Binding of Salivary Glycoprotein-Secretory Immunoglobulin A Complex to the Surface Protein Antigen of Streptococcus mutans

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The interaction between a surface protein antigen (PAc) of Streptococcus mutans and human salivary agglutinin was analyzed with a surface plasmon resonance biosensor. The major component sugars of the salivary agglutinin were galactose, fucose, mannose, N-acetylgalactosamine, N-acetylgalactosaminyne, and N-acetylneuraminic acid. Binding of salivary agglutinin to PAc was calcium dependent and heat labile and required a pH greater than 5. Binding was significantly inhibited by N-acetylenuraminic acid and α,β,6-linked sialic acid-specific lectin derived from Sambucus sieboldiana in a dose-dependent manner. Pretreatment of the salivary agglutinin with sialidase reduced the binding activity of the agglutinin to the PAc molecule. The agglutinin was dissociated into high-molecular-mass glycoprotein and secretory immunoglobulin A (sIgA) components by electrophoretic fractionation in the presence of 1% sodium dodecyl sulfate and 1% mercaptoethanol. Neither of the components separated by electrophoretic fractionation, high-molecular-mass glycoprotein or sIgA, bound to the PAc molecule. Furthermore, the high-molecular-mass glycoprotein strongly inhibited the binding of the native salivary complex to PAc. These results suggest that the complex formed by the high-molecular-mass salivary glycoprotein and sIgA is essential for the binding reaction and that the sialic acid residues of the complex play an important role in the interaction between the agglutinin and PAc of S. mutans.

Streptococcus mutans has been implicated as a prime causative organism of dental caries, one of the most common diseases in humans. Colonization of the surface of a tooth by S. mutans is thought to be initiated by attachment of the organism to acquired pellicles on tooth surfaces (21). A 190-kDa surface protein antigen, which has been variously designated as antigen I/II, B, IF, P1, SR, MSL-1, and PAc (37), is known to be one of the factors which mediates the binding of the organism to the tooth surface (3, 21, 23).

Various salivary components, such as secretory immunoglobulin A (sIgA) (36), β,γ-microglobulin (12), histidine-rich polypeptides (32), a 60-kDa glycoprotein (1), lysozyme (39), lactoferrin (43), and high-molecular-mass glycoproteins (4, 7, 13, 20), have been reported to bind to S. mutans and/or to induce agglutination of the organism. Much attention has also been focused on the interaction between PAc and salivary components (5, 8, 21, 27–29). Russell and Mansson-Rahemtulla (38) reported that antigen I/II binds to several salivary components, including basic proline-rich proteins with molecular weights of 28,000 and 38,000, lysozyme, and α-amylase separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Much remains to be learned about the interaction between native salivary agglutinin and PAc of S. mutans.

In this study, using a surface plasmon resonance biosensor, we isolated and characterized a complex of high-molecular-weight salivary glycoprotein and sIgA which binds to PAc. The data presented in this report suggest that the complex of salivary glycoprotein and sIgA is an important component in the interaction between salivary and the PAc protein in a native
TABLE 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT8148</td>
<td>Serotype c PAc</td>
<td>21</td>
</tr>
<tr>
<td>PAcEm-2</td>
<td>Serotype c Em' PAc ;  transformant of MT8148 with pPC12 Em'</td>
<td>21</td>
</tr>
<tr>
<td>PAcEm-3</td>
<td>Serotype c Em' PAc ; transformant of MT8148 with pPC12 Em'</td>
<td>21</td>
</tr>
<tr>
<td>TK18</td>
<td>Serotype c Em' PAc ; transformant of GS-5 with pSM1</td>
<td>21</td>
</tr>
<tr>
<td>Xc</td>
<td>Serotype c PAc</td>
<td>47</td>
</tr>
<tr>
<td>Xc31</td>
<td>Serotype c antigen' Em' PAc ; transformant of Xc carrying P15A replicon and Em inserted into gluA (a glucose-1-phosphate uridylyl-transferase gene)</td>
<td>51</td>
</tr>
</tbody>
</table>

