Characterization of a second cell-associated Arg-specific cysteine proteinase of Porphyromonas gingivalis and identification of an adhesin-binding motif involved in association of the prtR and prtK proteinases and adhesins into large complexes

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Porphyromonas gingivalis has been associated with the development of adult periodontitis and cysteine proteinases with Arg- and Lys-specific activity have been implicated as major virulence factors. In a cell sonicate of P. gingivalis W50, a complex of non-covalently associated proteins has been previously characterized. This complex is composed of a 45 kDa Arg-specific, calcium-stabilized cysteine proteinase (PrtR45), a 48 kDa Lys-specific cysteine proteinase (PrtK48) and seven sequence-related adhesins designated PrtR44, PrtR15, PrtR17, PrtR27, PrtK39, PrtK15 and PrtK44, with all proteins being encoded by the two genes prtR and prtK.

It has been proposed that these non-covalently associated complexes form extracellularly after autolytic processing of the PrtR and PrtK polyproteins, with the adhesins binding to the proteinases (PrtR45 and PrtK48) and autoaggregating. Another form of the cell-associated, Arg-specific, calcium-stabilized cysteine proteinase is described here. Designated PrtR1150, it is a discrete 50 kDa protein with no adhesin-association and has enzymic characteristics and an inhibitor/activator profile almost identical to PrtR45. The PrtR1150 proteinase is encoded as a preproprotein by a second gene, prtRII, with high sequence similarity to PrtR except that it lacks the C-terminal adhesin domains. A comparison of the deduced amino acid sequence of PrtR1150 with that of the adhesin-associated proteinases PrtR45 and PrtK48 revealed that PrtR1150 does not contain a C-terminal motif that is conserved in PrtR45 and PrtK48. Related motifs are also found in the adhesin domains of PrtR and PrtK. It is proposed that this conserved motif is an adhesin-binding motif (ABM) involved in association of the PrtR and PrtK proteinases and adhesins into large complexes, as the PrtR-PrtK proteinase-adhesin complex inactivated by N-alpha-p-tosyl-L-lysine chloromethyl ketone (TLCK) was shown to bind specifically to a synthetic peptide corresponding to the conserved motif in a competitive binding assay.

Keywords: Porphyromonas gingivalis, second Arg-specific cysteine proteinase, adhesin-binding motif

Abbreviations: ABM, adhesin-binding motif; Bz-1-Arg-pNA, benzoyl-L-Arg-p-nitroanilide; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DIPEA, diisopropylethylamine; DMF, N,N-dimethylformamide; EDT, ethanedithiol; HBTU, O-benzotriazole-N,N',N"-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; TIPS, triisopropylsilane; TLCK, N-alpha-p-tosyl-L-lysine chloromethyl ketone; z-L-Lys-pNA, benzylxycarbonyl-L-Lys-p-nitroanilide.

The GenBank accession number for the sequence reported in this paper is AF007124.
INTRODUCTION

Porphyromonas gingivalis has been implicated as a major aetiological agent in the development of adult periodontitis, a destructive inflammatory disease of the tooth-supporting tissues (Slots et al., 1986; Maiden et al., 1990; Socransky & Haffajee, 1992). This bacterium elaborates several putative virulence factors which are proposed to be involved in disease initiation and progression (Cutler et al., 1995). Of these virulence factors, extracellular Arg- and Lys-specific proteinases have been suggested to play a major role in disease pathogenesis (Smalley et al., 1989; Nakayama et al., 1995; Kesavalu et al., 1996) by dysregulation of the host defence and inflammatory responses (Kadowaki et al., 1994; Imamura et al., 1995a, b; Jagels et al., 1996) and by direct proteolytic degradation of host connective tissue and plasma proteins (Ciborowski et al., 1994; Pike et al., 1996).

We have recently characterized the cell-associated Arg- and Lys-specific proteinases of P. gingivalis W50 as non-covalently associated complexes of a 45 kDa Arg-specific, calcium-stabilized cysteine proteinase, a 48 kDa Lys-specific cysteine proteinase and several sequence-related adhesins (Bhogal et al., 1997). The 45 kDa Arg-specific cysteine proteinase, designated PrtR45, appeared similar to enzymes characterized in P. gingivalis culture supernatants (Chen et al., 1992; Curtis et al., 1996). We then further identified two genes, prtR and prtk, that encoded all the proteins of these proteinase–adhesin complexes (Slakeski et al., 1996; Bhogal et al., 1997). The prtR gene encodes a polypeptide with a preprofragment followed by the 45 kDa Arg-specific proteinase (PrtR45) and four sequence-related adhesin domains PrtR44, PrtR15, PrtR17 and PrtR27 in that order (Slakeski et al., 1996). The prtk gene is very similar in that it also encodes a polypeptide with a preprofragment followed by the 48 kDa Lys-specific proteinase (PrtK48) and three C-terminal adhesin domains, PrtK39, PrtK15 and PrtK44, all being sequence-related and similar to the PrtR adhesins (Bhogal et al., 1997). All domains of the PrtR and Prtk polypeptides are preceded by Arg or Lys residues – consistent with autolytic processing. It is proposed that the PrtR and Prtk proteins are secreted as mature 160 and 163 kDa proteins, respectively, after processing to remove their preprofragments. The mature PrtR and Prtk proteins associate or remain unassociated and are then autolytically processed to release the PrtR45 and Prtk48 proteinase domains as well as the seven sequence-related adhesins. The adhesins, by containing multiple repeat sequences, possess multiple binding sites that are capable of binding not only to host proteins and cells but also to the Arg- and Lys-specific proteinases and themselves to produce large non-covalently associated aggregates (Bhogal et al., 1997).

