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Comparative structural analysis of the glycosylation of salivary and buccal cell proteins: innate protection against infection by *Candida albicans*

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Mucosal epithelial surfaces, such as line the oral cavity, are common sites of microbial colonization by bacteria, yeast and fungi. The microbial interactions involve adherence between the glycans on the host cells and the carbohydrate-binding proteins of the pathogen. Saliva constantly bathes the buccal cells of the epithelial surface of the mouth and we postulate that the sugars on the salivary glycoproteins provide an innate host immune mechanism against infection by competitively inhibiting pathogen binding to the cell membranes. The structures of the *N*- and *O*-linked oligosaccharides on the glycoproteins of saliva and buccal cell membranes were analyzed using capillary carbon liquid chromatography-electrospray ionization MS/MS. The 190 glycan structures that were characterized were qualitatively similar, but differed quantitatively, between saliva and epithelial buccal cell membrane proteins. The similar relative abundance of the terminal glycan epitope structures (e.g. ABO(H) blood group, sialylation and Lewis-type antigens) on saliva and buccal cell membrane glycoproteins indicated that the terminal *N*- and *O*-linked glycan substructures in saliva could be acting as decoy-binding receptors to competitively inhibit the attachment of pathogens to the surface of the oral mucosa. A flow cytometry-based binding assay quantified the interaction between buccal cells and the commensal oral pathogen *Candida albicans*. Whole saliva and released glycans from salivary proteins inhibited the interaction of *C. albicans* with buccal epithelial cells, confirming the protective role of the glycans on salivary glycoproteins against pathogen infection.

Keywords: buccal epithelial cells / decoy / glycomics / mass spectrometry / saliva

Introduction

Glycans (or oligosaccharides) are the most abundant and diverse biopolymers in nature (Lowe and Marth 2003). These complex molecules cover the surface of cells by their attachment to membrane proteins and lipids and are sometimes secreted into the surrounding environment. Most infections are initiated by the interaction of the pathogen with the host, commonly mediated by surface lectins of pathogens binding to complementary surface carbohydrates on host cells (Beachey et al. 1988). In an age of increased microbial resistance to antibiotics and their decreased efficacy (Overbye and Barrett 2005), it is important to understand these interactions.

Saliva, which is produced specifically by the parotid, sublingual and submandibular glands, is a highly watery oral fluid containing mucus, glycoproteins and electrolytes (Amado et al. 2007). The functions of saliva include lubrication (Aguirre et al. 1989), digestion (Mandel 1987), coating of the oral surfaces and regulation of the oral flora (Lamont and Jenkinson 2010). Earlier studies of saliva focused mainly on specific protection against a single type of microorganism by the salivary immunoglobulins (Igs), although now it has become clear that there is also an innate immune system in addition to the acquired immune system (Amerongen and Veerman 2002). There has been strong evidence of the role played by peptides and glycoproteins in saliva as the first line of oral defense (Frohm Nilsson et al. 1999; Amado et al. 2007; Thongboonkerd 2007). Saliva contains many glycoproteins, some of which are highly glycosylated (the high molecular weight mucins MUC5B and MUC7) as well as salivary agglutinin (gp-340), secretory immunoglobulins (IgA), lactoferrin, amylase and proline-rich glycoproteins (Helmerhorst and Oppenheim 2007).

Candida albicans is a human commensal yeast that is also an opportunistic pathogen in immunocompromised subjects and can cause mucosal infections, including oral candidiasis and *Candida* vulvovaginitis (Cannon and Chaffin 1999). Adherence of *C. albicans* to buccal epithelial cells (BECs) is well documented and several adhesin–ligand interactions have been proposed, including glycan–protein, protein–glycan and protein–protein interactions (Cannon and Chaffin 1999). *In vitro* studies have also shown *C. albicans* adherence to salivary coated hydroxyapatite crystals (Cannon et al. 1995) and to salivary proteins immobilized on nitrocellulose membranes (O'Sullivan et al. 1997). Crude statherin, proline-rich salivary

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proteins (Cannon et al. 1995), basic protein fractions (O'Sullivan et al. 1997) and glycosylated salivary proteins (Hoffman and Haidaris 1993; Cannon et al. 1995) have all been shown to be receptors for *C. albicans* attachment. Specific glycans have also been reported to be involved in the binding of *C. albicans* to salivary glycoproteins with lectin-like interactions between the yeast and host receptor structures of Fucα1-2Gal (Brassartet al. 1991; Johansson et al. 2000), *N*-acetylglucosamine (GlcNAc; Critchley and Douglas 1987) and asialo-GM1 (Yu et al. 1994). Higher *C. albicans* carrier frequency was reported to occur in blood group O individuals and non-secretors (individuals who do not express the H-epitope in salivary glycans; Burford-Mason et al. 1988), although Shin et al. (2003) reported that there was no significant correlation between oral yeast burden and blood group or secretor status of ABO(H) or Lewis antigens.

Glycan receptors are displayed on both the salivary proteins and on the BEC mucosal surface that saliva constantly bathes in the oral cavity. We postulate that there is a competitive interaction of *C. albicans* between those sugar structures on the glycoproteins in the flow of saliva and the BEC surface glycoproteins. The specific glycan structures on salivary and BEC glycoproteins are described, and the inhibition of the interaction of the fungal pathogen with BEC by saliva and glycans released from salivary glycoproteins confirms their innate immune protective role.

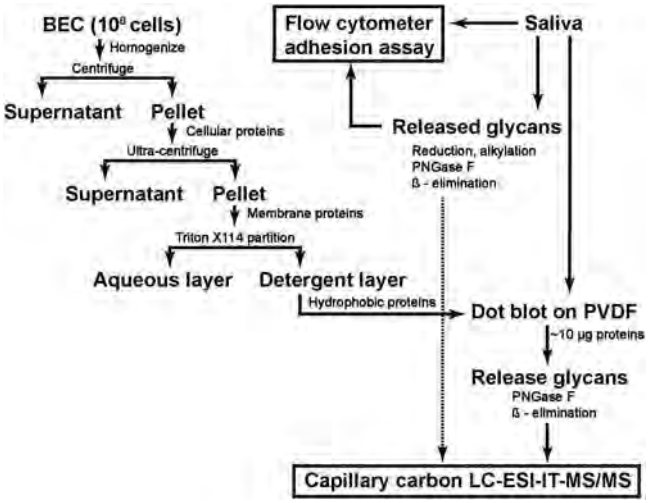

Fig. 1. Work flow for glycan analysis and the flow cytometry-based adhesion assay. Buccal cell membrane proteins were enriched by Triton X114 phase partitioning. *N*- and *O*-linked glycans were sequentially released by PNGase F and β-elimination respectively from BEC and salivary proteins dot blotted on to PVDF membranes or in solution. The glycan structures were analyzed by PGC LC-ESI-IT MS/MS. The flow cytometry-based fluorescent adhesion assay was used to quantify the effect of salivary glycans on the interaction of *C. albicans* with BECs.

Results

Characterization of N- and O-linked glycans from saliva and BECs

Saliva and buccal cell fractions were prepared from an oral swab of the same individual. Protein analysis of both fractions excluded the possibility of salivary contamination of the buccal cell filtrate, as the highly abundant salivary-specific proteins, secretory IgA, amylase and α-1-acid glycoprotein were not seen in the buccal cell fraction (data not shown). The strategy employed in the characterization of *N*- and *O*-linked glycans from saliva and BEC membrane proteins is shown in Figure 1. The released glycans were analyzed using porous graphitized carbon (PGC) liquid chromatography-electrospray ionization mass spectrometry (LC-ESI MS) in the negative-ion mode. PGC chromatography allows the separation of isobaric and isomeric structures and, using a combination of LC-ESI MS/MS fragmentation and retention time, the structure and sequence information of the oligosaccharides was deciphered.

Fragment ions generated in the negative-ion MS^2 fragmentation spectra were used for a detailed structural analysis of many of the glycans. Fragment masses can be diagnostic of such structural features as the position of fucose, the core type of *O*-glycans and the branching of *N*-glycan structures (Harvey 2005; Harvey et al. 2006, 2008). For example, as shown in the MS^2 spectrum in Supplementary data, Figure S4, the $[M\ -2H]^{2-}$ parent ion of *m/z* 1185.3, which has the monosaccharide composition $(Hex)_4(HexNAc)_4 + (Man)_3(GlcNAc)_2$, has an MS^2 spectrum with a dominant fragment ion (*m/z* 1714.5) corresponding to the loss of the Lewis y/b (*m/z* 656) fragment from one arm of the complex *N*-glycan. The signals at *m/z* 350, 553 and 2002.7 all indicate that the reduced glycan is core fucosylated.

This approach was used for the detailed structural analysis of the glycan sequences and structures of the 78 *N*-glycan (Supplementary data, Table S1) and 112 *O*-glycans (Supplementary data, Table S2) released from salivary and buccal cell membrane glycoproteins, and the relative percent composition of each structure was determined.

N-Glycans of whole saliva and buccal cell membrane proteins

The glycan profile in Figure 2 shows a representative summed MS spectra obtained from capillary carbon LC-ESI MS of *N*-glycans released from glycoproteins of whole saliva (Figure 2A) and from buccal cell membrane proteins (Figure 2B) from the same individual. Supplementary data, Table S1 summarizes the proposed *N*-glycan structures on the proteins of whole saliva and buccal cell membranes with their corresponding relative amounts as calculated from three separate LC-ESI MS analyses. A total of 78 unique *N*-glycans were characterized, with eight structures found to be unique to buccal cell membranes and three salivary-specific *N*-glycans.

