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PRB3 Null Mutations Result in Absence of the Proline-Rich Glycoprotein Gl and Abolish *Fusobacterium nucleatum* Interactions with Saliva In Vitro

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The glycosylated proline-rich glycoprotein (Gl or PRG), a product of the *PRB3* gene, is a major constituent of human parotid saliva. Important functions proposed for Gl include acting as a bacterial receptor. The Gl proteins of several subjects were typed by two polyacrylamide gel electrophoresis (PAGE) systems: acid-lactate PAGE followed by staining with the periodic acid-Schiff reagent and sodium dodecyl sulfate-PAGE followed by electrophoretic transfer and staining with amido black or concanavalin A. The results showed one subject who apparently lacked Gl. The four exons, including splice junctions, for both *PRB3* alleles of this subject were completely sequenced. Unexpressed (null) mutations were detected with an identical C nucleotide insertion in the same coding region of exon 3 of both alleles. This C nucleotide insertion leads to a frameshift with a premature termination codon that probably results in markedly reduced or absent *PRB3* gene expression. We then used a nitrocellulose blot overlay assay to assay the bacterial receptor activity of parotid saliva from the *PRB3*^{null} subject. No interactions with *Fusobacterium nucleatum*, shown previously to interact selectively with Gl, were detected. Together, these results suggest that this subject does not express the *PRB3* gene and that one of the consequences is an altered ability to interact with a bacterium known to colonize the oral cavity.

The Gl protein, or proline-rich glycoprotein (PRG), is a major component of human parotid saliva and, like other salivary proline-rich proteins (PRPs), is especially rich in proline, glycine, and glutamic acid. It has an isoelectric point greater than 8.2 and contains about 40% carbohydrate (18). The Gl protein is encoded by the *PRB3* gene, which is one of the six closely linked *PRP* genes on human chromosome 12pl3.2 that code for acidic, basic, and glycosylated PRPs (reviewed in references 6 and 23). Among the six *PRP* genes, *PRH1* and *PRH2* code for acidic PRPs and *PRB1*, *PRB2*, *PRB3* and *PRB4* code for basic and glycosylated PRPs. The *PRP* genes have been physically linked (16), and the sequences and evolutionary history of all six *PRP* genes have been reported (15).

Allelic size variants of the Gl proteins were described for whites and blacks (6), for the Japanese (23), and for the Chinese, Malays, and Indians (29). The polymorphic Ph proteins (14) are the same as two slow-mobility Gl variants (24). A disulfide-bonded and dimeric Gl variant (Gl 8) was found exclusively in Ashkenazi Jews (7). The Gl 8 monomer protein forms disulfide-bonded homodimers and heterodimers with salivary peroxidase (7). With the exception of the disulfide-bonded variant Gl 8, the different size variants of Gl proteins are due to different lengths of the tandemly repetitive exon 3 portions of the *PRB3* alleles (19). These different-length *PRB3* alleles are caused by frequent intragenic homologous and unequal crossovers within the tandemly repeated sequences of the third exon of *PRP* genes, and this results in frequent DNA length polymorphisms (20).

Unexpressed (null) alleles for Gl proteins have also been noted among whites, blacks, and Japanese, with population frequencies of approximately 0.046, 0.110, and 0.105, respectively. The probable molecular basis for a *PRB3* null allele was determined in a null heterozygote for an expressed allele (*PRB3*S^{cys}) that was productive for the cysteine-containing Gl 8 variant and for a null allele (*PRB3*M^{null}). DNA sequence analysis of exon 3 (encoding the major portion of the Gl protein) of the *PRB3*M^{null} allele showed a single C nucleotide insertion leading to a frameshift with a premature termination codon that probably caused the apparent lack of gene expression (7). Null alleles have also been described for other salivary PRPs (6, 24).

A number of functions have been described for the salivary PRPs, including binding to hydroxyapatite, calcium, and certain intraoral bacteria; suppressing hydroxyapatite crystal formation; mediating adherence of microorganisms to the coated tooth surface; forming part of enamel pellicle; and modifying the lubricative properties of saliva (reviewed in references 13 and 24). Of particular relevance to our current report are the studies of Gillece-Castro et al. (13), who determined the carbohydrate structure and bacterial receptor activity of the human PRG. They found that the gram-negative organism *Fusobacterium nucleatum*, a putative periodontal pathogen, binds selectively to different PRG size variants immobilized on solid support and the specificity for binding resides in a particular oligosaccharide structure in the PRG.

