INFECTION AND IMMUNITY, Aug. 1994, p. 3372–3380
0019-9567/94/$04.00+0
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Vol. 62, No. 8

Salivary Receptors for Recombinant Fimbrillin of *Porphyromonas gingivalis*

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Received 27 January 1994/Returned for modification 23 March 1994/Accepted 23 May 1994

Fimbriae are considered important in the adherence and colonization of *Porphyromonas gingivalis* in the oral cavity. It has been demonstrated that purified fimbriae bind to whole human saliva adsorbed to hydroxyapatite (HAP) beads, and the binding appears to be mediated by specific protein-protein interactions. Recently, we expressed the recombinant fimbrillin protein (*r*-Fim) of *P. gingivalis* corresponding to amino acid residues 10 to 337 of the native fimbrillin (A. Sharma, H. T. Sojar, J.-Y. Lee, and R. J. Genco, Infect. Immun. 61:3570–3573, 1993). We examined the ability of individual salivary components to promote the direct attachment of *r*-Fim to HAP beads. Purified *r*-Fim was radiolabeled with ^{125}I and incubated with HAP beads which were coated with saliva or purified individual salivary components. Whole, parotid, and submandibular-sublingual salivas increased the binding of ^{125}I-*r*-Fim to HAP beads. Submandibular-sublingual saliva was most effective in increasing the binding of ^{125}I-*r*-Fim to HAP beads (1.8 times greater than that to uncoated HAP beads). The binding of ^{125}I-*r*-Fim to HAP beads coated with acidic proline-rich protein 1 (PRP1) or statherin was four and two times greater, respectively, than that to uncoated HAP beads. PRP1 and statherin molecules were also found to bind ^{125}I-*r*-Fim in an overlay assay. The binding of intact *P. gingivalis* cells to HAP beads coated with PRP1 or statherin was also enhanced, by 5.4 and 4.3 times, respectively, over that to uncoated HAP beads. The interactions of PRP1 and statherin with ^{125}I-*r*-Fim were not inhibited by the addition of carbohydrates or amino acids. PRP1 and statherin in solution did not show inhibitory activity on ^{125}I-*r*-Fim binding to HAP beads coated with PRP1 or statherin. These results suggest that *P. gingivalis* fimbriae bind strongly through protein-protein interactions to acidic proline-rich protein and statherin molecules which coat surfaces.

Periodontal pocket formation is initiated by oral bacteria in dental plaques which form on the tooth surface (10, 42). *Porphyromonas* (*Bacteroides*) *gingivalis* is a gram-negative black-pigmented anaerobe associated with several periodontal diseases including adult periodontitis, generalized juvenile periodontitis, periodontal abscesses, and refractory periodontitis (51). *P. gingivalis*, accompanied by other bacteria including *Streptococcus oralis*, *Streptococcus sanguis*, and *Actinomyces viscosus*, is found in high proportions in dental plaques (11, 45, 46, 51). The adherence and colonization of this organism in the oral cavity are considered to be important as a first step in the pathogenic process of subgingival microbial infection and periodontal tissue destruction. *P. gingivalis* cells adhere to a variety of surfaces including epithelial cells (4, 22, 33), erythrocytes (2, 32, 33, 34), fibronectin-collagen complexes (30, 31), salivary components (15), and other bacteria (17, 26, 27, 40, 43, 48). Those elements which participate in the oral colonization process include bacterial surface adhesins, epithelial surface receptors, and salivary or crevicular fluids which coat the tooth and mucosal surfaces. The extent to which early colonization of *P. gingivalis* depends on salivary molecules is not known. The present studies are directed to evaluating the potential for *P. gingivalis* to bind to specific salivary molecules absorbed in pellicles on the tooth or mucous membranes in the oral cavity. Some cell surface components have been reported to contribute to the adherence properties of *P. gingivalis*, including fimbriae, vesicles, and hemagglutinins (27). Among those adherence-promoting proteins, fimbriae or fimbrillin (the structural subunit of fimbriae) mediate adherence of the organism to host epithelial cells (22) and other bacteria (17). Fimbriae are also involved in other interactions of *P. gingivalis* with host cells. *P. gingivalis* fimbriae can stimulate human gingival fibroblasts to produce thymocyte-activating factor (18) and activate mouse peritoneal macrophages and subsequently induce gene expression and production of interleukin-1 (19). Immunization of germ-free rats with fimbrillin protects against *P. gingivalis*-induced periodontitis (9).

Fimbriae have been purified and characterized biochemically and are considered important virulence determinants (47, 50). Fimbrillin, a 43-kDa monomeric structural subunit of *P. gingivalis* fimbriae, is a major component of the hair-like fimbrial organelle extending from the surface of *P. gingivalis* (47). We have shown previously that fimbriae are required for adherence of *P. gingivalis* to whole saliva-coated hydroxyapatite (HAP) beads (25). The binding of *P. gingivalis* to whole saliva-coated HAP beads was not inhibited by charged ions, amino acids, or carbohydrates such as monosaccharide, disaccharide, hexosamine, and sialic acid but was inhibited by synthetic fimbrial peptides based on the fimbrillin gene sequence of *P. gingivalis* 381. These results suggested that salivary components mediate the binding of *P. gingivalis* to surfaces coated with salivary pellicles. However, the specific salivary components that mediate the binding of *P. gingivalis* through its fimbriae have not been fully identified.

Recently, we expressed and purified a recombinant *P. gingivalis* fimbrillin protein (*r*-Fim), with an apparent molecular mass of 41 kDa, corresponding to the amino acid residues 10 to 337 of the native fimbrillin (44). This *r*-Fim showed immunological reactivity similar to that of purified fimbrillin and

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competitively inhibited up to 80% of *P. gingivalis* whole-cell binding to saliva-coated HAP beads. In this study, we examined the ability of individual purified salivary components to promote the direct attachment of *r*-Fim to HAP beads.

