Table 1. Numbering of amino acid residues refers to sequences described UniProt database. To express the EpaA domains in *E. coli*, the different EPAA domains were amplified with specific primers for each EPAA domain and genomic DNA of *C. glabrata* strain ATCC2001 as a template and inserted as NdeI/XhoI or NhEI/XhoI fragments into the vector pET-28(a)\(^{+}\). To express the different EPAA domains in *S. cerevisiae*, they were amplified with specific primers for each EPAA domain and genomic DNA of *C. glabrata* strain ATCC2001 as a template and subsequently inserted as SacII/Sacl fragments into plasmid BHUM2157. To obtain BHUM2157, the FLO11 secretion signal and a 3-fold hemagglutinin tag were amplified using Sall-SS-3HA-EpaXA and 1601-A2-Sacl SacII as primers and BHUM1760 as a template. Afterward, the PCR fragment and BHUM1964 were cut using the restriction enzymes Sall and Sacl and ligated together to obtain plasmid BHUM2157 carrying the following: (i) the PGK1 promoter; (ii) the FLO11 secretion signal spanning amino acid residues 1–30; (iii) a 3-fold hemagglutinin tag; (iv) the FLO11BC domain encompassing amino acids 214–1360, and (v) the FLO11 terminator.

**Recombinant Overproduction and Crystallization of Epa A Domains**—The wild type EpaA domains were overproduced using a previously developed low temperature protocol (23). The only modification to the protocol was the use of *E. coli* strain SHuffle T7 express (New England Biolabs GmbH, Frankfurt, Germany) instead of *E. coli* strain Origami 2. After lysis and clarification of the supernatant, the protein was purified by nickel-nitritotriacetic acid affinity chromatography (Qiagen, Hilden, Germany) and subsequent size exclusion chromatography using HiLoad Superdex 75 PG column (GE Healthcare, Munich, Germany), initially in AM-buffer (20 mM Tris/HCl, pH 8.0, 200 mM NaCl). Some of the EpaA domains interacted strongly with the Superdex 75 PG material under these conditions, resulting in very poor yields. This issue could be solved by adding either 50 mM lactose (AML-buffer) or 10 mM EDTA (AME-buffer) to the AM-buffer.

Initial crystal screening was performed in a 600-nl sitting drop setup using commercially available screens (Qiagen, Hilden, Germany) with a Digitalab Honeybee 963 dispensing system (Genomic Solutions, Huntingdon Cambridgeshire, UK) and yielded several positive conditions at 18 °C. Optimizations of original hits took place in a 2-μl hanging drop setup, which greatly improved crystal size. Drops were composed of 50% protein solution in AML-buffer (15 mg/ml) with 5 mM lactose and 50% reservoir solution for Epa6A. Crystals belonging to space group P2\(\text{1}\)2\(\text{1}\)2\(\text{1}\) were either yielded at 18 °C in conditions containing 80 mM sodium acetate, pH 4.6, 1.6 M ammonium sulfate, 20% glycerol or 10 mM sodium acetate, pH 4.6, 30% PEG 4000, 200 mM ammonium acetate as precipitant.

After size exclusion chromatography of Epa1A with AME-buffer, the protein solution was diluted by a factor of 10 with
AM-buffer to reduce EDTA concentration (1 mM). The subsequent crystallization screening was performed as described above, including 5 mM CaCl₂ and 5 mM T-Antigen (Dextra Laboratories, Reading, UK) in the protein solution. Crystals belonging to space group P4₁2₁2₁ were yielded at 18 °C in conditions containing 100 mM HEPES, pH 7, and 20% PEG 6000.

All crystals were flash-frozen in mother liquor supplemented with 20–30% (v/v) glycerol.

Soaking of Pre-grown Epa6A—The T-antigen complex, the complete soaking structure, was performed with PHASER (26); data processing was performed with XDS, XSCALE, PHENIX, and CCP4 (27–29) and refinement with alternating rounds of REFMAC (30) and Coot (31). Secondary structure assignments were performed with STRIDE (32), and manual inspection was done using the Epa1A structure as comparison (Protein Data Bank code 4AF9). Figures of protein structures were generated using the molecular graphics program PyMOL Version 1.4.1 (DeLano Scientific).

High Throughput Glycan Binding Assays—Recombinant EpaA domains were fluorescently labeled using an AlexaFluor 488 THF kit (Life Technologies, Inc.) and applied to CFG array chips at protein concentrations of 200 µg/ml. Chip surfaces were repeatedly washed, and the remaining fluorescence was measured and quantified.

Fluorescence Titration Spectroscopy—Fluorescence titrations of EpaA domains against respective carbohydrates were performed as described (23, 24). Binding was followed at an emission wavelength between 340 and 350 nm by excitation of intrinsic tryptophan fluorescence at 280 nm. Fluorescence quench was recorded during titration and fitted using a one-site quench model.

Adhesion of S. cerevisiae and C. glabrata to Human Epithelial Cells—For adhesion tests, the human epithelial cell line Caco-2 (American Type Culture Collection HTB-37) was used together with the S. cerevisiae strain BY4741 carrying plasmids with the appropriate pPGK-3HA-EPA11BC constructs (Table 1). The presence of EpaA domains at the S. cerevisiae cell surface was quantified by immunofluorescence microscopy.