During the analysis of the prtR and prtk genes we noted that a Southern blot of P. gingivalis W50 genomic DNA contained two BamHII and two HindIII restriction fragments which hybridized with an oligonucleotide probe designed using the N-terminal sequence of the PrtR45 Arg-specific proteinase (Slakeski et al., 1996). This result suggested the presence of a second closely related gene encoding another Arg-specific proteinase. Here we describe the purification and characterization of the second Arg-specific proteinase of P. gingivalis and the cloning and sequence analysis of its gene, prtkII.

METHODS

Materials. O-Benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HO-Bt), diisopropylethylamine (DIPEA), N,N-dimethylformamide (DMF), piperidine, trifluoroacetic acid (TFA) and 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids were obtained from Auspep (Melbourne, Australia). Tris-isopropylsilane (TIPS) and ethanedithiol (EDT) were obtained from Aldrich. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was obtained from Sigma. Phenol and diethyl ether were obtained from BDH. Unless otherwise stated chemicals were of peptide synthesis grade or its equivalent.

Bacterial strain and growth conditions. Lyophilized cultures of P. gingivalis W50 were kindly provided by Professor P. Marsh (PHLS, Centre for Applied Microbiology and Research, Wiltshire, UK). P. gingivalis W50 was grown anaerobically (Bhogal et al., 1997) and Escherichia coli JM109 and LE392 strains were grown as previously described (Slakeski et al., 1996).

Purification of the 50 kDa Arg-specific proteinase. P. gingivalis W50 was grown in batch culture (5 l) and harvested at late-exponential phase by centrifugation (5000 g, 20 min, 4°C). Cells were washed once with 150 ml TC buffer (20 mM Tris/HCl, pH 7.4, and 5 mM CaCl2) containing 50 mM NaCl and sonicated as described previously (Bhogal et al., 1997). The sonicate was centrifuged (10000 g, 30 min, 4°C) and the supernatant filtered (0.22 μm) prior to anion-exchange FPLC. The sonicate was applied to an anion-exchange column (Hiloak XK 16/10 Q-Sepharose, Pharmacia-LKB) cooled to 4°C, in multiple injections using a 50 ml superloop (Pharmacia-LKB). The sonicate was eluted using a linear gradient from 0 to 100% buffer B over 90 min at a flow rate of 0.3 ml min⁻¹. The fractionation and elution collected at 4°C in 6 ml fractions using a Frac 100 fraction collector (Pharmacia-LKB). Buffer A was TC buffer containing 50 mM NaCl and buffer B was TC buffer containing 500 mM NaCl. Fractions were analysed for proteolytic and amidolytic activity using azocasein (A-2765, Sigma), benzoyl-l-Arg-p-nitroanilide (Bz-l-Arg-pNA, Sigma) and benzoxacycarbolinyl-l-Lys-p-nitroanilide (z-l-Lys-pNA, Calbiochem) as described previously (Bhogal et al., 1997) except that fractions were preincubated with 10 mM cysteine for 10 min at 25°C before the addition of substrate. For the amidolytic assays A₄⁰₅ was monitored at as previously described (Bhogal et al., 1997) and the amidolytic activity expressed as U, where 1 U = 1 μmol substrate converted min⁻¹ at 25°C. Anion-exchange fractions eluting between 160 and 246 mM NaCl containing the highest ratio of Arg-specific to Lys-specific activity were washed and concentrated in TC buffer containing 150 mM NaCl using a Centriprep and Centricon-10 concentrators (Amicon) and applied to a gel-filtration column (Superose 12, HR 10/30, Pharmacia-LKB) using TC buffer containing 150 mM NaCl at a flow rate of 0.3 ml min⁻¹. A₄₀₅ was monitored and fractions collected at 4°C using a Frac 100 fraction collector. The molecular mass values of eluant peaks were determined using gel-filtration molecular mass standards (Pharmacia-LKB). The
peak eluting at 50 kDa containing only Arg-specific amidolytic activity was washed in TC buffer containing 50 mM NaCl using a Centricron-10 concentrator (Amicon) and applied to a Mono Q (HR 5/5) anion-exchange column using a 5 ml loop and eluted using a linear gradient of 0–100% buffer B at a flow rate of 1 ml min⁻¹. Buffer A was TC buffer containing 150 mM NaCl, buffer B was TC buffer containing 500 mM NaCl. A was monitored and fractions collected at 4 °C using a Frac-100 fraction collector.

