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Functions of Cell Surface-Anchored Antigen I/II Family and Hsa Polypeptides in Interactions of *Streptococcus gordonii* with Host Receptors

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Streptococcus gordonii **colonizes multiple sites within the human oral cavity. This colonization depends upon the initial interactions of streptococcal adhesins with host receptors. The adhesins that bind salivary agglutinin glycoprotein (gp340) and human cell surface receptors include the antigen I/II (AgI/II) family polypeptides SspA and SspB and a sialic acid-binding surface protein designated Hsa or GspB. In this study we determined the relative functions of the AgI/II polypeptides and Hsa in interactions of *S. gordonii* DL1 (Challis) with host receptors. For an isogenic mutant with the *sspA* and *sspB* genes deleted the levels of adhesion to surface-immobilized gp340 were reduced 40%, while deletion of the *hsa* gene alone resulted in >80% inhibition of bacterial cell adhesion to gp340. Adhesion of *S. gordonii* DL1 cells to gp340 was sialidase sensitive, verifying that Hsa has a major role in mediating sialic acid-specific adhesion to gp340. Conversely, aggregation of *S. gordonii* cells by fluid-phase gp340 was not affected by deletion of *hsa* but was eliminated by deletion of the *sspA* and *sspB* genes. Deletion of the AgI/II polypeptide genes had no measurable effect on *hsa* mRNA levels or Hsa surface protein expression, and deletion of *hsa* did not affect AgI/II polypeptide expression. Further analysis of mutant phenotypes showed that the Hsa and AgI/II proteins mediated adhesion of *S. gordonii* DL1 to human HEp-2 epithelial cells. Hsa was also a principal streptococcal cell surface component promoting adhesion of human platelets to immobilized streptococci, but Hsa and AgI/II polypeptides acted in concert in mediating streptococcal cell-platelet aggregation. The results suggest that Hsa directs primary adhesion events for *S. gordonii* DL1 (Challis) with immobilized gp340, epithelial cells, and platelets. AgI/II polypeptides direct gp340-mediated aggregation, facilitate multimodal interactions necessary for platelet aggregation, and modulate *S. gordonii*-host engagements into biologically productive phenomena.**

Streptococcus gordonii and related species of viridans streptococci, including *Streptococcus cristatus*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus parasanguinis*, and *Streptococcus sanguinis*, colonize most surfaces present in the human oral cavity. These streptococci make up 60% or more of the total bacteria cultivated from early plaque formed on clean enamel (47). The deposition of streptococci paves the way for development of complex microbial communities by providing new adhesion sites for other bacteria. This occurs through direct intermicrobial binding (coaggregation) (31) or by recognition of surface-adsorbed host molecules (32) or microbial factors, such as polysaccharides (2). If viridans streptococci enter the bloodstream, they have the ability to promote the formation of vegetations at cardiac sites that are characteristic of infective endocarditis (3).

S. gordonii expresses a range of cell surface adhesin proteins that are associated with colonization and virulence. Surface fibrillar structures on *S. gordonii* DL1 (Challis) are composed of CshA and CshB polypeptides which bind fibronectin and other oral microorganisms (43, 44), and they facilitate invasion of endothelial cells (50). ApbA and AbpB are polypeptides that bind α-amylase, which may promote adhesion of cells to the salivary pellicle, as well as provide a nutritional benefit by capturing a host enzymatic activity (56). There are two other families of adhesins that are involved in interactions of *S. gordonii* with human host receptors and with oral bacterial cell receptors. The first of these is the antigen I/II (AgI/II) family of polypeptides, which are produced by most oral viridans streptococci. *S. gordonii* expresses two AgI/II family polypeptides, designated SspA and SspB, from genes that are tandemly arranged on the chromosome and independently transcribed (11, 18). These polypeptides bind salivary agglutinin glycoprotein or gp340 (48), a member of the scavenger receptor cysteine-rich family of secreted host proteins that is highly glycosylated (20). SspA and SspB also bind collagen type I (40), which promotes invasion of root dentine (39), and they bind

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other oral microbial species, such as *Actinomyces naeslundii* (12) and *Porphyromonas gingivalis* (33), which facilitates biofilm formation. The second family of adhesins are serine-rich repeat polypeptides, which are represented by Hsa (2,178-amino-acid [aa] precursor) in *S. gordonii* Challis (52) and by GspB (3,072-aa precursor) in *S. gordonii* M99 (6, 55). Hsa is a sialic acid-binding protein that recognizes receptors on human erythrocytes and polymorphonuclear leukocytes (52, 53). These serine-rich repeat family polypeptides are glycosylated concomitant with export in streptococci (4), and GspB and Hsa have been shown to be involved in the binding of *S. gordonii* to human platelets (5, 6, 54). Both Hsa and AgI/II polypeptides have been implicated as factors involved in biofilm formation and oral colonization by *S. gordonii* (10, 38).

gp340 has a major role in modulating streptococcal colonization of the oral cavity since it binds to a wide range of viridans and nonviridans streptococci (37). There is strong evidence that gp340 displays different adhesive behaviors when it is present in the fluid phase than when it is immobilized on a surface (8, 23, 37). Diverse oral microbial species exhibit adhesion and aggregation phenotypes with gp340, and these phenotypes are likely to be important for determining whether bacteria are retained in the oral cavity or are aggregated and removed (37). In *S. gordonii*, gp340 binding is mediated by AgI/II polypeptides (18, 19, 23) and is also associated with expression of the Hsa glycoprotein (37). Recent evidence suggests that oral streptococci differ considerably in their affinities for binding fluid-phase or surface-immobilized gp340 and that the differences are related at least in part to the differential expression of AgI/II- or Hsa-like polypeptides (37). In this study we investigated the roles of the AgI/II polypeptides SspA and SspB and of Hsa in adhesion and aggregation interactions of *S. gordonii* Challis with gp340, epithelial cells, and platelets. We found that SspA and SspB are involved in binding gp340 in fluid and surface-associated phases, whereas Hsa specifically recognizes surface-bound gp340. Furthermore, we found that while Hsa mediates platelet binding to *S. gordonii*, the concerted activities of AgI/II polypeptides and Hsa are required for *S. gordonii*-induced platelet aggregation in plasma.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Wild-type strains of *S. gordonii* DL1 (Challis) and *S. mutans* NG8 serotype c were routinely cultured in brain heart infusion medium (Difco) containing 0.5% yeast extract (BHY medium) in sealed tubes or bottles incubated statically at 37°C. Streptococci were grown on BHY agar containing 5 g/liter Neopeptone and 15 g/liter Bacto agar (BHYN medium) in candle jars at 37°C. *S. gordonii* UB1360 Δ(*sspA sspB*) (18) was cultured in BHY medium containing 100 μg spectinomycin/ml, while *S. mutans* 834 *spaP*, an isogenic derivative of *S. mutans* NG8 deficient in production of the P1 (AgI/II) protein (34), was grown in BHY medium supplemented with 5 μg tetracycline/ml. Generation of *S. gordonii* strains UB1545 Δ*hsa* and UB1552 Δ*hsa* Δ(*sspA sspB*) is described below. Wild-type *Lactococcus lactis* MG1363 was cultured at 30°C without shaking in M17 medium containing 0.5% glucose (19). Plasmids derived from pUB1000 (see below) were maintained in *L. lactis* by including 5 μg erythromycin/ml in the growth medium.