Neutral N-glycans

The *N*-linked glycans of both saliva and BEC (Figure 2, Supplementary data, Table S1) showed a predominance of neutral biantennary and some triantennary fucosylated structures. Large biantennary structures were seen with 1–5 fucose residues (structures 14, 28 and 40). Most of the neutral glycans were core fucoslyated with the terminal antennae containing Lewis a/x, Lewis y/b and H-epitope-type fucosylation.

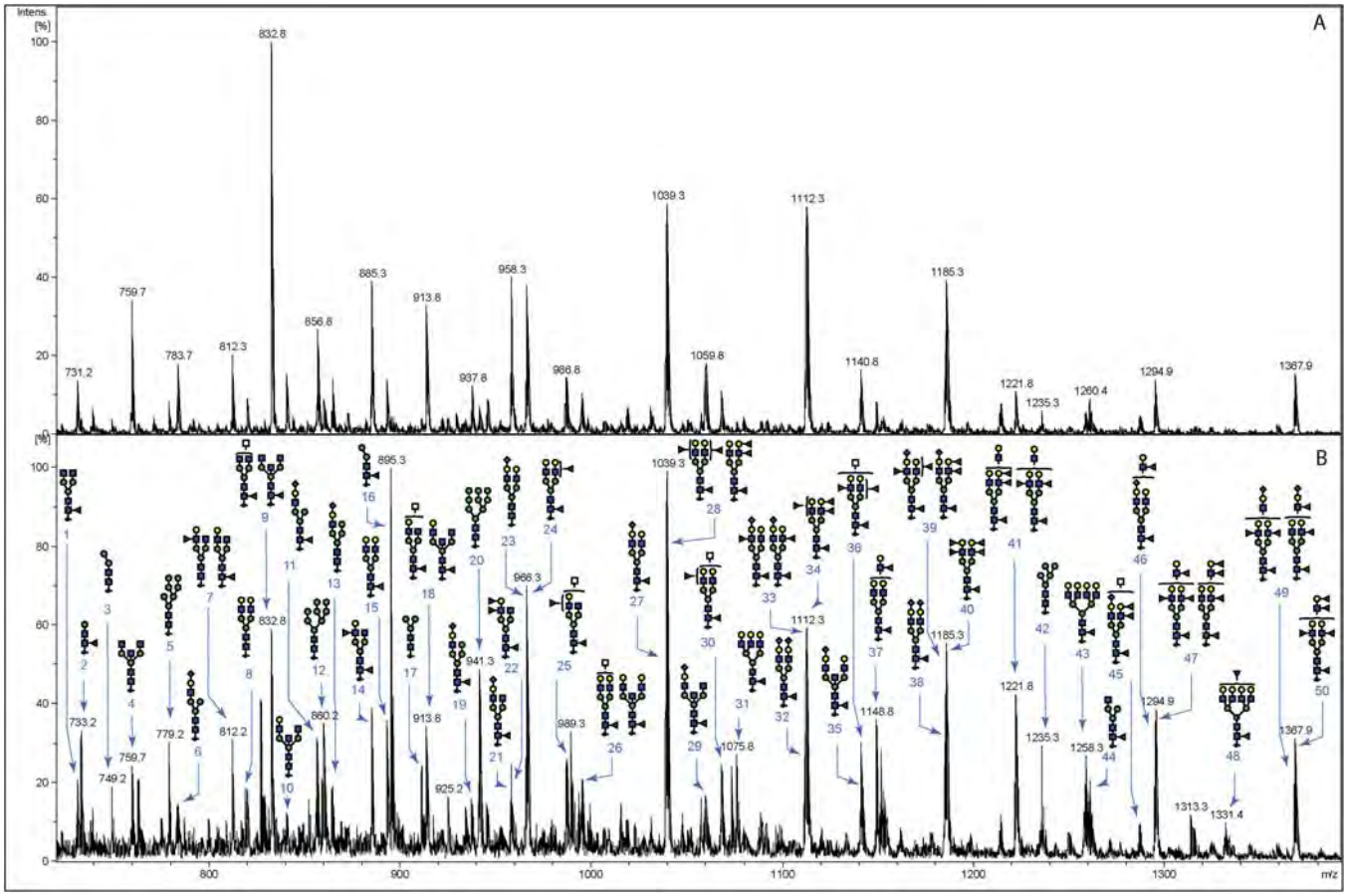

Fig. 2. Summed mass spectra of *N*-glycans released from salivary proteins (**A**) and buccal epithelial membrane proteins (**B**) and analyzed by capillary carbon LC-ESI-IT MS/MS. Numbers refer to the structures shown in Supplementary data, Table S1. Key of oligosaccharide symbols and linkage information is shown in Supplementary data, Figure S2.

Bisecting GlcNAc-type neutral glycans, with and without core fucosylation, were also present. An example of bisecting *N*-glycan characterization is illustrated in Supplementary data, Figure S9. Structure 9a, a bisecting agalactosyl biantennary structure (Figure 2, Supplementary data, Table S1), is the most dominant peak of the salivary *N*-glycans amounting to 17% of the total pool. The molecular masses of these structures often corresponded to isomeric and isobaric structures that were well separated by PGC and are listed in Supplementary data, Table S1 with their corresponding content. Both saliva and BEC were comprised of similar N-linked glycosylation structures but with different abundances; for example, the structure *m/z* 1039.3^{2-}, of composition $(Hex)_2(HexNAc)_2(Deoxyhexose)_3 + (Man)_3(GlcNAc)_2$ comprised four isomers, all of which were present in both saliva and BEC (28a–d, Supplementary data, Table S1). The most abundant isomer (3.7%) of this composition in BEC was structure 28a and the fragmentation spectrum of this structure indicated that the antennae contained the Lewis x/a epitope. On the other hand, structure 28c was the dominant isomer in saliva (3.2%) and the diagnostic ions indicated that one of the biantennary arms in this isomer contained the Lewis y/b antigen. Similarly, the separation and characterization of the four tetra-fucosylated biantennary *N*-glycan isomers (34a–d, Supplementary data, Table S1) is shown in Supplementary data, Figure S7. Tri- and tetra-antennary structures containing core and terminal fucosylation were seen in both samples.

Acidic N-glycans

A large proportion of salivary (21.6%) and BEC membrane (22.4%) glycoproteins was sialylated. MS^2 diagnostic ions (*m/z* 290 and 655) identified terminal sialic on galactose (Gal; Lowe and Marth 2003; Ito et al. 2007), and the retention time (using known bovine fetuin *N*-glycan structures as retention standards) was used to determine the linkage (α2-3 and α2-6 linked) of the sialic acid (Lowe and Marth 2003; Ito et al. 2007; Pabst et al. 2007). Based on these diagnostic features, only two of the 22 sialylated *N*-glycans were potentially α2-3 linked. The less abundance of α2-3 linked *N*-glycans is consistent with a previous study of *N*-glycans found on oral parotid gland glycoproteins (Guile et al. 1998).

Sialylated Lewis x/a antigens were also present on the *N*-glycans of whole saliva and BEC membrane proteins. For example, the glycan of composition $(Hex)_2(HexNAc)_2(Deoxyhexose)_2(NeuAc)_1 + (Man)_3(GlcNAc)_2$ with *m/z* 1111.8^{2-} (33a and b, Supplementary Table 1) showed two isomers in the extracted ion chromatogram that were both core fucosylated

biantennary glycans. Structure 33a had a terminal sialylated Lewis antigen (diagnostic MS/MS ion, *m/z* 801), whereas structure 33b had one antenna that was sialylated (diagnostic MS/MS ion *m/z* 655) with the other antenna containing a Lewis x/a (diagnostic MS/MS ion *m/z* 510) antigen.

High-mannose and hybrid glycans

BEC membrane protein glycosylation contained 10.4% high-mannose glycans of composition $(Man)_9(GlcNAc)_2$ to $(Man)_5(GlcNAc)_2$, while these structures comprised only 4.2% of the glycans on salivary proteins. The presence of high-mannose glycans of composition $(Man)_6(GlcNAc)_2$ and $(Man)_5(GlcNAc)_2$ in human parotid salivary gland has been reported previously (Guile et al. 1998). Hybrid *N*-glycans were also present to the same extent in both samples with saliva and BEC containing 4.6 and 4.5%, respectively. Most hybrid glycans were core fucosylated (structures 11, 19a and19b, Supplementary data, Table S1) with the arm containing the lactosamine either sialylated or fucosylated.

Paucimannose glycans

The most abundant *N*-glycan structure in the BEC membrane glycosylation, which is completely absent in the salivary glycan profile, was a paucimannose glycan of the composition $(Hex)_2(HexNAc)_2(Deoxyhexose)_1$, with a relative abundance of 11.5%. Other paucimannose structures were also seen only in BEC, including $(Hex)_1(HexNAc)_2(Deoxyhexose)_1$ and $(Hex)_2(HexNAc)_2$, and these unusual structures amounted to a total relative content of 14.3% of the total *N*-glycan pool. These structures were also not present in saliva. The negative-ion fragmentation spectra of these paucimannose glycans are shown in Figure 3.

More than 84% of the *N*-glycans of both saliva and BEC were fucosylated, with core fucosylation occurring on most structures. In summary, the major relative differences between the *N*-glycans of BEC and salivary glycoproteins were that of paucimannose structures being only present on BEC membranes (14.3%) and not salivary glycoproteins, and the higher content of bisecting *N*-glycans in saliva (30%) compared with BEC (9.8%).

