VimA is part of the maturation pathway for the major gingipains of *Porphyromonas gingivalis* W83

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The authors have shown previously that the *vimA* gene, which is part of the *bcp-recA-vimA* operon, plays an important role in protease activation in *Porphyromonas gingivalis*. The gingipain RgpB proenzyme is secreted in the *vimA*-defective mutant *P. gingivalis* FLL92. An important question that is raised is whether the *vimA* gene product could directly interact with the proteases for their activation or regulate a pathway responsible for protease activation. To further study the mechanism(s) of VimA-dependent protease activation, the *vimA* gene product was further characterized. A 39 kDa protein consistent with the size of the predicted VimA protein was purified. In protein–protein interaction studies, the VimA protein was shown to interact with gingipains RgpA, RgpB and Kgp. Immune sera from mice immunized with *P. gingivalis* immunoreacted with the purified VimA protein. Taken together, these data suggest an interaction of VimA with the gingipains and further confirm the role of this protein in their regulation or maturation.

INTRODUCTION

The expression of extracellular proteolytic activities is highly regulated in both prokaryotic and eukaryotic systems. This regulation can occur at multiple levels, including expression of the protease genes, secretion, processing of an inactive secreted precursor to its active form and/or the post-translational glycosylation of the proteins (Gallagher *et al.*, 2003; Bosques *et al.*, 2004). The multiple layers of regulation are vital to ensure that expression is tightly controlled in the appropriate temporal and spatial patterns.

Porphyromonas gingivalis, a black-pigmented Gram-negative anaerobic bacterium, is an important aetiological agent of chronic adult periodontitis and is associated with other systemic diseases (Amano, 2003; Amano *et al.*, 2000; Deshpande *et al.*, 1998; Grau *et al.*, 2004). While several virulence factors have been implicated in the pathogenicity of *P. gingivalis*, the high proteolytic abilities of this organism have been the focus of much attention, and appear to play an important role in virulence. The major proteases, called gingipains, are both extracellular and cell-associated. They consist of arginine-specific protease [Arg-gingipain (Rgp)] and lysine-specific protease [Lys-gingipain (Kgp)] (Nakayama, 2003).

The mechanism of gingipain regulation in *P. gingivalis* is unclear. We have shown that the *vimA* gene can modulate the phenotypic expression of the gingipains in *P. gingivalis* (Abaibou *et al.*, 2001; Olango *et al.*, 2003; Vanterpool *et al.*, 2005b). The *vimA* gene is part of the *bcp-recA-vimA* transcriptional unit (Fig. 1). A *vimA*-defective mutant strain designated *P. gingivalis* FLL92 was non-black pigmented and showed significant reductions in proteolytic, haemolytic and haemagglutinating activities (Abaibou *et al.*, 2001). While a reduction in Arg-X- and Lys-X-specific proteolytic activities was observed in *P. gingivalis* FLL92, transcription of the gingipain genes was unaltered in these mutants compared to that of the wild-type strain (Abaibou *et al.*, 2001). A similar phenotype of the gingipain genes was also seen in *P. gingivalis* FLL32, a *recA*- and *vimE*-defective isogenic mutant that had reduced Arg-X- and Lys-X-specific proteolytic activities (Abaibou *et al.*, 2000; Vanterpool *et al.*, 2004). While there was a unique late onset of Arg-X- and Lys-X-specific proteolytic activity in *P. gingivalis* FLL92, there was little or no observed change of proteolytic activity in stationary-phase in *P. gingivalis* FLL93, a *vimE*-defective mutant (Vanterpool *et al.*, 2004). Collectively, these observations have raised the question whether the regulation of proteolytic activity in *P. gingivalis* may occur by multiple mechanisms. Further, it is unclear if the *vimA* gene product can physically interact with the gingipains or regulate a pathway for protease maturation/activation. To investigate the mechanism of VimA-dependent gingipain biogenesis, the *vimA* gene product was further characterized. The results described here demonstrate an interaction

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<i>recA</i>	<i>vimA</i>	<i>vimE</i>	<i>vimF</i>
Plays a role in DNA damage and may play a role in regulating virulence	Play a role in regulating the maturation and activation of the major virulence factors Also play a role in carbohydrate biogenesis		Encodes a putative glycosyltransferase that plays a role in the maturation and activation of the major virulence factors

Fig. 1. The *recA-vimA-vimE-vimF* virulence-regulating locus of *P. gingivalis* and roles of the genes in this bacterium. See Vanterpool *et al.* (2005) for the role of *vimF*.

of VimA protein with the gingipains and further confirm a possible role of this protein in their regulation.

