Oral Microbiology Immunology 2009: 24: 162–169
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ORAL MICROBIOLOGY AND IMMUNOLOGY

Role of lysine in interaction between surface protein peptides of *Streptococcus gordonii* and agglutinin peptide

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Koba H, Okuda K, Watanabe H, Tagami J, Senpuku H. Role of lysine in interaction between surface protein peptides of Streptococcus gordonii *and agglutinin peptide. Oral Microbiol Immunol 2009: 24: 162–169.* © 2009 John Wiley & Sons A/S.

Introduction: *Streptococcus gordonii* interacts with the salivary pellicle on the tooth surface and plays an important role in dental biofilm formation. Reports show that the analog Ssp peptide (A11K; alanine to lysine at position 11 in the arranged sequence, ^{1}DYQAKLAAYQAEL^{13}) of SspA and SspB of *S. gordonii* increased binding to the salivary agglutinin (gp-340/DMBT1) peptide (scavenger receptor cysteine-rich domain 2: SRCRP2). To determine the role of lysine in the binding of the Ssp(A11K) peptide to SRCRP2, we investigated whether an additional substitution by lysine influenced the binding of Ssp(A11K) peptide to SRCRP2 using a BIAcore biosensor assay.
Methods: Six analogs of the Ssp peptide with positive charges in surface positions on the structure were synthesized using substitution at various positions.
Results: The binding activity of analog Ssp(A4K–A11K) peptide was significantly higher than the other Ssp analogs. The binding activity rose under low ionic strength conditions. The distance between positively charged amino acids in the Ssp(A4K–A11K) peptide between 4K and 11K was 1.24 ± 0.02 nm and was close to the distance (1.19 ± 0.00 nm) between Q and E, presenting a negative charged area, on SRCRP2 using chemical computing graphic analysis. The molecular angle connecting 1D–11K–4K in the Ssp(A4K–A11K) peptide secondary structure was smaller than the other peptide angles (1D–11K–XK). The Ssp(A4K–A11K) peptide showed higher inhibiting activity for *Streptococcus mutans* binding to saliva-coated hydroxyapatite than the (A11K) peptide.
Conclusion: The positioning of lysine is important for binding between Ssp peptide and SRCRP2, and the inhibiting effect on *S. mutans* binding to the tooth surface.

Key words: lysine; peptide; *Streptococcus gordonii; Streptococcus mutans;* SRCRP2

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Accepted for publication October 17, 2008

Many oral streptococci including *Streptococcus gordonii* colonize the dental plaque in large numbers and interact with the enamel salivary pellicle to form a biofilm on tooth surfaces. Early biofilm formation occurs after attachment and colonization and is dependent on both the bacterial species involved and the surface composition (4, 16, 33, 37). Mutans streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*) are known to be members of the principal bacterial flora on the tooth surface and are the predominant etiological agents of human dental caries (18, 30). The mutans streptococci are able to adhere as late colonizers to the tooth surface biofilm. The adhesive capabilities of mutans streptococci and *S. gordonii* are facilitated in part by using specific surface adhesins (22, 42, 50). Because streptococcal species are isolated from the same intraoral sites and express similar surface proteins, they may compete for binding to the same array of available host receptors (2, 27, 34).

Streptococcus mutans produces surface protein adhesins, PAc (also known as AgI/II, B, P1, SpaP and MSL-1) at 190 kDa (10, 15, 23, 35, 39, 41); *S. sobrinus* produces PAg (SpaA) at 170 kDa (26, 48); and *S. gordonii* produces SspB (SspA) at 180 kDa (2, 20). These interact with salivary components including lysozyme (40, 44), soluble immunoglobulin A (31, 47), amylase (40), proline-rich proteins of 18 and 38 kDa (40), and salivary agglutinin, a 300–400 kDa protein (9). The salivary agglutinin is a member of the scavenger receptor cysteine-rich (SRCR) superfamily (21, 29). Bikker et al. synthesized consensus-based peptides from the SRCR domain and SRCR-interspersed domains and found that one peptide, the SRCR domain peptide 2 (SRCRP2) bound to *S. mutans* cells (3). Oho et al. demonstrated recombinant PAc (rPAc) bound to SRCRP2 (36). The SspB (390–T400K–402) analog peptide from the A-region of SspB was derived from *S. gordonii* and was homologous to the PAc(365–377) peptide from *S. mutans* MT8148 (43, 46) and the SspA peptide from *S. gordonii* M5 (11). SspB (390–T400K–402) peptide showed the greatest binding response to salivary components and to SRCRP2 (19); and inhibited the binding of *Streptococcus sanguinis* to salivary components by more than 50%, measured using a BIAcore assay (19). The report suggests that the region containing lysine may have binding activity to the salivary components in *S. gordonii* and *S. sanguinis*. We speculate that this peptide, in addition to inhibiting *S. gordonii* and *S. sanguinis* adhesion, may inhibit colonization by *S. mutans*.

