Role of the Carboxyl-Terminal Region of *Porphyromonas gingivalis* Fimbrillin in Binding to Salivary Proteins

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Received 15 July 1996/Returned for modification 6 September 1996/Accepted 6 November 1996

Porphyromonas gingivalis fimbriae are considered to play an important role in the adherence and colonization of the bacteria in the oral cavity. In this study, we generated and purified three carboxyl-terminal variants of recombinant fimbrillin (r-FimA 224-337, r-FimA 266-337, and r-FimA 287-337, corresponding to amino acid residues 224 to 337, 266 to 337, and 287 to 337, respectively, of the 43-kDa fimbrillin of *P. gingivalis* 2561). They were used as inhibitors of *P. gingivalis* cell binding to human salivary protein-coated hydroxyapatite (HAP) beads. All of the carboxyl-terminal region polypeptides inhibited binding in a dose-dependent manner; however, the inhibitory effect of r-FimA 287-337 was less than that of the other two polypeptides when HAP beads were coated with whole saliva or purified salivary proline-rich protein 1 (PRP1). Assays of binding of a synthetic peptide corresponding to amino acid residues 266 to 286 of *P. gingivalis* 2561 fimbrillin to salivary proteins showed that this peptide bound strongly to whole saliva or PRP1 but only weakly to statherin. These results suggest that the carboxyl-terminal region corresponding to amino acid residues 266 to 337 of *P. gingivalis* fimbrillin plays an important role in binding to salivary proteins and that the domain corresponding to amino acids 266 to 286 is likely a major binding site for PRP1s and the domain corresponding to amino acids 287 to 337 is likely a major binding site for statherin.

Porphyromonas gingivalis is a putative pathogen associated with several periodontal diseases, including adult periodontitis, generalized juvenile periodontitis, periodontal abscesses, and refractory periodontitis (16, 26). The adherence and colonization of this organism in the oral cavity are considered to be essential first steps in the pathogenic process of subgingival microbial infection and periodontal tissue destruction (23). *P. gingivalis* fimbriae have been reported to play an important role in the adherence of this bacterium to salivary proteins (13), host epithelial cells (10), and other oral bacteria (8, 12, 18). Recently, it has been reported that a *fimA* gene-inactivated *P. gingivalis* mutant had significantly less ability to bind to saliva-coated hydroxyapatite (HAP) beads and to cause periodontal bone loss in a gnotobiotic rat model of periodontal disease than wild-type *P. gingivalis* (15).

We have previously shown that fimbriae are required for adherence of *P. gingivalis* whole cells to saliva-coated HAP through specific protein-protein interactions (13). Fimbrillin, a 43-kDa monomeric structural subunit of fimbriae, is a major component of the hair-like fimbrial organelle extending from the surface of *P. gingivalis* (25). We expressed and purified recombinant fimbrillin (r-FimA) corresponding to amino acid residues 1 to 337 of the native fimbrillin (1, 21), which showed immunological reactivity similar to that of the native fimbriae. Recently, salivary receptors for r-FimA have been demonstrated to be statherin and salivary acidic proline-rich protein 1 (PRP1) (2). It has been further shown that the carboxyl-terminal synthetic peptides strongly inhibited *P. gingivalis* whole-cell or r-FimA binding to whole saliva-, PRP1-, or

statherin-coated HAP. Further support for the importance of the C-terminal portion of fimbrillin comes from our studies showing that the C-terminally truncated variants of fimbrillin (corresponding to amino acid residues 1 to 265) are significantly less able to bind to statherin and PRP1 than the whole fimbrillin (1).

In this study, to further localize the binding regions within the C-terminal portion of the fimbrillin in *P. gingivalis* binding to salivary proteins, we constructed three C-terminal r-FimA polypeptides and measured their inhibitory effects on *P. gingivalis* whole-cell binding to whole saliva-, PRP1-, or statherin-coated HAP. Moreover, since the domain corresponding to amino acid residues 266 to 286 of the fimbrillin was considered to be a potent binding site for PRP1, the ability of a synthetic peptide corresponding to amino acid residues 266 to 286 of the fimbrillin to bind to salivary proteins was examined.

