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Saliva-Binding Protein (SsaB) from *Streptococcus sanguis* 12 Is a Lipoprotein

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Two lipoprotein consensus sequences (Leu-X-X-Cys) are found in the presumptive signal peptide region (positions 12 to 15 and 17 to 20) of saliva-binding protein (SsaB) from *Streptococcus sanguis* 12. Three analogs of SsaB containing Cys→Gly mutations were constructed by site-directed mutagenesis of pSA2, the recombinant plasmid expressing SsaB. [^{3}H]palmitate was incorporated into SsaB only when the native Cys-20 residue was present. These data show that SsaB is a lipoprotein and that Cys-20 is the critical site for acylation.

Incipient plaque formation has been attributed to the interactions of certain oral streptococci with a variety of salivary components that form the acquired tooth pellicle (2, 8, 17, 21) and to other oral bacteria (12). A small number of potentially adhesive proteins on the surfaces of these streptococci already have been identified and characterized. They include the 162-kDa sialic acid-binding lectin SSP-5 from *S. sanguis* M5 (3); the adhesin cloned from *S. gordonii* G9B (18); a 76-kDa lipoprotein (SarA) from *S. gordonii* Challis which is associated with aggregation in saliva and serum and coaggregation with several *Actinomyces* species (10, 11); the type 1 fimbrial protein from *S. sanguis* FW213 (4, 5); and the 34.7-kDa saliva-binding protein, SsaB, from *S. sanguis* 12 (6, 7). The last two proteins show more than 87% homology (6) and may be members of a group of functionally related adhesins (13).

Jenkinson (10) showed that the N-terminal amino acid sequence of SarA from *S. gordonii* Challis contains a lipoprotein consensus sequence (Leu-X-X-Cys) and that SarA is radiolabeled when [^{3}H]palmitate is added exogenously. Leu-X-X-Cys is the site where signal peptidase II cleaves a prolipoprotein signal peptide (9, 22). Examination of SsaB from *S. sanguis* 12 (6) indicates two putative lipoprotein consensus sequences (Leu-X-X-Cys) at the C-terminal end of the presumptive signal peptide. The present study was undertaken to examine whether SsaB is a lipoprotein and to determine which consensus sequence in the signal peptide region of SsaB is modified.

The *ssaB* gene is 927 bp long and codes for a 309-amino-acid protein, SsaB (6). Inspection of the sequence shows that SsaB contains two putative prolipoprotein consensus sequences (Leu-X-X-Cys) at amino acids 12 to 15 and 17 to 20. The codon for each of the cysteine residues in these sequences was chosen for site-directed mutagenesis. The sequence from the unique *Nco*I site (at −362) to the unique *Mlu*I site (at +74) of plasmid pSA2 (which encodes SsaB) was chosen for amplification by the polymerase chain reaction (PCR) (Fig. 1A). Oligonucleotides were prepared on a DNA synthesizer (PCR Mate; Applied Biosystems, Foster City, Calif.) and purified on oligonucleotide purification cartridges (Applied Biosystems). The GeneAmp Kit (Perkin Elmer Cetus, Norwalk, Conn.) and a thermocycler (Perkin Elmer Cetus) were used. The amplification involved denaturation at 94°C for 1 min, annealing at 55°C for 2.5 min, and extension at 72°C for 3 min, for 30 cycles (1). An oligonucleotide primer containing the *Nco*I site at its 5′ end was made as a common primer. Three oligonucleotide primers (GN1 to GN3) complementary to the sense strand were made with the *Mlu*I site at their 5′ end. Oligonucleotide primers GN1 and GN2 contained a single nucleotide change (from A to C) at the targeted position, as indicated in Fig. 1A, which would result in an amino acid change from cysteine to glycine at positions 20 and 15, respectively. The oligonucleotide primer GN3 has two changes, resulting in the substitution of the amino acid glycine for cysteine at both positions 15 and 20.

pSA2 was digested with *Nco*I and *Mlu*I restriction enzymes, and the products were dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim Corp., Indianapolis, Ind.). The three different PCR-amplified fragments were digested with *Nco*I and *Mlu*I. The deleted pSA2 fragment and the appropriate fragments from the restriction enzyme digests of the PCR products were purified on a low-melting-point agarose gel (FMC Bioproducts, Rockland, Maine), ligated overnight at 16°C, and used to transform *Escherichia coli* DH5α (Life Technologies, Inc., Gaithersburg, Md.) (19). Individual colonies were picked; plasmid DNA was purified by the Magic Miniprep method (Promega, Madison, Wis.) and was used for dideoxy sequencing (20), using Sequenase version 2.0 (United States Biochemical, Cleveland, Ohio) and [^{35}S]dATPαS (Dupont, NEN Research Products, Boston, Mass.) to verify the changes at specific amino acids (Fig. 1B). The three plasmids containing the altered codons specifying the indicated substitutions at residues 20, 15, and both 15 and 20 were designated pGN1, pGN2, and pGN3, respectively.

