11 Bacterial Peptides Targeting Periodontal Pathogens in Communities

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11.1 Introduction

The major, or more accurately keystone, pathogen in periodontitis is *Porphyromonas gingivalis*, an indigenous organism found in the oral cavity of most adults. *P. gingivalis* is well adapted to the subgingival ecosystem, where it can be located on the root surface, in the gingival crevicular fluid, as well as in and on gingival epithelial cells [1, 2]. A variety of properties underpin the success of *P. gingivalis* in the complex subgingival microenvironments. *P. gingivalis* expresses fimbrial and other surface protein adhesins that mediate attachment to other biofilm constituents and to host surfaces such as epithelial cells [2–7]. An asaccharolytic organism, *P. gingivalis*, secretes proteases, including the arginine- and lysine-specific gingipains, to provide peptides and amino acids for growth. Hemin released from heme-containing proteins such as hemoglobin is acquired by a number of hemin transport systems as the primary source of iron [1, 8, 9]. While normally in a homeostatic relationship with the host, if uncontrolled the factors that contribute to colonization and survival can also contribute to dysbiosis and tissue destruction. Moreover, the usual ecological context of *P. gingivalis* is a

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constituent of a polymicrobial biofilm, and other community inhabitants can exacerbate or restrain pathogenic potential [1, 5, 10]. Similarly, *P. gingivalis* as a keystone pathogen can modulate overall community pathogenicity, or nososymbiocity [10, 11], and it is the community that is the fundamental etiological unit initiating periodontal diseases. The concept of nososymbiocity gives rise to novel approaches for the control of the bacterial component in periodontal diseases. Community engineering to reduce nososymbiocity may be a more viable strategy than attempting to eliminate *P. gingivalis*, which is difficult to achieve as the organism is embedded in biofilms and within epithelial cells. Favoring the growth of organisms that are antagonistic to *P. gingivalis* and reducing the levels of synergistic or accessory pathogens would impede the transition to a homeostatic community to a pathogenic entity. More specifically, targeting the molecules that effectuate the interaction of *P. gingivalis* with other community participants and the increase in nososymbiocity have shown promise in both in vitro and in vivo studies.

11.2 *P. gingivalis* Community Development

The accumulation of periodontal bacteria into heterotypic communities is driven by co-adhesive and physiological compatibility. Thus, organisms that can provide nutrients for mutual growth tend to attach to each other and associate together, and this underlies structural partitioning within communities [2, 5]. As a strict anaerobe, *P. gingivalis* also benefits from the metabolic activity of the antecedent community which reduces oxygen levels. *P. gingivalis* binds avidly to the early biofilm colonizers such as actinomyces and streptococci, and the importance of attachment of *P. gingivalis* to a streptococcal substratum is evident from human studies. Oral introduction of *P. gingivalis* in human volunteers results in the organism initially localizing almost exclusively on streptococcal-rich supragingival plaque [12], and *P. gingivalis* is frequently detected in the supragingival plaque of healthy humans [13–23]. Oral streptococci also comprise a significant portion of the microbial population of subgingival plaque [24–34], and *P. gingivalis* is often isolated together with streptococci, including *S. gordonii,* from subgingival plaque samples [35]. Moreover, imaging studies of human dental plaque have revealed highly organized microbial consortia, within which *Porphyromonas* associates with streptococci and a number of other taxa [36, 37].

The molecular basis of attachment to *Streptococcus gordonii* has been defined in detail [38–40]. Co-adhesion is mediated by two sets of adhesin-receptor pairs: the FimA and Mfa1 component fimbriae of *P. gingivalis* which interact with streptococcal GAPDH and SspA/B surface proteins, respectively (Fig. 11.1). The accumulation of *P. gingivalis* also requires sensing of the streptococcal metabolite para-amino benzoic acid (pABA) [40] which is utilized in the tetrahydrofolate pathway. Both chemical sensing through pABA and physical attachment interface with a tightly regulated signal transduction cascade within *P. gingivalis* based on protein tyrosine (de)phosphorylation [41, 42] (Fig. 11.2) which controls the expression of fimbrial adhesins and the extent of community development [40]. Communities of *P. gingivalis-S. gordonii* are synergistically pathogenic in the murine model of alveolar bone loss [43], and hence the association of *P. gingivalis* with *S. gordonii* provides a therapeutic target for restricting the pathogenic potential of *P. gingivalis*.

