INFECTION AND IMMUNITY, May 1996, p. 1631–1637 Vol. 64, No. 5
0019-9567/96/$04.00+0

Structural Domains of *Porphyromonas gingivalis* Recombinant Fimbrillin That Mediate Binding to Salivary Proline-Rich Protein and Statherin

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Received 9 October 1995/Returned for modification 30 November 1995/Accepted 5 January 1996

Fimbriae (the oligomeric form of fimbrillin) are considered important in the adherence and colonization of *Porphyromonas gingivalis* in the oral cavity. In the present study, we have identified the structural domains of *P. gingivalis* fimbrillin that mediate the binding to salivary proline-rich protein 1 (PRP1) and statherin. A series of synthetic fimbrillin peptides were used to localize the active fimbrillin domains involved in the binding to PRP1 and statherin. The binding of ^{125}I-labeled 41-r-Fim (whole-length recombinant fimbrillin, amino acid [aa] residues 1 to 337) to PRP1-coated hydroxyapatite beads (HAP) was strongly inhibited by the fimbrillin C-terminal peptides corresponding to aa residues 266 to 286 and 318 to 337 (peptides 266-286 and 318-337, respectively), while the binding to statherin was inhibited by C-terminal peptides 266-286, 293-306, and 307-326. Peptide 126-146 also showed a weak inhibitory effect, about half that of other active peptides, on the binding to both PRP1 and statherin. *P. gingivalis* whole-cell binding to PRP1- or statherin-coated HAP was inhibited by more than 80% by the same active peptides. To confirm that the C-terminal portion of fimbrillin includes domains responsible for the binding, two C-terminally truncated variants of recombinant fimbrillin were generated and purified. These were designated 34.5-r-Fim, corresponding to aa residues 1 to 286, and 32-r-Fim, corresponding to aa residues 1 to 265. ^{125}I-34.5-r-Fim revealed 35 and 34% loss of binding ability to PRP1 and statherin, respectively. ^{125}I-32-r-Fim had significantly less binding ability to PRP1 and statherin than ^{125}I-34.5-r-Fim, which was reduced 78 and 73%, respectively. Whole-cell binding to PRP1-, statherin-, or whole saliva-coated HAP was inhibited up to 100% by 41-r-Fim, while 32-r-Fim also showed considerable inhibition, possibly due to the region of aa 126 to 146. Collectively, these results suggest that there are separate and multiple binding sites for PRP1 and statherin in the *P. gingivalis* fimbrillin, and the combination of all of these binding sites may be indispensable in establishing stable bacterial adherence to saliva-coated surfaces in the oral cavity.

Porphyromonas (*Bacteroides*) *gingivalis* is a gram-negative black-pigmented anaerobe considered a bacterium associated with several forms of periodontal disease such as adult periodontitis, generalized juvenile periodontitis, periodontal abscesses, and refractory periodontitis (28). This putative periodontopathogen can adhere to a variety of surfaces in the mouth, including epithelial cells, erythrocytes, fibronectin-collagen complex, salivary proteins, and other bacteria (20). This adherence is thought to be mediated by adhesive surface proteins of *P. gingivalis* such as fimbriae, vesicles, hemagglutinin, and proteases (18, 20). These elements would participate in the oral colonization process through epithelial cell receptors, other bacterial surface adhesins, and receptors of salivary and crevicular fluid proteins. The main habitat of *P. gingivalis* seems to be the periodontal pocket in patients with periodontitis and the subgingival flora of healthy subjects (28). *P. gingivalis* is also isolated from the tonsils, tongue, gingiva, and buccal mucous membranes in periodontitis patients (5, 28). Salivary proteins are absorbed in pellicles on the tooth, mucosus membrane, and the surfaces of other oral bacteria. Although the extent to which early colonization of *P. gingivalis* depends on salivary molecules is unclear, specific salivary molecules possessing strong binding abilities to *P. gingivalis* cells would play a critical role in the adherence to saliva-coated oral surfaces by this organism as the very first event in the pathogenic process of microbial infection and periodontal tissue destruction.

Fimbriae or fimbrillin (FimA, the structural subunit of fimbriae) are considered important in adherence and colonization (20). Fimbriae from *P. gingivalis* have been purified and characterized biochemically (24, 27) and reported to mediate the adherence of this organism to host epithelial cells, other oral bacteria, and salivary proteins (20). Recently, it has been reported that a *fimA* gene-inactivated *P. gingivalis* mutant had significantly less ability to bind to whole saliva-coated hydroxyapatite beads (HAP) and to cause periodontal bone loss in a gnotobiotic rat model of periodontal disease than wild-type *P. gingivalis* did (17). The fimbria-deficient mutant was also unable to bind to human gingival fibroblasts and epithelial cells (10).

We previously showed that fimbriae are required for the adherence of *P. gingivalis* cells to whole saliva-coated HAP (15). The binding of *P. gingivalis* to whole saliva-coated HAP was not inhibited by charged ions such as amino acids or carbohydrates such as monosaccharides, disaccharides, hexosamine, and sialic acid but was inhibited by some synthetic

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fimbrillin peptides corresponding to residues 49 to 68 (designated peptide 49-68, for example), 81 to 98, 126 to 146, 186 to 205, 206 to 225, 226 to 245, 266 to 286, 293 to 306, 307 to 326, and 318 to 337 (15). These results suggested that *P. gingivalis* and its fimbriae can likely bind to salivary proteins through protein-protein interactions and that fimbrillin may have specific binding domains for salivary proteins. r-Fim 10-337 (recombinant fimbrillin protein of *P. gingivalis* corresponding to amino acid [aa] residues 10 to 337 of native fimbrillin) has been generated in *Escherichia coli* and shown to be chemically, antigenically, and serologically identical to the native fimbrillin (23, 25). The r-Fim 10-337 bound to whole saliva-coated HAP to a greater extent than native fimbriae did and was thus used to identify salivary components that mediate the binding of *P. gingivalis* through its fimbriae. Salivary acidic proline-rich protein 1 (PRP1) and statherin showed strong binding abilities to r-Fim 10-337 among nine major salivary components in human submandibular-sublingual saliva (1). These two salivary molecules may promote the adherence of *P. gingivalis* to saliva-coated oral surfaces through specific interactions.