Recently, we discovered a unique subject who apparently lacked the Gl protein. Here, we report the results of studies designed to determine both the molecular-genetic basis for this defect and the bacterial binding characteristics of the subject's salivary proteins. The fact that no saliva-*F. nucleatum* interactions could be demonstrated suggests one possible biological consequence of this mutation.

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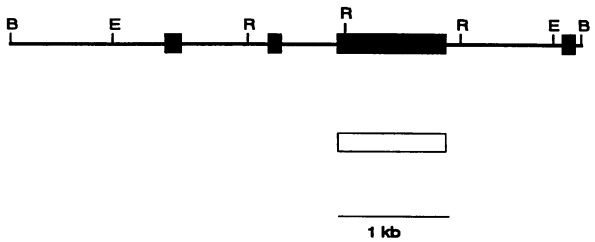

FIG. 1. The *PRB3* gene restriction map used for cloning *PRB3* alleles of a subject with the Gl 0 phenotype. *Bam*HI (B), *Eco*RI (E), and *Rsa*I (R) sites within and surrounding a prototype *PRB3* gene were used for cloning and subcloning the *PRB3*M^{null} and *PRB3*VL^{null} alleles from the subject with the Gl 0 phenotype. The exons are shown as solid boxes. The two alleles differ in length because of different numbers of tandem repeats in exon 3 (see text). The *Hin*fI 980 PRP probe (from exon 3 of *PRB1*, hybridizing to exon 3 of all six *PRP* genes [21]) was used for cloning, subcloning, and genomic Southern analysis and is shown as an open box.

MATERIALS AND METHODS

Electrophoretic analysis of parotid salivary PRPs of a subject with the Gl 0 phenotype. Parotid saliva was collected as ductal secretions from five donors, one of whom had the Gl 0 phenotype. The Gl proteins in these samples were typed by two polyacrylamide gel electrophoresis (PAGE) systems. The numerical genetic designation for the Gl proteins as used in this article was previously described (3, 7) and refers to 0, 1, or 2 allelic products in each sample. The first system was sodium dodecyl sulfate (SDS)-PAGE (17) followed by electrophoretic transfer and staining with amido black for proteins or with concanavalin A, which is more sensitive for glycoproteins (7, 9). The second was acid-lactate PAGE, and the gel was stained with the periodic acid-Schiff reagent (3).

Saliva from the subject with the Gl 0 phenotype was also typed for other PRP polymorphisms, including acidic PRPs (Pr, Db, Pa, and PIF) by isoelectric focusing PAGE (2) and basic PRPs (Ps, PmS, CON I, CON II, Pe, and Po) by SDS-PAGE (9, 10). One basic PRP, PmF, was typed by acid-lactate PAGE (1, 29).

Cloning and sequencing *PRB3* alleles from the subject with the Gl 0 phenotype. Terminology for the *PRB3* alleles in the subject with the Gl 0 phenotype is based in part on genomic Southern analysis (not shown) demonstrating different-length *PRB3* alleles (representing a length polymorphism due to different numbers of tandem repeats in exon 3). The *PRB3* alleles of this subject are designated *PRB3*M^{null} (medium) and *PRB3*VL^{null} (very large). The designation "null" for both alleles is based on data to be presented.

