Role of the Charged Tail in Localization of a Surface Protein Antigen of *Streptococcus mutans*

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To make clear the role of the C terminus of a surface protein antigen (Pac) of *Streptococcus mutans*, stepwise truncations beginning at the C terminus of Pac were performed by utilizing site-directed mutagenesis. A remarkable increase in the amount of cell-free Pac was observed upon deletion of four or more amino acid residues at the C terminus. On the other hand, the amount of cell surface Pac gradually decreased when increasing numbers (four or more) of amino acid residues were deleted at the C terminus, and deletion of six amino acids involving both the total charged tail and Leu, an amino acid residue immediately upstream of the charged tail, resulted in a drastic reduction in the amount of cell surface Pac. These results indicate that the cytoplasmic charged tail and Leu residue are required for cell surface localization of Pac in *S. mutans*.

It has been reported that a 190-kDa protein antigen of *Streptococcus mutans* variously designated as antigen I/II (25), B (26), IF (12), P1 (9), SR (1), MSL-1 (4), and Pac (21) is important in the attachment of the organism to tooth surfaces (14, 17). This protein antigen is considered to be covalently linked to the cell wall of *S. mutans* (11). The gene coding for Pac was cloned and sequenced, and the primary structure of the protein was deduced (13, 16, 21, 22). Pac 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). Recombinant Pac fragments containing the A-region have the ability to bind to salivary components (3, 10, 18, 19). In the C-terminal region of Pac, there is a potential anchor domain which consists of four features: a hydrophilic wall-spanning region rich in glycine and proline, a consensus LPXTG motif, a hydrophobic membrane-spanning region, and a cytoplasmic charged tail (13, 22). The anchor domain shares a consensus structure with various surface proteins of gram-positive cocci (6–8, 27).

Recently, it was reported that deletion of the entire C-terminal domain results in the release of most of the Pac (P1) in the culture medium (11). Schneewind et al. (27–29) reported that the signal both necessary and sufficient for cell wall anchoring of staphylococcal protein A consists of the LPXTG motif, a C-terminal hydrophobic domain, and a charged tail. However, details of the function of the C-terminal domain of Pac have not yet been elucidated. In the present study, we examined the role of the C-terminal domain of Pac in protein localization and found an important role of the cytoplasmic charged tail in the sorting of Pac to the cell surface.

Bacterial strains. Serotype c *S. mutans* MT8148 and its transformants were grown at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.). *Escherichia coli* XL1-Blue [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac F' proAB lacI^q ΔM15 Tn10 (Tet^r)*], JM109 [*recA1 endA1 gyrA96 thi-1 hsdR17(r_K⁻m_K⁺) supE44 relA1 Δ(lac-proAB) F' traD36*

proAB lacI^qΔM15], and HB101 [*recA13 hsdS20 (r_B⁻m_B⁻) supE44 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 ml-1 leuB6*] were used in DNA manipulations. BMH 71-18 *mutS* [*Δ(lac-proAB) supE thi-1 mutS215::Tn10 (Tet^r) F' traD36 proA⁺B⁺ lacI^qΔM15*] and CJ236 [*dut-1 ung-1 thi-1 relA1/pCJ105 (F' Cam^r)*] were used for site-directed mutagenesis. *E. coli* strains were grown in Luria-Bertani medium at 37°C. The following antibiotics were used at the indicated concentrations: ampicillin at 50 μg/ml and erythromycin at 100 μg/ml for *E. coli*; erythromycin at 10 μg/ml for *S. mutans*.

DNA manipulations. Isolation of plasmid DNA by a rapid alkaline lysis procedure, digestion with restriction endonucleases, ligation with T4 DNA ligase, transformation of *E. coli* with recombinant plasmids, agarose gel electrophoresis, and Southern blot analysis with a digoxigenin-labeled probe were performed as described previously (31). Transformation of *S. mutans* was carried out by the method of Perry et al. (23).