P. gingivalis fimbriae of various strains have been classified into four types (I to IV) on the basis of the first 20 N-terminal residue sequences (16). Recently, Fujiwara et al. (7) reported the deduced complete amino acid sequences of fimbrillins of nine *P. gingivalis* strains divided into four different types in agreement with the classification of Lee et al. (16). Purified type I fimbriae have been reported to have a much higher binding ability to whole saliva-coated HAP than purified type II and type III fimbriae (19). This discrepancy of the binding abilities may have a critical influence on the colonization process of numerous *P. gingivalis* strains in the oral cavity.

In this study, 41-r-Fim, corresponding to whole-length (aa 1 to 337) native fimbrillin, has been prepared. The localization of active binding domains of whole-length r-Fim to PRP1 and statherin has been examined by fimbrillin peptide inhibition studies. These findings have been verified by use of C-terminal truncated fimbrillin mutants. The amino acid sequences of the possible binding domains among the four types of fimbrillin are compared.

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are as follows: 41-r-Fim, whole-length r-Fim (molecular size, 41 kDa) corresponding to aa 1 to 337 of native fimbrillin of *P. gingivalis*; 34.5-r-Fim, C-terminally truncated r-Fim (molecular size, 34.5 kDa) corresponding to aa 1 to 286; 32-r-Fim, C-terminally truncated r-Fim (molecular size, 32 kDa) corresponding to aa 1 to 265; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; FMLP, *N*-formylmethionylleucyl phenylalanine.

Bacterial culture conditions. *P. gingivalis* 2561 was grown and radiolabeled with [^{3}H]thymidine as described previously (1). The cells were centrifuged at 4,000 × *g* for 10 min at room temperature, washed three times with 50 mM KCl containing 1 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ (pH 6.0; KCl buffer) (4), and prepared for the *P. gingivalis* binding assays as described previously (1). *E. coli* DH5α (Gibco BRL, Gaithersburg, Md.) and BL21 (Gibco BRL), which served as host cells for the expression of r-Fim, were cultured in LB broth or plates supplemented with ampicillin (50 μg/ml) (23).

Construction of the recombinant plasmid vectors. *P. gingivalis* 2561 genomic DNA was isolated as described previously (6) and used as a template for amplifying the *fimA* gene by PCR. PCR primers based on the *fimA* gene sequence (6) were selected to code for the corresponding amino acid residues of the fimbrillin. Primers were synthesized at the DNA Sequencing and Nucleotide Synthesis Facility of the State University of New York at Buffalo. The primer sequences with restriction sequences (underlined) are as follows: forward primer, 5′-GCGCCCATGGCTTTTGGAGTTGGCGATGAC-3′; reverse primer, 5′-CGGCGGATCCTCACCAAGTAGCATTCTGACC-3′. These primers were selected to amplify a DNA fragment encoding aa residues 1 to 337 of native fimbrillin (41-r-Fim). The forward primer incorporated a *Nco*I site, whereas the reverse primer incorporated the *Bam*HI site and a translational stop site TGA. Plasmid pET-11d (Novagen, Madison, Wis.) was used as the vector for the expression of fimbrillin polypeptide. PCR was performed under conditions described previously (23). The PCR products were resolved on 1.2% agarose gels with Tris-acetate buffer, and the amplified DNA fragment was extracted from the gel by a GeneClean procedure (Bio 101, Inc., La Jolla, Calif.). Subsequent cloning of the *fimA* gene into the pET-11d expression vector was carried out as described previously (23). 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. Primers for sequencing were based on the *fimA* gene.

Purification of r-Fim. 41-r-Fim was expressed in *E. coli* BL21 by a method reported previously (23) and purified by a modification of a method described previously (1). Briefly, cells were harvested by centrifugation at 2,000 × *g* for 15 min and washed three times with 50 mM Tris-HCl (pH 8.0). The cell pellet was suspended in the same buffer and lysed by seven cycles of a 1-min sonication treatment in an ice bath (Vibra Cell model VC 250; Sonic and Materials Inc., Danbury, Conn.). The disrupted *E. coli* cells were centrifuged at 10,000 × *g* for 30 min. The pellet was washed once with 1 M sucrose solution and then dissolved in 50 mM Tris-HCl (pH 8.0) containing 2% Triton X-100 and 10 mM Na_2EDTA at 4°C overnight. After centrifugation at 10,000 × *g* for 30 min, the pellet of inclusion bodies was solubilized in 50 mM Tris-HCl (pH 8.0) containing 8 M urea and 0.1 mM PMSF. The residual unsolubilized components were removed by centrifugation at 100,000 × *g* for 60 min, and the supernatant was used as a starting material for 41-r-Fim purification. The material 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 50 mM Tris-HCl buffer (pH 8.0) containing 8 M urea. Fractions containing 41-r-Fim were dialyzed extensively against cold 20 mM Tris-HCl (pH 8.0) containing 0.1 mM PMSF and then applied to a Q-Sepharose column (1.5 by 12 cm; Pharmacia LKB Biotechnology) equilibrated with 20 mM Tris-HCl (pH 8.0). The column was eluted with a linear gradient of NaCl (0 to 0.5 M) in the same buffer at a flow rate of 1 ml/min. Fractions containing 41-r-Fim were eluted at approximately 0.2 M NaCl and then dialyzed extensively against cold KCl buffer. The purified preparation was used for the binding experiments after the purity of the protein was assessed by SDS-PAGE followed by silver staining and N-terminal sequencing. N-terminal sequencing was carried out with an Applied Biosystems 477A/120 gas-phase automatic sequencer on which 500 pmol of purified protein was loaded as described previously (15, 16).

Collection of saliva samples. Unstimulated human whole saliva was collected by expectoration from a 35-year-old male donor into a chilled container. Ten percent (vol/vol) of the enzyme inhibitor solution (KCl buffer containing 2% Na_2EDTA, 10% 2-propanol, and 2 mM PMSF) (22) was added to the saliva. After centrifugation at 12,000 × *g* for 10 min at 4°C, the clarified saliva was dialyzed extensively against cold KCl buffer and stored at −20°C until use. PRP1 and statherin were prepared from human submandibular-sublingual saliva collected from a 33-year-old female donor as outlined in a previous study (22).