Controlling the dental plaque bacteria is important in the prevention and treatment of oral diseases. Therefore, the development of new antimicrobial compounds effective against oral microorganisms is important in oral health research leading to oral disease prevention strategies. Recently, various types of antimicrobial peptides to oral streptococci derived from the host have been reported (1, 14, 49). Another peptide originally isolated from tree frog skin is bactericidal for *S. mutans*, *S. sobrinus*, *Actinomyces viscosus*, *Fusobacterium nucleatum*, and *Escherichia coli*. Other small peptide candidates that combat infectious disease are the tripeptides inhibiting streptococcal infections *in vivo* (38) and the P1025 peptide inhibiting *S. mutans* colonization (24). Lactoferrin peptide, Lf(480–492), inhibited rPAc binding to SRCRP2 (36). Therefore, peptides derived from various sources may have multiple functions in antimicrobial and inhibiting activities; and the effects could be beneficial to oral ecology or their application may interfere with pathogenic microorganisms. However, specificity, stability, and safety studies are required before clinical use can be considered.

Here we determined the role of the lysine residue in the surface protein peptide of *S. gordonii* as it relates to binding to salivary components. The motif sequence of SspA and SspB is -Y—L–Y—L- and is important for its α-helical secondary structure (9, 12, 19, 45). Analog peptides with substitute lysine at various amino acid positions in the peptide were constructed and used to analyse binding activities to the agglutinin peptide, SRCRP2, in BIAcore biosensor experiments. Furthermore, secondary conformations were analysed using crystallographic analyses of the various analog peptides. Adhesion of the radiolabeled reference species to saliva-coated hydroxyapatite (sHA), an *in vitro* model of the tooth surface in the mouth, was measured in the presence of the peptides to determine if the peptides inhibit binding to salivary receptors. The inhibition activities using the analog peptides in *S. mutans* binding with sHA were also studied. Understanding the role of the lysine residue in the binding peptide with SRCRP2 may lead to products to prevent dental caries in the future.

Materials and methods

Bacterial strains and culture

Streptococcus mutans MT8148 was cultured in brain–heart infusion broth (BHI, Difco Laboratory, Detroit, MI) in an aerobic atmosphere of 5% CO_2, 75% N_2, and 20% O_2 (GasPack CO_2, Becton/Deckinson, Sparks, MD) at 37°C before incubation with sHA beads.

Peptide synthesis

The amino acid sequence (DYQAKLAAYQAEL) (Table 1) was identified from streptococcal homologous expressed amino acid sequences where the -Y—L–Y—L motif is important for the α-helix secondary structure (45) in SspA and SspB derived from *S. gordonii* (11); and is homologous to the PAc(365–377, TYEAALKQYEADL) peptide from *S. mutans* MT8148 (Table 1) (19). Various peptides were constructed with substituted lysine where they expressed two or three positive charges on the peptide surface using Molecular Operating Environment (MOE) analysis (Table 2 and Fig. 1) (19). A peptide analog of the Ssp peptide was synthesized by changing the A at positions 4 and 11 to K, yielding Ssp(A4K–A11K). The PAc(316–334, YQTELARVQKANADAKATY) peptide derived from *S. mutans* (43) was used as a control for the adhesion blocking assay to sHA beads. PAc(316–334) contains two lysines but not

Table 1. Identification of universal sequence from homologous Ssp peptides

Peptide	Amino acid sequence[a]												
SspA(162-174)	E	Y	E	A	K	L	A	Q	Y	Q	K	D	L
SspA(187-199)	D	Y	Q	N	K	L	S	A	Y	Q	A	E	L
SspA(269-281)	D	Y	Q	A	K	L	A	A	Y	Q	A	E	L
SspA(326-338)	T	Y	E	A	A	L	K	Q	Y	E	A	D	L
SspA(351-363)	D	Y	Q	T	K	L	A	E	Y	Q	T	E	L
SspA(408-420)	D	Y	E	A	K	L	A	K	Y	E	A	D	L
SspB(163-175)	E	Y	E	A	K	L	A	Q	Y	Q	K	D	L
SspB(188-200)	D	Y	Q	N	K	L	S	A	Y	Q	T	E	L
SspB(270-282)	D	Y	Q	A	K	L	A	A	Y	Q	T	E	L
SspB(352-364)	D	Y	Q	A	K	L	A	A	Y	Q	T	E	L
SspB(409-421)	D	Y	E	A	K	L	A	K	Y	E	A	D	L
Consensus sequence	[1]D	Y	Q	A	K	L	A	A	Y	Q	A	E	L[13][b]

[a]Various Ssp A and Ssp B sequences were selected in previous report (11).
[b]Number indicated position in 13 mer amino acid residues.
Major amino acid in box was selected at each position as a consensus sequence.

Table 2. Amino acid sequences of Ssp peptide substituted with lysine

Peptide	Amino acid sequence
Consensus sequence	[1]DYQAKLAAYQAEL[13a]
Ssp(A11K)	DYQAKLAAYQ**K**[b]EL
ssp(Q3K-A11K)	DY**K**AKLAAYQ**K**EL
Ssp(A4K-A11K)	DYQ**K**KLAAYQ**K**EL
Ssp(A7K-A11K)	DYQAKL**K**AYQ**K**EL
Ssp(A8K-A11K)	DYQAKLA**K**YQ**K**EL
Ssp(Q10K-A11K)	DYQAKLAAY**K**K**K**EL
Ssp(A4K-A11K)[2]	DYQ**K**KLAAYQ**K**EL–DYQ**K**KLAAYQ**K**EL

[a]Number indicated position in 13 mer amino acid residues.
[b]The substituted amino acid with lysine was indicated in bold.

