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Gingipain enzymes from *Porphyromonas gingivalis* preferentially bind immobilized extracellular proteins: a mechanism favouring colonisation?

Adrian D. McAlister, BSc (Hons)^{a,||}, Aneta Sroka, PhD^{b,||}, Rebecca E. Fitzpatrick, BSc (Hons)^a, Noelene S. Quinsey, PhD^a, James Travis, PhD^c, Jan Potempa, PhD^{b,||}, and Robert Neil Pike, Ph.D.^{a,||,*}

^aDepartment of Biochemistry and Molecular Biology and CRC for Oral Health Sciences, Monash University, Clayton, Victoria, 3800 ^bDepartment of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland ^cDepartment of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602. USA.

Abstract

Porphyromonas gingivalis, an anaerobic bacterium associated with adult periodontal disease, employs a number of pathogenic mechanisms, including protease/adhesin complexes (gingipains), fimbriae and haemagglutinins, to maintain attachment within colonised hosts. Here we show that the gingipains and whole, live *P. gingivalis* cells preferentially bind immobilised extracellular matrix proteins in the presence of soluble forms of the same proteins, which may constitute a colonisation mechanism in the oral environment. Fimbriae appeared to be redundant for adhesion to immobilised proteins in the presence of the gingipains, indicating that the protease-adhesins and hemagglutinins may be important for adhesion under these conditions. The data presented here provides evidence for a model of adhesion for *P. gingivalis* within the dynamic environment of the oral cavity.

Keywords

PORPHYROMONAS GINGIVALIS; HEMAGGLUTININS; PROTEASE-ADHESINS; GINGIPAINS; ADHESION; COLONISATION; FIMBRIAE

1. Introduction

The Gram negative, anaerobic bacteria *Porphyromonas gingivalis* is associated with adult periodontal disease in humans and uses a number of adhesin molecules to aid colonisation of the oral cavity, including fimbriae, hemagglutinins and protease/adhesin complexes [1,2,3,4]. Although each of these molecules possess a degree of functional redundancy with respect to one another, it is becoming apparent that each molecule may in fact have a preferred, discreet role under the competitive conditions found within the dynamic environment of the oral cavity *in vivo* [4,5,6,7,8,9].

*To whom correspondence should be addressed at: Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3800, Australia, Telephone: 61-3-9905 3923, Fax: 61-3-9905 4699, Email: E-mail: rob.pike@med.monash.edu.au.

^{||}These authors made equal contributions to this work.

The gingipain protease/adhesin complexes are believed to allow bacterial cells to intimately associate with proteins in the gum matrix, prior to the secretion and dissemination of the enzymes through the oral cavity, leading to the destruction of integral matrix proteins [3,10, 11]. It is likely that the gingipains are dominant factors in the physically destructive aspects of infection and associated disease [4].

The lysine- and arginine-specific protease/adhesin complexes (Kgp and HRgpA, respectively) consist of catalytic domains non-covalently associated with adhesin subunits that mediate gingipain binding to erythrocytes and the extracellular matrix proteins, fibrinogen, fibronectin and laminin [3,10,11,12,13]. Maturation of the Kgp and HRgpA pro-proteins requires *N*- and *C*-terminal proteolytic processing of the catalytic domains of both gingipains [14,15]. The *C*-terminal domains of HRgpA contain regions involved in adhesion to members of the oral biofilm, erythrocytes and fibrinogen, while the *C*-terminal domains of Kgp are required for successful folding and assembly of surface-expressed Kgp and are thought to aid bacterial adhesion to immobilised ligands [10,12,16,17].

The dominant surface-expressed hemagglutinin, HagA, shares homology to the *C*-terminal domains of HRgpA and Kgp [18]. Several open reading frames within the *P. gingivalis* genome have been identified as potential mediators of hemagglutination [19,20,21], and together with the gingipains, HagA, is involved in erythrocyte and haemoglobin binding, as well as heme acquisition, which is essential for survival [4].

