Activation of Blood Coagulation Factor X by Arginine-specific Cysteine Proteinases (Gingipain-Rs) from 
*Porphyromonas gingivalis*

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The effect of two arginine-specific cysteine proteinases (gingipain Rs) from *Porphyromonas gingivalis*, a causative bacterium of adult periodontitis, on human blood coagulation was investigated. Activated partial thromboplastin time and prothrombin time were shortened by these proteinases, with a 95-kDa gingipain R containing adhesin domains being 5-fold more efficient in comparison to a 50-kDa gingipain R containing the catalytic domain alone. The 50-kDa enzyme reduced each coagulation time in several plasmas deficient in various coagulation factors, while it was ineffective in factor X-deficient plasma unless reconstituted with this protein. Each proteinase activated factor X in a dose- and time-dependent manner, with Michaelis constants ($K_m$) being found to be lower than the normal plasma factor X concentration, strongly suggesting that factor X activation by gingipain Rs, especially the 95-kDa form which is strongly activated by phospholipids, could occur in plasma. This is the first report of factor X activation by bacterial proteinases and indicates that the gingipain Rs could be responsible for the production of thrombin and, indirectly, with the generation of prostaglandins, interleukin-1, etc., which have been found to be associated with the development of periodontitis induced by *P. gingivalis* infections. Furthermore, the data support the hypothesis that induction of blood coagulation by bacterial proteinases may be a causative agent in the pathogenesis of disseminated intravascular coagulation in sepsis.

Periodontitis is an infectious disease associated with a loss of connective tissue, resorption of alveolar bone, and formation of periodontal pockets. It is the most common cause of tooth loss in adults, primarily because of the declining incidence of dental caries in the general population (1, 2). Although the pathogenesis of periodontitis is not completely understood, prostaglandins (3, 4) and interleukin-1 (5, 6), which increase in gingival crevicular fluid in periodontal pockets, are considered to be predominant factors in the tissue destruction process associated with this disease. However, the mechanism of the production of these inflammatory mediators is still unclear.

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1. The abbreviations used are: gingipain R, arginine-specific gingipain; PT, prothrombin time; APTT, activated partial thromboplastin time; TLCK, tosyl-L-lysine chloromethylketone; factor Xa, activated coagulation factor X; DIC, disseminated intravascular coagulation.

2. J. Potempa, unpublished data.
mas deficient in factors VII, VIII, IX, X, XI, XII, or prekallikrein were purchased from Sigma. SIMPLASTIN®, AUTOMATED APTT, and Platelintm (rabbit brain phospholipids) were purchased from Organon Teknika, Corp. (Durham, NC) while d-Phe-Pro-Arg-chloromethylketone (FPR-ck) was obtained from Bachem Biosci. Inc. (King of Prussia, PA). Purified human factor Xa was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN) and purified human factor Xa was acquired from Kaketsukun (Kumamoto, Japan). t-Butyloxycarbonyl-i-isoceulyl-t-glutamyl-glycyl-t-arginine-4-methyl-coumaryl-7-amide (Boc-Ile-Glu-Gly-Arg-MCA) was purchased from the Peptide Institute (Minoh, Japan). p-Nitrophenyl-p'-guanidinobenzoate was from Nakarai Tesque (Kyoto, Japan). Normal human plasma was obtained by centrifugation of a mixture of 9 volumes of freshly drawn blood from healthy volunteers and 1 volume of 3.8% (w/v) sodium citrate.

**Proteinase Purification**—50-kDa gingipain R and 95-kDa gingipain R were isolated according to the method described by Pike et al. (23). The amount of active enzyme in each purified proteinase was determined by active site titration using FPR-ck. The concentration of active gingipain R was calculated from the amount of inhibitor needed for complete inactivation of the proteinase.

**Activation of Proteinases**—Each gingipain R form was activated with 10 mM cysteine in 0.2 M Hepes buffer, pH 8.0, containing 5 mM CaCl₂ at 37 °C for 10 min. The activated proteinase (2 μM) was then diluted with 10 mM Tris-HCl, pH 7.3, containing 150 mM NaCl (TBS) and 5 mM CaCl₂ prior to use.