**SDS-PAGE, protein transblot and N-terminal sequence analysis.** SDS-PAGE was performed using a Mini Protein II electrophoresis system (Bio-Rad) with 12% (w/v), 1 mm separating gels, overlaid with 5% stacking gels (Laemmli, 1970) and proteins transblotted and N-terminally sequenced as previously described (Bhogal et al., 1997).

**Cloning and nucleotide sequence analysis.** The P. gingivalis W50 LambdaGEM-12 genomic library, described previously (Slakeski et al., 1996), was screened using synthetic oligonucleotides derived from the nucleotide sequence of PrtR. Oligonucleotide probes were 5’-end labelled using [γ-³²P]ATP and T4 polynucleotide kinase. Approximately 1.5 x 10⁸ phage were screened by lifting onto Nylon membrane filters and hybridizing with radiolabelled oligonucleotides overnight in hybridization buffer: 6 x SSC (SSC is 15 mM sodium citrate, 150 mM NaCl, pH 8.0), 0.25% SDS, 5 x Denhardt’s solution (Sambrook et al., 1989) and 100 µg salmon sperm DNA ml⁻¹ at 49 °C. Filters were washed extensively in a solution of 2 x SSC containing 0.1% SDS (w/v) at 49 °C. Fragments were washed using standard procedures (Sambrook et al., 1989).

**Northern blot analysis.** Total RNA was isolated from P. gingivalis W50 as previously described (Slakeski et al., 1996). Northern blots were prepared as described by Sambrook et al. (1989) and the blotted membranes hybridized with the nucleotide sequence of PrtR. Oligonucleotide probes were 5’-end labelled using [γ-³²P]ATP and T4 polynucleotide kinase. Approximately 1.5 x 10⁸ phage were screened by lifting onto Nylon membrane filters and hybridizing with radiolabelled oligonucleotides overnight in hybridization buffer: 6 x SSC (SSC is 15 mM sodium citrate, 150 mM NaCl, pH 8.0), 0.25% SDS, 5 x Denhardt’s solution (Sambrook et al., 1989) and 100 µg salmon sperm DNA ml⁻¹ at 49 °C. Filters were washed extensively in a solution of 2 x SSC containing 0.1% SDS (w/v) at 49 °C. Fragments were washed using standard procedures (Sambrook et al., 1989). Phage DNA was digested with Eco72I and the resulting fragments ligated into SmaI-BAP pUC18 (Pharmacia), which was used to transform E. coli JM109 using the heat-shock procedure (Sambrook et al., 1989). Double-stranded template DNA was sequenced as described previously (Slakeski et al., 1996). PCR was used to amplify a 991 bp fragment containing the internal Eco72I site encoded by prtR using the two oligonucleotide primers 5’-CCGGTTCGCTGAAGTC-3’ (forward primer identical to bases 657–672 of PrtR sequence) and 5’-TGGCTACGATGACGATCATACGAC-3’ (reverse primer identical to bases 657–672 of PrtR sequence) and 5’-0.2 mM dNTPs, 1.5 mM MgCl₂, 100 pmol of each primer, 20 mM Tris/HCl, pH 8.4, 50 mM KCl and 2.5 U Taq DNA Polymerase (Gibco-BRL). The reaction mixture was heated at 95 °C for 3 min and then subjected to 25 cycles of DNA denaturation at 95 °C for 30 s, primer annealing at 40 °C for 1 min and extension at 72 °C for 2 min. Following cycling, the reaction mixture was finally heated at 72 °C for 5 min. Amplified DNA was purified using a PCR Spin cleanup Kit (Promega) and sequenced across the Eco72I site in both directions.

**Northern blot analysis.** Total RNA was isolated from P. gingivalis W50 as previously described (Slakeski et al., 1996). Northern blots were prepared as described by Sambrook et al. (1989) and the blotted membranes hybridized with the oligonucleotide probe anti-sense to the amino acid sequence RMIVIVA present in the N-terminal sequence of PrtR (Slakeski et al., 1996). Hybridization and washing conditions were identical to those described above.

**Purification of high-molecular-mass complexes of Arg-specific and Lys-specific proteinases and adhesins (PrtR-PrtK complexes).** The high-molecular-mass, cell-associated proteinase–adhesin complexes (PrtR-PrtK complexes) of P. gingivalis W50 were purified using a combination of anion-exchange, gel-filtration and Sephacryl S-200 affinity chromatography from a cell sonicate as described previously (Bhogal et al., 1997). The complexes were characterized using SDS-PAGE, transblotting and sequence analysis and assayed for enzyme activity using Bz-L-Arg-pNA and Z-L-Lys-pNA substrates (Bhogal et al., 1997).