P. gingivalis major (FimA) and minor (Mfa1) fimbriae are responsible for adhesion to a number of ligands in the oral environment, including matrix proteins, integrin receptors of fibroblasts and organisms found within the normal flora of the oral biofilm [1,2,22,23,24,25,26]. Proteolytic processing of the major fimbrial subunit protein, FimA, has been shown to depend on the arginine-specific gingipains, but not Kgp [4].

Here, we show that Kgp, HRgpA and whole *P. gingivalis* cells preferentially bind to immobilised human proteins, suggesting that, *in vivo*, the bacterium may prefer attaching to matrix proteins immobilised in gum tissue rather than those found in human body fluids, thus facilitating colonisation of the oral cavity.

2. Materials and Methods

2.1 Bacterial strains, fimbriae and purified enzymes

Sparsely (W83) and heavily (ATCC33277) fimbriated strains of *P. gingivalis* were routinely cultivated in Sheadler or brain heart infusion (BHI) broth, respectively [1,16]. Wild type Kgp and HRgpA were purified from culture supernatants of *P. gingivalis* strain HG66 as previously described [3,27]. Fimbriae from *P. gingivalis* strain ATCC33277 were purified according to a modified method originally described by Hamada *et al.* [1]. Briefly, *P. gingivalis* cells harvested from early stationary cultures by centrifugation were subjected to ultrasonication in an ice bath. Following centrifugation, fimbriae in the supernatant were purified by ammonium sulphate precipitation and chromatography on a DEAE-Sepharose CL-6B column. SDS-PAGE analysis of the final preparation showed a minor and major protein band of 67 kDa (Mfa1) and 41 kDa (FimA) (data not shown), respectively, indicating that a mixture of major (FimA) and minor (Mfa1) fimbriae was obtained.

2.2 Binding of Kgp to immobilised proteins

Kgp suspended in PBS was activated by 10 mM cysteine and then inhibited by the addition of 1mM α -p-Tosyl-L-lysine-chloromethyl ketone [TLCK] (Sigma, Sydney, Australia) at room temperature for 10 min. Matrix proteins (10 nM) were coated onto the wells of an ELISA plate (Nunc Maxisorp, Melbourne, Australia) and incubated at 4°C overnight. Plates were blocked

with 0.5% (w/v) BSA in PBS at room temperature for one hour. TLCK-inactivated Kgp (10 nM), chicken anti-Kgp IgY polyclonal antibody and anti-chicken IgY HRP-conjugated (Sigma, Sydney, Australia) antibodies were applied sequentially and incubated for one hour each. Plates were washed with 0.05% (v/v) Tween-20 in PBS three times between each protein application. Reactions were developed using tetramethylbenzidine (Sigma, Sydney, Australia) as a substrate.

2.3 Competition assays

Matrix proteins (10 nM) were coated and plates were blocked as above. TLCK-inactivated Kgp (10 nM) was added to the wells at the same time as human plasma or matrix proteins (0–100 nM) suspended in PBS, and incubated at room temperature for one hour. Kgp was detected and reactions were developed as above.

2.4 Whole cell binding assays

Whole cell *P. gingivalis* binding assays were conducted using the same techniques described above, except *P. gingivalis* cells were diluted to an OD₆₆₀ of 0.5 in PBS before being assayed. Cells were fixed using 0.4% (v/v) formaldehyde incubated overnight at 4°C and washed three times in PBS before use. Assays were conducted as described above.

3. RESULTS AND DISCUSSION

3.1 Binding of Kgp to immobilised proteins

The binding of proteolytically inactivated Kgp to immobilised fibrinogen (Fb), fibronectin (Fn) and vitronectin (Vn) was firstly examined here, essentially as described previously [10]. As shown previously, Kgp bound to Fb and Fn very effectively and here we show that the protease-adhesin was able to bind Vn, albeit with much lower signal from the ELISA employed (Fig. 1).