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, 5 μg of hemin, and 0.2 μg of menadione and buffered at pH 7.4 (25). For radiolabeling, the cells were incubated with 5 μCi of [^{3}H]thymidine per ml (Du pont, NEN Research Products, Boston, Mass.) at 37°C for 2 days in an anaerobic chamber (85% N_2, 10% H_2, and 5% CO_2). The cells were centrifuged at 4,000 × *g* for 10 min at room temperature, washed three times in 50 mM KCl containing 1 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$ buffered at pH 6.0 (buffered KCl), and suspended in the same buffer (5). The washed cells were gently agitated with a Pasteur pipette and allowed to stand for 30 s to 1 min to separate aggregated cell clumps from well-dispersed cells. The well-dispersed floating cells were used for *P. gingivalis* binding and binding inhibition assays.

Purification of recombinant fimbrillin. The recombinant fimbrillin (*r*-Fim 10-337) corresponding to amino acid residues 10 to 337 deduced from the fimbrillin gene of *P. gingivalis* 381 (7) was expressed as reported previously (44). *Escherichia coli* BL 21 containing a recombinant plasmid construct was harvested by centrifugation at 2,000 × *g* for 15 min and washed three times with 50 mM Tris-HCl buffer (pH 8.0). The cell pellet was suspended in the same buffer and lysed by three cycles of a 1-min sonication treatment at a 20-W output 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 50 mM Tris-HCl buffer (pH 8.0) containing 2 M urea and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and then solubilized in the same buffer containing 8 M urea and 0.5 mM dithiothreitol. The residual unsolubilized components were removed by centrifugation at 100,000 × *g* for 60 min, and the supernatant was used as the starting material for fimbrillin purification. 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 50 mM Tris-HCl buffered at pH 8.0 and containing 8 M urea. The flow rate was 8 ml/h, and the eluate was collected into 4-ml fractions. The fractions containing *r*-Fim were dialyzed against 20 mM Tris-HCl buffered at pH 8.0 and containing 0.1 mM PMSF. The dialysate was then applied to a Q-Sepharose column (1.5 by 12 cm; Pharmacia LKB Biotechnology) equilibrated with the same buffer. 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. The fractions containing *r*-Fim eluted at approximately 0.18 M NaCl and were collected and dialyzed against buffered KCl. The purified preparation of *r*-Fim was used for the following binding experiments after purity was confirmed by demonstration of a single band at 41 kDa when a sodium dodecyl sulfate (SDS)-polyacrylamide gel of 10 μg of the preparation was stained with silver.

Collection of saliva. Human saliva was obtained from a 34-year-old male donor. Human whole saliva (HWS) was collected by paraffin stimulation in an ice-chilled container and clarified by centrifugation at 12,000 × *g* for 10 min at 4°C. Citric acid-stimulated human parotid saliva (HPS) was obtained with a collecting device as described previously (6), and human submandibular-sublingual saliva (HSMSL) was collected and processed as reported previously (39). Ten percent (wt/wt) of an enzyme inhibitor solution containing 2% disodium EDTA, 10% 2-propanol, and 2 mM PMSF in 0.1 M Tris-HCl buffered at pH 7.5 was added to saliva samples immediately after collection. Fresh saliva samples were filtered through glass wool to remove cellular debris and used immediately to coat HAP beads after the total protein concentration was adjusted to 1.5 mg/ml.

Purification of salivary components. HSMSL was dialyzed extensively against distilled water at 4°C by using Spectra/Por 3 tubing (Spectrum Medical Industries Inc., Los Angeles, Calif.) and then freeze-dried. The lyophilized samples were reconstituted in 0.1 M Tris-HCl buffer (pH 7.5) containing 6 M guanidine-HCl and fractionated by gel filtration chromatography with a Sephadex G-200 column (1.5 by 100 cm; Pharmacia LKB Biotechnology) as described by Ramasubbu et al. (39). Four pools (A to D) were obtained, and the materials in pools A, C, and D were further fractionated to purify the major HSMSL salivary components. High-molecular-weight salivary mucin (MG1), low-molecular-weight salivary mucin isoform (MG2a), acidic proline-rich protein 1 (PRP1), neutral cystatin SN, statherin, and basic PRP were purified as described previously (39). α-Amylase was purified as described by Scannapieco et al. (41). Histatin-5 was synthesized by a solid-phase peptide synthesis technique and purified as described by Raj et al. (37). Secretory immunoglobulin A (secretory IgA) was purchased from Sigma Chemical Co. (St. Louis, Mo.). The purity of each protein was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and anionic PAGE (38, 39, 41), high-pressure liquid chromatography (37, 39), and protein sequencing (38). All samples were dialyzed against buffered KCl containing 0.04% NaN_3 prior to being used in binding assays.

Iodination of *r*-Fim. The purified *r*-Fim was radiolabeled with ^{125}I by the chloramine T method (21) with a modification reported previously (25). One hundred micrograms of the *r*-Fim (1 mg/ml) was labeled with 0.5 mCi of Na^{125}I (Amersham Corp., Arlington Heights, Ill.) in 0.5 M phosphate buffer (pH 7.2) in the presence of 10 μl of chloramine T (2 mg/ml) for 60 s. Iodination was terminated by adding 25 μl of a 4-mg/ml solution of sodium metabisulfite. After termination, 100 μl of the same buffer containing 10% sucrose and 10% potassium iodine was added, and the mixture was loaded onto a Sephadex G-75 column (1 by 25 cm; Pharmacia LKB Biotechnology) equilibrated with buffered KCl. The column was washed once extensively with buffered KCl containing purified *r*-Fim (100 μg/ml) to avoid nonspecific loss of *r*-Fim in the column before the iodinated mixture was loaded. The iodinated protein peak was collected and stored at −20°C until used. The specific activity of ^{125}I-*r*-Fim was 10.8 mCi/μmol.