To show that the mutated plasmids were expressing the proteins of interest, *E. coli* DH5α cells containing the four plasmids, pSA2, pGN1, pGN2, and pGN3, were grown overnight at 37°C. Cells were solubilized in 2× sodium dodecyl sulfate (SDS) solubilization mixture (50 mM Tris-HCl [pH 6.8] containing 2% SDS and 0.1% β-mercaptoethanol) by heating at 95°C for 5 min. Samples were analyzed by SDS–12% polyacrylamide gel electrophoresis (SDS-PAGE) (14). Gels were either stained with Coomassie brilliant blue (Fig. 2A) or blotted to a nitrocellulose membrane. Membranes were blocked with 0.05% Tween 20 in Tris-buffered

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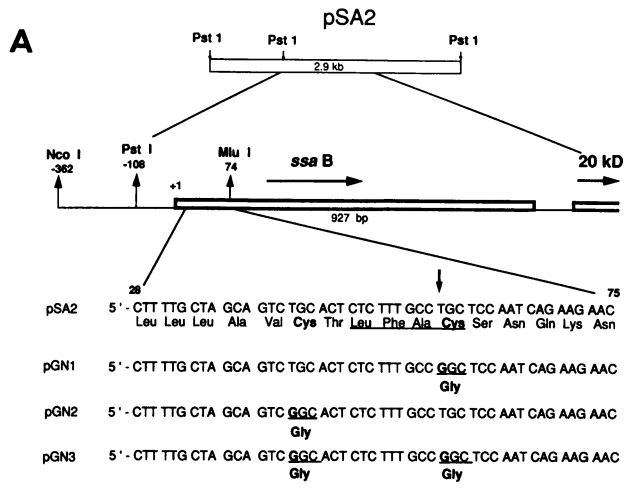

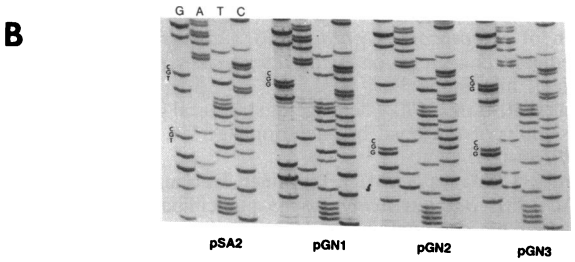

FIG. 1. (A) Partial restriction enzyme map of pSA2 and outline for site-directed mutagenesis. Only restriction enzyme sites used for PCR amplification for mutagenesis are indicated. Nucleotides 28 to 75 of *ssaB* and corresponding amino acids −10 to +6 are shown. The determined consensus sequence for signal peptidase II is underlined, and the cleavage site is indicated by a vertical arrow. The two cysteine residues chosen for mutagenesis are indicated by bold letters. The single T-to-G substitution and the resulting amino acid substituion are indicated by bold letters. (B) Autoradiograph of DNA sequencing gel of the parent plasmid pSA2 and the mutant plasmids pGN1, pGN2, and pGN3. Nucleotide changes that result in the substitution of cysteine to glycine are indicated in small letters adjacent to the G lanes.

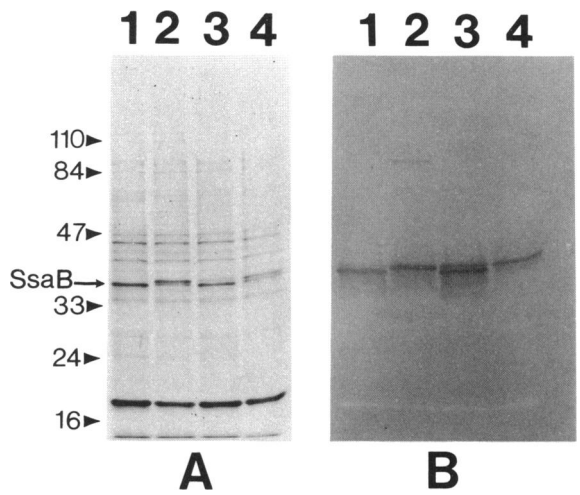

FIG. 2. Protein-stained gel and Western blot (immunoblot) of cell lysates of *E. coli*, harboring the parent and mutant plasmids, probed with anti-SsaB serum. (A) SDS–12% PAGE gel stained with Coomassie brilliant blue; (B) Western blot of the gel probed with anti-SsaB serum. Lane 1, pSA2; lane 2, pGN1; lane 3, pGN2; lane 4, pGN3. Prestained molecular weight markers (Bio-Rad Laboratories, Richmond, Calif.): phosphorylase *b* (110,000), bovine serum albumin (84,000), ovalbumin (47,000), carbonic anhydrase (33,000), soybean trypsin inhibitor (24,000), and lysozyme (16,000). The position of SsaB is indicated.