METHODS

Cloning of *vimA* into an expression vector. Oligonucleotide primers specific for the ORF of the *vimA* gene were synthesized (*vimA* forward, 5'-ATGCCCATCCCTCTATACCTG-3'; *vimA* reverse, 5'-TACCTGTTTTTGCTGACCGG-3') and used in PCR amplification of the *vimA* gene as previously described (Abaibou *et al.*, 2001). The fragment carrying the *vimA* gene was cloned into pCRT7/CT-TOPO expression plasmid (Invitrogen) carrying coding for a C-terminal His-tag. The recombinant plasmid, designated pFLL131, was transformed into competent BL21(DE3)pLysS *Escherichia coli*. The orientation was determined by restriction endonuclease digestion. The nucleotide sequence of the insert in pFLL131 was analysed by DNA sequencing to rule out any mutations.

Expression and purification of rVimA. *E. coli* BL21(DE3)pLysS carrying pFLL131 was grown to exponential phase (OD₆₀₀ 0.7) in Luria-Bertani broth in the presence of carbenicillin (50 µg ml⁻¹) and chloramphenicol (24 µg ml⁻¹). IPTG to a final concentration of 1 mM was added at exponential phase and the culture was incubated at 37 °C with shaking for a further 6 h. Cells were harvested by centrifugation and lysed by sonication as previously reported (Johnson *et al.*, 2004). Cell membranes and debris were then harvested by centrifugation, and the supernatant and pellet were analysed for the recombinant protein, rVimA, by SDS-PAGE. The insoluble rVimA was solubilized from the pellet with 6 M guanidine/20 mM sodium phosphate/0.5 mM NaCl. For large-scale preparation, rVimA was purified by FPLC using a HiTrap Chelating Ni-NTA column (GE Healthcare). rVimA was eluted using guanidine elution buffer (6 M guanidine, 20 mM sodium phosphate, 0.5 mM NaCl, 0.5 M imidazole). Eluate fractions were analysed for rVimA by SDS-PAGE. Desired fractions were then pooled and dialysed against 8 M urea buffer (8 M urea, 20 mM sodium phosphate, 0.5 mM NaCl) followed by a protein-binding buffer (20 mM sodium phosphate, 0.5 mM NaCl, 0.5 M L-arginine). For small-scale preparation, a Ni-NTA magnetic bead and magnetic separator (Qiagen) was used to purify the recombinant protein according to the manufacturer's recommendations. The presence of the poly-histidine tag was confirmed using the GelCode 6 × His Protein Tag kit according to the manufacturer's instructions (Pierce).

Preparation of extracellular fractions of *P. gingivalis*. *P. gingivalis* W83 was grown to stationary phase (OD₆₀₀ 1.5) from actively growing cells. Cells were harvested by centrifugation at 10 000 g for 30 min. The cell-free culture fluid was precipitated with 37.5% or 60% acetone (−20 °C), and the protein pellet was resuspended in 7 ml 100 mM Tris/HCl buffer (pH 7.4) in the presence of 1 mM N-p-tosyl-L-lysine chloromethyl ketone (TLCK), dialysed for 24 h against the same buffer and then stored on ice or at 0 °C.

Protein–protein interaction studies. Approximately 75 µg of the purified rVimA protein was incubated with the Ni-NTA-linked

magnetic beads. The beads with attached rVimA were washed with wash/interaction buffer (50 mM NaH₂PO₄, 300 mM NaCl, 50 mM imidazole and 0.005% Tween 20) and incubated with purified extracellular RgpB from *P. gingivalis* (Athens Research Technology) or with cell lysates or extracellular proteins from *P. gingivalis* W83 or FLL92. As a negative control, the lysates or extracellular fractions from *P. gingivalis* were incubated with the magnetic beads without the attached rVimA. After incubation, the unbound proteins were eliminated by repeated washing in wash/interaction buffer. Proteins were eluted off the beads under denaturing conditions [1 × lithium dodecyl sulfate (LDS) sample buffer incubated at 90 °C for 5 min].