11.3 Inhibition of Fimbrial Assembly

The fimbriae of *P. gingivalis* mediate most of the adhesive properties of the organism and can bind to other bacteria, host cells, and extracellular matrix proteins [5, 44, 45]. The Mfa1 fimbriae of *P. gingivalis*, which engage with the streptococcal SspA/B surface protein, are assembled by a unique mechanism that entails proteolytic processing of lipidated precursor subunits. Polymerization of processed subunits occurs on the cell surface by a donor strand exchange (DSE) mechanism which can involve either the N- or

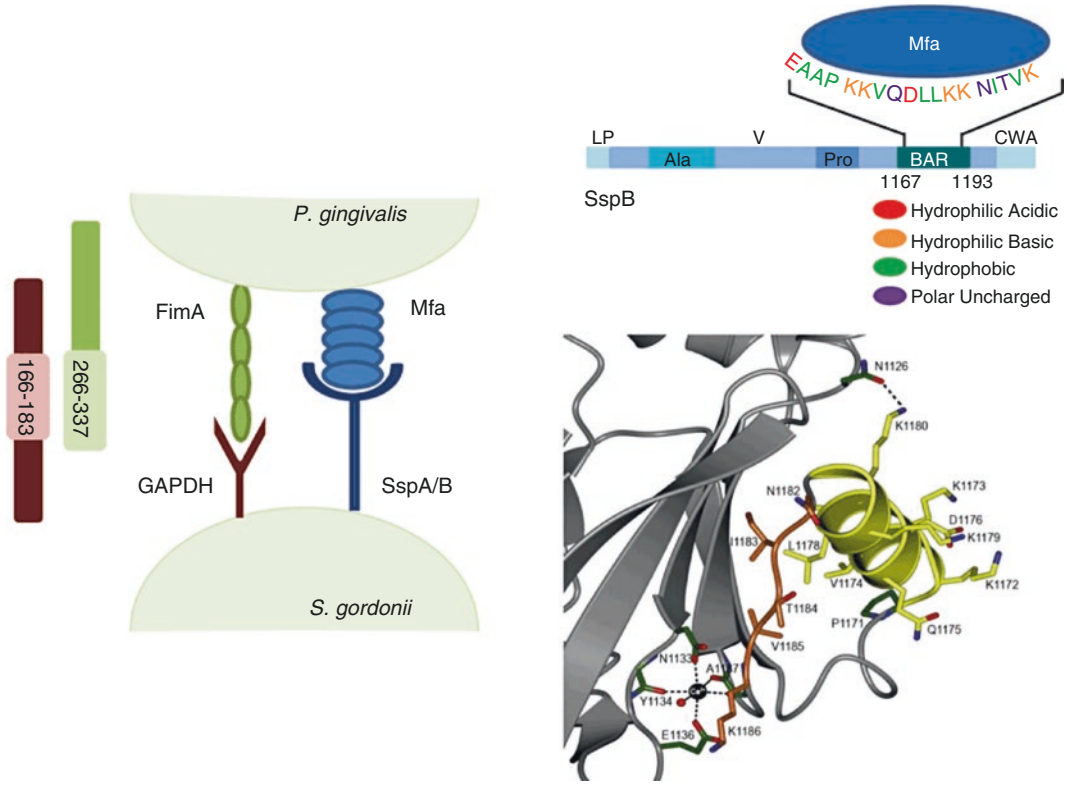

Fig. 11.1 Structures required for the maintenance and stabilization of *P. gingivalis–S. gordonii* co-adhesion. Center panel shows the FimA–GAPDH and Mfa1–SspA/B adhesin–receptor pairs. The left panel shows the interacting domains of the FimA and GAPDH proteins with the amino acid (aa) residues indicated. The upper right panel shows the domain structure of the SspB protein and the aa residues involved in recognition of Mfa1. *LP* leader peptide, *Ala* alanine-rich repeats, *V* variable region, *Pro* proline-rich repeats, *BAR* Mfa1-interacting domain, *CWA* cell wall anchor. BAR spans aa residues 1167–1193, and the EAAP, KKVQDLLKK, and NITVK sequences are involved in Mfa1 recognition. The lower right panel shows the structure of the SspB C-terminal region with the protruding BAR handle. Reproduced with permission from [44] https://doi.org/10.1111/j.1365-2958.2011.07707.x

C-terminal regions [46, 47]. Given their novel extracellular assembly mechanism and their critical role in *P. gingivalis* colonization, the Mfa1 fimbriae are potential targets for disruption or prevention of *P. gingivalis* community development. Alaei et al. [48] established that a 9-residue peptide derived from the Mfa1 C-terminal region significantly inhibited fimbrial polymerization through competing with the endogenous subunit pro region and donor strand for binding in the cognate hydrophobic groove. Moreover, the Mfa1 C-terminal peptide caused a significant reduction in *P. gingivalis* adhesion to *S. gordonii* in a dual species community model. Since the assembly of the Mfa1 fimbriae occurs on the extracellular surface, inhibitory molecules do not need to cross the bacterial membranes. Hence molecules based on the Mfa1 C-terminal region have promise for delivery in the oral cavity.