**Solid-phase peptide synthesis.** Peptides were synthesized manually using standard Fmoc solid-phase peptide synthesis protocols. The peptides were assembled as the carboxamide form using Fmoc/Pal/PEG/PS resin (PerSeptive Biosystems). Coupling was accomplished with HBTU/HEB activation using four equivalents of Fmoc-amino acid and six equivalents of DMAP. The Fmoc group was removed by 2% (v/v) DBU in DMF containing 2% (v/v) piperidine. Cleavage of peptides from the resin support was performed using TFA/phenoxypropanol/EDT/water (92:2:2:3, by vol.) cleavage cocktail for 2.5 h. After cleavage the resin was removed by filtration and the filtrate concentrated to approximately 1 ml under a stream of nitrogen. After the peptide products were precipitated in cold ether, they were centrifuged and washed three times. The peptide precipitate was then dissolved in 10 ml water containing 0.1% (v/v) TFA and insoluble residue was removed by centrifugation.

Synthesized peptides were purified using a Brownlee C18 Aquapore ODS column (250 x 100 mm) installed in a Waters HPLC system. Chromatograms were developed at a flow rate of 50 ml min⁻¹ using 0.1% (v/v) TFA in water (solvent A) and 0.1% (v/v) TFA in 90% aqueous acetonitrile (solvent B). Peptides were eluted with a gradient of 10–30% solvent B over 40 min. Analytical HPLC was carried out using a Brownlee C8 Aquapore RP-300 column (220 x 46 mm) installed in an Applied Biosystems HPLC system. Chromatograms were developed using solvent A and solvent B at a flow rate of 1 ml min⁻¹ and a 0–100% linear gradient of solvent B over 30 min. Material eluted from the columns was monitored by mass spectrometry using a PerSeptive Biosystems Voyager DE MALDI-TOF.

**Competitive binding assay.** Wells of flat-bottomed polyvinyl microtitre plates (Microtitre, Dynatech) were coated overnight at 4 °C using a solution (5 µg ml⁻¹) of the adhesin-binding motif (ABM) peptide in 0.1 M phosphate-buffered saline, pH 7.4, containing 0.1% (w/v) Tween 20 (PBST) and 0.1% (v/v) TFA in 90% aqueous acetonitrile (solvent B). Peptides were eluted with a gradient of 10–30% solvent B over 40 min. Analytical HPLC was carried out using a Brownlee C8 Aquapore RP-300 column (220 x 46 mm) installed in an Applied Biosystems HPLC system. Chromatograms were developed using solvent A and solvent B at a flow rate of 1 ml min⁻¹ and a 0–100% linear gradient of solvent B over 30 min. Material eluted from the columns was monitored at A₂₅₂. Peptides were analysed by mass spectrometry using a PerSeptive Biosystems Voyager DE MALDI-TOF.
RESULTS

PrtRII50 Arg-specific proteinase purification and characterization

The *P. gingivalis* W50 cell sonicate contained 0.36 mg protein ml⁻¹ and had activities of 2.4 and 1.1 μmol min⁻¹ (mg protein)⁻¹ with 1.0 mM Bz-L-Arg-pNA and Z-L-Lys-pNA as substrates, respectively, at 25 °C. The sonicate was subjected to Q-Sepharose anion-exchange FPLC, and proteolytic/amidolytic activity eluting between 160–246 mM NaCl was collected and concentrated using a Centriprep and Centricon-10 concentrator (Amicon). This fraction represented the leading edge of the main peak of proteolytic/amidolytic activity and contained the highest ratio of Arg-specific activity to Lys-specific activity. After concentration, the fraction was applied to a Superose 12 gel-filtration column (Fig. 1). Arg- and Lys-specific activity was associated with the high-molecular-mass eluting material corresponding to peaks with molecular masses of 0.6–2.0 × 10⁶ Da and 300 kDa, as reported previously (Bhogal et al., 1997). However, a lower-molecular-mass peak of 50 kDa was also observed, which displayed only Arg-specific activity and this peak was collected for further purification. The 50 kDa gel-filtration peak was applied to a Mono Q anion-exchange column and upon application of a NaCl gradient the Arg-specific activity eluted in a distinct peak at 200 mM NaCl with a 28-fold purification over the original crude sonicate (Table 1). The peak containing Arg-specific activity was subjected to SDS-

![Fig. 1. Gel-filtration FPLC of pooled and concentrated fractions eluting from Q-Sepharose anion-exchange FPLC. Anion-exchange fractions eluting between 160 and 246 mM NaCl and representing the leading edge of the main peak of proteolytic/amidolytic activity were pooled, equilibrated in TC buffer, pH 7.4, containing 50 mM NaCl, concentrated and applied to a Superose 12 HR 10/30 gel-filtration column using the same buffer at a flow rate of 0.3 ml min⁻¹. Fractions (0.5 ml) were assayed for proteolytic/amidolytic activity using azocasein, Bz-L-Arg-pNA and Z-L-Lys-pNA. Amidolytic activity of each 0.5 ml fraction with Bz-L-Arg-pNA is shown by the histogram.](image1)