*S. gordonii* ATCC 10589

were hydrolyzed in 4 M trifluoroacetic acid at 100°C for 3 h and dried at 50°C. Free amino groups were acetylated by adding 50 μl of a mixture of methanol-pyridine-water (6:3:2) and 2 μl of acetic anhydride. The solution was incubated at room temperature for 30 min. After the solution was dried by evaporation at 50°C, a coupling reagent (10 μl) was added (0.67 g of 2-aminopyridine per ml in acetic acid) and the mixture was heated at 90°C for 20 min. After the excess reagents were removed by evaporation under a stream of nitrogen gas at 60°C for 20 min, a reducing reagent (10 μl) was added (60 mg of borane-dimethylamine complex per ml in acetic acid) and the mixture was heated at 90°C for 35 min and dried under a stream of nitrogen gas at 50°C for 10 min. The sample was dissolved in 1 ml of distilled water and analyzed by HPLC with a PALPAK Type A column (4.6 by 150 mm; Takara Shuzo Co., Ohtsu, Japan) at a flow rate of 0.3 ml per min at 65°C.

Sialic acids, N-acetylneuraminic acid, and N-glycolylneuraminic acid were analyzed by HPLC according to the method of Haras et al. (16). A 1-μg sample was hydrolyzed at 80°C for 1 h in 200 μl of 50 mM HCl. After the solution was cooled, 200 μl of DMB solution (7 mM 1,2-diamino-4,5-methyleneoxetene, 0.75 M 2-mercaptoethanol, and 18 mM sodium hydrosulfite) was added. The mixture was heated at 60°C for 2.5 h in the dark to develop fluorescence. The sample was analyzed by HPLC with a Nakanopac ODS-A column (6 by 150 mm; Ajinomoto Co., Handa, Japan) at a flow rate of 1 ml per min at 40°C.

Sialic acid content was determined according to the method of Lowry et al. (26), with bovine serum albumin as a standard.

rPAc. Recombinant PAc (rPAc) was purified from the culture supernatants of transformant *S. mutans* TK18 by ammonium sulfate precipitation, chromatography on DEAE-cellulose, and subsequent gel filtration on Sepharose CL-6B (Pharmacia) (21).

Binding of salivary agglutinin to rPAc. The binding of salivary agglutinin to rPAc was determined with a BIAcore 2000 (Pharmacia), which permits real-time interaction analysis of two interacting macromolecules (19). rPAc was immobilized on the carboxymethylated dextran-coated gold surface of a CM5 sensor chip by the primary amino groups according to the method of Johnsson et al. (18). Thirty-five microliters of rPAc (300 μg/ml) in 10 mM potassium phosphate buffer (pH 4 to 7) containing 0.15 M NaCl was bound to the surface. The flow of PBS (pH 7.0) was maintained at 1 μl per min throughout the immobilizing process. Salivary agglutinin in the running buffer was exposed to the rPAc-immobilized surface at a flow rate of 10 μl/min. To examine the effect of pH on the binding of salivary components to rPAc, 10 mM potassium phosphate buffer (pH 4 to 7) containing 0.15 M NaCl and 1 mM CaCl₂ and 10 mM Tris-HCl buffer (pH 7 to 9) containing 0.15 M NaCl and 1 mM CaCl₂ were used as running buffers. To examine the divalent cation specificity, CaCl₂, MgCl₂, or MnCl₂ was added to PBS (pH 7.0) at a final concentration of 0 to 2.5 mM. The dissociation phase was followed by the injection of the same buffer at 10 μl per min. All binding experiments were performed at 25°C. The surface plasmon resonance signal in each binding cycle was expressed in resonance units (RU). A resonance of 1,000 RU corresponded to a shift of 0.1° in the resonance angle, which means a change in surface protein concentration of approximately 1 ng/mm² (44).