Iodination of r-Fim. Purified r-Fim was ^{125}I labeled by the chloramine T method as reported previously (1). The specific activities of ^{125}I-41-r-Fim, ^{125}I-34.5-r-Fim, and ^{125}I-32-r-Fim were 9.8, 8.6, and 6.7 mCi/μmol, respectively.

Preparation of synthetic fimbrillin peptides. Synthetic peptides were prepared on the basis of the predicted amino acid sequence of *P. gingivalis* 381 fimbrillin (6) and then purified and characterized as described previously (15, 16).

Binding assays of r-Fim and *P. gingivalis* cells to salivary protein-coated HAP. The binding assay of r-Fim or *P. gingivalis* cells to salivary protein-coated HAP was carried out by the method described previously (1). For assays, 150 μl of purified PRP1 (100 μg/ml), statherin (100 μg/ml), whole saliva (1.5 mg/ml), or lipid-free BSA (1 mg/ml; catalog no. A-7030; Sigma Chemical Co., St. Louis, Mo.) as a control was added to the tubes containing washed HAP, and the tubes were rotated in a Roto-torque rotator (model 7637; Cole-Parmer Instrument Co., Chicago, Ill.) at room temperature overnight. The mixtures were then washed twice with KCl buffer.

For the binding of r-Fim, different amounts of ^{125}I-r-Fim (0.125 to 1.25 nmol) in a total volume of 200 μl of KCl buffer were added to tubes containing salivary protein-coated HAP. The mixtures were incubated at 20 rpm in a rotator at room temperature for 1 h. The mixtures were then washed with KCl buffer containing 0.1 M NaCl and 0.01% Tween 20 and washed once more with KCl buffer. The amount of ^{125}I-r-Fim bound was determined as described previously (1). The binding level was calculated by subtracting the nonspecific binding in the presence of excess unlabeled r-Fim (50 nmol per tube).

For the binding study of whole cells, ^{3}H-labeled *P. gingivalis* 2561 (10^8 cells; specific activity, 1 cpm/10^4 cells) in 400 μl of KCl buffer was added to a series of tubes containing salivary protein-coated HAP. After incubation, the radioactivity of the cells bound was determined as described previously (1). The binding level was calculated by subtracting the nonspecific binding, which was determined by the addition of ^{3}H-labeled cells following the incubation of HAP with nonlabeled bacterial cells (10^8 cells per tube).

Inhibition studies using various agents including synthetic fimbrillin peptides were carried out by the simultaneous addition of inhibitors with ^{125}I-41-r-Fim or ^{3}H-labeled whole cells. The salivary protein-coated HAP in control tubes were equilibrated with KCl buffer. For the inhibition assays with 41-r-Fim, 100 μl of ^{125}I-41-r-Fim (10 nmol/ml) and 100 μl of each peptide (500 nmol/ml) were added simultaneously to the tubes containing salivary protein-coated HAP. Fatty-acid-free BSA, which is not a specific inhibitor for fimbrial binding to salivary protein (1, 15), was used as a control (100 μg per tube). For inhibition assays using whole

cells, 200 μl of ^{3}H-labeled *P. gingivalis* cells (5×10^8 cells per ml) and 200 μl of each peptide (500 nmol/ml) were mixed in the same way as described above. All assays were performed in triplicate on three separate occasions.

Generation and purification of C-terminally truncated r-Fims. Two C-terminally truncated r-Fim polypeptides corresponding to aa residues 1 to 286 (34.5-r-Fim) and aa residues 1 to 265 (32-r-Fim) were expressed in *E. coli* BL21. The PCR forward primer used was the same as that used for 41-r-Fim. The reverse primers used were as follows: 5′-CGGCGGATCCTCACTTAGGCGTATAATTGCT-3′ selected for 34.5-r-Fim and 5′-CGGCGGATCCTCAATAGGTCTTGCCTTCTGC-3′ selected for 32-r-Fim. Amplified DNAs with the expected sizes (817 and 880 bp) were observed for each primer pair set. The digested DNA with *Nco*I and *Bam*HI was cloned into the pET-11d expression vector, and DNA sequencing was performed to confirm the sequence of the *fimA* gene fragment. The subsequent expression of protein was carried out as described for 41-r-Fim. The solubilized crude materials of both of the C-terminally truncated r-Fims were subjected to Sepharose CL-6B column chromatography followed by a Q-Sepharose anion-change chromatography as described above. Fractions containing 34.5- and 32-r-Fim were eluted at approximately 0.25 and 0.3 M NaCl, respectively, and then dialyzed extensively against cold KCl buffer. The purity of the C-terminally truncated variant was assessed by the same methods as those used for 41-r-Fim. Binding and inhibition assays with 34.5-r-Fim and 32-r-Fim were performed as described above.

Analytical methods. SDS-PAGE was performed by the method of Laemmli (14). Samples were prepared for SDS-PAGE in 0.125 M Tris-HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, and 0.001% bromophenol blue with 5% β-mercaptoethanol at 100°C for 5 min. Gels were stained with Coomassie brilliant blue or silver. Low-molecular-weight markers (Bio-Rad Laboratories, Hercules, Calif.) were used to estimate the molecular mass of sample proteins. For immunoblot analysis, the proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (Trans-Blot; Bio-Rad Laboratories) with a semidry transfer system (Semi-phore TE-77; Hoefer Scientific Instruments, San Francisco, Calif.) as described in the manufacturer's instructions. The membrane was probed with antibodies to either *P. gingivalis* fimbriae (24) or synthetic fimbrillin peptide 287-306 (3). The protein content of samples was determined with a Bio-Rad protein assay kit with BSA as a standard (2). The contents of peptides and purified proteins were measured by amino acid analysis as described previously (15).

Overlay assay. The overlay assay was done as described previously (1). Salivary samples were dissolved in 0.125 M Tris-HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, and 0.001% bromophenol and incubated for 30 min at room temperature. Preparations separated on SDS-PAGE gels were transferred to nitrocellulose membranes (Trans-Blot). Unoccupied binding sites on the membranes were blocked by incubation for 1 h with KCl buffer containing 1% lipid-free BSA (Sigma; catalog no. A-7030) as a blocking agent (8).