Western blotting (immunoblotting). Lyophilized culture supernatants (10 mg) of *S. mutans* strains prepared as described previously (20) were suspended in 1 ml of 10 mM Tris-HCl (pH 6.8) containing 1% (wt/vol) sodium dodecyl sulfate, 1% (vol/vol) mercaptoethanol, and 20% (vol/vol) glycerol and heated for 5 min at 100°C. After the samples (10 μl) were electrophoresed at 25 mA per gel with a 7.5% (wt/vol) stacking gel (15), proteins were electrophoretically transferred to a nitrocellulose sheet by the Western blotting technique (2). The sheet was treated with rabbit anti-Pac serum (20). The antibodies bound to the immobilized replica proteins on the sheet were detected by a solid-phase immunoassay with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Zymed Laboratories, South San Francisco, Calif.).

ELISA. Cell surface localization of Pac was estimated by enzyme-linked immunosorbent assay (ELISA) (24). Whole cells of *S. mutans* strains were washed with phosphate-buffered saline (PBS), and the cell suspension was adjusted with PBS to an optical density at 600 nm (OD₆₀₀) of 0.2. The cell suspension (100 μl per well) was added to each well of a polystyrene microtiter plate, and the plate was centrifuged at 4,000 × g for 15 min. To fix the cells to the microtiter plate, 100 μl of 0.25% glutaraldehyde solution in PBS was added to each well, and the plate was then incubated for 1 h at room temperature. After the glutaraldehyde solution had been removed, the plate was

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FIG. 1. Construction of integration plasmids for deletion of the C-terminal region of PAC. (A) Appropriate DNA fragments from the *pac* gene of *S. mutans* MT8148 and the fragment containing a three-successive-termination-codon unit (indicated by triple asterisks), which was isolated from pQE-30, were ligated to pResEmMCS11. The resultant plasmids were designated pREB-A and pREB-B. (B) The fragment coding for the C terminus of PAC was mutated by site-directed mutagenesis to establish the termination codon at the desired position. The mutated fragments were introduced into pResEmMCS11. The resultant plasmids were designated pREB-Sp, -C6, -C5, -C4, -C3, -C2, and -C1. Amp, β -lactamase gene; Em, erythromycin resistance gene; *cat*, chloramphenicol acetyltransferase gene; MCS, multicloning site; pUC ori, pUC replication origin; P15A ori, P15A replication origin; Ac, *AccI*; Ba, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; Hi, *Hind*III; Nc, *Nco*I; No, *Not*I; Sa, *Sac*I; Sm, *Sma*I; Sp, *Spe*I.

washed three times with PBS. Coated plates were incubated with rabbit anti-PAC serum which was diluted 1:2,000 with PBS (100 μ l per well) and alkaline phosphate-conjugated goat anti-rabbit immunoglobulin G (Zymed Laboratories). The substrate, *p*-nitrophenyl phosphate (1 mg/ml) in 0.01 M diethanolamine buffer (pH 9.6), containing 2.5 mM MgCl₂ (100 μ l per well), was added to each well of the plate. After incubation for 60 min at 37°C, the OD₄₀₅ was measured by a microplate reader.

Truncation of C terminus of PAC. To delete the C terminus of the PAC protein, chimeric plasmids were constructed to be integrated into the *S. mutans* chromosome. These were composed of pResEmMCS11 (30) and parts of the *pac* gene, which cannot replicate in *S. mutans* (Fig. 1). The 3.7-kb *SacI* fragment, the 2.4-kb *AccI* fragment, and the 3.3-kb *SacI*-*Bam*HI fragment of the *pac* gene were prepared from pPC41 (21). The fragments, except for the 3.7-kb *SacI* fragment, were blunt ended with T4 DNA polymerase. The 3.7-kb *SacI* fragment

