Salivary Agglutinin, Which Binds Streptococcus mutans and Helicobacter pylori, Is the Lung Scavenger Receptor Cysteine-Rich Protein GP-340*

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This paper is an equal contribution of two research groups with two senior authors (SJF and NS) and three first authors (AP, FX and VMH).
SUMMARY

Salivary agglutinin is a high-molecular-mass component of human saliva that binds Streptococcus mutans, an oral bacterium implicated in dental caries. To study its protein sequence, we isolated the agglutinin from human parotid saliva. After trypsin digestion, a portion was analyzed by matrix assisted laser/desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), which gave the molecular mass of 14 unique peptides. The remainder of the digest was subjected to high performance liquid chromatography, and the separated peptides were analyzed by MALDI-TOF/postsource decay; the spectra gave the sequences of 5 peptides. The molecular mass and peptide sequence information showed that salivary agglutinin peptides were identical to sequences in lung (lavage) gp-340, a member of the scavenger receptor cysteine-rich protein family. Immunoblotting with antibodies that specifically recognized either lung gp-340 or the agglutinin confirmed that the salivary agglutinin was gp-340. Immunoblotting with an antibody specific to the sialyl Le\(^\varepsilon\) carbohydrate epitope detected expression on the salivary, but not the lung glycoprotein, possible evidence of different glycoforms. The salivary agglutinin also interacted with Helicobacter pylori, implicated in gastritis and peptic ulcer disease, S agalactiae, implicated in neonatal meningitis, and several oral commensal streptococci. These results identify the salivary agglutinin as gp-340 and suggest it binds bacteria that are important determinants of either the oral ecology or systemic diseases.
INTRODUCTION

Human saliva has several critical functions, including lubrication (1-3), digestion (4), formation of a bioactive semipermeable barrier (pellicle) that coats oral surfaces (5-8), and regulation of the composition of the oral flora. Saliva fulfills the latter function by virtue of its antimicrobial activity (9,10) and by promoting selective microbial clearance or adherence (11-14). The diverse functions attributed to saliva are allocated among its many components, which include amylases, cystatins, proline-rich proteins, proline-rich glycoproteins, carbonic anhydrases, peroxidases, statherins, histatins, lactoferrin, lysozyme, sIgA, mucins and salivary agglutinin. Protein sequences have now been deduced for all the major salivary components except the agglutinin (15-18).

Salivary agglutinin was identified as a protein fraction that mediates specific adhesion and aggregation of *Streptococcus mutans* (19-22). Monoclonal antibodies (mAb^1^) to agglutinin block adherence of *S. mutans* to experimental pellicles and aggregation of the bacterial cells by parotid saliva (23,24). Several studies have related the levels of agglutinin in saliva to the numbers of *S. mutans* in dental plaque (19,25), the rate of plaque formation (26), and the susceptibility to dental caries (27). Other studies have not found these associations (28,29).
In spite of a potentially important role of agglutinin in regulating the composition of the oral flora, very little is known about the chemical nature of the molecule. When isolated from parotid saliva, it behaves as a $5 \times 10^6$ Da oligomeric complex which contains a major 440 kDa glycoprotein (20). Immunoblotting experiments show that this protein is recognized by the same mAb that blocks *S. mutans* adherence and its parotid saliva-induced aggregation *in vitro* (20,23).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) shows that the complex contains other proteins, including secretory IgA, and minor, as yet unidentified, components (20,21,30). In the absence of additional structural information about the salivary agglutinin, we isolated the glycoprotein and analyzed its peptide portion by using mass spectrometry techniques. The results showed identity with gp-340 discovered in airway secretions by virtue of its affinity for surfactant protein D (31,32). We also obtained additional interesting information about its glycosylation and bacterial binding properties.
EXPERIMENTAL PROCEDURES

Materials—All chemicals, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose membranes (0.45 µm) were obtained from Schleicher & Schuell (Keene, NH). Polyvinylidene difluoride membranes (Immobilon-P, 0.45 µm) were obtained from Millipore (Bedford, MA). Human lung gp-340 was purified from a lavage pellet as described (33). Rabbit polyclonal antibodies that recognized a synthetic peptide of gp-340 (anti-gp-340-1) and a purified human lung gp-340 (anti-gp-340-3), were prepared as described (31-33). A mAb that recognized human purified lung gp-340 [anti-gp-340-2 (Hyb 213-6)] was prepared as described (31,32). We used two mAbs that specifically reacted with salivary agglutinin: mAb 143 and mAb 303 (34). The latter two antibodies were from Dr. Daniel Malamud (University of Pennsylvania, Philadelphia, PA). The anti-sLe^a mAb was prepared by culturing the hybridoma cell line CSLEX-1, obtained from the American Type Culture Collection (Rockville, MD), in RPMI 1640 medium, supplemented with 15% fetal bovine serum. The culture supernatant containing the desired mouse IgM was collected after 48 h. Horseradish peroxidase-conjugated goat anti-rabbit IgG (heavy and light chains) was purchased from Bio-Rad Laboratories (Hercules, CA) and DAKO (Glostrup, Denmark). Goat anti-mouse IgG (heavy and light chains) and goat anti-mouse IgM, both conjugated to horseradish peroxidase, were purchased from Jackson Immuno Research Laboratories Inc. (West Grove, PA). High purity trypsin was from Promega (Madison, WI). Fast-stain® (Coomassie blue) was from Zoion
Biotech (Shrewsbury, MA). Brucella agar and yeast extract were from Difco Laboratories (Detroit, MI). IsoVitalex, trypticase soy broth and Columbia-II-agar were from Becton Dickinson Microbiology Systems (Cockeysville, MD). Chemiluminescence detection reagents (SuperSignal® Substrate) were purchased from Pierce (Rockford, IL).