RESULTS

Generation and purification of 41-r-Fim. In our previous study (1), we used r-Fim 10-337 missing 9 aa residues in the N terminus to identify salivary components which bind strongly to *P. gingivalis* fimbriae. To localize domains of fimbrillin corresponding to the binding to PRP1 and statherin, 41-r-Fim was prepared in this study. The PCR-amplified DNA observed to have the expected size (1,033 bp) on an agarose gel was digested with *Nco*I and *Bam*HI and cloned into the pET-11d vector as described previously (23). DNA sequencing was performed to confirm the sequence of the cloned *fimA* gene fragment. The recombinant plasmid containing the correct in-frame coding sequence of fimbrillin was transformed for expression in *E. coli* BL21 as described previously (23). Following SDS–12% PAGE and silver staining, the purified 41-r-Fim migrated as a single component with an apparent molecular mass of 41 kDa and an electrophoretic mobility similar to that of native fimbrillin from *P. gingivalis* 2561 (data not shown). On immunoblot analysis, purified 41-r-Fim reacted with antiserum against native fimbriae (data not shown). The N terminus of r-Fim was Ala, which is the same as that of native fimbrillin. There was no difference observed between the binding levels of ^{125}I-41-r-Fim and ^{125}I-r-Fim 10-337 (1) to PRP1- and statherin-coated HAP.

Binding inhibition by synthetic peptides. A series of synthetic peptides (15) corresponding to the deduced amino acid sequence of the cloned *fimA* gene were used as inhibitors to localize the active binding domains of fimbrillin. As shown in Fig. 1A, peptides 266-286 and 318-337 significantly inhibited the ^{125}I-41-r-Fim binding to PRP1-coated HAP by 84 and 72%, respectively, while peptides 266-286, 293-306, and 307-326 inhibited ^{125}I-41-r-Fim binding to statherin-coated HAP by 65, 58, and 62%, respectively. Peptides 126-146 and 266-286 were effective in inhibiting ^{125}I-41-r-Fim binding to both PRP1- and statherin-coated HAP. Although the inhibitory effect of peptide 126-146 was less than half that of peptide 266-286, the inhibitory level was significant in comparison with that of BSA. ^{125}I-41-r-Fim binding to whole saliva was inhibited by the five peptides described above and peptide 226-245.

In the inhibition study of ^{3}H-labeled *P. gingivalis* cells, the five peptides significantly inhibited whole-cell binding to PRP1 as well as ^{125}I-41-r-Fim binding (Fig. 1B). However, their inhibition levels reached 95% and were greater than those in ^{125}I-41-r-Fim binding. Peptide 226-245 had an inhibitory effect on the binding to PRP1 and whole saliva. Nonspecific binding levels of ^{125}I-41-r-Fim and ^{3}H-labeled cells were less than 5% of the total binding of both.

Preparation of C-terminally truncated r-Fim. The results of the inhibition studies using 41-r-Fim showed that active domains of binding of fimbrillin to PRP1- and statherin-coated HAP are primarily confined to the C-terminal region from aa residues 266 to 337. To confirm this, 3′-truncated *fimA* vector plasmids were constructed and C-terminally truncated r-Fims were generated to assess their ability to bind to PRP1- and statherin-coated HAP. On the basis of the findings of the peptide inhibition assays, 32-r-Fim (aa 1 to 265) was designed to contain one possible binding domain, aa 126 to 146, that showed a weakly inhibitory effect in the 41-r-Fim binding, while 34.5-r-Fim (aa 1 to 286) was designed to contain aa residues 126 to 146 and one additional, possibly strong binding domain, aa 266 to 286. The two polypeptides were expressed and purified to homogeneity by the methods described above (Fig. 2). Both polypeptides reacted with antibodies to native fimbriae but did not react with antibodies to synthetic fimbrillin peptide 287-306, which is a missing region in both C-terminally truncated variants (Fig. 2).

Binding of C-terminally truncated r-Fim to salivary proteins. The binding levels of the C-terminally truncated variants to salivary protein-coated HAP were compared with that of 41-r-Fim (Fig. 3). ^{125}I-34.5-r-Fim bound to PRP1- or statherin-coated HAP by 35 and 34% less than 41-r-Fim did, respectively, while the binding of ^{125}I-32-r-Fim was 78% less to PRP1-coated HAP and 73% less to statherin-coated HAP than the binding of 41-r-Fim. The reductions in the binding level of 34.5-r-Fim and 32-r-Fim were also observed with whole saliva-coated HAP. The binding of 32-r-Fim to PRP1- and statherin-coated HAP was almost equivalent to the levels of all r-Fims to BSA-coated HAP, which could be regarded as background. The domain of aa 126 to 146 in 32-r-Fim seemed to have little effect on the direct binding of r-Fim to salivary proteins. Nonspecific binding levels of all ^{125}I-labeled r-Fims were less than 5% of the total binding amount.

Overlay assay. Autoradiography of the membrane blots of PRP1 and statherin incubated with each of the ^{125}I-labeled r-Fims was performed. ^{125}I-34.5-r-Fim reacted with salivary protein on the membrane was seen as clear radioactive bands corresponding to the positions of PRP1 and statherin as well as ^{125}I-41-r-Fim (Fig. 4). On the other hand, the intensities of the radioactive bands of ^{125}I-32-r-Fim were identical to the background as a result of the interaction of ^{125}I-32-r-Fim with the BSA used as a blocking agent, and the reaction bands with ^{125}I-32-r-Fim were not detected.

