Saliva-Binding Region of Streptococcus mutans Surface Protein Antigen

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A 190-kDa surface protein antigen (PAc) of Streptococcus mutans binds to human salivary components. For detection of specific binding of the PAc protein to human salivary components, a simple sandwich assay was used. Microtiter plates precoated with recombinant PAc (rPAc), PAc fragments, or S. mutans whole cells were allowed to react with human whole saliva and then were incubated with biotinylated rPAc. The biotinylated rPAc bound to salivary components was detected by use of alkaline phosphatase-conjugated streptavidin and p-nitrophenylphosphate. In this assay, the binding of whole cells of S. mutans and purified rPAc to salivary components was confirmed. For determination of a saliva-binding region of the PAc molecule, 14 truncated PAc fragments were constructed by use of the polymerase chain reaction and an expression vector, pAX4a+. The binding of these truncated PAc fragments to human salivary components was determined by the sandwich assay. Among the truncated PAc fragments, fragments corresponding to residues 39 to 864 and residues 39 to 1000 of PAc showed a high ability to bind to salivary components. Shorter recombinant fragments corresponding to residues 39 to 217, residues 200 to 481, residues 470 to 749, and residues 688 to 864 did not exhibit any binding ability. The fragment that corresponds to a proline-rich repeating region (residues 828 to 1000) bound directly to the PAc protein. These results suggest that residues 39 to 864 of the PAc molecule are important in the binding of the surface protein to human salivary components, and the proline-rich repeating region of the PAc protein may contribute to spontaneous self-aggregation of the PAc protein.

Dental caries is one of the most common diseases in humans. Among numerous members of the oral flora, Streptococcus mutans is considered to be a major causative agent of human dental caries (11, 20). A 190-kDa protein antigen, PAc, which is also known as I/II (28), B (30), P1 (8), or MSL-1 (5), has been given much attention as a possible dental caries vaccine. Subcutaneous immunization of monkeys with purified PAc (I/II or B) (18, 19, 31) and local, passive immunization of monkeys (17) and humans (21) with anti-PAc (I/II) monoclonal antibodies (MAbs) were found to confer significant protection against dental caries or inhibition of S. mutans colonization.

The molecular structure of PAc (P1) of S. mutans serotype c was clarified by cloning (15, 24) and sequencing (12, 25) of the gene coding for this protein. The PAc protein possesses two internal repeating amino acid sequences; one is rich in alanine and is located in the N-terminal region (A region), and the other is rich in proline and is located in the central region (P region). The biological function of the PAc protein remains unclear. However, there is suggestive evidence that PAc contributes to the salivary agglutinin-mediated aggregation and adherence of S. mutans. It is well known that whole cells of S. mutans aggregate in the presence of human whole saliva or salivary agglutinin (5, 7, 9). Koga et al. (13) and Lee et al. (16) have constructed isogenic mutants of S. mutans deficient in PAc and have revealed that the PAc-defective mutants do not aggregate in the presence of whole saliva or salivary agglutinin. Ogier et al. (22, 23) have cloned and sequenced the gene coding for a saliva receptor protein of S. mutans serotype f and have found that the molecular struct-

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MATERIALS AND METHODS

Bacterial strains. S. mutans MT8148 was used as a representative strain of S. mutans serotype c. S. mutans TK18 is a recombinant strain that produces a large amount of PAc (13). S. mutans PACEm-2 is a PAc-defective mutant (24).

rPAc and PAc fragments. Recombinant PAc (rPAc) protein was purified from the culture supernatants of transformant S. mutans TK18 by ammonium sulfate precipitation and chromatography on DEAE-cellulose (13). Truncated PAc and its polypeptide fragments were prepared as fusion proteins with β-galactosidase by use of an expression plas-
mid vector, pAX4a+ (Medac, Hamburg, Germany). Truncated pac gene fragments were amplified by the PCR (32). Primers used in the PCR are listed in Table 1. The amplified DNAs were digested with KpnI and SalI and ligated to the KpnI-SalI-digested plasmid vector. The ligated DNAs were transformed into E. coli NM522. Fusion proteins were extracted from whole cells of the transformants by sonication and purified by p-aminophenyl-1-thio-β-d-galactopyranoside (APTG) affinity chromatography in accordance with the instructions of the manufacturer (Medac). The amino acid positions in the PAC molecule of these rPAC fragments are summarized in Fig. 1. Fusion proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14).

Saliva. Paraffin-stimulated whole saliva was collected from five healthy donors into an ice-chilled plastic tube and clarified by centrifugation for 10 min at 12,000 × g. Whole saliva that was obtained from one donor (donor A; see Fig. 6) and that exhibited a relatively high ability to bind to the PAC protein was mainly used in the present study.