Collection of Saliva—Human parotid and submandibular/sublingual (SM/SL) salivas were collected as the ductal secretions as previously described (11,35). The samples were either used immediately or mixed with an equal volume of loading buffer and stored at -20°C before the experiment.

SDS-PAGE—Parotid and SM/SL saliva samples, purified salivary agglutinin preparations and purified lung gp-340 were electrophoretically separated on polyacrylamide gels (36). Three types of gels were used. One had a 3% stacking gel and 10% running gel and the others were gradient gels, either 4-15% or 10-20% (Bio-Rad, Hercules, CA). Protein bands were visualized by staining with either Fast-stain® or Coomassie brilliant blue.

Biochemical Purification of Salivary Agglutinin—Four hundred µl of fresh parotid saliva from one donor was concentrated to 25 µl by using a Centricon-Plus 20 filter (Millipore, Marlborough, MA), then mixed with an equal volume of loading buffer. The concentrated sample was electrophoretically separated; the stacking gel contained 3% acrylamide and the
running gel contained 10% acrylamide. After electrophoresis, the protein bands were visualized with Fast-stain®. Under these conditions the salivary agglutinin was well separated from other proteins. This allowed excision of the $M_r \sim 350$ kDa band.

**Affinity Purification of Salivary Agglutinin**—Agglutinin was purified from fresh parotid or SM/SL saliva using published methods (20,30). Briefly, *S. mutans* strain Ingbritt was maintained as described below. The cultures were harvested in exponential phase, washed once with phosphate-buffered saline (PBS; 10 mM $K_2HPO_4/KH_2PO_4$, 0.15 M NaCl), pH 6.8, and suspended at a concentration of $5 \times 10^9$ cells/ml. Equal volumes (100 ml) of bacterial suspension and parotid or SM/SL saliva (diluted 1:1 in PBS, pH 6.8) were mixed and incubated at 37°C for 60 min. The aggregated bacteria were pelleted by centrifugation (15,000 x g) for 30 min at 4°C. After the supernatant was discarded, agglutinin was released by adding 6 ml of 20 mM EDTA in PBS, pH 6.8, at room temperature. Then the sample was centrifuged (30,000 x g) for 30 min at 4°C. The EDTA extraction and centrifugation steps were repeated once. The two supernatants were pooled and subjected to gel filtration on a Superdex 200 26/60 column (Pharmacia, Uppsala, Sweden) which was eluted with 10 mM PBS, pH 6.8. The agglutinin-containing void volume (40 ml) was concentrated to a final volume of 600 µl by using Centriprep-10 centrifugal concentrators (Amicon, Beverly, MA). The protein concentration was estimated by using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA) with bovine albumin as a standard. The void volume fraction was also analyzed by gradient SDS-PAGE (4-15% acrylamide).
Mass Spectrometry of Peptides Isolated from the Biochemically Purified Agglutinin—The excised agglutinin band was macerated in a solution of high purity trypsin (0.05 µg/µl of 25 mM ammonium bicarbonate). The proteolytic digestion was allowed to continue at 37°C for 16 h. The resulting peptides were eluted from the gel with a solution of 50% acetonitrile and 5% trifluoroacetic acid (TFA) in distilled water and concentrated in a SpeedVac. First, the eluate was analyzed by matrix assisted laser/desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Portions of unseparated tryptic digests were cocrystallized in a matrix of α-cyano-4-hydroxycinnamic acid and analyzed by using a PerSeptive Biosystems DE-STR MALDI-TOF mass spectrometer equipped with delayed extraction operated in the reflector mode.

Alternatively, peptide samples were subjected to high performance liquid chromatography (HPLC) separation prior to mass spectrometry. The apparatus was fitted with a Michrom Bioresources MagicMS C18 column (0.2 x 50 mm; 5 µm particle size; 200 Å pore size) which was equilibrated with 7% acetonitrile/0.1% TFA in H₂O. A flow rate of 1 µl/min was established by using an Eldex Micropro pump. Peptides were eluted isocratically for 10 min followed by a linear gradient (0.875%/min) to a final mobile phase composition of 63% acetonitrile/0.082% TFA in H₂O. One to 2 µl HPLC fractions were spotted directly onto a
MALDI target with 1.5 µl of α-cyano-4-hydroxycinnamic acid. Postsource decay (PSD) sequencing was done as previously described (37). MS/MS sequencing was by quadrapole orthogonal time-of-flight mass spectrometry (Q-TOFMS; Micromass, Manchester, UK).

