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 polypeptides, 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 PrtrII50, 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 PrtrII50 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 PrtrII50 with that of the adhesin-associated proteinases Prtr45 and Prtk48 revealed that PrtrII50 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*- α -*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-L-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',N'*-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; TIPS, triisopropylsilane; TLCK, *N*- α -*p*-tosyl-L-lysine chloromethyl ketone; z-L-Lys-pNA, benzyloxycarbonyl-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 polyprotein 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 polyprotein 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 polyproteins 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 *Bam*HI and two *Hind*III 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, *prtRII*.

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). Triisopropylsilane (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 (100 000 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 (HiLoad 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 2.0 ml min⁻¹. A₂₈₀ was monitored and eluate 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-*p*NA, Sigma) and benzyloxycarbonyl-L-Lys-*p*-nitroanilide (z-L-Lys-*p*NA, 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 A₄₁₀ was monitored 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. A₂₈₀ 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 Centricon-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 1.0 ml min⁻¹. Buffer A was TC buffer containing 150 mM NaCl, buffer B was TC buffer containing 500 mM NaCl. A₂₈₀ 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 Protean 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 (Bhagal *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* (Slakeski *et al.*, 1996) corresponding to the N-terminal sequence of PrtR45. Oligonucleotide probes were 5' end-labelled using [γ -³²P]ATP and T4 polynucleotide kinase. Approximately 1.5 × 10⁴ phage were screened by lifting onto Nylon membrane filters and hybridizing with radiolabelled oligonucleotides overnight in hybridization buffer: 6 × SSC (SSC is 15 mM sodium citrate, 150 mM NaCl, pH 8.0), 0.25% SDS, 5 × 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 × SSC containing 0.1% SDS (w/v) at 49 °C. Phage from positively hybridizing plaques were purified using standard procedures (Sambrook *et al.*, 1989). Phage DNA was digested with *Eco*72I and the resulting fragments ligated into *Sma*I-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 *Eco*72I site encoded by *prtR*II using the two oligonucleotide primers 5'-CGGCTTCCGTAAAGTC-3' (forward primer identical to bases 657–672 of *PrtR*II sequence) and 5'-TGGCTACGATGACGATCATAACGAC-3' (reverse primer with 96% identity to bases 1624–1647 of *prtR*II). The PCR was carried out in a final volume of 100 µl and each reaction mixture contained 100 ng *P. gingivalis* W50 genomic DNA, 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 Spinclean Kit (Progen) and sequenced across the *Eco*72I 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 PrtR45 (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 Arg-Sepharose affinity chromatography from a cell sonicate as described previously (Bhagal *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 (Bhagal *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 carboxyamide form using Fmoc/Pal/Peg/PS resin (PerSeptive Biosystems). Coupling was accomplished with HBTU/HOBt activation using four equivalents of Fmoc-amino acid and six equivalents of DIPEA. 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/phenol/TIPS/EDT/water (92:2:2:2:2, 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 × 100 mm) installed in a Waters HPLC system. Chromatograms were developed at a flow rate of 5.0 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 × 4.6 mm) installed in an Applied Biosystems HPLC system. Chromatograms were developed using solvent A and solvent B at a flow rate of 1.0 ml min⁻¹ and a 0–100% linear gradient of solvent B over 30 min. Material eluted from the columns was monitored at A₂₁₄. Peptides were analysed 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% (v/v) Tween 20 (PBST) and 0.1% (w/v) sodium azide. After removal of the coating solution, 2% (w/v) skim milk powder in PBST was added to block the remaining uncoated plastic for 1 h at room temperature and then washed (4 × PBST). A solution (1 µg ml⁻¹) of the PrtR–PrtK proteinase–adhesion complex (inactivated with 1 mM TLCK) was incubated with known concentrations of ABM peptide, control peptide and casein for 1 h and then transferred to the microtitre plates coated with the ABM peptide. Following incubation for 2 h at 37 °C the plates were washed (5 × PBST). A 1:10000 dilution of rabbit anti-PrtR–PrtK antiserum in PBST containing 1% (w/v) skim milk powder was then added to the washed wells and incubated for 2 h at 37 °C. Bound antibody was detected by incubation with horseradish-peroxidase-conjugated goat immunoglobulin (Ig) directed against rabbit Ig (Bio-Rad) for 1.5 h at 37 °C. After washing (5 × PBST), substrate (0.4 mM 3,3',5,5'-tetramethylbenzidine in 0.1 M sodium acetate/citric acid buffer containing 0.004% v/v, hydrogen peroxide) was added and colour development was stopped by addition of 2 M H₂SO₄. A₄₅₀ was measured using a Bio-Rad microplate reader model 450.

