Binding of *Porphyromonas gingivalis* Fimbriae to Proline-Rich Glycoproteins in Parotid Saliva via a Domain Shared by Major Salivary Components

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*Porphyromonas gingivalis*, a putative periodontopathogen, can bind to human saliva through its fimbriae. We previously found that salivary components from the submandibular and sublingual glands bind to *P. gingivalis* fimbriae and that acidic proline-rich protein (PRP) and statherin function as receptor molecules for fimbriae. In this study, we investigated the fimbria-binding components in parotid saliva. Fractionated human parotid saliva by gel-filtration chromatography was immobilized onto nitrocellulose membranes for the overlay assay following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The salivary components on the membrane were incubated with fimbriae purified from *P. gingivalis* ATCC 33277, and the interacted fimbriae were probed with anti-fimbria antibodies. The fimbriae were shown to bind to two forms of proline-rich glycoproteins (PRGs) as well as to acidic PRPs and statherin. Moreover, fimbriae bound to several components of smaller molecular size which appeared to be acidic PRP variants and basic PRPs. Fimbriae bound strongly to the purified PRGs adsorbed onto hydroxyapatite (HAP) beads. In contrast, PRGs in solution failed to inhibit the fimbrial binding to the immobilized PRGs on the HAP beads. These findings suggest that the appearance of binding site(s) of PRGs can be ascribed to their conformational changes. We previously identified the distinct segments within PRP and statherin molecules that are involved in fimbrial binding. The peptides analogous to the binding regions of PRP and statherin (i.e., PRP-C and STN-C) markedly inhibit the binding of fimbriae to PRP and statherin immobilized on the HAP beads, respectively. The PRP-C significantly inhibited the binding of fimbriae to PRG-coated HAP beads as well as to PRP on HAP beads. The peptide did not affect the binding of fimbriae to statherin, whereas the STN-C showed no effect on the fimbrial binding to PRPs or PRGs. In the overlay assay, the PRP-C clearly diminished the interactions between the fimbriae and the various salivary components, including PRPs, the PRGs, and the components with smaller molecular sizes but not statherin. These results strongly suggest that fimbriae bind to salivary components (except statherin) via common peptide segments. It is also suggested that fimbriae bind to saliva through the two distinct binding domains of receptor salivary components: (i) PRGs and PRPs and (ii) statherin.

*Porphyromonas gingivalis*, a gram-negative anaerobic rod, has been well recognized as a major etiologic agent of periodontal diseases (32). Dental plaque accumulation around the gingival crevice and other oral surfaces is a predisposing factor for the initiation of periodontal diseases. Among plaque bacterial species, *P. gingivalis* has been shown to prevail in various saliva-coated surfaces of oral matrices such as mucosal membrane (9), healthy crevices (8), and supragingival plaques (35). Therefore, saliva would be a critical factor for the organism to adhere to and colonize the oral cavity. Among the various adhesive factors of the organism, fimbriae are thought to play a major role in its interaction with saliva (19). We previously searched for submandibular and sublingual salivary components that specifically interact with *P. gingivalis* fimbriae (4). We found that fimbriae strongly bind to acidic proline-rich protein (PRP) and statherin that had been immobilized onto nitrocellulose membranes or hydroxyapatite (HAP) beads. These bindings occur via protein-protein interactions through definitive domains of the fimbriae (3) and salivary proteins, i.e., PRP and statherin (2, 16).

Human salivary PRPs (SPRPs) are composed of heterogeneous molecules that are rich in saliva from the parotid and submandibular and sublingual glands. SPRPs comprise about 75 to 80% of the parotid salivary components (5) and are classified into three groups: acidic (molecular mass, ≤16 kDa), basic (molecular mass, 6 to 9 kDa), and glycosylated (molecular mass, 39 kDa) (31). These SPRPs are coded for by a multigene family of six genes, resulting in more than 20 SPRPs by both differential RNA splicing and posttranslational modification (proteolytic cleavages) after secretion (24). Acidic PRPs have been reported to act as salivary receptors for several plaque-forming bacteria (4, 10, 13).