The peptide mass and peptide fragment-ion data were used, with the MS-Fit and MS-Tag programs, which are available on the World Wide Web (http://prospector.ucsf.edu), to search databases to determine peptide identity.

Mass Spectrometry of Peptides Isolated from the Affinity-purified Agglutinin —The analyses were performed essentially as previously described (38). First, the sample was subjected to SDS-PAGE on a 10-20% gradient gel (Bio-Rad, Hercules, CA) and stained with Coomassie brilliant blue. A single band was visible near the origin of the gel. This band was excised and digested with trypsin. The peptides were analyzed by MALDI-TOF mass spectrometry on a TofSpec E instrument (Micromass, Manchester, UK) with delayed extraction and in reflectron mode, using a matrix of α-cyano-4-hydroxycinnamic acid. For MS/MS, the sample was analyzed on a Q-TOF instrument (Micromass, Manchester, UK) and the m/z 1459 ion selected for fragmentation analysis. The results were used to search sequence databases using the MS-Fit and MS-Tag programs as described above.
Immunoblotting—Following SDS-PAGE, samples were transferred to nitrocellulose or Immobilon-P membranes as previously described (39). Briefly, non-specific binding was blocked by incubating blots for 1 h in PBS containing 0.05% Tween-20 (T-PBS) and 5% Carnation non-fat dried milk (T-blotto). The blot was then incubated for 2 h with one of five antibodies that specifically recognized either gp-340 or salivary agglutinin: Anti-gp-340-1 was raised against a synthetic peptide that corresponds to a region of gp-340 (33). Anti-gp-340-2 and anti-gp-340-3 were raised against a gp-340 preparation purified from lung (31,32). mAbs 143 and 303 were raised against a salivary agglutinin preparation purified from saliva. An earlier study showed that mAb 143 recognizes the agglutinin protein core, whereas mAb 303 recognizes the Le$^\alpha$ epitope in the context of the agglutinin protein core (34). All the antibodies were diluted in T-blotto (v:v) as indicated: anti-gp-340-1, 1:1,000; anti-gp 340-2, 1:10,000; anti-gp-340–3, 1:2,000; mAb143, 1:60,000; and mAb303, 1:60,000. The blots were washed three times in T-PBS, 5 min each. Binding of primary polyclonal antibodies was detected after incubation for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:2,000 (v:v) in T-blotto. Binding of mAbs was detected after incubation for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:2,000 (v:v) in T-blotto. After washing, bands were detected either by staining the blots with 3,3’-aminobenzidine (6 mg in 100 ml PBS containing 0.03% hydrogen peroxide) or chemiluminescence detection reagents (SuperSignal®Substrate). Proteins that carried the sLe$^\alpha$ determinant were identified by staining nitrocellulose transfers with mouse monoclonal anti-sLe$^\alpha$ IgM (diluted 1:5, v:v in T-blotto) essentially as described above. The
horseradish peroxidase-conjugated, isotype-specific secondary antibody used for detection of the IgM (source) was diluted 1:2,000 (v:v) in T-blotto.

*Bacteria and Culture Conditions*—The origin of *Streptococcus mutans* strain Ingbritt was as previously described (24). All other streptococcal strains (see Table II) were from Dr. Mogens Kilian (Aarhus University, Denmark). The streptococcal cells were grown as previously described (24). The cultures were harvested at exponential phase, washed two times in PBS and suspended at a concentration of either $2 \times 10^9$ or $5 \times 10^9$ cells/ml in PBS, pH 6.8. *Helicobacter pylori* strain CCUG17875 was grown as previously described (40). Briefly, the cells were maintained and grown on Brucella agar supplemented with 10% bovine blood, 1% IsoVitalex, amphotericin B (4 mg/L), vancomycin (10 mg/L) and trimetropin (5 mg/L). After incubation for 48 h at 37°C in microaerophilic conditions (10% CO$_2$, 5% O$_2$, 85% N$_2$), the cells were harvested, washed two times in PBS and suspended at a concentration of $2 \times 10^9$ cells/ml in PBS.

*Aggregation of Bacterial Cells by Purified Agglutinin*—The ability of affinity-purified parotid agglutinin to agglutinate a panel of streptococci (Table II) and *H. pylori* strain CCUG17875 was assessed as described earlier (20). Briefly, 1 ml of purified agglutinin (~ 2 µg/ml) in PBS, pH 6.8, was mixed with 1 ml of bacteria suspended at a concentration of $5 \times 10^9$ cells/ml in PBS, pH 6.8. Aggregation was recorded by measuring, at 1-min intervals for 1 h, the OD$_{700}$ using a Beckman$^\text{®}$ DU-50 Series spectrophotometer (Beckman, Stockholm, Sweden).
Binding of Agglutinin to Bacterial Cells—4 ml of either an *H. pylori* strain CCUG17875 or an *S. mutans* Ingbrit cell suspension (2 x 10⁹ cells/ml PBS) was incubated with an equal volume of fresh parotid saliva diluted 1:1 in PBS (experimental) or PBS alone (control). Binding took place at room temperature for 1 h. The cells were then pelleted by centrifugation, washed twice with PBS and suspended in 150 µl of SDS-PAGE loading buffer. After 30 min, the cells were pelleted. An aliquot of the supernatant (~ 25 µl) was subjected to SDS-PAGE and immunoblotting with anti-gp-340-1 as described above.
RESULTS

