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Variant size- and glycoforms of the scavenger receptor cysteine-rich protein gp-340 with differential bacterial aggregation

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Abstract Glycoprotein gp-340 aggregates bacteria in saliva as part of innate defence at mucosal surfaces. We have detected size- and glycoforms of gp-340 between human saliva samples (n=7) and lung gp-340 from a proteinosis patient using antibodies and lectins in Western blots and ELISA measurements. Western blots of saliva samples, and of gp-340 purified, from the seven donors using a gp-340 specific antibody distinguished four gp-340 size variants, designated I to IV (n=2,2,2 and 1). While saliva gp-340 variants I to III had single bands of increasing sizes, variant IV and lung gp-340 had double bands. Purified I to IV proteins all revealed a N-terminal sequence TGGWIP upon Edman degradation. Moreover, purified gp-340 from the seven donors and lung gp-340 shared N-glycans, sialylated Galβ1-3GalNAc and (poly)lactosamine structures. However, the larger size gp-340 grouping II/III (n=4) and smaller size grouping I/IV correlated with a secretor, Se(+), and a non secretor, Se(−), dependent glycoform of gp-340, respectively (p=0.03). The Se(+) glycoforms contained ABH, Le^b, Le^y and polylactosamine structures, while the Se(−) glycoforms lacked ABH antigens but expressed Le^a, Le^x and lactosamine structures. By contrast, lung gp-340 completely lacked ABH, $Le^{a/b}$, $Le^{x/y}$ or sLe^x structures. Gp-340 and secretor typing of saliva from additional donors (n=29) showed gp-340 glycoforms I to IV for 6, 16, 4 and 0 donors, respectively, and 3 non-typeable donors, and verified that gp-340 glycoforms I and II/III correlate with Se(−) and Se(+) phenotypes, respectively (p<0.0001). The glycoforms of saliva and lung gp-340 mediated differential aggregation of Le^b- (*Helicobacter pylori*), sialylpolylactosamine- (*Streptococcus suis*) or sialic acid- (*Streptococcus mutans*) binding bacteria. In conclusion, variant size- and glycoforms of gp-340 are expressed by different individuals and may modulate the biological properties of gp-340 pertinent to health and disease.

Keywords Saliva · Gp-340 · DMBT1 · Secretor status · Glycoforms

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Introduction

Saliva contains a wide range of proteins that bind to bacteria or toxic molecules as part of innate defence at mucosal surfaces. While salivary acidic proline-rich polypeptides

(PRPs) neutralize dietary tannins [22], glycoproteins, such as secretory IgA (S-IgA) [40], mucins [36] or agglutinins [10, 37], aggregate micro-organisms in saliva. The agglutinin which aggregates *Streptococcus mutans* [10], implicated in dental caries, was recently identified as a homologue to the scavenger receptor cysteine rich protein gp-340 from lung tissues [37]. In saliva, agglutinin/gp-340 is an oligomeric complex with S-IgA [10, 24] and is, consequently, linked to both innate and immune defences.

Gp-340 and DMBT1 are spliced protein variants encoded by the *dmbt1* gene (deleted in malignant brain tumour, [14, 15, 33]. The *dmbt1* gene encodes orthologs in rabbit (hensin [43]), mouse (CRP-ductin, [29]) and rat (Ebnerine [23]). The gp-340/DMBT1 proteins contain 14 highly homologous scavenger receptor cysteine-rich (SRCR) domains, separated by 13 SIDs (SRCR interspersed domains), two CUB (Clr/Clq Uegf Bmp1) domains and a ZP (Zona Pellucida) domain [15]. The SRCR, SID, CUB and ZP domains participate in protein ligand binding, oligomerization and pattern recognition [2, 18, 20]. Besides aggregating micro-organisms, gp-340 activates PMN cells and macrophages [45] and binds to surfactant proteins D and A [25], lactoferrin [30], S-IgA [24], MUC5B [44] and to complement component C1q [5]. Gp-340/DMBT1 may thus represent pattern recognition receptors in a variety of host innate defences [20]. The DMBT1 protein is up-regulated in inflammation and thought to regulate differentiation to proliferation switching processes in gastrointestinal cells through interactions with the extra cellular matrix [19, 31]. Moreover, DMBT1 is a tumour surveillance or suppressor candidate molecule [31, 33]. Gp-340 and DMBT1 are expressed in the respiratory tract (trachea and lung), the alimentary tract (stomach, small intestine and salivary gland) and the brain [15].

Fluid phase gp-340, or derivatives thereof, aggregates a wide array of pathogenic (e.g. *S. mutans, Streptococcus suis, Streptococcus pyogenes* and *H. pylori*) and commensal (e.g. *Streptococcus gordonii* and *Streptococcus sanguinis*) bacteria [4, 17, 28, 37] or viruses [11]. Surface phase gp-340, on the other hand, mediates adhesion of commensal and cariogenic streptococci to hydroxyapatite surfaces [8, 28]. A microbial pattern recognition by gp-340 is suggested by (1) the differential recognition by fluid and surface phase gp-340 of different adhesion phenotypes in a given streptococcal species [28] and (2) the multiple receptor–adhesin pairs used by commensal *S. gordonii* to interact with gp-340, as opposed to the AgI/II (Pac or SpaP)-mediated gp-340 interaction for pathogenic *S. mutans* [17, 28]. Moreover, gp-340-mediated adhesion of *S. mutans* to salivary pellicles coincide with caries development and the PRP profile of saliva [42]. However, little is known about individual polymorphisms of salivary agglutinin/gp-340 as relates to bacterial aggregation or adhesion.

Protein polymorphism is a function of variation in both the protein core and carbohydrate portion of a glycoprotein. The SRCR-SID region of gp-340/DMBT1 is thought to define a multi allele system that together with SNP polymorphisms varies between healthy and cancer subjects [31, 32]. In human tear fluid two protein variants of DMBT1 were recently detected and found to differ somewhat in glycosylation [41]. Gp-340/DMBT1 exhibits a high density of potential O-glycosylation sites in the SIDs, and 14 potential N-glycosylation sites, particularly localized in the CUB and ZP domains [15, 33]. sLe^x receptors for selectins on PMN cells exist in salivary agglutinin/gp-340 [36, 37], and structural analyses have revealed sialyl-T, disialyl-T, sialylated *N*-acetyl-lactosamine, and sialyl Le^a structures in DMBT1 from human tear fluid [41]. The carbohydrates are mediators of many of the gp-340-mediated bacterial adhesion and aggregation reactions, such as sialic acid oligosaccharides for *S. gordonii*, *S. suis* or *Actinomyces odontolyticus* and Galβ1-3 structures for *Actinomyces naeslundii* [17, 28]. Moreover, gp-340 contain Le^b blood group antigens for binding of *H. pylori* [37]. The expression or lack of expression of ABH and Lewis blood group antigens on secreted molecules or mucosal cells is dependent on the Se(+) and Se(−) phenotypes, which are due to an active or inactive α(1-2) fucosyltransferase (*FUT2*) gene, respectively [12, 35]. While *H. pylori* [16], *Candida albicans* [3] and Norwalk virus [26] bind to Se(+) phenotypes, uropathogenic *Escherichia coli* [38] binds to Se(−) phenotypes. A relationship between secretor status and infection susceptibility has accordingly been a long-standing proposal [26]. However, little is known about iso- and glycoforms, or individual polymorphisms involving the secretor phenotype, as relates to agglutinin/gp-340 in saliva.