We estimated the stoichiometry of binding and the equilibrium dissociation constant for each interaction using the results of an ELISA in which increasing concentrations of Kgp were applied to the immobilised proteins (Fig. 2). The stoichiometry appeared to be equivalent to one, based on the concentration of the protein immobilised and the concentration of Kgp at which maximal binding was approached. Thus, for Fb and Fn, 10 nM of the protein was immobilised in each case and the maximal signal was approached at 10 nM, indicating a 1:1 interaction. This was also seen for Vn, where 50 nM was immobilised in order to maximise the signal. We tested the amount of protein left in coating solutions after immobilisation and it appeared that all protein had bound to the plate, indicating the stoichiometry estimated to most likely be correct. A single site binding equation fitted the curve for each protein, further indicating a stoichiometry of one and allowing the determination of the equilibrium dissociation constants for the interactions. We found that Kgp had the highest affinity for Fn with a K_d value of 0.65 nM, closely followed by Fb with a K_d value of 1.5 nM and the K_d value for Vn was 3.1 nM (all estimates of K_d values had standard errors of less than 10%).

The K_d value for Kgp binding to immobilised Fb obtained here was approximately 3-fold lower than that determined previously for Kgp binding to soluble Fb [13]. This indicated that Kgp bound immobilised Fb with three times the affinity of soluble Fb. If this were indeed the case *in vivo*, it would have interesting implications for binding of the extracellular proteins as a colonisation/invasion mechanism, whereby the protease/adhesins of *P. gingivalis* might preferentially bind host proteins in their matrix bound form compared to their soluble forms, which are also found for each of the proteins examined here. Interestingly, these K_d values are 2-fold lower than the K_d value for the binding of a RgpA-Kgp complex to Fb and Fn [28],

implying that association of both gingipains into a complex may somewhat decrease their affinity for Fb and Fn.

3.2 Purified Kgp preferentially binds to immobilised matrix proteins

In order to examine the preferential binding of immobilised proteins further, we investigated whether soluble forms of each of the proteins could compete for binding of purified Kgp in the ELISA format. For Fb, even a 10-fold higher concentration of soluble Fb was only able to reduce the signal for binding of the immobilised protein by less than 0.1 of an absorbance unit (Fig. 3a). Soluble Fn and Vn were similarly ineffective in reducing binding to immobilised Fb (Fig. 3a). Soluble Fb was more able to compete for binding to immobilised Fn, with an equal concentration reducing binding by approximately 25%, and a 10-fold excess reducing binding by just over 50% (Fig. 3b). Soluble Fn was also somewhat more effective in reducing the binding to immobilised Fn, although this was clearly less effective than soluble Fb (Fig. 3b). Soluble Fb and Fn very effectively competed for binding to Vn, with no binding detected in the presence of Fb and Fn above equimolar concentrations and soluble Vn was unable to effectively compete for binding to immobilised Fb and Fn (Fig. 3c). The results indicate that Kgp has a distinct preference for immobilised proteins over soluble proteins and that different extracellular matrix proteins are likely to be recognized with differing affinities by the same or overlapping domains of the gingipain molecule. Similar results to all of the above for Kgp were obtained for the HRgpA protease/adhesin (data not shown).

The above results indicate that it is likely that the change in conformation of the proteins when bound to a surface [a phenomenon that has been shown for fibronectin, for instance [29]] further exposes or changes the conformation of the binding site for the adhesins on the protein such that the affinity increases.

3.3 The preference for immobilised proteins is also exhibited by whole bacteria

We used live *P. gingivalis* strains W83 and ATCC33277 to examine whether soluble Fb could compete for binding of the bacterium to immobilised Fb. We found that there was no disruption of binding to the immobilised protein by soluble Fb (Fig. 4), indicating that the finding with the purified protein could also be seen with whole bacteria. The same results were found for fixed bacteria (results not shown).