Sandwich assay. Ninety-six-well flat-bottom microtiter plates (Sumitomo Bakelite Co., Tokyo, Japan) were coated with rPAC (1 μg), fusion PAC fragments (5 μg), or whole cells of S. mutans (10 μg) in 10 μl of 50 mM carbonate buffer, (pH 9.6) overnight at 4°C. After three washes with phosphate-buffered saline containing 1 mM CaCl2 (Ca-PBS), 1% (wt/vol) bovine serum albumin (BSA) in Ca-PBS was added, and the mixture was incubated overnight at 4°C. Excess BSA was removed by three washes with Ca-PBS, and then human whole saliva diluted with Ca-PBS was added (100 μl per well). After overnight incubation at 4°C, the plates were washed three times with Ca-PBS. Biotinylated rPAC (5 μg/ml) in Ca-PBS containing 1% BSA was added (100 μl per well), and the mixture was incubated overnight at 4°C. After three washes with Ca-PBS, alkaline phosphatase-conjugated streptavidin (BRL) diluted 1:1,000 with Ca-PBS containing 1% BSA was added (100 μl per well). The plates were incubated for 2 h at 20°C and washed with Ca-PBS, and the substrate, p-nitrophenylphosphate (1 mg/ml) in 0.1 M diethanolamine buffer (pH 9.6), was added (100 μl per well). After 1 h of incubation with the substrate, the optical density at 405 nm (OD405) was measured with a microplate reader (MRP A4; Tosoh Co., Tokyo, Japan).

![Diagram](https://via.placeholder.com/150)

**FIG. 1.** Cloning and expression of PCR-amplified truncated pac gene fragments. PCR-amplified gene fragments were ligated to the KpnI-SalI-digested pAX4a+ vector. The nucleotide sequences of the primers are listed in Table 1. E. coli transformants harboring the chimeric plasmids expressed truncated PAC fragments as β-galactosidase fusion proteins.
Glucose, galactose, mannose, fucose, lactose, glucosamine, galactosamine, N-acetylglucosamine, N-acetylgalactosamine, and N-acetyleneuraminic acid were tested for their ability to inhibit the binding of biotinylated rPAc to salivary components. Biotinylated rPAc (5 μg/ml in Ca-PBS containing 1% BSA) was mixed with the sugar being tested (0.2 M), and the mixture was added to wells of plates and incubated overnight at 4°C as described above.

RESULTS

Sandwich assay. A sandwich assay was used to examine the interaction between the PAc protein and salivary components. Binding of whole cells of PAc-producing S. mutans MT8148 to salivary components was detected by this procedure. On the other hand, a PAc-defective mutant, S. mutans PAcEm-2, showed a negative reaction, indicating that binding in this assay is PAc specific (Fig. 2). A panel of sugars (glucose, galactose, mannose, fucose, lactose, glucosamine, galactosamine, N-acetylglucosamine, N-acetylgalactosamine, and N-acetyleneuraminic acid) was assayed for inhibition of the binding of PAc to salivary components. No sugar significantly inhibited the binding of PAc to salivary components (data not shown).

Binding of rPAc fragments to salivary components. For determination of the salivary-binding region of the PAc molecule, we prepared 14 PAc fragments by using PCR technology and an expression vector, pAX4a+ (Table 1 and Fig. 1). Fusion proteins expressed by recombinant E. coli were purified by APTG affinity chromatography and used for the sandwich assay. Figure 3 shows SDS-PAGE patterns of crude and purified fusion proteins. Proteins purified by APTG affinity chromatography showed two bands. The 116-kDa protein in the purified samples was a β-galactosidase nonfusion protein produced by host E. coli.

rPAc (residues 39 to 1531) exhibited a positive reaction in the sandwich assay, and β-galactosidase did not bind to salivary components (Fig. 4). The truncated PAc fragments, PAc-14 (residues 39 to 864) and PAc-15 (residues 39 to 1000) bound to salivary components, whereas the shorter fragment PAc-12 (residues 39 to 481) did not. On the other hand, PAc-27 (residues 200 to 1531), PAc-37 (residues 470 to 1531), and PAc-57 (residues 828 to 1531) exhibited a positive reaction in the sandwich assay with saliva (Fig. 4A). However, biotinylated rPAc bound directly to PAc-37 and PAc-57 in the absence of saliva (Fig. 4B). Collectively, these results suggest that residues 39 to 864 of the PAc molecule are important in the binding of the PAc protein to salivary components and that the C-terminal region of the PAc molecule is capable of binding directly to the PAc protein.

We prepared shorter truncated PAc fragments, PAc-1 to PAc-7 (Fig. 1), and used them for the sandwich assay. None of these fragments, except for PAc-5 (residues 828 to 1000), bound to salivary components (Fig. 5A). PAc-5 bound directly to biotinylated rPAc (Fig. 5B).