saline (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) and then incubated with rabbit anti-SsaB immunoglobulin (6) for 18 h at 4°C. The membranes were then washed and incubated with alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Promega) for 1 h and developed with Promega color development reagent (Fig. 2B). SsaB as well as mutant SsaB proteins were expressed in *E. coli* DH5α cells. A site-directed mutation affecting the cysteine in position 20 in plasmids pGN1 and pGN3 reduced the rate of migration of the proteins SsaBgly20 and SsaBgly15gly20 in SDS-PAGE (Fig. 2, lanes 2 and 4, respectively) compared with the migration rate of SsaB expressed by either the parent plasmid pSA2 (Fig. 2, lane 1) or plasmid pGN2 (SsaBgly15) (Fig. 2, lane 3). The slower migration rate of proteins SsaBgly20 and SsaBgly15gly20 is presumably due to the uncleaved signal peptide containing the additional 19 amino acids. SsaBgly15 expressed by pGN2 (Fig. 2B, lane 3) appears as an immunoreactive doublet. Presumably the change of Cys-15 to Gly-15 interferes with cleavage by signal peptidase II, resulting in incomplete formation of mature SsaB.

To determine whether the SsaB protein is a lipoprotein and which consensus sequence is being acylated, a 1-ml portion of each of the four *E. coli* cultures harboring the four plasmids was incubated for 1 h with [9,10-^{3}H]palmitic acid (20 μCi/1.3 nmol/ml) (Dupont, NEN Research Products) (15). Solubilized proteins were resolved by SDS–12% PAGE. The gel was then treated with En^{3}Hance (Dupont), dried onto Whatman 3MM paper under vacuum, and exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C overnight. The results showed that SsaB had radioactivity from the exogenously added [^{3}H]palmitate (Fig. 3, lane 1, arrow). Like SsaB, the mutant SsaBgly-15 protein also was labeled by [^{3}H]palmitate (Fig. 3, lane 3). In contrast, an amino acid change at Cys-20 resulted in no labeling by [^{3}H]palmitate (Fig. 3, lanes 2 and 4). Weak labeling of lower-molecular-size material in all four lanes was seen and was probably due to acylation of host *E. coli* proteins. Incorporation of [^{3}H]palmitate at the amino-terminal cysteine of the mature SsaB protein supports the previous observation that SsaB is blocked because of an N-terminal modification (6). These results provide evidence that SsaB is a lipoprotein and indicate that the consensus sequence of residues 17 to 20 is recognized for protein modification. The lipid moiety of SsaB might be used to anchor the protein by the N-terminal end in the cell membrane, which could allow other parts of the protein to interact with salivary receptors.

Lipid modification of the lipoproteins in bacteria occurs by addition of an acyl glyceride residue onto an appropriate cysteine residue (9, 22). Subsequent cleavage of the protein by signal peptidase II occurs in both gram-positive and gram-negative bacteria (9, 15, 16, 22).

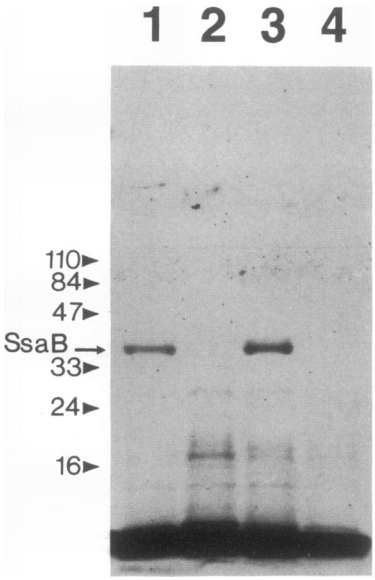

FIG. 3. Radiolabeling of *E. coli* harboring the parent and mutant plasmids with [^{3}H]palmitic acid. Cell lysates were separated by SDS–12% PAGE. The gel was subsequently treated with En^{3}Hance, dried, and fluorographed. Lane 1, pSA2; lane 2, pGN1; lane 3, pGN2; lane 4, pGN3. Molecular weight markers are as indicated in the legend to Fig. 2. The position of SsaB is shown.

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