The aim of this study was to investigate the presence of variant size- and glycoforms of gp-340 among multiple individual saliva samples as compared to a lung tissue sample of gp-340. We found variant size- and glycoforms of saliva gp-340 with deviating aggregation of bacteria and that the saliva glycoforms of gp-340 are associated with the secretor phenotype of the donor.

Materials and methods

Materials All chemicals, unless otherwise stated, were obtained from Sigma. Antibodies to blood group ABH antigens, Le^x, α-chain or secretory component of S-IgA and peroxidase conjugated anti-mouse IgG and ortho-phenylenediamine (OPD) substrate were purchased from DAKO (Carpinteria, CA) and monoclonal antibodies to Le^a and Le^b from Immucor (Rodermark, Germany) or to Le^y from Signet Laboratories (Dedham, MA) or to sLe^x

from Seikagagku (Tokyo, Japan). Monoclonal antibody mAb143 was provided by D. Malamud (University of Pennsylvania). mAb213-1 was purchased from Antibodyshop (Gentofte, Denmark) and horseradish peroxidase-conjugated goat anti-mouse IgG from Nordic Biosite (Stockholm, Sweden). All biotinylated lectins, except for Tomato and MAA, were from Vector Laboratories (Burlingame, CA). Streptavidin conjugated peroxidase and chemiluminescence SuperSignal substrate were from Pierce (Rockford, IL).

Bacterial strains and culturing H. pylori strain 17-1 (CCUG17875), provided by T Borén (Umeå University), was cultured in 5% O_2 and 10% CO_2 on *Brucella* agar plates over night at 37°C [16]. *S. mutans* strains Ingbritt and LT11 [28] and *S. suis* KU5 [27], provided by J. Finne (Åbo University), were grown in Jordans broth at 37°C overnight as described [28]. *E. coli* MS 506 [46], provided by A Wold (Göteborg University), was cultured in LB broth containing 10 μg/ml tetracycline. *Lactococcus lactis* strains expressing Pac or SpaP were constructed and cultured as described [17].

Collection of saliva Parotid saliva was collected on ice as ductal secretions from various donors using Lashley cups and acid stimulation. The saliva samples were either used immediately or stored at −80°C prior to the experiments.

Purification of agglutinin/gp-340 from individual or pooled saliva samples Fresh samples of parotid saliva from the individual donors A to G (each 50–100 ml) or pooled from multiple donors (about 1 litre) were used for purification of gp-340 as previously described [37]. Briefly, equal volumes of a *S. mutans* Ingbritt suspension (5×10^9 cells/ml) and parotid saliva diluted 1:1 in 10 mM phosphate buffered saline (PBS, K_2HPO_4/KH_2PO_4, 150 mM NaCl), pH 6.8, were mixed and aggregated at 37°C for 60 min. After release of agglutinin/gp-340 by 50 mM EDTA from the pelleted aggregates, gp-340 was purified using gel filtration (Superdex 200 26/60: Pharmacia, Uppsala, Sweden). The gp-340-containing void volume was analysed for purity and amount by SDS-PAGE and densitometry of Coomassie blue-stained gels and by the DC Protein Assay (Bio-Rad laboratories, Hercules, CA).

Purification of gp-340 from lung lavage Lung gp-340 was purified from human brochioalveolar lavage from a patient (donor H) suffering from pulmonary alveolar proteinosis obtained by centrifugation of one litre of lavage was dissolved in buffer containing 10 mM EDTA, dialyzed and purified by sequential separation on a column of Mono-Q Fast Flow followed by a Resource Q column and finally by gel filtration on a Superose 6 prepgrade column. The purified gp-340 was analysed for purity by gel filtration and for protein concentration by adsorption at 280 nm.

N-terminal sequence analysis of gp-340 I to IV glycoforms The purified gp-340 I to IV saliva glycoforms were analyzed by Edman degradation using an Applied Biosystems Procise HT sequencer instrument.

Carbohydrate composition analysis The carbohydrate composition of gp-340 was determined by high pH anion-exchange chromatography on a Dionex HPLC system equipped with a pulsed amperometric detector (Dionex, Sunnyvale, CA) as described [21].

SDS-PAGE and molecular mass estimation Saliva and gp-340 samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on premade 5% or 4–15% polyacrylamid gels (Bio-Rad). The running buffer was 25 mM Tris, 192 mM glycin, 0.1% SDS, pH 8.3. Samples were reduced by heating at 100°C for 5 min in 5 mM DTT and sample buffer (62.5 mM Tris, 10.1% glycerol, 2% SDS, 0,01% pyronin). Unreduced samples were heated in sample buffer. Proteins were stained by Coomassie, glycan detection according to the manufacters instructions (Roche, Mannheim, Germany) or by immunoblotting.

The molecular masses of the saliva gp-340 variants I to IV were estimated from 5% SDS-PAGE gels immunoblotted with mAb143 using the Rainbow RPN800 molecular mass markers 250, 160, 105 and 75 kDa and the Gel-Pro Analyzer 3.1 software.

Immunoblotting with antibodies or lectins After SDS-PAGE, samples were transferred to an Immobilon-P transfer membrane, pore size 0.45 μm, (Millipore, Billerica, MA) using 65 mA/membrane for 60 min for detection with antibodies or lectins (Table 1).