11.4 Mimicking the Streptococcal Receptor for *P. gingivalis*

The SspA/B adherence-mediating domain (designated BAR, SspB Adherence Region) is also well characterized, and spans amino acid (aa) residues 1167–1193. BAR is fully conserved between SspA and SspB [49] and contains three distinct functional domains that encompass aa residues 1182–1186 (NITVK), 1174–1178 (VQDLL), and 1168–1171 (EAAP). Within

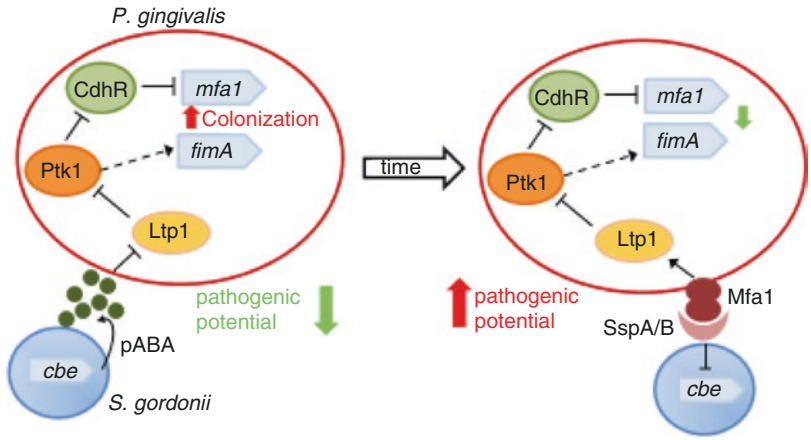

Fig. 11.2 Communication in *P. gingivalis-S. gordonii* communities. Interactions between *P. gingivalis* and *S. gordonii* resulting from metabolite (pABA) perception (left) and direct contact (right). pABA secreted by *S. gordonii* inactivates the *P. gingivalis* tyrosine phosphatase Ltp1. Dephosphorylation and inactivation of the tyrosine kinase Ptk1 are thus reduced. Ptk1 phosphorylates and inactivates the transcription factor CdhR, which is a repressor of the *mfa1* gene. Ptk1 activity also converges on the expression of the *fimA* gene. The expression of both fimbrial adhesins is increased, and in this mode *P. gingivalis* is primed for attachment to *S. gordonii*. However, nososymbiocity is reduced, and pABA-treated *P. gingivalis* cells are less pathogenic in animal models. The engagement of Mfa1 with the streptococcal SspA/B surface protein increases Ltp1 and reverses information flow through the Ltp1–Ptk1 axis. In addition, Mfa1–SspA/B binding suppresses the expression of chorismate binding enzyme (Cbe), which is responsible for pABA production. Prolonged physical interaction between *P. gingivalis* and *S. gordonii* leads to increased nososymbiocity, and dual infection of animal models causes more alveolar bone loss than *P. gingivalis* infection alone. Reproduced with permission from [2] https://doi.org/10.1038/s41579-018-0089-x