**Proteinase Activity Assay**—The amidolytic activity of the gingipain R was determined using benzoyl-L-arginine-p-nitroaniline. Samples were preincubated at 37 °C for 5 min in 0.1 M Tris-HCl buffer, pH 7.6, containing 200 mM Gly-Gly, 5 mM CaCl₂, and 10 mM cysteine, pH 7.6, and then assayed for amidolytic activity using 1 mM substrate. The formation of p-nitroaniline was monitored spectrophotometrically at 405 nm.

**Clotting Assay**—Clotting time was measured with COAG-A-MATE® XC (General Diagnostics, Morris Plains, NJ) following the manufacturer’s instructions. For prothrombin time (PT) assay, 90 μl of plasma and 10 μl of a given proteinase were incubated in a plastic cell at 37 °C for 3 min, followed by addition 200 μl of SIMPLASTIN® to initiate coagulation. For activated partial thromboplastin time (APTT) assay, 90 μl of plasma and 10 μl of proteinase were incubated in a plastic cell at 37 °C for 1 min, followed by addition of 100 μl of AUTOMATED APTT®, incubation at 37 °C for 3 min, and addition of 100 μl of 25 mM CaCl₂ to initiate coagulation.

**Kinetic Analysis of Factor X Activation**—Factor X, dissolved in 450 μl of 0.1 M Tris-HCl, pH 7.6, containing 0.15 M NaCl, 5 mM CaCl₂ and 45 μg of phospholipids, was incubated with 50 μl of either of the gingipain R (0.5 mM) at 37 °C for 30, 60, 90, 120, or 150 s. One-hundred ml of the solution was then added to 500 μl of the same buffer supplemented with 1.5 μM antipain, to completely inhibit cysteine proteinase activity but not the amidolytic activity of factor Xa at the concentration used. This was followed by addition of 20 μl of a factor Xa-specific substrate, Boc-Ile-Glu-Gly-Arg-MCA (10 mM). The amount of t-aminomethyl coumarin (AMC) released by factor Xa at 37 °C was measured fluorometrically with a fluorescence spectrophotometer (Model 650–40, Hitachi), the fluorescence at 440 nm with excitation at 380 nm being monitored with a recorder. The factor Xa concentration produced by each proteinase was calculated by using a standard the amidolytic activity of purified factor Xa which had been active site-titrated with p-nitro-p'-guanidinobenzoate (28). The initial velocity of factor Xa production at various factor X concentrations was determined by the best fit line for each factor Xa concentration at the five incubation periods mentioned above. Several factor X concentrations in a range from 35 to 425 mM were used for the kinetic study.

The values of the Michaelis constant (Kₘ) and the maximum velocity (Vₘₐₓ) in the Michaelis-Menten equation were obtained using three different plots, [S]/v versus [S]₀, v versus U/[S]₀ and v versus [S]₀/[v], where [S]₀ and [v] denote the catalytic rate and the initial substrate concentration, respectively, where the best fit values were determined by the method of least squares with Taylor expansion, described by Sakoda and Hiroi (29).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis**—Analysis by SDS-polyacrylamide gel electrophoresis was performed with 15% slab gels according to the method of Laemmli (30), with 0.8% Coomassie Brilliant Blue R-250 being used for protein staining.

**Amino-terminal Sequence Analysis**—Automatic sequence analysis was performed with a pulse-liquid phase sequencer (model 477A Pro-

3 J. Potempa, R. Pike, and J. Travis, manuscript in preparation.

**FIG. 1. Activated partial thromboplastin time of normal human plasma incubated with the 50- or 95-kDa gingipain R.** Ninety microliters of normal plasma was incubated with 10 μl of a proteinase for 1 min at 37 °C in a plastic cell, followed by addition of 100 μl of APTT reagent (AUTOMATED APTT). After incubation at 37 °C for 5 min, 100 μl of 25 mM CaCl₂ was added to the mixture and measured the clotting time with COAG-A-MATE® XC. The value is expressed the average ± S.D. in triplicate assay. The proteinase concentrations in plasma are shown. ○, 50-kDa gingipain R; ◇, 95-kDa gingipain R; ●, TLCK-inactivated 50-kDa gingipain R; ▲, TLCK-inactivated 95-kDa gingipain R. Dashed lines are borders of controls which were assayed using 10 mM Tris-HCl, pH 7.3, containing 150 mM NaCl (TBS) instead of the 50-kDa gingipain R. In the case of clotting within 20 s, this assay is not available.