The membranes intended for antibody detection were blocked with 5% non fat dried milk in TBS-T (50 mM Tris, 150 mM NaCl and 0.05 % Tween 20), pH 7.4, overnight at 4°C. The membranes were then incubated for 1 h with either mAb143 (1:100000), mAb213-1 (1:250), anti α-chain (1:2000) or anti secretory component (1:1000) in TBS-T with 5% non fat dried milk or with Le^a (1:200), Le^b (1:200) in 10 mM PBS-T ($Na_2HPO_4/NaH_2PO_4\times H_2O$, 150 mM NaCl, 0,05% Tween-20), pH 7.2, with 1% BSA. The membranes were washed four times in TBS-T, followed by 1 h incubation with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies in TBS-T containing 5% non fat dried milk, with the exeption for Le^x and Le^y where 10 mM PBS, pH 7.2, with 1% BSA was used. The membranes were

washed four times. Bands were detected using chemiluminescence SuperSignal substrate (Pierce, Rockford, IL).

The membranes intended for detection with a panel of lectins (selected based on low background binding) were blocked with 3% BSA in 10 mM PBS-T, pH 7.2, overnight at +4°C. The membranes were incubated with biotinylated lectins UEA, 1,0 μg/ml; RCA-1, 0.25 μg/ml; ACA, 0.025 μg/ml or ConA, 0.1 μg/ml in lectin buffer (10 mM PBS pH 7.2, 0.1 mM $CaCl_2$, and 0.01 mM $MgCl_2$). The membrane was washed four times in 10 mM PBS-T. Detection of bound lectins was done using streptavidin conjugated peroxidase, in 10 mM PBS, pH 7.2, with 1% BSA, followed by chemiluminescence SuperSignal Substrate (Pierce, Rockford, IL).

Table 1 Lectin and antibodies used in the present study

Sugars[a]	Lectin/antibody	Specificity[a]
Lectin		
N-linked glycans	PHA-E	Mannose-containing N-glycans
	PHA-L	Mannose-containing N-glycans
	PSA	Mannose-containing N-glycans with core fucose
	LCA	Mannose-containing N-glycans with core fucose
	ConA	Mannose-containing N-glycans
O-linked core 1	PNA	Galβ1-3GalNAc
	ACA	SiaGalβ1-3GalNAc
Gal/GalNAc	DBA	GalNAcα1-(3GalNAcβ−)
	GSL I	Gal(NAc)α−
	SBA	Gal(NAc)
	SJA	Galβ1-3/4GalNAcβ−
	RCA-1	Galβ1-4GlcNAc
GlcNAc	WGA	GalNAcα/GlcNAc/Sia
	sWGA	
Polylactosamine	Tomato	$(Galβ1\text{-}4GlcNAcβ1\text{-}3)_n$
Fucose	UEA-1	Fucα1-2Gal
Sialic acid	SNA	Siaα2-6
	MAA II	Siaα2-3
Antibody		
A	81FR2.2	GalNAcα1-3(Fucα1-2) Galβ1-3/4GlcNAc
B	E37	Galα1-3(Fucα1-2)Galβ1-3/ 4GlcNAc
H	92FR-A2	Fucα1-2Galβ1-3/4GlcNAc
Le^a	LM112/ 161	Galβ1-3(Fucα1-4)GlcNAc
Le^b	LM129/ 181	Fucα1-2Galβ1-3(Fucα1-4) GlcNAc
Le^x	C3D-1	Galβ1-4(Fucα1-3)GlcNAc
Le^y	F3	Fucα1-2Galβ1-4(Fucα1-3) GlcNAc
SLe^x	KM-93	Neu5Acα1-2Galβ1-4(Fucα1-3) GlcNAc

[a] Sugar or sequence recognized by the lectin or antibody.

Saliva gp-340 I to IV phenotyping Typing of saliva samples into the gp-340 I to IV phenotypes was done by separation of unreduced samples on 5% gels and immunoblotting with mAb143. Saliva from donors showing stable gp-340 I to III banding patterns served as typing references. The majority of gp-340 I to IV phenotypes, including all I and III phenotypes, was distinguished in a single electrophoresis analysis. Some phenotypes, in particular II, required one or two additional electrophoresis runs to be distinguished from III, and for three non typeable saliva samples the gp-340 banding patterns could not be classified into the variant I to IV proteins.

ELISA-like analysis Antibody and lectin mapping (Table 1) of gp-340 was done in an ELISA-like fashion. A volume (200 μl, 0.2 μg/ml) of purified gp-340 dissolved in 50 mM carbonate buffer (Na_2CO_3/$NaHCO_3$), pH 9.6, was coated on a 96-well Nunc-immuno™ microtiter plate with MaxiSorp™ surface (Roskilde, Denmark) and agitated overnight at 4°C. After six washes with 10 mM PBS-T, pH 7.2, unspecific binding sites were blocked with 0.5% BSA in PBS-T for 1 h at room temperature. After six washes of the plates with PBS-T, antibodies to antigens A (1:25), B (1:25), H (1:1000), Le^a (1:25), Le^b (1:50), Le^x (1:25), Le^y (1:50) or sLe^x (1:1000) or biotinylated lectins (2, 0.2 and 0,02 μg/ml) in lectin buffer were added. After incubation for 4 h at room temperature, the wells were washed six times with PBS-T and horseradish peroxidase-conjugated goat anti-mouse IgG or peroxidase conjugated streptavidin, both diluted 1:4000, were added. After 1 h incubation at room temperature, plates were washed six times with PBS-T and OPD dissolved in substrate buffer (0,05 M Citric acid, 0.10 M Na_2HPO_4), pH 5.0, were added. The reaction was arrested with 4.5 M H_2SO_4 and scored by measuring the absorbance at 490 nm:—marks an absorbance 0–0.5, +0.5–1.0, ++1.0–2.0, +++2.0–3.0 and ++++ an absorbance >3.0.

Secretor typing of parotid salivas Parotid salivas, diluted 1:10 in PBS, were secretor typed using the ELISA method and anti H- (cross-reacting with A and B antigens), Le^a- and Le^b- antibodies (Table 1). Se(+) and Se(−) salivas were defined as Le(b+) and ABH(+) or Le(a+) and ABH (−), respectively.

Aggregation of bacteria by gp-340 Aggregation of bacteria by gp-340 was measured as described [28, 37]. Briefly, washed bacterial cells were suspended in 10 mM PBS, pH 6.8, supplemented with 1 mM $CaCl_2$ to give an optical density at 700 nm [OD_{700}] of 1.0. Gp-340 was added at a final concentration of 1 μg/ml. Aggregation was recorded by measuring the OD_{700} at 1-min intervals over 90 min with a Beckman DU-50 series spectrophotometer. The extent of aggregation was expressed as a percentage after 60 min and

evaluated with the formula $[(t_0 \text{ at } A_{700} - t_{60} \text{ at } A_{700})/t_0 \text{ at } A_{700}] \times 100$.