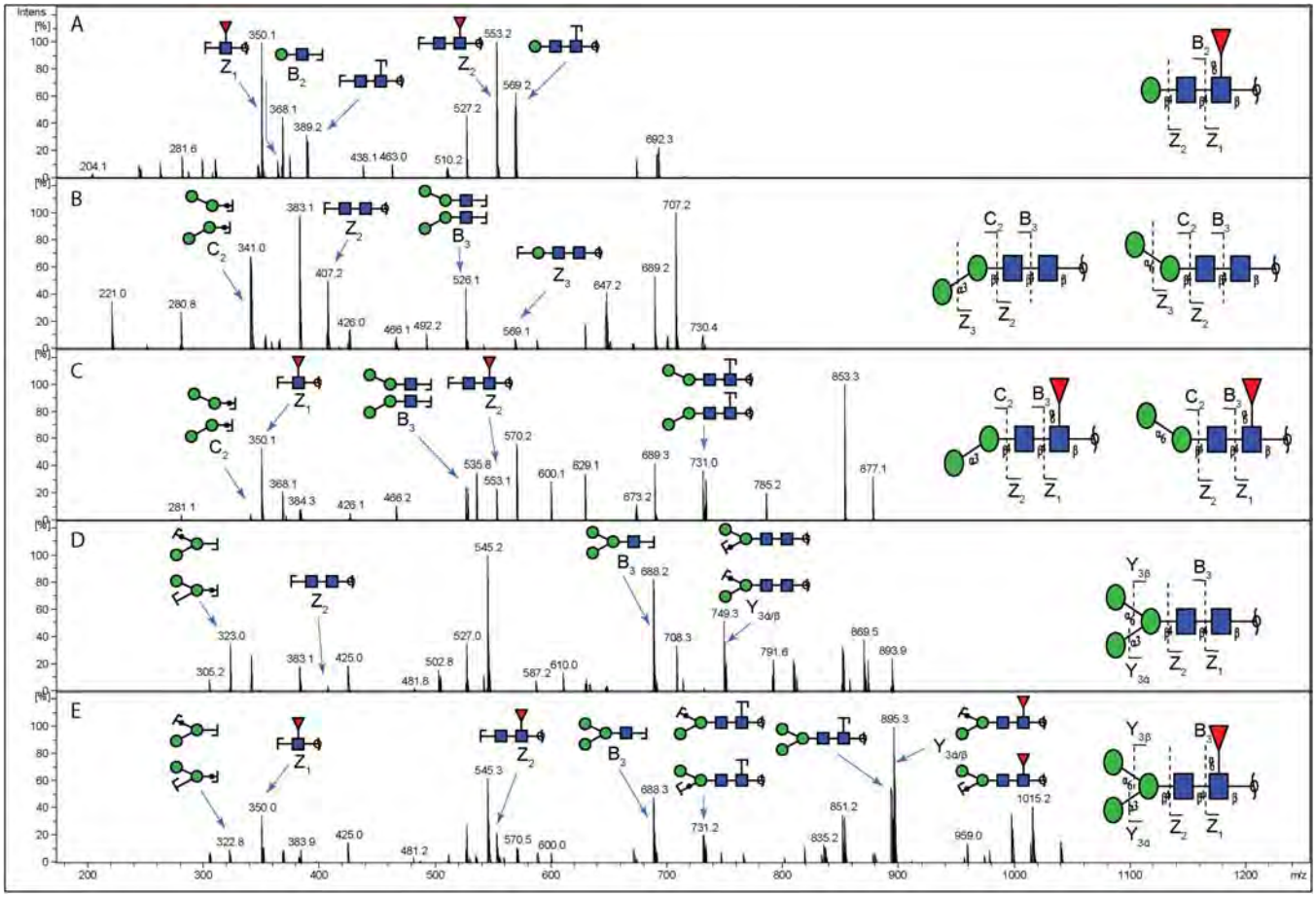

Fig. 3. Singly charged MS^2 negative-ion fragmentation spectra of paucimannose structures corresponding to *m/z* 733 (**A**), *m/z* 749 (**B**), *m/z* 895 (**C**), *m/z* 911 (**D**) and *m/z* 1057 (**E**). Fragments are labeled according to the Domon and Costello (1988) scheme. Identified structures with key fragments are shown. Key of oligosaccharide symbols and linkage information is shown in Supplementary data, Figure S2.

O-Glycans of whole saliva and buccal cell membrane proteins

The *O*-glycan profile in Figure 4 shows the summed MS spectra obtained from capillary carbon LC-ESI MS of the total *O*-glycans released from saliva (Figure 4A) and buccal cell membrane (Figure 4B) proteins. Supplementary data, Table S2 summarizes the proposed *O*-glycan structures of whole saliva and buccal cell membrane proteins with their corresponding relative quantities.

The *O*-linked oligosaccharides released from saliva and BEC membrane proteins of the same individual displayed a heterogeneous mixture of neutral and acidic oligosaccharides, detected both as singly and doubly negatively charged ions (Figure 4, Supplementary data, Table S2). In total, 112 glycan structures were separated by PGC chromatography. Most of the abundant structures were sialylated and highly fucosylated, such as structures 14, 29 and 30 (Supplementary data, Table S2).

The MS^2 fragmentation spectra of the reduced *O*-linked oligosaccharides contained diagnostic fragment ions characteristic of core type, blood group epitope, Lewis type and *N*-acetyllactosamine elongation (Karlsson et al. 2004; Robbe et al. 2006) that facilitated structure determination. The total *O*-glycan profile (Figure 4) demonstrated that most structures from saliva and BEC membrane glycoproteins were of the core -1 and core 2-type *O*-glycans. Among the eight core 3 structures, five were common to both saliva and BEC, whereas the other three were unique to saliva. Core 4-type *O*-glycans were also present and were higher in saliva (22%) relative to BEC (13%). Most structures involved the addition of LacNAc units to simple core 1- and/or core 2-type structures consistent with known glycosylation pathways and previously reported salivary glycoprotein structures of MUC5B (Thomsson et al. 2005), MUC7 (Karlsson and Thomsson 2009) and $DMBT^{SAG}$ (Issa et al. 2010). These LacNAc units were mostly fucosylated on both the GlcNAc and Gal residues, thereby yielding H-type substructures (Fucα1-2Galβ1-), Lewis x/a-type fucosylation (Fucα1-3/4GlcNAcβ1-) and Lewis y/b-type fucosylation [Fucα1-2Galβ1-4/3(Fucα1-3/4)GlcNAcβ1-]. Type 1 and Type 2 LacNAc extensions act as scaffolds and are often modified with sialic acid and fucose residues (Zheng et al. 2011). Neutral fucosylated structures were the most abundant structures (70%) in the *O*-glycan profiles of both salivary and BEC membrane glycoproteins.

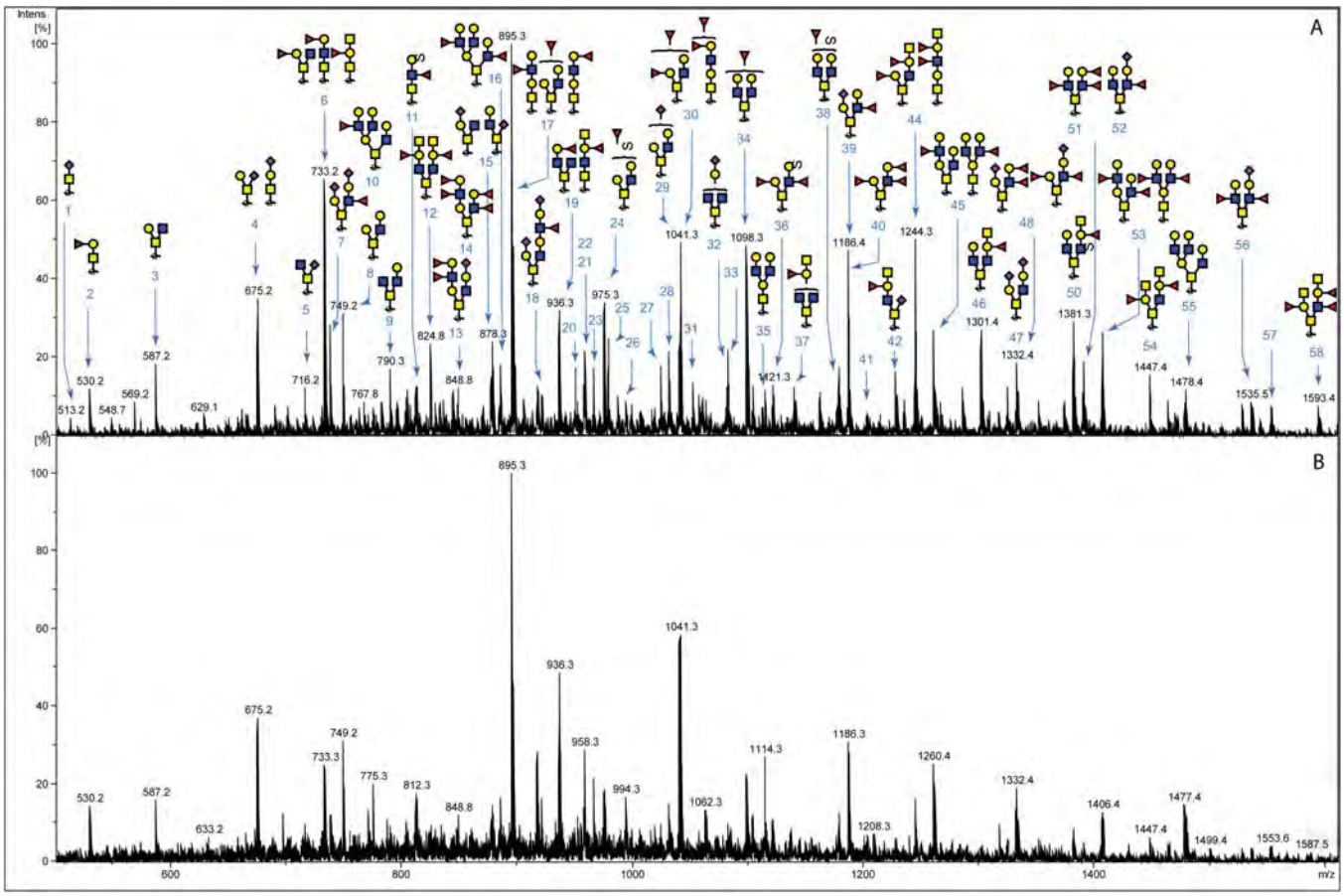

Fig. 4. Summed mass spectra of *O*-glycans released from salivary proteins (**A**) and buccal epithelial membrane proteins (**B**) and analyzed by capillary carbon LC-ESI-IT MS/MS. Numbers refer to the structures shown in Supplementary data, Table S2. Key of oligosaccharide symbols and linkage information is shown in Supplementary data, Figure S2.