Isolation of Agglutinin for Mass Spectrometry Analyses—The salivary agglutinin was isolated by two methods. The first was biochemical. Whole parotid saliva was electrophoretically separated on a SDS-polyacrylamide gel and the band corresponding to the molecular mass of the salivary agglutinin was excised (Fig. 1A, arrow head). The second method exploited the known biological properties of the molecule. Specifically, the glycoprotein was purified by selective adsorption from whole parotid saliva to S. mutans bacteria, followed by release of bound proteins with EDTA-containing buffer, and then fractionation of the released proteins on a Superdex 200 column. SDS-PAGE of the void volume fraction showed only a single band of ~350 kDa under non-reducing conditions (Fig. 1B, lane 1, arrow head), which had a slightly lower molecular mass under reducing conditions (Fig. 1B, lane 2). Finally, we also purified agglutinin from SM/SL saliva by selective adsorption to S. mutans cells. SDS-PAGE of the SM/SL agglutinin showed a single band with the same electrophoretic properties as the parotid agglutinin under both non-reducing (Fig. 1B, lane 3) and reducing conditions (Fig. 1B, lane 4).

Analysis of the Peptide Portion of the Salivary Agglutinin by Mass Spectrometry—Initially, both agglutinin preparations were subjected to in-gel trypsin digestion. An aliquot of each unseparated digest was analyzed by MALDI-TOF MS, which yielded a peptide mass fingerprint. A total of 15 peptides and 14 unique sequences were obtained from the biochemically purified
sample (Table I). The sequence of 5 of these peptides was determined by MALDI-TOF/PSD (bold italic typeface in Table I). As an example, the MALDI-TOF spectrum of one of the digests is shown in Fig. 2A. A PSD spectrum and fragmentation of the peptide $m/z$ 1459.8 is shown in Fig. 2B, together with the interpretation and deduced amino acid sequence. The peptide mass fingerprint and PSD fragment-ions (Table I) were used for database searching. The results showed that the peptides were identical to regions found within both gp-340 and its splice variant DMBT1, both members of the scavenger receptor cysteine-rich protein family.

The tryptic digest of the affinity-purified agglutinin sample was also analyzed by MALDI-TOF MS. The results showed a dominant peptide of $m/z$ 1459.8. MS/MS analyses of this peak showed that the sequence was identical to that of the peptide of the same mass that was analyzed from the biochemically purified sample. Other ions corresponding to the peptides marked with an asterisk in Table I were also identified, as well as two additional ions at $m/z$ 1082.2 and 2739 that correspond to peptide regions that lie toward the C-terminus of both gp-340 and DMBT1. Together, these results show that the samples isolated by the two different methods are very likely to be the same protein.

*Immunoblot Analyses of Salivary Agglutinin with Antibodies Specific for either gp-340, Salivary Agglutinin, or the sLe$^\alpha$ Epitope*—Next, we determined whether antibodies raised against gp-340 cross-react with the salivary agglutinin. Fig. 3A shows immunoblot analyses (anti-gp-340-1) of
whole parotid saliva samples from 6 donors, as well as purified gp-340 isolated from a human lung lavage pellet as a control. The sample of lung gp-340 reacted with the antibody (lane 1). Immunoreactive bands of ~350 kDa were also observed in the parotid saliva samples obtained from all 6 donors (lanes 2-7). Additional immunoblot experiments were performed with anti-gp-340-2. The latter antibody recognized single bands of ~350 kDa in samples of whole parotid saliva (Fig. 3B, lane 1) and affinity-purified agglutinin (Fig. 3B, lane 2) only under non-reducing conditions, as did anti-gp-340-3. Bands of the same estimated molecular mass in both samples also reacted with mAb 143, which was raised against the agglutinin protein core (data not shown). Together, the data from the immunoblot experiments suggested that the salivary agglutinin and gp-340 contained the same immunogenic regions.

During the course of the immunoblot analyses we noted that mAb 303, which recognizes the Le$^\alpha$ epitope in the context of the agglutinin protein core (34), reacted with a single band in parotid saliva and in the affinity-purified agglutinin sample (data not shown). This result suggested the presence of specialized oligosaccharide structures. To obtain additional information about glycosylation of this molecule we surveyed expression of the sLe$^\alpha$ epitope, which is carried by the low-molecular-weight salivary mucin and which interacts with L-selectin (42,43). Interestingly, immunoblotting showed that the salivary agglutinin reacted with anti-sLe$^\alpha$, whereas gp-340 purified from lung did not (Fig. 3C). This result suggested the possibility of different gp-340 glycoforms due to differences in the genotypes of the donors.
Salivary Agglutinin Interacts with a Variety of Bacteria—Next, we analyzed interactions between salivary agglutinin and a panel of commensal and pathogenic bacteria (Table II). The agglutinin mediated strong aggregation of *S. mutans* (3 out of 3 strains), implicated in dental caries, and *S. agalactiae* (2 out of 2 strains), implicated in neonatal meningitis, but not *S. pyogenes* (2 out of 2 strains), implicated in tonsillitis and invasive infections. Furthermore, while agglutinin mediated strong aggregation of some strains of *S. oralis, S. mitis* and *S. intermedius*, several other strains of the same bacteria, as well as other streptococci that colonize dental and oral mucosal surfaces (see Table II), were not subject to aggregation.