Disruption of *hsa* in *S. gordonii* UB1545 and UB1552. Routine DNA manipulations were performed as described by Sambrook et al. (49). The *hsa* gene encoding Hsa glycoprotein was disrupted by allelic exchange with the *aphA3* kanamycin resistance determinant. For the following PCR primer sequences, EcoRI sites (underlined) were incorporated where appropriate to facilitate subsequent cloning. Primers hsaF1 (5′-AGCTGTTCCTATCCTTTATCC-3′) and hsaR1 (5′-GGAAGCTGTCCTTTAGGAATTCCACTGGATTTGCTCC-3′), based on the *hsa* sequence determined for *S. gordonii* DL1 (GenBank accession no. AB029393), were used for PCR amplification of a 692-bp fragment from *S. gordonii* DL1 (Challis) comprising 394 bp of *hsa* and 298 bp of upstream sequence (bp 19 to 710 in GenBank accession no. AB029393). A 646-bp fragment, including 135 bp of the *hsa* gene and 511 bp downstream (positions 6,719 to 7,364 in GenBank accession no. AB029393) was amplified with primers hsaF2 (5′-GGAGCAAATCCAGTGGAATTCCTAAAGGACAGCTTCC-3′) and hsaR2 (5′-AACACTCTGTTGGAAAGTCAC-3′). Equal amounts of products from these reactions were mixed and reamplified with the hsaF1 and hsaR2 primers, which yielded a 1,315-bp fragment containing a roughly centrally located EcoRI site. This fragment was cloned in pGEM-T, generating plasmid pGEM-*hsa*. An approximately 1.5-kbp fragment comprising *aphA3* was amplified from a pUC19-derived plasmid (25) using primers aphF1 (5′-GACGGCCAGTGAATTCGAGC-3′) and aphR1 (5′-GTGGAATTCTGAGCGGATAAC-3′). This region was cloned into the unique EcoRI site in pGEM-*hsa*. The *hsa*::*aphA3* construct (~2.8 kb) was amplified using primers hsaF1 and hsaR2, gel purified, and used for transformation of *S. gordonii* DL1 to generate strain UB1545 Δ*hsa* or for transformation of *S. gordonii* UB1360 to obtain strain UB1552 Δ*hsa* Δ(*sspA sspB*). The correct allelic replacement mutagenesis constructs were confirmed by PCR, DNA sequencing, and Southern blotting.

RNA extraction and quantitative RT-PCR. RNA was extracted from streptococcal cells as described elsewhere (21). RNA was treated with RQ1 DNase I (Promega UK, Southampton, United Kingdom) according to the manufacturer's instructions, and 1 μg was used as a template for reverse transcription (RT)-PCR with avian myeloblastosis virus reverse transcriptase and *Tfl* DNA polymerase (Promega). Primers for amplification of *tpx* (TX1 [5′-CATCTAGAAGTAGGCGACACAGC-3′] and TX2 [5′-GCTATTGCCGGATCCTAGTCAGG-3′]) and *scaR* (SR1 [5′-GTCCACCATCTAGACTATACCAC-3′] and SR2 [5′-CTTGCGGATCCACTTCAAGAGA-3′]) have been described previously (21). The primers used for amplification of *hsa* were hsaF3 (5′-CTGGTAATGCTACTGTACA-3′) and hsaR3 (5′-GAAGATCTTTAACTACTCTG-3′), and the primers used for amplification of *gly* were glyF1 (5′-CAGTCTATCTATCGTGAGG-3′) and glyR1 (5′-TGAGACTTGGCTTCCAGC-3′). The primers used for *sspA* and *sspB* were sspABf1 (5′-TATGAAGCAGATTTGGCAGC-3′) and sspABr1 (5′-ATAGTGGAAATGAACTGTCGG-3′). Reverse transcription was performed at 48°C for 45 min, and the PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 15 to 35 cycles of 94°C for 30 s, 52°C for 45 s, and 68°C for 80 s (2.5 min for *sspA* and *sspB*). For each sample, a control reaction was performed without reverse transcriptase. RNA extraction and RT-PCR were performed twice for each sample, with similar results. Images of gels were captured using a Kodak EDAS 290 imaging system, and band intensities were quantified with the Bio-Rad Quantity1 image analysis software.

Western immunoblotting and whole-cell dot blots. Proteins were extracted from *S. gordonii* strains by incubation with mutanolysin (21) and were separated on continuous 5% polyacrylamide gels under denaturing conditions. Proteins were transferred onto Hybond ECL membranes (Amersham Bioscience, Buckinghamshire, United Kingdom) at 100 V for 1.5 h. For whole-cell dot blots, cells cultured in BHY medium for 16 h were harvested by centrifugation at 3,500 × *g* for 7 min at 4°C, washed once, and suspended in TBS (10 mM Tris-HCl, 150 mM NaCl; pH 7.6). Samples containing approximately 1×10^6 cells/ml and doubling dilutions of these samples were applied under a vacuum to prerinsed Hybond ECL membranes using a Bio-Dot SF transfer apparatus (Bio-Rad, Hemel Hempstead, United Kingdom). The membranes were probed with rabbit polyclonal antisera against sodium lauroyl sarcosinate (SLS) extracts of *S. gordonii* cell wall proteins (1:500 dilution) (24) or *S. mutans* AgI/II (1:10,000 dilution) (18), and the results were detected with peroxidase-linked swine immunoglobulins to rabbit immunoglobulin (IgG) as described previously (28). Alternatively, blots were incubated with 2 μg/ml succinylated wheat germ agglutinin (sWGA), and results were detected with 0.2 μg/ml peroxidase-conjugated streptavidin.

Expression of AgI/II proteins in *L. lactis*. Plasmid pUB1000 was employed for constitutive expression of AgI/II polypeptides. This vector carries a lactococcal P1 promoter and the signal peptide coding sequence of *L. lactis usp45* followed immediately by a SalI site (18). Just downstream of the SalI site there is a sequence from the C-terminal coding region of *sspA* that contains a BamHI site, a sequence encoding the sortase recognition motif LPxTG for cell wall anchorage, and a stop codon with a rho-independent transcription terminator. BamHI restriction sites are conserved approximately 100 bp upstream of the sequence encoding the LPxTG motif in the *S. gordonii sspA* and *sspB* genes and in *S. mutans spaP*. Insertion in frame of the *S. gordonii* DL1 *sspA* or *sspB* PCR-amplified fragment between the SalI and BamHI sites results in expression and anchorage of the AgI/II polypeptides on the lactococcal cell surface (18). To express *S. mutans* SpaP (AgI/II) in *L. lactis*, a 4,363-bp *spaP* gene fragment was amplified from *S. mutans* NG8 with primers spaPF1 (5′-ACGCGTCGACACA

CAAACTGGAAATCCAGCG-3′; SalI site underlined) and spaPR1 (5′-ATCT TGCGGATCAGTAGGGTCTGC-3′). The latter primer is complementary to the *spaP* gene sequence immediately downstream of the BamHI site. Cloning of the PCR-amplified fragment into the SalI and BamHI sites of pUB1000 resulted in cell surface expression and anchorage of SpaP (PAc) (23). Expression of SspA, SspB, or SpaP on the surface of *L. lactis* was confirmed by Western immunoblot analysis of cell wall extracts with antibodies raised to AgI/II (18, 19).