MATERIALS AND METHODS

Bacterial culture conditions and radiolabeling. *P. gingivalis* 2561 was grown in half-strength (18-mg/ml) brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 5 mg of yeast extract per ml, 5 µg of hemin per ml, and 0.2 µg of menadione per ml. For radiolabeling, the cells were incubated with 5 µCi of [³H]thymidine (NEN Research Products, Boston, Mass.) per ml. After the culture was incubated at 37°C for 2 days in an anaerobic chamber (85% N₂, 10% H₂, and 5% CO₂), the cells were harvested by centrifugation at 4,000 × g for 10 min at 4°C, washed three times in 50 mM KCl (pH 6.5) containing 1 mM KH₂PO₄, 1 mM CaCl₂, and 0.1 mM MgCl₂ (KCl buffer), and suspended in the same buffer. The washed cells were gently agitated with a Pasteur pipette and allowed to stand for 30 s to separate aggregated cells from well-dispersed cells. The well-dispersed floating cells were used for binding and binding inhibition assays. *Escherichia coli* DH5α (Gibco BRL, Gaithersburg, Md.) and BL21 (Gibco BRL), which served as host cells for the expression of r-FimA polypeptides, were cultured in Luria-Bertani (LB) broth (Gibco BRL) or LB agar containing 50 µg of ampicillin per ml.

Construction of the pET-11d plasmid containing the recombinant fimbrillin gene. *P. gingivalis* 2561 genomic DNA was isolated as described previously (6) and used as a template for amplifying the *fimA* gene by PCR. The primers used for PCR were constructed on the basis of the *fimA* gene sequence (6). The primer sequences with restriction sequences (underlined) are as follows: forward

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primers, 5'-GCGCCCATGGCTTTTGG-AGTTGGCGATGAC-3' (selected for r-FimA 1-337, corresponding to amino acid residues 1 to 337 of the fimbriin), 5'-GCGCCCATGGGAATTCAT-CCGACAATCCTG-3' (selected for r-FimA 224-337, corresponding to amino acid residues 224 to 337 of the fimbriin), 5'-GCGCCCATGGCCTACCTGT-ATTGGTAAAC-3' (selected for r-FimA 266-337, corresponding to amino acid residues 266 to 337 of the fimbriin), and 5'-GCGCCCATGGCCAATAAAAT-TGAGCGTAAC-3' (selected for r-FimA 287-337, corresponding to amino acid residues 287 to 337 of the fimbriin); reverse primer, 5'-CGGCGGATCCTCA-CAAAGTAGCATTCTG ACC-3' (selected for all of the recombinant fimbriin polypeptides). These primers were synthesized at the DNA Sequencing and Nucleotide Synthesis Facility of the State University of New York at Buffalo. Plasmid pET-11d (Novagen, Madison, Wis.) was used as the vector for the expression of r-FimA polypeptides. PCR and subsequent cloning of the *fimA* gene into the pET-11d expression vector were carried out as described previously (21). Each amplified DNA was digested with *Nco*I and *Bam*HI (New England Biolabs, Beverly, Mass.), purified from an agarose gel by a Gene Clean procedure (Bio 101 Inc., La Jolla, Calif.), and ligated into the pET-11d vector linearized with restriction enzymes *Nco*I and *Bam*HI (20). The ligated vector was transformed into *E. coli* DH5 α by calcium chloride treatment (20), and the colonies were selected on LB agar plates containing 50 μ g of ampicillin per ml. Plasmid DNA was obtained by the miniprep plasmid method described by Birnboim and Doly (3). DNA sequencing was performed on the double-stranded plasmid DNA at the DNA Sequencing and Nucleotide Synthesis Facility of the State University of New York at Buffalo. Each recombinant plasmid construct was transformed into *E. coli* BL21 by calcium chloride treatment (20), and the colonies were selected as described above.