Results

Different size-variants of gp-340 in saliva and lung secretions Western blots of parotid saliva from seven donors (A to G) probed with mAb143 (directed to the gp-340 protein core) revealed four different size-variants of gp-340, designated I to IV (Fig. 1a). I to III had single broad bands of about 345, 375 and 389 kDa masses, respectively, and variant IV a double band (345 and 287 kDa). The same banding patterns occurred for purified gp-340 from the same donors when stained for carbohydrates, although variants I to III stained stronger than IV (Fig 1b). The same banding patterns also occurred when the purified gp-340 proteins were stained with mAb143, except that the lower band of the variant IV double band was hard to detect (Fig. 1c).

Gp-340 was also purified from lung lavage of a proteinosis patient (donor H) and separated on 5% gels, Western blotted and reacted with mAb143 or stained for carbohydrates (Fig. 1b and c). Lung gp-340 behaved similarly to the saliva gp-340 variant IV in its double band character, migration and low carbohydrate content (Fig. 1b and c).

Identical N-terminal sequences for gp-340 variants I to IV Edman degradation of all gp-340 variants I to IV purified from saliva revealed similar data. For each, the N-terminal sequence TGGWIP was found which corresponds to a start at residue 20 of the amino acid sequence deduced from cDNA data [15].

Saliva gp-340 variants I to IV share core carbohydrates To compare the glycosylation of gp-340 from the different donors, the purified gp-340 proteins were coated on microtiter wells and probed with lectins and antibodies in an ELISA-assay (Tables 1 and 2). All gp-340 preparations from donors A to G carried sialylated Galβ1-3GalNAc (ACA), α2-6 and α2-3 -linked sialic acids (SNA and MAA, respectively) and N-glycans (e.g. PHA-E and ConA). Moreover, all gp-340 preparations carried type 2 lactosamine or polylactosamine structures (RCA-1 and Tomato lectin, respectively) and stained positive with ABH, $Le^{a/b}$, $Le^{x/y}$ or sLe^x specific antibodies, verifying the presence of fucosylated type 1 and 2 lactosamine or polylactosamine structures in saliva gp-340. The most abundant sugars were sialylated Galβ1-3GalNAc (ACA), lactosamine (RCA-1), mannose-containing N-glycans (PHA-E) and sialic acid (SNA), as only the ACA, RCA-1, PHA-E and SNA lectins stained gp-340 also at 10 to 100-fold lower lectin concentrations (data not shown). None of the gp-340 preparations except for donor B showed moderate staining with PNA specific for Galβ1-3GalNAc or with SJA specific for Galβ1-3/4GalNAc.

Saliva gp-340 size variants coincide with different glycoforms The saliva gp-340 variant groupings II/III and I/IV each displayed a particular glycoform (Table 2), related to the secretor status of the donor as determined by ABH and Le antigen typing of parotid salivas from the A to G donors [35]. Thus, the donors C/D and E/F with variant II and III, respectively, were all typed as Se(+) and their gp-340 carried the expected ABH and Le^b, whereas donors A, B and G with the smaller gp-340 I and IV proteins were Se (−) and their gp-340 did not express ABH and Le^b but Le^a instead (p=0.03) (Table 2). However, the gp-340 variant II from the Se(+) donor D carried ABH but not Le^b, Le^x or sLe^x. Moreover, the secretor glycoforms of gp-340 stained generally well with the fucose specific UEA-1 lectin, PHA-L and Le^y antibodies or with the polylactosamine specific Tomato or terminal GalNAcα-specific GSL1 and SBA lectins. By contrast, the non secretor gp-340 glycoforms stained generally less, if at all, with the same lectins, and the gp-340 variant I proteins stained comparably well with LCA specific to mannose and with Le^x antibodies.

The different glycosylations of the purified saliva gp-340 variant I to IV proteins from donors A to G were confirmed in Western blots with selected lectins (UEA-1 and RCA-1) or antibodies (anti Le^a and Le^b) (Fig. 2). Accordingly, while the Le^b antibody selectively bound gp-340 II and III proteins except for gp-340 from the Le(a-b-) donor D, the Le^a antibody primarily bound gp-340 variant I and IV proteins. Similarly, UEA-1 and RCA-1 detected the appropriate gp-340 variant, and some further banding heterogeneity in gp-340 (e.g. IIIE staining with RCA), but no other protein bands except for a weak staining by UEA-1 of a yet unidentified protein component. Thus, gp-340 is the primary target recognized by lectins or antibodies.

Prevalence of the gp-340 variant proteins I to IV and dependence on secretor status of the donor To estimate the prevalence of the gp-340 variants I to IV among parotid saliva donors, saliva samples from additional donors (n= 29) were classified as gp-340 I to IV phenotypes using Western blots with mAb143 (Fig. 1a). Twenty six of the donors could be classified and 6 had variant I, 16 variant II and 4 variant III. The remaining three donors had unique patterns not typeable as I, II, III or IV proteins. Some variant II and III phenotypes required repeated runs to be distinguished.

To verify the correlation between gp-340 variant I to III proteins and secretor status, we secretor typed the gp-340 I

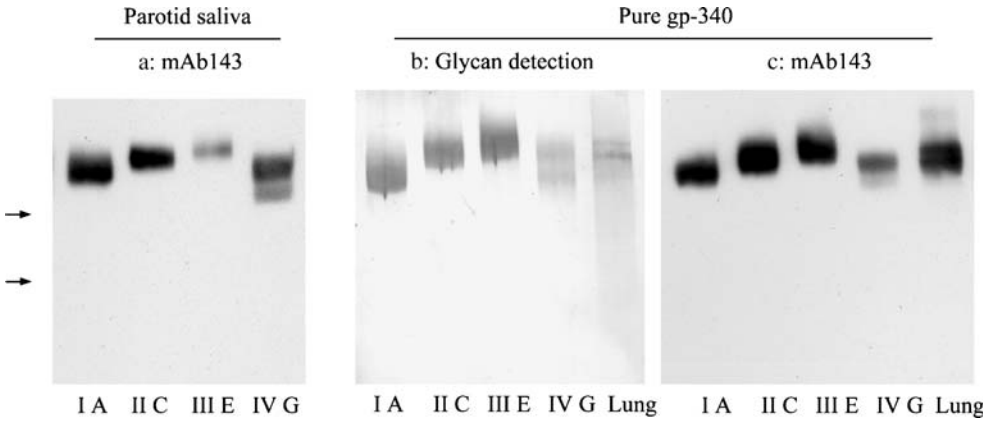

Fig. 1 Size-variants of gp-340 in saliva and lung secretions. Size variants of saliva gp-340, designated I to IV, between human donors (*A*, *C*, *E* and *G*) and banding pattern of lung gp-340 from a proteinosis patient (marked lung). Unreduced parotid saliva and purified gp-340 samples from the saliva and lung donors were separated by SDS-PAGE on 5% gels, Western blotted and stained with mAb143 or glycan detection. *Arrows* mark the positions of the 250 and 160 kDa molecular mass markers