**RESULTS**

**Activation of Blood Coagulation by Gingipain R**—To investigate the effect of gingipain R on blood coagulation, normal human plasma was incubated with either of the proteinases and examined for APTT. Both proteinases decreased APTT in a dose-dependent manner at concentrations as low as 0.1 nM, with the 95-kDa gingipain R being approximately 5-fold more effective than that of the 50-kDa gingipain R (Fig. 1). Since TLCK-treated proteinases did not shorten APTT (Fig. 1), the effect of gingipain R is dependent on their proteolytic activity. To investigate the mechanism for reducing APTT by gingipain R, the effect observed was further studied using plasmas deficient in a given factor found in the intrinsic coagulation pathway. The 50-kDa gingipain R shortened APTTs of plasmas deficient in factor XII, prekallikrein, XI, IX, or VIII in a dose-dependent manner (Table I). However, this enzyme did not affect the APTT of factor X-deficient plasma, unless it was activated by the clotting time with COAG-A-MATE® XC. The value is expressed the average ± S.D. in triplicate assay. The proteinase concentrations in plasma are shown. ○, 50-kDa gingipain R; ◇, 95-kDa gingipain R; ●, TLCK-inactivated 50-kDa gingipain R; ▲, TLCK-inactivated 95-kDa gingipain R. Dashed lines are borders of controls which were assayed using 10 mM Tris-HCl, pH 7.3, containing 150 mM NaCl (TBS) instead of the 50-kDa gingipain R. In the case of clotting within 20 s, this assay is not available.

**TABLE I**

| Proteinase | APTT (sec) |
|------------|------------|
| Control    | 70 ± 5     |
| 50-kDa R   | 30 ± 2     |
| 95-kDa R   | 20 ± 1     |
| TLCK-treated 50-kDa R | 70 ± 5 |
| TLCK-treated 95-kDa R | 70 ± 5 |

**Factor X Activating Proteinases from P. gingivalis**

16063
plasma, unless it was reconstituted with factor X (Table II). These data again suggest that gingipain R decreased PT through factor X activation and that accelerated blood coagulation induced by gingipain R is specifically due to activation of this factor.

**Activation of Factor X by Gingipains Rs**—To investigate factor X activation by gingipain Rs, we incubated these cysteine proteinases with purified factor X and measured the factor Xa activity produced. Both proteinases generated factor Xa in a dose- and time-dependent manner (Fig. 3, A and B). Consistent with the data shown in Figs. 1 and 2, the 95-kDa gingipain R produced more factor Xa than the 50-kDa gingipain R (Fig. 3, A and B). TLCK-treated gingipain Rs did not induce factor X activation (Fig. 3A).

**Cleavage of Factor X by the 50-kDa Gingipain R**—To investigate the mechanism of factor X activation by gingipain R in more detail, we examined the pattern of cleavage during this process on an SDS-polyacrylamide gel, followed by amino-terminal sequence analysis of the fragments generated. Treatment with proteinase did not shift the 21-kDa molecule, which had an amino-terminal sequence ANSFLXMMK (X is presumed to be ε-carboxyglutamic acid) identical to that of the factor X light chain (Fig. 4). This indicates that cleavage had only occurred within the heavy chain which contains the catalytic domain blocked by the activation peptide. Our data also suggests that the first cleavage by gingipain R resulted in a shift in molecular mass from 47 to 45 kDa (lane b) without affecting the amino-terminal sequence (SVQAATSSSG-), presumably because of hydrolysis between Arg<sub>278</sub>-Gly<sub>288</sub> and release of a 19-amino acid peptide. This is consistent with the fact that the molecular mass of the final form of the heavy chain is slightly smaller than that of the factor Xa heavy chain (lanes d and e). The amino-terminal sequences of the 35- and 32-kDa molecules were GDNLTRIVG- and IVGGQECRDG-, respectively. From the amino acid sequence of human factor Xa (32), the generation of the 35-kDa fragment must have occurred through cleavage between Arg<sub>44</sub> and Gly<sub>45</sub> within the modified heavy chain to release a 45-amino acid activation glycopeptide. Finally, cleavage must occur between Arg<sub>59</sub> and Ile<sub>58</sub> to release the remainder of the activation peptide, also glycosylated. All of these proteolysis sites are in agreement with the fact that gingipain Rs only cleave polypeptide chains after arginine residues (22, 23) and at positions which are consistent with the activation of Factor X to Factor Xa.