We were also interested in whether the agglutinin interacts with other medically important bacteria that are likely to pass through, rather than permanently colonize, the oral cavity. We were particularly interested in *H. pylori*, an organism implicated in gastritis and peptic ulcer disease (44,45). Results of these experiments showed that the salivary agglutinin also mediated aggregation of *H. pylori* to nearly the same extent as whole parotid saliva (Table II).

We used a second method to confirm this potentially important observation. *S. mutans* and *H. pylori* were incubated with parotid saliva and bound agglutinin was detected by immunoblotting with anti-gp-340-1 as described in Experimental Procedures. The results are shown in Fig. 4. As expected, parotid saliva (*lane 1*) showed an ~350 kDa immunoreactive band. Components
of the extract of control *S. mutans* cells that were incubated in PBS failed to react with the antibody (Fig. 4, lane 2), but cells incubated in parotid saliva showed an immunoreactive band of the anticipated molecular mass (Fig. 4, lane 3). When *H. pylori* cells were incubated with parotid saliva or PBS, an antibody reactive band of ~350 kDa was detected only in the extract prepared from cells that were incubated with parotid saliva (Fig. 4, compare lanes 4 and 5).
Here we report that salivary agglutinin is very likely gp-340. The primary structure of gp-340, recently established by molecular cloning, shows a polypeptide chain of 2413 amino acids (32). The N-terminus consists of a signal peptide and a sequence of 69 unique amino acids. Residues 95 to 1741 contain highly repetitive regions consisting of 13 scavenger receptor cysteine-rich (SRCR) domains. These domains are separated by SID (SRCR interspersed domain) sequences, of which there are 12. The interval between residues 1742 and 2134 contains a Ser-Thr-Pro-rich region and an additional SRCR domain flanked by two CUB (C1r/C1s Uegf Bmp1) domains, protein modules initially found in complement subcomponents C1r/C1s, Uegf, and bone morphogenetic protein-1. The remaining residues contain a hydrophobic zona pellucida domain.

The 14 unique agglutinin peptides we detected by mass spectrometry were scattered throughout the entire protein and occurred within all the major domains (see Table I). The identification of salivary agglutinin as the gp-340 protein was also suggested by the results of immunoblotting experiments. The ~350 kDa protein we isolated from parotid saliva, by using either biochemical or affinity methods, reacted with antibodies raised against salivary agglutinin (34) and antibodies raised against lung gp-340 (31-33). Moreover, the size of the salivary molecule was the same as that of gp-340 isolated from lung lavage, evidence that the agglutinin is the full-length molecule rather than the splice variant DMBT1, which lacks 628 amino acids consisting of 5 paired SRCR
and SID domains (32,46). However, we have not ruled out the possibility that the salivary molecule is a differentially glycosylated form of DMBT1. Agglutinin in parotid saliva obtained from all 6 individuals of diverse ethnic backgrounds reacted specifically with anti-gp-340 (Fig. 3A), suggesting that expression is probably widespread in the population. However, there could be genetic variability in the expression and/or splicing of this molecule, an issue we have yet to address. In addition, differences among individuals in glycosylation seem highly likely, since the agglutinin reacted with anti-sLe\(^\alpha\), an oligosaccharide epitope whose expression depends on genotype. Functional heterogeneity exists as well. For example, previous studies demonstrated differences among subjects with regard to agglutinin binding of *S. mutans* (21,47) and that these differences coincide with susceptibility and resistance to dental caries (47). Finally, the presence of this glycoprotein in saliva is consistent with reverse transcription-polymerase chain reaction analyses that show the main sites of gp-340 expression are lung, trachea, salivary glands, small intestine and stomach (32).

Knowing the structure of salivary agglutinin will greatly facilitate experiments to understand its various biological roles. One important general function likely arises from the glycoprotein’s ability to interact with bacteria. The agglutinin-mediated aggregation of *S. mutans*, implicated in dental caries, and *S. agalactiae*, implicated in neonatal meningitis, suggests a role in microbial clearance of potentially pathogenic microorganisms. Some strains of *S. oralis*, *S. gordonii* and *S. mitis* were also aggregated by the agglutinin, suggesting that this glycoprotein interacts with
commensal organisms as well. In either case, the outcome of agglutinin binding may depend on
the microbial ligand and its method of interaction, as well as whether the interaction occurs in
solution, which probably favors clearance, or on oral surfaces where adherence becomes
possible. In this regard, it is interesting to note that the agglutinin-binding adhesin AgI/II of S.
mutans possesses different domains that are involved in either agglutinin-mediated adhesion to
hydroxyapatite surfaces or aggregation in solution (48). Finally, since gp-340 is an opsonin
receptor for surfactant proteins A and D (32), future studies should investigate whether this
interaction influences the bacteria-binding properties of the salivary molecule.