Binding inhibition by r-Fims. Full-length r-Fim and C-terminally truncated r-Fims were compared for their ability to

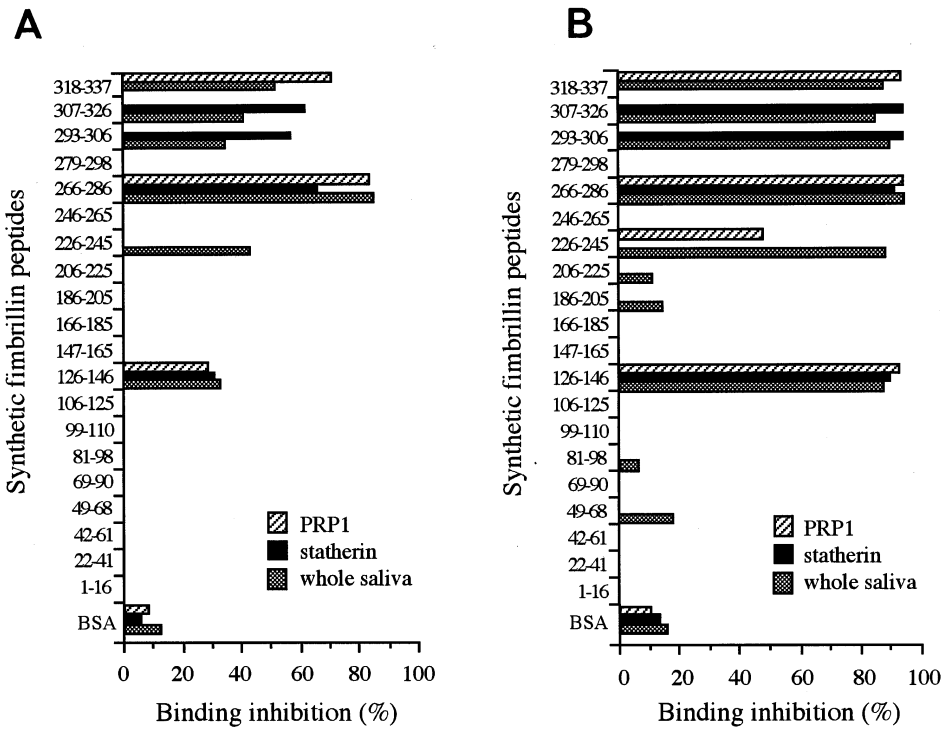

FIG. 1. Inhibitory effects of synthetic fimbrillin peptides in fimbrial binding. HAP coated with PRP1, statherin, or whole saliva were incubated with 41-r-Fim or *P. gingivalis* cells in the presence of each peptide. (A) For the inhibition assay of 41-r-Fim binding, 100 μl of ^{125}I-41-r-Fim (10 nmol/ml) and 100 μl of each peptide (500 nmol/ml) were added simultaneously to the tubes containing salivary protein-coated HAP. (B) For the inhibition assay of whole cells, 200μl of ^{3}H-labeled *P. gingivalis* cells (5×10^8 cells per ml) and 200 μl of each peptide (500 nmol/ml) were mixed the same way as ^{125}I-41-r-Fim. Fatty-acid-free BSA (100 μg per tube) was used as a control inhibitor.

inhibit ^{3}H-labeled whole-cell binding to salivary protein-coated HAP (Fig. 5). The inhibition by 41-r-Fim was significantly effective, and whole-cell binding to any salivary protein-coated HAP was almost completely inhibited by the addition of 1 nmol of protein. 34.5-r-Fim inhibited the binding to PRP1 by 80%, and 32-r-Fim inhibited the binding to PRP1 by 68%. One nanomole of 34.5-r-Fim inhibited binding to statherin and whole saliva by 90 and 70%, respectively, whereas 1 nmol of 32-r-Fim inhibited binding statherin and whole saliva by 60 and 70%, respectively. 32-r-Fim, which showed little direct binding ability, clearly inhibited the binding of all salivary proteins used. BSA as a control showed no significant inhibitory effect in any case.

DISCUSSION

At the beginning of the present investigation, the structural domains of *P. gingivalis* fimbrillin involved in binding to PRP1 and statherin were investigated by a binding inhibition experiment using synthetic peptides. However, small synthetic peptides might be able to inhibit fimbrial binding to salivary proteins by nonspecific interactions such as hydrophobic, hydrophilic, and/or ionic interactions. The three-dimensional structure is also important in exposing or concealing the biologically active region of proteins, whereas the structural conformation of small peptides could be different from that of intact protein. Therefore, two r-Fim variants (34.5-r-Fim and 32-r-Fim) were subsequently generated and used in the binding study to confirm the findings of the peptide inhibition study.

Three peptides, 126-146, 266-286, and 318-337, inhibited the binding of 41-r-Fim to PRP1. The inhibitory ability of peptide 126-146 was less than half that of the other two peptides. In the direct binding experiment of r-Fim variants, 34.5-r-Fim showed a binding level intermediate to those of 41- and 32-r-Fim. 32-r-Fim missing regions of aa 266 to 286 and 318 to 337 revealed a marked reduction in binding ability to PRP1. The qualitative study by overlay assay also showed a significant loss of the binding ability of 32-r-Fim. These results confirm the participation of regions of aa 266 to 286 and 318 to 337 in the fimbrillin binding to PRP1. The region of aa 126 to 146 ap-

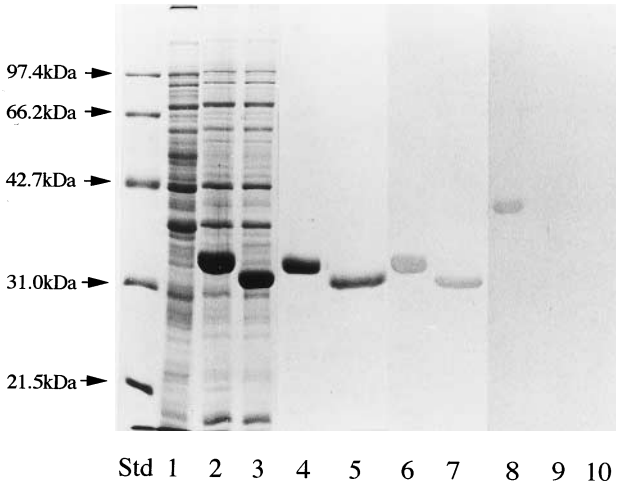

FIG. 2. SDS–12% PAGE of the expressed 34.5- and 32-r-Fim and immunoblot. Lanes: Std, molecular mass standard protein; 1, extracts of cells containing the pET-11d vector (control); 2, extracts of cells in which 34.5-r-Fim is expressed; 3, extracts of cells in which 32-r-Fim is expressed; 4, purified 34.5-r-Fim; 5, purified 32-r-Fim; 6, immunoblot of purified 34.5-r-Fim probed with rabbit anti-fimbrial antibodies; 7, immunoblot of purified 32-r-Fim probed with rabbit anti-fimbrial antibodies; 8, immunoblot of purified 41-r-Fim probed with rat anti-fimbrillin peptide 287-306; 9, immunoblot of purified 34.5-r-Fim probed with rat anti-fimbrillin peptide 287-306; 10, immunoblot of purified 32-r-Fim probed with rat anti-fimbrillin peptide 287-306. The proteins in lanes Std, 1, 2, and 3 are stained with Coomassie brilliant blue, and the proteins in lanes 4 and 5 are stained with silver.