**Effect of Phospholipids on Gingipain R Factor X Activation**—Phospholipids are important cofactors in blood coagulation and accelerate the cascade reaction of coagulation factors. We, therefore, studied the effect of phospholipids on factor X activation by gingipain Rs. 95-kDa gingipain R factor Xa production increased in a phospholipid concentration-dependent manner with the effect reaching a plateau at concentrations above 40 μg/ml, and yielding a 7- to 8-fold increase in factor X activation at enzyme concentrations of both 0.02 and 0.05 nM (Fig. 5). Interestingly, phospholipids did not stimulate the activation of factor X by the 50-kDa gingipain R (Fig. 5). Phospholipids did not augment 95-kDa gingipain R factor X activation in the absence of calcium, while factor X activation by the two proteinases was not affected by calcium in the absence of phospholipids (Fig. 5). It is likely that the calcium ion-mediated binding of the adhesin domains to phospholipids, in addition to factor X binding, is involved in augmentation of the factor X activation.

**Kinetics of Factor X Activation by Gingipain Rs**—To investigate the kinetics of factor X activation by gingipain Rs, the values of $K_m$ and $k_{cat}$ were determined for the interaction of purified factor X with either enzyme at a phospholipid concent-
Factor X Activating Proteinases from P. gingivalis

**TABLE III.**

| Proteinase      | $K_m$ (M) | $k_{cat}$ (s⁻¹) |
|----------------|-----------|---------------|
| 95-kDa gingipain R | 0.56      | 1.25          |
| 50-kDa gingipain R | 0.26      | 0.50          |
| TLCK-inactivated 95-kDa gingipain R | 0.50      | 0.25          |
| TLCK-inactivated 50-kDa gingipain R | 0.26      | 0.50          |

**DISCUSSION**

In this study we have provided experimental evidence to support the hypothesis that gingipain Rs can induce blood coagulation through activation of factor X. Although snake venom enzymes are known to activate factor X (35, 36), no bacterial proteinase has been reported so far to perform this same function. Indeed, staphylococagulase is the only bacterial coagulase which can induce human plasma coagulation, appar-
PT of factor VII-deficient plasma but not of factor X-deficient plasma (Table II). Thus, it seems likely that the factor X activation by gingipain Rs is the primary site through which blood coagulation is induced by these proteinases.

The \( K_m \) values of gingipain Rs (Table III) are lower than the factor X concentration in normal plasma (around 10 \( \mu \)g/ml, 170 nM) (27), which, together with their effects on APTT and PT (Figs. 1 and 2), supports the hypothesis that factor X activation by gingipain Rs can occur in plasma. Since phospholipids and calcium ions are ubiquitous cell membrane components and ions, respectively, their stimulatory effect on 95-kDa gingipain R-induced factor X activation (Fig. 5) can occur in vivo. Together with the fact that the \( k_{cat}/K_m \) value is close to the value of RVV-XCP (Table III), a strong factor X activator, it is likely that the high molecular weight gingipain R is physiologically a more important factor X activator than the smaller form.

We previously reported the binding activity of the adhesin domains in high molecular mass forms of gingipains to various proteins (40). The fact that the phospholipids augment factor X activation only by 95-kDa gingipain R (Fig. 5), complexed with adhesin domains, suggest the binding of the adhesin domains to phospholipids. Exoenzyme S, recently confirmed as an adhesin for *Pseudomonas aeruginosa* (41), binds to phosphatidylethanolamine specifically in the presence of 5 mM CaCl\(_2\) (42) and lipid receptors have been shown to be involved in the binding of *Helicobacter pylori* and *Burkholderia* (Basonym *Pseudomonas*) capsules to epithelial cells (42, 43). Thus, it may be natural for 95-kDa gingipain R adhesin domains to bind to phospholipids. However, phospholipids augmented neither Boc-Ile-Glu-Gly-Arg-MCA hydrolysis, prekallikrein activation, nor fibrinogen degradation by the complexed form, hence the phospholipids effect appears to be specific for factor X activation. The augmentation of 95-kDa gingipain R factor X activation by phospholipids, together with the lower \( K_m \) and much higher \( k_{cat} \) values of 95-kDa gingipain R than those of the 50-kDa form (Table III), indicates a further function for the adhesin domains in increasing the affinity to factor X as well as facilitating substrate cleavage.