The proline-rich glycoprotein (PRG) is a basic protein (with an isoelectric point of >8.2) that is especially rich in proline, glycine, and glutamic acid. PRG contains 40% carbohydrate and is found abundantly only in parotid saliva (7, 30). The role of PRG has been demonstrated in lubrication, a property of oral pellicles which may protect surfaces against mechanical disruption or abrasion (7). PRG can bind to *Streptococcus oralis, Streptococcus gordonii* (7), and *Fusobacterium nucleatum* (14) in a lectin-like fashion and to *Actinomyces viscosus* by protein-protein interactions (6). Although PRG appears to be able to bind to various oral bacteria, little information is available concerning the binding mechanisms involved.

We recently determined that the minimum active domain of
PRP1 (a major variant of acidic PRP) for the binding to *P. gingivalis* fimbrae is Pro-Gln-Gly-Pro-Pro-Gln (PGQPPQ) (16). The synthetic PRP1 peptide (i.e., PRP-C analogus to the carboxyl-terminal 21 amino acid residues containing PGQPPQ and PGQPPPQ showed almost 100% inhibition in the binding of fimbrae to PRP1 (16). We describe here the finding that PRG is specific for the binding to *P. gingivalis* fimbrae via the common domain found in various SPRP variants as a typical repeating sequence (PGQPPQ or PGQPPPQ). It was also found that the peptide containing the repeating sequence inhibits fimbrial binding to all salivary proteins except statherin.

**MATERIALS AND METHODS**

**Bacterial culture and purification of fimbrae.** Fimbrae were mechanically detached from *P. gingivalis* ATCC 33277 cells grown anaerobically and purified chromatographically by the method of Yoshimura et al. (34). The iodination of purified fimbrae was performed as described previously (4). The specific activity of iodinated protein was 1.8 mCi/µmol of fimbritin.

**Preparation of salivary components.** Human parotid saliva was obtained from nine 22-year-old male donors by citric acid stimulations with collecting devices as described previously (6). The individual saliva samples were mixed together. Ten nine 22-year-old male donors by citric acid stimulations with collecting devices as described previously (6).

**Overlay assay.** Saliva samples were dissolved in 0.125 M Tris-HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, and 0.01% bromophenol blue and were incubated for 30 min at room temperature. Preparations separated on SDS-PAGE gels were transferred to nitrocellulose membranes (Trans-Blot, 0.45 µm pore size; Bio-Rad Laboratories, Hercules, Calif.). Unoccupied binding sites were blocked by incubating the membranes for 1 h with the same buffer containing 1% casein and bovine serum albumin (BSA). The inhibition assay was carried out with various reagents; however, no significant inhibitors were found among the inhibitors tested.

**RESULTS**

**Binding of parotid saliva components to *P. gingivalis* fimbrae.** The overlay assay was performed to detect salivary components capable of binding to fimbrae. The parotid saliva was fractionated by gel filtration chromatography, and 10 peak fractions were obtained. These 10 fractions and the parotid saliva were separated by SDS-PAGE (Fig. 1A), and a replica was prepared by the transference of salivary components to a nitrocellulose membrane (Fig. 1B). The parotid saliva components in lanes 3 to 7 and 10 clearly bound to fimbrae (Fig. 1B); the protein bands c and e shown in Fig. 1 were PRP1 and statherin, whereas the broad bands a and b were novel fimbria-binding proteins. The latter two bands belonged to PRGs as judged by the relative mobility and pink-violet coloration with CBB R-250. Other reacting bands d with molecular weights lower than that of PRP1 were also observed in lanes 6 and 7. The relative mobilities of these bands and the pink-violet coloration with CBB R-250 indicate that these components are acidic PRP variants.

**Purification of PRG variants and binding to fimbrae.** The PRGs were newly found to bind to *P. gingivalis* fimbrae and were purified to homogeneity. The purities of the final preparations of PRGs with a higher molecular size (H-PRG) and a lower molecular size (L-PRG) were confirmed by SDS-PAGE results stained by CBB (data not shown). The abilities of the H- and L-PRGs to bind to fimbrae were then compared to those of PRP1 and statherin by using HAP beads (Fig. 2). The binding levels of 125I-labeled fimbrae to H- and L-PRG-coated HAP beads were very similar. In addition, the binding abilities of fimbrae to PRP1, statherin, and whole saliva were higher than those to PRGs, whereas the fimbrial bindings to the H- and L-PRGs were significantly greater than the binding to the BSA. The inhibition assay was carried out with various reagents; however, no significant inhibitors were found among amino acids (0.1 M N-acetylgalactosamine, N-acetylgalacosamine, galactosamin, and glucosamin), neutral sugars (0.1 M glucose, mannos, galactose, fucose, fructose, lactose, and maltose), and amino acids (0.1 M l-arginine and l-lysine).