the NITVK domain, the N1182 and V1185 residues are essential for recognition by Mfa1 [50]; however, it is the physical properties of the amino acids occupying the 1182 and 1185 positions which dictate activity. Substitution of basic amino acids for N1182 and substitution of hydrophobic residues for V1185 enhance binding to Mfa1, indicating that both electrostatic and hydrophobic interactions contribute to the BAR-Mfa1 interaction [51]. The VQDLL motif of BAR constitutes an α-helix that resembles the eukaryotic nuclear receptor box domain which is involved in protein–protein interactions through a hydrophobic or amphipathic α-helical motif [52]. The VQDLL domain is flanked on either side by two lysine residues which may stabilize the initial binding through a charge clamp mechanism. EXXP, which is a Ca^{2+} binding motif, is represented in BAR by EAAP [43], and crystallography data show that Pro1171 constrains the amphipathic VQDLL α-helix such that hydrophobic residues V1174, L1177, and L1178 face a hydrophobic contact surface [53]. Further, the crystal structure shows that the NITVK and VQDLL motifs protrude from the polypeptide in a configuration described as an attachment "handle," and this structure is stabilized by Ca^{2+} [53]. A synthetic peptide based on the BAR region inhibits *P. gingivalis-S. gordonii* co-adhesion and community development and diminishes the pathogenicity of *P. gingivalis* in a mouse model of periodontitis. In order to develop clinical applicability, mechanisms that allow the delivery of sustained high concentrations of BAR have been explored [54]. The incorporation of BAR into poly (lactic-co-glycolic acid) (PLGA) and methoxy-polyethylene glycol PLGA (mPEG-PLGA) nanoparticles (NPs) resulted in sustained release of active BAR concentrations [55]. The BAR-encapsulated NPs were capable of inhibiting *P. gingivalis-S. gordonii* community formation and disrupting pre-existing communities [55]. BAR-encapsulated NPs may thus provide a potent platform to disrupt the association between *P. gingivalis* and *S. gordonii*. However, the use of peptides as topically applied therapeu-

tic agents in the oral cavity is problematic due to the cost of peptide synthesis and susceptibility to proteases which are produced in abundance by oral bacteria. Small-molecule mimetics of BAR are therefore an attractive alternative. Patil et al. [56] synthesized stable small-molecule mimetics of the BAR region by joining mimics of VXXLL and NITVK through the "click" reaction. Molecules with a 2,4,5-trisubstituted oxazole framework blocked *P. gingivalis* adherence to *S. gordonii* in vitro when "click" coupled with substituted arylalkynes. Moreover, community development was also inhibited in the presence of *Fusobacterium nucleatum*, which can bind to both *P. gingivalis* and *S. gordonii*, indicating the potential for efficacy in the more complex multispecies plaque biofilm environment in vivo [57]. Significantly, several of the BAR-derived compounds reduced alveolar bone loss in mice infected with *P. gingivalis*-*S. gordonii* communities. The most-active compounds were compact molecules with short intraatomic distances between the fluorine and methoxy groups.

11.5 Antagonistic Interactions Among Oral Bacteria

In addition to polymicrobial synergy, antagonistic interactions among oral organisms also occur. Interbacterial antagonistic interactions may arise through competition for nutrients or space, or can be mediated by specific toxic compounds such as bacterocins and antibiotics, or by type VI secretion systems. In the oral cavity, pH is also important and *Streptococcus mutans* which is strongly acidogenic can inhibit the growth of *Treponema denticola* and *P. gingivalis* [58]. Hydrogen peroxide produced by oral streptococci is also toxic to other organisms such as *Aggregatibacter actinomycetemcomitans* [59, 60]. Another example of rivalry is illustrated by *P. gingivalis* and *Streptococcus mitis*. *P. gingivalis* is able to induce DNA fragmentation and death in *S. mitis* [61], consistent with the overarching keystone pathogen concept that *P. gingivalis* can orchestrate the composition and function of oral microbial communities.