Adhesion to immobilized gp340. Salivary agglutinin glycoprotein gp340 was purified from pooled parotid saliva (48), diluted in coating buffer (20 mM Na_2CO_3, 20 mM $NaHCO_3$; pH 9.3), and adsorbed onto wells (0.2 μg per well) of an Immobilon (Nunc) microtiter plate (MTP) at 4°C for 16 h. The wells were washed once with TBS containing 5 mM $CaCl_2$ (TBSC), blocked with 1% bovine serum albumin for 1 h at 25°C, and washed with TBSC. Bacteria were cultured in TYG (0.5% tryptone, 0.5% yeast extract, 0.5% glucose, 2 g/liter K_2HPO_4, adjusted to pH 7.5) for 16 h, harvested by centrifugation, washed once with TBSC, and suspended in TBSC at an optical density at 600 nm (OD_{600}) of 1.00 (approximately 10^9 cells/ml). Cells (0.1-ml portions) were added to MTP wells and incubated for 2 h at 37°C. The wells were washed twice with TBS, and adherent bacteria were fixed with 25% formaldehyde for 30 min at 25°C. After two washes with TBS, the cells were stained with 0.5% crystal violet at 25°C for 1 min. The wells were washed three times with TBS, the remaining dye was dissolved in 7% acetic acid, and the absorbance at 595 nm (A_{595}) was measured. The numbers of cells bound were calculated from standard plots relating numbers of streptococcal cells fixed to plastic microtiter wells to A_{595} following crystal violet staining (23). In some experiments, immobilized gp340 was treated with 0.001 U neuraminidase (Sigma type X from *Clostridium perfringens*) in 0.1 M sodium acetate buffer (pH 5.0) containing 2 mM $CaCl_2$ for 2 h at 37°C prior to the assay for bacterial adhesion.

gp340-mediated aggregation. Bacteria were cultured for 16 h in BHY medium, washed twice in PBSC (0.1 M NaCl, 0.05 M KCl, 1 mM $CaCl_2$, 10 mM Na_2HPO_4-KH_2PO_4, pH 7.2), and suspended in PBSC at an OD_{600} of 0.7. To assay for aggregation, purified gp340 (final concentration, 1.2 μg/ml) was added, and triplicate samples were thoroughly mixed and incubated at 37°C. The optical density at 600 nm was measured at 10-min intervals for up to 3 h.

Adhesion to HEp-2 cells. HEp-2 cells, derived from a human larynx carcinoma (46), were cultured in Dulbecco's modified Eagle's medium (DMEM)/Nut mixture F-12 (Invitrogen Ltd., Paisley, United Kingdom) supplemented with 10% fetal calf serum, 0.6 μg L-glutamine/ml, and 0.5 μg hydrocortisone/ml. Cells were grown at 37°C under 5% CO_2 in a humidified environment and split every 3 to 4 days by trypsinization. For assays of bacterial adhesion, HEp-2 cells were suspended in DMEM at a density of approximately 10^5 cells/ml, seeded onto 24-well cell culture plates (Greiner Bio-One Ltd., Stonehouse, United Kingdom), and grown to confluence (48 to 72 h). Monolayers were washed with phosphate-buffered saline (PBS) prior to addition of bacteria.

Bacterial cells were cultured to the early exponential phase (OD_{600}, 0.3 to 0.4) in BHY medium. Approximately 10^9 cells were harvested, washed once in PBS, and suspended in PBS. Tenfold dilutions (20 μl) were plated onto BHYN medium for enumeration of input cells. For adhesion assays, approximately 10^7 bacterial cells/ml DMEM (1 ml) were added to the epithelial cells (multiplicity of infection, ~100) and incubated under 5% CO_2 at 37°C for 1 h. Following incubation, the plates were washed three times with PBS to remove nonadherent bacteria. Trypsin-EDTA (0.1 ml) was added, and the plates were incubated at 37°C under 5% CO_2 for 15 min to disrupt the epithelial cell monolayers. Bacteria were dissociated from the HEp-2 cells by addition of 0.001% Triton X-100 (0.4 ml) and 15 min of incubation at 37°C in the presence of 5% CO_2. Samples were serially diluted in PBS, and the numbers of CFU were determined by measuring viable counts on BHYN agar.

Platelet preparation. Collection of platelets and adhesion assays were performed as described elsewhere (30). Briefly, blood was drawn from healthy human volunteers who had not taken nonsteroid anti-inflammatory drugs within the previous 10 days. Ethical approval for collection of plasma was obtained from the Royal College of Surgeons in Ireland Ethics Committee. To prevent coagulation, 1.5 volumes of acid-citrate-dextrose was added to 8.5 volumes of blood, and centrifugation of the blood at 150 × *g* for 10 min yielded platelet-rich plasma (PRP). The pH of approximately 10 ml PRP was adjusted to 6.5 using acid-citrate-dextrose, and apyrase (1 U/ml) and prostaglandin E_1 (2 μM) were added. Platelets were harvested by centrifugation at 650 × *g* for 10 min, the supernatant (platelet-poor plasma) was removed, and the pellet was suspended in 2 ml modified HEPES Tyrode's buffer (JNL buffer) (6 mM dextrose, 130 mM NaCl, 9 mM $NaHCO_3$, 10 mM sodium citrate, 3 mM KCl, 0.8 mM KH_2PO_4, 0.9 mM $MgCl_2$, 2 mM $CaCl_2$; pH 7.4). Platelets were gel filtered through a Sepharose 2B-300 column, and the platelet concentration in pooled platelet-rich fractions was determined using a Sysmex-100 particle counter.

Platelet adhesion assays. For adhesion assays, bacteria were cultured in BHY medium for 16 h and harvested by centrifugation. Cells were washed and suspended in PBS, and the concentration was adjusted to approximately 3×10^9 cells/ml. Bacterial suspensions (0.1 ml) were applied to wells of a Costar polystyrene MTP and incubated at 37°C for 2 h. The MTP was washed and blocked with 1% bovine serum albumin at 37°C for 1 h. After a further two washes in modified JNL buffer, 50 μl gel-filtered platelets (2×10^8 cells per ml) was added and incubated at 37°C for 30 min. Nonadherent platelets were removed by three washes with JNL buffer, and bound platelets were lysed at 37°C for 2 h in acid phosphatase detection buffer (0.1 M sodium acetate [pH 5.5], 0.1% Triton X-100, 10 mM *p*-nitrophenol phosphate). The reaction was stopped by addition of 1 M NaOH, and the color was read at 410 nm with a microplate reader (Wallac Victor^2; Perkin-Elmer, Cambridge, United Kingdom). For each sample, adhesion was measured in three separate assays using platelets from different donors.

Platelet aggregation. Platelet aggregation was assessed by monitoring light transmission using a PAP-4 platelet aggregometer (Bio/Data Corp., Horsham, PA). Bacteria were prepared as described above for the platelet adhesion assays, and 50 μl of bacterial cells was mixed with 450 μl PRP. The light transmission of PRP without added bacteria and the light transmission of platelet-poor plasma were defined as 0% and 100% light transmission, respectively, and platelet aggregation was expressed as a final percentage of light transmission after 30 min. Five independent assays were performed for each strain.