3.4 Fimbriae are redundant for *P. gingivalis* adhesion to surfaces coated with extracellular matrix proteins

To assess the contribution of fimbriae to binding by the bacterium, we investigated the effect of purified fimbriae on the adhesion of *P. gingivalis* strains W83 and ATCC33277 to immobilised Fb. We found that both pre-incubation of Fb coated surfaces with fimbriae and the presence of fimbriae during binding did not affect adherence of *P. gingivalis* (Fig. 4). In contrast, similar treatments with soluble, inactivated Kgp decreased attachment of ATCC33277 by approximately 3-fold. Adhesion of strain W83 bacteria was only slightly affected by even the highest concentration of soluble Kgp. The significance of fimbrial adhesion in pathogenesis is becoming more clearly defined in the current body of literature. *P. gingivalis* fimbriae have been shown to compete for interactions with $\alpha v \beta 3$ and $\alpha 5 \beta 1$ integrin receptors, eliminating interactions with their ligands, vitronectin and fibronectin, respectively, in a dose-dependent manner [6]. Although purified fimbriae can interact with a variety of matrix proteins, including vitronectin and fibronectin, pre-incubation and competition of purified fimbriae did not disrupt interaction of whole live cells with immobilised Fb, suggesting that matrix proteins tend to not be the preferred ligand of fimbriae in a competitive environment, such as is likely to be found *in vivo* [6,25].

3.5 A suggested mechanism for colonisation

In vivo, the preferential binding of immobilised protein by adhesins from *P. gingivalis* may permit the bacterium to maintain attachment to the periodontal substratum during host colonisation, despite the presence of soluble forms of its ligands in the human body fluids bathing the environment, suggesting a mechanism for host colonisation. Further investigations into the binding site on Kgp for these extracellular proteins could lead to the production of inhibitors of colonisation, which could constitute highly effective agents for the prevention of periodontal disease.

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References

1. Hamada N, Watanabe K, Sasakawa C, Yoshikawa M, Yoshimura F, Umemoto T. Construction and characterization of a fimA mutant of *Porphyromonas gingivalis*. Infect. Immun 1994;62:1696–1704. [PubMed: 7909537]
2. Lamont RJ, Bevan CA, Gil S, Persson RE, Rosan B. Involvement of *Porphyromonas gingivalis* fimbriae in adherence to *Streptococcus gordonii*. Oral Microbiol. Immunol 1993;8:272–276. [PubMed: 7903442]
3. Pike R, McGraw W, Potempa J, Travis J. Lysine- and arginine-specific proteinases from *Porphyromonas gingivalis*. Isolation, characterization, and evidence for the existence of complexes with hemagglutinins. J. Biol. Chem 1994;269:406–411. [PubMed: 8276827]
4. Shi Y, Ratnayake DB, Okamoto K, Abe N, Yamamoto K, Nakayama K. Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. Construction of mutants with a combination of rgpA, rgpB, kgp, and hagA. J. Biol. Chem 1999;274:17955–17960. [PubMed: 10364243]
5. Mezyk-Kopec R, Bzowska M, Potempa J, Jura N, Sroka A, Black RA, Bereta J. Inactivation of membrane tumor necrosis factor alpha by gingipains from *Porphyromonas gingivalis*. Infect. Immun 2005;73:1506–1514. [PubMed: 15731048]
6. Nakagawa I, Amano A, Inaba H, Kawai S, Hamada S. Inhibitory effects of *Porphyromonas gingivalis* fimbriae on interactions between extracellular matrix proteins and cellular integrins. Microbes Infect 2005;7:157–163. [PubMed: 15716056]
7. Ogawa T, Asai Y, Hashimoto M, Uchida H. Bacterial fimbriae activate human peripheral blood monocytes utilizing TLR2, CD14 and CD11a/CD18 as cellular receptors. Eur. J. Immunol 2002;32:2543–2550. [PubMed: 12207338]
8. Sroka A, Sztukowska M, Potempa J, Travis J, Genco CA. Degradation of host heme proteins by lysine- and arginine-specific cysteine proteinases (gingipains) of *Porphyromonas gingivalis*. J. Bacteriol 2001;183:5609–5616. [PubMed: 11544223]
9. Yilmaz O, Watanabe K, Lamont RJ. Involvement of integrins in fimbriae-mediated binding and invasion by *Porphyromonas gingivalis*. Cell Microbiol 2002;4:305–314. [PubMed: 12027958]
10. Pavloff N, Pemberton PA, Potempa J, Chen WC, Pike RN, Prochazka V, Kiefer MC, Travis J, Barr PJ. Molecular cloning and characterization of *Porphyromonas gingivalis* lysine-specific gingipain. A new member of an emerging family of pathogenic bacterial cysteine proteinases. J. Biol. Chem 1997;272:1595–1600. [PubMed: 8999833]
11. Pavloff N, Potempa J, Pike RN, Prochazka V, Kiefer MC, Travis J, Barr PJ. Molecular cloning and structural characterization of the Arg-gingipain proteinase of *Porphyromonas gingivalis*. Biosynthesis as a proteinase-adhesin polypeptide. J. Biol. Chem 1995;270:1007–1010. [PubMed: 7836351]