Table 2 Binding of lectins and antibodies to saliva gp-340 variants I to IV and to lung gp-340

Sugars	Lectin[d] /antibody	Gp-340 variants[abc]							
		I		II		III		IV	Lung gp-340
		A (Se–)	B (Se–)	C (Se+)	D (Se+)	E (Se+)	F (Se+)	G (Se–)	
N- linked glycans	PHA-E	++++	++++	++++	++++	++++	++	++++	++++
	PHA-L	–	–	+++	+++	++	–	–	–
	PSA	+++	++++	++++	++	++++	+	++	++++
	LCA	+++	+++	++	++	++	–	+	++++
	ConA	++++	++++	++++	++++	++++	++++	++++	++++
O-linked core 1	PNA	–	++	–	–	–	–	–	–
	ACA	++++	++++	++++	++++	++++	++++	++++	++++
Gal/GalNAc	DBA	+	+++	++++	++	++++	++	+	+
	GSL I	++	++	++++	++++	++++	++++	++	–
	SBA	+	++	++++	++++	++++	–	+	–
	SJA	–	–	–	+	+	–	–	+
	RCA-1	++++	++++	++++	++++	++++	++++	++++	++++
GlcNAc	WGA	++++	++++	++++	++++	++++	++++	++++	++++
	sWGA	–	–	–	++	++	–	–	+
Polylactosamine	Tomato	++	++	++++	++++	+++	+	+++	+++
Fucose	UEA-1	–	–	++++	++++	++++	++++	–	–
Sialic acid	SNA	++++	++++	++++	++++	++++	++++	++++	++++
	MAA	++++	++	++++	+++	+++	++++	+	++++
A	81FR2.2	–	–	–	–	++	–	–	–
B	3E7	–	–	–	+	–	–	–	–
H	92FR-A2	–	–	++++	+	++++	+++	–	–
Le^a	LK112/161	+++	++	–	–	–	+	++++	–
Le^b	LM129/181	–	–	++++	–	++	++++	–	–
Le^x	C3D-1	++	+	–	–	–	+	–	–
Le^y	F3	–	–	++++	++	++++	++++	–	–
Sle^x	KM-93	++++	+++	+++	–	+++	+++	++	–

[a] – marks no binding, + weak, ++ moderate, +++ strong and ++++ very strong binding (see Materials and methods).
[b] Purified gp-340 variant I to IV proteins from parotid saliva of different donors, A to G.
[c] Secretor status, Se(+) or Se(−), as defined by the ABH and Lewis antigen profile of the donor saliva.
[d] Results from 2 μg/ml of lectin. At 0.2 μg/ml and 0.02 μg/ml of lectin, only RCA-1, ACA, SNA and PHA-E were capable of staining the gp-340 preparations.

to III saliva phenotypes (n=26) by ABH and Le antigen typing of their parotid saliva's. All gp-340 I saliva phenotypes (n=6) except for one Se(+) donor were classified as Se(−) subjects (5 out of 6), and all gp-340 II and III saliva phenotypes were classified as Se(+) subjects (20 out 20) (p<0.0001).

Saliva gp-340 I to III variants differ in aggregation of bacteria To identify differences in aggregation of bacteria by the gp-340 variant I to III proteins, pure gp-340 I to III proteins from donors B to E were tested for ability to aggregate a panel of bacteria (Fig. 3). While *S. suis* KU5 with specificity for sialylpolylactosamine was aggregated in the order of variant IICD > IIIE and IB, *H. pylori* 17-1 with specificity for Le^b was aggregated with the Le(b+) glycoforms IIC, IID and IIIE but not with Le(b−) glycoform IB. By contrast, *S. mutans* Ingbritt and *Lactococcus lactis* expressing Pac or SpaP (AgI/II) polypeptides from *S. mutans* were aggregated to a similar extent by the gp-340 I to III proteins. *E. coli* 506 with specificity for high mannose N-glycans was not aggregated by gp-340 I to III proteins (data not shown).

Glycosylation and bacteria-binding properties of lung gp-340 from a proteinosis patient We next investigated the glycosylation of lung gp-340 from a proteinosis patient by analysing its monosaccharide composition and lectin and antibody staining patterns (Tables 2 and 3). Lung gp-340 had a lower saccharide content than saliva gp-340 (variant II from donor D) and a high relative content of Man but lower Fuc/GlcNAc and Gal/GalNAc ratios (Table 3). Thus, the lung gp-340 preparation may differ in its relative content of mannose-containing N-glycans and short chain core 1 type or fucosylated (poly)lactosamine saccharides.

The presence of N-glycans in lung gp-340 was verified by its staining with mannose specific lectins (PHA-E, ConA, PSA and LCA), although lung gp-340 was more strongly stained by the PSA and LCA lectins than saliva gp-340 (Table 2). Moreover, both lung and saliva gp-340 carried sialylated Galβ1-3GalNAc (ACA), but not Galβ1-3GalNAc (PNA), verifying sialylated core 1 O-glycans in both preparations. Moreover, both lung and saliva gp-340 carried type 2 lactosamine or polylactosamine (RCA-1 and Tomato lectin, respectively) and α2-6 and α2-3 linked sialic acid (SNA and MAA, respectively) structures. By contrast, lung gp-340 differed from saliva gp-340 in not carrying terminal GalNAcα (GSL1 or SBA) or fucose (UEA-1) residues, and the absence of fucosylated lactosamine structures in lung gp-340 was verified by its lack of staining with ABH and Lewis blood group antigen specific antibodies. Accordingly, lung gp-340 was not stained by any antibody specific for ABH, $Le^{a/b}$, $Le^{x/y}$ or sLe^x antigens.