Inhibition assay. The effects of sugars and lectins on the binding of the salivary component to rPAc were examined. Either fucose, galactose, mannose, N-acetyl-galactosamine, N-acetylglucosamine, or N-acetylgalactosamine was added to the salivary agglutinin suspension (25 μg/ml) in PBS (pH 7.0) for the BIAcore assay at a final concentration of 0.1 M. Varying amounts of Maackia amurensis agglutinin (MAM; Honen Corp., Tokyo, Japan), which recognizes α2,6-linked sialic acid residues (49), or *Sambucus sieboldiana* agglutinin (SSA; Honen Corp.), which recognizes α2,6-linked sialic acid residues (40), were added to the salivary agglutinin suspension for the BIAcore assay. The percent inhibition in the BIAcore assay was calculated as follows: percent inhibition = 100 × [(a – b)/a], where a is the mean RU without inhibitor and b is the mean RU with inhibitor.

Heat and sialidase treatments. Salivary agglutinin was heated at 50 to 100°C for 15 min and subjected to the BIAcore assay. For sialidase treatment, the component was dissolved in 200 μl of 50 mM sodium acetate buffer (pH 4.5) for sialidase from Clostridium perfringens and pH 5.5 for sialidases from *Arthrobacter ureafaciens* and *Vibrio cholerae*. For sialidase from *V. cholerae*, 10 mM CaCl₂ and 0.1 M NaCl were also added. Each mixture was incubated overnight at 37°C and dialyzed against PBS at 4°C.

Aggregation assay. Whole streptococcal cells were suspended in aggregation buffer at an optical density at 550 nm (OD550) of approximately 1.5. Either clarified whole saliva (10 μl) or 10 μl of salivary agglutinin (0.5 mg/ml) was mixed with 1 ml of the cell suspension, and the total volume of the reaction mixture was adjusted to 1.5 ml with aggregation buffer, CaCl₂, was also added to the mixture of salivary agglutinin at a final concentration of 1 mM. Bacterial aggregation was determined by monitoring the change of OD550 at 37°C for 60 min with a visible-UV recording spectrophotometer equipped with a CPS-240A cell positioner (UV-2200; Shimadzu Co., Kyoto, Japan).

PAGE, Western blotting, and lectin blotting. Native PAGE and SDS-PAGE were performed in 3 to 15% gradient polyacrylamide slab gels according to the methods of Davis (9) and Laemmli (22), respectively. After electrophoresis, the gels were stained with silver. Electrophoresis calibration kits (Pharmacia) were used for molecular mass markers.

For Western blotting and lectin blotting, salivary samples were subjected to native PAGE or SDS-PAGE and transferred electrophoretically to nitrocellulose membranes according to the method of Burnette (6). After blocking with 1% bovine serum albumin in Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl [pH 7.5]) plus 1% Triton X-100 (TBS-Triton), the membranes were treated with rabbit antibody against human IgA (Zymed Laboratories, South San Francisco, Calif.). After washing with TBS-Triton, the antibodies bound to proteins immobilized on the membrane were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed Laboratories). The membrane was also treated with a 4-μg/ml solution of biotinylated MAM or SSA (Honen Corp.) in TBS-Triton, and the lectins bound to glycoproteins immobilized on the membrane were detected with horseradish peroxidase-conjugated avidin D (Vector Laboratories).

Statistical analysis. Differences between the control and the test sample in the binding of salivary components to rPAc were determined by Student’s t test.

RESULTS

Analysis of salivary agglutinin. Salivary agglutinin, which bound to whole cells of *S. mutans*, was purified by gel filtration on Superdex 200 HR and analyzed by native PAGE and SDS-PAGE. Native PAGE analysis of salivary agglutinin bound to whole cells of *S. mutans* showed diffuse high-molecular-mass bands of more than 669 kDa (Fig. 1A, lane 2). The high-molecular-mass bands reacted with anti-human IgA(α) anti-