12. Curtis MA, Aduse-Opoku J, Slaney JM, Rangarajan M, Booth V, Cridland J, Shepherd P. Characterization of an adherence and antigenic determinant of the ArgI protease of *Porphyromonas gingivalis* which is present on multiple gene products. *Infect. Immun* 1996;64:2532–2539. [PubMed: 8698476]
13. Pike RN, Potempa J, McGraw W, Coetzer TH, Travis J. Characterization of the binding activities of proteinase-adhesin complexes from *Porphyromonas gingivalis*. *J. Bacteriol* 1996;178:2876–2882. [PubMed: 8631676]
14. Veith PD, Chen YY, Reynolds EC. *Porphyromonas gingivalis* RgpA and Kgp proteinases and adhesins are C terminally processed by the carboxypeptidase CPG70. *Infect. Immun* 2004;72:3655–3657. [PubMed: 15155679]
15. Veith PD, Talbo GH, Slakeski N, Dashper SG, Moore C, Paolini RA, Reynolds EC. Major outer membrane proteins and proteolytic processing of RgpA and Kgp of *Porphyromonas gingivalis* W50. *Biochem. J* 2002;363:105–115. [PubMed: 11903053]
16. Sztukowska M, Sroka A, Bugno M, Banbula A, Takahashi Y, Pike RN, Genco CA, Travis J, Potempa J. The C-terminal domains of the gingipain K polypeptide are necessary for assembly of the active enzyme and expression of associated activities. *Mol. Microbiol* 2004;1393–1408. [PubMed: 15554977]
17. Kamaguchi A, Ohyama T, Sakai E, Nakamura R, Watanabe T, Baba H, Nakayama K. Adhesins encoded by the gingipain genes of *Porphyromonas gingivalis* are responsible for co-aggregation with *Prevotella intermedia*. *Microbiol* 2003;149:1257–1264.
18. Han N, Whitlock J, Progulske-Fox A. The hemagglutinin gene A (hagA) of *Porphyromonas gingivalis* 381 contains four large, contiguous, direct repeats. *Infect. Immun* 1996;64:4000–4007. [PubMed: 8926061]
19. Lepine G, Progulske-Fox A. Duplication and differential expression of hemagglutinin genes in *Porphyromonas gingivalis*. *Oral Microbiol. Immunol* 1996;11:65–78. [PubMed: 8941757]
20. Progulske-Fox A, Tumwasorn S, Holt SC. The expression and function of a *Bacteroides gingivalis* hemagglutinin gene in *Escherichia coli*. *Oral Microbiol. Immunol* 1989;4:121–131. [PubMed: 2700777]
21. Progulske-Fox A, Tumwasorn S, Lepine G, Whitlock J, Savett D, Ferretti JJ, Banas JA. The cloning, expression and sequence analysis of a second *Porphyromonas gingivalis* gene that codes for a protein involved in hemagglutination. *Oral Microbiol. Immunol* 1995;10:311–318. [PubMed: 8596675]
22. Hamada N, Sojar HT, Cho MI, Genco RJ. Isolation and characterization of a minor fimbria from *Porphyromonas gingivalis*. *Infect. Immun* 1996;64:4788–4794. [PubMed: 8890240]
23. Lamont RJ, Hsiao GW, Gil S. Identification of a molecule of *Porphyromonas gingivalis* that binds to *Streptococcus gordonii*. *Microb. Pathog* 1994;17:355–360. [PubMed: 7723662]
24. Nakagawa I, Amano A, Kuboniwa M, Nakamura T, Kawabata S, Hamada S. Functional differences among FimA variants of *Porphyromonas gingivalis* and their effects on adhesion to and invasion of human epithelial cells. *Infect. Immun* 2002;70:277–285. [PubMed: 11748193]
25. Nakamura T, Amano A, Nakagawa I, Hamada S. Specific interactions between *Porphyromonas gingivalis* fimbriae and human extracellular matrix proteins. *FEMS Microbiol. Lett* 1999;175:267–272. [PubMed: 10386378]
26. Njoroge T, Genco RJ, Sojar HT, Hamada N, Genco CA. A role for fimbriae in *Porphyromonas gingivalis* invasion of oral epithelial cells. *Infect. Immun* 1997;65:1980–1984. [PubMed: 9125593]
27. Chen Z, Potempa J, Polanowski A, Wikstrom M, Travis J. Purification and characterization of a 50-kDa cysteine proteinase (gingipain) from *Porphyromonas gingivalis*. *J. Biol. Chem* 1992;267:18896–18901. [PubMed: 1527017]
28. Pathirana RD, O'Brien-Simpson NM, Veith PD, Riley PF, Reynolds EC. Characterization of proteinase-adhesin complexes of *Porphyromonas gingivalis*. *Microbiol* 2006;152:2381–2394.
29. Wolff A, Lai CS. Fluorescence energy transfer detects changes in fibronectin structure upon surface binding. *Arch. Biochem. Biophys* 1989;268:536–545. [PubMed: 2913946]