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

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.

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.

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 $0.6\text{--}2.0 \times 10^6$ 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-

Table 1. Purification of the 50 kDa Arg-specific proteinase PrtrII50

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

* Amidolytic activity using 1.0 mM Bz-L-Arg-pNA; 1 unit = µmol min⁻¹ at 25 °C.

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      230                               275
PrtrII50  ..YTPVEEKENG..RMIVIVPKKYEDIEDFVDWKNQRGLRTEVKVAEDI
Prtr45    ..*****Q*..*****A***G**K*****
PrtrK48    DV**DHGDLV*TPV**L*VAGA*FK*ALKPWLT**A*K*FYLD*HYTDEA

      276                               321
PrtrII50  ASPVTANAIQQFVKQEYEKE...GNDLTYVLLVGDHKDIPA.KITPGIKS
Prtr45    *****I*****
PrtrK48    EVGT*NASIKA*IHKK*NDGLAASAAPVFLA***TDV*SGE*GKKT*V

      322                               371
PrtrII50  DQVYGQIVGNHDYNEVFIGRFSCESKEDLKTQIDRTIHYERNITTEDKWL
Prtr45    *****
PrtrK48    TDL*YSA*DG*YFP*MYTF*M*AS*P*E*TNI**KVL**KATMPDKSY*

      372                               421
PrtrII50  GQALCIAAEGGPSADNGESDIQHENIIANLLTQYGYTKIICKYDGPVTF
Prtr45    *****V*****
PrtrK48    EKV*L**G*DYSWNSQV*QPT*KYG.MQYYNQEH***DVYNYLKAPY*

      422                               471
PrtrII50  KNIIDAFNGGISLANYTCHGSETAWGTSHFGTTHVKQLTNSNQLPFIFDV
Prtr45    *****
PrtrK48    .GCYSHL*T*V*F****A*****ADPLLT*SQL*A***KDKYFLAIGN

      472                               521
PrtrII50  ACVNGDFLYNVPCFAEALMRAQKDGKPTGTVAIIASTINQSWASPMRGQD
Prtr45    ..*****FSM*****
PrtrK48    C*ITAQ*D*VQ***G*VIT...V*EK*AY*Y*G*SP*SY*GEDYYWSV

      522                               561
PrtrII50  EMNEILCEKHP..NNIKRTFGGVTMNGMFAMVEKYKKDGEKM.....
Prtr45    *****
PrtrK48    GA*AVFGVQPTFEGTSMGSYDATFLEDSYNTVNSIMWA*NLAATHAGNIG

      562                               601
PrtrII50  .....LDTWTVFGDPSLLVRTLVPTKMQVTAPANISASAQTFEVA
Prtr45    .....*****Q*NLTDASVN*S
PrtrK48    NITHIGAHHYWEAYH*L*G*VMPYRAM*KNTNY*L**SLPQNQASYSIQ

      602                               650
PrtrII50  CDYNGAIATLSDDGDMVGTAIVK.DGKAIKLNESIADETNLTLTVVGVN
Prtr45    *****I*AN*K*F*S*V*E.N*T*T*N*.TGLTN*ST*****
PrtrK48    ASAGSYV*.I*K**VLY**GVANAS*V*TVSMTKQ*TENG*YDVVITRS*

      651                               699
PrtrII50  KVTVIKDVKEGTSIA.DVANDKPYTVAVSGKTITVESPAAGLTIFDMNG
Prtr45    *E***TINTN*EPNPYQPVSNLTA*TGQOKV*LKWDA*STKTNATNTA
PrtrK48    YLP***QIQ*.*EPSPYQPVSNLTA*TGQOKV*LKWA*S*KKAEGSREV

      700                               736
PrtrII50  RRVATAKNRMVFEAQNGVYAVRIATEGKTYTEKVIVK
Prtr45    *SVDGIRELVLLSVSDAPELL*.....
PrtrK48    K*IGDG...L*VTIEPAND*.....

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Fig. 3. Alignment of the deduced amino acid sequences of PrtrII50, Prtr45 Arg-specific proteinase and PrtrK48 Lys-specific proteinase with optimized similarity. The amino acyl residues of PrtrII50 are numbered from the Met initiation codon. An asterisk indicates an identical residue to that in PrtrII50. The underlined residues indicate the putative ABM. The putative catalytic His and Cys residues are boxed.

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 YTPVEEKENG RMIVIVPKKYEDIED. 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 *Bam*HI 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 *Bam*HI fragment, was chosen for further analysis and DNA was isolated from this clone and digested with *Eco*72I and randomly cloned into plasmid *Sma*I-BAP pUC18. Adjacent 3.3 and 1.2 kb *Eco*72I 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*72I 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 (PrtrII50). The N-terminal sequence of the mature protein, like Prtr45, is immediately preceded by an Arg residue in the profragment.