Preparation of HAP beads coated with salivary proteins. Spherical HAP beads (BDH Chemicals, Poole, England) were coated with purified individual salivary components as well as HWS, HPS, and HSMSL by the method of Clark et al. (5). Two milligrams of HAP beads was added to a siliconized borosilicate culture tube (12 by 75 mm; PGC Scientific, Gaithersburg, Md.), washed once with distilled water to remove fine particles, and placed in 1.5 ml of buffered KCl containing 0.04% NaN_3. The beads were equilibrated by gentle oscillation in a Rototorque rotator (model 7637; Cole-Parmer Instrument Co., Chicago, Ill.) at room temperature overnight. The HAP beads were then washed twice with buffered KCl. Each salivary preparation (150 μg; 10 ng to 1.5 mg of protein per ml) was added to the tubes containing washed HAP beads and rotated at room temperature overnight. HAP beads coated with sali-

vary preparations were washed three times with buffered KCl. Because of the large variation in the molecular masses of salivary components, the protein concentrations of individual salivary components used to coat HAP beads were estimated on the basis of their molecular weights (MG1, 1,000,000; MG2a, 125,000; IgA, 390,000; amylase, 55,000; PRP1, 16,000; cystatin SN, 14,056; basic PRP, 7,000; statherin, 5,380; histatin-5, 3,037).

Assay for measuring the binding of *r*-Fim and *P. gingivalis* cells to coated HAP beads. The *r*-Fim binding assay using HAP beads was carried out by the method of Lee et al. (25). Briefly, a final preparation of ^{125}I-*r*-Fim (0.4 mg of protein per ml; 1.62 mCi/μmol) in a working dilution of buffered KCl was prepared by mixing the unlabeled *r*-Fim (1 mg/ml) with the ^{125}I-*r*-Fim, fivefold-concentrated buffered KCl, and distilled water in a ratio of 4:1.5:2:2.5 (by volume). For initial studies, different amounts of ^{125}I-*r*-Fim (5 to 70 μg) in a total volume of 200 μl of buffered KCl were added to a series of tubes containing saliva-coated HAP, and uncoated HAP beads were used as a control. The reaction mixture was incubated at 20 rpm in a rotator at room temperature for 1 h. After incubation, the HAP beads were washed three times with buffered KCl and gently transferred to a new tube with a Pasteur pipette. The radioactivity of the beads was determined with a gamma counter after one additional washing with buffered KCl. To assess the ability of individual salivary components to promote *r*-Fim binding, 50 μg of ^{125}I-*r*-Fim in 200 μl of a working dilution was added to each tube containing HAP beads coated with individual salivary components.

The binding assay of intact *P. gingivalis* cells to HAP was performed by the method described by Lee et al. (25). [^{3}H]thymidine-labeled *P. gingivalis* cells of strain 2561 (2.5×10^8 cells per ml) in 400 μl of buffered KCl were added to a series of tubes containing HAP beads previously coated with individual salivary components. The mixture was incubated in the rotator at room temperature for 1 h. The tubes were also gently agitated by hand for 5 s every 10 min to disperse aggregates and to maximize binding of *P. gingivalis* cells. After an incubation time of 1 h, the reaction mixture was layered onto 1.5 ml of 100% Percoll (Sigma) in a new siliconized borosilicate tube to separate cells which were free from those bound to the HAP beads. Unbound, free *P. gingivalis* cells floating on the Percoll layer were removed by aspiration with a Pasteur pipette, and the beads with bound cells and the wall of the tube were then washed once with 0.5 ml of Percoll after two washings with 1 ml of buffered KCl. One milliliter of buffered KCl was added to the tube, and the beads were transferred to a vial to determine their radioactivity. No radioactivity was detected on the Pasteur pipette used to transfer the beads. The assay was performed in triplicate, and the experiments were repeated at least twice on different days for each set of conditions. Since similar results were obtained in repeat experiments, the values are shown as the means of the nine replicates in three experiments.

Inhibition studies of *r*-Fim binding to HAP beads were carried out with various agents including PRP1 and statherin in solution. A suspension of 100 μl of ^{125}I-*r*-Fim (500 μg/ml) and 100 μl of each agent at different concentrations was incubated with saliva-coated HAP beads for 1 h.

Analytical methods. SDS-PAGE was performed by the method of Laemmli (24). 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 for protein with either Coomassie brilliant blue or silver stain. Low-molecular-weight markers (Bio-Rad Laboratories, Hercules, Calif.) were used to estimate the molecular mass of sample proteins. The protein content was determined by the method of Bradford with a Bio-Rad protein assay kit (Bio-Rad Laboratories) with bovine serum albumin (BSA) as a standard (3).

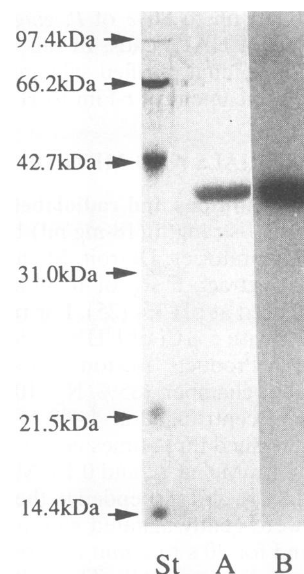

FIG. 1. SDS-PAGE of the purified *r*-Fim and ^{125}I-*r*-Fim on 12.5% polyacrylamide gels. Lanes: St, molecular mass standard proteins; A, purified *r*-Fim (10 μg) stained with silver; B, autoradiogram of ^{125}I-*r*-Fim after SDS-PAGE.