We next compared the ability of lung and saliva gp-340 (variant II from donor D) to aggregate strains of *S. mutans*, *S. suis* and *S. gordonii* with different receptor or gp-340 binding specificities [28] (Table 4). Lung and saliva gp-340

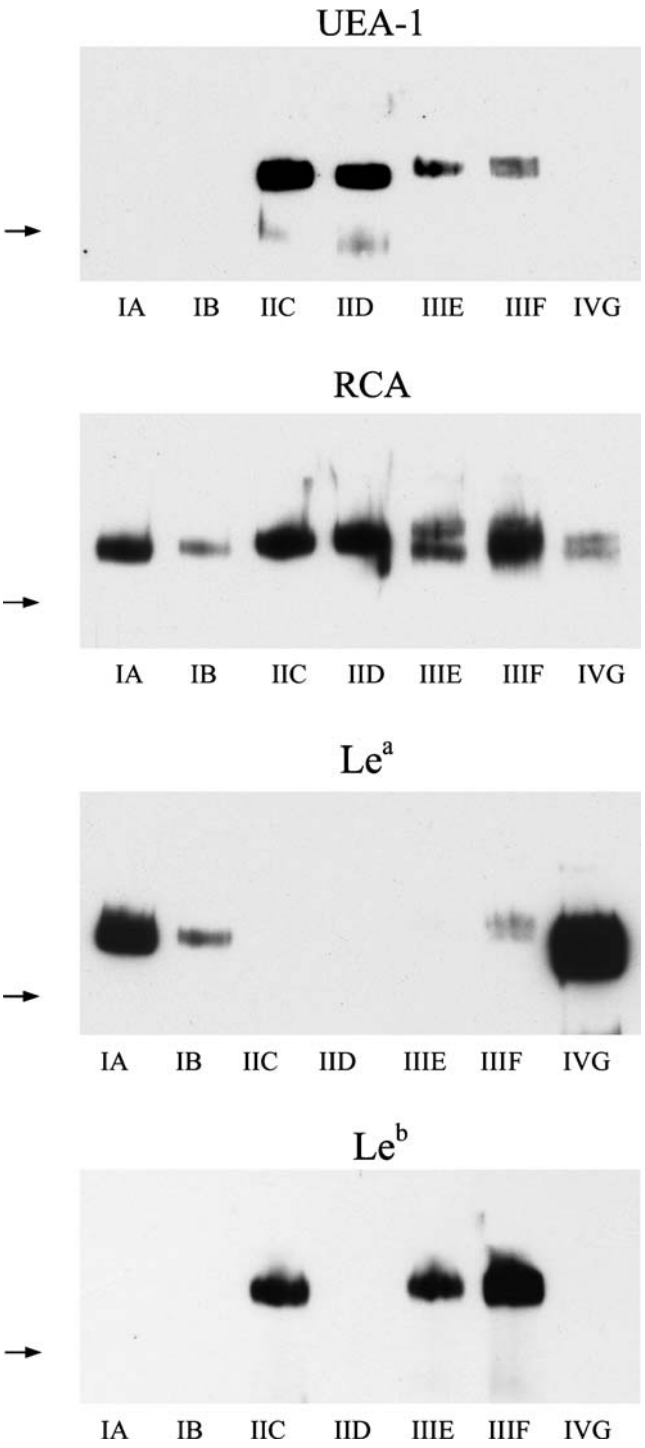

Fig. 2 Overlay of gp-340 purified from saliva donors A to G with lectins or antibodies. Unreduced samples were separated on 4–15% SDS-PAGE gels, Western blotted and reacted with lectins or antibodies. Variant I to IV proteins and donors are indicated at the bottom of each overlay. The *arrow* marks the 250 kDa molecular mass marker

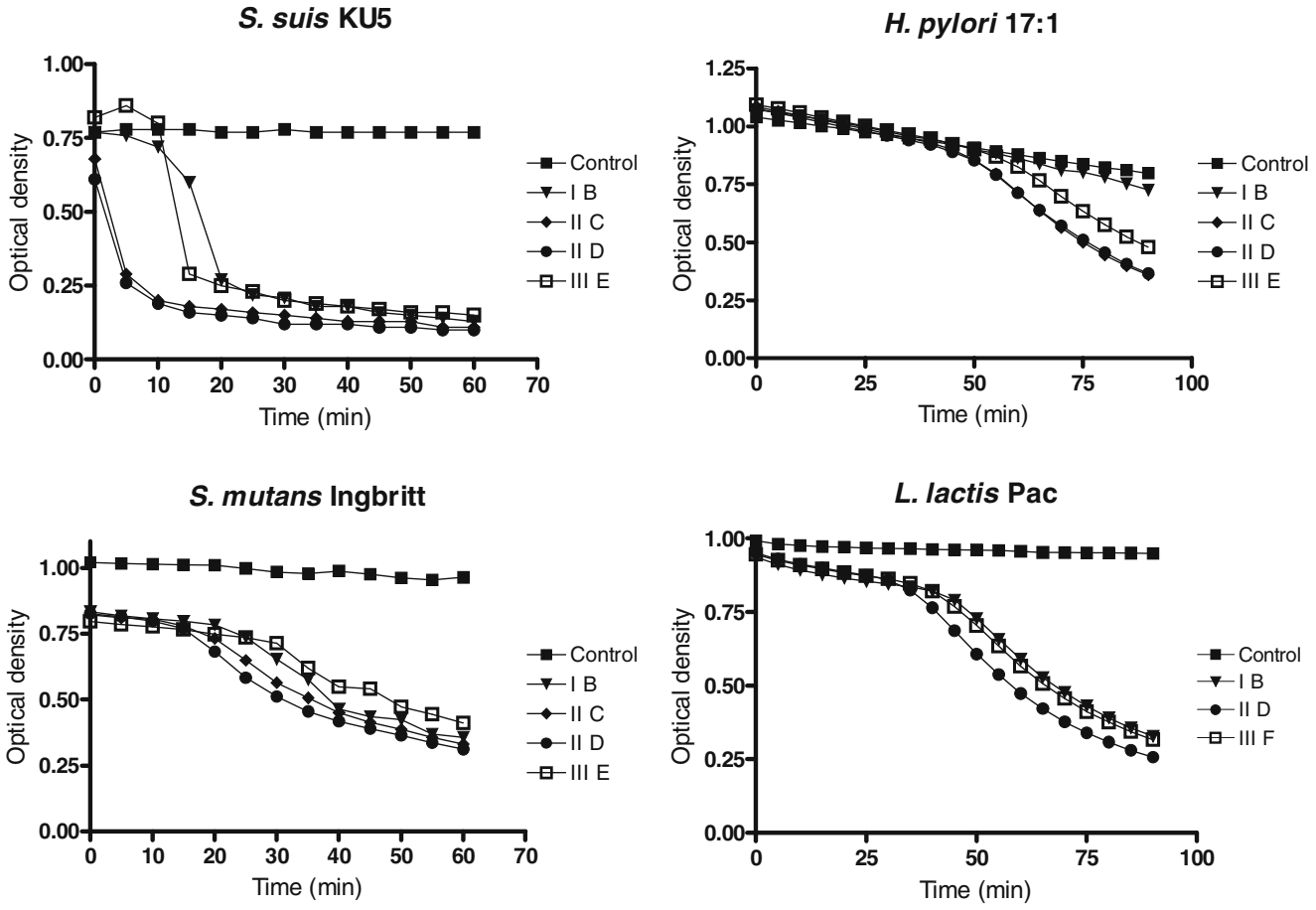

Fig. 3 Aggregation of bacteria by gp-340 variant I to III proteins from individual saliva donors. Aggregation of bacteria was done by mixing gp-340 (1 μg/ml) and bacterial cells (OD_{700} of 1.0). The individual saliva donors (*B*, *C*, *D*, *E*) are indicated. *L. Lactis* expressing Pac or SpaP generated similar results