TABLE 1. Primers used

Primer	Sequence ^a	Position (bp) ^b
Sp	CTTAGTTACTT <u>GAATG</u> AATTCGCTTGGTTT	4650–4679
C6	TTTGCTTGGTTAAGAGCTCAAGAAAGATT	4668–4696
C5	GCTTGGTTTATAGGCGAATTCAGATTGACAG	4671–4701
C4	TGGTTTAAAGTGAGAGCTCGATTGACAGC	4674–4702
C3	TTTAAAGGCTTAGGAATTCGACAGCATA	4677–4705
C2	AAAGGCTAAGTAAGAATTCAGCATAGAC	4680–4708
C1	GGCTAAGAAATAGTGAGCTCATAGACATTA	4683–4712

^a Altered nucleotides are indicated by asterisks. The consequent stop codon is double underlined, and restriction sites generated are underlined (*Eco*RI, GAATTC; *Sac*I, GAGCTC).

^b Numbering begins at the start codon of the *pac* gene of *S. mutans* MT8148 (22).

was ligated into the *Sac*I site of pResEmMCS11, and the resultant plasmid was designated pREB-C0. The other fragments were ligated to pResEmMCS11 at the *Sma*I site (Fig. 1A). The orientation of the fragments was checked by restriction pattern. The plasmids in which the *Not*I and *Sac*I sites were located in the region downstream of the 3' end of the *pac* gene were selected. The three-successive-termination-codon unit on the *Nco*I-*Sac*I fragment derived from pQE-30 (Quigen GmbH, Hilden, Germany) was introduced into the *Not*I and *Sac*I sites of the selected plasmids. Before introducing the termination codon unit, the *Not*I and *Nco*I sites were blunt ended with T4 DNA polymerase. The resultant plasmids were designated pREB-A and pREB-B for the 2.4-kb *Acc*I fragment and the 3.3-kb *Sac*I-*Bam*HI fragment derivatives, respectively (Fig. 1A).

Plasmids for C-terminal truncation of PAc, deleting up to 12 amino acid residues, were constructed by site-directed mutagenesis (Fig. 1B). The 1.0-kb *Hind*III-*Eco*RI fragment coding for the C terminus of PAc was subcloned into pUC118, and the single-stranded DNA template containing uracil was prepared from *E. coli* CJ236 harboring the plasmid. To generate termination codons in the sequences encoding amino acid residues after the 13th, 7th, 6th, 5th, 4th, 3rd, and 2nd amino acid residues from the C terminus of PAc and the *Eco*RI or *Sac*I site immediately downstream from the region of the generated termination codon, seven phosphorylated oligonucleotides were prepared as shown in Table 1. The oligonucleotides (10 pmol each) were annealed to the uracil-containing single-stranded DNA template (0.5 pmol) in an annealing buffer containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, and 1 mM dithiothreitol. The reaction mixture was incubated for 1 min at 90°C and then allowed to cool to room temperature. The complementary strand was synthesized in a reaction mixture of T7 DNA polymerase, a mix of the four deoxynucleoside triphosphates (10 mM each), and the oligonucleotides used as primers. T4 DNA ligase was used to seal the new-strand DNA to the 5' ends of the oligonucleotides. *E. coli* BMH 71-18 *mutS* was then transformed with the synthesized plasmid DNA. Plasmid DNA was isolated from presumptive mutants. The *Eco*RV-*Eco*RI fragments were isolated from the plasmids which were constructed with primers Sp, C5, C3, and C2, and ligated into pResEmMCS11 at the *Sma*I and *Eco*RI sites. On the other hand, the *Eco*RV-*Sac*I fragments were isolated from the plasmids which were constructed with primers C6, C4, and C1, and ligated into pResEmMCS11 at

the *Sma*I and *Sac*I sites. The resultant plasmids were designated pREB-Sp, pREB-C6, pREB-C5, pREB-C4, pREB-C3, pREB-C2, and pREB-C1 for plasmids which were constructed with primers Sp, C6, C5, C4, C3, C2, and C1, respectively (Fig. 1B).