Generation and purification of recombinant fimbriin. r-FimA polypeptide expression was induced with isopropyl- β -D-thiogalactopyranoside (IPTG), and the polypeptides were purified by a modification of a method described previously (1). Briefly, an overnight culture of the transformed *E. coli* BL21 (10 ml) was inoculated into 500 ml of fresh LB broth containing 50 μ g of ampicillin per ml. The culture was incubated at 37°C with vigorous shaking until the optical density at 590 nm reached 0.4, and then IPTG was added to a final concentration of 1 mM. After an additional 3 h of incubation, the cells were harvested by centrifugation at 2,000 $\times$ g for 15 min at 4°C and washed three times with 50 mM Tris-HCl, pH 8.0 (TB). The cell pellet was suspended in the same buffer and lysed by 10 cycles of 1 min of sonication treatment in an ice bath (Vibra Cell model VC 250; Sonic and Materials Inc., Danbury, Conn.). After the disrupted *E. coli* cells were centrifuged at 10,000 $\times$ g for 30 min at 4°C, the pellet was agitated in TB containing 1 M sucrose overnight at 4°C and then dissolved in TB containing 2% Triton X-100 and 10 mM Na₂EDTA for 4 h at 4°C. Following centrifugation at 10,000 $\times$ g for 30 min at 4°C, the pellet of inclusion bodies was solubilized in TB containing 8 M urea and 2 mM phenylmethylsulfonyl fluoride. The residual insoluble components were removed by centrifugation at 40,000 $\times$ g for 1 h at 4°C, and the supernatant was subjected to gel filtration chromatography with a Sepharose CL-6B column (2.5 by 150 cm; Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) equilibrated with TB containing 8 M urea. Fractions of 4 ml were collected at room temperature at a flow rate of 6 ml per h. Fractions containing r-FimA polypeptides were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out as described below. The fractions containing r-FimA polypeptides were dialyzed extensively against distilled water and lyophilized. The material containing r-FimA 1-337, corresponding to amino acid residues 1 to 337 of the fimbriin, was applied to the same column repetitively. The material containing r-FimA 224-337, r-FimA 266-337, or r-FimA 287-337, corresponding to amino acid residues 224 to 337, 266 to 337, or 287 to 337, respectively, was loaded onto Sephadex G-50 columns (1.4 by 140 cm; Pharmacia LKB Biotechnology Inc.), which were equilibrated with TB containing 8 M urea. Fractions of 6 ml were collected at room temperature at a flow rate of 9 ml per h. The purified r-FimA polypeptides were dialyzed extensively against cold KCl buffer and used for *P. gingivalis* whole-cell binding inhibition assays. The purity of the r-FimA polypeptides was assessed by SDS-PAGE and amino-terminal amino acid sequencing. Amino-terminal sequencing was carried out with an Applied Biosystems 477A/120 gas-phase automatic sequencer as described previously (13, 14).

Collection of saliva. Unstimulated human whole saliva was collected by expectoration from a 32-year-old male donor into a chilled container. Ten percent (vol/vol) enzyme inhibitor solution (KCl buffer containing 2% Na₂EDTA, 10% 2-propanol, and 2 mM phenylmethylsulfonyl fluoride) was added to the saliva to minimize protease and glycosidase degradation (22). After centrifugation at 12,000 $\times$ g for 30 min at 4°C, the clarified saliva was dialyzed extensively against cold KCl buffer and stored at -20°C until use. PRP1 was purified from human submandibular-sublingual saliva and human parotid saliva, and statherin was prepared from human submandibular-sublingual saliva by a method reported previously (5, 19).