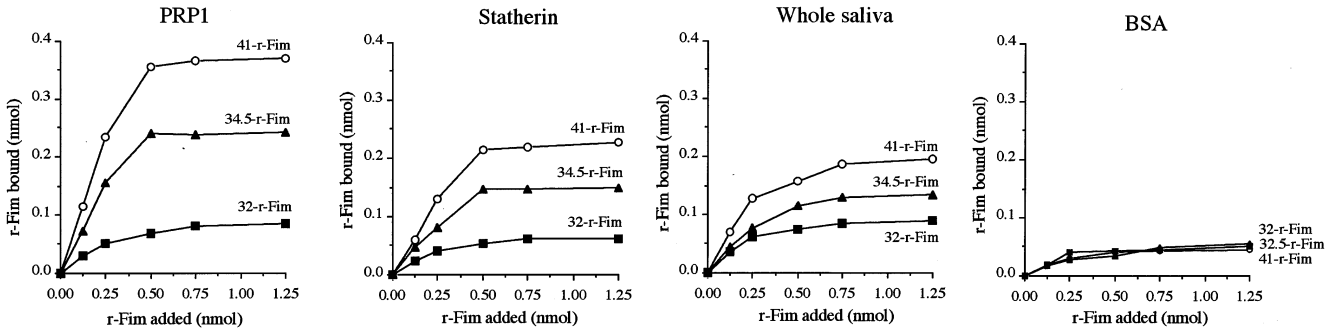

FIG. 3. Binding curves of ^{125}I-r-Fim (41-, 34.5-, and 32-r-Fim) to HAP coated with salivary proteins. Different amounts of ^{125}I-r-Fim (0.125 to 1.25 nmol) in a total volume of 200 μl were added to tubes containing HAP coated with PRP1, statherin, or whole saliva. Lipid-free BSA was used to coat HAP as a control. The reaction mixture was incubated at room temperature for 1 h. After incubation, HAP were washed with buffered KCl containing 0.1 M NaCl and 0.01% Tween 20.

peared to have little effect on direct binding. However, peptide 32-r-Fim had a clearly inhibitory effect on the binding of whole cells to PRP1. On the basis of these findings, all three regions would be involved in the fimbrillin binding to PRP1. 32-r-Fim might fail to bind PRP1 because of the low affinity of the region of aa 126 to 146 to salivary proteins, or a potential provided by only one binding domain may be insufficient to maintain the binding. In a comparison of the amino acid sequences of three possible binding domains to PRP1, the regions of aa 266 to 286 and 318 to 337 proved to have a common sequence, VLVXXN, which may be one of the structural elements involved in the binding (see Fig. 6).

Four peptides, 126-146, 266-286, 293-306, and 307-326, had inhibitory effects on r-Fim and whole-cell binding to statherin. The importance of the regions of aa 266 to 286, 293 to 306, and 307 to 326 was confirmed by the direct binding experiment of r-Fim variants. With respect to the region of aa 126 to 146, 32-r-Fim showed little ability to bind to statherin; however, peptide 126-146 and 32-r-Fim clearly inhibited the whole-cell binding to statherin as well as to PRP1. Therefore, all four domains would have an important role in the binding to statherin. The regions of aa 126 to 146, 266 to 286, and 293 to 306, which are possible statherin binding sites, also have the common sequence XYDXXXT, which may be one of many elements involved in binding (Fig. 6).

In the study of inhibition of binding to whole saliva, the five peptides which inhibited the binding to PRP1 and/or statherin as well as peptide 226-245 showed inhibitory effects. The peptide inhibited whole-cell binding to PRP1, too. The inhibitory effect observed may be due to whole saliva, which is a crude protein preparation. Whole saliva and PRP1 incubated with whole cells could contain proteolytic fragments produced by host- and bacterium-derived proteases (8). The proteolysis of the salivary proteins might expose a hidden receptor region(s) of the salivary proteins (9). Such a receptor region might have reacted with peptide 226–245. It is also possible that there exists a third molecule in parotid saliva that binds strongly to fimbrillin.

It appears that the region of aa residues 318 to 337 is a specific site that mediates fimbrillin-PRP1 interaction and that the region of aa residues 293 to 326 is specifically responsible for fimbrillin-statherin interaction. Two regions, aa residues 126 to 146 and 266 to 286, are likely to act as multifunctional binding sites for both PRP1 and statherin. These results suggest that the active binding sites in fimbrial binding to PRP1 and statherin are located in the C-terminal region of aa residues 266 to 337 and possibly in the region of aa residues 126 to 146 and that the fimbrillin protein moiety has multiple, separate sites for PRP1 and statherin molecules. Collectively, it is proposed that there exist three or more binding sites stereochemically on fimbrillin protein and that the combination of all of these binding sites would be indispensable for binding to salivary proteins and for the stable holding of the fimbrillin moiety on PRP1 and statherin. Taken together, the inhibition of bacterial binding to salivary proteins can be achieved by coverage or interference on only one of multiple binding sites of salivary protein.