The separation potential of graphitized carbon also permitted the separation of many isomers with a specific order of elution and retention time, providing information about the core-type and/or elongation of *O*-glycans. For example, structure 17 (Supplementary data, Table S2) of *m/z* 895 is present as two isomers in BEC (15 and 1%) and four isomers in saliva (6.2, 3.5, 1.7 and 0.6%) that have different retention times (23, 24, 29 and 34 min), respectively. The different structural isomers were characterized based on diagnostic MS/MS fragment ions (core 2/3/4: *m/z* 407, core 1/2: *m/z* 366, Fucα1-2Gal: *m/z* 325, etc.; Karlsson et al. 2004; Robbe et al. 2006) that assign the core types (core 1/core 2) and fucose linkage to either Gal or GlcNAc.

The presence of sialic acid was confirmed by MS^2 analysis with the *m/z* 291 ion loss diagnostic of the presence of *N*-acetylneuraminic acid (NeuAc). The loss of sialic acid followed by a loss of hexose indicated that the sialic acid was attached to terminal Gal. Linkage analysis of the sialic acids was not performed to differentiate α2-3 from α2-6, although previous studies (Prakobphol et al. 1998; Thomsson et al. 2005; Karlsson and Thomsson 2009) have shown that the majority of the *O*-linked sialylated oligosaccharides on salivary mucins were of the α2-3 linkage with a low abundance of α2-6 linked sialic acid oligosaccharides such as the T-antigen [Galβ1-3(NeuAcα2-6)GalNAcol]. Our data show that there were few low abundance glycans with NeuAc α2-6 linked to the core GalNAcol (structures 5, 15 and 42; Figure 4; Supplementary data, Table S2). These structures were predominantly seen on salivary glycans and not on BEC *O*-linked membrane glycoproteins.

Sulfate containing *O*-linked glycans were also found on both the whole saliva and BEC membrane glycoproteins. Distinguishing sulfates from phosphates is difficult by the MS techniques used in this study, but previous reports have shown that salivary mucins are typically modified by sulfates and not phosphates (Prakobphol et al. 1998; Thomsson et al. 2002, 2005; Karlsson and Thomsson 2009). The location of the sulfate on either the Gal or the GlcNAc was established by the fragment ions *m/z* 241 (HSO_3-3Galβ1-) and *m/z* 262 (HSO_3-GlcNAcβ1). For example, structure 11 (Supplementary data, Figure S6 and Table S2) consists of isomers where the sulfate is attached to either the terminal Gal (structure 11b) or GlcNAc (structure 11a). Salivary glycoproteins had a higher abundance of sulfated glycans than that attached to BEC membrane proteins. Sulfated Lewis structures, which are known to be high-affinity epitopes for L-selectin, were seen in both the glycan profiles of salivary and BEC membrane proteins.

A significant amount of the *O*-glycans of salivary (54.6%) and BEC membrane (29.4%) proteins carried the blood group H-epitopes (Fucα1-2Galβ1-). The terminal blood group H is usually modified by a single monosaccharide residue, β1-3 linked Gal or *N*-acetylgalactosamine (GalNAc), that determines blood group ABO(H). The structures found in this report were from an individual of blood group A and the terminal structures of glycans 12, 34, 37, 44 and 58 (Figure 4, Supplementary data, Table S2) have HexNAc attached to the blood group H-epitope, indicating expression of blood group A antigen [GalNAcα1-3(Fucα1-2)Galβ1-] on both their salivary and BEC glycoproteins (Supplementary data, Figure S10b). The sequence elucidated from MS^2 glycosidic cleavages indicated the presence of this blood group antigen, although there were no truly diagnostic cross ring fragments to determine the exact linkage of the ABO(H)-type epitopes under the conditions used (Karlsson et al. 2004). Thomsson et al. (2005) have reported that ABO(H) blood group-specific sequences were found on the glycoforms of salivary MUC5B resulting in distinct glycosylation that varied extensively between individuals, with non-secretors having a higher degree of sialylation than blood group secretors.

Our data also show the extension of the blood group H substructure by LacNAc (Galβ1-4GlcNAc) disaccharides such as seen on structure 30 (Figure 4, Supplementary data, Table S2). These unusual structures have been previously reported on salivary MUC7 (Slomiany et al. 1993; Karlsson and Thomsson 2009) and recently on $DMBT^{SAG}$ (Issa et al. 2010). We identified these structures on salivary (3.6%) and BEC membrane (6.8%) glycoproteins (structures 16, 10, 35 and 55; Figure 4; Supplementary data, Table S2). These structures were characterized based on their MS/MS fragmentation patterns as described by Karlsson et al. (2004) (Supplementary data, Figure S10c).

Terminal glycan epitopes on *N*- and *O*-glycans from salivary and BEC membrane glycoproteins

The terminal structures on the glycans attached to proteins, such as blood groups, Lewis antigens, sialylation, sulfation, high mannose and lactosamine, have all been implicated as the receptors for bacterial adhesion (Gagneux and Varki 1999; Osswald et al. 2003). These different terminal glycan epitopes on the *N*- and *O*-glycans of salivary and BEC membrane proteins were quantified and compared using the relative MS ion intensities of each of the glycan structures carrying these substructures. Figure 5 shows the relative percent abundance of these epitopes, clearly demonstrating that glycans from the salivary and BEC membrane proteins from the same person, at the same time, have essentially the same terminal epitopes differing in relative abundance. This is interesting as glycan diversity is cell-type-specific (Varki 2006); salivary proteins are produced by the cells of the major (parotid, submandibular and sublingual) and minor (tongue, palate and buccal and labial mucosa) glands (Amado et al. 2007), yet their glycosylation profiles are remarkably similar to that of the glycoproteins produced by the BECs. Both displayed different levels of the same pathogen-binding epitopes.

Sialylation of the *N*-glycan terminal structures was of similar abundance in saliva (13.8%) and BEC (14.9%), whereas the *O*-glycan terminal structures in BEC (24.4%) had higher sialylation than that of saliva (15.2%). The major terminal antigen on salivary and BEC membrane glycoproteins, in both their *N*-glycan (25.5 and 32.6%, respectively) and *O*-glycan (26 and 27.5%, respectively) profiles, was the Lewis x/a antigen. H-type antigens were present in high abundance on the *O*-glycans of saliva (16.1%) and BEC (12.6%) when compared with their presence on *N*-glycan termini [saliva (6.9%) and BEC (2.4%)]. Terminal LacNAc residues on