We examined whether H- and L-PRGs functioned as inhibitors on the binding of fimbrae to the HAP beads pretreated with H- and L-PRGs, respectively (Fig. 3). Although increasing amounts of PRGs were added (up to 1,000 µg/ml), no significant inhibition of the fimbrial binding to the PRG-immobilized HAP surfaces was observed, a finding that is probably due to cryptic receptors.

**Inhibition studies by synthetic peptides (PRP-C and STN-C).** The bindings of fimbrae to the immobilized PRP1 and statherin have been shown to be strongly inhibited by synthetic peptides containing the binding sites of PRP1 and statherin for fimbrae. Synthetic peptides, PRP-C (the carboxyl-terminal 21-amino-acid segment of PRP1 [PGQPPQGGRPGQPGQPOQPGQ]), and STN-C (the carboxyl-terminal 15-amino-acid segment of statherin [LYPQPYPOYOYQYTF]), were used to determine whether the fimbria-PRG interactions would be mediated by the same mechanism as those of PRP1 or statherin.
As shown in Fig. 4, PRP-C strongly inhibited the binding of fimbriae to the immobilized PRP1, whereas it was ineffective for the fimbrial binding to statherin (Fig. 4A). On the other hand, STN-C, but not PRP-C, inhibited the fimbrial binding to the statherin-coated HAP beads (Fig. 4B). In the interaction between fimbriae and immobilized PRGs, PRP-C was markedly inhibitory, whereas STN-C showed no inhibitory effect (Fig. 4C and D). These results suggest that P. gingivalis fimbriae recognize the domain common to H- and L-PRGs and to PRP1 and that the binding site(s) of statherin for fimbriae is distinct from that of PRP or PRG.

Inhibitory effect by PRP-C in the binding of fimbriae to salivary components. Fimbriae bound to several unknown salivary components which appeared to be acidic and basic PRP variants (protein bands d in Fig. 1). These variants have been reported to possess the typical repeating amino acid sequences, PQGPPQ and PQGPPPQ, as well as SPRP (5). Therefore, the effects of PRP-C on the binding of fimbriae to these components with smaller molecular sizes were examined. As shown in Fig. 5, the overlay assays were performed with or without the

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addition of PRP-C. The salivary proteins that had reacted with the fimbriae were clearly probed with anti-fimbria antibodies (Fig. 5B). The addition of PRP-C (100 nmol/ml) diminished the binding of fimbriae to most of the saliva proteins, including the smaller molecular size components, the PRGs and PRP1, whereas the fimbrial binding to statherin was not affected (Fig. 5C). These results suggest that *P. gingivalis* fimbriae recognize a common peptide segment shared by various salivary components (except statherin).

**DISCUSSION**

The flow of saliva in the oral cavity may contribute to the promotion and/or retardation of the adherence of oral bacteria to oral and tooth surfaces. Many bacterial species are known to be agglutinated in the presence of saliva, which results in the detachment of oral bacteria and their eventual clearance from the oral cavity (30). It was reported that several saliva components, including the SPRPs, mucin, amylase, and statherin can bind to various oral bacteria (30). For the clearance of bacterial cells from the oral cavity, flowing saliva components must interact with the organisms. However, PRG, PRF, and statherin in flowing saliva are unlikely to bind to *P. gingivalis* cells. PRP1, a major variant of acidic PRP, has been thought to possess unique hidden receptors termed “cryptitopes” that promote bacterial adherence (11, 12). The PRP1 molecule in solution shows little ability to bind to bacteria. However, once adsorbed to HAP surfaces, the protein undergoes a conformational change. As a result, the cryptitopes are exposed, resulting in the promotion of bacterial adherence to the surfaces. Similarly, it was found that PRGs and statherin also bound to fimbriae only when immobilized to the solid surface of HAP beads or a nitrocellulose membrane. All of these phenomena may be ascribed to the appearance of hidden receptors caused by a conformational change of the saliva proteins. Although the nature of these cryptitopes remains to be elucidated, it is suggested that salivary cryptitopes enhance the adherence of the pathogenic bacteria but not the host defense.