Statistical analyses. Statistical significance was determined using Student's *t* test.

RESULTS

Construction of AgI/II- or Hsa-deficient mutants of *S. gordonii* DL1 (Challis). The *sspA* and *sspB* genes encoding AgI/II polypeptides are tandemly arranged on the *S. gordonii* DL1 chromosome (11). The products SspA (1,575 aa) and SspB (1,499 aa) are exported via the major Sec pathway and become covalently linked to cell wall peptidoglycan at the C terminus following sortase processing. *S. gordonii* UB1360 Δ(*sspA sspB*) was constructed as described elsewhere (18) and carries an allelic replacement of the entire *sspA sspB* coding region (9,774 bp) with *aad9* encoding spectinomycin resistance.

The genetic structure of the *hsa* locus in *S. gordonii* Challis has been derived from sequencing of the *S. gordonii* CH1 (Challis) genome by The Institute for Genome Research. The two different Challis strain designations, CH1 and DL1, are simply different laboratory designations for lineages of the same original Challis strain. The 5′ end of the *hsa* locus comprises the *hsa* gene and a putative upstream promoter encoding a polypeptide consisting of 2,178 aa. Downstream of the *hsa* gene in *S. gordonii* CH1 (5, 54) are eight open reading frames that are believed to be involved in glycosylation, processing, and secretion of Hsa (Fig. 1A). The Hsa protein does not have a conventional leader peptide but does contain the C-terminal motif for sortase-dependent cell wall anchorage. Evidence from work on the orthologous *gspB* locus in *S. gordonii* M99 suggests that glycosylation of GspB occurs prior to export (4). As recently reported (54), the *gly* gene that is immediately downstream of *hsa* and encodes a putative glycosyltransferase in *S. gordonii* CH1 (Challis) (Fig. 1A) carries a frameshift mutation that may result in production of a truncated polypeptide. We confirmed the presence of this frameshift in our laboratory stock of *S. gordonii* DL1 (Challis), although the effect, if any, of this mutation on Hsa surface structure and function is not known. To construct an isogenic mutant deficient in production of Hsa, most of the *hsa* coding region was deleted and replaced by the *aphA3* gene encoding kanamycin resistance, as described in Materials and Methods (Fig. 1A). To confirm that the deletion in strain UB1545 did

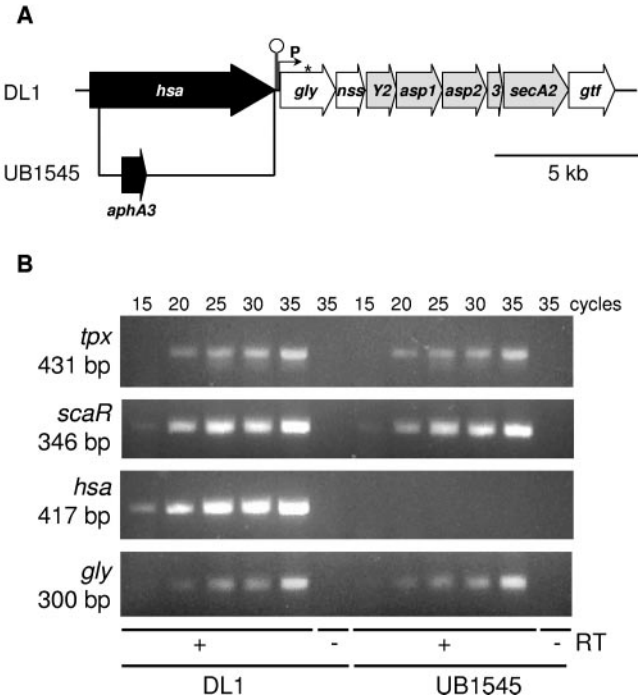

FIG. 1. (A) Organization of the *hsa* locus in *S. gordonii* Challis (strain DL1) and insertion site of *aphA3* for generating strain UB1545 Δ*hsa*. The arrangement of genes (derived from the genomic sequence of *S. gordonii* CH1 at www.tigr.org) potentially involved in Hsa post-translational processing (open arrows) and secretion (shaded arrows) is shown (also see references 5 and 54). The glycosyltransferase (*gly*) gene contains a frameshift mutation (asterisk) predicted to result in production of a truncated polypeptide. A stem-loop (rho-independent transcriptional terminator) and a putative promoter element (P) are present between the *hsa* and *gly* genes. (B) Transcription of *gly* in strains DL1 and UB1545 was monitored by quantitative RT-PCR. No product was observed in the absence of reverse transcriptase (RT). Measurements of thiol peroxidase (*tpx*) and ScaR metallorepressor protein (*scaR*) gene transcript levels were included as controls.

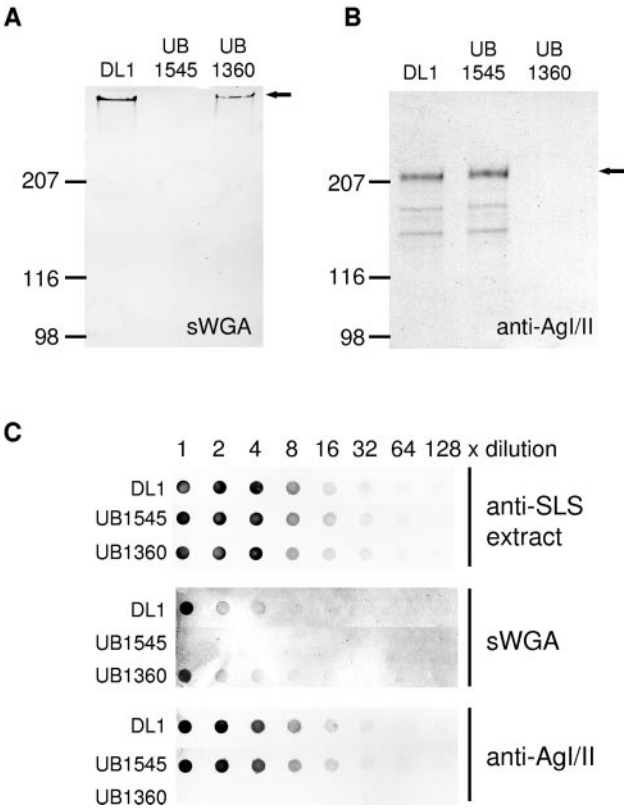

FIG. 2. Expression of Hsa and AgI/II polypeptides by *S. gordonii* DL1, UB1545 Δ*hsa*, and UB1360 Δ(*sspA sspB*). Cell wall protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotted onto a nitrocellulose membrane. (A) sWGA detection of Hsa, indicated by an arrow. (B) SspA and SspB (AgI/II family) proteins visualized with polyclonal antibodies to AgI/II, indicated by an arrow (the two lower-molecular-mass bands are fragments). (C) Twofold dilutions of intact cells dot blotted onto nitrocellulose and reacted with antibodies to surface proteins (SLS extract) of *S. gordonii* DL1, with sWGA, or with polyclonal antibodies to AgI/II.

not affect transcription from the putative promoter region immediately downstream of *hsa*, we utilized quantitative RT-PCR to measure levels of *gly* mRNA in strains DL1 and UB1545. These levels were compared with the levels of expression of two control genes, *tpx* (encoding thiol peroxidase) and *scaR* (encoding an Mn^{2+}-dependent transcriptional repressor protein) (22). Expression of *tpx* is regulated in response to oxidative stress (22), while expression of *scaR* appears to be constitutive (21). We expected, therefore, that these genes would provide suitable mRNA controls likely to be relatively unaffected by deletion of a surface protein adhesin gene. As shown in Fig. 1B, the relative levels of the *gly*, *tpx*, and *scaR* mRNAs in *S. gordonii* UB1545 were similar to wild-type levels, confirming that the allelic replacement of *hsa* did not affect expression of the downstream *gly* gene.