![Fig. 2. SDS-PAGE (boiled/reduced conditions) of the anion-exchange (Mono Q) peak eluting at 200 mM NaCl and containing only Arg-specific activity. Lane 1, Pharmacia low-molecular-mass standards (molecular masses, in kDa, are indicated); lane 2, purified 50 kDa Arg-specific proteinase, PrtRII50.](image2)

### Table 1. Purification of the 50 kDa Arg-specific proteinase PrtRII50

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Proteolytic activity (U⁺)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Purification (-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonicate</td>
<td>54.0</td>
<td>128</td>
<td>2.4</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>FPLC (Q-Sepharose) anion-exchange</td>
<td>2.70</td>
<td>28</td>
<td>10.4</td>
<td>4.3</td>
<td>22</td>
</tr>
<tr>
<td>Gel-filtration (Superose 12) FPLC</td>
<td>0.80</td>
<td>10</td>
<td>12.5</td>
<td>5.2</td>
<td>8</td>
</tr>
<tr>
<td>FPLC (mono Q) anion-exchange</td>
<td>0.12</td>
<td>8</td>
<td>66.7</td>
<td>27.8</td>
<td>6</td>
</tr>
</tbody>
</table>

* Amidolytic activity using 1.0 mM Bz-L-Arg-pNA; 1 unit = μmol min⁻¹ at 25 °C.
Second Arg-specific proteinase of *P. gingivalis*

PAGE, which confirmed a single 50 kDa protein band (Fig. 2). The 50 kDa band was transblotted and subjected to N-terminal sequence analysis, which provided the amino acyl sequence YTPVEEKENGRMIVIVPKKYEED. The specificity of the 50 kDa proteinase for arginyl residues was confirmed by the enzyme cleaving Bz-L-Arg-pNA but not z-L-Lys-pNA. The Arg-specific 50 kDa enzyme was activated by thiols (particularly cysteine), not inhibited by the serine proteinase inhibitors PMSF or 4-(2-aminoethyl)-benzenesulfonyl fluoride but inhibited by thiol-directed reagents, leupeptin and EDTA at similar concentrations to those which inhibited the PrtR45 (Bhogal et al., 1997). Inhibition with EDTA could be reversed by the addition of excess Ca\(^{2+}\) and the pH optimum of the enzyme was 8.0 with minimal activity below pH 6.0.

**Molecular cloning and sequence analysis of the prtrII gene**

Screening of the *P. gingivalis* genomic library using oligonucleotide probes specific for the N-terminus of PrtR45 identified several positive clones. The DNA from these clones was extracted and subjected to Southern analysis to identify those containing the 12 kb BamHI fragment previously proposed to correspond to the gene encoding the second Arg-specific proteinase (Slakeski et al., 1996). Lambda clone 18, containing a 12 kb BamHI fragment, was chosen for further analysis and DNA was isolated from this clone and digested with Eco**I**I and randomly cloned into plasmid SmaI-BAP pUC18. Adjacent 3.3 and 1.2 kb Eco**I**I genomic fragments were sequenced in both directions to generate the entire *prtrII* nucleotide sequence (GenBank accession no. AF007124). A 991 bp PCR fragment was generated and sequenced to confirm the sequence encompassing the internal Eco**I**I site.

The *prtrII* ORF comprises 2208 bp (736 aa residues) and encodes a preproprotein consisting of a putative leader sequence and a profragment followed by the mature Arg-specific proteinase (507 aa residues) containing the exact N-terminal amino acyl sequence obtained for the purified 50 kDa enzyme (PrtRIIS0). The N-terminal sequence of the mature protein, like PrtR45, is immediately preceded by an Arg residue in the profragment.
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The prtRII gene exhibits a high degree of similarity with the 5' two-fifths of the prtR gene which encodes PrtR45 and its associated adhesins (Figs 3 and 4). A comparison of the two translated sequences shows an overall similarity of 76% and 80% for the translated profragment and the proteinase domain, respectively. The prtRII gene, however, does not encode any of the C-terminal haemagglutinin/adhesin domains encoded by the prtR and prtK genes (Slakeski et al., 1996; N. Slakeski and others, unpublished data), this being consistent with the finding that the purified PrtRII50 proteinase was not associated with adhesins. The molecular mass of the PrtRII50 mature proteinase deduced from the translated prtRII gene sequence is 55.6 kDa, which is consistent with the 50 kDa obtained by SDS-PAGE (Fig. 2) and is slightly larger than the deduced molecular mass of 53.9 kDa for PrtR45 (Bhogal et al., 1997).