Overlay assay. For the overlay assay, salivary protein samples were prepared for SDS-PAGE in 0.125 M Tris-HCl, buffered at pH 6.8, containing 2% SDS, 10% glycerol, and 0.001% bromophenol blue (without β-mercaptoethanol) at room temperature for 30 min. Preparations separated on an SDS-polyacrylamide gel were transferred to membranes by Western blot (immunoblot) with a semidry transfer system (Semi-phore TE-77; Hoefer Scientific Instruments, San Francisco, Calif.) Three kinds of membranes were used for Western blot: Trans-Blot (nitrocellulose membrane; Bio-Rad Laboratories), Immobilon-P (polyvinylidene difluoride membrane; Millipore Ltd.), and Problot (modified nitrocellulose membrane; Applied Biosystems). Unoccupied binding sites on the membranes were blocked by incubation for 1 h with Tris-buffered saline (20 mM Tris-HCl, 0.5 M NaCl [pH 7.5]) containing 0.5% BSA. The membranes was incubated with 10 ml of ^{125}I-*r*-Fim (100 μg/ml) for 1 h at room temperature and overnight at 4°C. The membranes were washed five times with Tris-buffered saline and dried. Autoradiography of the membrane was performed at −70°C for 24 h with X-Omat low-sensitivity film (Eastman Kodak Co., Rochester, N.Y.) by using an intensifying screen.

RESULTS

Purification of *r*-Fim. The amount of the purified protein obtained was 31.88 mg from 1 liter of culture. The purified *r*-Fim displayed a single 41-kDa protein band by SDS-PAGE (Fig. 1). The position of the single band was not affected by β-mercaptoethanol or heat treatment. However, the purified protein showed a broad peak of 41 kDa, ranging from 40 to 90 kDa, when the molecular weight was estimated by gel filtration chromatography using a Superose 12 column (Pharmacia

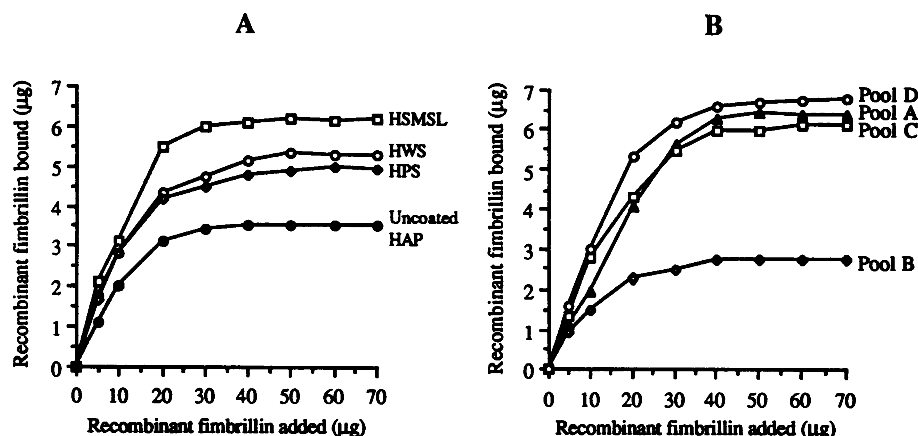

FIG. 2. (A) Binding curves of ^{125}I-*r*-Fim to uncoated HAP beads or beads coated with 150 µl (1.5 mg of protein per ml) of HWS, HPS, or HSMSL. (B) Binding curves of ^{125}I-*r*-Fim to HAP beads coated with 150 µl (0.2 mg/ml) of HSMSL pools A to D. Different amounts of ^{125}I-*r*-Fim (5 to 70 µg) in a total volume of 200 µl of buffered KCl were added to a series of tubes containing saliva-coated HAP beads; uncoated HAP beads were used as a control. The radioactivity of the beads was determined after they were washed with buffered KCl. The values are shown as the means of nine replicates from three experiments. The standard deviation of each value is less than ±5%.

LKB) equilibrated with 50 mM Tris-HCl, buffered at pH 8.0, in a fast protein liquid chromatography system. Thus, the protein was likely mainly a monomer of the fimbrillin subunit; however, a small portion may dimerize under conditions of gel filtration. Neutral sugars were not detected in the purified protein by the phenol-sulfuric acid method using glucose as a standard (8). The purity of ^{125}I-*r*-Fim was confirmed by SDS-PAGE autoradiography, which showed a single band of radioactivity at 41 kDa (Fig. 1).

***r*-Fim binding to saliva-coated HAP beads.** The binding of *r*-Fim to HAP beads coated with HWS, HPS, or HSMSL was compared with binding to uncoated HAP beads equilibrated with buffered KCl (Fig. 2A). The *r*-Fim bound to saliva-coated and uncoated HAP beads in a concentration-dependent manner. Regardless of salivary preparations, all batches of HAP beads showed a saturation level of *r*-Fim binding to 2 mg of HAP beads when about 30 µg of *r*-Fim was added. The salivary preparations adsorbed to HAP beads clearly enhanced the binding of *r*-Fim to HAP beads. The binding of *r*-Fim to HSMSL was slightly higher than the other two saliva preparations, reaching a level at saturation which was 1.8 times greater than binding to uncoated HAP beads.

***r*-Fim binding to fractions of HSMSL.** Since, of the saliva samples, HSMSL showed the highest enhancing effect on *r*-Fim binding, HSMSL was chosen for further study and fractionated by gel filtration (39) into four pools, A to D, which were tested for their ability to promote the binding of *r*-Fim. Major salivary components found in each pool were as follows: MG1, MG2a, secretory immunoglobulins, and amylase in pool A; 43-kDa protein that inhibits *P. gingivalis* adhesion to *Streptococcus gordonii* (48) in pool B; acidic PRPs and cystatins in pool C; and statherin and basic PRPs in pool D. HAP beads were incubated overnight with each HSMSL pool (200 µg of protein per ml). The results are shown in Fig. 2B. Pools A, C, and D promoted the binding of *r*-Fim to a greater extent than HSMSL, whereas the binding of *r*-Fim to HAP beads coated with pool B was at the same level as that of *r*-Fim to uncoated HAP beads.