Preparation of synthetic fimbriin peptides. Synthetic fimbriin peptides were prepared based on the predicted amino acid sequence of *P. gingivalis* fimbriin (6, 7) and then purified and characterized as described previously (13, 14). The purified synthetic peptide corresponding to amino acid residues 266 to 286 of *P. gingivalis* 2561 fimbriin (p-FimA 266-286 type I) was ¹²⁵I labeled by the chloramine T method as reported previously (specific activity, 7.4 $\times$ 10⁶ cpm/ μ g) (9).

Binding and binding inhibition assays. The assays of inhibition of *P. gingivalis* whole-cell binding to salivary protein-coated HAP by the carboxyl-terminal r-FimA polypeptides were performed by a method described previously (1, 2). Briefly, 2 mg of spherical HAP beads (BDH Chemicals, Poole, England) was added to a siliconized borosilicate culture tube (12 by 75 mm; PGC Scientifics, Gaithersburg, Md.), washed once with distilled water to remove fine particles, and equilibrated in 1 ml of KCl buffer containing 0.04% NaN₃ with a gentle oscillating motion in a Roto-torque rotator (Cole-Parmer Instrument Co. model 7637) at room temperature overnight. The HAP beads were washed twice with KCl buffer; treated with 150 μ l of whole saliva (1.5 mg of protein per ml), PRP1 (100 μ g of protein per ml), or statherin (100 μ g of protein per ml); rotated in the rotator at room temperature overnight, and then washed twice with KCl buffer. Four hundred microliters of ³H-labeled *P. gingivalis* 2561 (2 $\times$ 10⁸ cells; specific activity, 1 cpm/1 $\times$ 10⁴ cells) in KCl buffer and 100 μ l of inhibitors at various concentrations were added to a series of tubes containing salivary protein-coated HAP simultaneously. The mixture was incubated at 20 rpm in the rotator at room temperature for 1 h. The tubes were agitated gently for 5 s every 15 min. After incubation, the reaction mixture was layered on 1 ml of Percoll (Sigma Chemical Company, St. Louis, Mo.) in a new siliconized borosilicate tube to separate cells which were free from those bound to the salivary protein-coated HAP beads. Unbound *P. gingivalis* cells floating on the Percoll layer were removed by aspiration with a Pasteur pipette. The HAP beads with bound *P. gingivalis* whole cells were washed once with 0.5 ml of Percoll and twice with 1 ml of KCl buffer. Five hundred microliters of KCl buffer was added to the tube, the beads were transferred to a vial, and the radioactivity of the beads was determined.

For the binding assay with p-FimA 266-286 type I, different amounts of ¹²⁵I-labeled p-FimA 266-286 type I in a total volume of 200 μ l of KCl buffer were added to a series of tubes containing salivary protein-coated HAP. The mixture was incubated as described above and washed three times with KCl buffer. Five hundred microliters of KCl buffer was added to the tube, the beads were transferred to a vial, and the radioactivity of the beads was determined with a gamma counter. All assays were performed in duplicate or triplicate and repeated twice on different days.

Dot blot assay. For the dot blot assay, 10 μ l of salivary protein (500 μ g of protein per ml) was spotted on a nitrocellulose membrane (Trans-Blot; Bio-Rad Laboratories, Hercules, Calif.) square (1.5 by 1.5 cm). After drying completely, the membrane was blocked with 0.1% filtered casein in TB containing 0.9% sodium chloride (TBS) at room temperature for 1 h and then incubated with ¹²⁵I-labeled p-FimA 266-286 type I at room temperature overnight. On the following day, the membrane was washed three times with TBS and dried. Autoradiography of the membrane was performed at -70°C for 1 day with X-Omat film (Eastman Kodak Co., Rochester, N.Y.) by using an intensifying screen.