Dissecting the interactions between salivary agglutinin and various bacteria at a molecular level
offers an interesting opportunity to restore, in the case of relevant disease states, the normal
balance found in healthy individuals. With regard to H. pylori, the results from our study, which
show an affinity of this bacterium for the salivary agglutinin, support the hypothesis of oral
transmission which could include transient adherence in the mouth (49). It is interesting that
salivary mucins also interact with this bacterium (Prakobphol, Borén and Fisher; unpublished
observations). The latter observation is in accord with two previous studies. First, we showed
that salivary mucins carried the fucosylated blood group antigens \textit{(i.e.} the ABO and Lewis type)
(39). Second, the Le\textsuperscript{b} and H-1 histo-blood group antigens mediate adherence of H. pylori to
human gastric mucosa (41). Eradication of H. pylori infection with antibiotic treatment has
proved to be difficult in patients who harbor H. pylori in the oral cavity/dental plaque (50). The
results of this study suggest new, pharmacological strategies for inhibiting infections by this organism.

With regard to interactions with cells of the host immune system, we found that the salivary agglutinin can carry the sLe\(^x\) epitope. This suggests that this molecule, like the low-molecular-weight salivary mucin (43), could tether both bacteria and leukocytes, a potentially important consideration for immune interactions in the oral cavity. Also with regard to immune function, it is interesting to consider data that show gp-340 from lung stimulates random migration (chemokinesis) of alveolar macrophages (33). This activity could also enhance bacterial interactions with cellular components of the host immune system. Finally, studies by Nagashunmugam et al. (51) suggest that salivary agglutinin has HIV-neutralizing properties. Thus, the agglutinin could be a component of the molecular system that prevents oral transmission of the virus. Understanding the relationship between the structure of the salivary agglutinin and the many interesting functions it may carry out is the focus of our future experiments.
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Abbreviation and Textual Footnote

The abbreviations used are: mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SM/SL, submandibular/sublingual; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; MALDI-TOF MS, matrix assisted laser/desorption ionization time-of-flight mass spectrometry; HPLC, high performance liquid chromatography; PSD, postsource decay; Q-TOFMS, quadrapole orthogonal time-of-flight mass spectrometry; SSCR, scavenger receptor cysteine-rich; SID, SRCP interspersed domain
FIGURE LEGENDS

Fig. 1. **SDS-PAGE of a parotid saliva sample containing the salivary agglutinin, and purified agglutinin samples isolated from parotid and SM/SL saliva by adsorption to S. mutans.** A, a sample of whole parotid saliva was separated on a 10% polyacrylamide gel as described in “Experimental Procedures.” The band of 350 kDa, denoted by the arrow head, was excised and subjected to an in-gel trypsin digest, prior to analysis by mass spectrometry (see Fig. 2 and Table I). B, salivary agglutinin (single band denoted with an arrow head) was also isolated from saliva by adsorption to S. mutans. SDS-PAGE (4-15% gradient gel) of the glycoproteins that were purified from parotid (lanes 1 and 2) and SM/SL saliva (lanes 3 and 4). Under both non-reducing (B, lanes 1 and 3) and reducing conditions (B, lanes 2 and 4) the agglutinin appeared as a single band, with a slightly lower molecular mass under reducing conditions. Proteins were visualized by staining with either Fast-stain® (A) or Coomassie brilliant blue (B). The top and bottom of the stacking gels are marked with arrows.

Fig. 2. **The trypsin digest of the biochemically purified salivary agglutinin included a peptide with a molecular mass and sequence identical to an area within the cysteine-rich region of the scavenger receptor gp-340.** A, a band corresponding to the estimated molecular mass of the salivary agglutinin was excised from an SDS-PAGE gel (see Fig. 1 A) and digested with trypsin. MALDI-TOF MS analysis of the digest revealed a peptide that gave a particularly...
strong signal at \( m/z \) 1459.8. B, this peptide was sequenced by PSD. The fragment ions were used to search databases, which allowed assignment of the amino acid sequence shown above the spectrum. This sequence is identical to a region found within the scavenger receptor, cysteine-rich region of gp-340. Two classes of ions are labeled in the spectrum, immonium and related ions which are found in the low mass region and b- and y-ions as indicated. The \( b_{6-18} \) and \( b_{8-18} \) ions were observed due to loss of \( \text{H}_2\text{O} \). C, peptides also present in a negative control, a portion of the polyacrylamide gel that did not stain for protein; T, trypsin autoproteolysis peptides which were used to internally calibrate the spectrum.

