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Molecular interactions of alanine-rich and proline-rich regions of cell surface protein antigen c in *Streptococcus mutans*

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Introduction: *Streptococcus mutans* has been implicated as a primary causative agent of dental caries in humans, and its cell surface protein antigen c (PAc) is known to be associated with sucrose-independent adhesion to tooth surfaces. PAc is composed of several domains, including an N-terminal signal sequence, an alanine-rich repeat region (A-region), a proline-rich repeat region (P-region), and an anchor region.
Methods: To investigate the functions of each domain, an A-region-deficient mutant strain of *S. mutans* was constructed, and recombinant PAc and A- and P-region proteins were also constructed. The interactions of each domain with the recombinant proteins were analyzed using surface plasmon resonance spectroscopy with a biomolecular interaction analyzing system.
Results: The A-region-deficient mutant strain showed the lowest levels of adherence to saliva-coated hydroxyapatite. Furthermore, findings in an immunoblot assay indicated that the A-region protein reacted strongly with proline-rich proteins in saliva, while the recombinant P-region protein interacted more quickly with PAc than the recombinant A-region protein.
Conclusion: These results suggest that the A-region has a strong relationship with adhesion to tooth surfaces, while the P-region has a high affinity for PAc.

Key words: functional domain; protein antigen c; *Streptococcus mutans*

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Streptococcus mutans has been implicated as a primary causative agent of dental caries in humans (9) and possesses a number of virulence factors that enable it to colonize tooth surfaces. The cell surface protein antigen c (PAc), one of the major surface proteins of *S. mutans* and known by a number of other names, including SpaP (13), antigen I/II (26) and B (25), P1 (7), and MSL-1 (6), is correlated to the virulence of the organism in the developmental of dental caries. PAc is known to participate in bacterial adherence to teeth via interaction with the salivary pellicle (12). The gene for PAc of *S. mutans* serotype *c* has been cloned and sequenced (13, 23), and includes an N-terminal signal sequence, a region with a series of three 82-residue alanine-rich repeats (A-region) within the N-terminal third of the molecule and a region with a series of three 39-residue proline-rich repeats (P-region) in the central portion of the molecule (23). Furthermore, C-terminal sequences characteristic of wall- and membrane-spanning domains of streptococcal surface proteins have also been reported (11).

Human salivary proline-rich proteins (PRPs), composed of heterogeneous molecules, are abundant in saliva produced

from the parotid, submandibular, and sublingual glands (3). They comprise about 80% of parotid salivary components and are classified into three groups: acidic (molecular mass <16 kDa), basic (molecular mass <6 to 9 kDa), and glycosylated (molecular mass 39 kDa) (27). PRPs are coded by a multi-family of six genes, and more than 20 PRPs have been discovered by both differential RNA splicing and proteolytic cleavages after secretion (17). Acidic PRPs have been reported to act as salivary receptors for several plaque-forming bacteria (2), while glycosylated PRPs have also been shown to interact with several types of microorganisms, such as *Fusobacterium nucleatum* (27).

Several studies have reported that the proline-rich region may bind to PAc; however, the binding specificities and underlying mechanisms remain unknown. In the present study, we constructed PAc-deficient mutants by inserting an erythromycin-resistance gene into the A-region of the *pac* gene of *S. mutans* strain MT8148. In addition, recombinant PAc, and A- and P-region proteins were generated. We then analyzed the binding specificities of each domain and attempted to determine the biological function of each region of PAc using these recombinant proteins and PAc-deficient mutants.

Materials and methods

Bacterial strains and plasmids

Table 1 lists the bacterial strains and the plasmids utilized in the present study. We used *S. mutans* strain MT8148 (serotype *c*) (24). For the saliva-coated hydroxyapatite adherence assay, ^{3}H-labeled cells were prepared by culturing in brain–heart infusion broth (Becton Dickinson Co., Franklin Lakes, NJ) containing [^{3}H]thymidine (0.1 MBq/ml; GE Health Bio-Science Corp., Piscataway, NJ) for 16 h at 37°C. *Escherichia coli* strains used for plasmid manipulation and maintenance were grown aerobically at 37°C on Luria–Bertani agar or broth.