Phenotypic analysis of mutants. Cell wall extracts, prepared by mutanolysin digestion of *S. gordonii* wild-type or mutant strains, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. The presence of Hsa was detected with sWGA, which reacts with *N*-acetylglucosamine residues that decorate Hsa (5). In our surface protein extracts, Hsa was the only protein component of the *S. gordonii* DL1 cell wall extract that bound sWGA (53). The presence of AgI/II polypeptides was detected with polyclonal AgI/II antibodies that cross-react with SspA and SspB (18). Wild-type strain DL1 contained a single band at an apparent molecular mass of >300 kDa that reacted with sWGA (Fig. 2A). This band was present in strain UB1360 Δ(*sspA sspB*) but absent from strain UB1545 Δ*hsa* (Fig. 2A). In a corresponding immunblot reacted with AgI/II antiserum, the SspA and SspB (AgI/II family) proteins in the wild-type strain extract comigrated at approximately 200 kDa (Fig. 2B). The two lower-molecular-mass bands were AgI/II fragments that characteristically appear following extraction of the proteins from cells (28). These AgI/II antibody-reactive bands were absent from extracts of strain UB1360 cells, but their production by strain UB1545 was apparently not affected (Fig. 2B). We also generated double-mutant strain UB1552 Δ*hsa* Δ(*sspA sspB*) by transforming the *hsa*::*aphA3* construct into strain UB1360. Western blots of cell wall protein extracts prepared from double-mutant strain UB1552 cells showed no bands reactive with sWGA or with AgI/II antibodies (results not shown).

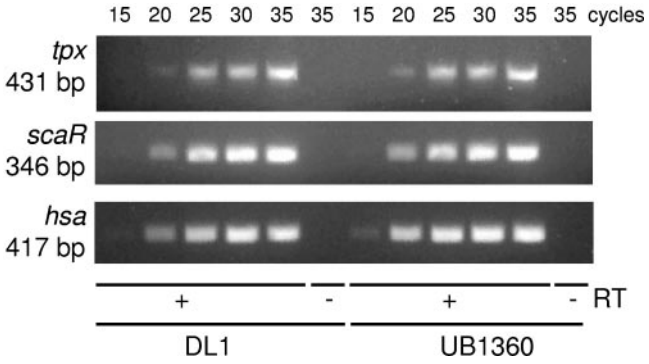

FIG. 3. Transcription of the *hsa* gene in *S. gordonii* DL1 and strain UB1360 Δ(*sspA sspB*) assessed by quantitative RT-PCR. Amplification products from *tpx* and *scaR* mRNAs were included as controls. Bands were subjected to densitometric analyses, and *hsa* mRNA product levels were calculated relative to the *tpx* and *scaR* mRNA product levels in the corresponding strain. Experiments were repeated three times, and a representative gel from one experiment is shown.

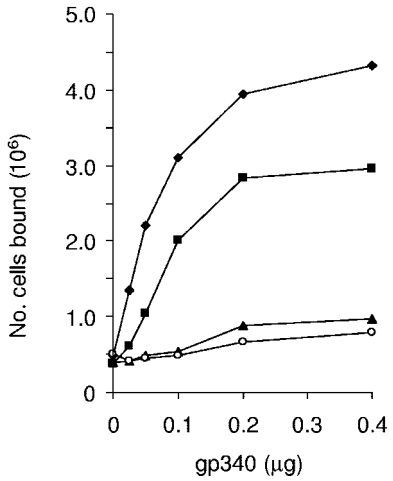

FIG. 4. Adhesion of *S. gordonii* strains to immobilized gp340. Binding of DL1 (◆), UB1360 Δ(*sspA sspB*) (■), UB1545 Δ*hsa* (▲), or UB1552 Δ*hsa* Δ(*sspA sspB*) (○) to gp340 immobilized onto plastic wells was determined following crystal violet staining of bound cells (see Materials and Methods). Experiments were repeated at least three times, and the results of one representative experiment are shown; the data points are means for triplicate samples, and the standard deviations were within ±10%.

Expression of Hsa and AgI/II polypeptides on the cell surface was determined with serial twofold dilutions of wild-type, UB1360, and UB1545 cells applied to nitrocellulose (Fig. 2C). These experiments confirmed the absence of surface expression of Hsa and AgI/II polypeptides in mutant strains UB1545 Δ*hsa* and UB1360 Δ(*sspA sspB*), respectively. It was also possible to determine, from densitometric analyses, that the surface accessible levels of Hsa in UB1360 Δ(*sspA sspB*) were not significantly different from those in wild-type strain DL1 cells. Likewise, the surface-expressed levels of AgI/II polypeptides in strain UB1545 were not significantly different from those in the wild type (Fig. 2C). Identical reactivities of DL1, UB1545, and UB1360 cells with a control antiserum raised to an SLS extract of *S. gordonii* wild-type cells (24) confirmed that equivalent amounts of wild-type and mutant cells were applied to the nitrocellulose.

As a final check on whether deletion of the *sspA* and *sspB* genes affected transcription of *hsa*, we utilized quantitative RT-PCR to determine levels of expression of *hsa* mRNA in UB1360 Δ(*sspA sspB*) compared with those in the wild type. Densitometric analysis of the bands indicated that during the linear amplification phase of PCR (cycles 15 to 25), there was no major difference between the amounts of *hsa* mRNA in strains UB1360 and DL1 (Fig. 3) relative to the amounts for the corresponding *tpx* and *scaR* mRNA controls. In separate experiments (data not shown) we also determined by RT-PCR the levels of expression of *sspA* (1,848-bp internal fragment) and *sspB* (1,632-bp internal fragment) mRNAs in strain UB1545 Δ*hsa* compared with those in strain DL1. Again, we found no major differences (<0.8- to >1.2-fold) between the expression levels. Thus, deletion of the *sspA* and *sspB* genes does not appear to affect the levels of transcription of the *hsa* gene or the surface-expressed levels of the Hsa protein compared to the wild-type levels. Likewise, deletion of *hsa* does not detectably affect the *sspA* and *sspB* mRNA levels or surface expression of the AgI/II polypeptides in strain UB1545 Δ*hsa* compared with wild-type strain DL1.