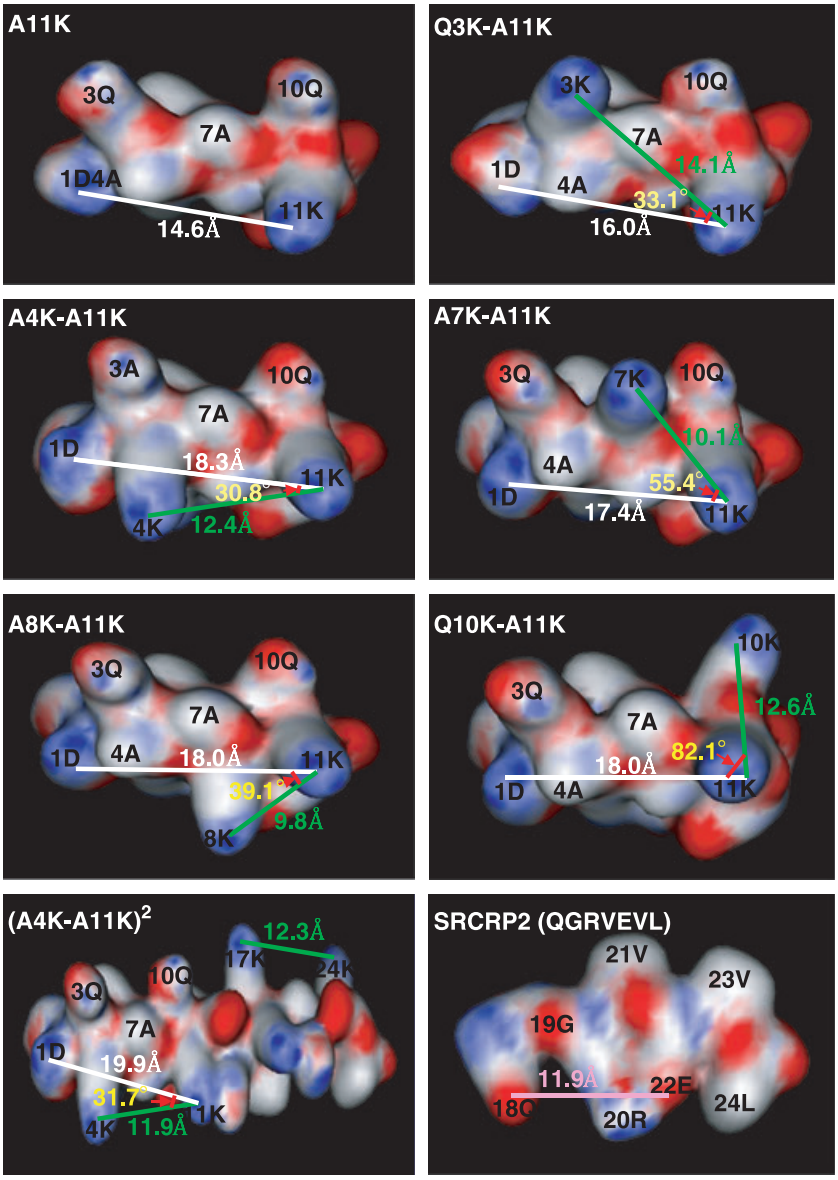

Fig. 1. Schematic representation of Ssp peptides and SRCRP2 peptides. Models of the secondary structure and surface charge (positive [blue] and negative [red]) in the peptides were constructed using MOE software with CHEMICAL COMPUTING GRAPHICS. Position numbers and the amino acid residues on the surfaces of the peptides are indicated. White and green lines indicate distances between 1D and 11K, and XK and 11K on α-helical structure in Ssp peptides. Pink line indicates a distance between 18Q and 22E on the β-sheet in SRCRP2. Arrow indicates angle between three positive charge residues.

the sequence (TYEAALKQYEADL) that is important for binding activity to salivary components. The scavenger receptor cysteine-rich domain peptide 2 (SRCRP2; QGRVEVLYRGSWGTVC) of the agglutinin/gp340/DMBT1 was used as the receptor for the synthesized bacterial peptides and was also synthesized. All peptides were synthesized using the stepwise solid-phase procedure at Scrum Inc. (Tokyo, Japan). The synthesized peptide samples were subsequently purified using reverse-phase high-performance liquid chromatography (HPLC) with a SunFire^{TM} column (4.6 × 150 mm; Nihon Waters K.K., Tokyo, Japan) using a 5–45% acetonitrile gradient in 0.1% trifluoroacetic acid for 50 min at a flow rate of 5 ml/min. Purity was determined to be greater than 95% using HPLC. The peptides were dissolved in sterile demineralized water at 1 mg/ml; and aliquots were freeze-dried and stored at −20°C.