The binding of PAc, PAc-14, PAc-12, and β-galactosidase to saliva from five donors was examined with the sandwich assay (Fig. 6). PAc and PAc-14 bound to salivary components from all the donors, but PAc-12 and β-galactosidase did not. These results suggest that the salivary components that the region including residues 39 to 864 reacts with are present in all saliva samples. However, the reactivity of the saliva differed with each donor (Fig. 6).
truncated PAC fragments to salivary components. The present study suggested that residues 39 to 864 of the PAC molecule play an important role in the binding of the protein to salivary components (Fig. 4). Shorter fragments, such as PAC-12 (residues 39 to 481), PAC-3 (residues 470 to 749), and PAC-4 (residues 688 to 864), exhibited no binding activity. This finding may support the idea that a rather large region of the PAC molecule is required for reaction with salivary components. The N terminus of the PAC protein is clearly involved in binding to salivary components. However, it is difficult to rule out the possibility that the C-terminal region also contains a binding site, as the ability of PAC-14 (residues 39 to 864) to bind to salivary components was lower than that of PAC-15 (residues 39 to 1000) (Fig. 4). It is possible that structural changes result from truncation of the PAC peptide or its expression as a fusion protein with β-galactosidase and that such changes can influence the interpretation of results.

Salivary agglutinin-induced aggregation of S. mutans is reported to be inhibited by fucose and lactose (5). On the other hand, Russell and Mansson-Rahemtulla (29) showed that the binding of 125I-PAC (I/II) to parotid gland salivacoated hydroxyapatite is inhibited by glucosamine, galactosamine, mannosamine, and N-acetylgalactosamic acid. In the sandwich assay, no sugar significantly inhibited the binding of PAC to saliva components. Although glucosamine and galactosamine (0.2 M) weakly inhibited the binding of PAC, the degree of inhibition was not significant. Brady et al. (3) used an array of 11 anti-PAC (P1) MAbs in a salivary agglutinin-induced aggregation inhibition assay and found that 6 MAbs markedly inhibited the salivary agglutinin-induced aggregation of S. mutans. Among these MAbs, one MAb recognized the truncated PAC fragment of residues 39 to 480, two MAbs recognized a fragment of residues 480 to 612, one MAb recognized a fragment of residues 612 to 1218, and two MAbs recognized a fragment of residues 1307 to 1561. Their findings suggest that the functional domain involved in the agglutinin-induced aggregation of S. mutans is located throughout the entire PAC (P1) molecule. On the other hand, the present study suggested that the saliva-interacting region of the PAC molecule might be the region between residues 39 to 864. We cannot compare simply our results with those reported by Brady et al. (3). We used whole saliva in the present study, but Brady et al. (3) used agglutinin purified from whole saliva. Moreover, the sandwich assay used in this study seems more like adherence than aggregation. It is possible that inhibitory antibodies react with a number of sites that all compose a functional domain when the PAC protein is properly configured. These sites may appear to be far apart on the amino acid sequence but actually may be closely related spatially.

The present study revealed that PAC-5 (residues 828 to 1000), corresponding to the P region of the PAC protein, bound to the whole PAC protein (Fig. 5). Moreover, binding to the P region was observed with PAC-57 (residues 828 to 1531) and PAC-37 (residues 470 to 1531) but not with PAC-27 (residues 200 to 1531) (Fig. 4B). These results suggest that binding to the P region is counteracted by the N-terminal portion of the PAC molecule. The biological significance of PAC protein binding to the P region is unknown. Our preliminary study, however, indicated that the native PAC protein formed aggregates in solution, and the molecular mass of the aggregates was estimated to be more than 1,000 kDa by gel filtration, suggesting that the PAC protein in solution may be a 5-mer or a 6-mer (unpublished data). Therefore, it is possible that the P region contributes to self-aggregation of the PAC protein. In addition, saliva
inhibited self-binding of the PAC-5 fragment (P region) (Fig. 5), suggesting that this region may also possess a functional domain for salivary binding.

* S. mutans* can be bound and/or agglutinated by various salivary molecules, such as secretory immunoglobulin A (27), β₂-microglobulin (6), histidine-rich polypeptides (26), a 60-kDa glycoprotein (24), and high-molecular-weight glycoproteins (2, 7, 10). Russell and Mansson-Rahemtulla (29) reported that cell surface protein antigen PAC (I/II) of *S. mutans* binds to several salivary components, such as basic proline-rich salivary proteins with molecular weights of 28,000 and 38,000, lysozyme, and α-amylase. The present study showed that salivary components bound to a rather large N-terminal region of the PAC molecule. However, it is possible that these salivary components differ from one another with regard to their binding regions on the PAC molecule. Further work is needed to determine the precise region on the PAC molecule that is bound by each salivary component.

As suggested by Russell and Mansson-Rahemtulla (29), the interaction between the PAC protein and salivary components may be of a low affinity and a weak specificity. However, the sandwich assay developed in the present study seemed to be useful and sensitive enough for studying the interaction between the PAC protein and salivary components. In addition, the truncated PAC fragments may be very useful for characterizing the biological and immunological properties of the PAC protein of *S. mutans*.

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ADDENDUM

After submission of this paper, a paper by Crowley et al. (3a) appeared. Their results indicate that the A-region of the PAC (P1) molecule interacts directly with salivary agglutinin.

REFERENCES