For this purpose, cultures of plasmid carrying strains were grown in low fluorescence yeast medium to an optical density at 595 nm of 1, before cells were washed three times in PBS, 1% BSA. Then, cells were incubated with a monocular mouse
### TABLE 2

**Data collection statistics for Epa1A and Epa6A complexes**

Values for the highest resolution shell are shown in parentheses.

|                | Epa1A-T-antigen | Epa6A-lactose | Epa6A-T-antigen | Epa6A-N-acetyl-α-lactosamine | Epa6A-lacto-N-biose | Epa6Aα1-3-galactobiose |
|----------------|-----------------|---------------|-----------------|-----------------------------|---------------------|-----------------------|
| **PDB code**   | 4D3W            | 4COU          | 4COW            | 4COY                        | 4COZ                | 4COV                  |
| **X-ray source** | 14.1, BESSY     | 14.1, BESSY   | 14.1, BESSY     | 14.1, BESSY                 | 14.1, BESSY         | 14.1, BESSY           |
| **Wavelength (Å)** | 0.918409       | 0.918409      | 0.826606        | 0.918409                    | 0.826606            | 0.918409              |
| **Resolution (Å)** | 24.60-1.50      | 28.77-1.48    | 34.24-2.00      | 41.58-1.80                  | 53.12-2.25          | 41.58-1.50            |
| **Observed reflections** | 384,090         | 174,129       | 109,348         | 146,305                     | 120,749             | 257,032               |
| **Unique reflections** | 35,383          | 45,573        | 18,879          | 27,537                      | 13,420              | 47,367                |
| **Completeness (%)** | 100 (100)       | 99.7 (99.9)  | 99.8 (99.7)     | 99.5 (99.5)                 | 99.9 (99.9)         | 100                   |
| **Multiplicities** | 109 (10.6)      | 3.8 (3.8)    | 11.9 (2.7)      | 17.1 (4.4)                  | 12.1 (3.5)          | 5.4 (5.2)             |
| **Rmerge (%)** | 10.1 (50.5)     | 4.4 (57.1)   | 12.3 (60.4)     | 18.2 (22.3)                 | 17.3 (64.7)         | 46.2 (50.5)           |
| **Rwork/ Rfree (%)** | 15.8/18.3       | 14.4/17.2    | 17.7/23.0       | 26.12/26.0                  | 17.8/22.0           | 15.0/17.6             |
| **Total reflections** | 33528           | 44337         | 14534           | 26129                       | 11930               | 46100                 |
| **Test set reflections** | 1767            | 1173          | 740             | 1378                        | 634                 | 1223                  |
| **No. of atoms (total)** | 2183            | 2193          | 2109            | 2056                        | 2193                | 2219                  |
| **Mean B value (Å²)** | 2.15            | 1.92          | 1.98            | 1.97                        | 2.39                | 2.02                  |
| **Root mean square deviations bond lengths (Å)** | 0.012           | 0.008         | 0.010           | 0.001                       | 0.008               | 0.007                 |
| **Root mean square deviations bond angles (°)** | 1.611           | 1.295         | 1.319           | 1.384                       | 1.209               | 1.134                 |

**Note:** All proteins were crystallized using hanging vapor diffusion at 18 °C. After three wash steps, cells were detected in a room temperature of 1:1000 in PBS/1% BSA for 30 min at room temperature. After three wash steps, cells were detected in a room temperature of 1:1000 in PBS/1% BSA for 30 min at room temperature.
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A = CFU (adherent cells)/CFU (adherent cells)
\quad + CFU (non-adherent cells) \quad (\text{Eq. 1})

Bioinformatic Analysis—Hierarchical clustering analysis of EpaA domains was performed using the Cluster 3.0 software (35–37) and the centroid linkage clustering algorithm. For this purpose, relative glycan binding values (absolute binding values divided by the binding value of the best bound glycan) for each EpaA domain toward the 610 glycans present on the CFG array Version 5.1 were calculated, and clustering results were visualized by using the Java TreeView software (35–37). Pie chart analysis of glycan binding profiles was done as described previously (14).

Figures of protein structures were generated with the molecular graphics software PyMOL Version 1.4.1 (38). To model the structures of further Epa family members, the Modeller 9v7 software was used (39). As templates, the structures of Epa1A (4ASL) and Epa6A (4COU) were used. The respective sequences were collected from the Candida Genome Database (40). To analyze structures for their conserved residues and regions, the Consurf server was used with Epa1A as template (41, 42). The alignment was constructed using T-coffee (43). Molecular evolutionary genetic analysis was performed and visualized using the MEGA6 software (44). Where indicated, 500 bootstrap replications were included.

Data Deposition—The glycan array data from this publication have been deposited at the Consortium for Functional Glycomics and assigned the identifiers cfg_rRequests 2554, 2645, 2737, and 2854. The atomic coordinates and structure factors have been deposited in the Protein Data Bank and assigned the accession codes 4COU, 4COV, 4COW, 4COY, 4COZ, and 4D3W.