Hsa and AgI/II polypeptides mediate adhesion of *S. gordonii* to immobilized gp340. Salivary agglutinin glycoprotein (gp340) is a major glycosylated receptor for streptococcal AgI/II family polypeptides (26). The relative roles of the Hsa and AgI/II polypeptides in mediating adhesion of *S. gordonii* DL1 to gp340 were determined by measuring levels of binding of wild-type and mutant cells to purified gp340 (0 to 0.4 μg) immobilized onto plastic microwell plates. Cells of *S. gordonii* UB1360 Δ(*sspA sspB*) showed an approximately 40% reduction in adhesion to gp340, while the levels of adhesion to gp340 for UB1545 Δ*hsa* and UB1552 Δ*hsa* Δ(*sspA sspB*) cells were both >80% reduced (Fig. 4). Thus, while the AgI/II family proteins SspA and SspB contribute to the overall adhesion of wild-type strain DL1 cells to gp340, the major adhesion-mediating factor under these conditions appears to be Hsa.

Adhesion of *S. gordonii* DL1 to gp340 is sialidase sensitive. Since it is well documented that Hsa binds sialic acid residues (52), we determined the effect of sialic acid depletion (sialidase treatment) of gp340 on adhesion of *S. gordonii*. The adhesion of wild-type DL1 cells to desialylated gp340 was >85% reduced (Fig. 5A). A similar reduction in the level of adhesion of strain UB1360 Δ(*sspA sspB*) cells was observed (Fig. 5A). These results contrast with those obtained for adhesion of *S. mutans* NG8 cells to gp340, which showed that the adhesion level was not significantly reduced following desialylation of gp340 (Fig. 5B). Furthermore, inactivation of the *spaP* gene encoding the AgI/II polypeptide in *S. mutans* 834, an isogenic derivative of NG8 (34), resulted in elimination of cell adhesion to native or desialylated gp340 (Fig. 5B). Thus, the adhesion of *S. mutans* NG8, which does not carry an *hsa*-like gene, to gp340 is directed principally by the AgI/II polypeptide and is sialidase insensitive. Conversely, adhesion of *S. gordonii* DL1 to gp340 is highly sensitive to sialidase and is mediated primarily through Hsa.

In separate experiments we compared directly the sialidase sensitivity of binding of the *S. gordonii* SspA and SspB polypep-

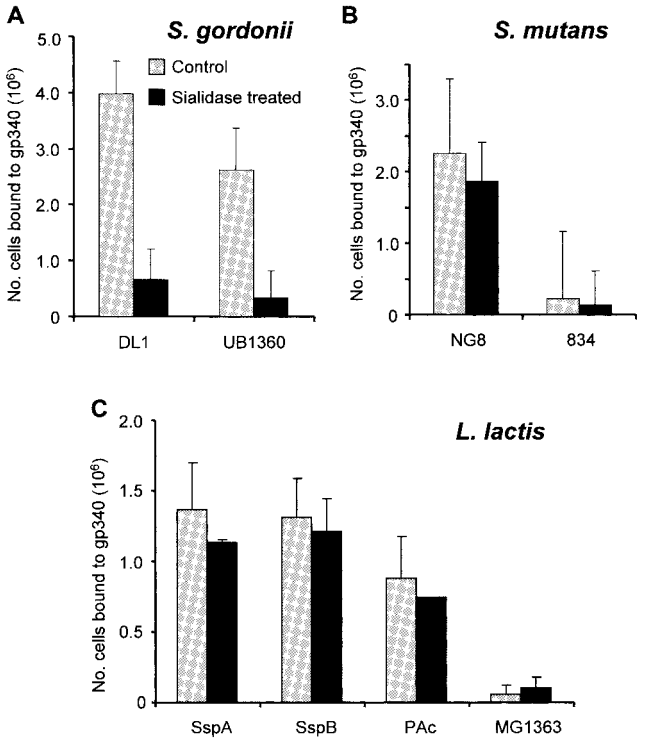

FIG. 5. Modulation of streptococcal adhesion to gp340 by sialidase. (A) *S. gordonii* DL1 and UB1360 Δ(*sspA sspB*) mutant; (B) *S. mutans* NG8 and 834 Δ*spaP*; (C) *L. lactis* MG1363 wild type or strains expressing AgI/II polypeptide SspA, SspB, or PAc. Bacterial adhesion to 0.2 μg immobilized gp340, pretreated with buffer (control) or sialidase, was quantified by crystal violet staining of bound cells. Means and standard deviations of three independent experiments are shown.

tides and of the *S. mutans* SpaP polypeptide to gp340. The genes encoding these polypeptides were expressed on the surface of *L. lactis* MG1363, and the levels of adhesion of lactococci to gp340 were determined. In each case expression of the AgI/II polypeptide conferred adhesion of lactococci to gp340 (Fig. 5C), with SspA and SspB promoting slightly higher levels of adhesion than SpaP. Treatment of gp340 with sialidase, which resulted in a >85% reduction in the adhesion of *S. gordonii* DL1 cells, only marginally reduced (approximately 10%) the levels of adhesion of all lactococci expressing AgI/II polypeptides (Fig. 5C). The lower levels of adhesion were not statistically significant, as determined by Student's unpaired *t* test ($P > 0.05$ for all comparisons). These results confirm that the AgI/II polypeptides directly bind gp340 but suggest that their major interactions with gp340 are not sialidase sensitive.

AgI/II polypeptides mediate *S. gordonii* cell aggregation by fluid-phase gp340. *S. gordonii* wild-type DL1 cells were rapidly aggregated over a 3-h period in the presence of 1.2 mg/ml gp340 (Fig. 6). No aggregation was observed in buffer-only controls over a similar period. Deletion of *hsa* in UB1545 had no effect on the aggregation rate of UB1545 cells in the presence of gp340 (Fig. 6). By contrast, deletion of the *sspA* and *sspB* genes resulted in approximately 85% reductions in gp340-mediated aggregation of strain UB1360 and UB1552 cells (Fig. 6).

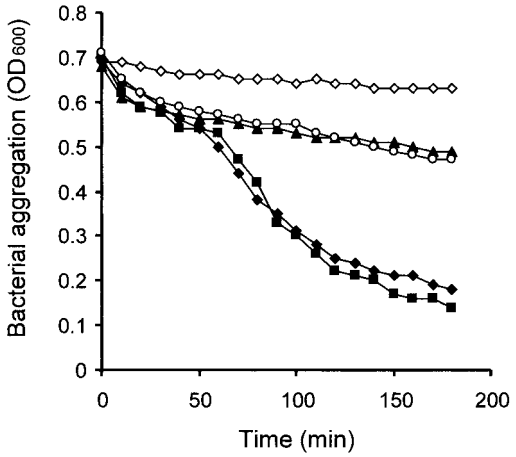

FIG. 6. Aggregation of *S. gordonii* by fluid-phase gp340. Purified gp340 (1.2 μg) was added to bacterial cell suspensions (1×10^9 cells/ml), and the extent of aggregation (reduction in OD_{600}) at 37°C was measured at intervals. Symbols: ◆, DL1; ▲, UB1360; ■, UB1545; ○, UB1552; ◇, DL1 control (no gp340). Samples were run in triplicate in three experiments, and the results from one representative experiment are shown.