S. mutans MT8148 was transformed with the circular pREB-A, pREB-B, pREB-S, pREB-C6, pREB-C5, pREB-C4, pREB-C3, pREB-C2, pREB-C1, and pREB-C0. The resultant erythromycin-resistant transformants were designated strains ΔA, ΔB, ΔS, ΔC6, ΔC5, ΔC4, ΔC3, ΔC2, ΔC1, and ΔC0, respectively (Fig. 2). The C terminus of PAc was truncated in all transformants except for strain ΔC0 by Campbell-type homologous recombination. Strain ΔC0, which produced the full length of PAc, was used as a control. The truncated *pac* gene in strain ΔB expressed the protein possessing one-third of the cell wall-spanning region, and that in strain ΔA expressed the PAc protein lacking the entire anchor domain. The PAc protein translated in strain ΔS, which had 12 amino acid residues deleted at the C terminus, had the entire cell wall-spanning domain involving the LPXTG motif and two-thirds of the membrane-spanning region. Stepwise truncations of the cytoplasmic charged tail, which is composed of five amino acid residues (KAKKD), were accomplished in strains ΔC1, ΔC2, ΔC3, ΔC4, and ΔC5, and PAc produced in strain ΔC6 lacked the entire cytoplasmic charged tail and Leu, an amino acid residue immediately upstream of the charged tail (Fig. 2).

A small amount of 190-kDa protein which reacted with anti-PAc serum was found in the culture supernatant of strain MT8148. Strains ΔC0 and ΔC1 did not show a significant change in the amount of cell-free 190-kDa protein which reacted with the antiserum (Fig. 3A, lanes 1 to 3). The amount of the protein in the culture supernatants of strains ΔC2 and ΔC3 increased slightly (Fig. 3A, lanes 4 and 5), and a greater increase of the protein in the culture supernatants was observed when PAc lacked four or more amino acids at the C terminus (Fig. 3A, lanes 6 to 11). The protein which was detected with anti-PAc serum in the culture supernatant of strain ΔA showed a molecular mass of 180 kDa (Fig. 3A, lane 11).

Deletion of a C-terminal amino acid from PAc in strain ΔC1 had no significant effect on cell surface localization of the protein antigen as compared with those in strains MT8148 and ΔC0 used as controls (Fig. 3B). A significant decrease of cell surface PAc was not observed in strain ΔC2 or ΔC3 (Fig. 3B). Deletion of four or more C-terminal amino acids from PAc resulted in a significant decrease of cell surface PAc. The amount of the cell surface PAc gradually decreased when an increasing number (four or more) of amino acid residues were deleted at the C terminus. Deletion of six or more amino acid residues at the C terminus showed a drastic reduction in the amount of the protein on the cell surface. No statistically significant difference in the cell surface localization of PAc was observed between any pairs of strains ΔC6, ΔS, ΔB, and ΔA, in which PAc lacked six or more amino acids at the C terminus (Fig. 3B).

Homonylo-McGavin and Lee (11) have recently demonstrated that deletion of the entire C-terminal anchor domain results in secretion of almost all the PAc (P1) into the culture supernatant. In addition, they showed that PAc (P1) which carries only the cell wall-spanning region is partially associated with the cell surface, suggesting that the cell wall-spanning region may play some role in the intercalating of PAc (P1) to the cell wall. In the present study, we could not find any significant difference between the amount of cell surface PAc in strain ΔC6 and that in strain ΔA (Fig. 3B). PAc which was produced in strain ΔC6 has the entire cell wall-spanning region as well as almost all of the membrane-spanning region,

B

FIG. 2. (A) Diagrammatic representation of Pac structure and C-terminal deletion. A-region, V-region, and P-region denote an alanine-rich region, a variable segment, and a proline-rich region, respectively. The numbers in parentheses represent amino acid residue numbers of full-length and truncated Pac proteins (22). The termination codon newly established in the charged tail region by site-directed mutagenesis is indicated by an asterisk. (B) Amino acid sequence of the anchor domain of Pac. The LPXTG motif is underlined.

whereas Pac which was produced in strain ΔA does not have any cell wall-spanning region (Fig. 2). These findings suggest that the cell wall-spanning region of Pac is unlikely to play an important role in its cell surface localization.