Modeling of the secondary structure by surface charge

Modeling of the secondary structures using the surface charges of Ssp(A11K) was performed using a MOE with the developing platform of PPh4Dock and the MMFF94s force-field subroutines, which are used for energy evaluation in this program (17). Ssp(A3K–A11K), Ssp (A4K–A11K), Ssp(A7K–A11K), Ssp(A8K–A11K), Ssp(Q10K–A11K), and doubling the synthesis peptide of Ssp(A11K) were evaluated and analysed on the basis of an α-helical structure using the MOE system (version 2006–2008, Chemical Computing Group Inc., Montreal, Quebec, Canada) (Fig. 1). An α-helical structure was constructed using a ϕ-angle of −65° and a ψ-angle of −39° with energy minimization of possible structures in these peptides; construction was performed using atom size, bond stretch, angle bend, stretch-bend, out-of-plane, torsion, and charge. Conversely, SRCRP2 is composed of two short β-sheets joined by a β-turn (3) and SRCRP2 (QGRVEVL) in a β-sheet was also constructed in MOE (Fig. 1). The distance between XK and 11K, and the angle among positive charge residues were independently analysed three times.

Immobilizing the ligand

The binding of analog peptides to SRCRP2 was determined using a BIAcore Biosensor System (BIAcore 2000, BIAcore AB, Uppsala, Sweden). We used a standard CM5 sensor chip that has a carboxymethylated

dextran-coated gold surface on the sensor chip and was activated by injecting 70 μl of a solution containing 400 mM *N*-ethyl-*N*′-(3-diethylaminoprophyl) carbodiimide and 100 mM *N*-hydroxysuccinimide at a flow rate of 10 μl/min. Following activation, 70 μl of 0.75 mg SRCRP2 peptide per milliliter in 10 mM sodium acetate buffer (pH 5.0) was applied to the chip and immobilized on its surface. Residual *N*-hydroxysuccinimide esters were then inactivated using 70 μl of 1 M ethanolamine hydrochloride. A flow rate of HBS-EP buffer saline [0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA), 0.005% Surfactant P20] was maintained at 10 μl/min throughout the immobilization procedure. Three millimolar EDTA and 0.005% surfactant P20 were included in the buffer condition to optimize peptide binding to SRCRP2 because excess bound peptide was not completely regenerated by elution buffer and disturbed the next response in the buffer without them. Moreover, the buffer is suitable for the sensitive, stable responses recorded for the molecular binding to the receptors.

Interaction of the peptides with SRCRP2

The peptide solutions at 0.625, 1.25, and 2.5 mM in HBS-EP were exposed to the immobilized ligand on the CM5 sensor chip (flow rate, 10 μl/min); and the dissociation phase was followed by injection of HBS-EP at a rate of 10 μl/min. All binding experiments were conducted at 25°C (19, 43). At the end of each binding cycle, the surface of the sensor chip was regenerated using 50 mM glycine–NaOH (pH 9.5) for 60 s. Ssp peptide solutions at 0.625 mM were prepared with pH 4, 5, 6, and 7.4 in HBS-EP and exposed to an SRCRP2-immobilized CM5 sensor chip surface at a flow rate of 10 μl/min. Moreover, to compare the binding activities of Ssp(A4K–A11K) and Ssp(A4K–A11K)^2 (TABLE 2), peptide solutions at 0.625 mM and 1.25 mM of Ssp(A4K–A11K) and Ssp(A4K–A11K)^2 peptides were exposed to the immobilized ligand. Association (K_a) and dissociation (K_d) rate constants were analysed from the responses of all peptides to SRCRP2 using BIAEVALUATION software (BIAcore AB).

Human saliva collection

Whole saliva from five human volunteers (27–44 years old) was stimulated by chewing paraffin gum; and collected and pooled into ice-chilled sterile bottles over 5 min. The saliva was clarified by centrifugation at 10,000 ***g*** for 10 min at 4°C; filter sterilized; and used immediately to coat HA for the binding assays.

Inhibiting effects of the analog Ssp peptides on binding of *S. mutans* to whole saliva-coated HA

The sHA binding assay employed was originally described by Liljemark et al. (28) and Koga et al. (25), and modified for our research. We used Ssp(A11K) peptide and Ssp(A4K–A11K) peptide, which showed the highest binding activity to SRCRP2 in the BIAcore biosensor assay to assess binding inhibition of *S. mutans* MT8148 to sHA. Consensus sequence peptide and PAc(316–334) peptide, which did not show significant binding activities in the BIAcore assay, were also applied as a control for the binding inhibition assay using sHA. Twenty milligrams of HA (SANGI Co., Ltd, Tokyo, Japan) was equilibrated with phosphate-buffered saline (PBS); soaked in 1.5 ml whole saliva; incubated for 60 min at room temperature; and washed twice with sterilized PBS. Saliva-treated HA was suspended and incubated for 60 min at 37°C in 0, 0.125, 0.25, 0.375, 0.5, 0.625, and 0.75 mM of peptide solution in PBS. *Streptococcus mutans* were grown at 37°C for 18 h in BHI broth containing [methyl-^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) at a final concentration of 10 μCi/ml to a specific activity of between 2.5×10^{-2} and 0.6×10^{-3} counts per min (c.p.m.)/cell. The bacteria were harvested and washed with sterilized PBS three times; suspended in PBS; sonicated on ice for 10 s; and adjusted to 5×10^7 colony-forming units in a 1.5-ml bacterial solution. The saliva-coated (sHA) specimens were suspended in the ^{3}H-labeled bacterial solution and incubated with shaking for 90 min at 37°C. Unattached cells were removed; and beads with bound ^{3}H-labeled bacteria were washed five times with sterilized PBS and transferred to a scintillation vial. After the addition of 10 ml Ultimagold Scintillation Cocktail (Packard Co., Downers Grove, IL), the radioactivity was determined using a liquid scintillation counter (LSC-5000, Aloka Co. Ltd., Tokyo Japan). Unattached cells were also pooled and their radioactivity was determined. In the input of 7.5×10^7 cells, 60% of cells attached to the sHA and showed radioactivity ranging from 36,500 to 42,500 c.p.m. in the assay without the peptide. Background values were less than 100 c.p.m.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney's *U*-test. Differences at the 0.05 level or less were considered to be statistically significant.