Results

Diversity of Epa Family Ligand-binding Patterns—We first determined the carbohydrate specificity profiles of 17 Epa adhesins encoded in the genome of C. glabrata strain ATCC 2001/CBS138 (5, 21). For this purpose, corresponding A domains were purified from E. coli and analyzed for in vitro binding to 610 different carbohydrates using a mammalian glycan array from the CFG. The 17 binding profiles obtained by this approach (Fig. 1) provided 10,370 data points, which were further analyzed by hierarchical clustering using the Cluster 3.0 and TreeView software (35–37). This analysis revealed that each of the EpaA domains possesses its own specific binding profile and that the Epa family can be broadly divided into three separate functional classes (Fig. 2). Class I consists of five EpaA adhesins (Epa1A, Epa7A, Epa3A (CAGL0E06688g), Epa10A (CAGL0A01284g), and Epa9A (CAGL0A01366g)), whose best-bound glycans are β1–3 or β1–4 linked galactosides; class II contains six proteins that best bind to either β1–3– or α1–6-linked (Epa6A, Epa13A (CAGL0L13332g), and Epa22A (CAGL0K00170g)) or sulfated galactosides (Epa12A (CAGL0M00132g), Epa15A (CAGL0J11968g), and Epa23A (CAGL0L00220g)); and class III consists of six members, which best bind to acidic sugars (Epa19A (CAGL0A00110g), Epa2A (CAGL0E06666g), Epa20A (CAGL0E06666g), and Epa21A (CAGL0D06732g)) and sulfated galactosamines (Epa8A (CAGL0C00847g)) or α1–3–linked galactosides (Epa11A (CAGL0L1329g)). Generally, the preferred ligands of class I and class II were bound with apparently medium to high affinity as based on data obtained by the glycan array analysis (Fig. 1), whereas binding values obtained for members of class III indicated mostly weak and hence less specific binding.

To further assess and compare the different ligand recognition patterns, we also classified the 17 EpaA adhesins with respect to the best-bound terminal sugars in combination with the linkage type to the penultimate carbohydrate moiety. This analysis also leads to a tripartite classification of the Epa family that is comparable with the one obtained by unbiased hierarchical clustering (Fig. 3). It also shows the following within class I: (i) Epa7A and Epa3A are highly specific for terminal galactose-linked via a β1–3 glycosidic bond to the penultimate sugar moiety (Galβ1–3); (ii) Epa1A best binds to Galβ1–3 and Galβ1–4; (iii) Epa10A recognizes Galβ1–3 and to some degree also Galα1–4; and (iv) Epa9A is able to bind Galβ1–3 and Galβ1–4 as well as Galα1–3 and Galα1–4. In class II, the preferred ligands contain terminal α-linked galactose (Epa6A, Epa13A, Epa12A, and Epa22A) or terminal (6S)-galactose units connected by a β-type linkage (Epa12A, Epa22A, Epa15A, and Epa23A). Finally, Epa proteins of class III prefer nongalactosides as terminal residues (Epa8A, Epa19A, Epa2A, Epa20A, and Epa21A) with the exception of Epa11A that binds to both nongalactosides and Galα1–3 with comparable strength.

In summary, our comprehensive glycan profiling shows that the 17 Epa members encoded in the genome of strain CBS138 (i) all appear to be well folded and functional in vitro, (ii) all possess individual ligand-binding patterns, (iii) together cover a wide variety of glycosidic ligands containing terminal α- and β-linked galactosides as well as nongalactosidic sugars, and (iv) display highly variable ligand binding affinities.

Epithelial Cell Adhesion by EpaA Domains—We next directly compared in vivo functionality of the different EpaA domains by measuring their adhesion activity to human colo-rectal and epithelial Caco-2 cells. For this purpose, all 17 EpaA domains analyzed in vitro were individually expressed in non-adhesive S. cerevisiae cells using a flocculin gene-based expression system as described previously (14). Successful expression and presentation of the EpaA domains on the cell surface were first monitored by fluorescence microscopy. Here, we found significant amounts of A domains in the case of 15 Epa paralogs (Fig. 4A). Corresponding S. cerevisiae strains were further assayed for in vivo epithelial cell adhesion along with appropriate control strains and a C. glabrata strain (Fig. 4B). As expected, strains that lack an EpaA domain did not adhere to Caco-2 cells. The 15 A domain presenting strains exhibited variable epithelial cell adhesion and roughly divide the Epa adhesins into three groups. The first group consisting of Epa1A, Epa7A, and Epa6A conferred very efficient cell adhesion, which was between 13- and 17-fold higher than the activity measured in the absence of an A domain, but did not fully reach the adhesion strength observed for C. glabrata (Fig. 4B). Epa9A, Epa12A, Epa15A, Epa23A, and Epa8A compose a second group and mediate adhesion with values that are between 3- and 6-fold higher than the control measurement. All other EpaA
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domains constitute a third class and confer adhesion that is less than 3-fold better than the control. In summary, we find that of the 15 Epa adhesins tested about 20% (3/15) mediate efficient and 35% (5/15) confer mediocre epithelial cell adhesion, whereas the other members of the Epa family (7/15) mediate only very weak binding to Caco-2 cells.