A restriction map of a prototype *PRB3* gene used in cloning and sequencing is shown in Fig. 1, and it is based on the data of Kim et al. (15). Most of the Gl protein (except for 12 amino acids in exons 1 and 2) is coded in exon 3, where the protein terminates. Both *PRB3* alleles were cloned from the size-fractionated genomic leukocyte DNA, isolated from a blood sample donated by the subject with the Gl 0 phenotype, as approximately 5.4-kbp *Bam*HI fragments in lambda DL10 (32). Recombinant bacteriophage clones and subclones were detected by hybridization to the *Hin*fI 980 PRP probe (open box in Fig. 1) (from the tandemly repeated sequences of exon 3 of *PRB1* [5]), which hybridizes to exon 3 of all six *PRP* genes (21). The *Bam*HI fragments containing two *PRB3* alleles, *PRB3*M^{null} and *PRB3*VL^{null}, could be identified in the genomic library by characteristic polymorphic length differences, and these *Bam*HI fragments from the phage clones were subcloned into plasmid Bluescript (Stratagene, La Jolla, Calif.). Other fragments for sequencing were further subcloned from the *Bam*HI fragments into plasmid Bluescript. These fragments included the approximately 4-kbp *Eco*RI fragments, which cover exons 1, 2, and 3, and the two adjacent *Rsa*I fragments (a 5′ constant-length fragment of 864 bp and a 3′ variable-length fragment of around 1 kbp), which cover exons 2 and 3. All four exons (including splice junctions) of both alleles were sequenced twice as double-stranded DNA by using Sequenase (United States Biochemical Corp., Cleveland, Ohio) by dideoxy chain termination (28). Extended sequence was obtained for some fragments by deletion with exonuclease III and mung bean nuclease (Stratagene methods) to generate overlapping series of subclones. Thus, the 3′ *Rsa*I fragment was deleted from the 3′ end to cover the bulk of exon 3. The *Bam*HI fragment was deleted from the 3′ end to cover exon 4, and the *Eco*RI fragment was deleted from the 5′ end to sequence exon 1.

Sequence data were analyzed with software provided by the Genetics Computer Group (12).

Overlay method for assessing bacterial binding to glycoproteins. Saliva was collected from the subject with the Gl 0 phenotype and two additional donors who were chosen because they possessed size-variant Gl proteins (13). Parotid saliva was collected as ductal secretions from all the donors. Whole saliva was collected by expectoration into an equal volume of loading buffer, pH 7.0, containing 6 M urea, 1% SDS, and 1% β-mercaptoethanol.

Saliva samples were separated on 7.5% slab gels (17). Molecular weight standards were as follows (M_rs): 29,000, carbonic anhydrase; 45,000, ovalbumin; 66,000, bovine plasma albumin; 97,400, phosphorylase *b*; 116,000, β-galactosidase; 205,000, myosin. Since proline-rich proteins migrate anomalously with respect to molecular mass in SDS-PAGE gels, standards were used only as markers of relative electrophoretic mobility. Duplicate gels containing identical samples were run. One gel was used to demonstrate proteins by staining with silver (25). The other gel was transferred by blotting to nitrocellulose membranes according to the procedure of Towbin et al. (30). After blotting, gels were routinely silver stained in order to estimate the efficiency of transfer.

F. nucleatum was metabolically labeled with [^{35}S]methionine and allowed to attach to the nitrocellulose blots as previously described (27). To demonstrate bands supporting attachment of bacteria, the nitrocellulose membranes were air dried and autoradiography was performed with Hyperfilm.

RESULTS

Electrophoretic studies of salivary Gl proteins. Electrophoretic transfers (after SDS-PAGE) of salivary proteins were stained for glycosylated proteins with concanavalin A (Fig. 2A) or, less sensitively, for protein with amido black (Fig. 2B). The SDS-PAGE was optimized for separating the large-molecular-weight Gl proteins, and many smaller proteins are not shown. The electrophoretic pattern of control parotid salivas (lanes 1, 3, and 4) shows heterozygote phenotypes for the Gl protein size variants: samples in lanes 1 and 3 show the Gl 1-3 phenotype, and that in lane 4 shows the Gl 1-4 phenotype. The Gl protein is not seen in the saliva of the subject with the Gl 0 phenotype (lane 2).