The sequence alignment of the deduced amino acyl sequence of PrtRII50 with the PrtR45 Arg-specific proteinase and the PrtK48 Lys-specific proteinase (Slakeski et al., 1996; Bhogal et al., 1997) shows that PrtRII50 displays high sequence similarity (97.5% identity) to the adhesin-associated PrtR45 proteinase except for the C-terminal 80 amino acyl residues (Fig. 3). In fact, this C-terminal 80 residue sequence of PrtRII50 is similar (47% identity) to the C-terminal 80 residues of the PrtR27 adhesin domain, the last domain of PrtR (Fig. 4). In contrast to the high sequence identity of the PrtRII50 and PrtR45 proteinases, there is lower overall similarity (25% identity) between the two adhesin-

Fig. 4. Schematic representation of the prtRII, prtR and prtK genes. The PrtR nascent polyprotein consists of a leader sequence and a profragment followed by the PrtR45 Arg-specific proteinase and the PrtR44, PrtR15, PrtR17 and PrtR27 adhesin domains. The PrtK nascent polyprotein contains no associated adhesins and consists of a leader sequence, a profragment, the PrtK48 Lys-specific proteinase and the PrtK39, PrtK15 and PrtK44 adhesin domains. The PrtRII nascent polyprotein contains no associated adhesins and consists of a leader sequence, a profragment and the PrtRII50 Arg-specific proteinase only. B indicates the relative positions of the putative ABMs. H and C represent the location of the putative catalytic His,Cys dyad of the proteinases.

Fig. 5. Alignment of the putative ABMs found in the PrtR45 Arg-specific proteinase, the PrtK48 Lys-specific proteinase, the PrtR44 and PrtR17 adhesins, and the PrtR39 and PrtK44 adhesins. Residues identical with PrtR45 (aa 659-687) are boxed and the amino acyl residues, shown in parentheses, are numbered from the Met initiation codon of all three gene products.

Fig. 6. Competitive binding assay demonstrating binding of the TLCK-inactivated PrtR-PrtK proteinase–adhesin complex to the synthetic peptide corresponding to the putative ABM. ○, ABM synthetic peptide PYQPVSNLTATTQGQKVTLKWDAPSTK; □, control peptide FNGGSLANYTGHGSETAWGT, corresponding to residues 199–219 of PrtR45; ▲, casein. See Methods for details.

The associated PrtR45 and PrtK48 proteinases except around the C-terminal region where the motif GEPNPYQPV-SNLATTQGQKVTTLKWDAPSTK (underlined in Fig. 3) is almost identical in both proteinases but is absent in PrtRII50. Similar motifs also occur in the PrtR44, PrtR17, PrtR39 and PrtK44 adhesin domains of PrtR and PrtK (Figs 4 and 5); this finding has led us to propose that this motif is an ABM involved in the association of the PrtR and PrtK proteinases and adhesins into large complexes.

**Binding of the PrtR–PrtK complex to a synthetic peptide corresponding to a putative ABM**

A peptide (ABM peptide) corresponding to the proposed ABM PYQPVSNLTATTQGQKVTTLKWDAPSTK was synthesized and used to measure binding of the PrtR–PrtK complex. Specific binding of TLCK-in-
activated PrtR–PrtK complex to the ABM peptide was demonstrated in a competitive binding assay, where a 5–100-fold molar excess of the ABM peptide in solution was required to inhibit binding of the complex to the ABM peptide adsorbed onto the microtitre plate (Fig. 6). A control peptide, FNGGISLANYTGHGSETAWGT, corresponding to residues 428–448 of PrtR45, as well as casein did not inhibit the binding of the TLCK-inactivated PrtR–PrtK complex to the adsorbed ABM peptide. The anti-PrtR–PrtK antiserum did not bind to the ABM peptide in the absence of the PrtR–PrtK complex. The inactivation with TLCK ensured that the active sites of the proteinases. This was also confirmed by lack of binding of the PrtR–PrtK complex to casein and a non-specific peptide of similar size and lysine content to the ABM peptide but of unrelated sequence. These results demonstrating specific binding of the TLCK-inactivated PrtR–PrtK complex to the ABM peptide therefore are consistent with the proposed role of this conserved motif in the association of the PrtR and PrtK proteinases and adhesins into large complexes.

Northern analysis

Northern analysis using the oligonucleotide probe antisense to an N-terminal amino acyl sequence of the proteinases of prtR and prtRII revealed two positively hybridizing transcripts of 5.8 kb and 2.6 kb (Fig. 7).