***r*-Fim binding to purified salivary components.** To precisely identify the individual salivary component(s) which promote the binding of *r*-Fim to HAP beads, purified salivary components represented in pools A, C, and D were tested. Acidic PRP1 significantly increased the binding of *r*-Fim to HAP beads as shown in Fig. 3. PRP1 increased *r*-Fim binding to HAP beads by 290% at a concentration of 2×10^{-9} mol of protein per ml, which saturated *r*-Fim binding to PRP1-coated HAP beads. Statherin also enhanced the binding of *r*-Fim, which was increased by 38% at a concentration of 2×10^{-9} mol of protein per ml and reached a 90% increase at saturation levels when the beads were coated with 3.7×10^{-8} mol of protein per ml. No effect was observed when less than 2×10^{-10} mol of statherin per ml was used for coating HAP beads;

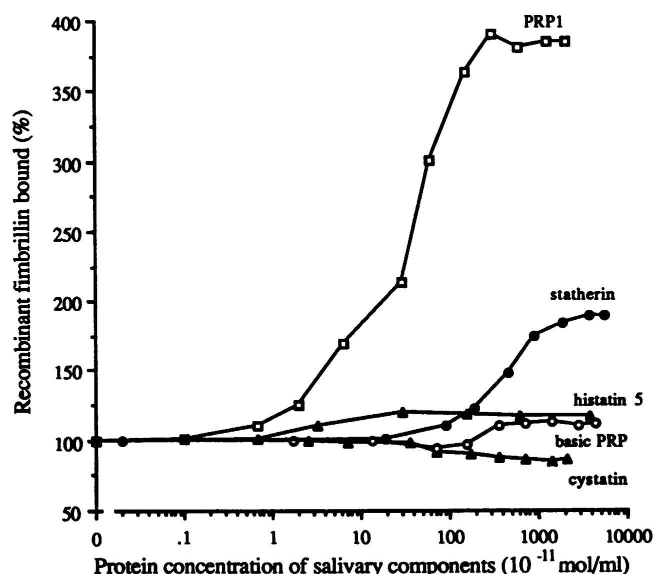

FIG. 3. Binding curves of ^{125}I-*r*-Fim to HAP beads coated with purified salivary components. Fifty micrograms of ^{125}I-*r*-Fim in 200 µl of buffered KCl was added to each tube containing HAP beads coated with 150 µl of individual salivary components. The values are shown as the means of nine replicates from three experiments. The standard deviation of each value is less than ±5%. The values of MG1, MG2a, IgA, and amylase ranged between those of histatin-5 and cystatin.

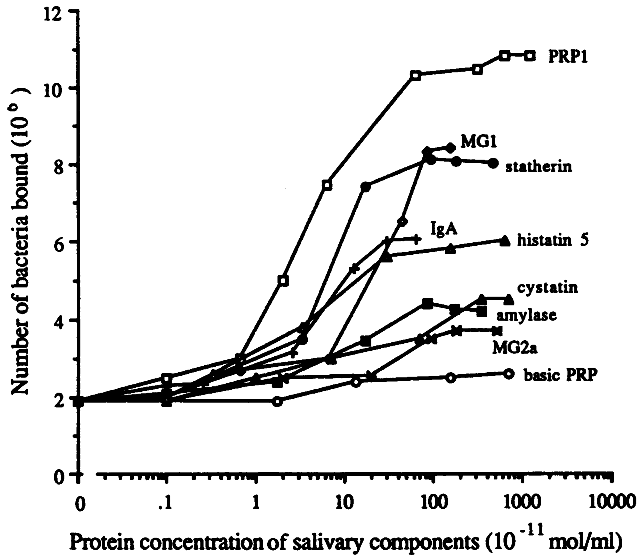

FIG. 4. Binding curves of [^{3}H]thymidine-labeled *P. gingivalis* 2561 to HAP beads coated with various concentrations of purified salivary components. [^{3}H]thymidine-labeled *P. gingivalis* 2561 (10^8 cells) in 400 μl of buffered KCl was added to a series of tubes containing HAP beads coated with 150 μl of individual salivary components. After an incubation time of 1 h, the reaction mixture was layered onto 1.5 ml of 100% Percoll in new siliconized borosilicate tubes to separate cells which were free from those bound to the HAP beads.

however, PRP1 showed 130% enhancement of *r*-Fim binding when a concentration of 2×10^{-10} mol of protein per ml was used. The binding of *r*-Fim to other purified salivary components adsorbed to HAP beads was not significantly greater than binding to uncoated HAP beads. MG2a, amylase, IgA, histatin-5, and basic PRP slightly increased the binding of *r*-Fim to 24, 25, 16, 14, and 13% at the saturation level, respectively. In contrast, MG1 and cystatin slightly decreased the binding by 12 and 15%, respectively. Except for PRP1 ($P < 0.001$) and statherin ($P < 0.001$), none of the other salivary components affected binding in a statistically significant manner ($P > 0.2$).