FIG. 1. Native PAGE (A) and Western blot (B) analyses of human salivary agglutinin. (A) Salivary samples were subjected to native PAGE (3 to 15% polyacrylamide), and the gel was stained with silver. The molecular mass markers used were bovine serum albumin (67 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). (B) Salivary components on the gel were electrophoretically transferred to a nitrocellulose membrane, and the membrane was reacted with rabbit antibody against human IgA(α). Lanes: 1, whole saliva (2 μg); 2, salivary agglutinin (1 μg).
body in the Western blots (Fig. 1B, lane 2). SDS-PAGE analysis of salivary agglutinin showed diffuse 145- to 630-kDa bands (Fig. 2A, lane 2). Reducing treatment of the diffuse 145- to 630-kDa component with 2-mercaptoethanol resulted in its separation into diffuse 145- to 420-kDa bands, three bands corresponding to components of sIgA (25, 59, and 88 kDa), and a faint band of 80 kDa (Fig. 2A, lane 5). sIgA purified from clarified saliva by using jacalin-agarose was also subjected to SDS-PAGE. It showed three bands of 25, 59, and 88 kDa after reducing treatment (Fig. 2A, lane 6). Antibody to the heavy chain of human IgA (α chain) reacted with the nonreduced 145- to 630-kDa bands and reduced the 59-kDa band in Western blots (Fig. 2B, lanes 1 and 2). Salivary glycoprotein-sIgA complex was separated by SDS-PAGE in the reduced state and transferred to a nitrocellulose membrane, and the complex was separated by electrophoretically transferred to nitrocellulose membranes, and the membranes were reacted with biotinylated SSA (lanes 1 and 2) or biotinylated MAM (lanes 1 and 3). Salivary sIgA purified in a serotype antigen-defective transformant of strain Xc (strain Xc31), reacted with the nonreduced salivary agglutinin (1 μg); 3, nonreduced sIgA (0.5 μg); 4, reduced whole saliva (2 μg); 5, reduced salivary agglutinin (1 μg); 6, reduced sIgA (0.5 μg). (B) Salivary components on the gel were electrophoretically transferred to nitrocellulose membranes, and the membranes were reacted with rabbit antibody against human IgA (Fig. 2A, lane 5). sIgA purified from clarified saliva by using jacalin-agarose was also subjected to SDS-PAGE. As mentioned above, the salivary agglutinin eluted from saliva-treated whole saliva and salivary agglutinin induced aggregation of the serotype c antigen-defective transformant of strain Xc.

**Binding of salivary agglutinin to rPAc.** The interaction between salivary agglutinin and the rPAc molecule was analyzed by the BLAcore assay. The addition of CaCl₂ to the running buffer enhanced the binding of salivary agglutinin to rPAc (Table 3). The maximum increase of RU was obtained when 1 mM CaCl₂ was added to PBS. The other divalent cations, magnesium and manganese, were not effective. The binding capacity of salivary agglutinin to rPAc was stable up to 60°C, but it was lost when salivary agglutinin was heated at 80°C for 15 min (Fig. 3A). The optimal pH for binding of salivary agglutinin was 7.0, and binding did not occur below pH 5 (Fig. 3B). Based on these data, PBS (pH 7.0) supplemented with 1 mM CaCl₂ was used as the running buffer for the BLAcore assay in subsequent experiments.

**Chemical composition of salivary components.** As mentioned above, the salivary agglutinin eluted from saliva-treated whole cells of *S. mutans* is composed mainly of high-molecular-mass glycoprotein and sIgA. We tried to dissociate these components by using electrophoretic fractionation in the presence of 1% SDS and 1% 2-mercaptoethanol. Fractions containing high-molecular-mass (145- to 420-kDa) glycoprotein and fractions containing sIgA components (25, 59, and 88 kDa) were pooled separately and used as the preparation of high-molecular-mass glycoprotein and that of sIgA components, respectively.

![Figure 2](image)

**TABLE 2. Aggregation of streptococcal cells induced by whole saliva and salivary agglutinin**

<table>
<thead>
<tr>
<th>Strain</th>
<th>No additives</th>
<th>Whole saliva</th>
<th>Salivary agglutinin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em></td>
<td>MT8148</td>
<td>&lt;0.01</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>PAcEm-2</td>
<td>&lt;0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>PAcEm-3</td>
<td>&lt;0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Xc</td>
<td>&lt;0.01</td>
<td>0.44 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Xc31</td>
<td>&lt;0.01</td>
<td>0.36 ± 0.02</td>
</tr>
</tbody>
</table>

* S. gordonii ATCC 10558. Both whole saliva and salivary agglutinin induced aggregation of the serotype c antigen-defective transformant of strain Xc.