Analytical methods. SDS-PAGE was carried out in gels of 15% acrylamide according to the method of Laemmli (11). Samples were prepared for SDS-PAGE in 0.125 M Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol, and 0.001% bromophenol blue with 5% mercaptoethanol at 100°C for 10 min. Gels were stained with Coomassie brilliant blue or silver. Low-molecular-weight markers (Bio-Rad Laboratories) and prestained molecular weight markers (Low range kit; Integrated Separation Systems, Natick, Mass.) were used to estimate the molecular masses of r-FimA polypeptides. For immunoblot analysis, the r-FimA polypeptides separated by SDS-PAGE were transferred to a nitrocellulose membrane (Trans-Blot; Bio-Rad Laboratories) with a semidry transfer system (Semi-phor TE-77; Hoefer Scientific Instruments, San Francisco, Calif.) according to the manufacturer's instructions. Prestained molecular weight markers (Mid range kits SDS-200P; Diversified Biotech, Newton Centre, Mass.) were used for immunoblot analysis. The membrane was probed with antibodies to either *P. gingivalis* fimbriae or r-FimA corresponding to amino acid residues 10 to 337 of *P. gingivalis* fimbriin (21, 25). The protein contents of all samples except PRP1 were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories) with bovine serum albumin (Sigma Chemical Company) as a standard (4). The protein content of PRP1 was calculated by amino acid analysis as described previously (13).

RESULTS

Generation and purification of recombinant fimbriin. In this study, to further define the binding regions within *P. gingivalis* fimbriin, we generated three C-terminal r-FimA polypeptides (r-FimA 224-337, r-FimA 266-337, and r-FimA 287-337 [the numbers indicate the amino acid residues expressed]) in *E. coli* BL21 and examined the purified r-FimA polypeptides for the role of the C-terminal domain in *P. gingivalis* whole-cell binding to salivary protein-coated HAP. Following SDS-PAGE and Coomassie brilliant blue staining, the purified r-FimA migrated as a single component with an apparent molecular mass of 43, 12, 6, or 4.5 kDa for r-FimA 1-337, r-FimA 224-337, r-FimA 266-337, or r-FimA 287-337,

FIG. 1. SDS-PAGE and immunoblot analysis of purified r-FimA polypeptides. (A) SDS-15% PAGE. Lanes: 1, r-FimA 1-337; 2, r-FimA 224-337; 3, r-FimA 266-337; 4, r-FimA 287-337. Molecular size markers are labeled in the left margin. (B) Immunoblot. Electrophoresis was performed as described in Materials and Methods, and polypeptides were transferred to a nitrocellulose membrane. The blot was probed with rabbit anti-rFimA 10-337 antibody followed by horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. The bound antibodies were visualized by the addition of 4-chloro-1-naphthol color development reagent. The samples (except for the molecular size markers) and lanes are as described for panel A.

respectively (Fig. 1A). A similar result was obtained with silver staining. All of the purified r-FimA polypeptides reacted with antiserum against r-FimA 10-337 on immunoblots (Fig. 1B). When antiserum against native fimbriae was used, r-FimA 1-337 and r-FimA 224-337 reacted, but r-FimA 266-337 and r-FimA 287-337 did not (data not shown). This result suggests that immunodominant domain of native fimbriae is located in amino acid residues 1 to 265 of the fimbrillin. The authenticity

of the purified r-FimA polypeptides was confirmed by the findings that the N-terminal 15-amino-acid sequences of the r-FimA polypeptides were identical to amino acid sequences expected based on the *fimA* gene sequence, the r-FimA polypeptides reacted with antibody to r-FimA 10-337, and the C-terminal DNA sequences were identical to DNA sequences expected based on the *fimA* gene sequence.