Binding of *P. gingivalis* cells to salivary components. Of the salivary components tested, PRP1 and statherin were the most effective in increasing binding of *r*-Fim, which was used as a protein model of *P. gingivalis* fimbriae. To determine whether salivary components act as receptors for *P. gingivalis* whole cells, *P. gingivalis* cell binding to HAP beads coated with individual purified salivary components was examined. In the case of *P. gingivalis* whole-cell binding, all salivary components tested, except basic PRP, enhanced the binding of *P. gingivalis* to HAP beads to some extent (Fig. 4). PRP1 and statherin strongly promoted binding of *P. gingivalis* to HAP beads similarly to the way they promoted *r*-Fim binding. However, no significant difference between PRP1 and statherin was observed. PRP1 increased the whole-cell binding by 440% at a concentration of 6.3×10^{-10} mol/ml. Statherin increased the binding by 316% at a concentration of 8.0×10^{-10} mol/ml. The saturation levels of *P. gingivalis* binding to PRP1 and statherin reached almost the same concentrations for both proteins (6.3×10^{-10} to 9.3×10^{-10} mol/ml). In the binding of *r*-Fim, salivary components other than PRP1 and statherin did not show significant effects. However, MG1 increased the binding of *P. gingivalis* cells by 342% in a concentration-dependent manner, while IgA and histatin-5 increased the binding of *P. gingivalis* cells by slightly over 200% when coated on HAP beads. Amylase, cystatin, and MG2a showed the least enhancement of binding of whole *P. gingivalis* cells when coated on HAP beads.

Overlay assay. Pools A to D of HSMSL and purified PRP1 and statherin were subjected to SDS-PAGE and then transferred to membranes. All samples were dissolved in sample buffer containing SDS at room temperature. The acidic PRPs migrated as two characteristic violet bands (39) when stained with Coomassie brilliant blue on SDS-polyacrylamide gels (Fig. 5A). In the case of statherin, two protein bands, corresponding to the dimeric and monomeric forms of the protein, were seen in pool D. The binding of ^{125}I-*r*-Fim to salivary proteins on the membrane were seen as clear radioactive bands corresponding to the positions of acidic PRPs and statherin on Trans-Blot (Fig. 5B). No other bands were observed on the film. The binding of ^{125}I-*r*-Fim to PRP1 was observed only when PRP1 was adsorbed onto Trans-Blot. PRP1 transferred onto either Immobilon-P or Problot failed to bind ^{125}I-*r*-Fim. However, ^{125}I-*r*-Fim bound to statherin adsorbed to all three membranes.

Inhibition of *r*-Fim binding. To examine the nature of interactions involved in *r*-Fim binding to salivary proteins, the inhibitory effects of various agents were evaluated (Table 1). Binding to HAP beads coated with pools A and B was inhibited by arginine and BSA. In the case of other pools or with individual purified salivary components, no significant inhibition was observed when charged amino acids or BSA was added to the mixture. No significant inhibition of the binding was observed when *r*-Fim was incubated with calcium ions, amino sugars, or neutral sugars during binding to saliva-coated HAP beads. Moreover, the presence of PRP1 in solution, even at a concentration of 1,000 μg/ml, did not significantly inhibit the binding of *r*-Fim to PRP1-coated HAP beads (Fig. 6). Similarly, statherin in solution, even at high concentrations, did not exhibit significant inhibition of *r*-Fim binding.

DISCUSSION

We recently described a method for the expression and purification of *r*-Fim from insoluble inclusion bodies by solubilization with urea and gel filtration chromatography (44). However, this procedure sometimes required repetitive chromatography to ensure the purity of the *r*-Fim, which resulted in lower yields. In this study, we modified the previous method by simplifying the preparation of insoluble inclusion bodies and added an anion-exchange chromatography step after the gel filtration. This modification gave yields of over 30 mg of *r*-Fim from 1 liter of culture.

In our previous report, it was shown that *P. gingivalis* binding to whole saliva-coated HAP beads was inhibited by the purified fimbriae and several synthetic peptides corresponding to the C-terminal fimbrillin sequence (25). These studies suggested that some domains in the fimbrillin protein are responsible for *P. gingivalis* binding to one or several salivary components. In the present study, we used *r*-Fim as a tool to examine the ability of individual salivary components to promote the binding of fimbriae to HAP. PRP1 and statherin were found to significantly enhance the binding of *r*-Fim to HAP beads. The same level of binding enhancement was achieved by PRP1 when its concentration was 1% that of statherin. Other major salivary components did not show significant enhancement of binding of *r*-Fim to HAP beads. The effect of PRP1 and statherin on *r*-Fim binding was confirmed by an overlay assay. Negligible effects of charged amino acids, EDTA, and $CaCl_2$ on the binding of *r*-Fim to PRP1 and statherin was observed. These