**TABLE 3. Effects of cations on the binding of salivary agglutinin to rPAc**

<table>
<thead>
<tr>
<th>Cation</th>
<th>Binding of salivary agglutinin to rPAc at indicated concn (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Calcium</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Magnesium</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Manganese</td>
<td>6 ± 1</td>
</tr>
</tbody>
</table>

*S* Salivary agglutinin (25 μg/ml) in PBS (pH 7.0) containing various amounts of CaCl₂, MgCl₂, or MnCl₂ was allowed to react with rPAc immobilized on a sensor chip.

* Binding is expressed as RU determined by BLAcore assay. The values are the means ± standard deviations of triplicate assays.
The composition of the salivary agglutinin preparation was 74.8% protein, 4.0% fucose, 4.5% galactose, 0.7% mannose, 4.2% N-acetylgalactosamine, 5.5% N-acetylglucosamine, and 5.3% N-acetylneuraminic acid. The high-molecular-mass glycoprotein separated by electrophoretic fractionation was 73.0% protein, 4.4% fucose, 4.9% galactose, 0.8% mannose, 4.6% N-acetylgalactosamine, 5.8% N-acetylglucosamine, and 5.8% N-acetylneuraminic acid. The preparation of IgA components separated by electrophoretic fractionation was 90.2% protein, 1.1% fucose, 2.0% galactose, 0.5% mannose, 0.5% N-acetylgalactosamine, 3.5% N-acetylglucosamine, and 1.5% N-acetylneuraminic acid.

Inhibition of binding of salivary agglutinin to rPAc. The inhibitory effects of various sugars on the binding of salivary agglutinin to the rPAc molecule were examined. N-Acetylneuraminic acid, which is one of the component sugars of salivary agglutinin, caused the highest inhibition (98%), whereas fucose, galactose, and mannose only caused weak inhibition (Table 4). Other aminosugars, such as N-acetylgalactosamine and N-acetylglucosamine, had negligible inhibitory effect.

The effects on binding of two types of lectin, MAM and SSA, which recognize α2,3-linked and α2,6-linked sialic acid residues, respectively, were also examined. Binding of salivary agglutinin to rPAc was strongly inhibited in a dose-dependent manner by the addition of SSA, but the degree of inhibition by MAM was very low (Fig. 4). Treatment of salivary agglutinin with sialidases from A. ureafaciens and V. cholerae resulted in approximately 50% inhibition of binding to rPAc (Table 5). On the other hand, the treatment of salivary agglutinin with sialidase from C. perfringens showed a small inhibitory effect.

**FIG. 3.** Heat stability of salivary agglutinin (A) and the effect of pH on the binding of the agglutinin to rPAc (B). (A) After salivary agglutinin (25 μg/ml) was treated at 25 to 100°C for 15 min, the samples were subjected to BIAcore analysis. (B) Reactions were carried out with salivary agglutinin (25 μg/ml) in 10 mM potassium phosphate buffer (pH 4 to 7) containing 0.15 M NaCl and 1 mM CaCl2 (○) and 10 mM Tris-HCl buffer (pH 7 to 9) containing 0.15 M NaCl and 1 mM CaCl2 (●). The binding of salivary agglutinin to rPAc is expressed as RU determined by BIAcore assay. Values are given as the means ± standard deviations of triplicate assays.