DISCUSSION

Using a P. gingivalis W50 cell sonicate we have purified and characterized a second cell-associated, Arg-specific, calcium-stabilized cysteine proteinase that is almost identical to the previously characterized Arg-specific cysteine proteinase PrtR45 (Bhogal et al., 1997). However, despite the almost identical enzymic characteristics and inhibitor/activator profile to PrtR45, the second enzyme exhibits a number of key differences. Firstly, the second enzyme, designated PrtRII50, is a discrete enzyme not associated with adhesins. The Arg-specific cysteine proteinase, PrtR45, is a 45 kDa component of a large multi-protein complex of Arg- and Lys-specific proteinases and adhesins (Bhogal et al., 1997). Secondly, PrtRII50 is slightly larger than PrtR45 on SDS-PAGE (molecular mass 50 kDa) and thirdly there are four amino acid substitutions in the first 25 N-terminal residues of PrtRII50. PrtRII50 has a Glu at position 8 instead of Gln, a Pro at position 17 instead of Ala, a Glu at position 22 instead of Gly and a Glu at position 25 instead of the Lys in PrtR45 (Fig. 3). These differences in size and the N-terminal amino acyl sequence were confirmed with the cloning and sequence analysis of the gene prtRII encoding the second Arg-specific proteinase.

The deduced amino acid sequence of the prtRII gene exhibits 98% identity with that of the recently reported rgpB gene from P. gingivalis ATCC 33277 (Nakayama, 1997), suggesting that these two genes represent the same locus in two different strains. However, the sequence for the mature proteinase of the rgpB gene does not contain three of the N-terminal amino acyl substitutions found in the prtRII gene product and only has the Gln → Glu substitution at position 8. The substitutions at positions 17, 22 and 25 found in PrtRII50, that enabled the gene product to be unequivocally differentiated by N-terminal sequence analysis from the mature PrtR45 proteinase of prtR (rgpA), were not found in rgpB. In the current study the differences in N-terminal sequence and size of the mature proteinases enabled the differentiation of the discrete 50 kDa Arg-specific proteinase (PrtRII50) from the 45 kDa Arg-specific proteinase (PrtR45) found associated with adhesins. The assignment of the two proteinases (PrtR45 and PrtRII50) to the two genes (prtR and prtRII respectively) has enabled identification of a conserved motif in the two adhesin-associated proteinases (PrtR45 and PrtK48) not found in the discrete PrtRII50. As the conserved motif was also found in several adhesins of the prtR and prtK proteinases and adhesins into large complexes. This proposition is supported by the demonstration that a synthetic peptide corresponding to the conserved motif specifically binds to the TLCK-inactivated PrtR–PrtK complex.

Recently, Rangarajan et al. (1997) purified and characterized a second Arg-specific cysteine proteinase from the culture supernatant of a P. gingivalis W50 isogenic mutant lacking a functional prpRI (equivalent to prtR).
This second Arg-specific proteinase was of similar size (50–55 kDa), exhibited the same N-terminal sequence and enzyme characteristics as PrtRII50 and was identified as a discrete enzyme not associated with adhesins. The results of Rangarajan et al. (1997) therefore are consistent with the results presented here that the cell-associated form of the second Arg-specific enzyme PrtRII50 is a discrete enzyme and not adhesin-associated.

The high sequence similarity (97.5%) between PrtRII50 and the adhesin-associated PrtR45 suggests that prtR has arisen by gene duplication and recombination of ancestral prtRII and adhesin genes, or, alternatively, that prtRII arose by gene duplication and deletion of the adhesin domains from an ancestral prtR as previously suggested by Nakayama (1997) for the rgpA and rgpB genes of P. gingivalis ATCC 33277.

On further examination of the alignment of the deduced amino acyl sequence of PrtRII50 with the catalytic domains of the PrtR45 Arg-specific proteinase and the PrtK48 Lys-specific proteinase (Slakeski et al., 1996; Bhogal et al., 1997; N. Slakeski and others, unpublished data) some further interesting areas of similarity were revealed (Fig. 3). Although these three cysteine proteinases from P. gingivalis have no similarity with any of the other known families of cysteine proteinases, it is possible to speculate on the identity of the catalytic residues since only one His residue and two Cys residues are conserved in the three sequence-related enzymes. The catalytic Cys, His dyad of these enzymes therefore is likely to consist of H140 of PrtRII50, the only conserved His in the three proteinases. The identification of H140 as a catalytic residue is also suggested by the sequence T X H140 G XX TA of the three proteinases, conforming to part of the Prosite consensus signature sequence for the catalytic His of cysteine proteinases (Appel et al., 1994). The catalytic Cys is also likely to be one of the two conserved cysteiny1 residues C473 and C484 in the three sequence-related proteinases, although neither have flanking sequences that conform to the Prosite consensus signature sequence for the catalytic Cys of cysteine proteinases (Appel et al., 1994). Using active-site labelling of a 44 kDa Arg-specific proteinase of P. gingivalis with N-[2-3H]acetyllysine chloromethyl ketone, Nishikata & Yoshimura (1995) identified a labelled peptide in an endoproteinase Asp-N digest with an N-terminal sequence Asp-Val-Ala-Cys. This cysteine corresponds to C473 of PrtRII50 (Fig. 3), which is consistent with this residue being involved in catalysis.