Generation of PAc, A-region, and P-region recombinant proteins

Recombinant proteins were generated using a method described previously (18). Briefly, DNA fragments encoding total-PAc (PAc), the A-region of PAc, and the P-region of PAc in *pac* (23) (GenBank accession no. X14490) were amplified from the genomic DNA of strain MT8148 using a polymerase chain reaction (PCR) technique with AmpliTaq® polymerase (Applied Biosystems, Foster City, CA). The amplified fragments of the *pac* gene were subcloned into a pGEM-T Easy Vector (Promega, Madison, NJ), which was constructed based on the sequences shown in Table 2 (total PAc: forward, V2-1; reverse, V2-2; A-region: forward, G21; reverse, G22; P-region: forward, G25; reverse, G26), after which appropriate restriction sites were introduced for subcloning (*Bam*HI at the 5′ end of the upper primer and *Sal*I at the 5′ end of the lower primer). The presence of an insert was confirmed by *Bam*HI and *Sal*I digestions, followed by gel electrophoresis and purification with a QIAEX gel extraction kit (Qiagen, Chatsworth, CA). The purified fragments were subcloned into the expression vector pET42a(+) (Novagen, Darmstadt, Germany) and named pV12, pG212, and pG256, respectively. The plasmids were then transformed into *E. coli* BL21 (DE3) cells and those harboring each recombinant plasmid were grown in Luria–Bertani broth (800 ml) to the middle log phase at 37°C. Isopropylthio-β-D-galactoside (Wako Pure Chemical Industries, Osaka, Japan) was added to a final concentration of 1.0 mM, then the cultures were incubated for an additional 3 h to induce protein synthesis. The cells were harvested by centrifugation, then pelleted cells were suspended in 20 mM imidazole buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl, 20 mM imidazole, pH 7.4) and sonicated on ice. Recombinant PAc (rPAc), A-region (rG12), and P-region (rG56) proteins were obtained after centrifugation at 12,000 ***g*** for 30 min. The supernatants were subjected to a HiTrap chelating affinity chromatography column (GE Health Bio-Science) and eluted with 0.5 M imidazole buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl, 0.5 M imidazole, pH 7.4). The purified rPAc, rG12, and rG56 proteins were dialyzed with MilliQ water and stored at −80°C.

Rabbit anti-PAc serum was generated using a method described previously (22). Purified rPAc protein was mixed with Freund's complete adjuvant (Becton Dickinson) and injected intramuscularly into adult white rabbits twice with a 14-day

Table 2. Primers used in this study

Primer	Sequence (5'–3')	Position
V2-1	GCC ATG GTA GCA GCA GTC TCT TGT AGC AGGA	69–98
V2-2	GTC GAC ATC TTT CTT AGC CTT TAA ACC AAG	4671–4700
G21	CTG GAT CCG CCG AGO TTG AAC GCA T	547–571
G22	TCT GCG TCG ACT TTT TGA TAT TTG G	1378–1402
G25	GAA GGT TGT GGA GGT AGA CTA GGT CAG CTG	2507–2531
G26	TTT GGA AAC ATT GTC GAC ACG GAT TTT ACC	2950–2974
Erm (SacI)-F	AAT TAG GAG CTC AAA ATT TGT AAT TAA GAA	
Erm (SacI)-R	AAG CGA CTC ATA GAG CTC TTT CCT CCC GTT	

Endonuclease recognition sequences are underlined.

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source/reference
Strains		
MT8148	Wild type	24
PDG12	A-region of PAc deletion mutant strain derived from MT8148; Em^r	This study
Plasmids		
pGEM-T Easy	Cloning vector; Amp^r	Promega
pET42a (+)	Vector for expression of GST fusion protein	Promega
pV12	pET42a (+) derived plasmid containing full length of PAc, Km^r	22
pG212	pET42a (+) derived plasmid containing PCR-amplified DNA encoding amino acid 182 to 467 of PAc; Km^r	This study
pG256	pET42a (+) derived plasmid containing PCR-amplified DNA encoding amino acid 835 to 991 of PAc; Km^r	This study
pG312	pGEM-T Easy derived plasmid containing PCR-amplified DNA encoding amino acid 182 to 467 of PAc; Amp^r	This study
pE312	pG312 inserted erythromycin resistance cassette at the medium of PAc; Amp^r , Em^r	This study

Amp^r, ampicillin resistance; Km^r, kanamycin resistance; Em^r, erythromycin resistance.

interval. One week after the second injection, blood was drawn, then antiserum was collected and stored at −20°C.