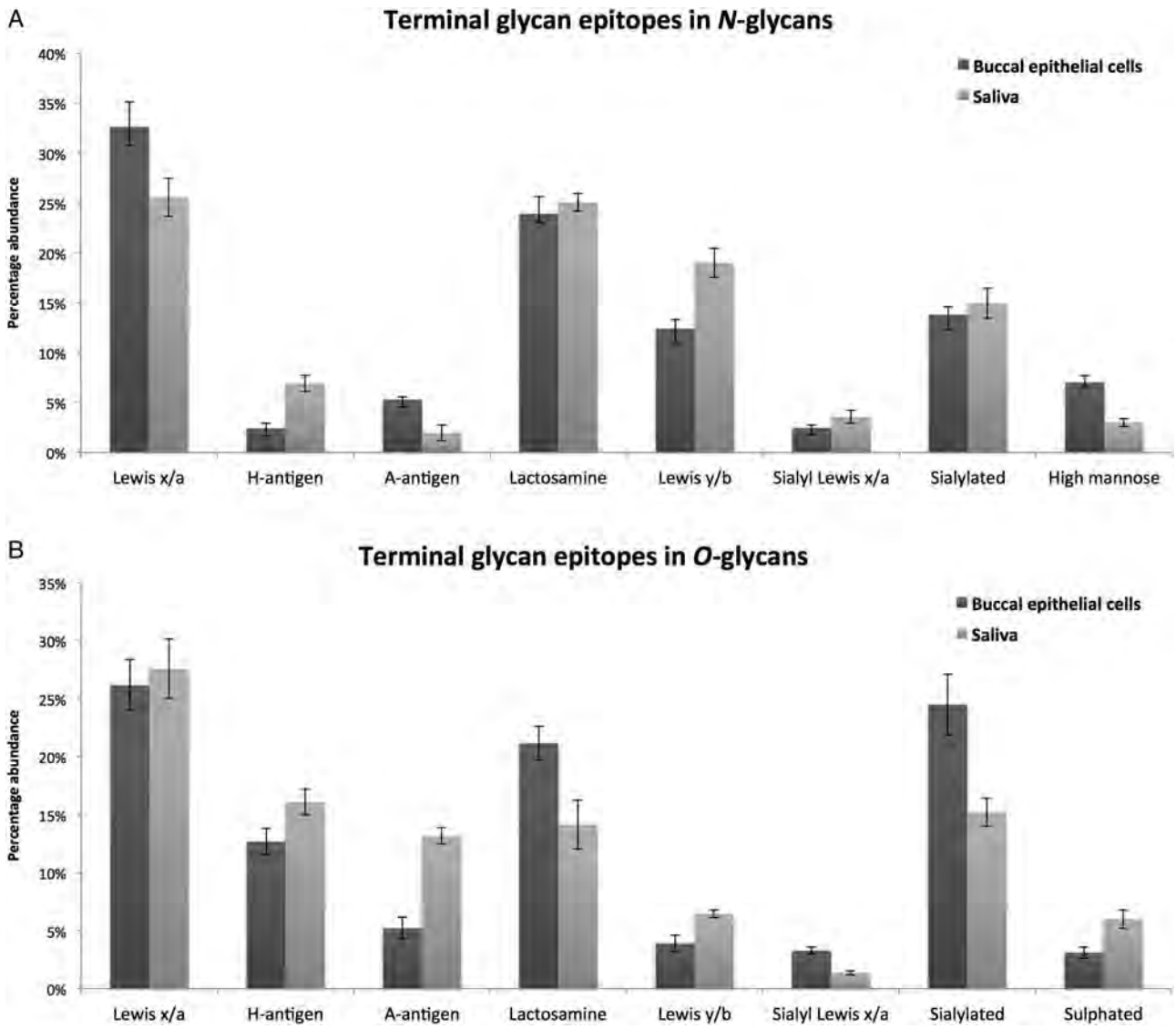

Fig. 5. Relative amounts of terminal glycan epitopes on (**A**) *N*-glycans and (**B**) *O*-glycans of salivary and BEC membrane proteins. The calculation is based on relative MS ion intensities of the glycans carrying these structures. The data represents the mean ± standard error of triplicate analysis of saliva and buccal cell membrane glycans from an individual of blood group A secretor status.

N-glycans were of similar abundance in saliva (23.9%) and BEC (25.1%), though *O*-glycans of BEC (21.1%) have a higher abundance than that of saliva (14.1%). Sulfated structures were only present on the *O*-glycans of both salivary (6%) and BEC (3.1%) glycoproteins. As the saliva and BEC were collected from an individual of blood group A, the *O*-glycans also showed the presence of terminal blood group A antigens. Salivary *O*-glycans had a higher blood group A-antigen abundance of 13.1% compared with the *N*-glycans (1.9%), whereas BEC membrane protein *N*- and *O*-glycans had similar A-antigen expression (5.2 and 5.3% respectively). High-mannose *N*-glycans were lower on salivary glycoproteins (2.9%) than on BEC membrane proteins (7%).

Variation of *N*- and *O*-glycans between individuals

To compare the variation in glycans between individuals, 12 abundant *N*- and *O*-glycan masses from saliva and BEC (Supplementary data, Figure S5) of five secretor individuals of blood group A were plotted by a box-and-whisker plot as shown in Figure 6. Although there is variation among individuals, the same abundant glycan masses are seen in every individual's salivary (Figure 6A and C) and BEC (Figure 6B and D) glycan profiles, demonstrating that salivary glycans mimic the BEC membrane glycans. Additionally, Supplementary data, Figure S5f shows the glycan profile of a non-secretor blood group A individual. The inability of non-secretors to synthesize blood group H-antigen results in the absence of glycan masses that correspond to structures with the H-antigen.

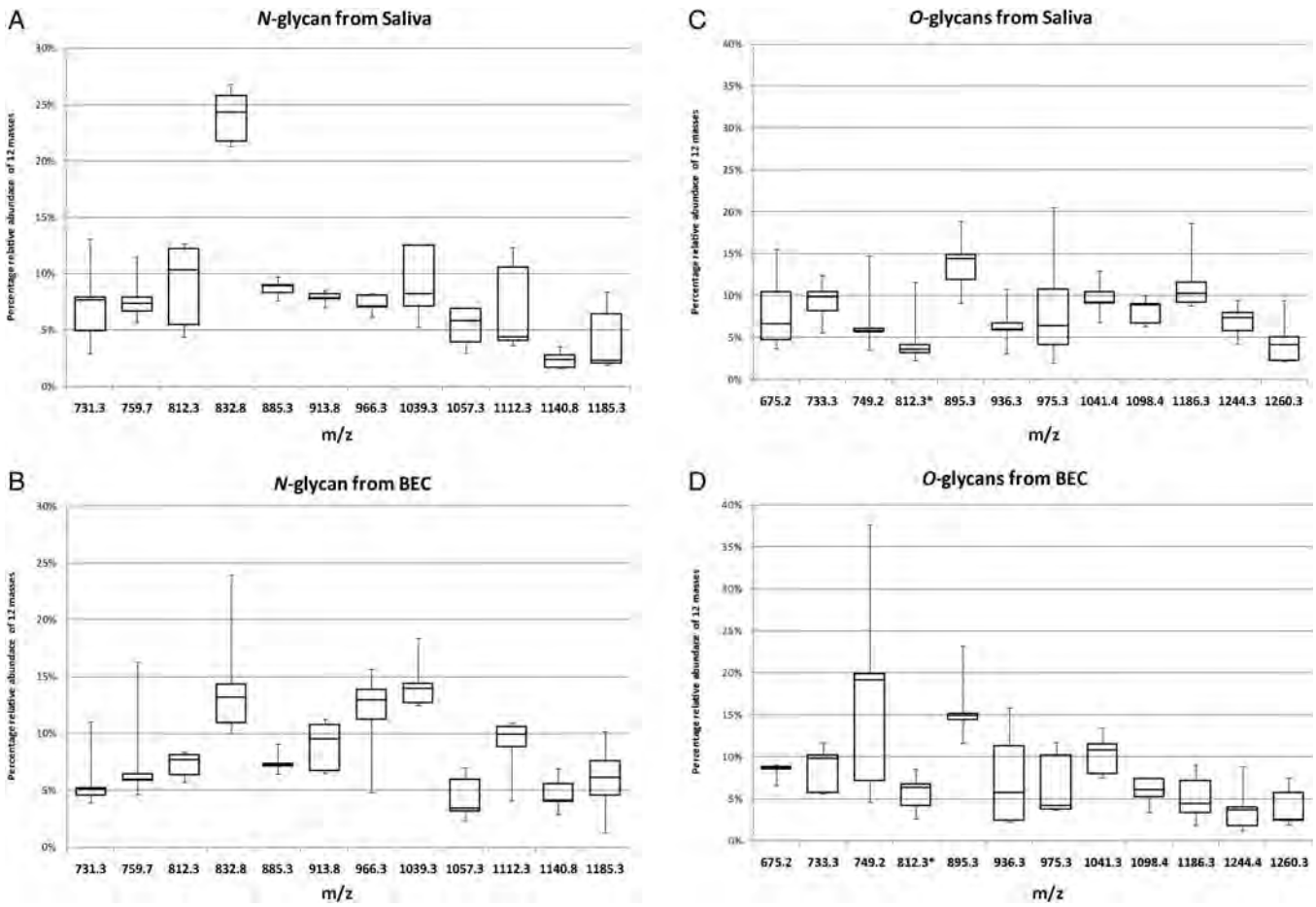

Fig. 6. Box and whisker plot representing the relative abundances of 12 *N*- and *O*-linked abundant glycan masses of five blood group A secretor individuals. All *N*-glycan masses are doubly charged, while all *O*-glycan masses are singly charged except for *m/z* 813.3.

For example, m/z 530^{1-} from *O*-glycan and *m/z* 1112.4^{2-} from *N*-glycans are completely absent in non-secretor individual's saliva and BEC profiles (Supplementary data, Figure S5f). Quantitative monosaccharide analysis was carried out by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) to compare the relative contribution of the *N*- and *O*-glycans to the overall glycosylation of the saliva. The neutral monosaccharide composition (GlcNAc, GalNAc, Gal, NeuAc and Fuc) of the *N*- and *O*-glycans released from the same quantity of saliva is shown in Supplementary data, Figure S1. In saliva, the total monosaccharide composition of *O*-glycans (3.8 nmol/μg) is six times higher than that of the *N*-glycans (0.6 nmol/μg) released from the same quantity of salivary proteins.

Flow cytometry-based adhesion assay

The yeast *C. albicans* has been reported to have numerous adhesins to mediate its binding to host receptors, including lectin-like adhesins that bind to oligosaccharides (Fukazawa and Kagaya 1997; Cannon and Chaffin 1999). Fluorescently labeled *C. albicans* (3×10^7 cells/mL), when incubated with BEC (1×10^6 cells/mL) for 60 min at room temperature, was seen by confocal microscopy to adhere to the BEC (Figure 7).

The adherence of the yeast to the BEC was quantitated by flow cytometry. The BECs that had been scraped from the oral cavity emit clear intrinsic autofluorescence under laser excitation (mainly by 407 and 488 nm) and owing to their large size fall in the upper quadrants of the dot plot where their population is gated and quantified in the FITC channel (Figure 8A). The *C. albicans* were fluorescently labeled internally with carboxyfluorescein succinimidyl ester (CFSE) to not disturb the cell membrane and were mixed with BEC. The non-adherent yeast were distinguished by their size difference by forward scattering channel (FSC) vs side scattering channel (SSC) and were gated as a different population compared with that of the BEC. The yeast adhering to the BEC contributed to an increase in the fluorescence intensity by up to 2 orders of magnitude of the BEC population (Figure 8B). When the *C. albicans* was pre-incubated with the mixture of *N*- and *O*-glycans released from 0.5 mL of saliva before mixing with the BEC, the interaction of the yeast was reduced by ~45% as reflected by the gated BEC population showing decreased (55%) fluorescence intensity (Figure 8C).