Fig. 3. Immunoblotting showed that the salivary agglutinin reacted with anti-gp-340 and anti-sLe\(^x\). Immunoblotting was carried out as described in “Experimental Procedures.” The transfers shown in A and C contained the same samples: gp-340 purified from lung lavage (lane 1) and parotid saliva samples from six individuals (lanes 2-7). The transfer shown in B contained a sample of parotid saliva (lane 1) and the agglutinin glycoprotein purified from parotid saliva by adsorption to \( S. \text{mutans} \) (lane 2). A, polyclonal anti-gp340-1 reacted with both lung lavage gp-340 and bands of \( \sim 350 \) kDa in all the parotid saliva samples. B, monoclonal antibody anti-gp-340-3 (Hyb 213-6) reacted with a band of \( \sim 350 \) kDa in parotid saliva and with the affinity-purified agglutinin from parotid saliva. C, anti-sLe\(^x\) did not react with lung gp-340 (lane 1), whereas all the samples of whole parotid saliva contained a single high molecular mass band that stained (lanes 2-7). The top and bottom of the stacking gels are marked with arrows.
Fig. 4. **Salivary agglutinin binds to H. pylori.** The bacterial cells were incubated with fresh parotid saliva and washed several times with PBS. Bound salivary components were eluted, separated by SDS-PAGE, transferred to nitrocellulose and visualized by immunoblotting with anti-gp-340-1. The transfer contained the following samples: lane 1, parotid saliva; lane 2, extract from control *S. mutans* cells that were incubated with PBS; lane 3, extract from experimental *S. mutans* cells that were incubated with parotid saliva; lane 4, extract from control *H. pylori* cells that were incubated with PBS; lane 5, extract from experimental *H. pylori* cells that were incubated with parotid saliva. Anti-gp 340-1 stained only the agglutinin in parotid saliva (lane 1) and a band of the same estimated molecular mass in extracts prepared from bacterial cells that were incubated with parotid saliva (lanes 3 and 5). No antibody-reactive bands were seen in the control cell extracts (lanes 2 and 4). Arrows indicate the top and the bottom of the stacking gel.
TABLE I

Summary of MS analyses of tryptic peptides from salivary agglutinin.

The biochemically purified salivary agglutinin was subjected to in-gel trypsin digestion and an aliquot of the unseparated digest was analyzed by MALDI-TOF MS, which gave the molecular mass of 15 peptides. The remainder of the digest was subjected to high performance liquid chromatography, and the separated peptides were analyzed by MALDI-TOF/PSD; the spectra showed the sequences of 5 peptides (bold italicized typeface).

<table>
<thead>
<tr>
<th>Peptide sequence</th>
<th>Start-end</th>
<th>Monoisotopic mass ± 0.05 Da</th>
<th>gp-340 domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEVLYR</td>
<td>247-252, 376-381, 507-512, 615-620, 746-751, 875-880, 1135-1140, 1264-1269, 1393-1398, 1653-1658</td>
<td>778.4</td>
<td>SRCR2, SRCR3, SDSR4, SRCR5, SRCR6, SDCR7, SRCR9, SRCR10, SRCR11, SRCR13</td>
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<tr>
<td>VEILYR</td>
<td>115-120</td>
<td>792.5</td>
<td>SRCR1</td>
</tr>
<tr>
<td>MTIHFR</td>
<td>1855-1860</td>
<td>804.4</td>
<td>CUB1</td>
</tr>
<tr>
<td>FPSVYLKR</td>
<td>2353-2359</td>
<td>881.5</td>
<td>ZP</td>
</tr>
<tr>
<td>AFHFLNR</td>
<td>2346-2352</td>
<td>904.5</td>
<td>ZP</td>
</tr>
<tr>
<td>GRVEVLYR</td>
<td>245-252, 1651-1658</td>
<td>991.6</td>
<td>SRCR2, SRCR13</td>
</tr>
<tr>
<td>FISDHSITR</td>
<td>2099-2107</td>
<td>1075.6</td>
<td>CUB2</td>
</tr>
<tr>
<td>qIFTSSYNR*</td>
<td>1846-1854</td>
<td>1098.5</td>
<td>CUB1</td>
</tr>
<tr>
<td>QIFTSSYNR*</td>
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<td>1115.5</td>
<td>CUB1</td>
</tr>
<tr>
<td>GSFTSSSNFMSIR</td>
<td>2086-2098</td>
<td>1420.7</td>
<td>CUB2</td>
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<tr>
<td>DDTYGPYSSPSLR</td>
<td>2326-2338</td>
<td>1457.7</td>
<td>ZP</td>
</tr>
<tr>
<td><strong>FGQGSGPIVLDDVR</strong></td>
<td>418-431, 549-562, 657-670, 788-801, 917-930, 1048-1061, 1177-1190, 1306-1319, 1435-1448, 1695-1708</td>
<td>1459.8</td>
<td>SRCR3, SDSR4, SRCR5, SRCR6, SDCR7, SRCR8, SRCR9, SRCR10, SRCR11, SRCR13</td>
</tr>
<tr>
<td>QPGCGWAMSAPGNAR</td>
<td>1033-1047</td>
<td>1559.7</td>
<td>SRCR8</td>
</tr>
<tr>
<td><strong>SAPGNAQFGQGSGPIVLDDVR</strong></td>
<td>282-302, 1557-1577</td>
<td>2085.0</td>
<td>SRCR2, SRCR12</td>
</tr>
<tr>
<td><strong>SAPGNAWFQGQGSGPIALDDVR</strong></td>
<td>150-170</td>
<td>2115.0</td>
<td>SRCR1</td>
</tr>
</tbody>
</table>

* Position of the amino acid residue in the deduced peptide sequence of gp-340.