Construction of deletion mutants

DNA fragments encoding the A-region from *S. mutans* MT8148 were amplified by PCR with Ampli Taq® (Applied Biosystems) using primers (forward, G21; reverse, G22), as shown in Table 2. The DNA fragments were subcloned into a pGEM-T Easy Vector (Promega) and named pG312. The DNA fragment encoding the Em^r cassette from pVA838 (16) was amplified by PCR with Ampli Taq®. PCR primers were constructed based on the sequences shown in Table 2, which added the restriction enzyme site of *Sac*I at the 5′ and 3′ ends of the fragment. Fragments of the Em^r cassette were digested using the appropriate enzyme, then ligated into pG312 and named pE312. The plasmid was digested with *Eco*RI, then linearized and transformed into *S. mutans* MT8148 using the method of Lindler and Macrina (14). Strain MT8148 was grown overnight in brain–heart infusion broth and then transferred to Todd–Hewitt broth (Becton Dickinson) containing 10% heat-inactivated horse serum (Gibco BRL, Rockville, MD). After the resultant culture was incubated at 37°C for 2 h, 200 μg pE312 plasmid DNA was added to separate cultures, which were allowed to stand at 37°C for 2 h, then plated on Mitis Salivarius agar (Becton Dickinson) containing erythromycin (final concentration, 10 μg/ml). The A-region-deficient mutant strain PDG12 was confirmed by PCR amplification, which determined the nucleotide sequences of the *erm* and *pac* genes, as well as by Western blotting of whole cells of the mutants with PAc-specific rabbit antiserum.

Saliva-coated hydroxyapatite adherence assay

Sucrose-independent adhesion to saliva-coated hydroxyapatite (SHA) was assayed using the method of Matsumoto et al. (19), with some modifications. Paraffin-stimulated whole saliva was collected from a healthy subject; it was placed into a beaker placed on ice and clarified by centrifugation at 12,000 ***g*** for 15 min. Thereafter, the saliva was stored at −20°C and thawed before use. Five milligrams of spheroidal hydroxyapatite (HA; BDH Chemicals Ltd, Poole, UK) was placed in 3-ml plastic tubes and washed three times with buffered KCl (5 mM KCl, 2 mM K_2PO_4, 1 mM $CaCl_2$; pH 6.0) to remove the fines. Clarified saliva (1 ml) was mixed at room temperature with the washed HA on a rotator (RT-50; Taitec Corporation, Koshigaya, Japan) at a speed of 1.1×10^{-3}**g** for 60 min. Each SHA suspension was added to an individual cup in a vacuum filtration device (1225 Sampling Manifold; Millipore, Bedford, MA) containing a mixed cellulose–ester membrane (0.45-μm pore size; Toyo Roshi Kaisha Ltd., Osaka, Japan) and a vacuum was applied. The membrane was washed three times in buffered KCl to remove the loosely bound SHA, which produced a monolayer of beads. After washing the SHA-binding membrane, radiolabeled MT8148 and the mutant strain were added individually to the cup and reacted at room temperature for 30 min. Unbound cells were decanted, after which the SHA-binding membrane was washed four times in buffered KCl. The radioactivity of the SHA-binding membrane was measured using a liquid scintillation spectrometer.

Proline-rich protein fraction of saliva

Paraffin-stimulated whole saliva was collected from a healthy subject and dialyzed extensively against distilled water at 4°C using a cellophane tube (Dialysis Membrane size 36; Wako), then freeze-dried. Lyophilized samples were reconstituted in 0.1 M Tris–HCl buffer (pH 7.5) containing 6 M guanidine–HCl and fractionated by gel filtration chromatography with a Sephacryl S-200 column (2.5 × 100 cm, Bio-Rad Laboratories, Hercules, CA), using the method described by Strömberg et al. (28). The flow rate was 0.4 ml/min at 4°C and the elution was collected as 4-ml fractions. Fractions containing PRPs were dialyzed and freeze-dried.

Dot blot assay

Each PRP fraction was labeled with biotin using an ECL Biotinylation Kit (GE Health Bio-Science). The rPAc, rG12, and rG56 (100 μg) were blotted on nitrocellulose membranes using a 96-well dot blot manifold (Bio-Rad) and reacted with biotin-labeled PRPs. The wells were washed twice with 200 μl of phosphate-buffered saline (PBS), then the filters were removed from the apparatus and blocked with PBS containing 5% skim milk and 0.25% Triton X-100. Horseradish peroxidase-conjugated streptavidin was added to the wells and development was performed with a 4-chloro-1-naphthol solution.