Hsa and AgI/II polypeptides mediate adhesion of *S. gordonii* to human epithelial cells. Since Hsa and AgI/II polypeptides have been shown to interact with receptors on erythrocytes and monocytes (13, 53), we investigated the effects of inactivating Hsa or AgI/II polypeptide genes on adhesion of *S. gordonii* to HEp-2 human epithelial cells. At a multiplicity of infection of 100:1 (ratio of streptococci to HEp-2 cells) about 27% of the input wild-type DL1 bacteria adhered to the epithelial cell monolayers (Fig. 7). The levels of adhesion of UB1360 Δ(*sspA sspB*) cells were reduced by approximately 45%, while the levels of adhesion of UB1545 or UB1552 cells carrying deletions in *hsa* were reduced by 85% (Fig. 7).

Hsa and AgI/II polypeptides mediate interactions of *S. gordonii* with human platelets. It has recently been shown that Hsa plays a major role in adhesion of *S. gordonii* to human

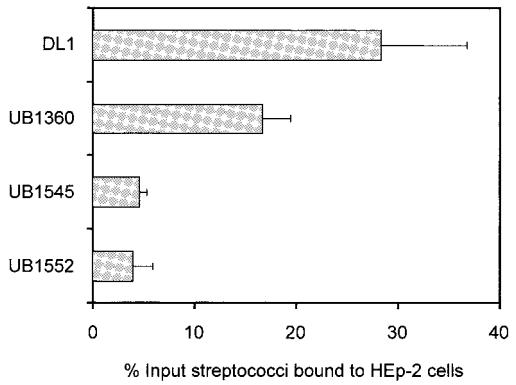

FIG. 7. Adhesion of *S. gordonii* DL1 (wild type), UB1360 Δ(*sspA sspB*), UB1545 Δ*hsa*, and UB1552 Δ*hsa* Δ(*sspA sspB*) to HEp-2 epithelial cells. The percentages of streptococcal cells (input, 10^5 cells/ml) that were bound after 2 h of incubation at 37°C were determined by viable counting. The values are the means and standard deviations of three independent experiments.

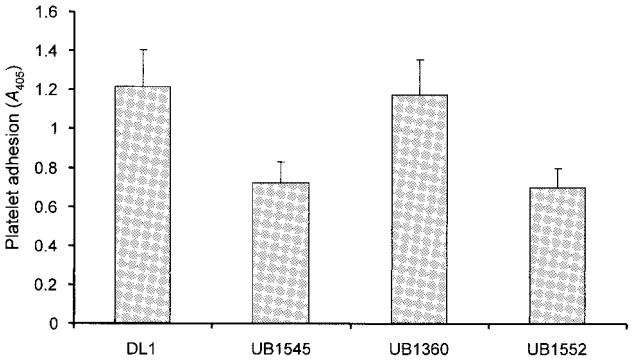

FIG. 8. Platelet adhesion by *S. gordonii* DL1 (wild type), UB1360 Δ(*sspA sspB*), UB1545 Δ*hsa*, and UB1552 Δ*hsa* Δ(*sspA sspB*). Equivalent numbers of streptococcal cells (3×10^8 cells) were immobilized onto microtiter plate wells, and the adhesion of purified human platelets (2×10^8 cells/ml) was measured by an enzymatic assay as described in Materials and Methods. The data are the means and standard deviations of three independent experiments.

platelets and that the adhesion is sialidase sensitive (54). We extended these analyses and measured the quantitative effects of Hsa or AgI/II polypeptide gene inactivation on adhesion of platelets to immobilized bacteria and on aggregation of platelets in plasma by bacterial suspensions. Deletion of *hsa* resulted in a 50% reduction in the levels of platelet adhesion to UB1545 cells and to UB1552 cells compared with controls (Fig. 8). On the other hand, inactivation of the *sspA* and *sspB* genes had no effect on platelet adhesion to immobilized strain UB1360 bacteria (Fig. 8). In platelet aggregation assays, however, different effects were observed. The abilities of strains UB1545 and UB1360 to mediate platelet aggregation in plasma were reduced by about 10% and 20%, respectively (Fig. 9). These reductions were not statistically significant, as determined by Student's paired *t* test ($P > 0.05$). However, deletion of the *hsa*, *sspA*, and *sspB* genes in strain UB1552 resulted in elimination of platelet-aggregating activity (Fig. 9). These results thus revealed a concerted activity for Hsa and AgI/II polypeptides in the platelet-aggregating phenotype of *S. gordonii* DL1.

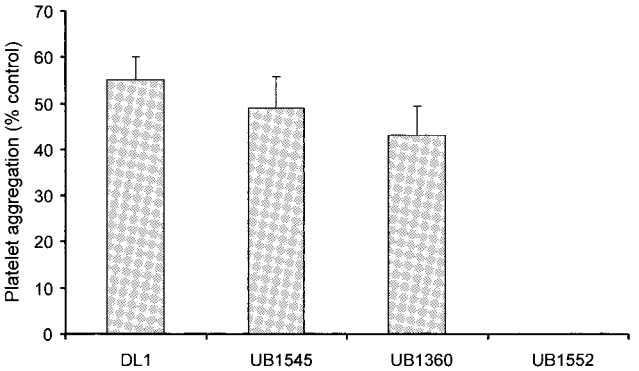

FIG. 9. *S. gordonii*-mediated platelet aggregation. *S. gordonii* cells (7×10^9 cells/ml) were mixed with human platelets (2×10^8 cells/ml) in plasma, and aggregation was expressed as a percentage of the control sample aggregation induced with arachidonic acid. Means and standard deviations are shown, based on five independent assays. No aggregation occurred with strain UB1552 Δ*hsa* Δ(*sspA sspB*).

DISCUSSION

Salivary agglutinin glycoprotein, or gp340, has long been recognized as an important agglutinating factor for oral streptococci. It is found as a high-molecular-mass complex (20) in association with secretory IgA (36), and it is thought that this protein is involved in the clumping and clearance of microbial cells from the oral cavity (7, 14, 48). It has also been shown recently that it aggregates other gram-positive bacteria, as well as gram-negative bacteria, such as *Helicobacter pylori* (7, 42, 48), and functions as a scavenger receptor at mucosal surfaces (17). However, when adsorbed to surfaces in the oral cavity, such as teeth, epithelium, or microorganisms, gp340 provides a receptor for bacterial adhesion. gp340 is a major receptor for streptococcal AgI/II polypeptides, and evidence suggests that AgI/II presents a carbohydrate-binding (lectin-like) domain in the central V region of the polypeptide (57). However, the structure of the epitope(s) on gp340 recognized by AgI/II proteins is not known. In this study we generated mutants of *S. gordonii* with deletions in the *hsa* gene, encoding a sialic acid-binding protein, and in the *sspA* and *sspB* genes, encoding AgI/II polypeptides. We established that deletion of *hsa* had no effect on expression of the *gly* gene immediately downstream. This was important because an effect on expression of *secA2*, which is further downstream from *gly* (Fig. 1) and encodes an alternate secretion system component, could potentially influence secretion of multiple proteins (35). Moreover, we obtained no evidence that deletion of *hsa* affected levels of AgI/II polypeptide gene expression or AgI/II surface protein production or that deletion of *sspA* and *sspB* affected Hsa production. However, these results do not rule out the possibility that a complex of the Hsa and AgI/II polypeptides or other adhesins is formed on the cell surface and that this complex is required for functionally correct outcomes of binding to gp340. Evidence for concerted action of multiple adhesins, either in complexes or disparately, in the interactions of *S. gordonii* DL1 with host receptors comes from (i) the reductions in several adhesive interactions with gp340, epithelial cells, and platelets as a result of deletions in genes encoding the Hsa or AgI/II polypeptides and (ii) elimination of platelet aggregation in plasma only by deletion of genes encoding both the Hsa and AgI/II polypeptides. In contract to *S. gordonii*, *S. mutans* NG8 seems to exhibit gp340 interactions that are mediated predominantly by AgI/II polypeptides (34). *S. mutans* NG8 does not express an Hsa-like protein (Jakubovics, unpublished data), and the sequenced genome of *S. mutans* UA159 (1) does not carry *hsa*-like sequences. However, higher levels of AgI/II surface protein expression in *S. mutans* NG8 are evident compared with the levels in *S. gordonii* (23). This presumably accounts for the similar overall levels of adhesion to gp340 observed for *S. mutans* and *S. gordonii* cells.