11.6 Inhibition of Virulence Expression in *P. gingivalis* by *S. cristatus*

Thee expression of the virulence genes is regulated in *P. gingivalis* in response to precarious environments. Noteworthy results from oral bacterial cell-cell communication studies indicate that the expression of FimA, the major subunit protein of the long fimbriae of *P. gingivalis,* is suppressed in the presence of *S. cristatus*. Consequently, *P. gingivalis* binds poorly to the surface of *S. cristatus* and the two organisms do not accumulate into dual species communities [62]. These data suggest that *S. cristatus* may have an ability to re-direct the development of pathogenic dental biofilms by preventing *P. gingivalis* attachment and colonization. This concept is supported by the findings that, unlike the case for *S. gordonii* [43], oral infection of experimental animals with *P. gingivalis* and *S. cristatus* together results in less alveolar bone loss compared to *P. gingivalis* alone [63]. Additionally, the examination of patients with periodontitis demonstrated an inverse relationship between the number of *S. cristatus* and *P. gingivalis* cells in dental plaque [64].

The investigation of the molecular basis of the antagonistic interactions between *P. gingivalis* and *S. cristatus* identified a surface protein of *S. cristatus*, arginine deiminase (ArcA), as the signaling molecule to which *P. gingivalis* responds by repressing the expression of the *fimA* gene [65]. ArcA catalyzes the hydrolysis of L-arginine to L-citrulline and ammonia, and the latter is believed to be important for oral biofilm pH homeostasis and also caries prevention [66]. The reduction in arginine levels can also impede the ability of *P. gingivalis* to form communities with oral streptococci [67].

Many species of streptococci are arginine deiminase-positive [68]; however, the expression of the *arcA* gene varies significantly. Under standard growth conditions, *S. cristatus* transcript levels are almost 15-fold higher than those in *S. gordonii* [69]. Relatively lower ArcA expression in *S. gordonii* compared to *S. cristatus* may be one factor that contributes to the different relationships

of each of these organisms with *P. gingivalis*. The inhibition of FimA production in *P. gingivalis* may require a relatively high expression of *arcA*, and the limited expression of *arcA* in *S. gordonii* consequently is not able to induce a response in *P. gingivalis*. The mechanistic basis of differential expression of *arcA* in various streptococcal species is not well defined, but sequence variation in the promoter regions of *arcA* likely plays a key role in dictating the level of gene expression [69]. ArcA from *S. intermedius* also downregulates *fimA* transcription as well as the expression of the *mfa1* gene encoding the Mfa1 minor fimbrial adhesive subunit protein [67, 70]. Despite the overall similarity in outcomes, the mechanisms of action of ArcA in *P. gingivalis* antagonism may differ between *S. intermedius* and *S. cristatus*. In the case of *S. intermedius* the enzymatic function of ArcA is necessary for antagonistic activity. For *S. cristatus,* the converse may be the case as the arginine deiminase inhibitors, aminoguanidine and L-lysine, while being able to block the arginine deiminase activity of ArcA, had no effect on the inhibition of *fimA* expression [65]. Moreover, transwell experiments show that the *P. gingivalis*–*S. cristatus* antagonistic interaction requires direct cell–cell contact [71].

A systematic analysis of the active domains of ArcA from *S. cristatus* identified an 11-mer peptide with the native amino acid sequence (NIFKKNVGFKK) which could bind tightly to the surface of *P. gingivalis* [71]. Strikingly, in addition to the inhibition of *fimA* transcription, this peptide also inhibited the expression of several virulence genes in *P. gingivalis*, including *mfa1* and genes encoding the gingipain proteases (*rgpA/B* and *kgp*), with half-inhibitory concentrations (IC50) of around 10 μM. Consistent with the suppression of transcripts, the production of fimbrial proteins and gingipains was also significantly reduced when *P. gingivalis* was treated with this peptide, which thereby was designated Streptococcal-derived Anti-*P. gingivalis* Peptide (SAPP).