To quantitate the extent to which salivary glycans can inhibit the yeast adhesins from binding to the glycans on the buccal cells, a mixture of *N*- and *O*-glycans was released from saliva that was collected from the same individual on

three separate occasions and was pre-incubated with *C. albicans* before measuring their interaction with BECs that were collected at the same time (Figure 9). *Candida albicans* incubated with phosphate-buffered saline (PBS) buffer alone was used as a control. Pre-incubation of the yeast with 0.5 mL of whole saliva (based on a normal flow of saliva of 0.5 mL/min) resulted in ~75% inhibition of binding of the yeast to the BEC (Figure 9C). Interestingly, pre-incubation with only the combined *N*- and *O*-linked salivary glycans released from this volume of whole saliva was able to inhibit the interaction of the yeast with the buccal cells by 55% (Figure 9D). Increasing the quantity of salivary glycans by 10-fold (equivalent to that in 5 mL of saliva) reduced the interaction of the *C. albicans* with the buccal cells to 5% of the control (Figure 9E). This concentration-dependent inhibition (Supplementary data, Figure S3) was non-linear, perhaps indicating the saturation of the yeast-binding sites. A comparison of the inhibition of binding by either *N*- or *O*-linked glycans released from the same quantity of salivary proteins (0.5 mL) showed that the *O*-linked glycans alone had a greater inhibitory effect (45%) on binding than the *N*-linked glycans (30%) which correlates with the greater *O*- than *N*-glycosylation of salivary proteins (Supplementary data, Figure S1). These data thereby show the potential of a complex mixture of glycans to significantly inhibit the interaction of *C. albicans* with the cells lining the oral cavity.

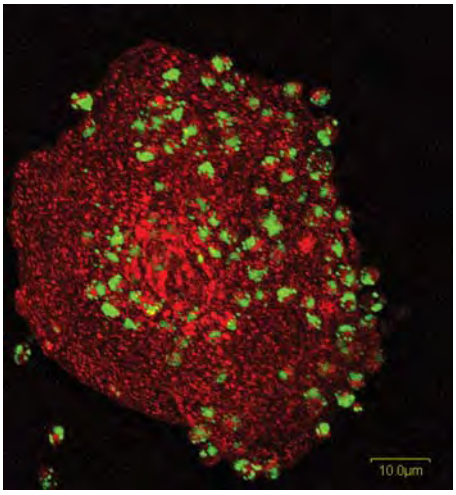

Fig. 7. Adhesion of *C. albicans* to BECs as visualized by a Olympus Fluoview 300 confocal microscope. The yeast was CFSE (green) labeled and the BECs were labeled with FM 64 lipophilic styryl dye (red).

Discussion

In this study, we have taken the oral cavity as a model system to study the potential of salivary glycoproteins to inhibit

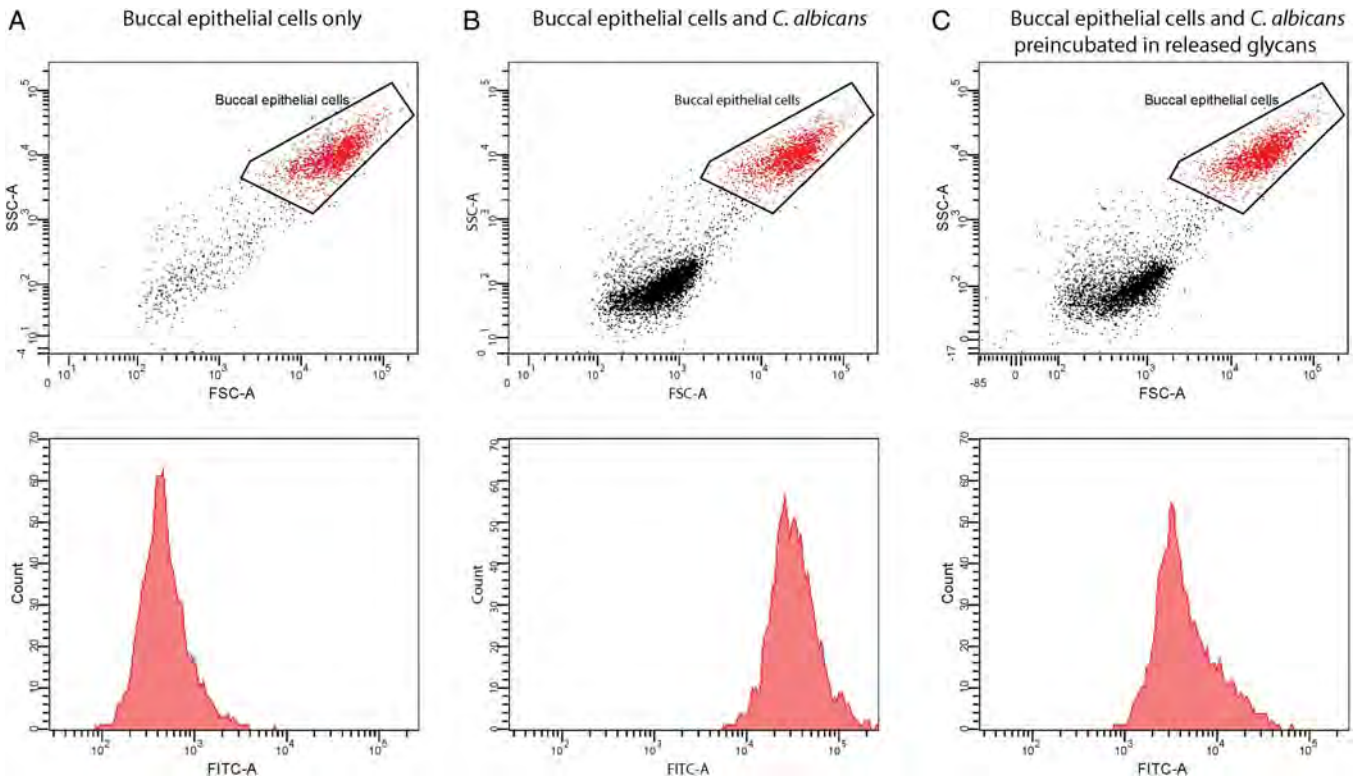

Fig. 8. Flow cytometry assay of BECs binding to the yeast *C. albicans* on the basis of cell geometry and granularity. The dot plots at the top show the FSC–SSC distribution, while the corresponding histograms of BEC count vs fluorescence intensity in the FITC channel is shown below. (**A**) Autofluorescence of buccal cells alone; (**B**) fluorescently labeled yeast *C. albicans* incubated with BEC; (**C**) *C. albicans* pre-incubated with salivary *N*- and *O*-glycans before mixing with BEC.

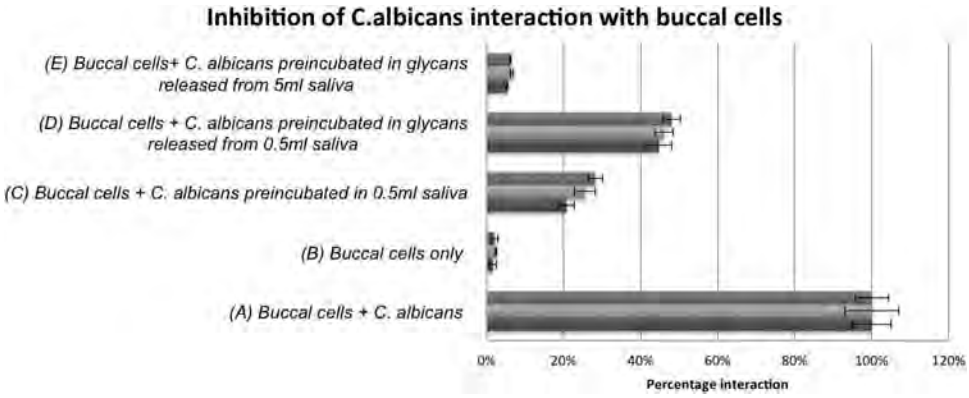

Fig. 9. Quantitation of adhesion of *C. albicans* to BEC as measured by flow cytometry. Interaction between the yeast and the BEC (**A**) is normalized to 100% with the autofluorescence of buccal cells (**B**) at ~2%. The extent of inhibition of adhesion was determined by pre-incubation with (**C**) whole saliva (0.5 mL); (**D**) *N*- and *O*-glycans released from saliva (0.5 mL); (**E**) *N*- and *O*-glycans released from saliva (5 mL). The data represent the mean ± standard error of three independent biological replicates and their technical triplicates.

infection by the pathogen *C. albicans*. Specifically, we have investigated the role of the BEC membrane oligosaccharides in the binding of the pathogen and have compared their detailed structural composition with the oligosaccharides on the salivary proteins that constantly bathe these oral cells. Although other studies have shown that *C. albicans* adheres to oligosaccharide receptors on BEC as well as to salivary glycoproteins (Brassart et al. 1991; Cameron and Douglas 1996; de Repentigny et al. 2000; Johansson et al. 2000; Shin et al. 2003), the inference that salivary glycans could mimic the glycan receptors displayed on the BEC membranes, and thus compete with their binding as an innate immune defense mechanism, has not been established previously.

Using the high-resolution separation obtained by PGC together with the ESI MS/MS fragmentation spectra of the separated glycans, we were able to identify hundreds of protein bound *N*- and *O*-linked oligosaccharides in whole saliva as well as on BEC membranes.

The *N*-linked oligosaccharides of saliva were highly fucosylated with terminal epitopes containing Lewis and H-type antigens, which is consistent with previous studies of the salivary glycans secreted by the parotid gland (Guile et al. 1998). The most abundant glycan in whole saliva was the bisecting agalactosyl biantennary structure. The BEC *N*-glycan profile was seen to have qualitatively similar, but quantitatively different, glycan structures to that of saliva. Overall, the *N*-glycan profile of BEC membrane proteins was comparatively more heterogeneous than saliva, with a larger number of isomeric structures and a higher degree of sialylation. In contrast to the salivary proteins, the expression of the ABO(H) blood group antigen was limited to the terminus of only a few structures, although there were a number of structures terminating with the H-epitope. To the best of our knowledge, this is the first report to characterize the glycosylation of BEC membrane proteins.