**FIGURE 1.** Glycan binding profiles. Binding profiles of 17 EpaA domains from *C. glabrata* strain CBS138 (ATCC 2001) were obtained by using the CFG mammalian glycan array Version 5.1 and fluorescently labeled EpaA proteins purified from *E. coli* at a concentration of 200 μg/ml. Fluorescence units measured for the different EpaA domains and the 610 different glycans present on the array are shown as black lines. Glycans with unambiguous terminal disaccharide types, which were best bound by the different EpaA domains, are indicated with numbers according to their position on the CFG array. Profiles are depicted according to their classification (roman numbers) obtained by the hierarchical clustering analysis presented in Fig. 2.

**FIGURE 2.** Glycan profile-based relationship of the Epa family. A, hierarchical clustering analysis. The 17 Epa paralogs from *C. glabrata* strain CBS138 were clustered according to the relative *in vitro* binding strength of their A domains purified from *E. coli* toward a total of 610 different glycans present on the CFG array Version 5.1 using Cluster 3.0 and Java TreeView software. Relationships among the 17 Epa adhesins as given by their binding profiles are shown by the tree on the left; glycans are clustered as shown on the top according to the similarity of their recognition by different Epa paralogs. Relative binding strength is shown in color with values ranging from 0 (yellow) to 100 (dark blue). Classification is indicated by roman numbers and dotted lines. B, comparison of relative binding strength of best bound glycan ligands. The left part shows the terminal residues of all glycan structures with unambiguous terminal disaccharide types best bound by the individual EpaA domains. Numbers label the different polyglycan types according to their position on the CFG array. The right side shows the relative binding strength of these glycans to all EpaA domains. CFG array numbers are indicated. Relative binding is shown in color as in A.
Quantification of Ligand Binding by Epa1A, Epa6A, and Epa7A—We next wanted to quantify the ligand binding specificities for a number of selected EpaA domains in more detail. For this purpose, we focused on Epa1A, Epa6A, and Epa7A, because (i) these adhesins are closely related at the protein sequence level (12, 14), and (ii) we here found these adhesins to confer the most efficient epithelial cell binding (Fig. 4B). We performed fluorescence titration analysis and determined the in vitro binding constants ($K_D$ values) for these proteins with five different disaccharides, α1–3-galactobiose (Galα1–3Gal), β1–3-galactobiose (Galβ1–3Gal), T-antigen (Galβ1–3GalNAc), lacto-N-biose (Galβ1–3GlcNAc), and N-acetyl-D-lactosamine (Galβ1–4GlcNAc). Comparison of the different $K_D$ values not only reveals the differences in ligand binding affinities but also provides the discrimination ratios with respect to β1–4-linked versus β1–3- and β- versus α-linked disaccharides and with regard to the nature of the sugar moiety attached to galactose, namely Gal, GalNAc, or GlcNAc. We found that Epa1A and Epa7A only minimally discriminate between β1–4- and β1–3-linkages as exemplified by the comparison of Galβ1–4GlcNAc and Galβ1–3GlcNAc, although the affinity of Epa1A for these ligands is generally 2.4–3.0-fold higher (Fig. 5A). However, Epa1A and Epa7A strongly discriminate between β- and α-glycosidic linkages as illustrated by the Galβ1–3Gal/Galα1–3Gal ratios of 5.6 and 9.4, respectively. This efficient β/α discrimination is further emphasized by the glycan array values obtained for these disaccharides with even higher ratios of 16 (Epa1A) and 56 (Epa7A). Moreover, both proteins exhibit similarly high affinities toward Galβ1–3Gal with $K_D$ values of about 1 μM, but Epa7A binds the Galα1–3Gal isomer 2.5-fold less efficient than Epa1A. With respect to the nature of the second hexose moiety, we further find that Epa1A and Epa7A clearly prefer Gal and GalNAc over GlcNAc. In contrast to Epa1A and Epa7A, Epa6A is almost unspecific with regard to discriminating between different types of glycosidic linkages and significantly less specific with respect to discriminating between Gal, GalNAc, and GlcNAc at the secondary position of disaccharides (Fig. 5B). Remarkably, however, the overall affinity of Epa6A to the diverse ligands is comparable with Epa1A and Epa7A.