proteins aggregated *S. mutans* Ingbritt with a sialidase-sensitive gp-340 interaction similarly, while only saliva gp-340 aggregated *S. mutans* LT11 with a sialidase-insensitive gp-340 interaction. Similarly, both saliva and lung gp-340 aggregated *S. suis* 836 with a Galα1-4Gal specificity, while only saliva gp-340 aggregated *S. suis* KU5 with a polylactosamine specificity. Lung and saliva gp-340 aggregated strains of *S. gordonii* belonging to three different gp-340 interaction modes similarly.

Both gp-340 monomer and S-IgA are glycosylated components present in the gp-340/agglutinin complex

Agglutinin/gp-340 is suggested to be an oligomeric complex of gp-340 and S-IgA in saliva [24]. To verify the presence of glycosylated gp-340 monomers and S-IgA components in the agglutinin/gp-340 complex, gp-340 purified from multiple saliva donors was analysed in Western blots using specific antibodies and lectins under non-reducing and reducing conditions (Fig. 4, Table 5). Both gp-340 monomer and S-IgA, but no other protein components, were detected in the agglutinin/gp-340 complex by the antibodies and lectins (Fig. 4). While some lectins (ACA, GSL1 and RCA-1) stained the gp-340 monomer under both conditions, other lectins (UEA-1, WGA and PSA) stained gp-340 under non-reducing conditions only. Moreover, the mannose specific PSA, PHA-E, LCA and ConA lectins stained the heavy chain and secretory component of S-IgA under reducing conditions.

Table 3 Sugar composition of gp-340 from saliva and lung lavage[a]

[a] Measured by high pH anion-exchange chromatography.
[b] Donor IIC.

Sample	Total saccharides (nmol)	Gal %	GlcNAc %	GalNAc %	Fuc %	Man %
Parotid gp-340[b]	132.9	28.8	30.2	7.1	30.0	3.9
Lung gp-340	6.9	55.1	13.0	21.7	0	10.1

Table 4 Aggregation of bacteria by lung gp-340

Receptor sugar	Bacteria	Aggregation[a]	
		Saliva gp-340[b]	Lung gp-340
Sialic acid	*S. mutans* IB[c]	56	51
Unknown	*S. mutans* LT11	51	7
sLe^x /3′SL[c]	*S. gordonii* DL1	37	40
Sialic acid	*S. gordonii* SK12	5	2
Sialic acid	*S. gordonii* M5	27	35
Sia2-3PL[c]	*S. suis* KU5	60	3
Galα1-4Gal	*S. suis* 836	81	77

[a] Numbers represent the percentage of aggregating cells, measured by decrease in OD_{700}, of the total number of added bacterial cells.
[b] Saliva gp-340 variant II from subject D.
[c] 3′SL=Sialylα2-3lactose, Sia2-3PL=sialylα2-3polylactosamine, IB=Ingbritt.

These findings verifies that the saliva agglutinin/gp-340 complex contains both glycosylated gp-340 monomer and S-IgA components.

Discussion

The present results suggest the presence of different size- and glycoforms of gp-340 among individual saliva's, and that the saliva glycoform for groupings II/III and I correlate with the secretor and non secretor type of glycosylation, respectively. Moreover, the size- and glycoforms of gp-340 aggregated certain bacterial ligands differently. These findings, taken together with the various

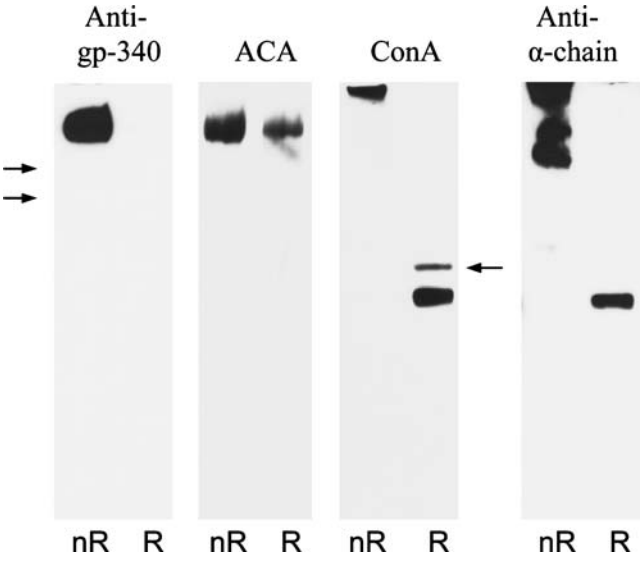

Fig. 4 Overlay of gp-340 purified from multiple saliva donors with S-IgA or gp-340 specific reagents under non reducing (*nR*) or reducing (*R*) conditions. Samples were separated on 4–15% SDS-PAGE gels, Western blotted and reacted with ACA, ConA lectins, anti gp-340 (mAb213-1) or anti α–chain S-IgA antibodies, as indicated. The *arrow* marks the position of the secretory component of S-IgA

Table 5 Overlay of agglutinin/gp-340 with lectins

Lectin/Antibody	Treatment	Binding to[a]		
		Gp-340	sc[b]	α-chain[b]
ACA	Unreduced	+	–	–
	reduced	+	–	–
GSL I	unreduced	+	–	–
	reduced	+	–	–
RCA-1	unreduced	+	–	–
	reduced	+	–	–
UEA-1	unreduced	+	–	–
	reduced	–	–	–
WGA	unreduced	+	–	–
	reduced	–	–	–
PSA	Unreduced	+	–	–
	reduced	–	+	+
PHA-E	unreduced	–	–	–
	reduced	–	+	+
LCA	unreduced	–	–	–
	reduced	–	+	+
ConA	unreduced	–	–	–
	reduced	–	+	+

[a] – marks no binding and + distinct binding to purified gp-340.
[b] Secretory component, sc, and α-chain of S-IgA.

gp-340/DMBT1 innate properties and long standing proposal for a role of secretor status in infection susceptibility, suggest a potential role for gp-340/DMBT1 polymorphisms not only in cancer but also in susceptibility toward infectious diseases.