In Fig. 2C, the salivary Gl proteins of control donors and the subject with the Gl 0 phenotype are shown after acid-

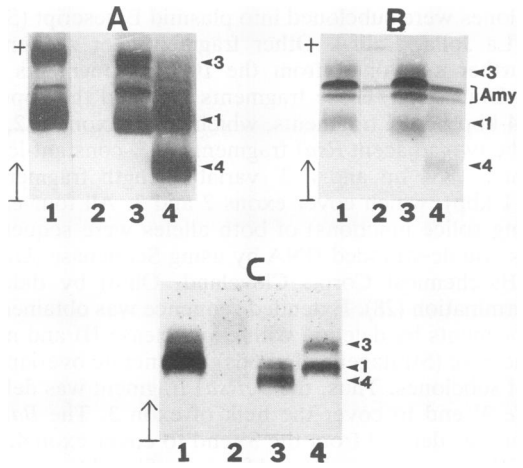

FIG. 2. Results of electrophoretic studies of salivary Gl proteins in the subject with the Gl 0 phenotype and control donors. Electrophoretic transfers of SDS gels were stained for glycoproteins with concanavalin A (A) or for proteins with amido black (B). The SDS-PAGE was optimized for detecting large-molecular-weight Gl proteins, and smaller proteins are not shown. Because of the marked sensitivity of the concanavalin A stain, the samples in panel A contain 7 μl of dried parotid saliva reconstituted in Laemmli sample buffer, whereas the samples in panel B contain 20 μl of dried saliva in the same buffer. Lanes in panels A and B: 1, Gl 1-3; 2, Gl 0; 3, Gl 1-3; 4, Gl 1-4. Amy, amylase. (C) Parotid salivary glycoproteins (0.6 ml of dried saliva reconstituted in 60 μl of sample buffer) were stained with the periodic acid-Schiff reagent after acid-lactate PAGE. Lane 1, Gl 1; lane 2, Gl 0; lane 3, Gl 1-4; lane 4, Gl 1-3.

lactate PAGE and periodic acid-Schiff staining. The control sample in lane 1 shows the Gl 1 phenotype and those in lanes 3 and 4 show Gl 1-4 and Gl 1-3 heterozygote phenotypes, respectively. Again, the Gl protein is not seen in the saliva of the subject with the Gl 0 phenotype (lane 2). An ill-defined smear is seen in lane 2, but this is noted for most samples, even heterozygote samples in which both *PRB3* alleles are expressed to give two different size-variant Gl proteins (lanes 3 and 4).

The saliva of the subject with the Gl 0 phenotype was typed for other PRP polymorphisms, and the results are as follows: Ps 2, +; PmF, −; PmS, −; CON I, +; CON II, −; Pe, +; Po, +; Pr 1-1, +; Db, +; Pa, −; PIF, + (not shown). Thus, the saliva of the subject with the Gl 0 phenotype contains a number of other PRPs.

DNA sequence analysis shows the same putative null mutation in both *PRB3* alleles of the subject with the Gl 0 phenotype. The four exons, including splice junctions, for *PRB3*M^{null} and *PRB3*VL^{null} alleles of the subject with the Gl 0 phenotype were completely sequenced and compared with those of Gl-productive *PRB3* alleles (7, 15, 19). The length difference between the *PRB3*M^{null} and *PRB3*VL^{null} alleles was due to two additional 63-bp tandem repeats (located upstream from the null mutation) in exon 3 of the larger *PRB3*VL^{null} allele (exon 3 portions of the alleles are 848 and 974 bp in length, respectively). The only potential null mutation detected was an identical C nucleotide insertion at nucleotides 621 and 747 in the same coding region of exon 3 of the *PRB3*M^{null} and *PRB3*VL^{null} alleles, respectively (nucleotides are numbered from the beginning of exon 3), and the insertions were located 228 bp from the ends of the exons. This C nucleotide insertion led to a frameshift with a premature termination codon (TAA) that occurred 42 nucleotides downstream from the insertion, and this probably results in markedly reduced or absent *PRB3* gene expression. All of the splice junctions and other exons of the two *PRB3* alleles of the subject with the Gl 0 phenotype were normal.

Although there is no evidence for expression, the *PRB3*M^{null} allele would code for a truncated 237-amino-acid protein (220 amino acids in exon 3 and 17 amino acids in exons 1 and 2). Similarly, the *PRB3*VL^{null} allele would code for a truncated 279-amino-acid protein (262 amino acids in exon 3 and 17 amino acids in exons 1 and 2). The normally encoded Gl proteins for *PRB3*M^{null} and *PRB3*VL^{null} alleles (without the C insertions) would be 292 and 334 amino acids in length, and the truncated proteins would be 19 and 16% smaller than the full-length proteins, respectively.