Binding inhibition by carboxyl-terminal recombinant fimbrillin polypeptides. Inhibitory effects of full-length r-FimA and C-terminal r-FimA polypeptides on *P. gingivalis* 2561 whole-cell binding to salivary protein-coated HAP were tested. Figure 2A, B, and C show the results of the inhibition assays when HAP beads were coated with whole saliva, PRP1, and statherin, respectively. In every case, all of the tested r-FimA polypeptides inhibited the binding in a dose-dependent manner. Among those tested, recombinant fimbrillin 1-337 showed the greatest inhibition and r-FimA 287-337 had the least inhibition activity. Although there was a slight difference in inhibitory effects between r-FimA 224-337 and r-FimA 266-337 when HAP beads were treated with PRP1, a difference was not observed when they were coated with whole saliva or statherin. A difference in inhibitory effects between r-FimA 266-337 and r-FimA 287-337 was observed clearly when HAP beads were coated with whole saliva or PRP1. At a concentration of 5 μ M, r-FimA 266-337 inhibited the binding by 97 and 79%, while the inhibition by r-FimA 287-337 was 77 and 65%, when HAP beads were coated with whole saliva and PRP1, respectively.

Binding of a synthetic peptide corresponding to amino acid residues 266 to 286 of *P. gingivalis* 2561 fimbrillin to salivary proteins. From the results of inhibition studies using the C-terminal r-FimA polypeptides, the domain corresponding to amino acid residues 266 to 286 was considered to play an important role in *P. gingivalis* binding to PRP1. Therefore, we examined the binding ability of a synthetic peptide corresponding to this domain (p-FimA 266-286 type I [YPVLVNFNSN NYTYDSNYTPK]) to salivary proteins (Fig. 3). This peptide strongly bound to whole saliva- or PRP1-coated HAP but had significantly less ability to bind to statherin.

FIG. 2. Inhibitory effects of carboxyl-terminal r-FimA polypeptides on *P. gingivalis* whole-cell binding to salivary protein-coated HAP beads. 3 H-labeled *P. gingivalis* 2561 (2×10^8 cells) in 400 μ l of KCl buffer was added to a series of tubes containing whole saliva-coated HAP (A), PRP1-coated HAP (B), or statherin-coated HAP (C). Assays of inhibition by r-FimA polypeptides (r-FimA 1-337 [○], r-FimA 224-337 [●], r-FimA 266-337 [△], r-FimA 287-337 [▲], and p-FimA 1-16 [□] [control]) were carried out by the simultaneous addition of 100 μ l of each inhibitor at various concentrations. After incubation at room temperature for 1 h, HAP beads were washed and their radioactivities were determined. Values represent the means from three replicates $\pm$ standard deviations. Standard deviations for some mean values were small enough to be within the height of the symbols and are not evident in the figure.

FIG. 3. Binding of ^{125}I -p-FimA 266-286 type I to salivary protein-coated HAP beads. Different amounts of ^{125}I -p-FimA 266-286 type I in a total volume of 200 μl were added to a series of tubes containing whole saliva-coated HAP (○), PRP1-coated HAP (●), or statherin-coated HAP (△). The reaction mixture was incubated at room temperature for 1 h. After incubation, HAP beads were washed and their radioactivities were determined. Values represent the means from three replicates $\pm$ standard deviations. Standard deviations for some mean values were small enough to be within the height of the symbols and are not evident in the figure.

Dot blot assay. To confirm the above-described result, a dot blot assay was done. Autoradiography of membrane blots of whole saliva, PRP1, and statherin incubated with ^{125}I -labeled p-FimA 266-286 type I was performed. ^{125}I -labeled p-FimA 266-286 type I binding to whole saliva or PRP1 was observed clearly, whereas that to statherin was not detected (Fig. 4).

Binding inhibition by synthetic peptides corresponding to amino acid residues 266 to 286 of *P. gingivalis* type II fimbriin. We synthesized a peptide corresponding to amino acid residues 266 to 286 of *P. gingivalis* type II fimbriin based on the DNA sequence reported by Fujiwara et al. (7) (p-FimA 266-286 type II [YPVLVNFNSNNYTYDNGYTPK]). This peptide also inhibited *P. gingivalis* whole-cell binding to whole saliva- or PRP1-coated HAP as strongly as p-FimA 266-286 type I (Fig. 5). At 10 μg , both of them inhibited the binding by more than 90%.