Remarkably, *N*-linked paucimannose structures were only present on the BEC membrane glycoproteins, with the most abundant *N*-glycan of the BEC membrane being a paucimannose structure of the composition $(Hex)_2(HexNAc)_2(Deoxyhexose)_1$. The lack of detection of any paucimannose structures in the saliva also confirms the effective separation of the saliva from the buccal cells. The possibility of these glycans originating from microorganisms adhering to BEC was ruled out, since some of the paucimannose structures were core fucosylated and were enzymatically released by PNGase F, indicating the presence of the mammalian α1-6 core fucose linkage. Degradation of the *N*-glycans giving rise to paucimannose structures as a sample preparation artifact was also ruled out, since the same preparation of membrane protein glycans from other cell types (Lee et al. 2011; Nakano et al. 2011) did not result in any paucimannose structures. The high turnover of BECs could potentially give rise to degraded *N*-glycans as paucimannosidic structures. These structures have rarely been reported in vertebrates except for quail mucoid (Natsuka 2005), human non-secretory ribonucleases (Lawrence et al. 1993) and recently in human embryonic stem cells (Satomaa et al. 2009) and are thought to be synthesized by an alternate glycosylation pathway (Natsuka 2005). The presence of the same paucimannose structures was also verified on membrane glycoproteins prepared from the BEC of 10 other individuals (Supplementary data, Figure S8).

The heterogeneous *O*-glycan profiles of both the salivary glycans and BEC membrane proteins illustrate the diversity of oligosaccharides usually associated with mucin-type glycosylation. The functions of these highly glycosylated, high molecular weight proteins are thought to involve the provision of binding sites for pathogens and leukocytes, as well as providing viscous and adhesive properties to saliva (Zalewska et al. 2000). The *O*-linked glycoproteins of saliva are mainly MUC5B and MUC7, agglutinin and IgA; Levine et al. 1987; Helmerhorst and Oppenheim 2007). The glycosylation on these proteins purified from saliva have been characterized extensively (Prakobphol et al. 1998; Karlsson and Thomsson 2009; Issa et al. 2010). MUC5B is glycosylated differently to the other major salivary mucin MUC7, containing longer and more diverse oligosaccharides (Thomsson et al. 2002, 2005) and is the largest carrier of blood group antigens in the saliva (Prakobphol et al. 1993; Thomsson et al. 2005). Characterization of the highly abundant glycans on MUC7 (Reddy et al. 1985; Prakobphol et al. 1998; Karlsson and Thomsson 2009) showed a novel branched I-antigen type structural epitope [GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-], with the

branch point usually initiated on core 1 and core 2 Gal residues, terminated by sialyl type 2 and sialyl Lewis x epitopes. A recent study by Issa et al. (2010) showed that DMBT ^{SAG} is hyperfucosylated with up to 11 fucoses on core 1 and core 2 structures with up to five *N*-acetyllactosamine units. Furthermore, they showed that DMBT^{SAG} carries siayl-Lewis x and Lewis b structures that are known to interact with *Helicobacter pylori*. The *O*-linked glycans of sIgA are complex and display many different glycan epitopes (Royle et al. 2003). The function of the glycosylation of sIgA has been thought to protect the protein from proteolytic activity and there is evidence to suggest that sIgA may participate both in adaptive immunity, via the protein components, as well as in innate immunity, via the glycans (Crottet and Corthesy 1998; Royle et al. 2003). Our data on the global *O*-glycan structural characterization of saliva are thus in agreement with the structures seen on these individual salivary glycoproteins reported in previous studies.

Other than the identification of two membrane associated mucins, MUC1 and MUC4 in BECs, very little is known about the *O*-linked glycans or their attached proteins in BEC (Offner and Troxler 2000). In our study, BEC membrane proteins had a visibly less complex and heterogeneous O-glycosylation profile than that of whole saliva. Some of the highly abundant structures in saliva, such as the blood group I structure, ABO(H) blood group antigens and oligosaccharides of core type 4 were present at a lower abundance on the glycans attached to BEC membrane proteins compared with salivary proteins. Saliva also possesses larger *O*-linked structures that are predominantly fucosylated and has a greater array of isomers for some compositions than BEC glycoproteins. The BEC membrane glycoprotein *O*-glycan profile in this study reveals that they reflect secretor status similarly to saliva, although the relative quantity of expressed H-epitopes is lower. The presence of blood group antigens on BEC has been previously reported by immunohistochemical methods (Navas et al. 1993; Dabelsteen 2002).

The adherence of the opportunistic pathogen *C. albicans* to host receptors is a critical first step in its infection process. The yeast expresses a family of eight known ALS (agglutinin-like sequence) glycoproteins that are involved in adhesion to mammalian cells (Lee et al. 2011; Zheng et al. 2011). The binding specificities of these adhesins are broad, as they bind to both mammalian glycoproteins and glycolipids (Seymour et al. 2006). Homology modeling and experimental studies of the N-terminal domains of the ALS proteins reveal that they belong to the Ig superfamily (Zheng et al. 2011). Deletion of one or more of the ALS genes affected *C. albicans* adhesion, growth and biofilm formation (Seymour et al. 2006). Recently, Donohue et al. (2011) have shown by surface plasmon resonance and microarray interaction studies that the N-terminal part of Als1 protein from *C. albicans* specifically binds fucose-containing glycans. The Als1 selectively bound to multiple ligands containing terminal fucose; the most intense interaction was with structures containing type 2 H-antigens, followed by fucose α1-3/4 linked to *N*-acetylglycosamine (Donohue et al. 2011). Interestingly, our in-depth glycan analysis of *N*- and *O*-glycans from saliva and BEC shows a high degree of fucosylation with glycan structures containing H- and Lewis-type terminal epitopes.

A reliable and highly reproducible flow cytometric cell adhesion assay was developed to provide information on the cell surface adhesion of *C. albicans* to the BEC host receptors. The fluorescently labeled yeast adhered to the BECs and the ability of saliva to inhibit this interaction (Hoffman and Haidaris 1993; Johansson et al. 2000) were confirmed and quantified by this assay. Importantly, the glycans released from the salivary proteins were able to inhibit *C. albicans* interaction with BEC on their own, thus demonstrating that the salivary glycans are recognized as binding epitopes by the yeast. The high similarity between the glycans of the soluble glycoproteins of saliva and the BEC membrane protein surface supports the idea that the glycans on salivary proteins could act as decoys to deter pathogens from binding to their target cells. Specifically, both the saliva and the host BEC glycans show common epitopes of the ABO(H) blood group, Lewis, sialylated and LacNAc antigens that are known to be binding sites for pathogens. For example, sialylated oligosaccharides are known to bind strongly to various oral bacteria (Prakobphol et al. 1999) and gut pathogens that enter via the mouth, such as *H. pylori*, have affinity to the multivalent Lewis y/b epitope, whereas *Campylobacter jejuni* is known to bind to blood group H-type structures (Ilver et al. 1998; Ruiz-Palacios et al. 2003; Aspholm et al. 2006). The detailed analysis of the specific oligosaccharide structures on salivary proteins, presented here, strongly supports the previously suggested notion that secreted glycoproteins, such as mucins, can act as decoys (Gagneux and Varki 1999; Perrier et al. 2006). Free human milk oligosaccharides have also been postulated to competitively inhibit gut pathogens and have structural homology similar to host cell receptors (Newburg et al. 2005). These observations open the possibility of using sugars as anti-adhesives to mimic and avoid the interaction of pathogens with the host glycan surface receptors.

The concept of carbohydrate-based anti-adhesive drugs has been around for three decades (Ofek et al. 1996). In an age of reduced efficacy of antibiotics and increased resistance of microbial pathogens, this approach provides an appealing alternative. It has the added benefit that the pathogen is unlikely to develop resistance to carbohydrate-based anti-adhesives, since a loss of binding to these carbohydrates would result in the loss of their binding to the carbohydrate receptors on host surfaces (Zopf and Roth 1996), a prerequisite to infection. There have been some clinical trials in humans to test the use of free oligosaccharides as such anti-adhesive drugs. One such trial involved the treatment of children with the NeuAc(α2-3)Galβ1-4GlcNAc glycan which failed to reduce the nasopharyngeal colonization with *Streptococcus pneumoniae* and *Haemophilus influenzae* over a 3-month period (Ukkonen et al. 2000). In another trial human patients treated with NeuAc(α2-3)Galβ1-4Glc failed to clear the gastric colonization of *H. pylori* (Parente et al. 2003). The failure of these trials suggests that the dose, or the use of a single oligosaccharide structure, may not have been sufficient to inhibit the initial binding stage of infection. The natural infective process involves a range of pathogen adhesins and host receptors such that a cocktail of oligosaccharides may be required to achieve

a therapeutic effect (Mulvey et al. 2001; Sharon 2006). Our study shows that such a mixture of oligosaccharides was sufficient to inhibit the binding of *C. albicans* to the host receptor cell.