In human plasma, gingipain Rs generate bradykinin (24), a potent vascular permeability enhancing peptide. In parallel, and through factor X activation thrombin is also generated (Fig. 1 and 2). Thus, in blood there is likely to be the simultaneous production of each by gingipain Rs, and for this reason it is believed that both may also be involved in the production of gingival crevicular fluid (24) and of prostaglandins and interleukin-1 (13–15), respectively, during infectious episodes. Hence, the elevated levels of prostaglandins and interleukin-1 in gingival crevicular fluid of adult periodontitis patients (3, 4, 6) may be connected to the thrombin produced by *P. gingivalis* proteinases. In addition, thrombin and fibrinopeptide B, released from fibrinogen by thrombin are each neutrophil chemo- tactic factors (44, 45) and may contribute to infiltration of these phagocytic cells at periodontitis sites (46). Finally, factor Xa also enhances macrophage interleukin-1 production (15) and is mitogenic for endothelial cells (47), smooth muscle cells (48), and lymphocytes (49). Therefore, the data described here would imply that gingipain R-induced activation of blood coagulation through conversion of factor X to Xa can easily be involved in the development of periodontitis.

In septic patients, disseminated intravascular coagulation (DIC) occurs frequently. Recent studies indicate that the initial activation of coagulation in sepsis is primarily dependent on activation of the extrinsic pathway, initiated by tissue factor expressed on monocytes (50). Since endotoxin, itself, or endo-

\[ \text{Factor X Activating Proteinases from } P. \text{ gingivalis} \]

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**TABLE III**

| Enzymes            | \( K_m \) (\( \mu \)M) | \( k_{cat} \) (10\(^{-3}\) M\(^{-1}\) s\(^{-1}\)) | \( k_{cat}/K_m \) (10\(^{-3}\) M\(^{-1}\) s\(^{-1}\)) |
|--------------------|-----------------------|-----------------|-----------------|
| 50-kDa gingipain R | 1.4 \times 10\(^{-7}\) | 1.2 \times 10\(^{-2}\) | 8.9 \times 10\(^{4}\) |
| 95-kDa gingipain R | 9.8 \times 10\(^{-8}\) | 0.4             | 4.1 \times 10\(^{9}\) |
| Factor IXa-VIIa    | 5.5 \times 10\(^{-8}\) | 1.3             | 2.4 \times 10\(^{8}\) |
| RVV-XCP\(^a\)     | 6.0 \times 10\(^{-8}\) | 17              | 2.8 \times 10\(^{9}\) |
|                    | 2.4 \times 10\(^{-7}\) | 2.3             | 9.6 \times 10\(^{8}\) |

\(^a\) Data obtained from Ref. 32.

\(^b\) Data obtained from Ref. 34. Factor VIIa-TF was activated factor VII complexed with tissue-factor apoprotein; factor IXa-VIIa, activated factor IX complexed with activated factor VIII.

\(^c\) RVV-XCP, Russell’s viper venom factor X coagulant protein.

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\(^d\) T. Imamura, unpublished data.
Factor X Activating Proteinases from P. gingivalis

16067

toxin-induced tumor necrosis factor-α can stimulate monocytes to express tissue factor (51, 52), the tendency has been to recognize such factors as the primary bacterial agents involved in the induction of DIC coagulation in sepsis. However, our finding that gingipain Rs can also readily induce blood coagulation suggests the possibility of bacterial proteinase-induced DIC coagulation in sepsis. It has been reported that a patient with an acute dentoalveolar abscess developed septicemia of *Bacteroides melaninogenicus*, a bacterium similar to *P. gingivalis*, and died with DIC (53). The fact that no plasma proteinase inhibitor effectively inactivates gingipain Rs (data not shown) supports this possibility. If so, inhibitors of such enzymes could be a therapy for DIC.

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