Figures 3A and B show the sequencing gel autoradiograms with nucleotides surrounding and including the C nucleotide insertions (circled) in the two *PRB3* null alleles of the subject with the Gl 0 phenotype. Figure 3C shows the normal sequence from the same region of exon 3 for the Gl-productive *PRB3*S^{cys} allele (7). The same normal sequence was also seen in two other Gl-productive *PRB3* alleles (19).

Saliva from the subject with the Gl 0 phenotype does not support adherence of *F. nucleatum*. Previously, we showed, using a nitrocellulose blot overlay technique, that radiolabeled *F. nucleatum* binds preferentially to a component of parotid saliva and that this receptor is the product of the *PRB3* gene (13, 27). We now investigated whether *F. nucleatum* would interact with saliva from the *PRB3*^{null} subject. Figure 4A shows the results of ^{35}S-labeled *F. nucleatum* overlay of nitrocellulose blots of parotid saliva collected from this individual, as well as parotid and whole salivas collected from two additional donors. No interaction of the labeled organisms with saliva collected from the subject with the Gl 0 phenotype (lane 1) was detected. In contrast, adherence of ^{35}S-labeled *F. nucleatum* to a low-molecular-weight (M_r, 36,900) PRG (donor 1; lanes 2 and 4) and a high-molecular-weight (M_r, 89,000) PRG (donor 2; lanes 3 and 5) in both parotid and whole salivas was demonstrated. Differences in the abilities of the samples from the various subjects to support adherence of *F. nucleatum* were seen despite the fact that silver staining of the samples (Fig. 4B, lanes 1 to 3) showed similar amounts of parotid saliva, as evidenced by the staining intensity of amylase, a major constituent of parotid saliva. Furthermore, bands corresponding in molecular weight to the low- and high-molecular-weight variant PRGs (Gls) were demonstrated in the salivas of donors 1 (lane 2) and 2 (lane 3). As described above for periodic acid-Schiff staining of acid-lactate gels, silver staining of parotid salivas from the subject with the Gl 0 phenotype (lane 1) and donor 2 (lane 3), both of which lacked a low-molecular-weight PRG, showed evidence of a much less intensely staining, diffuse band of a molecular mass similar to that of the low-molecular-weight Gl glycoprotein. The identity of this component(s) is currently unknown.

DISCUSSION

There is growing evidence that some PRPs and other salivary proteins serve as receptors for oral microorganisms. For example, certain oral streptococci bind with various degrees of specificity to different salivary glycoproteins. Pertinent to this report, *Streptococcus sanguis*, *Streptococcus gordonii*, and *Streptococcus oralis* bind to PRGs (among other salivary proteins [26]). The bacterium *F. nucleatum*, a