DISCUSSION

In this study, we generated subpeptides of the peptide consisting of amino acids 266 to 337 (peptide 266-337) to further localize the binding region for PRP1 and statherin. We also selected the domain corresponding to amino acid residues 224 to 337 to examine the role of the disulfide bond in the binding. We found no significant difference between the inhibitory effects of r-FimA 224-337 and r-FimA 266-337, suggesting that the disulfide bond between cysteines existing in r-FimA 224-337 is not important in binding of *P. gingivalis* fimbriae. The binding inhibition assay showed that all of r-FimA polypeptides inhibited *P. gingivalis* whole-cell binding to whole saliva-, PRP1-, or statherin-coated HAP. The full-length r-FimA (r-FimA 1-337) showed the strongest inhibitory effect of all r-FimA polypeptides. This likely results from the fact that the full-length r-FimA has amino acids 126 to 146, which have been reported to inhibit *P. gingivalis* or full-length r-FimA binding to salivary proteins (1, 13), as well as the C-terminal region. Since the C-terminal r-FimA polypeptides did not con-

tain this portion, their inhibitory effects may be less than that of r-FimA 1-337. Especially when HAP beads were coated with PRP1, the inhibitory effects of the C-terminal r-FimA polypeptides were much less than that of r-FimA 1-337; therefore, the domain corresponding to amino acid residues 126 to 146 of the fimbriin may be important in *P. gingivalis* binding to PRP1. Moreover, recently it has been reported that this domain strongly bound to fibronectin (24); therefore, this domain may be one of the important binding sites of *P. gingivalis* fimbriae. Further study is necessary to understand the role of this domain.

In our previous study (1), three candidate domains of *P. gingivalis* fimbriin (peptides 126-146, 266-286, and 318-337, respectively) for involvement in PRP1 binding were proposed based on synthetic peptide studies. In the present study, using truncated recombinant proteins as a separate approach, the reason all of the recombinant C-terminal variants inhibited the binding is that they all contain amino acid residues 318 to 337. However, we found differences in the abilities of r-FimA 266-337 and r-FimA 287-337 to inhibit *P. gingivalis* binding to PRP1 and statherin. As shown in Fig. 2, r-FimA 266-337 and r-FimA 287-337 differed in their abilities to inhibit PRP1 binding (the former being stronger than the latter). On the other hand, comparable inhibition was observed for statherin binding for r-FimA 266-337 and r-FimA 287-337. r-FimA 287-337 was more effective in inhibiting statherin binding than in inhibiting PRP1 binding. From these results, we concluded that peptide 266-286 is a stronger PRP1-binding domain than peptide 318-337. Although a role of peptide domain 126-146 in PRP1 binding cannot be ruled out, it may play a minor role, since this domain was less effective in inhibiting fimbriin binding to PRP1 than C-terminal domains (1). In this study, therefore, we focused on peptide domain 266-286 as the major binding site for PRP1. In our previous study (1), peptide 266-286 was utilized as an inhibitor of *P. gingivalis* binding to PRP1, indirectly pointing to its role in PRP1 binding. In the present study, however, we have performed a direct binding assay and a dot blot assay.

In regard to statherin, we previously showed that synthetic peptides 126-146, 266-286, 293-306, and 307-326 inhibited *P. gingivalis* binding to statherin-coated HAP (1). In the present study, all of the C-terminal r-FimA peptides showed similar inhibition. They all inhibited up to 90% at 5 μg , indicating that peptide domains 293-306 and 307-326 are stronger statherin-binding domains than peptide domains 126-146 and 266-286.