**TABLE 4.** Effect of various sugars on the binding of salivary agglutinin to rPAc

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Binding (RU)a</th>
<th>% Inhibitionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>775 ± 27</td>
<td>-</td>
</tr>
<tr>
<td>Fucose</td>
<td>720 ± 25</td>
<td>7.1</td>
</tr>
<tr>
<td>Galactose</td>
<td>619 ± 20c</td>
<td>20.1</td>
</tr>
<tr>
<td>Mannose</td>
<td>664 ± 22</td>
<td>14.3</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>752 ± 28</td>
<td>3.0</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>781 ± 31</td>
<td>0</td>
</tr>
<tr>
<td>N-Acetylneuraminic acid</td>
<td>15 ± 1b</td>
<td>98.1</td>
</tr>
</tbody>
</table>

a Salivary agglutinin (25 μg/ml) in PBS (pH 7.0) containing a monosaccharide (0.1 M) and 1 mM CaCl2 was allowed to react with rPAc immobilized on a sensor chip.

b Binding is expressed as RU determined by BIAcore assay. The values are the means ± standard deviations of triplicate assays.

c Percent inhibition in the BIAcore assay was calculated as follows: percent inhibition = 100 × [(a – b)/a], where a is the mean RU without inhibitor (control) and b is the mean RU with inhibitor.

d P < 0.01 compared with control.

e P < 0.001 compared with control.

FIG. 4. Dose-dependent inhibition of the binding of salivary agglutinin to rPAc by the sialic acid-specific lectins MAM (○) and SSA (●). Salivary agglutinin (25 μg/ml) was allowed to react with rPAc immobilized on a sensor chip in the presence of various amounts of lectin (0 to 100 μg/ml). The binding is expressed as RU determined by BIAcore assay. Values are given as the means ± standard deviations of triplicate assays. Single asterisk, P < 0.01; double asterisk, P < 0.001 (compared with control [no addition of lectin]).
TABLE 5. Effect of sialidase treatment on the binding of salivary agglutinin to rPAc

<table>
<thead>
<tr>
<th>Origin of sialidase</th>
<th>Binding (RU)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>810 ± 28</td>
<td></td>
</tr>
<tr>
<td>A. ureafaciens</td>
<td>421 ± 19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48</td>
</tr>
<tr>
<td>C. perfringens</td>
<td>647 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20</td>
</tr>
<tr>
<td>V. cholerae</td>
<td>378 ± 26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53</td>
</tr>
</tbody>
</table>

*Salivary agglutinin (25 μg/ml) was treated with each sialidase (115 μU/ml) and then allowed to react with rPAc immobilized on a sensor chip.

<sup>a</sup> Binding is expressed as RU determined by BIAcore assay. The values are the means ± standard deviations of triplicate assays.

<sup>b</sup> The percent inhibition was calculated as follows: percent inhibition = 100 × (a − b)/a, where a is the mean RU induced by native salivary agglutinin (control) and b is the mean RU induced by sialidase-treated salivary agglutinin.

<sup>c</sup> P < 0.01; double asterisk, P < 0.001 compared with control.

FIG. 6. Effects of salivary components on the binding of salivary agglutinin to rPAc. Salivary agglutinin (25 μg/ml) was allowed to react with rPAc immobilized on a sensor chip in the absence (control) or presence of various amounts of high-molecular-mass salivary glycoprotein separated by electrophoretic fractionation (●) or sIgA components separated by electrophoretic fractionation (○) (0 to 400 μg/ml). The binding is expressed as RU determined by BIAcore assay. Values are given as the means ± standard deviations of triplicate assays. Single asterisk, P < 0.01; double asterisk, P < 0.001 (compared with the control).

Several salivary components are known to bind to and/or agglutinate S. mutans. Among these components, a high-molecular-mass agglutinin is thought to bind to the PAc of S. mutans (5, 8, 15, 27, 28). On SDS-PAGE, salivary agglutinin that binds to S. mutans appeared as diffuse bands with molecular masses of about 400 kDa, suggesting that it may be a complex of several types of glycoprotein with different molecular masses. The variation in mass may depend on the length or number of carbohydrate chains covalently associated with each polypeptide. This study revealed that native PAc-binding salivary agglutinin is a complex of a high-molecular-mass glycoprotein, sIgA, and a minor component. We have recently shown that all of the whole-saliva preparations from 10 healthy subjects between 27 and 42 years old contained the agglutinin complex, which is bound to whole cells of S. mutans (31), suggesting that the agglutinin may be widely distributed in the human population.