The active peptides efficiently inhibited whole-cell binding by 95% to both PRP1 and statherin. However, the peptide inhibition levels in ^{125}I-41-r-Fim binding were less effective than those in whole-cell binding. The concentration of the inhibitory peptide was 50-fold higher (50 nmol) than that of 41-r-Fim (1 nmol), which could be a concentration large enough to show maximum inhibition effects of specific peptides. If PRP1 or statherin had only one site on each protein to bind fimbrillin, an additional competitive peptide should inhibit the ^{125}I-41-r-Fim binding by almost 100% by attachment and coverage of its binding site on PRP1 or statherin. The results in this study suggest that PRP1 and statherin also have multiple binding sites corresponding to each fimbrillin domain represented by inhibitory synthetic peptides. If one binding site of PRP1 or

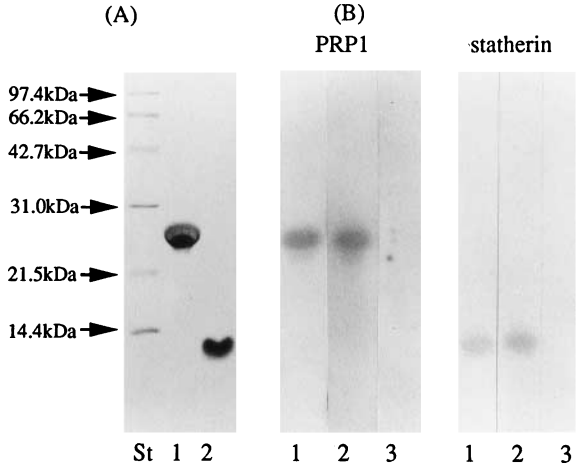

FIG. 4. Overlay assay of the binding of ^{125}I-r-Fim (41-, 34.5-, and 32-r-Fim) to PRP1 and statherin. (A) SDS-PAGE analysis of purified PRP1 and statherin on a 15% gel stained with Coomassie brilliant blue. Lanes: St, molecular mass standard markers; 1, PRP1; 2, statherin. (B) Autoradiography of PRP1 and statherin reacted with ^{125}I-r-Fim. PRP1 and statherin were transferred onto nitrocellulose membranes following SDS-PAGE. The membranes were incubated with ^{125}I-41-r-Fim, ^{125}I-34.5-r-Fim, or ^{125}I-32-r-Fim. Autoradiography was performed after washing the membrane. Lanes: 1, membrane incubated with ^{125}I-41-r-Fim; 2, membrane incubated with ^{125}I-34.5-r-Fim; 3, membrane incubated with ^{125}I-32-r-Fim.

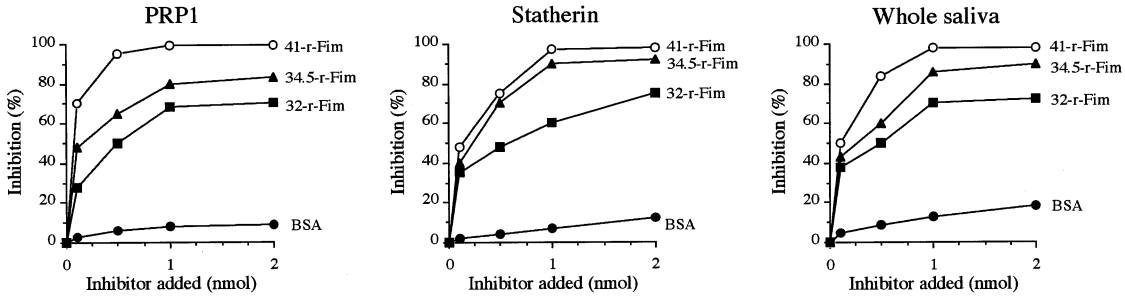

FIG. 5. Inhibitory effects of nonlabeled r-Fim (41-, 34.5-, and 32-r-Fim) in the binding of whole cells. ^{3}H-labeled *P. gingivalis* 2561 (5×10^{8} cells per ml) in 200 μl of buffered KCl was added to a series of tubes containing HAP with PRP1, statherin, or whole saliva. Inhibition studies with inhibitors such as 41-r-Fim, 34.5-r-Fim, 32-r-Fim, or BSA (0.1, 0.5, 1, and 2 nmol) were carried out by the simultaneous addition of 200 μl of each inhibitor at various concentrations as described for ^{125}I-41-r-Fim. After incubation at room temperature for 1 h, HAP were washed with Percoll, and their radioactivity was determined.

statherin could be blocked by a competitive synthetic peptide, the other binding sites could provide the situation to hold the fimbrillin protein on the salivary proteins. The discrepancy in the inhibition levels of synthetic peptides for ^{125}I-41-r-Fim and whole-cell binding could be elucidated by the difference in the molecular masses of whole cells and 41-r-Fim. It is likely that the *P. gingivalis* cells are too large to maintain stable binding to salivary proteins (PRP1 and statherin) without a complete set of binding bridges. On the other hand, 41-r-Fim, a much smaller-molecular-mass species, is able to bind salivary proteins. As described above, there might exist a third salivary molecule binding fimbrillin through the region of aa residues 226 to 245. However, 32-r-Fim containing the region of aa 226 to 245 showed a reduced binding ability to whole saliva as well as PRP1 and statherin. If a third molecule exists, it could have the same binding mechanism as PRP1 and statherin, and binding sites other than aa 246 to 245 would be located in the region of aa 266 to 337.