Measurement of molecular interactions with the BIAcore system

Interactions of the A-region and P-region with the PAc fragments were analyzed using a BIAcore Model 1000 system (BIAcore International, Uppsala, Sweden), according to the manufacturer's instructions. Briefly, a dextran layer on a sensor chip covalently attached to a carboxymethylated dextran gold surface (CM5 sensor chip, BIAcore International) was activated by injection of a mixture of *n*-hydroxysuccinimide (NHS) and carbodiimide (EDC) for 10 min, creating a reactive ester on the surface. Subsequently, rPAc in 10 mM of sodium acetate buffer (pH 4.5) was immobilized on the CM5 sensor chip. Excess binding sites containing non-reacted ester groups of the CM5 matrix were blocked with 1 M ethanolamine–HCl. One flow cell was left blank and used as a reference. The rG12 and rG56 were dissolved in PBS, which was also used as the running buffer for this experiment. Various concentrations of rG12 or rG56 were injected at a flow rate of 10 μl/min at 25°C, and the interactions were monitored as resonance units (RU) using a sensorgram. The results of each specific interaction are presented as the specific dissociation rate constant (k_d), association rate (k_a), and association constant (K_A, where $K_A = k_a/k_d$), which were determined using BIA EVALUATION software version 3.02, as described previously (1).

Statistical analysis

Intergroup differences of various factors were analyzed by a statistical analysis of variance (ANOVA) for factorial models. Fisher's protected least-significant differences test was used to compare individual groups.

Results

Expression and purification of rPAc

As for the recombinant proteins, analysis using sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed only a single band each for PAc, the A-region protein (rG12), and the P-region protein (rG56), which corresponded to 200, 60, and 75 kDa, respectively (Fig. 1).

Biological properties of PAc-deficient mutant strains

In the SHA binding assays of A-region-deficient mutant strains, PDG12 showed a significantly lower binding level than

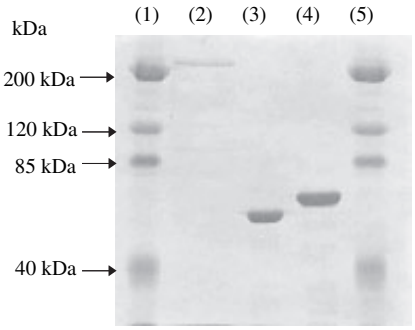


Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis with Coomassie blue staining. Lane 1, molecular marker; lane 2, rPAc; lane 3, rG12; lane 4, rG56; and lane 5, molecular marker.

MT8148 ($P < 0.01$, data not shown). In addition, the adherence of the PDG12 strain became elevated when rAr (1 mg/ml) was added, but it did not change when rPr (1 mg/ml) was added (Fig. 2A). Furthermore, the adherence of PDG12 was dependent on the concentration of rAr (Fig. 2B).

Interaction of recombinant PAc and salivary PRPs

PRPs were fractionated from whole saliva and the molecular sizes ranged from 20 to 25 kDa (Fig. 3A). Dot blot assay findings showed that the recombinant A-region protein (rG12) bound to the salivary PRPs strongly, whereas a band for the recombinant P-region protein (rG56) was not clearly detected (Fig. 3B).

Estimation of P-region binding affinity to PAc using BIAcore system

BIAcore analysis revealed that rG56 had a significantly lower k_d value than rG12, suggesting that the P-region was resistant to dissociation from PAc (Fig. 4A). Furthermore, the affinity of rG56 to PAc showed a more rigid and stable binding mode as compared with that of rG12. The K_a values, which indicated total binding affinity, were 5.17×10^5 for rG56 and 5.98×10^2 for rG12, suggesting a much greater adhesive affinity of the P-region to PAc (Fig. 4B).

Discussion

Several studies have demonstrated the functions of PAc using truncated recombinant proteins of *S. mutans* and have analyzed the function of each PAc domain using *E. coli* organisms that secrete PAc. However, those findings were merely from observations of the interactions between PAc and the target proteins, and did not analyze the functions of the cells (5). A possible reason for the limited findings presented in those reports may be linked to difficulties in the production of PAc deletion mutants. In the present study, we successfully constructed A-region-deleted mutant strains of *S. mutans* PDG12 and each showed different cariogenic properties *in vitro*.