Materials and methods

Collection of saliva and BECs

Unstimulated saliva (10 mL) was collected from a healthy donor in 2 mL of PBS supplemented with protease inhibitor cocktail (3 mg antipain–dihydrochloride, 0.5 mg aprotinin, 0.5 mg bestatin, 1 mg chymostatin, 3 mg E-64, 10 mg EDTA-Na_2, 0.5 mg leupeptin, 20 mg Pefabloc SC, 0.5 mg pepstatin and 3 mg phosphoramidon) one tablet per 100 mL of buffer (Roche Diagnostics, Mannheim, Germany) and 1% (v/v) Gibco antibiotic-antimycotic, containing 100 units/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B. The saliva sample was clarified by centrifugation at $3000 \times g$ at 4°C for 20 min. The supernatant containing saliva was further concentrated 10 times using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore, Bedford, MA) and stored at −80°C. Protein quantification of the stock saliva was performed by Bradford Assay (Sigma-Aldrich, MO). The pellet contains mainly BEC, oral microorganisms and some leukocytes (Xie et al. 2008). The BEC was enriched as described previously by Osswald et al. (2003); briefly, the cell pellet was resuspended in PBS and filtered with a 10 µm filter (Becton Dickinson), separating the larger BEC from the leukocytes and microorganisms.

Membrane preparation of BECs

The BEC fraction was further washed three times with PBS by centrifugation at $1000 \times g$ at 4°C for 20 min and cells counted with a haemocytometer. The pellet (2×10^8 cells) was resuspended in 2 mL of lysis buffer (pH 7.4) containing 50 mM Tris HCl, 0.1 M NaCl, 1 mM EDTA and protease inhibitor cocktail (Roche Diagnostics). The BEC membrane proteins were prepared as previously described by Lee et al. (2009). In brief, the cell pellet in lysis buffer was homogenized and the homogenate was centrifuged. The supernatant containing the cellular proteins was further diluted with Tris–HCl buffer and sedimented by ultracentrifugation. The membrane protein pellet was suspended in Tris HCl buffer containing 1% (v/v) Triton X-114 and the pellet homogenized completely. The samples were heated at 37°C and phase partitioned by centrifugation. The Triton X-114 detergent layer containing the membrane proteins were precipitated with 9 volumes of ice-cold acetone by incubating the sample overnight at −20°C. The precipitated membrane proteins were resolublilized in 8 M urea and protein quantification was performed by a Bradford Assay (Sigma-Aldrich, Sydney, Australia).

N- and O-linked glycan release for mass spectrometry analysis

N- and *O*-linked glycans were released from salivary and BEC membrane glycoproteins according to Wilson et al. (2002). Triplicate samples (10 µg) of proteins were immobilized by dot blotting on to a primed Immobilon-P PVDF membrane (Millipore). The *N*-linked glycans were released by incubation with 3 U of PNGase F (*Flavobacterium meningosepticum*, Roche Diagnostics) overnight at 37°C. The released *N*-linked glycans were incubated with 100 mM NH_4COOH, pH 5 (final concentration 15 mM), for 60 min at room temperature, and subsequently dried to completeness in a vacuum centrifuge. The samples were reduced with 20 µL of 1 M $NaBH_4$ in 50 mM KOH at 50°C for 3 h. The reduction was quenched with 1 µL glacial acetic acid and the *N*-linked glycans were desalted as described in the *O*-glycan desalting section.

O-Linked glycans were subsequently released from the same PVDF membrane spots by reductive β-elimination by incubating overnight with 20 µL of 0.5 M $NaBH_4$ in 50 mM KOH at 50°C. The reduction was quenched with 1 µL glacial acetic acid.

The released glycans were desalted using homemade cation exchange columns comprising 30 µL of AG50W-X8 cation-exchange resin (BioRad, Hercules) packed on top of µC18 ZipTips (Schulz et al. 2002). The residual borate from the glycan samples was removed by repeated addition of methanol (200 µL) and drying under vacuum. The glycans were resuspended in 10 µL of water and subjected to PGC-LC-ESI MS/MS separation and analysis.

Mass spectrometry

Both *N*- and *O*-glycan alditols were separated using a Hypercarb PGC (5 µm Hypercarb, 180 µm ID × 100 mm, Thermo Scientific, Waltham, MA) under the following gradient conditions. The separation for *N*-glycans was performed over an 85 min gradient of 0–45% CH_3CN in 10 mM NH_4HCO_3. *O*-Glycans were separated using a 45-min gradient of 0–90% CH_3CN in 10 mM NH_4HCO_3. The flow rate for both of the *N*- and *O*-glycans was set at 3 µL/min, using a HPLC system (Agilent 1100, Agilent Technologies, Palo Alto, CA) which was connected directly to an ESI source (Agilent 6330). The capillary voltage was set at 3 kV, and the dry gas maintained at 300°C. The MS spectra were obtained in the negative-ion mode. The scan range was between *m/z* 200 and *m/z* 2200.

Data analysis was carried out in ESI-Compass 1.3 (Bruker Daltonics, Bremen, Germany); the glycan peaks were semi-quantified using the extracted ion chromatogram peak area. Terminal epitopes from *N*- and *O*-glycans [Lewis x/a, Sialyl Lewis x/a, Lewis y/b, LacNAc, Sialyl LacNAc, blood group antigens (ABO(H)]), high mannose and sulfated terminals] were semi-quantified based on the peak area of epitope-carrying glycans.

N- and O-linked glycan release for inhibition assay

Glycans were released on a large scale from salivary glycoproteins in solution. Salivary proteins (1 ml) were reduced with 10 mM DTT for 45 min at 56°C and alkylated with 55 mM iodoacetamide in the dark for 30 min at room temperature. PNGase F (10 U/mg of protein, Roche Diagnostics) was added and incubated overnight at 37°C. The *N*-glycans released from the salivary glycoproteins were isolated by a 10 kD cutoff spin filter (Millipore). The flow through filtrate containing the *N*-glycans was desalted on a cation exchange column (200 µL) packed on the top of 1 mL Sep-pak C18 cartridges (Waters, Milford, MA) as described above. The retained filtrate

containing the proteins was dried and suspended in 100 µL of 0.5 M $NaBH_4$ in 50 mM KOH at 50°C overnight to release the *O*-glycans by β-elimination. The released *O*-glycans were desalted as above. The *N*- and *O*-glycans were further purified using graphitized carbon as a solid phase extraction cartridge as described by Packer et al. (1998). A fraction (2 µL) of these purified glycans was analyzed by MS and monosaccharide analysis to determine composition.

Monosaccharide analysis of released N- and O-linked glycans from salivary proteins

Monosaccharide analysis was performed to quantitate the released *N*- and *O*-glycans from saliva. The released glycans were hydrolyzed with 2 M TFA (100°C for 4 h, neutral sugars), 4 M HCl (100°C for 6 h, amino sugars) and 0.1 M TFA (80°C for 40 min, sialic acids). The monosaccharides were separated isocratically by HPAEC-PAD on a Dionex CarboPac PA10 column (Thermo Scientific, Waltham, MA, 2 × 250 mm) using 12 mM NaOH and quantified using an internal standard of 2-deoxyglucose.

Yeast isolate, culture conditions and fluorescent labeling

The *C. albicans* strain WM0.2.19 was a clinical isolate kindly provided by A/Prof. Wieland Meyer (Westmead Molecular Mycology Research Laboratory Collection, Westmead Hospital, Sydney, Australia). Yeast cells were grown on Sabouraud dextrose agar medium (Difco Laboratories, Detroit, MI) at 28°C for 36 h, resuspended in 2 mL of sterilized adhesion buffer (0.01 M PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) and washed twice by centrifugation (2500 × *g*, 5 min each) with the adhesion buffer. The yeast cells were fluorescently labeled with CellTrace™ CFSE Cell Proliferation Kit (Invitrogen Molecular Probe, Carlsbad, CA) according to the manufacture's protocol. CFSE passively diffuses into cells and is non-fluorescent until the acetate groups are cleaved by intracellular esterases. The succinimidyl ester group reacts with intracellular amines forming dye-protein fluorescent adducts (peak excitation wavelength of 492 nm and a peak emission wavelength of 517 nm) that are retained by the cells (Bronner-Fraser 1985) and not transferred to adjacent cells (Hodgkin et al. 1996). The yeast cells were resuspended in adhesion buffer to a density of 1–3 × 10^7 cells/mL. Microscopic examinations confirmed that the cells were fluorescently labeled, in yeast phase without germ tubes or pseudo-hyphae. For the adhesion assay, fresh BEC samples were gently scrapped from the oral cavity using flocked swabs (Microrheologics SRL, Brescia, Italy).

Flow cytometry

The BD FACSAria (BD Biosciences, San Jose, CA) flow cytometer was employed to statistically quantify the binding interaction of *C. albicans* to single BECs. The cells were excited with the argon laser emitting at a fixed wavelength of 488 nm. The FSC discriminates the particles at different sizes as the *x*-axis, whereas the SSC in the *y*-axis discriminates the different cellular granularity. The obvious difference in size and granularity allowed precise FSC and SSC gating to isolate the BEC populations from *C. albicans* populations. Data were recorded for 2000 BEC events using the CELLQuest software (BD Biosciences), each event signifying a BEC. The fluorescence intensities of gated BEC single-cell population were quantitatively detected at the FITC channel (band pass filter at 530/30 nm). The gated BEC population was plotted over a FITC histogram and the mean fluorescence intensity analyzed.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

ALS, agglutinin-like sequence; BEC, buccal epithelial cells; CFSE, carboxyfluorescein succinimidyl ester; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; FSC, forward scattering channel; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HPAEC-PAD, high-performance anion exchange chromatography with pulsed amperometric detection; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; LacNAc, *N*-acetyllactosamine; LC-ESI MS, liquid chromatography-electrospray ionization mass spectrometry; Man, mannose; MS/MS or (MS^2), tandem mass spectrometry; NeuAc, *N*-acetylneuraminic acid; PBS, phosphate-buffered saline; PGC, porous graphitized carbon; PVDF, polyvinylidene fluoride; SSC, side scattering channel; TFA, trifluoroacetic acid.

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