Results

Identification of the native amino acid sequence

The consensus amino acid sequence was studied in 11 streptococcal homologous sequences of SspA and SspB to PAc(365–377) (Table 1). The 13 amino acid residues were re-aligned from No. 1 to No. 13. Position 1 showed a negatively charged amino acid, a D in six sequences, and neutral and negative amino acids (E and Q) and T in the other four sequences where D was selected as a major amino acid at position 1. Position 3 showed Q in six sequences and E in five sequences where Q was selected as a major amino acid at position 3. In the same way, amino acids at all positions were selected to yield the consensus sequence: (^{1}DYQAKLAAYQ-AEL^{13}) (Table 1). The sequence differed at position 400 in amino acid residue from SspB(390–402) (DYQAKLAAYQTEL) as previously reported (9). The analog substituted from A to K at position 11 (A11K) was identical with the SspB(390–T400K–402), which was previously reported as the highest binding peptide to SRCRP2 using the BIAcore assay (19). Therefore, we considered that the Ssp(A11K) peptide was a universal binding analog peptide of SspA and SspB to the salivary agglutinin, SRCRP2.

Binding of the Ssp peptides to SRCRP2

To determine the role of lysine in the binding of Ssp(A11K) peptide to SRCRP2, we investigated whether additional substitutions of lysine influenced the binding of the Ssp(A11K) peptide to SRCRP2. Each peptide (0.625, 1.25, and 2.5 mM) was applied to the sensor chip after it was immobilized with SRCRP2. K_a and K_d were observed using BIAEVALUATION software (Fig. 2). The K_a ($7.6 \pm 1.1 \times e^3$) of Ssp(A4K–A11K) peptide to the immobilized SRCRP2 on the sensor-chip was significantly higher than that of the Ssp(A11K) peptides ($3.1 \pm 1.2 \times e^3$) (Fig. 2). Ssp(A4K–A11K) peptide showed dose-dependent binding activities (data not shown). In other lysine-substituted peptides, the K_a ($2.2 \pm 0.6 \times e^3$, $6.8 \pm 4.5 \times e^3$, and $3.8 \pm 1.9 \times e^3$) of Ssp(Q3K–A11), Ssp(A7K–A11K), and Ssp(A8K–

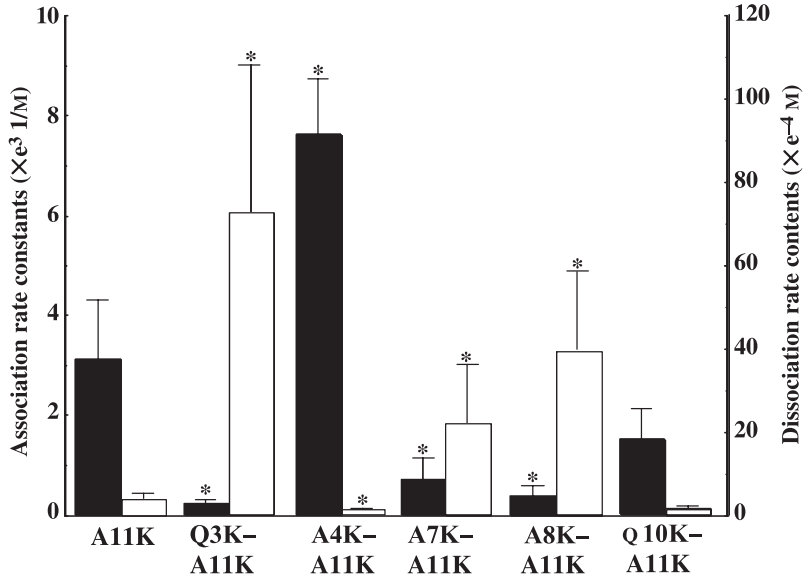

Fig. 2. Binding of various Ssp peptides to immobilized SRCRP2. The peptide solutions (0.625, 1.25, and 2.5 mM) were exposed to the immobilized CM5 sensor chip with SRCRP2 in HBS-EP pH 7.4. The association (K_a, ■) and dissociation (K_d, □) rate constants were analysed using BIAEVALUATION software. The results were expressed as mean ± standard deviation for four independent assays. The asterisks indicate significant difference as follows: one asterisk, $P < 0.01$ compared with the Ssp(A11K) peptide.