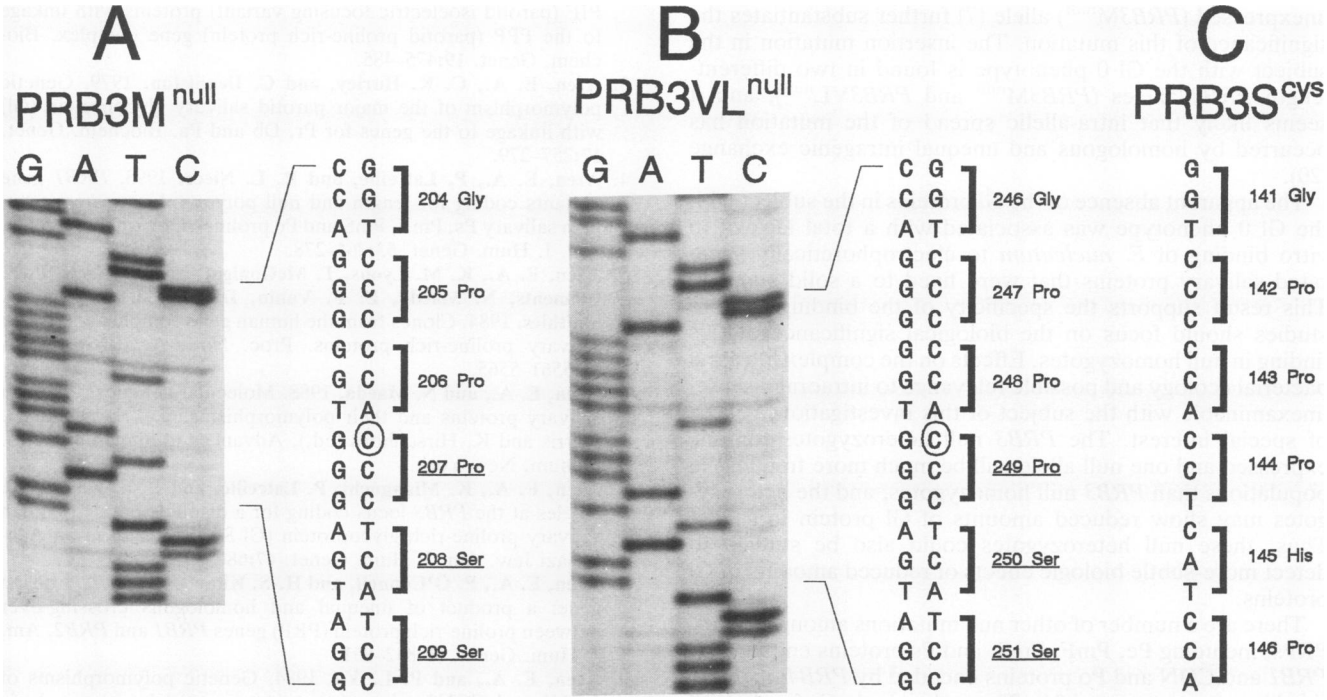

FIG. 3. DNA sequencing gel results for *PRB3* null alleles of the subject with the Gl 0 phenotype. Although the DNA sequences in panels A to C are from the same region of exon 3, the amino acids are numbered differently because of different numbers of tandem repeats upstream from this region in the *PRB3* alleles shown. (A and B) *PRB3*M^{null} and *PRB3*VL^{null} alleles of the subject with the Gl 0 phenotype. The sequences are from the noncoding strand. The coding-strand sequences and deduced amino acids, which are numbered according to their positions in exon 3, are also shown. Note the identical C nucleotide insertion mutations (circled) in the same region of exon 3. Frameshifted amino acids are underlined. (C) Gl-productive *PRB3*S^{cys} allele of a control subject (7). The coding-strand sequence and deduced amino acids from the same region of exon 3 as that shown in panels A and B are given for comparison.

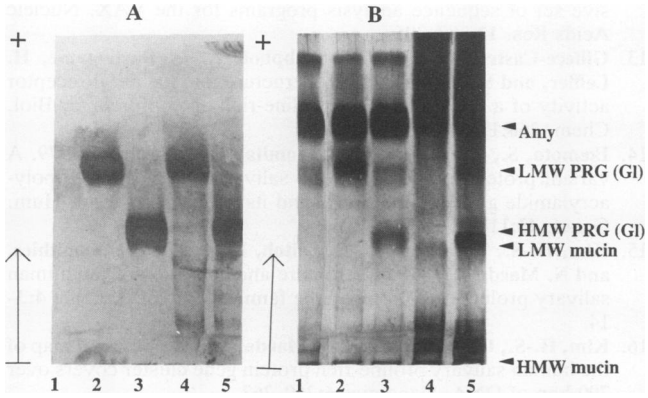

FIG. 4. Adherence of *F. nucleatum* to saliva samples from the subject with the Gl 0 phenotype and two donors with size-variant Gl proteins. (A) Autoradiogram. Lanes 1 to 3, parotid salivas from the *PRB3*^{null} donor, from donor 1 (showing low-molecular-weight PRG), and from donor 2 (showing high-molecular-weight PRG), respectively; lane 4, whole saliva from donor 1; lane 5, whole saliva from donor 2. Samples were separated by SDS-PAGE, transferred to nitrocellulose, and overlaid with ^{35}S-labeled *F. nucleatum*. Bacterial adherence was detected by autoradiography. Only the saliva samples from the subject with the Gl 0 phenotype (lane 1) failed to support adherence. (B) SDS-PAGE. Proteins from an identical gel were stained with silver. Electrophoretic mobilities are indicated on the right. LMW and HMW, low and high molecular weights, respectively; Amy, amylase.