Gel electrophoresis and immunoblot analysis. SDS-PAGE was performed with a 4–12% Bistris separating gel in MOPS-SDS running buffer (NuPAGE Novex gels; Invitrogen) according to the manufacturer's instructions. Samples were prepared (65% sample, 25% 4 × NuPAGE LDS sample buffer, 10% NuPAGE reducing agent), heated at 72 °C for 10 min and then electrophoresed at 200 V for 65 min in the XCell SureLock Mini-Cell System (Invitrogen). The protein bands were visualized by staining with Simply Blue Safe stain (Invitrogen). The separated proteins were then transferred to BioTrace nitrocellulose membranes (Pall Corporation) and processed at 15 V for 25 min with a Semi-Dry Trans-blot apparatus (Bio-Rad). The blots were probed with gingipain-specific antibodies (Potempa *et al.*, 1998). The secondary antibody used was immunoglobulin G (heavy plus light chains)–horseradish peroxidase conjugate (Zymed Laboratories). Immunoreactive proteins were detected using the Western Lighting Chemiluminescence Reagent Plus kit (Perkin-Elmer Life Sciences).

Identification of proteins that can physically interact with rVimA. Proteins in the eluates from the Ni-NTA magnetic beads (with or without the attached rVimA) were separated by SDS-PAGE and stained. Bands were excised using a gel cutter and dried in a Speed Vac (Savant Instruments) for 1 h. Dried gel bands were reduced with 20 µl 20 mM tri(2-carboxyethyl)phosphine (TCEP) at 56 °C and then alkylated with 20 µl 40 mM iodoacetamide for 30 min at 23 °C. Alkylated samples were then washed twice with 100 mM ammonium bicarbonate and dried using the Speed Vac for 1 h. Fifteen microlitres of digestion buffer (15 µl of 0.05 µg µl⁻¹ trypsin stock diluted in 3% acetic acid) was added to the dried gel slices and incubated for 10 min on ice. Excess digestion buffer was removed and 10 µl 100 mM ammonium bicarbonate was added to cover the gel slices in order to prevent drying. The gel slices were then incubated for 16 h at 30 °C. After 16 h, an additional 10 µl 100 mM ammonium bicarbonate was added to gel slices followed by incubation for an additional 30 min at 30 °C. Proteins were then trapped, washed and eluted using ZipTip_{C18} according to the manufacturer's instructions (Millipore). Eluted peptides were dried in the Speed Vac for 5–10 min and resuspended in 0.05% trifluoroacetic acid (TFA) in MS-grade water. All buffers were prepared in 100 mM ammonium bicarbonate. Tryptic peptides were separated and analysed on a Picoview model PV-500 Nanospray ESI unit (New Objective) coupled to an LCQ Deca XP ion trap mass spectrometer (Thermo Electron) using a four-event programme consisting of a

full MS scan followed by three MS/MS events for the most intense ions on full MS. A 75 $\mu\text{m} \times 10\text{ cm}$ capillary column packed with 5 μm C-18 coated silica was developed with a 40 min gradient elution programme of 2–90 % acetonitrile buffered with 0.5 % acetic acid and 0.005 % TFA at a flow rate of 300 nl min^{-1} . Data were collected with the Xcalibur software (Thermo Electron) and screened using Bioworks 3.1 Turboquest software (Thermo Electron) against a pgin.fasta database downloaded from the Los Alamos National Laboratory (<http://www.oralgen.lanl.gov>) website. Peptide tandem mass spectra were screened to filter out low/poor-quality spectra. Individual peptide matches were also confirmed manually using the BLAST database at <http://www.oralgen.lanl.gov>. Proteins were considered to be identified if at least two different peptides were identical matches.