A11K) peptide, respectively, were significantly lower than that of Ssp(A11K) peptide (Fig. 2). For the K_d, the inverse relationship of responses between lysine-substituted peptide and Ssp(A11K) compared with K_a was observed (Fig. 2). Control peptide, PAc(316–334) peptide and consensus sequence peptide did not show significant binding activity, and K_a and K_d were not analysed. Moreover, Ssp(A4K–A11K) peptide showed a higher K_a at a low pH than Ssp(A11K) and Ssp(A8K–A11K) peptides (Table 3). Ssp(A8K–A11K) peptide showed a lower K_a than Ssp(A11K) peptide at only pH 6.0 in the buffer. To determine the mechanisms of Ssp(A4K–A11K) peptide binding to SRCRP2, the binding activity of synthetic double peptide Ssp(A4K–A11K)^2 was compared with that of the single peptide of Ssp(A4K–A11K). The K_a ($6.6 \pm 3.0 \times e^3$) and K_d ($1.9 \pm 0.8 \times e^{-4}$) binding activities of Ssp(A4K–A11K)^2 peptide were similar to those ($6.9 \pm 3.2 \times e^3$ and $1.7 \pm 0.9 \times e^{-4}$, respectively) of Ssp (A4K–A11K) peptide. Therefore, the binding between Ssp(A4K–A11K) and SRCRP2 may be dependent on the single interaction receptor and ligand, and on molecular size.

Secondary structure and surface charge

To assess the binding shape of the molecule of the analog peptides to SRCRP2, their secondary structures and surface charges were visually analysed using chemical computing analysis with MOE (Fig. 1). Ssp(A4K–A11K) peptide had the highest binding activity to SRCRP2 (Fig. 2) and showed three surface positive charge areas at residues 1(D), 4(K), and 11(K). The distance between XK and 11K, and angles of 1D–11K–XK in these peptides are shown in Table 3. The distance between 4K and 11K was 1.24 ± 0.02 nm close to the distance (1.19 ± 0.00 nm) between the amino acids (Q and E) presenting a negative charge area in the β-sheet of SRCRP2 (Fig. 1; Table 4). Aspartic acid is not positively charged; however, a surface positive charge area at position 1 of Ssp(A4K–A11K) peptide is partially shown in the crystallographic analysis (Fig. 1) (19). This suggests that the main chain (amidohydrogen) in D is expressed on the surface of the α-helical peptide structure at N-terminal head. The angle among three positive charged residues of 1D–11K–4K was $30.8 \pm 4.4°$ and was smaller than in the Ssp(A7K–A11K), Asp(A8K–A11K) and Ssp(Q10K–A11K) peptides (Fig. 1; Table 4). The angle was similar in the Ssp(Q3K–A11K) peptide and Ssp(A4K–A11K); but the distance between the 3K and 11K was longer in Ssp(Q3K–A11K) than in the Ssp(A4K–A11K) peptide. The expressive direction of the positive charge area at 1D in Ssp(Q3K–A11K) peptide differed from that in the Ssp(A4K–A11K) peptide (Fig. 1). In contrast, the Ssp(Q10K–A11K) peptide showed a close distance between the amino acids presenting a positive charge area compared with between the amino acids presenting a negative charge area on the β-sheet in SRCRP2; but the angle of 1D–11K–10K was higher than those in the other peptides (Table 4). The Ssp(A7K–A11K) and Ssp(A8K–A11K) peptides showed a lower distance in XK–11K than 4K–11K in Ssp(A4K–A11K). The (A4K–A11K)^2 peptide showed a similar distance in XK–11K compared with the Ssp(A4K–A11K) peptide; but the angle of 1D–XK–11K was higher than that of the Ssp(A4K–A11K) peptide.

Effects of Ssp analog peptide on sHA

To observe whether the binding ability of Ssp(A4K–A11K) peptide affected *S. mutans* binding to sHA, a binding assay

Table 3. Association (Ka) and dissociation (Kd) rate constants of three peptides against SRCRP2

	Ka (l/M)			Kd (M)		
	A11K	A4K-A11K	A8K-A11K	A11K	A4K-A11K	A8K-A11K
pH4	$3.9 \pm 1.9 \times e^3$	$9.7 \pm 2.5 \times e^3$*	$4.1 \pm 1.0 \times e^3$	$3.1 \pm 1.3 \times e^{-4}$	$1.1 \pm 0.3 \times e^{-4}$*	$2.6 \pm 0.8 \times e^{-4}$
pH5	$4.6 \pm 1.8 \times e^3$	$9.5 \pm 2.1 \times e^3$*	$4.5 \pm 1.4 \times e^3$	$2.5 \pm 1.1 \times e^{-4}$	$1.1 \pm 0.3 \times e^{-4}$*	$2.2 \pm 0.6 \times e^{-4}$
pH6	$1.7 \pm 0.9 \times e^3$	$6.4 \pm 2.3 \times e^3$*	$6.5 \pm 4.8 \times e^2$**	$8.3 \pm 6.3 \times e^{-4}$	$1.8 \pm 1.0 \times e^{-4}$**	$2.5 \pm 2.1 \times e^{-3}$**
pH7.4	–	$1.4 \pm 0.9 \times e^{-4}$	–	–	$1.3 \pm 1.2 \times e^{-3}$	–

The peptide solutions (0.625 mM) were applied to the immobilized CM5 sensor chip with SRCRP2 in HBS-EP pH4, 5, 6 and 7.4. Ka and Kd were analysed by BIAEVALUATION software. The results are expressed as mean ± SD for six independent assays. The asterisks indicate significant difference as follows: one and two asterisks, $P < 0.01$ and $P < 0.05$ compared the Ssp(A11K) peptide in 4, 5, 6 or 7.4 pH condition.
–, Data were not analysed because of low value in BIAcore.