The ability of SAPP to prevent community development has been demonstrated through in vitro and ex vivo studies. Treatment of *P. gingivalis* with SAPP significantly reduced the formation of monospecies biofilms of *P. gingivalis* strains including both fimbriated and afimbriated lineages. This result is significant as strains of *P. gingivalis* devoid of fimbriae have been be isolated from periodontal pockets. SAPP can also inhibit the development of heterotypic *P. gingivalis-S. gordonii* communities and attachment of *P. gingivalis* to saliva-coated surfaces [71]. Another important finding is that SAPP not only inhibits *P. gingivalis* attachment to saliva-coated or to streptococcal surfaces but also disrupts established *P. gingivalis-S. gordonii* biofilms. Although the mechanism is unclear, it is speculated that SAPP inhibits re-entry of the detached *P. gingivalis* cells into the biofilm. Dispersed *P. gingivalis* cells will then have a greater chance of being eliminated from the oral cavity due to swallowing or expectoration. SAPP also works effectively to impede multi-species biofilm formation. In an ex vivo study, dental plaque retrieved from periodontitis patients was grown in the presence or absence of SAPP [72]. Bacteria bound to culture well surfaces were collected, and the number of sessile bacteria was determined using qPCR. The amount of *P. gingivalis* detected in complex multi-species biofilms cultured with SAPP was significantly lower than that found in wells without SAPP. This inhibitory activity is not strain-specific, as the effects of SAPP on the inhibition of *P. gingivalis* attachment were found with four distinct fimA genotypes (I, II, III, and IV). More remarkable was the finding that along with *P. gingivalis*, other chronic periodontitis-associated bacteria, *Tannerella forsythia* and *Treponema denticola*, were also significantly reduced in abundance in biofilms in the presence of SAPP. However, there was no significant difference in the numbers of streptococci in biofilms cultured in the presence or absence of SAPP. These data suggest that SAPP may affect colonization of other periodontal pathogens in highly orchestrated oral microbial biofilms and that *P. gingivalis*, as a keystone bacterium, plays an important role in modulating the composition of multispecies biofilms, as has been demonstrated in animal studies [11].

Another important aspect to the pathoecology of *P. gingivalis* in the gingival compartment

is the ability of the organism to enter into gingival epithelial cells and reside intracellularly for extended periods [73]. The invasion of *P. gingivalis* is initiated following the stimulation of epithelial cell integrin receptors by the FimA adhesin [74]. The invasion efficiency of *P. gingivalis* strains grown with SAPP at 2 × 50% of inhibitory concentration of *fimA* expression was reduced by approximately five-fold compared with *P. gingivalis* without SAPP treatment. Intracellular *P. gingivalis* impinge on a number of eukaryotic signal transduction pathways some of which converge on cytokine and chemokine expression, a phenomenon known as immune paralysis [2, 75, 76]. Further, *P. gingivalis* gingipains can degrade several cytokines/chemokines. Another feature of SAPP is its ability to reverse impaired secretion of cytokines and chemokines induced by *P. gingivalis*. The accumulation of IL-6, IL-8, and MCP-1 (Monocyte chemoattractant protein 1), in the extracellular milieu of gingival epithelial cells, was restored when *P. gingivalis* was pretreated with SAPP (Fig. 11.3). IL-8 and MCP-1 are both well-known chemoattractant cytokines. MCP-1 plays important roles in regulating the migration and infiltration of monocytes/macrophages [77], while IL-8 is involved in the recruitment of neutrophils to the site of infection [78]. By preventing *P. gingivalis* antagonism of these compounds, SAPP may be able to reduce the impairment of host immunity by *P. gingivalis*, which in turn may facilitate the maintenance of periodontal tissue homeostasis.