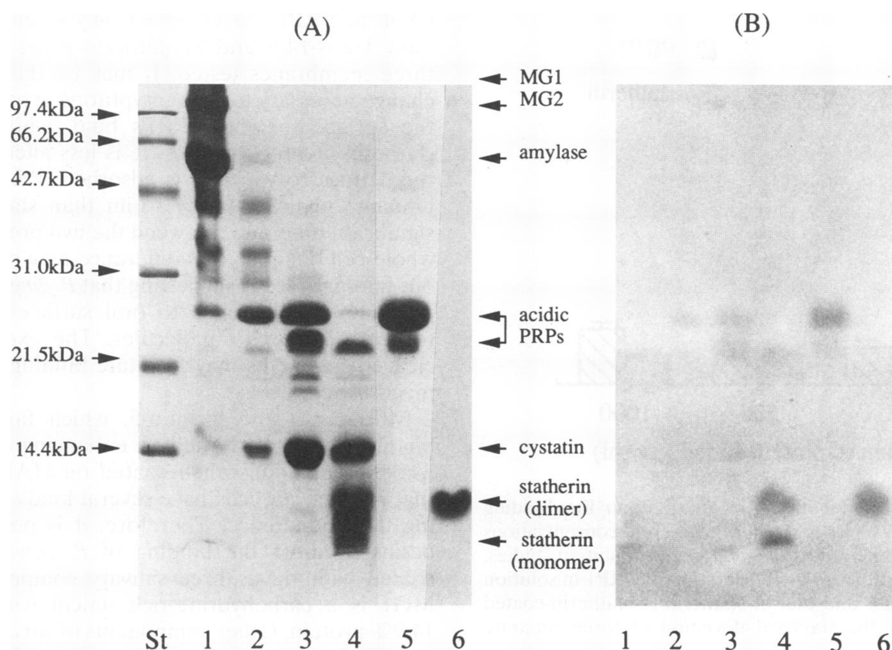

FIG. 5. Overlay assay of the binding of ^{125}I-*r*-Fim to individual salivary components. (A) SDS-PAGE analysis of pools A to D from HSMSL, purified PRP1, and statherin on a 15% gel stained with Coomassie brilliant blue. Lanes: St, molecular mass standard markers; 1, pool A; 2, pool B; 3, pool C; 4, pool D; 5, PRP1; 6, statherin. (B) Autoradiography of ^{125}I-*r*-Fim bound to salivary components transferred onto a nitrocellulose membrane following SDS-PAGE. The membrane was incubated with ^{125}I-*r*-Fim for 1 h at room temperature and then overnight at 4°C. Lanes: 1, pool A; 2, pool B; 3, pool C; 4, pool D; 5, PRP1; 6, statherin.

results suggest that this binding does not depend on ionic interactions. Moreover, judging by the fact that BSA and sugars including fucose did not significantly affect the *r*-Fim binding to PRP1 and statherin, the binding reaction is not likely associated with major hydrophobic or lectin-like interactions. The binding of ^{125}I-*r*-Fim to proteins on a nitrocellulose membrane was not affected by washing with a high-ionic-strength solution (1 M NaCl) or mild detergent solution (Tween 20). These results suggest that PRPs and statherin have specific receptors for *P. gingivalis* fimbrillin through protein-protein interactions.

Acidic PRPs and statherin are unique acidic and carbohydrate-free phosphoproteins which are characteristically present in saliva. PRPs contribute a complex family of salivary molecules which are composed of acidic, basic, and glycosylated species (1, 20). A number of these components, including

TABLE 1. Effect of various agents on the binding of recombinant fimbrillin to HAP beads coated with HSMSL fractions by gel filtration (pools A to D), PRP1, and statherin[a]

Agents	Final concn	Inhibition (%)[b]					
		Pool A[c]	Pool B[c]	Pool C[c]	Pool D[c]	PRP1[d]	Statherin[d]
Buffered KCl (1 mM $CaCl_2$), control		0	0	0	0	0	0
Buffered KCl without $CaCl_2$, EGTA treated		26	28	18	(−)	(−)	(−)
Buffered KCl with 5 mM $CaCl_2$		14	(−)	(−)	(−)	(−)	(−)
L-Lysine	0.1 M	22	17	(−)	8	(−)	(−)
L-Arginine	0.1 M	76	51	37	24	10	15
L-Histidine	0.1 M	9	10	34	38	8	27
Glycine	0.1 M	−10	7	8	11	(−)	(−)
Leucine	0.1 M	−7	(−)	(−)	(−)	(−)	(−)
Alanine	0.1 M	(−)	(−)	(−)	(−)	(−)	(−)
Threonine	0.1 M	(−)	6	(−)	(−)	(−)	(−)
Poly-L-lysine	0.01 M	−17	−30	(−)	−6	(−)	(−)
Poly-L-arginine	0.01 M	−23	−16	−8	−12	−8	−13
BSA	0.01 M	42	33	9	11	9	12
Defatted BSA	0.01 M	48	38	8	7	12	10
Fucose	0.1 M	10	−9	21	7	19	(−)
EDTA	2 mM	(−)	(−)	(−)	(−)	(−)	(−)

[a] Amino sugars (0.1 M *n*-acetylgalactosamine, *n*-acetylglucosamine, galactosamine, and glucosamine) and neutral sugars (0.1 M glucose, mannose, galactose, fructose, lactose, and maltose) showed no effect on *r*-Fim binding.
[b] (−); differential value ≤±5%.
[c] HAP beads were coated with 200 μg of each pool protein per ml overnight.
[d] HAP beads were coated with 100 μg of each protein per ml overnight.

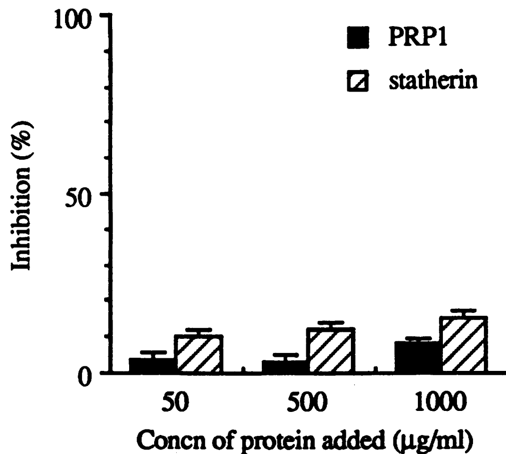

FIG. 6. Effect of PRP1 and statherin in solution on *r*-Fim binding to HAP beads coated with PRP1 or statherin. Different concentrations of PRP1 and statherin in buffered KCl were used for inhibition studies. Inhibition studies were performed by the addition of PRP1 in solution to PRP1-coated HAP beads and that of statherin to statherin-coated HAP beads. Bars signify the standard deviation of three separate determinations.

the acidic PRPs and statherin, have been shown to bind strongly to HAP or enamel and therefore form part of the acquired dental pellicle (23, 28). Acidic PRPs and statherin have been shown to promote the attachment of *A. viscosus* type 1 fimbriae (6, 13, 15) and of *Streptococcus mutans* cells (14, 15). Acidic PRPs are also reported to mediate *P. gingivalis* 381 whole-cell binding to HAP beads (15). Our result is consistent with the finding that the binding of *P. gingivalis* whole cells to HAP beads is promoted by PRPs. However, in the same study, statherin failed to promote *P. gingivalis* binding (15). To confirm the effect of statherin on the binding of *P. gingivalis*, we compared the binding of *P. gingivalis* 2561 with that of *A. viscosus* T14V to HAP beads coated with statherin. Statherin enhanced the binding of *P. gingivalis* as much as that of *A. viscosus*. This difference between our results and those of the previous study might be due to strain variations of *P. gingivalis* or culture conditions. It has been reported that the expression of *P. gingivalis* fimbriae could vary with culture conditions (36, 49). Oyston and Handley reported that the expression of fimbriae of *Bacteroides fragilis* depends on growth phase variation and temperature (35). We also had similar findings that variation in culture conditions, such as temperature and composition and concentration of culture media, affect the expression of *P. gingivalis* fimbriae.