Table 4. Substituted Ssp peptides' analysis utilizing MOE

Ssp peptide	Distance (Å) between positive charges 1D-11K	Distance (Å) between positive charges on lysine XK-11K		Angle (°) among positive charge 1D-11K-XK	
A11K	14.6 ± 0.3				
Q3K-A11K	16.0 ± 0.3	3K-11K	14.1 ± 0.2	1D-11K-3K	33.1 ± 3.6
A4K-A11K	18.3 ± 0.6	4K-11K	12.1 ± 0.2	1D-11K-4K	30.8 ± 4.4
A7K-A11K	17.4 ± 0.5	7K-11K	10.1 ± 0.2	1D-11K-7K	55.4 ± 0.8
A8K-A11K	18.0 ± 0.9	8K-11K	9.8 ± 0.5	1D-11K-8K	39.1 ± 0.5
Q10K-A11K	18.0 ± 0.8	10K-11K	12.6 ± 0.0	1D-11K-10K	82.1 ± 0.0
$(A4K-A11K)^2$	19.9 ± 0.8	4K-11K	16.9 ± 0.4	1D-11K-4K	31.7 ± 5.0

The results were analysed in chemical computing graphic (Fig. 1) and expressed as mean ± SD for three times analysis.

of *S. mutans* using saliva-coated HA beads was performed. Cell adherence by *S. mutans* was significantly inhibited by pretreatment with 0.625 mM Ssp(A11K) peptide on the sHA but not by pretreatments with less than 0.625 mM of the peptide (Fig. 3). The binding was also inhibited by pretreatment using the Ssp(A4K–A11K) peptide at 0.5, 0.625, and 0.75 mM (Fig. 3). However, the consensus sequence peptide and control peptide, PAc(316–334), did not show a significant inhibition in application of peptide at 0.625 mM (Fig. 3). This suggests the Ssp(A4K–A11K) peptide has a higher inhibition activity in binding *S. mutans* to sHA than the Ssp(A11K) peptide.

Discussion

In the N-terminal region (residues 1–429) of *S. gordonii* M5 cells, the surface adhesins, SspB and SspA, are 96% identical and show homology with SpaP (PAc) at 63% and 60% identity, respectively (10). The A-region is composed of three long and two incomplete repeating sequences (33). Therefore, it is considered that the some sequences that are homologous to the amino acid sequence ^{365}TYEAALKQYEADL^{377} of PAc(365–377) (43, 45) exist in SspA and SspB. In the present study, the consensus sequence was found by arrangement and might be an important sequence for the initial attachment of *S. gordonii* to the tooth surface. The sequence includes only one lysine, at position 5 where its binding activity is weak. However, the analog, Ssp(A11K) peptide, has a substitute A to K at position 11 in the native protein and shows the highest binding to the salivary components and SRCRP2 (19); it may also have an inhibiting effect on competing with colonizers such as *S. gordonii*, *S. sanguinis*, and *S. mutans*. Here we observed the inhibiting effects of Ssp(A11K) peptide in the binding assay of *S. mutans* to sHA (Fig. 3). The inhibiting effects were also observed in the binding assay of *S. gordonii* but not that of *Streptococcus mitis* (data not shown). *Streptococcus gordonii* may compete with *S. mutans* but not *S. mitis* for salivary adhesion sites such as the homologous SspA and SspB regions and PAc(365–377). Additional substitution by lysine at position 4 in Ssp(A11K) peptide elevated the binding and bacterial inhibiting activities of the Ssp(A11K) peptide. Furthermore, the binding activity rose at low ionic strength using the BIAcore assay (Fig. 3). The data suggest that the presence of an additional positive surface charge by substituting two lysines may have an important role in the binding of the peptide and may lead to stronger inhibition of bacteria to the tooth surface. Additionally, the data demonstrate that of the three cationic lysine residues, only the residues Lys-4 and Lys-11 contribute to the binding to SRCRP2. Further analysis shows that Lys-4 in the α-helical structure makes a significant contribution to the surface positive potential of the peptide.

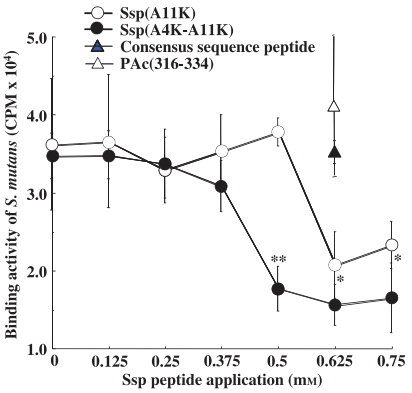

Fig. 3. Inhibition by Ssp peptides of *Streptococcus mutans* adherence on saliva-coated hydroxyapatitie (sHA). The adherence levels (counts per minute) of *S. mutans* MT8148 to sHA treated with 0, 0.125, 0.375, 0.5, 0.625, and 0.75 mM of Ssp(A11K) and Ssp(A4K–A11K) peptides, respectively, are shown. Treatments with 0.625 mM, consensus sequence peptide and PAc(316–334) peptide were performed as controls. The results are expressed as mean ± SD for three independent assays.