N-Acetyllneuraminic acid showed significant inhibitory effect on the binding of salivary agglutinin to the rPAc molecule. Lectin blotting revealed that both high-molecular-mass salivary glycoprotein and sIgA contain α2,6-linked sialic acid residues. In addition, lectin SSA, which recognizes α2,6-linked sialic acid residues, significantly inhibited the binding of salivary agglutinin to the rPAc molecule. These results suggest that the α2,6-linked sialic acid residues of salivary agglutinin are mainly responsible for its binding to the PAc molecule. Surface protein SSP-5 from S. gordonii M5 has the lectin-like property of binding to the α2,3-linked sialic acid residues of salivary agglutinin in the presence of calcium ions (11). In this study, whole saliva induced aggregation of S. gordonii ATCC 10558, but salivary agglutinin that binds to S. mutans did not induce aggregation of that strain (ATCC 10558). The sialic acid residues of salivary agglutinin that are recognized by the cell surface proteins of oral streptococci may differ with different species.

The binding of salivary agglutinin to rPAc was calcium dependent. Calcium ions are thought to be required as a bridge between negatively charged groups on the bacterial surface and similarly charged groups on the agglutinin (33), although some lectins require calcium ions as integral active-site constituents (10) or for specific conformational changes that are recognized by other molecules (14). Further studies are required to clarify the mechanism of calcium involvement in the interaction between salivary agglutinin and PAc.

In this study, nonreduced high-molecular-mass salivary ag-
glutinin reacted with antibody against human IgA, suggesting that the agglutinin was a complex of glycoprotein and sIgA. Oligosaccharides of sIgA are known to be receptors for the lectin-mediated adhesions of oral bacteria, such as *Actinomyces naeslundii* and *S. gordonii* (35). To clarify the roles of salivary glycoprotein and sIgA, we tried to dissociate salivary agglutinin by using solutions such as 6 M guanidine-HCl, 8 M urea, and 1% SDS. All of these treatments failed to separate the two components. Salivary glycoprotein and sIgA were separated by the treatment of salivary agglutinin with 1% SDS and 1% 2-mercaptoethanol, suggesting that these components are linked by disulfide bonds. Once separated by electrophoretic fractionation, both of these components no longer bound to rPAc. Moreover, the addition of high-molecular-mass glycoprotein separated by electrophoretic fractionation significantly decreased the RU value in the binding of salivary agglutinin to rPAc in a dose-dependent manner in the BIAcore assay. These results suggest that high-molecular-mass salivary glycoprotein plays an important role in the interaction between the native salivary complex and the rPAc molecule.

Recently, Senpuku et al. (39) reported that lysozyme binds strongly to the rPAc molecule in the BIAcore assay. They observed an increase of approximately 40 RU when 20 µl of lysozyme (1.05 × 10⁻³ M) was injected onto the surface of a sensor chip. Although almost the same amount of lysozyme, purified in a native condition by gel filtration on Superdex 200 HR, was injected onto the surface of a sensor chip in this study, only a very small increase in RU (approximately 5 to 10 RU) was observed when 20 µl was injected. The difference in RU seen in these two studies seems to be caused by such differences.

Human salivary mucins have been shown to interact with many oral bacteria and to protect against oral diseases (41, 46). Human submandibular and sublingual saliva contains both high- (more than 1,000 kDa) and low-molecular-mass (200 to 300 kDa) mucins (MG1 and MG2, respectively) (41). Highly glycosylated mucins can form homotypic and heterotypic complexes because of their negatively charged sialic acid and sulfate residues, as well as their hydrophobic domains (30, 50). The salivary agglutinin isolated in this study is similar to salivary mucins in molecular size and several other characteristics. Salivary agglutinin, however, contains fewer carbohydrates than salivary mucins, and the sialylation pattern involves α2,6 linkage, while salivary mucins have both α2,3 and α2,6 sialylation (41). Salivary agglutinin and mucins may be secreted by the same cells in salivary glands, but they may be hydrolyzed by different kinds of bacterial protease and glycosidase. The salivary agglutinin isolated in this study may be an assembly of cleaved mucins.