Based on the findings summarized above, we speculated that host and commensal bacterial cells may benefit from multimodal binding events prior to eliciting host innate defenses or bacterial cell proliferation. This is probably reflected in the dependence on SspA, SspB, Hsa, and potentially other adhesin factors in aggregation and adhesion of *S. gordonii* by gp340.

However, while we have found that the Hsa, SspA, and SspB polypeptides are all involved in adhesion of *S. gordonii* to gp340 immobilized in plastic microwells, it is difficult to infer from these in vitro experiments the relative importance of these adhesins in in vivo colonization. Aggregation of *S. gordonii* DL1 with gp340, which predominantly involves SspA and SspB, appears also to be mechanistically complex. This is shown by the fact that the gp340-mediated aggregation curve is biphasic (Fig. 6), with residual aggregation activity even after deletion of all three genes encoding the Hsa and AgI/II polypeptides. The ability to utilize multiple adhesins in recognizing gp340 could provide a selective advantage for an organism such as *S. gordonii* in competition with *S. mutans*. *S. gordonii* expresses at least three adhesins for gp340, while *S. mutans* produces only one major adhesin. This could explain, at least in part, why antibody and peptide inhibitors of AgI/II binding to gp340 were found to be especially effective in preventing recolonization of the human mouth by *S. mutans* (29, 41). The inhibitors of *S. mutans* AgI/II do not appear to affect the levels of recolonization of *Actinomyces* species (29) or of sanguinis group streptococci, which express Hsa-like proteins (9).

In addition to colonizing saliva-coated hard surfaces in the mouth, oral viridans streptococci bind to a variety of epithelial cells associated with the tongue, buccal surfaces, throat, and larynx (27). In *Streptococcus salivarius*, adhesion to epithelial cells is mediated through the production of a class of surface fibrils composed of antigen C, a glycosylated protein having a molecular mass of >450 kDa (58). However, the components present on the surfaces of most other oral viridans streptococci that mediate epithelial cell adhesion are unknown. In this work we found that the Hsa and AgI/II proteins function in mediating adhesion of *S. gordonii* to HEp-2 cells. These proteins appear to cooperate in binding to epithelial cells in a way similar to the way in which they cooperate in binding immobilized gp340. Interactions of *S. gordonii* DL1 with platelets seem, however, to be more complex. The binding of platelets to immobilized *S. gordonii*, such as the binding that might occur at an endocardial site initially colonized by streptococci, is directed principally by Hsa (54). In this reaction it is likely that Hsa recognizes the extracellular portion of the platelet membrane glycoprotein Ibα (5). Expression of the AgI/II proteins by *S. gordonii* DL1 does not seem to play a significant role in this adhesive interaction. However, in our experiments, strain UB1545 Δ*hsa* cells retained ~50% of the platelet-binding activity, so additional factors must be involved in binding to platelets under these conditions. We considered the possibility that the CshA and CshB surface fibrillar proteins (43), which have been implicated in interactions of *S. gordonii* with host cells (44, 50), are also involved in *S. gordonii*-platelet interactions. However, it has been shown that the *cshA cshB* mutant strain OB277 (45) is not significantly altered in platelet binding (C. McManus and D. Cox, unpublished data), so these polypeptides do not appear to be essential in this interaction.

Compared to the adhesion of platelets to streptococci in buffer, streptococcus-induced platelet aggregation in plasma involves a more complex array of interactions between platelet receptors, plasma components, and bacteria (30). Streptococci vary in the mechanisms by which they interact with platelets. Typically, for the strains of viridans streptococci that induce aggregation, there is a lag time of between approximately 5 min and 22 min following addition of bacteria to PRP before the first recognizable signs of aggregation. During this period, streptococci and platelets interact with or bind a number of plasma components, including fibrinogen, IgG, and complement (15, 16, 51). Platelet aggregation thus involves a combination of the following processes (30): fibrinogen binding to its receptor, GPIIb/IIIa; IgG binding to platelet Fc receptor $Fc_{\gamma}RIIA$; complement assembly on the streptococcal cell surface; binding of streptococci to platelet GPIb; and thromboxane-dependent platelet activation. In contrast to platelet binding to immobilized streptococci in the absence of plasma, *S. gordonii*-induced aggregation of platelets in plasma was found to depend upon expression of the Hsa and AgI/II polypeptides. Platelet aggregation by *S. gordonii* DL1 occurred after a lag time of 15 to 21 min (data not shown). Aggregation was not significantly affected by deletion of the *hsa* gene alone or by deletion of the AgI/II genes alone. Strikingly though, the ability to aggregate platelets was eliminated in strain UB1552, which is deficient in expression of the Hsa and AgI/II polypeptides. Thus, the concerted activities of Hsa, SspA, and SspB are necessary for assembly of the molecular apparatus that is integral to *S. gordonii*-induced platelet aggregation. Apart from the interaction of Hsa with GPIb, it is not known which of the other putative recognition events (e.g., binding of fibrinogen, IgG, or complement) are dependent upon the Hsa and AgI/II proteins or if these streptococcal proteins bind additional as-yet-uncharacterized platelet receptors. Studies to investigate these possibilities are in progress.

In summary, this work demonstrated that the Hsa, SspA, and SspB polypeptides on the cell surface of *S. gordonii* play a variety of structurally independent, but functionally integrated roles in binding of *S. gordonii* cells to human receptors. The Hsa protein recognizes sialic acid residues on gp340 and on human cells (52, 54), but the availability of the sialic acid receptors may be conformationally sensitive. Expression of receptors on immobilized gp340 provides for binding mediated principally by Hsa. However, sialic acid receptors are not sufficient or are not accessible to promote multivalent interactions that are associated with gp340-induced aggregation of bacteria in suspension. These interactions appear to require functional AgI/II proteins. Moreover, the AgI/II proteins are essential determinants in the development of *S. gordonii* communities within dentinal tubules (40) and salivary biofilms (10) in association with other oral bacteria, such as *A. naeslundii* (23) and *P. gingivalis* (33). Their multiple roles in determining the development of oral microbial communities suggests that further development of inhibitors of AgI/II functions are worthwhile strategies for modulating biofilm formation in the human host.

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