The physical interaction of these cationic peptides to anionic interfaces was previously investigated for other antimicrobiological agents (5, 6, 8, 32). Many naturally occurring antimicrobial peptides consist of linear molecules with the potential to adopt an amphipathic α-helical confirmation and interact with lipid bilayers in membranes (13, 51). Davies et al. reported that the α-helical structure region containing three lysine residues in a linear fatty acid-binding protein interacted with the anionic interface as a result of initial electrostatic interaction (8). Only those cationic residues contribute significantly to this binding. Other investigators suggest that apoliprotein A-I lysine positive charges play an important role in the specific recognition of negatively charged phospholipids on the surface of an ABCA1 (5). These results support our data that the positively charged amino acid residue, i.e. lysine, is essential for binding activity on the peptide surface, the positively charged α-helical structure, to the negatively charged interface on SRCRP2.

Cheng et al. suggest that ion-pairing (lysine and negative charged functional groups) interactions are important for protein stabilization (7). The C-terminal neighbor of lysine at position 4 and 11 was Q, having a negatively charged amino acid at positions 3 and 10 in the Ssp(A4K–A11K) peptide (Table 2). Other substituted peptides did not show such ion-pairing in addition to Q and K at position 10 and 11. Therefore, it is considered that the substitutions of two lysines, which induce a significant binding activity to SRCRP2, form durable interactions with the negatively charged amino acid; Q. The stability may influence the inhibition activity of *S. mutans* binding to sHA.

The differences in the secondary structure between Ssp(A4K–A11K) peptide and other analogous peptides were visualized on the basis of the α-helical structure; and the surface charges were plotted using chemical computing graphics (Fig. 1). The binding activity may be associated with the molecular distance between surface positive charges in the α-helical structure and may be close to the distance between the amino acids presenting surface negative charges in the SRCRP2 β-sheet. In contrast, the K_a of Ssp(Q3K–A11K), Ssp(A7K–A11K), and Ssp(A8KA11K) peptides (which were observed to have different distances between the positive charges and between the negative charges from SRCRP2), were lower than those of

Ssp(A4K–A11K) and Ssp(A11K) peptides (Table 3). Therefore, double lysine-substituted Ssp peptides excepting Ssp(A4K–A11K) might change the conformation of the Ssp(A11K) peptide, which was required for binding activity. The expressive directions of some of the positive charges in the Ssp(A4K–A11K) peptide were different from the other peptides (Fig. 1). The angle connecting the three positively charged residues (1D–11K–4K) in the Ssp(A4K–A11K) peptide was smaller than the other peptide angles (1D–11K–XK) (Fig. 1; Table 3). These results indicated the peptide-positioning amino acid with a positive charge, the distance between positively charged residues, the expressive direction of the positive charge, and the linear-like connection with the positively charged residues on the α-helical confirmation may be necessary for the inhibition of *S. mutans* adherence on the tooth surface.

Competitive binding of Ssp(A4K–A11K) peptide is strongly suggested to occur through interactions with a salivary pellicle constituent(s) and the adhesion to sHA is maintained even in the presence of a competing peptide. We speculate that the Ssp(A4K–A11K) peptide binds to SRCRP2 and interferes with the attachment of *S. mutans* to the salivary components. Another possibility that cannot be ruled out is that the Ssp(A4K–A11K) peptide inhibits binding of *S. mutans* to other salivary receptors. The binding of fluorescein isothiocyanate-labeled peptide to the sHA can be visualized using a microscope (data not shown) and the inhibiting level of Ssp(A4K–A11K) peptide was about 50%. In other reports, anti-PAc(365–377) antibodies and monoclonal antibody from mice immunized with PAc(365–377) peptide show 47.2% and 56.6% binding inhibition to salivary components and tooth surface in rats (43, 46). The homologous region to PAc(365–377) in SspA/B and PAc might not contribute completely to the binding of *S. gordonii* and *S. mutans* to salivary components. Taken together, the inhibition by Ssp (A4K–A11K) peptide may be restricted by the potential of interactions between SspA/B and salivary receptors and the affinity levels of peptides to the salivary receptors.

Our data suggest that the binding to salivary agglutinin and the specific inhibition of *S. mutans* adherence required the positioning of a positive charge on lysine at positions 4 and 11 in the Ssp consensus sequence and three surface positive charge connections on α-helical structure. The Ssp(A4K–A11K) peptide may be employed as an antiadherence peptide in future therapy of oral infections. It may, therefore, be useful for the prevention of dental caries in clinical treatments.

Acknowledgments

This work was supported in part by a grant-in aid for the Development Scientific Research (19659559) from the Ministry of Education, Science, and Culture of Japan, and by a grant from the Ministry of Health, Labor and Welfare (H19-Medical Services-007).

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