Human salivary mucins are known to make complexes with other salivary proteins, such as sIgA and lysozyme (24). Complexes of mucins with such protective proteins are believed to help concentrate these components at various tissue-environment interfaces. On the other hand, Brack and Reynolds (4) reported that rat salivary mucin complex loses agglutination activity with *S. mutans* after dissociation treatment with 6 M urea. Biesbrock et al. (2) also showed that mucin no longer binds to *Pseudomonas aeruginosa* and *Staphylococcus aureus* after sIgA is removed from the mucin-sIgA complex under dissociating conditions. Such dissociation treatments of mucins or complexes of mucin and other salivary proteins may destroy the interaction capability of mucins with bacteria. In this study, the individual components (high-molecular-mass glycoprotein and sIgA) of the salivary agglutinin complex did not interact as effectively with rPAc as did the intact complex itself. Furthermore, the interaction of the agglutinin complex with rPAc was heat labile. The binding of the agglutinin complex to rPAc was, however, inhibited by 98% in the presence of the monosaccharide N-acetyleneuraminic acid, and the binding was significantly inhibited by treatment of the agglutinin complex with either sialidases or α2,6-linked sialic acid-specific lectin. Treatment of N-acetyleneuraminic acid with heat (at 100°C for 5 min in the presence of 1% SDS and 1% 2-mercaptoethanol or at 80°C for 15 min) had no effect on the ability of the monosaccharide to inhibit the binding of the agglutinin complex to rPAc. Degradation of the agglutinin complex with endogenous salivary proteases (41) and/or conformational changes and denaturation of the polypeptide chain that could occur during the dissociation or heat treatment might hide or damage the N-acetyleneuraminic acid residue-containing PAc-binding epitope on the agglutinin macromolecule. The possibility also exists that the naked nonglycosylated region as well as the glycosylated region may play a role in the interaction of the agglutinin macromolecule with PAc of *S. mutans*.

The serotype c-specific polysaccharide antigen of *S. mutans* is composed of a backbone structure of 1,2- and 1,3-linked rhamnolysosamine polymer with side chains consisting of a terminal α-linked glucose unit (25). We recently cloned four genes (*rmlA*, *rmlB*, *rmlC*, and *rmlD*) involved in dTDP-l-rhamnose synthesis and a gene (gluA) encoding glucose-1-phosphate uridylyltransferase from *S. mutans* serotype c and showed that these genes are involved in the biosynthesis of the serotype c antigen (47, 48, 51). We have insertionally inactivated the *rml* genes and the *glu* gene of *S. mutans* Xc. All of the *rml*-inactivated mutant strains, but not the *glu* mutant strain, were observed by the naked eye to clump in the absence of human whole saliva. Therefore, we examined whether human whole saliva and salivary agglutinin induce aggregation of the serotype c antigen-deficient transformant Xc51, in which the *glu* gene was insertionally inactivated. Whole saliva and salivary agglutinin induced strong aggregation of the transformant, suggesting that the serotype c antigen does not interact with salivary agglutinin.

Rosan et al. (34) compared the saliva-mediated *S. mutans*-aggregating and adhesion-promoting activities in a group of caries-susceptible individuals and a group of caries-resistant individuals. They demonstrated that there is a significant increase in *S. mutans*-aggregating activity in saliva of the caries-resistant group versus that of the caries-susceptible group and a significant decrease in *S. mutans* adhesion-promoting activity in saliva of the caries-resistant group. Furthermore, Slomiany et al. (42) reported that the bacterial aggregating epitope of salivary mucins is expressed to a greater extent in caries-resistant individuals than in caries-susceptible individuals. These findings suggest that salivary components may play an important role in the resistance to dental caries. However, further work is needed to determine the role of the salivary agglutinin isolated in this study in oral health.

In conclusion, this study suggests that the high-molecular-mass salivary glycoprotein-sIgA complex binds to PAc of *S. mutans* via α2,6-linked sialic acid residues and that the complexing of glycoprotein and sIgA in a native condition is essential for the accomplishment of this function.

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