SAPP is an 11-mer peptide with molecular weight of 1322.62 Da and theoretical isoelectric point (pI) of 10.48. The secondary structure of SAPP as determined by circular dichroism (CD) spectroscopy shows it exists as a random coil in water. SAPP also appears to have no significant helical propensity, as helix structures were not formed in the presence of tri-fluoroethanol, a helix-inducing agent [72]. SAPP is soluble in

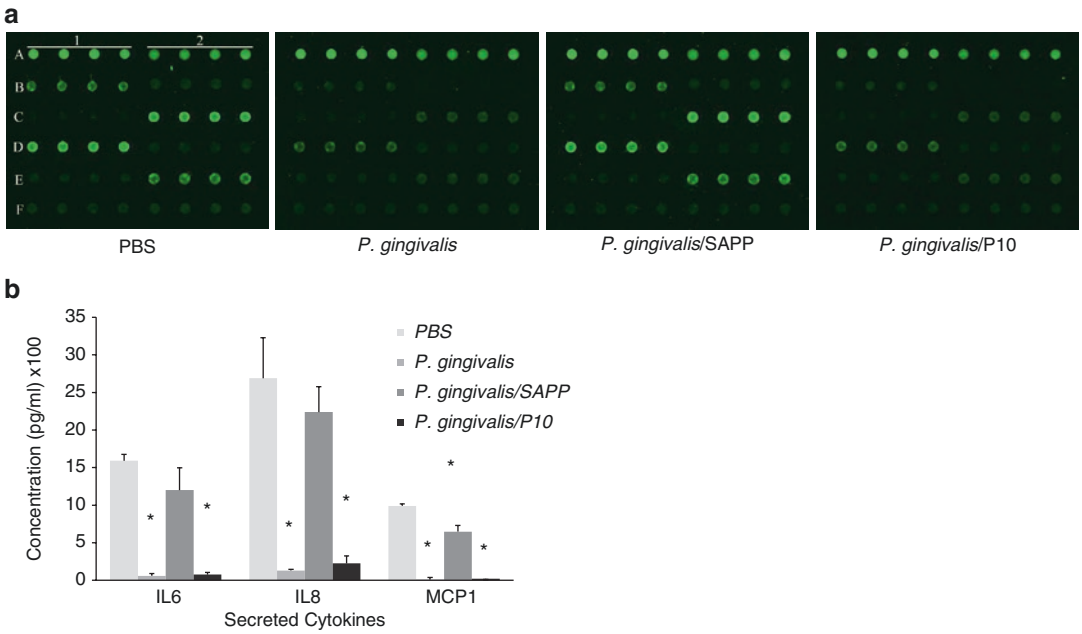

Fig. 11.3 The role of SAPP in reversing *P. gingivalis* induced immune paralysis. (**a**) Gingival epithelial cells were reacted with PBS, *P. gingivalis* 33277, *P. gingivalis* treated with SAPP (30 μM), or *P. gingivalis* treated with a SAPP analog (P10). Levels of 10 selected cytokines secreted into the media were determined by a protein array. Each antibody is printed in quadruplicate horizontally: A1 and A2 are biotin-labelled IgG (positive control); B1 is IL-1α; B2 is IL-1β; C1 is IL-4; C2 is IL-6; D1 is IL-8; D2 is IL10; E1 is IL-13; E2 is MCP-1; F1 is INF-γ; and F2 is TNF-α. (**b**) Quantitation of array data for three selected cytokines. Asterisks indicate significant differences in the levels of cytokines detected with or without *P. gingivalis* treatment (*t* test, $p < 0.05$)