Lee et al. (14) have classified *P. gingivalis* fimbriae of various strains into four types (types I, II, III, and IV) on the basis of the sequences of the first 20 N-terminal amino acids. Recently, Fujiwara et al. (7) reported the deduced complete amino acid sequences of fimbriins of nine *P. gingivalis* strains, in agreement with the grouping reported by Lee et al. (14). On the other hand, there was a report that *P. gingivalis* ATCC 53977 (A7A1-28) fimbriae (type II fimbriin) had less ability to bind to saliva-coated HAP than *P. gingivalis* ATCC 33277 fimbriae (type I fimbriin) (17). Therefore, we synthesized peptides

FIG. 4. Dot blot analysis. Five-microgram portions of salivary proteins were spotted on 1.5- by 1.5-cm squares of a nitrocellulose membrane and blocked with casein. The membrane was then incubated with ^{125}I -labeled p-FimA 266-286 type I. Autoradiography was performed after the membrane was washed. Lanes: 1, whole saliva; 2, PRP1; 3, statherin; 4, KCl buffer (control).

FIG. 5. Inhibitory effects of p-FimA 266-286 type I and type II on *P. gingivalis* whole-cell binding to whole saliva- or PRP1-coated HAP beads. (A) ^3H -labeled *P. gingivalis* 2561 (2×10^8 cells) in 400 μl of KCl buffer was added to a series of tubes containing whole saliva-coated HAP (A) or PRP1-coated HAP (B). Assays of inhibition by p-FimA 266-286 type I ($\circ$), p-FimA 266-286 type II ($\bullet$), and p-FimA 1-16 ($\triangle$) (control) were carried out by the simultaneous addition of 100 μl of each inhibitor at various concentrations. After incubation at room temperature for 1 h, the HAP beads were washed and their radioactivities were determined. Values represent the means from three replicates $\pm$ standard deviations. Standard deviations for some mean values were small enough to be within the height of the symbols and are not evident in the figure.

corresponding to amino acid residues 266 to 286 of type I fimbriin (p-FimA 266-286 type I) and type II fimbriin (p-FimA 266-286 type II) on the basis of the result reported by Fujiwara et al. (7). In this region, only two amino acid residues are different: amino acid residues 281 and 282 are Ser-Asn and Asn-Gly for p-FimA 266-286 type I and p-FimA 266-286 type II, respectively. In the binding inhibition assays, both of them strongly inhibited *P. gingivalis* whole-cell binding to whole saliva- or PRP1-coated HAP. Therefore, the difference in these two amino acid residues seems not to have contributed to the difference in the binding abilities of type I and type II fimbriin. Since these peptides had strong inhibitory effects on *P. gingivalis* binding to whole saliva or PRP1, the domain included in amino acids 266 to 286 may be a potent candidate for a vaccine to prevent *P. gingivalis* from adhering and colonizing in the oral cavity.

There is a possibility that the difference in molecular mass affects the inhibitory ability of r-FimA polypeptides. However, since r-FimA 224-337 and r-FimA 266-337 showed similar inhibition activities, the difference in the number of amino acids seems not to be the main contribution to the difference in the inhibitory effects. Although the disulfide bond between two cysteines in r-FimA 224-337 may not be important for *P. gingivalis* binding, as described above, there is a possibility that the three-dimensional structure may be important for binding of *P. gingivalis* fimbriae. The structural conformation of the C-terminal r-FimA polypeptides could be slightly different from that of the intact protein. Further structural study of fimbriae or fimbriin, by X-ray crystallography or nuclear magnetic resonance, may be useful for understanding the mechanism of *P. gingivalis* binding in detail.

Taken together, the data indicate that there are specific binding domains in *P. gingivalis* fimbriae for salivary proteins, amino acid residues 266 to 286 are a potent binding domain for PRP1, and the domain corresponding to amino acid residues 287 to 337 contains a major binding site for statherin. Therefore, these peptides could be good candidates for a vaccine to prevent periodontal diseases associated with *P. gingivalis*.

ACKNOWLEDGMENTS

We thank Darlene Badgett for her assistance in the preparation of this article.

This study was supported by U.S. Public Health Service grants DE08240 and DE04898.

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Editor: J. R. McGhee