Lee et al. (13) reported that PAc protein was not detected in any cellular fractions of a PAc-deficient mutant strain, while the protein was detected in the culture medium. They speculated that either a frame shift of the *pac* gene occurred by the insertional mutation procedure or one of the genetic functions had changed. As a result, the LPXTG wall anchor motif could not be expressed and then an incomplete PAc of the mutant strain was translocated into the supernatant. In the present study, the PDG12 strain showed the lowest value of adherence to SHA among all of the tested strains, while the recombinant A-region protein complemented the adherence to SHA by the A-region deletion mutant strain (Fig. 2) These results indicate that the A-region is strongly related to adherence to SHA and suggest that the recombinant A-region protein enhances binding of the PAc-deficient mutant strain to SHA. A previous study reported that incomplete PAc was found in 4% of

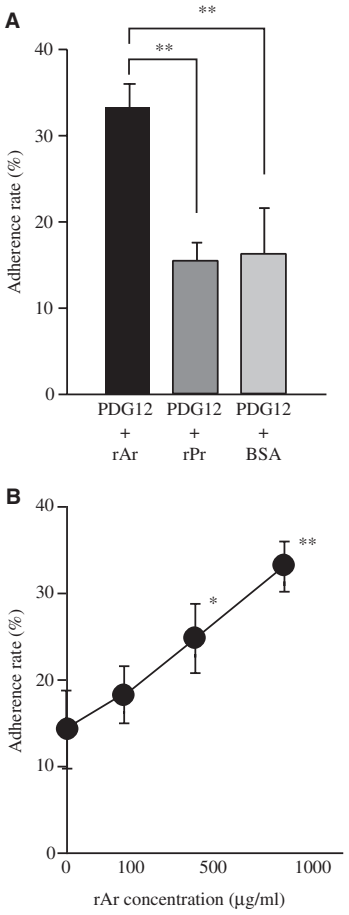


Fig. 2. Bacterial adherence to saliva-coated hydroxyapatite by A-region deletion mutant strain PDG12. (A) Recombinant A-region (rAr) and P-region (rPr) proteins were added to the PDG12 strain. (B) Various concentrations of rAr were added to the PDG12 strain. There were statistically significant differences between each of the protein added strains and between the concentrations of rAr (**$P < 0.01$; Fisher's protected least-significant differences analysis).

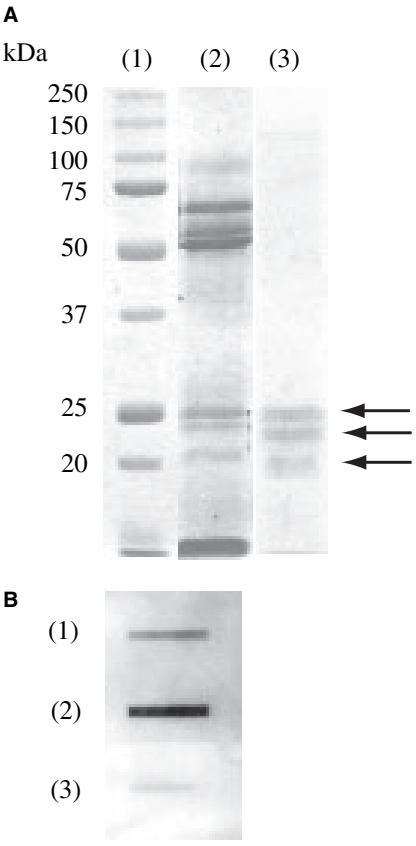
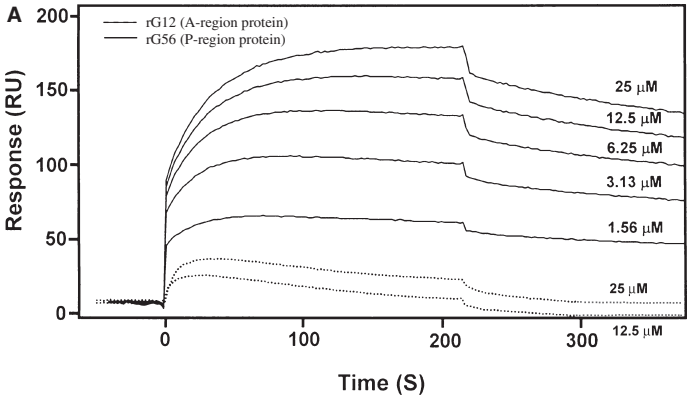


Fig. 3. Binding of salivary proline-rich proteins (PRPs) to *Streptococcus mutans* PAc. (A) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of fractional components. The fraction containing salivary PRP was dissolved in SDS-loading buffer. It should be noted that the PRPs migrated in an anomalous manner with respect to molecular mass in the SDS–PAGE gels. Lane 1, molecular marker; lane 2, whole saliva; and lane 3, fraction containing PRPs (arrows indicated the acidic PRPs). (B) rPAc, rG12 (A-region protein), and rG56 (P-region protein) were blotted onto nitrocellulose membranes and reacted with biotin-labeled PRPs. The wells were then exposed to horseradish peroxidase-conjugated streptavidin. Well 1, rPAc; well 2, rG12; and well 3, rG56.