Immunization of ApoE mice with *P. gingivalis*. Mating pairs of C57BLK mice (male ApoE^{-/-}) were purchased from Jackson Laboratories. Breeding of ApoE^{+/-} mice was performed at the Animal Care Facility of Loma Linda University. Male and female mice were pair-housed (monogamous pairing: one male and one female). The litters were weaned between 21 and 28 days and separated into male and female groups (5 animals per group). The animals were challenged with *P. gingivalis* according to the method described by Baker *et al.* (2000). At 10 weeks, the animals were infected by oral gavage three times at 2 day intervals with 10⁹ c.f.u. of live *P. gingivalis* W83 or *P. gingivalis* FLL92 in 100 μl PBS containing 2 % carboxymethylcellulose. Controls included sham-infected mice, which received the carboxymethylcellulose gavage without *P. gingivalis* (W83/FLL92). At 14 or 24 weeks after the first gavage, mice were euthanized using CO₂. The blood was collected by cardiac puncture. Sera collected were stored at -80 °C. Similarly, the sera of unimmunized (baseline) animals were collected and stored at -80 °C. These experiments were performed under authorization of a Loma Linda University approved animal use protocol (OSR #83045).

RESULTS

Bioinformatic analysis of VimA

BLAST and homology searches of VimA failed to yield matches to any known or conserved proteins or enzymic domains in the databases (<http://www.oralgen.lanl.gov> or <http://www.ncbi.nlm.nih.gov>). However, hydrophobicity scaling (<http://www.tigr.org>) of the VimA protein showed approximately 20 aa residues located at the N-terminus to be hydrophobic. This could suggest that this protein is a putative membrane protein.

rVimA interacts with the major gingipains of *P. gingivalis* W83

We have previously shown that inactivation of the *vimA* gene alters the maturation of the gingipains (Olango *et al.*, 2003; Vanterpool *et al.*, 2005b; Fig. 1). Expression of the cloned His-tagged *vimA* ORF in *E. coli* BL21(DE3)pLysS cells as described in Methods, followed by purification using a Ni-NTA column, showed that *vimA* encodes the expected 39 kDa recombinant protein (data not shown). To determine if VimA can interact with the gingipains, rVimA attached to Ni-NTA magnetic beads was incubated with cell lysates from *P. gingivalis* W83 or FLL92. As a negative control, the lysates were incubated with the

magnetic beads without the recombinant protein. As shown in Fig. 2(a), multiple proteins that interacted with rVimA were detected. Western blot analysis of the eluates using anti-RgpA and anti-Kgp antibodies showed immunoreactive bands consistent with the catalytic and haemagglutinin domains of RgpA and Kgp (Fig. 2b, c). In addition, an immunoreactive 50 kDa band and bands ranging from 70 to 90 kDa representing the catalytic RgpB and membrane-type RgpB, respectively, were observed (Fig. 2d). To determine if rVimA could interact with the mature processed gingipain, magnetic beads with rVimA attached were incubated with purified RgpB. As shown in Fig. 3, a 50 kDa band, consistent with the size of RgpB, was observed only when VimA was incubated in the presence of the extracellular fraction from *P. gingivalis*.

rVimA interacts with possible regulatory proteins

To identify other proteins that may interact with rVimA, protein bands from the eluates were analysed by LC-MS. In addition to two sugar metabolism proteins, β -lactamase and a putative sialidase, several hypothetical proteins and the HtrA homologue were also identified to interact with rVimA *in vitro* (Table 1). LC-MS analysis of interacting partners of rVimA interestingly demonstrated a unique 26 aa peptide residue (RACEMEGIHITFDFEGSMLEGIEGFFR) present on the hypothetical protein PG1833 and also the periplasmic serine protease HtrA. This 26 aa residue is not part of the predicted sequence of these proteins (<http://www.oralgen.lanl.gov>) but is identical to the amino acid sequence near the C-terminus of the VimA protein (see <http://www.oralgen.lanl.gov> for the full sequence).

VimA is antigenic in an ApoE mouse model

To determine if the VimA protein is exposed to the host's immune system, sera from mice challenged with the parent strain or the *vimA*-defective isogenic mutant were examined. Western blot analysis using sera from animals immunized with *P. gingivalis* W83 exhibited immunoreactivity with rVimA (Fig. 4). In contrast, sera from animals not immunized with *P. gingivalis*, or immunized with the *vimA*-defective mutant FLL92, exhibited no immunoreactivity with the rVimA protein. Taken together, these results suggest that the *vimA* gene product is immunogenic in the ApoE mice.