The gp-340 glycoform groupings II/III and I of multiple subjects (donors A to F and the additional 26 donors) correlated with secretor Se(+) and a non secretor Se(−) status, respectively. Accordingly, the proportion of gp-340 I versus II/III among the 26 donors (23%/77%) coincides with the prevalence of Se(−)/Se(+) subjects (20%/80%) with an inactive and active FUT2 fucosyltransferase, respectively [26]. The gp-340 IV double band observed in saliva from donor G, on the other hand, was difficult to verify with purified gp-340 protein or among multiple saliva donors. Although the predominant gp-340 I to III size variants displayed the same N-terminal sequences, and coincided with Se dependent glycoforms, further studies are required to exclude the involvement also of genetic and other protein polymorphisms. The double bands of saliva variant IV and lung gp-340 may result from splice variation or post-translational proteolysis. Moreover, whether other tissues expressing gp-340 and FUT2 or other glycosyltransferases also harbour subject specific size- and glycoforms of gp-340 remains unknown. Similar to previously typed salivary proteins from Se(+) and Se(−) subjects [35], the Se(+) glycoform of gp-340 was ABH, Le(b+) and Le(y+) and the Se(−) glycoform ABH negative but Le(a+) and Le(x+). Moreover, the Se(+), as opposed to the Se(−),

glycoforms of gp-340 generally were rich in polylactosamine, N-glycans recognized by PHA-L and GalNAcα structures. Although this could suggest extensive differences in FUT2-related glycosylation patterns between gp-340 groupings I and II/III, we do not know the carbohydrate and/or protein polymorphisms generating the gp-340 size variants II and III. Nevertheless, the selective detection of the Se(+) dependent gp-340 glycoform by PHA-L is interesting in view of its T lymphocyte mitogenic activity [6], and that the SRCR protein WC1/T19 shows a differential utilization of SRCR domains on different T cell populations [34]. It has previously been shown that the secretor dependent ABH and Lewis antigens are selectively expressed on salivary mucin MG1 [35] and, as presently shown, on salivary gp-340. Gp-340 and MG1 may accordingly constitute the primary ABH and Lewis antigen-carrying molecules in saliva. Hypothetically, the ABH and Lewis antigens could be selectively expressed on particular SRCR/SID domains or other domains of gp-340.

Salivary gp-340 interacts with micro-organisms in a pattern recognition-like fashion, and the present results show a differential aggregation of bacteria by the size- or glycoforms of gp-340. The Se(+) gp-340 glycoforms II and III with Le^b and polylactosamine structures, but not the Se(−) glycoform I, aggregated *H. pylori* and *S. suis* with Le^b and polylactosamine specificity, respectively. By contrast, the variant I, II and III proteins aggregated *S. mutans* and lactococci expressing AgI/II adhesins similarly, suggesting a shared gp-340 carbohydrate or peptide receptor motif. There are further arguments for a potential role of size- and glycoforms of gp-340 in individual host defences and disease susceptibility. First, since gp-340 and DMBT1 interacts with bacterial and epithelial cells and with immune or innate effector cells or molecules, size- or glycoforms of gp-340/DMBT1 could influence the host defence system at multiple levels. Second, the Se(+) or Se(−) glycosylation modes are known to correlate with infection susceptibility as proposed for gastritis by *H. pylori* [16], candidosis by *C. albicans* [3], urinary tract infections by *E. coli* [38] and for viral infections [26]. Actually, Se(−) subjects homozygously recessive for the α(1-2)fucosyltransferase gene (*FUT2*) are resistant to Norwalk virus infections as well as lack binding of the virus by saliva [26]. The mechanisms for an involvement of secretor status in infection susceptibility may besides aggregation or adhesion of microbes reside in deviating behaviours of innate molecules such as gp-340. Third, dental caries coincides with secretor status and caries prone subjects are non secretor phenotypes [1, 13]. Caries prone subjects differ in saliva mediated adhesion of *S. mutans* to hydroxyapatite surfaces, a phenomenon involving agglutinin/gp-340, and, consequently, susceptibility to caries may involve gp-340 polymorphisms and misbehaviours at the pellicle adhesion or other innate defence levels. In future studies we will evaluate such potential roles of gp-340 size- or glycoforms in biofilm formation and susceptibility to infectious diseases.

The present study suggests still further aspects about agglutinin/gp-340 and its glycosylation. First, while saliva gp-340 contains fucosylated blood group antigens and the sialyl Le^x leukocyte homing receptor, lung gp-340 from a proteinosis patient was totally devoid of ABH and Lewis related antigens. At present, however, we do not know if this absence of ABH and Lewis antigens in lung gp-340 relates to a tissue specific glycosylation or splice variation or to the proteinosis disease of the donor. Second, both the gp-340 monomer and S-IgA are glycosylated components present in the agglutinin/gp-340 complex from saliva, as Western blotting with antibodies and lectins primarily detected gp-340 monomer or S-IgA components. At present, however, we can not fully exclude the presence of additional minor glycoproteins in the native agglutinin/gp-340 complex, but suggest that both gp-340 and S-IgA contribute to its mosaic of carbohydrates. Third, some carbohydrate and S-IgA epitopes or components, however, may be cryptic in the gp-340/agglutinin complex. Such a cryptic nature of PNA binding sites may be suggested from our previous notion of binding of PNA to salivary S-IgA in Western blots [7] but present lack of binding of PNA to most gp-340/agglutinin preparations in ELISA. Similarly, the inability of gp-340/agglutinin to aggregate mannose- and S-IgA-binding *E. coli* may reflect epitope masking, or particular requirements of the mannose specific adhesin. Inter- or intramolecular interactions or foldings may mask S-IgA or carbohydrate epitopes in gp-340/agglutinin, although various ligand or surface interactions of gp-340/agglutinin may unmask and functionally activate the same components [39]. Such a cryptic and dynamic behaviour of carbohydrates have been reported for both S-IgA [39] and for agglutinin/gp-340 [28], which exposes different carbohydrate receptors in fluid as opposed to surface phase [28]. Finally, even though the DMBT1/gp-340 protein has been expressed as a recombinant glycoprotein [9], the present findings with S-IgA and size and glycoforms of gp-340 stresses the importance of also studying the native protein or protein complex in resolving the structure and biology of gp-340/DMBT1.

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