The size variants of the PRG molecule are expressed due to different lengths of the tandemly repetitive exon 3 portions of the allele (*PRB2*) and the differences of glycosylation (20, 21, 24). It has been reported that the electrophoretic patterns of PRG variants are expressed to give two size-variant PRG proteins (15, 33). Various variants of the SPRP family have been found, the SPRP-coding genes have been identified, and the whole amino acid sequences of the core proteins have been deduced by analyzing the genes, including *PRH1* and *PRH2* and *PRB1* to *PRB4* (22–24). Many of the characteristics of the
repeating amino acid sequences are observed in the SPRPs (5). For example, a typical repeating sequence in acidic PRPs is Pro-Gln-Gly-Pro-Pro-Gln-Gly (PQGPPPQG), while the sequence PQGPPPQGQ dominates in basic and glycosylated PRPs. The segment PQGPPPQG has been shown to be an active domain of PRP1 to bind to fimbriae, whereas the peptide PQGPPPQGQ is also inhibitory for the fimbrial interaction with PRP1 (16). It is of interest that the majority of SPRPs possess the characteristic repeating sequences which would act as the binding domains for P. gingivalis fimbriae. Although it is not yet known whether all of the purified SPRPs bind to fimbriae, the findings in this study suggest that smaller-molecular-size components, presumably acidic and basic PRP variants, also bind to fimbriae via the repeating sequences. It is likely that SPRPs act as anchoring proteins for P. gingivalis to oral surfaces, including dental pellicle and plaque. It is unknown whether the binding sites of the smaller-molecular-size components are masked. These protein components might agglutinate P. gingivalis and interfere with the adherence to the oral cavity by the organism. Further study is necessary to understand the biological function of the smaller acidic and basic PRP variants in the interaction with fimbriae.

The findings obtained so far strongly suggest that fimbriae bind to salivary components (except statherin) via a common receptor domain. The active peptide region of statherin for binding to fimbriae has been demonstrated to be the PGG region (13, 30), few studies on the mechanisms of these interactions are available. It has been demonstrated that S. gordonii recognizes the PQ residues located at the carboxyl-terminal end of PRP1 and that the PQ sequence of the internal residues of PRP1 does not promote the adhesion (11). Various interactions between bacteria and salivary components are known (30). Further analyses of these interactions will increase our understanding of the diversity of adherence of many species of oral organisms to different oral surfaces.

Fimbriated P. gingivalis strains exhibit higher binding abilities for collagen type I and IV (28). Since human collagen alpha (types I and IV) contains multiple repeating regions of PQGPP, it is reasonable to speculate that the binding of P. gingivalis to collagen might be mediated by the interaction between fimbriae and the PQGPP regions found in collagen. It would be of interest to determine whether P. gingivalis fimbriae can recognize these characteristic PRP regions ubiquitously present in various host tissues, including those of the periodontal area.

The fimbrial binding to salivary proteins is not inhibited by the addition of L-arginine (4). However, Kontani et al. (17, 18) recently reported the presence of cryptic receptors in fibronectin and the surface components of fibroblasts for P. gingivalis fimbriae, in which the arginine residue plays a critical role. They showed that the adherence of fimbriae to the fibroblasts and fibronectin immobilized on the culture plates was significantly enhanced by the treatment of proteinase of the organism that split the arginine residue. The proteolysis likely exposes a cryptitope in the host matrix proteins, i.e., the carboxy-terminal Arg residue, so that the fimbriae can bind to the cryptitope through a fimbria-Arg interaction. In this regard, the aggregation of S. oralis cells induced by fimbriae was inhibited by L-arginine (1). Fibrinogen is a potent inhibitor of P. gingivalis coaggregation with S. oralis, and chemical modification of the Arg residues of fibrinogen markedly reduced the inhibitory ability (27). Histatin likely binds to the fimbriae and hemagglutinin of P. gingivalis, and arginine residues of histatin seem to be critically important for the inhibition of coaggregation (25). Although little is known regarding cryptic receptors in the coaggregation, fibrinogen seems to possess cryptic receptors for fimbriae (26). These cryptic receptors might mediate the selective colonization of P. gingivalis in the oral cavity.

In conclusion, it has been shown that PRGs, which are found...
only in parotid saliva, can bind to P. gingivalis fimbriae via the common cryptic peptide segment shared with SPRPs, the major salivary components. It is also suggested that fimbriae can bind to saliva through the two distinct binding domains of receptor salivary components, SPRPs, and statherin.

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REFERENCES