In the cysteine proteinase papain, the catalytic mechanism has been proposed to involve, as well as the catalytic Cys22, His16 thiolate/imidazolium ion pair, stabilization of the oxyanion transition state by the NH2 group of the Gln19 side chain and stabilization of the thiolate/imidazolium ion pair by the side chain of Asn75 (Otto & Schirmeister, 1997). In the alignment of the amino acyl sequences of the three sequence-related cysteine proteinases from P. gingivalis, one Gln (Q28) and seven Asn (N218, N228, N248, N461, N516, N524 and N539) residues of PrtRII50 are conserved in both PrtR45 and PrtK48 (Fig. 3). None of the conserved asparaginyl residues have flanking sequences that conform to the Prosite consensus signature sequence for the active site N of cysteine proteinases (Appel et al., 1994), so it is difficult to predict which Asn, if any, may be involved in catalysis.

Previous Northern analysis of P. gingivalis RNA hybridized with an oligonucleotide specific for the PrtR45 N-terminal region showed a single positively hybridizing transcript of approximately 5.3 kb which corresponded to the transcript of the prtR gene (Slakeski et al., 1996). In the present study, the N-terminal sequence analysis of the purified PrtRII50 revealed differences to the N-terminal sequence of PrtR45, which was used to design the probe for the Northern analysis. Considering these differences, the Northern analysis of P. gingivalis RNA was repeated with less stringent hybridization and washing temperatures. The repeated Northern analysis showed a second positively hybridizing transcript in the RNA preparations analysed (Fig. 7). In addition to the 5.8 kb transcript (previously estimated as 5.3 kb) corresponding to prtR, the presence of a second transcript of approximately 2.6 kb is appropriately sized to indicate transcriptional activity of prtRII.

The absence of the proposed ABM on PrtRII50 would explain why this enzyme is not found associated with adhesins like PrtR45 and PrtK48 in the large proteinase–adhesin complexes (Bhogal et al., 1997). The adhesins, by containing multiple repeat sequences (Slakeski et al., 1996), would possess multiple binding sites capable of binding to host proteins and to this C-terminal motif on the PrtR45 and PrtK48 proteinases as well as to themselves. The presence of the ABM would explain why after autolytic processing of the PrtR and PrtK polyproteins, the domains, consisting of the two proteinases PrtR45 and PrtK48 and the seven sequence-related adhesins (Fig. 4), remain associated in large, non-covalently associated aggregates (Bhogal et al., 1997). The association of the adhesins with the proteinases is likely to be a mechanism by which the proteolytic activity is targeted to the desired substrates and the non-covalent nature of the association allows release of the proteinase once localized (Bhogal et al., 1997). It is also possible that the association of the cell-associated adhesins with the proteinases facilitates cell adhesion by the proteolytic activity exposing buried binding sites (cryptitopes) on host substrates (Kontani et al., 1996). Further, the multiple binding sites and autoaggregation of the adhesins would facilitate the formation of large complexes capable of cell–cell interaction and agglutination.

The lack of the putative ABM on the second Arg-specific proteinase, PrtRII50, could indicate that this enzyme’s role is primarily as a periplasmic processing enzyme. Observations that would be consistent with this speculation are that the enzyme is found in a periplasmic extract of chloroform-treated cells (P. Bhogal and others, unpublished data) and several workers have...
implicated Arg-specific proteolytic activity in the processing of P. gingivalis extracellular proteins (Okamoto et al., 1996; Nakayama et al., 1996; Tokuda et al., 1996). However, as Rangarajan et al. (1997) have purified and characterized a second Arg-specific cysteine proteinase equivalent to PrtRII50 in the culture supernatant of a P. gingivalis isogenic mutant lacking a functional prpR (equivalent to prtR), then this result may suggest that PrtRII50 is both cell-associated and released into the culture fluid, as are the mature PrtR and PrtK such that this second Arg-specific proteinase may also play a direct role in the pathogenic potential of P. gingivalis.

We have previously reported that several groups have characterized structurally different genes encoding apparently the same Arg-specific proteinase from P. gingivalis with characteristics equivalent to PrtR45 (Slakeski et al., 1996). The genes characterized have been designated prtR (Slakeski et al., 1996), rgp (Pavloff et al., 1995), prpR (Aduse-Opoku et al., 1995), agp (Okamoto et al., 1995) and cpgR (Gharbia & Shah, 1995). Upon comparison it is apparent that the genes prtR, rgp and revised prpR represent strain variations of the same genetic locus and the genes agp and cpgR represent internally deleted and truncated versions, respectively, that may be a consequence of cloning and sequencing artefacts, or alternatively may represent a closely related gene at a second locus. In this paper we have characterized the sequence-related Arg-specific proteinase of the second genetic locus and the size and sequence of the gene are not consistent with agp or cpgR.

Concluding remarks

In conclusion, we have identified a second Arg-specific, calcium-stabilized cysteine proteinase of P. gingivalis which is a discrete 50 kDa protein with no adhesion association and which is encoded by the gene designated prtR.

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