Structural Basis for Ligand Discrimination by Epa1A and Epa6A—To better understand the different binding patterns obtained with members of the Epa1 subfamily, we aimed at obtaining crystal structures of Epa1A and Epa6A in complex with different galactosides assayed for in vitro binding. For Epa1A, we have previously solved the structure in complex with different disaccharides (Galβ1–3GalNAc, PDB code 4ASL; and Galβ1–3Glc, PDB code 4AF9) by soaking the crystals obtained before by co-crystallization with lactose (14). Here, we obtained a structure of Epa1A in complex with the T-antigen (Galβ1–3GalNAc, PDB code 4D3W, Table 2) by co-crystallization in a new space group at a resolution of 1.5 Å, which does not significantly differ from the previous structure (root mean square deviation of 0.31 Å for 172 Ca positions). This finding not only underscores the fact that EpaA domains are structurally quite rigid, it also validates the previously used soaking protocol. Therefore, we used the same crystallization approach to determine the structures of Epa6A in complex with lactose and Galβ1–3GalNAc, which were obtained at a resolution of 1.56 and 2.15 Å (Table 2; PDB codes 4COU and 4COW), respectively. The overall structural fold of Epa6A is highly comparable with Epa1A with a root mean square deviation of 0.37 Å for 177 Ca positions (Fig. 6). The structure is composed of a β-sand-
wich that is derived from the PA14 domain of the B. anthracis protective antigen (22). It harbors two consecutive aspartate residues connected by a cis-peptide bond, as has been found in EpA1A and the A domains of the S. cerevisiae flocculins Flo5A and Lg-Flo1A (14, 23, 45). Ligand binding in EpA6A is achieved by coordination via a Ca$^{2+}$ ion, which is fixed by the DcisD motif and an asparagine residue in CBL2 (Fig. 6B). In addition, the binding pocket harbors residues of the calcium-binding loop CBL2 and a tryptophan residue situated in a flexible loop above the pocket, which both mediate crucial interactions and

FIGURE 4. In vivo binding of EpA domains. A, expression of different EpA domains using a heterologous S. cerevisiae expression system. S. cerevisiae strain BY4741 carrying either of the plasmids B2445 (control), BHUM2157 (no A), BHUM1983 (EpA1A), BHUM1985 (EpA2A), BHUM2150 (EpA3A), BHUM2018 (EpA6A), BHUM1988 (EpA7A), BHUM2051 (EpA8A), BHUM2020 (EpA9A), BHUM2152 (EpA11A), BHUM2153 (EpA12A), BHUM1987 (EpA13A), BHUM2154 (EpA15A), BHUM2155 (EpA19A), BHUM2156 (EpA20A), BHUM1993 (EpB21A) or BHUM1988 (EpA23A) was grown to logarithmic phase before EpA domains were visualized at the cell surface by immunofluorescence microscopy using anti-HA primary and Cy3-conjugated secondary antibodies. Specific signals were quantified, and the values obtained are shown in percentage relative to the strain expressing the carrier domain alone (no A) set to 100. Scale bar corresponds to 10 μm. B, epithelial cell adhesion conferred by 15 EpA domains. Adhesion was determined using S. cerevisiae strains shown in A or a C. glabrata strain (triple-auxotrophic derivative of CBS138) after 2 h of incubation with a monolayer of Caco-2 cells. Adhesion of S. cerevisiae strains expressing no adhesin (control) or only the carrier domain (no A) is shown in black. For S. cerevisiae strains, EpA-specific adhesion is indicated by the white part of bars that results from subtracting the value obtained by the strain expressing the carrier domain alone (no A) from the total adhesion. Error bars indicate standard deviation obtained by 10 independent experiments. The functional classification is shown at the bottom. DIC, differential interference contrast.
are responsible for specificity and affinity. The binding pocket is also shielded from the surrounding solvent by the flexible loops L1 and L2, which are connected by a highly conserved disulfide bridge formed by Cys-78 and Cys-119. Finally, the overall orientation of Gal\textsubscript{1–3Gal} in the Epa6A binding pocket is comparable with the orientation of the same ligand in Epa1A (Fig. 7, A and B). However, the precise ligand interaction patterns of the two proteins clearly differ with respect to two residues in CBL2. In the case of Epa6A, ligand binding involves Asp-227 and Asn-228, whereas the corresponding positions in Epa1A are represented by Glu-227 and Tyr-228 (Fig. 7, A and B).

To better understand how individual EpaA domains discriminate between different ligands, we solved the structures of Epa6A in complex with three further glycans, Gal\textsubscript{1–3Gal} (PDB code 4COV), Gal\textsubscript{1–4GlcNAc} (PDB code 4COY), and Gal\textsubscript{1–3GlcNac} (PDB code 4COZ) (Fig. 7, C, E, and F; Table 2). This set of high resolution structures allows delineation of the structural features, which contribute to the differences of specific ligand binding. First, a comparison of the Epa6A-Gal\textsubscript{1–3Gal} complex with the corresponding model of an Epa1A-Gal\textsubscript{1–3Gal} complex reveals that the CBL2 region seems to determine the efficiency by which these two proteins are able to discriminate between \( \text{GalNAc} \)- and \( \text{Gal} \)-linked carbohydrates (Fig. 8A). Although a shorter aspartate residue (Asp-227) can be found at position II of its CBL2 region, Epa1A harbors a glutamate (Glu-227) at this position. This suggests that the shorter residue in Epa6A enables more efficient binding of \( \text{Gal} \)-linked glycosides than Epa1A due to reduced steric hindrance (Fig. 8A). In addition, a hydrogen bond is observed between Gal\textsubscript{1–3Gal} and the CBL2 position III (Asn-228) of Epa6A. This ligand interaction is absent in the Epa1A-Gal\textsubscript{1–3Gal} complex, suggesting that the efficient binding of \( \text{Gal} \)-linked galactosides is further fostered by interaction with CBL2 position III. This conclusion is also supported by our previous finding that a change of the CBL2 region of Epa1A to its Epa6A counterpart is sufficient to increase the binding to \( \text{Gal} \)-linked galactosides (14).