Fig. 1.

Binding of Kgp to immobilised fibrinogen, fibronectin and vitronectin. Each protein was coated at 10 nM (black bars) and 1 nM (grey bars). Following blocking, wells were incubated with 10 nM TLCK-inactivated Kgp, washed and bound Kgp was detected in an ELISA using as described previously [19].

Fig. 2.

Determination of the equilibrium binding concentrations for Kgp binding to fibrinogen, fibronectin and vitronectin. A range of concentrations of TLCK-inactivated Kgp were applied to plates coated with 10 nM Fb, Fn and Vn. A single site binding equation was used to fit a line to the data using non-linear regression (GraphPad Prism version 3) and thereby yield a K_d value for the binding interaction.

Fig. 3.

Competition assays to determine whether soluble proteins can prevent binding to immobilised forms of fibrinogen, fibronectin and vitronectin. Soluble forms of Fb, Fn and Vn suspended in PBS competed for interactions to TLCK-inactivated Kgp against each of the same three immobilised proteins using an ELISA-based system. **A)** 10nM immobilised Fb (light grey bars) and Fn (dark grey bars), and 50nM immobilised Vn (black bars) were competed against increasing concentrations of soluble Fb. **B)** 10nM immobilised Fb (light grey bars) and Fn (dark grey bars) and 50nM of immobilised Vn (black bars) were competed against increasing concentrations of soluble Fn. **C)** 10nM immobilised Fb (light grey bars) and Fn (dark grey

bars) and 50nM immobilised Vn (black bars) were competed against increasing concentrations of soluble Vn.

Fig. 4. Comparison of adherence of sparsely (W83) [dotted bars] and heavily fimbriated (ATCC33277) [hatched bars] strains of live *P. gingivalis* whole cells to immobilized Fb in the presence of soluble proteins at the indicated concentrations.