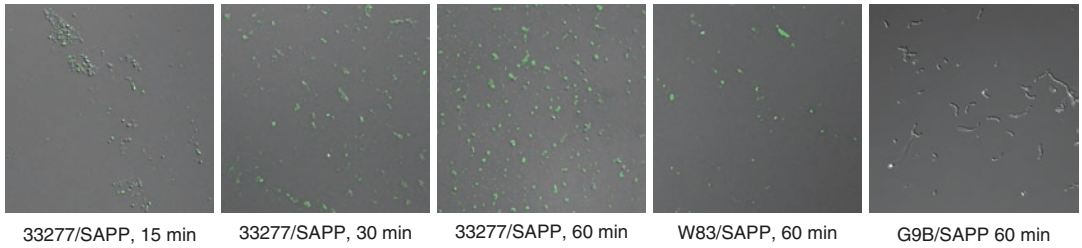

Fig. 11.4 Specific interaction of SAPP and *P. gingivalis* strains. Biofilms of *P. gingivalis* 33277, W83, and *S. gordonii* G9B were formed on saliva-coated glass and exposed to FITC-labeled SAPP (5 μM) for 15, 30, or 60 min. Bacterial-associated SAPP (green) was visualized using confocal microscopy

water and PBS, and is relatively stable for at least 24 h at 25 °C. However, gradual degradation of SAPP occurs in diluted whole saliva within a 24 h period. Toxicology studies detected no plasma membrane impairment in gingival epithelial cells or in human periodontal ligament fibroblasts (HPLFs) after exposure to SAPP at concentrations as high as 800 μM (approximately 80 times greater than the IC50 for *fimA* and *rgpA* expression) for as long as 48 h. Moreover, SAPP treatment also did not induce apoptosis or necroptosis of these cell types. These properties of SAPP make it a promising agent for therapeutic development against chronic periodontitis.

The mechanism by which SAPP initiates signal transduction in *P. gingivalis* is currently not clear, although direct interaction between SAPP and *P. gingivalis* cells appears to be required. Fluorescently labeled SAPP can be detected on *P. gingivalis* surfaces after a 15 min exposure, and a time-dependent increase in SAPP binding was observed during a 1 h exposure (Fig. 11.4) [72]. Two functionally inactive SAPP derivatives that have one or two residues substituted with alanine failed to bind efficiently to *P. gingivalis* cells. SAPP binds specifically to *P. gingivalis*, and as shown in Fig. 11.4, SAPP interacts with both fimbriated and afimbriated stains (33277 and W83) but not with *S. gordonii*. Two surface proteins of *P. gingivalis*, a major immunodominant antigen (RagB, PGN_0294) and a protein of the MotA/TolQ/ExbB proton channel family (PGN_0806), were identified as putative receptors of SAPP. The deletion of the genes encoding these proteins significantly reduced the *P. gingivalis* response to SAPP. Previously, the *Pseudomonas aeruginosa* TonB–ExbB–ExbD protein complex was reported to be involved in signal transduction [79]. Moreover, RagA, which is thought to associate with RagB on the *P. gingivalis* surface, is a TonB-dependent receptor [80–82]. Hence TonB-dependent transportation may be necessary for SAPP action.

11.7 Conclusion

Treatment of chronic periodontitis conventionally involves surgical and nonsurgical mechanical therapies that physically remove the plaque biofilm. In cases of severe and refractory manifestations of the disease, the treatments are sometimes supplemented with systemic or local administration of antibiotics [83–85]. Concerns regarding the use of antibiotics include the disruption of symbiotic or mutualistic relationships between the host and commensal microbiota [86], and the emergence of oral bacteria resistance to antibiotics [87]. In either event, currently available treatments for periodontitis are often only temporarily effective and recurrence of the disease is common [88, 89], possibly due to incomplete pathogen elimination [90]. Therefore, therapeutic agents that specifically interfere with the colonization of periodontal pathogens are an attractive and advantageous alternative over conventional antibiotics. Compounds that target fimbrial assembly or the Mfa1-Ssp interaction show substantial promise, particularly with regard to the development of active small-molecule mimetics. The advantage of SAPP is that it inhibits surface attachment, host invasion, and biofilm formation

of *P. gingivalis* in vitro by repressing the expression of a plurality of virulence factors including the fimbrial adhesins and gingipain proteases. Importantly, SAPP has a broad, though species-specific, activity against a spectrum of *P. gingivalis* strains but not oral commensal bacteria. Selectively targeting *P. gingivalis* has the added advantage of preventing the pathogenic influence of the keystone pathogen on the entire community, and thus potentially maintaining a healthy oral microbiota.

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