DISCUSSION

VimA is a putative membrane protein that appears to play a role in virulence regulation in *P. gingivalis* via gingipain biogenesis (Abaibou *et al.*, 2001; Olango *et al.*, 2003; Vanterpool *et al.*, 2005b). If *vimA* is part of a pathway(s) that is involved in the maturation and/or activation of the gingipains, then it is likely that VimA could interact with the gingipain. Our studies have indicated that rVimA may indeed interact with the gingipains from *P. gingivalis*. The fact that rVimA interacted with RgpB only in the presence of

Fig. 2. rVimA interacts with gingipains RgpA, RgpB and Kgp. (a) Eluates from interaction studies with cell lysates of *P. gingivalis* W83 and FLL92 in the exponential phase (E) or stationary phase (S) were separated by SDS-PAGE and stained with SimplyBlue Safestain. (b–c) Eluates from protein interactions with lysates prepared from cells grown to late exponential phase were analysed by immunoblotting using gingipain-specific antibodies: anti-RgpA (b), anti-Kgp (c) or anti-RgpB (d). W83 (–), *P. gingivalis* W83 incubated with beads only; FLL92 (–), *P. gingivalis* FLL92 incubated with beads only (negative controls).

other extracellular proteins may suggest that this interaction occurs via a protein complex. The presence of the 70 kDa RgpB in the interaction studies using cell lysates and its absence using RgpB from extracellular fractions indicates that, in addition to the 50 kDa matured form of RgpB, rVimA can also interact with the heavily glycosylated membrane-type RgpB, which is not found in the extracellular fractions.

Several other proteins were observed to interact with rVimA. These proteins in other systems are known to be involved in post-translational regulation. The multifunctional periplasmic HtrA protein is a heat-shock-induced serine protease which is important in many organisms. At low temperatures it can provide chaperone function. However, proteolytic activity is induced at high temperature. HtrA has also been shown to degrade misfolded proteins; it can play a role in oxidative stress resistance and can regulate growth at high temperature (az-Torres & Russell, 2001; Cortes *et al.*, 2002; Foucaud-Scheunemann & Poquet, 2003; Lipinska *et al.*, 1990; Lyon & Caparon, 2004; Pallen & Wren, 1997; Poquet *et al.*, 2000). In addition, HtrA is involved in the maturation of proteins, including cysteine protease (Poquet *et al.*, 2000; Foucaud-Scheunemann & Poquet, 2003; Lyon & Caparon, 2004). The inactivation of HtrA has also resulted in attenuated virulence in several organisms (Cortes *et al.*, 2002; Lyon & Caparon, 2004). The regulatory role(s) of HtrA in *P. gingivalis* is unclear. Our preliminary studies have shown that HtrA can interact with and regulate the gingipains under environmental stress conditions (data

not shown). Thus, the interaction of VimA with HtrA further confirms a regulatory role of VimA in *P. gingivalis*; however, its specific mechanism is unclear and is currently under investigation.

Glycosylation is a post-translational regulatory mechanism that is important in gingipain biogenesis in *P. gingivalis* (Gallagher *et al.*, 2003; Curtis *et al.*, 1999; Vanterpool *et al.*, 2005a, b). Further, the post-translational addition of carbohydrates to the gingipains is highly variable, thus implicating a role for multiple factors in this process. In this study, the VimA protein was also observed to interact with the β -lactamase and sialidase proteins, which are known to play a role in sugar metabolism (Ishikura *et al.*, 2003; Smayevsky *et al.*, 2001; Brook, 1989; Hedberg & Nord, 1996; Sechi *et al.*, 2004). Although sialylation can be involved in protein maturation, its role, if any, in gingipain biogenesis is unknown. Furthermore, we cannot rule out the possibility that the VimA protein may regulate the function of these proteins via a common mechanism shared with the gingipains. Carbohydrates biogenesis was altered in a *P. gingivalis* vimA-defective mutant (Vanterpool *et al.*, 2005b). The presence of sialidase in many organisms suggests that sialidase activity may be important for colonization and/or pathogenicity. In *P. gingivalis*, similar to many organisms, the sialidase may cleave selected sugar moieties from tissues, leaving them susceptible to cleavage by proteolytic enzymes, such as the gingipains. The physical interaction of the sialidase with VimA could further suggest a role for VimA in the coordinate regulation of several of the virulence factors