**Figure 5.** In vitro binding of selected EpaA domains. **A,** dissociation constants (\( K_d \) values) for binding of Epa1A, Epa6A, and Epa7A to five different galactosides (\( \alpha\text{1–3-galactobiose} = \text{Gal}_{1–3}\text{Gal}; \beta\text{1–3-galactobiose} = \text{Gal}_{1–3}\text{Gal}; \text{T-antigen} = \text{Gal}_{1–3}\text{GalNAc}; \text{lacto-N-biose} = \text{Gal}_{1–3}\text{GlcNAc}; \text{N-acetyld-lactosamine} = \text{Gal}_{1–4}\text{GlcNAc} \) were determined by fluorescence titration analysis and are shown in regular letters, including values for standard deviations. Binding values obtained for Epa1A, Epa6A, and Epa7A and the five galactosides on glycan arrays (corresponding array numbers are indicated in parentheses) are shown below \( K_d \) values in italic letters. B, discrimination ratios with respect to different linkage types (\( \beta\text{1–4} / \beta\text{1–3} \) and \( \beta / \alpha \)) and with regard to the nature of the sugar moiety attached to galactose (Gal/GalNAc and GalNAc/GlcNAc) were calculated from the corresponding association constants (\( 1/K_d \)) obtained by fluorescence titration (\( ft \) analysis or from the glycan array fluorescence signals (\( array \)) shown in A.

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**Table 2.** Dissociation constants (\( K_d \) values) for binding of Epa1A, Epa6A, and Epa7A to five different galactosides (\( \alpha\text{1–3-galactobiose} = \text{Gal}_{1–3}\text{Gal}; \beta\text{1–3-galactobiose} = \text{Gal}_{1–3}\text{Gal}; \text{T-antigen} = \text{Gal}_{1–3}\text{GalNAc}; \text{lacto-N-biose} = \text{Gal}_{1–3}\text{GlcNAc}; \text{N-acetyld-lactosamine} = \text{Gal}_{1–4}\text{GlcNAc} \).
Our structural analysis further reveals that the binding poses of lactose, N-acetyl-d-lactosamine, and lacto-N-biose bound to the Epa6A domain (Fig. 7, D–F) show different orientations of the second hexoses in the outer binding site despite a common recognition of the galactose moiety within the inner subsite. This is facilitated by alternative H-bonding interactions.
between Asp-227 and the 4-OH group of GlcNAc in Galβ1–3GlcNAc, the 3-OH of GlcNAc in Galβ1–4GlcNAc, and the 6-OH of the Glc moiety in lactose, respectively (Fig. 8, B and C). Like the corresponding position II residue in Epa1A, the side chain conformation of Asp-227 in Epa6A is stabilized by an H-bond to the peptide group of Cys-119 in loop L2 (Fig. 8B). Interestingly, the bulky N-acetyl moiety is found to be well ordered independent of its contacts either with the backbone of Cys-119 in the Epa6A-Galβ1–4GlcNAc structure or with the indole group of Trp-198 in the Epa6A-Galβ1–3GlcNAc structure. A comparison of the binding mode of the β1,4- and β1,3-linked Gal-GlcNAc disaccharides reveals that the orientation of the GlcNAc moiety is rotated by nearly 180° (Fig. 8B). This leads to an exchange of the interaction pattern regarding the Cys-119 backbone. Although the 6-OH group of the β1,3-linked glycan is interacting with that residue, the β1,4-linked glycans interacts via its N-acetyl moiety. The latter is also forming an additional salt bridge with Asn-228 by its 6-hydroxy group. Thus, the detailed binding patterns of β1,4- and β1,3-linked Gal-GlcNAc disaccharides clearly differ, even though the involved amino acid residues are nearly conserved. Finally, comparison of Epa6A-Galβ1–3GalNAc with Epa6A-Galβ1–4Glc reveals highly deviating binding patterns. Here, the secondary carbohydrate is rotated by 90°, and different amino acid residues are employed for interaction (Fig. 8C). This strongly suggests that the energetic contribution of the second hexose to the overall binding of the Gal-based ligands appears to be independent of the mode of its accommodation in the outer subsite.

Discussion

We here have performed a comprehensive in vitro and in vivo functional analysis of the large family of epithelial adhesins of the human pathogenic yeast C. glabrata. Our study reveals that most Epa paralogs possess individually tailored ligand binding properties. Moreover, our data permit us to directly compare the structural and functional relationships of this large family of medically relevant fungal virulence factors. As shown in Fig. 9, such a comparison clearly reveals that the phylogenetic relationship, as based on primary sequence and overall three-dimensional structural features of the A domains, does not mark-edly correlate with a functional classification that is based on the ligand-binding patterns. Members of the functional class II, for instance, are widely scattered on the phylogenetic tree and can be found in several distantly related branches (Fig. 9B). Thus, the A domains of functionally closely related members, such as Epa6 and Epa13 or Epa1 and Epa3, are structurally quite diverse. Vice versa, phylogenetically closely related adhesins, such as Epa6 and Epa7 or Epa3 and Epa22, possess markedly distinct ligand binding specificities. Our study therefore sug-
gests that functionally related Epa variants might have repeatedly developed independently.