An example of multiple domains being involved in ligand-receptor binding has been reported in the study of FMLP receptor. Multiple domains of FMLP receptor on phagocytes have been found to be required for high-affinity ligand binding by use of recombinant *N*-formyl peptide receptor variants (21). The binding is completed with a major determinant located in the first extracellular loop (aa residues 40 to 145) and several adjacent transmembrane domains located at aa residues 241 to 351. The cell surface protein isolated from *Streptococcus mutans*, termed streptococcal antigen I/II (SAI/II), has been thought to mediate the bacterial adherence to salivary receptors (12). The interaction has been reported to be promoted through more than one receptor-binding region such as aa residues 1005 to 1024, 1025 to 1044, 1085 to 1104, and 1095 to 1114 in the intact SAI/II (12). Many investigations of the binding through fimbriae of a number of bacteria such as *E. coli*, *Neisseria gonorrhoeae*, and *Pseudomonas aeruginosa* have been reported (reviewed in reference 12). However, unlike protein-

```
      1                                                                  66
Type  I AFGVGDDESK VAKLTVMVYN GEQQEAIKSA ENAT---KVE DIKC-SAGQR TLVVMANTGA MELVGKTLAE
     II :::E--:::: :::::::::: :::::::::: ::::---::: ::::-::::: ::::N::::E :K:A::::::
    III :::NAG::A: :::::::::K :::::::::: ::::---::: N:::-::::: :::::::::G :::A::::::
     IV :V:DGLADA: IT:::A:::A :QI::G::TV :E:DGVL::: G:P:K:GAN: V:::V::H-N YE:T::S:N:

     67                                                           126      134
       VKALTTELTA ENQEAAGLIM TAEPKTIVLK AGKNYIGY-S GTGEGNHIEN D-PLKIKRVH ARMAFTEIKV
       :::::::::: :::::::::: ::::VEVT:V ::N::Y::-D :SQG::Q:SQ :T::E::::: ::::::::::
       :::::::::E G:::::::::: ::::VEVT:V ::N::Y::-D :SQG::Q:SQ :T::E::::R ::I:::K:E:
       :E::::S::: :::N:KN::: :GKSAAFTI: P:S:HY::PD ::TSD:LVSA GT::AVT::: :GIS:AGVE:

     135          146                                                      202
       QMSAAYDNIY TFVP--EKIY GLIAKKQSNL FGATLVNADA NYLTGSLTTF NGAYTPANYA NVPWLSRNYV
       :::PS:V:K: N:A:--:N:: A:V:::E::: :::S:A:S:D A:::::::N: ::::S::::T H:D::G:D:T
       T::QS:A:K: N:A:--:N:: A:V:::K::: :::S:A:N:D A:::::::T: :::::::::T H:D::G:DFT
       N:ATQ:Q:Y: S:N:ADAK:A A:V:::D:KI ::NS:VSNTN A::Y:VQ:P- A:L:::DAAG ETYE:EASLN

     203                                                              266 269
       APAADAPQGF YVLENDYSAN GGTIHPTILC VYGKLQKN-G ADLAGADLAA AQAANWV--D AEGKTYYPVL
       E:SNN::::: ::::ST:AQ: A:-LR::::: :K:::T:HD: TP:SSEEMT: :FN:G:IVA: NNPT::::::
       E:SNN::::: ::::ST:AQ: A:-LR::::: IK:::T:HD: TP:SSEEMT: :FN:G:IVAN NDPT::::::
       -TNYAVGA:: ::::SK:DAS NE-LR::::: I::::LDKD: NP:TEPA:TD :IN:GFC--: GD:T::::::

     270              286      293          306          318      326          337
       VNFNSNNYTY DSNYTPKN-K IERNHKYDIK LTITGPGTNN PENPITESAH LNVQCTVAEW VLVGQNATW
       :::::::::: :NG:::::-: :::::::::: :::::::::: :::::::::: :::::::::: :::::::::
       :::::::::: TGEAVE:G-: :V::::F::N :::::::::: :::::::::: :::N:V::A: KG:V::VI:
       ::YDG:G:I: SGAI:QGQN: :V::NHYK:T :N:::::::T ::::QPVQ:N :::T:Q:TP: :V:N:A:::
```

FIG. 6. Sequence homologies among type I, II, III, and IV fimbrillin of *P. gingivalis*. The deduced amino acid sequences of type I fimbrillin (6), type II fimbrillin of *P. gingivalis* HW24DI, type III fimbrillin of *P. gingivalis* ATCC 49417, and type IV fimbrillin of *P. gingivalis* HG564 (7) are aligned. Spaces (–) are inserted to maximize the sequence homologies. Amino acids identical to those in the type I sequence are represented by colons. Arrows show putative binding domains defined by the peptide inhibition study illustrated in Fig. 3. Symbols: ←——→, synthetic peptide inhibiting fimbrial binding to both PRP1 and statherin; ←- - - -→, synthetic peptide inhibiting fimbrial binding to statherin; ←— — —→, synthetic peptide inhibiting fimbrial binding to PRP1.

protein interactions involved in the fimbria-mediated binding of *P. gingivalis*, the fimbria-associated binding of other bacteria such as *E. coli* is sugar inhibitable (12).

P. gingivalis fimbriae have been divided into four different types on the basis of those amino acid sequences (7, 16). Purified type I native fimbriae have a much higher binding ability to whole saliva-coated HAP than purified type II and type III native fimbriae do (19). The deduced sequences of type I (fimbrillin in this study), type II (HW24DI), type III (ATCC 49417), and type IV (HG564) are listed in Fig. 6. Although amino acid sequence homologies of type I and IV fimbrillin are low, type I, II, and III fimbrillin have considerably high sequence homology. In spite of significant differences in the binding of type I, II, and III fimbrillin to saliva-coated HAP, binding domains among these fimbrillin types do not show significant amino acid changes. However, the sequence heterogeneity observed in a potential supplementary binding site (aa residues 126 to 146) and in the intermediate region (residues 147 to 265) could explain this discrepancy. It is plausible that the intermediate region (residues 147 to 265), together with the potential binding domain (residues 126 to 146), are necessary to form a complete binding bridge. The intermediate region gives a characteristic secondary structure for each type of fimbrillin from the Chou-Fausman prediction reported by Hamada et al. (11). In light of these findings, *P. gingivalis* fimbrial binding to salivary proteins could depend not only on the presence of its specific binding domains but also on their presentation to salivary proteins. The conformation of the intermediate region could be responsible for the availability of binding sites to salivary proteins and hence their binding. Structural variations of assembled fimbrillin in fimbria formation could also be related to the ability to bind to salivary proteins. It has been suggested that 50- and 80-kDa proteins encoded by genes downstream from *fimA* of type I are associated with fimbriae (26). These fimbria-associated proteins might play a role in their assembly and adhesion.

Although further investigation is necessary to understand the fimbrial binding mechanism in detail, the polypeptide corresponding to the C-terminal region of fimbrillin which possesses binding domains for salivary proteins could be used as a vaccine to block fimbria-mediated adherence and possibly other fimbria-host interactions to prevent *P. gingivalis*-associated periodontal disease.

ACKNOWLEDGMENTS

We thank Rose Parkhill and Darlene Badgett for their assistance in the preparation of this article and Satoshi Shizukuishi for his valuable suggestions.

This study was supported by grants DE08240 and DE04898 from the National Institutes of Health.

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Editor: B. I. Eisenstein