Schneewind et al. (27) have demonstrated that cell wall sorting of surface proteins in *Staphylococcus aureus* consists of four distinct steps: (i) the export of the polypeptide into or across the cytoplasmic membrane, (ii) the retention of the polypeptide, (iii) the proteolytic cleavage of the polypeptide

FIG. 3. (A) Western blot analysis of culture supernatants of strain MT8148 and its derivatives. The antigens immobilized on a nitrocellulose sheet were allowed to react with rabbit anti-Pac serum, and bound antibodies were detected by solid-phase immunoassay with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. (B) Estimation of cell surface localization of full-length and truncated Pac proteins by ELISA. Whole cells of *S. mutans* were suspended in PBS to an OD₆₀₀ of 0.2. The cell suspension (100 μl per well) was added to each well of microtiter plates. Rabbit anti-Pac serum diluted 1:2,000 with PBS was added to the wells of coated plates. Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. The OD₄₀₅ was measured after 1 h of incubation with the substrate. Each value represents the mean ± standard deviation of triplicate cultures. The significant difference (*, $P < 0.01$) between ELISA values of strain MT8148 and that of other strains was determined by Student's *t* test. Numbers along x-axis: 1, strain MT8148; 2, strain ΔC0; 3, strain ΔC1; 4, strain ΔC2; 5, strain ΔC3; 6, strain ΔC4; 7, strain ΔC5; 8, strain ΔC6; 9, strain ΔS; 10, strain ΔB; 11, strain ΔA.

chain between Thr and Gly residues of the LPXTG motif with a sortase, and (iv) the linkage of the carboxyl of threonine to the free amino group in the pentaglycine cross-bridge of the staphylococcal cell wall. In addition, they showed that the sorting signal sufficient for the cell wall sorting of staphylococcal protein A consists of the LPXTG motif, a C-terminal hydrophobic domain, and a charged tail (29). Although the C-terminal anchor domains of cell surface proteins in gram-positive bacteria are well conserved, there is heterogeneity in their structures and functions. The charged tail of protein A of *S. aureus* is composed mainly of an Arg residue (29), whereas that of Pac is composed mainly of a Lys residue (13, 22). Moreover, the spacing distances between the LPXTG motif and the charged tail of WapA of *S. mutans* (5) and between the motif and the charged tail of Pac are 29 and 30 residues, respectively. The spacing length in WapA or Pac is shorter than that in protein A (28). The anchor domain of the WapA protein of *S. mutans* does not display cell wall sorting activity in *S. aureus* (28). Mutagenesis analysis of protein A revealed that the retention of the protein from the export pathway depends on the

presence of at least two Arg residues, which are charged amino acids, spaced 31 to 33 residues from the LPXTG motif (28). In this study, a significant reduction of cell surface PAC was observed in strain $\Delta C4$, and further truncation of the C terminus of PAC in strains $\Delta C5$ and $\Delta C6$ resulted in a drastic decrease of the cell surface protein (Fig. 3B). These results suggest that the presence of Leu, Lys, and Ala residues at the C terminus probably functions as a PAC cell surface sorting signal. A charged amino acid, Lys, at the C terminus, spaced 30 residues from the LPXTG motif, and the adjacent hydrophobic amino acids, Leu and Ala, may function as a stop transfer signal to position the LPXTG motif at the surface of the membrane to be acted upon by a sortase (27). Further work, however, is needed to clarify the precise anchor mechanism of PAC in *S. mutans*.

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