How could the Epa family have evolved? A scenario becomes evident when including a comparison of the variability/conervation of amino acid residues located on the protein surfaces of the A domains of different Epa members or within their ligand binding pockets (Fig. 9, A and C). This analysis reveals that although all EpaA domains have conserved PA14/Flo5-like cores, their surface composition is highly variable. Important exceptions, however, are found in the ligand binding pockets.
and include the highly conserved DcisD motif of CBL1 and an asparagine of CBL2, both of which confer coordination of the Ca$^{2+}$ ion, and we would like to refer to these as the “DD-N” signature (Fig. 9, A and C). In addition, highly conserved surface features include a tryptophan from loop L3 and an arginine at position I of CBL2 (14), which form a corner of the inner binding pocket, and we refer to them as the “W-R” corner signature. Together, the DD-N and W-R signatures constitute an invariable core of the binding pocket and are essential for the efficient binding of the terminal hexose moiety in most C. glabrata EpaA domains (Fig. 9C). It is interesting to note that the DD-N calcium-binding signature can be found in over 85% of the estimated 200 Epa-like domains that are currently present in the known fungal genome sequences.\(^5\) In contrast, the W-R signature is highly restricted to the Epa-like adhesins of the “globra group” of Nakaseomyces species, including C. glabrata, C. bra- carensis, C. nivariensis, and N. delphensis (19), and can be found in only very few Epa-like orthologs from other fungi. Therefore, the W-R signature is a hallmark of the globra group and might have evolved in a close ancestor of this group of Nakaseomyces.

Apart from the conserved core module, the binding pockets of the different C. glabrata EpaA domains also contain three highly variable residues that are located within the CBL2 region (Fig. 9C). We have previously defined these residues as CBL2 positions II–IV and suggested that they significantly contribute to Ca$^{2+}$ and asparagine of CBL2, both of which confer coordination of the Ca$^{2+}$ ion, and we would like to refer to these as the “DD-N” signature (Fig. 9, A and C). In addition, highly conserved surface features include a tryptophan from loop L3 and an arginine at position I of CBL2 (14), which form a corner of the inner binding pocket, and we refer to them as the “W-R” corner signature. Together, the DD-N and W-R signatures constitute an invariable core of the binding pocket and are essential for the efficient binding of the terminal hexose moiety in most C. glabrata EpaA domains (Fig. 9C). It is interesting to note that the DD-N calcium-binding signature can be found in over 85% of the estimated 200 Epa-like domains that are currently present in the known fungal genome sequences.\(^5\) In contrast, the W-R signature is highly restricted to the Epa-like adhesins of the “globra group” of Nakaseomyces species, including C. glabrata, C. bra- carensis, C. nivariensis, and N. delphensis (19), and can be found in only very few Epa-like orthologs from other fungi. Therefore, the W-R signature is a hallmark of the globra group and might have evolved in a close ancestor of this group of Nakaseomyces.

Apart from the conserved core module, the binding pockets of the different C. glabrata EpaA domains also contain three highly variable residues that are located within the CBL2 region (Fig. 9C). We have previously defined these residues as CBL2 positions II–IV and suggested that they significantly contribute to the ligand binding specificity of different Epa members (14). Our current functional analysis, which reveals distinct binding patterns for the 17 Epa paralogs from C. glabrata strain CBS138, supports this hypothesis, because the CBL2 regions of nine members carry unique sequence motifs at positions II–IV (Fig. 9, A and B). Moreover, four pairs of Epa adhesins exist with two members each that carry identical CBL2 sequence motifs (Epa1 and Epa7; Epa2 and Epa19; Epa3 and Epa22; and Epa9 and Epa10). In the case of three of these pairs, both members belong to the same functional class. Thus, our study further emphasizes a strong correlation between CBL2 sequences and functional specificity in the case of 15 Epa family members. Moreover, 12 of the 16 Epa-like orthologs found in C. bra- carensis, C. nivariensis, and N. delphensis, which contain a DD-W-NR core structure, carry CBL2 motifs not present in C. glabrata (Fig. 10), suggesting that their binding specificities differ from C. glabrata Epa adhesins. Thus, specific adaptation of the CBL2 motifs of Epa adhesins might have evolved after the DD-N and W-R core motifs and account for the differences in host specificities observed for the different members of the glabrata group of Nakaseomyces (19, 46). It is important to point out, however, that residues outside the CBL2 region must also contribute to ligand binding specificity, given the fact that we found four cases in which two different Epa proteins have identical CBL2 motifs but distinct ligand-binding patterns. The identity of such residues remains to be determined, but they might well reside within loops L1, L2 or L3, which also form part of the binding pocket (14) or that can interact with residues of the CBL2 region (see below).