B

Recombinant protein	k_{as}(1/Ms)	k_{ds} (1/s)	K_a (1/M)	K_d (M)
rG12 (A-region protein)	20.4	$3.41\text{x}10^{-2}$	$5.98\text{x}10^{2}$	$1.67\text{x}10^{-1}$
rG56 (P-region protein)	$4.77\text{x}10^{3}$	$9.22\text{x}10^{-3}$	$5.17\text{x}10^{5}$	$1.93\text{x}10^{-6}$

Fig. 4. Binding affinity of rG12 and rG56 to PAc analyzed using a BIAcore system. (A) The binding ability of rG56 to PAc was monitored and is presented as a sensorgram. The same molar amount of each recombinant PAc was immobilized on the matrix of the chip. rG12 and rG56 were injected at a flow rate of 10 μl/min for 240 s. For kinetic studies, rG56 was used with increasing concentrations. (B) Binding constants of rG12 and rG56 to rPAc.

clinical *S. mutans* isolates from Japanese children (22). Thus, the fragment size of PAc or the presence of the A-region may indicate the level of cariogenicity of these strains.

Several studies of *S. mutans* have shown that the recombinant peptide fragment derived from the A-region binds salivary agglutinin (5) and salivary glycoproteins (20), while the binding activity of that from the P-region is lower than that from the A-region. Another study also reported that the P-region bound to the PAc molecule itself and proposed that this segment may contribute to spontaneous self-aggregation of the molecule (21). Proline-rich sequences are frequently repetitive and generally form extended structures (30), and the fibronectin-binding proteins of *Streptococcus pyogenes* have very similar proline-rich sequences adjacent to their fibronectin-binding domains (29). Repetitive proline-rich segments are also present in the sequence encoded by genes of the immunoglobulin A Fc binding protein of group B streptococci (10), as well as numerous proteins expressed by a wide variety of bacterial species.

PRPs are thought to possess unique hidden receptors, termed cryptitopes, which promote bacterial adherence (8). Several oral bacteria have been observed to bind to adsorbed PRPs on dental enamel surfaces, while agglutinin and glycoprotein are considered to bind to the A-region of PAc (5, 20). Thus, we investigated the interactions between PRPs and each PAc domain in the present experiments. Our immunoblot assay findings showed that the A-region strongly reacted with PRPs (Fig. 3), indicating that they may bind specifically to the A-region in PAc. On the other hand, the P-region protein does not adhere to tooth surfaces, though the proline-rich region is known to be involved in a variety of intra- and intermolecular protein–protein interactions (30). In addition, a fragment of PAc that included the central proline-rich repeat (amino acid residues 816 to 1213) was reported to be protective against recolonization with *S. mutans* in human passive immunization experiments (15). In addition, the P-region protein is an integral component that contributes to the conformation of the central region of PAc and its presence is necessary for surface expression of the molecule on *S. mutans* (4).

In the present study, incomplete PAc was translocated to the supernatants of all PAc-deletion mutant strains, which led us to consider that the P-region may have another biological function that is kept stable by conformation. Proline-rich sequences are frequently repetitive, and generally form extended structures and flexible regions (30). We employed surface plasmon resonance technology with a biomolecular interaction analyzing system (BIAcore) for kinetic analysis of the P-region with PAc. A strong interaction of the P-region with PAc was shown in our results of surface plasmon resonance spectroscopy obtained with the BIAcore, while real-time observations revealed that the P-region protein interacted more quickly with PAc than the A-region protein. The significant association constant (k_a) values obtained by BIAcore analysis demonstrated that the interaction between the P-region protein and PAc was specific. Furthermore, the P-region protein possessed a significantly higher affinity with PAc compared to the A-region protein. We consider that this high level of affinity may promote swift adhesion and aggregation.

In summary, the A-region of PAc of *S. mutans* was shown to bind to proline-rich proteins, which are salivary components, while the P-region also demonstrated a high affinity to PAc. Nevertheless, further investigations are needed to clarify the relationship of PAc and self-aggregation. Additional findings may lead to elucidation of the function of each domain of PAc in *S. mutans* and their involvement in biofilm formation.

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