component of subgingival plaque flora of patients with periodontal disease, also binds specifically to PRGs (13). The biological significance of these binding activities is unclear, but these interactions may lead to localization of bacteria on tooth or mucosal surfaces if the proteins are components of adhesive surface coatings. Also, soluble binding proteins that are not adsorbed to these surfaces could prevent this bacterial colonization by competing with surface-bound receptors and/or promoting agglutination. The microbial ecology of the oral cavity may be strongly influenced by these microbe–salivary-protein interactions.

The salivas of individuals with electrophoretically determined null phenotypes for specific salivary PRPs may be useful as negative controls to confirm the identity and specificity of microbe-PRP interactions. It is first necessary to establish that the electrophoretically missing protein is truly absent or markedly reduced in amount and is not present in some alternative form (possibly allelic) that is not electrophoretically recognized. This important criterion was met for the Gl protein in our subject whose two different-length *PRB3* alleles contain the same insertion mutation of a C nucleotide in the exon 3 coding regions. Such insertion frameshift mutations leading to premature termination codons may result in variably reduced or absent steady-state mRNA levels, as has been seen in a number of other genes (reviewed in references 11 and 31), and this mechanism probably contributed to the electrophoretically absent Gl protein in the subject with the Gl 0 phenotype. The presence of the same insertion mutation in a previously described Gl-null heterozygote with one expressed (*PRB3*S^{cys}) and one

unexpressed (*PRB3*M^{null}) allele (7) further substantiates the significance of this mutation. The insertion mutation in the subject with the Gl 0 phenotype is found in two different-length *PRB3* alleles (*PRB3*M^{null} and *PRB3*VL^{null}), and it seems likely that intra-allelic spread of the mutation has occurred by homologous and unequal intragenic exchange (20).

The apparent absence of the Gl proteins in the subject with the Gl 0 phenotype was associated with a total lack of in vitro binding of *F. nucleatum* to electrophoretically separated salivary proteins that were fixed to a solid support. This result supports the specificity of the binding. Future studies should focus on the biological significance of this finding in null homozygotes. Effects on the complex intraoral bacterial ecology and possible relevance to intraoral disease, unexaminable with the subject of this investigation, will be of special interest. The *PRB3* null heterozygotes for one expressed and one null allele will be much more frequent in populations than *PRB3* null homozygotes, and the heterozygotes may show reduced amounts of Gl protein in saliva. Thus, these null heterozygotes could also be studied to detect more-subtle biologic effects of reduced amounts of Gl proteins.

There are a number of other null mutations among salivary PRPs, including Pe, PmF, PmS, and Ps proteins encoded by *PRB1* and CON and Po proteins encoded by *PRB4* (summarized in references 6 and 24). The molecular basis for the null phenotypes has been established for several of these proteins. First, unequal and homologous crossovers between *PRB1* and *PRB2* may lead to a fusion gene (*PRB2/1*) that is missing the coding region of *PRB1*. Thus, *PRB2/1* homozygotes are totally lacking all *PRB1*-encoded proteins (Ps, Pe, PmF, and PmS [8]). Second, a premature stop [CGA(Arg)→TGA (stop)] mutation in the Ps protein-coding region of the *PRB1* gene results in absence of Ps protein and a markedly reduced amount of Pe protein (4). Third, precursor proteins from all of the *PRP* genes (except *PRB3*) may be proteolytically cleaved to generate a variety of smaller PRPs (22), and mutations at these cleavage sites could cause a lack of cleavage and electrophoretic absence of the smaller PRPs in saliva (19). Such mutations [CGA(Arg)→TGA (stop) and CGA(Arg)→CAA (Gln)] have been described and may account for absence of PmF and PmS proteins in salivas of some individuals (4, 19).

The possible binding activities of microbial organisms to these other PRPs (which show frequent null phenotypes) have not yet been established. However, if such activities exist, additional opportunities will occur for in vivo and in vitro studies of salivary-protein–microbe interactions which could be important in establishing individual differences in the intraoral microbial ecology and in clinical disease susceptibilities.

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