Fig. 4. Schematic representation of the *prtRII*, *prtR* and *prtK* genes. The PrtR nascent polypeptide 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 polypeptide similarly consists of a leader sequence, a profragment, the PrtK48 Lys-specific proteinase and the PrtK39, PrtK15 and PrtK44 adhesin domains. The PrtR15 nascent polypeptide contains no associated adhesins and consists of a leader sequence, a profragment and the PrtR15 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.

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 pre-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 PrtR150 proteinase was not associated with adhesins. The molecular mass of the PrtR150 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 (Bhagal *et al.*, 1997).

The sequence alignment of the deduced amino acyl sequence of PrtR150 with the PrtR45 Arg-specific proteinase and the PrtK48 Lys-specific proteinase (Slakeski *et al.*, 1996; Bhagal *et al.*, 1997) shows that PrtR150 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 PrtR150 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 PrtR150 and PrtR45 proteinases, there is lower overall similarity (25% identity) between the two adhesin-

PrtR45 (659-687)	GEPNPYQPVSNLTTATTO	GQKVTCLKWDAPS
PrtK48 (681-709)	GEPSPYQPVSNLTTATTO	GQKVTCLKWEAPS
PrtR44 (919-947)	EGSNEFAPVQNLTGSAV	GQKVTCLKWDAPN
PrtK39 (940-968)	EGSNEFAPVQNLTGSSV	GQKVTCLKWDAPN
PrtR17 (1375-1403)	VNSTQFNPVKNLKQAPD	GGDVVLKWEAPS
PrtK44 (1393-1423)	VNSTQFNPVQNLTAEQAPNSMDAILKWNABA	

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 PrtK39 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 PYQPVSNLTTATTOGQKVTCLKWDAPSTK; ■, control peptide FNGGISLANYTGHGSETAWGT, corresponding to residues 199-219 of PrtR45; ▲, casein. See Methods for details.

associated PrtR45 and PrtK48 proteinases except around the C-terminal region where the motif GEPNPYQPVSNLTTATTOGQKVTCLKWDAPSTK (underlined in Fig. 3) is almost identical in both proteinases but is absent in PrtR150. Similar motifs also occur in the PrtR44, PrtR17, PrtK39 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 PYQPVSNLTTATTOGQKVTCLKWDAPSTK was synthesized and used to measure binding of the PrtR-PrtK complex. Specific binding of TLCK-in-

Fig. 7. Northern analysis of *P. gingivalis* RNA hybridized with an oligonucleotide specific for the PrtR45 N-terminal region which shows two positively hybridizing transcripts of 2.6 and 5.8 kb. The relative positions of the RNA molecular size markers (kb) are indicated.

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 complex was not binding to the peptide through 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 PrtR1150, 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, PrtR1150 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 PrtR1150. PrtR1150 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 PrtR1150, 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 (PrtR1150) from the 45 kDa Arg-specific proteinase (PrtR45) found associated with adhesins. The assignment of the two proteinases (PrtR45 and PrtR1150) 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 PrtR1150. As the conserved motif was also found in several adhesins of *prtR* and *prtK* we propose that it is an ABM involved in association 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 PrtR45 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 PrtR45 is a discrete enzyme and not adhesin-associated.

The high sequence similarity (97.5 %) between PrtR45 and the adhesin-associated PrtR45 suggests that *prtR* has arisen by gene duplication and recombination of ancestral *prtR* and adhesin genes or, alternatively, that *prtR* 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 PrtR45 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 H⁴⁴⁰ of PrtR45, the only conserved His in the three proteinases. The identification of H⁴⁴⁰ as a catalytic residue is also suggested by the sequence T X H⁴⁴⁰ 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 cysteinyl 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-[α -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 PrtR45 (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 Cys²⁵, His¹⁵⁹ 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 Asn¹⁷⁵ (Otto & Schirmeister, 1997). In the alignment of the amino acyl sequences of the three sequence-related cysteine proteinases from *P. gingivalis*, one Gln (Q³³) and seven Asn (N²³⁸, N⁴²⁹, N⁴³⁶, N⁴⁶¹, N⁵¹⁰, N⁵²⁴ and

N⁶⁵⁰) residues of PrtR45 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 PrtR45 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 *prtR*.

The absence of the proposed ABM on PrtR45 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 polypeptides, 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 auto-aggregation 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, PrtR45, 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 *prpRI* (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), *prpRI* (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 *prpRI* 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 adhesin association and which is encoded by the gene designated *prtRII*.

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