Fig. 3. Interaction of VimA with matured processed RgpB. Purified RgpB and extracellular proteins of *P. gingivalis* W83 were incubated with Ni-NTA beads in the presence or absence of rVimA. Eluates of the protein interaction were separated by SDS-PAGE and subjected to immunoblot analysis with RgpB-specific antibodies as probes. Lanes: 1, purified RgpB in the presence of VimA; 2, purified RgpB in the absence of VimA; 3, *P. gingivalis* W83 extracellular proteins in the presence of VimA; 4, *P. gingivalis* W83 extracellular proteins in the absence of VimA.

of this organism. The role of this sialidase gene in *P. gingivalis* is being further evaluated in our laboratory.

Many cellular processes are facilitated by supramolecular complexes (Sheng & Sala, 2001). Several protein domains have been described in both eukaryotic and prokaryotic systems that are involved in protein–protein interactions in these complexes (Pawson *et al.*, 2002; Ponting, 1997; Sheng & Sala, 2001; Agrawal & Kishan, 2002).

Fig. 4. VimA is antigenic in a mouse model. Serum was drawn from *P. gingivalis*-challenged ApoE mice after immunization. Sera from challenged mice were then used as primary antibodies against purified rVimA protein during immunoblotting.

We demonstrated the presence of a 26 aa residue of VimA on two *P. gingivalis* proteins, HtrA and hypothetical protein PG1833, which would be consistent with the hypothesis that these proteins can interact. This 26 aa sequence may be a direct interaction site, but we cannot rule out the possibility that its presence on HtrA and PG1833 is due to heavy contamination from the rVimA bait. The HtrA protein has PDZ domains that are known to interact with

Table 1. Proteins that interact with recombinant VimA

Gene ID	Protein	Description
PG1101	Alanyl-tRNA synthase	Translation
PG0535	HtrA	Serine periplasmic protein
PG1833	Conserved hypothetical protein	Unknown
PG1337	Hypothetical protein	Unknown
PG0324	Conserved hypothetical protein (putative sialidase)	Unknown
PG1768	RgpA	Arg-gingipain
PG0461	RgpB	Arg-gingipain
PG1605	Kgp	Lys-gingipain
PG0010	β -Lactamase	Energy/sugar metabolism

the C-terminus of other proteins. Similar to the HtrA protein in other organisms, this protein in *P. gingivalis* shares a similar structure (<http://www.oralgen.lanl.gov>; <http://www.tigr.org>) (Ponting, 1997). A preliminary structural analysis (data not shown) of the protein encoded by the PG1833 gene shows a transmembrane protein with a domain that is conserved among several other proteins of unknown function (<http://www.oralgen.lanl.gov>; <http://www.tigr.org>). The function of this domain and its importance in the interaction of VimA are currently being investigated in our laboratory. Collectively, however, these data may suggest that a complex of proteins could be involved in gingipain biogenesis in *P. gingivalis*. It is unclear whether VimA may have an 'adaptor' protein function similar to what occurs during caspase activation (Boatright *et al.*, 2003; Boatright & Salvesen, 2003a, b). The gingipain RgpB has been shown to have a caspase-like fold (Eichinger *et al.*, 1999).

Finally, the ability of sera from animals immunized with *P. gingivalis* to recognize VimA indicates that this protein is exposed to the immune system during the course of an infection. This would be consistent with a putative membrane location for VimA and could have therapeutic implications.

In summary, our data have demonstrated an interaction of VimA protein with the gingipains and other proteins that could affect its maturation/activation. This further supports a possible role of this protein in their regulation. The specific mechanism of VimA-dependent regulation in *P. gingivalis* is being further investigated in our laboratory.

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