It was demonstrated that PRPs and statherin have unique hidden receptor(s) called cryptitope(s) which are responsible for the binding of these components to *A. viscosus*. (13, 15). PRP1 and statherin in solution did not bind to *A. viscosus* cells (13, 15). It is recognized that the cryptitope that promotes the binding of bacteria is exposed upon adsorption of these components to HAP beads or after absorption to other surfaces. Recently, it was reported that the Pro-Gln segment of the cryptitope in the C-terminal region of PRP molecules is associated with bacterial binding (16). In the present study, the *r*-Fim binding to saliva-coated HAP beads was not inhibited when PRP1 and statherin in solution were used as competitive inhibitors; hence, *P. gingivalis* fimbrillin appears to bind to PRPs and statherin only when they coat a surface such as HAP. Further studies are necessary to confirm this. The binding of *r*-Fim to PRP1 was observed only when PRP1 was adsorbed onto Trans-Blot and to statherin when bound to any of the three membranes tested. It may be that the conformational change necessary to cause cryptitope exposure is dependent on the surface to which PRPs bind, while expression of the statherin cryptitope exposure is less affected by the nature of the surface to which it is adsorbed. PRP1 showed a greater binding enhancement of *r*-Fim than statherin. However, no significant difference between the two proteins was observed in whole-cell binding. This difference may be explained by previous findings (4, 15) suggesting that *P. gingivalis* proteases could promote its adherence to oral surfaces by exposing cryptitope(s) of receptor molecules. The exposed cryptitopes on cleaved fragments may facilitate binding to the tooth or oral mucosal cells.

MG1, IgA, and histatin-5, which failed to promote the binding of *r*-Fim, showed an enhancing effect on *P. gingivalis* whole-cell binding when coated on HAP beads. It is known that *P. gingivalis* cells have several kinds of molecules mediating their adherence. Therefore, it is possible that other adhesins promote the binding of *P. gingivalis* to HAP beads coated with these three salivary components. For example, MG1 is a carbohydrate-rich mucin which consists of only 14.9% protein. Other components of this molecule, such as the carbohydrate moieties, may interact with adhesins of *P. gingivalis* which act in a lectin-like fashion. It has been reported that histatin significantly inhibits hemagglutinin activity and coadhesion of *P. gingivalis* (29). Histatin-5 likely binds to *P. gingivalis* hemagglutinin to mediate coadhesion with other bacteria. *P. gingivalis* proteases are associated with its adherence to *A. viscosus* cells (26). It is also possible that proteases may have participated in adhesive interactions to salivary proteins on HAP beads. Overall, it appears that the nonfimbrial adhesins of *P. gingivalis* do not mediate adhesion reactions as strongly as fimbriae since the enhancing effects on *P. gingivalis* whole-cell binding by PRP1 and statherin are greater than those by MG1, IgA, and histatin-5. Furthermore, *r*-Fim inhibits up to 80% of *P. gingivalis* whole-cell binding to saliva-coated HAP beads (44).

It has been reported that adhesion of *A. viscosus* to *P. gingivalis* is inhibited by arginine (26) and defatted BSA, an inhibitor of hydrophobic interactions (40). This interaction has been suggested to be mediated through non-lectin-like interaction by coadhesins such as fimbriae and proteases of *P. gingivalis* (17). However, the binding of *r*-Fim to PRP1 and statherin is not inhibited by defatted BSA. Fimbrillin protein may have different domains responsible for each of these adhesive interactions, or other protein component(s) related to coaggregation may associate with fimbrillin. Further study is necessary to understand this difference.

Pool C from HMLSL, rich in PRPs, did not effectively promote the binding of *r*-Fim to HAP beads. Johnson and coworkers mentioned that an effective monolayer is reached when the surface coverage is such that additional molecules are more likely to interact with those already adsorbed rather than with the free surface (23). PRPs and statherin have been shown to bind strongly and rapidly to HAP or enamel and therefore form part of the acquired dental pellicle (23, 28). These receptor molecules may have been be masked by other overlying salivary components in pool C. The coating of HAP beads with salivary proteins, including HWS, HSMSL, and HPS, significantly enhanced the binding of *r*-Fim. The differential values of the binding of *r*-Fim to various batches of HWS, HSMSL, and HPS depended to a large extent on the individuals from whom the salivary samples were collected (data not shown). This phenomenon was attributed not only to

the variations of the concentration of PRPs and statherin but also to individual variation in the concentration of salivary components which may block the binding or function of PRP1 or statherin on the surface of HAP beads.

Purified *P. gingivalis* fimbriae and synthetic fimbrillin peptides used as vaccines have been shown to prevent periodontal tissue destruction associated with *P. gingivalis* infection in gnotobiotic rats (9). Hence, *r*-Fim is a strong candidate for a vaccine against periodontal tissue destruction. The present study showing that *r*-Fim binds to PRPs and statherin will help delineate the epitopes of the fimbrillin responsible for binding to the salivary proteins. These epitopes of *P. gingivalis r*-Fim could be used as a vaccine to block fimbria-mediated adherence and possibly other fimbrial-host interactions to prevent *P. gingivalis*-associated periodontal disease.

ACKNOWLEDGMENTS

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

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