How exactly do structural features of the binding pockets of different Epa adhesins determine specific ligand binding? Our study contributes to answering this challenging question by providing a set of crystal structures from Epa1 and Epa6, two structurally closely related but functionally distinct Epa variants. Here, we have found compelling evidence that the residues at positions II and III of the CBL2 region are directly determining the efficiency with which Epa1 and Epa6 are able to discriminate between α- and β-linked glycosides. However, our study also suggests that residues outside of the CBL2 region must account for ligand discrimination efficiency, as exemplified by Epa1 and Epa7. Although both proteins carry identical CBL2 motifs, Epa7 significantly better discriminates between α- and β-linked 1–3-galactosiose as well as between Galβ1–3GalNAc and Galβ1–3GlcNAc, respectively. Close to their binding site, these two Epa variants only differ by single residues in loops L1 and L2 (Epa1A, Phe-70 and Gin-1; Epa7A, Leu-70 and Glu-122). These residues could indirectly affect the conformation of CBL2 and the fine structure of the binding pocket. However, our previous studies on the flocculin Flo5A have demonstrated that it is often difficult to rationalize such long range effects on the specificity profile, because changes in loop dynamics and hydration can elude structural analysis (23). Nevertheless, our study indicates that residues at specific positions within the L1 and L2 loops are crucial for determining the precise ligand binding specificity, e.g. by conferring fine-tuning of the binding pocket in the case of EpaA domains with identical CBL2 motifs.

A similar discrepancy is found for the domain pair Epa3A and Epa22A, which differ by only 20 of overall 230 amino acids. Here, the Epa3A domain can be clearly assigned to group I by its

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\(^5\) R. Diderrick, M. Kock, M. Maestre-Reyna, P. Keller, H. Steuber, S. Rupp, L.-O. Essen, and H.-U. Mösch, unpublished results.
binding specificity to Galβ1–3-linked glycans, whereas the Epa22A domain exerts a broad specificity profile that is characteristic for group II EpaA domains (Fig. 3). Interestingly, the CBL1 and CBL2 regions of Epa3 and Epa22 are identical, including the replacement of the otherwise conserved W-R core motif by W-I (Figs. 9A and 10). The only significant difference close to the carbohydrate-binding site is found for residue 106 in loop L2, which is a tyrosine in Epa3 but a phenylalanine in Epa22. L2 is folded around this residue that makes additional contacts with CBL2. Furthermore, all group I EpaA domains harbor a tyrosine or at least histidine (Epa10) in this position, whereas group II and III members have replacements by Phe, Leu, Ala, or Ser (Fig. 9A).

For comparison, in the flocculin Flo5 from S. cerevisiae such long range effects of single site mutations were likewise found to broaden the specificity from primary mannose to glucose ligands as well (23).

A further open question concerns the precise function of the EpaA domains of class III. Our glycan profiling suggests that these adhesins are able to bind diverse nongalactosides with low affinity and therefore might contribute to host cell adhesion by acting as low specificity lectins. However, it might well be that these Epa adhesins are able to bind with high affinity and specificity to yet unknown glycan structures that are not present on the arrays used in this study. This hypothesis is supported by the fact that all class III members contain the DD-N and W-R core motifs as well as individual CLB2 motifs that are not found in other Epa family members or related adhesins of the glabrata.

FIGURE 10. Phylogenetic analysis of Epa family members and related PA14 domain-containing adhesins. A structure-guided alignment of the Epa family and Epa-related orthologs from C. glabrata, C. bracarensis, C. nivariensis, N. delphensis, N. bacillisporus, and C. castellii was carried out together with other PA14 domain-harboring adhesins (Pwp proteins from C. glabrata and Flo adhesins from S. cerevisiae) using appropriate sequences (19, 40) and the three-dimensional structure of Epa1A (14) as described for Fig. 9A. Molecular evolutionary genetic analysis was then performed and visualized using the MEGA software (44). PA14-like A domains in the tree are colored with respect to the following structural motifs: no DD-N (gray), only DD-N (black), DD-N and W-R (purple), or DD-N and W-I (pink). For A domains containing W-R or W-I motifs, the residues at CBL2 positions II–IV are shown in turquoise. Details on sequence motifs are described in Fig. 9A and in the text.
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group of Nakaseomyces (Fig. 10). Clearly, more complex glycan arrays will be required to address this issue in the future (47).

In summary, our study indicates that C. glabrata has developed an extensive array of functionally diverse lectin-like adhesins that might be crucial as a whole for efficient host invasion and dissemination. Importantly, our study permits us to assess the potential host-ligand binding capacity of C. glabrata without knowing the precise EPA gene expression patterns before or during infection. These patterns and underlying regulatory mechanisms are likely to be highly complex and appear to involve both global mechanisms through silencing of subtelomeric regions as well as gene-specific mechanisms (48, 49).

As such, our study might contribute to the development of novel antimiycotics (50), for example the design of anti-adhesive multivalent carbohydrates (51), to effectively combat emerging fungal pathogens of the C. glabrata clade (19, 46).

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