* q, pyro-glutamic acid.

* Peptide masses that were also detected in the affinity-purified agglutinin sample.
TABLE II

Aggregation of streptococci and *H. pylori* by purified salivary agglutinin.

The affinity-purified salivary agglutinin sample was incubated with a bacterial cell suspension and aggregation was recorded by measuring, at 1-min intervals for 1 h, the OD$_{700}$ using a spectrophotometer.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Aggregation</th>
<th>Origin and characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em></td>
<td>SK765</td>
<td>2+</td>
<td>dental plaque, caries-associated</td>
</tr>
<tr>
<td></td>
<td>SK773, SK771</td>
<td>3+, 4+</td>
<td></td>
</tr>
<tr>
<td><em>S. mitis</em> biovar 1</td>
<td>SK601, SK611, SK614</td>
<td>5+</td>
<td>dental plaque, potentially cariogenic</td>
</tr>
<tr>
<td></td>
<td>SK155, SK595, SK616, SK674</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK138, SK597</td>
<td>3+, 4+</td>
<td></td>
</tr>
<tr>
<td><em>S. mitis</em> biovar 2</td>
<td>SK34</td>
<td>1+</td>
<td>dental plaque potentially cariogenic</td>
</tr>
<tr>
<td><em>S. mitis</em></td>
<td>SK282</td>
<td>-</td>
<td>dental plaque</td>
</tr>
<tr>
<td></td>
<td>SK322</td>
<td>2+</td>
<td></td>
</tr>
<tr>
<td><em>S. oralis</em></td>
<td>SK23</td>
<td>-</td>
<td>dental plaque</td>
</tr>
<tr>
<td></td>
<td>SK105, SK262</td>
<td>1+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK113</td>
<td>3+</td>
<td></td>
</tr>
<tr>
<td><em>S. salivarius</em></td>
<td>SK554, SK619</td>
<td>-</td>
<td>oral cavity, oral mucosa</td>
</tr>
<tr>
<td><em>S. gordonii</em></td>
<td>SK700</td>
<td>-</td>
<td>oral cavity, associated with endocarditis</td>
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<tr>
<td><em>S. crista</em></td>
<td>SK231</td>
<td>-</td>
<td>oral cavity</td>
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<tr>
<td><em>S. parasanguis</em></td>
<td>SK438</td>
<td>-</td>
<td>oral cavity</td>
</tr>
<tr>
<td></td>
<td>SK236</td>
<td>1+</td>
<td></td>
</tr>
<tr>
<td><em>S. vestibularis</em></td>
<td>SK240</td>
<td>-</td>
<td>oral mucosa</td>
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<tr>
<td><em>S. pyogenes</em></td>
<td>SK360, SK361</td>
<td>1+</td>
<td>pharynx, associated with tonsillitis and invasive infections</td>
</tr>
<tr>
<td><em>S. intermedius</em></td>
<td>SK443, SK507, SK508</td>
<td>-</td>
<td>subgingival plaque</td>
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<td></td>
<td>SK498</td>
<td>2+</td>
<td></td>
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<tr>
<td><em>S. anginosus</em></td>
<td>SK509</td>
<td>-</td>
<td>subgingival plaque</td>
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<tr>
<td></td>
<td>SK503</td>
<td>1+</td>
<td></td>
</tr>
<tr>
<td><em>S. agalactiae</em></td>
<td>SK870, SK873</td>
<td>3+</td>
<td>neonatal meningitis</td>
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<tr>
<td><em>H. pylori</em></td>
<td>CCUG17875</td>
<td>2+</td>
<td>human stomach, associated with gastritis and peptic ulcer disease</td>
</tr>
</tbody>
</table>

*a* - marks no aggregation after 1 hour; 1+, marks 50% decrease of OD$_{700}$ after 1 hour; 2+, marks 50% decrease within 1 hour; 3+, marks 50% decrease within 40 min; 4+, marks 50% decrease within 20 min.

*b* A typical origin of the bacterial strains and some characteristics.

*c* Strong self-aggregation of bacterial cells in the absence of agglutinin.

*d* Weak self-aggregation of bacterial cells in the absence of agglutinin.

*e* The aggregation score of *H. pylori* CCUG 17875 with whole parotid saliva was 3+. 
Salivary Agglutinin, Which Binds Streptococcus mutans and Helicobacter pylori, Is the Lung Scavenger Receptor Cysteine-Rich Protein GP-340*

Akraporn Prakobphol, Feng Xu, Van M Hoang, Thomas Larsson, Jorgen Bergstrom, Ingegard Johansson, Lars Frangsmyr, Uffe Holmskov, Hakon Leffler, Christina Nilsson, Thomas Boren, Joe Rae Wright, Niklas Stromberg and Susan J Fisher

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