Activation of blood coagulation factor IX by gingipains R, arginine-specific cysteine proteinases from *Porphyromonas gingivalis*

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The effect of two arginine-specific cysteine proteinases (gingipains R) from *Porphyromonas gingivalis*, an aetiological factor of adult periodontitis, on the activation of human factor IX was investigated in the presence of ethylene glycol, an activity enhancer of activated factor IX (factor IXa), with the use of a fluorogenic oligopeptide substrate. Each gingipain R rapidly activated factor IX but the 95 kDa proteinase complex (HRgpA) that contains both haemagglutinin/adhesion and catalytic domains was 2.4-fold more efficient than the single-chain 50 kDa gingipain R (RgpB), which has only a catalytic domain. SDS/PAGE and N-terminal sequence analysis of factor IX digestion fragments indicated that, like all endogenous activators, gingipains R also produce factor IXa β via an IXa intermediate. Significantly, phospholipids augmented the activation of factor IX by HRgpA but not by RgpB in a Ca²⁺-dependent manner. In the presence of both cofactors the kinetic efficiency of HRgpA to activate factor

IX ($k_{\text{cat}}/K_m = 1.9 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) was 8.5-fold higher than that of RgpB ($k_{\text{cat}}/K_m = 2.3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) and double that of the factor VIIa–tissue factor complex, but 8-fold lower than that for factor XIa. A comparison of the relative activation rates of factor IX, factor X and prothrombin directly in plasma by HRgpA suggests a significant contribution for factor IX conversion in blood coagulation induced by gingipains R. Taken together, gingipains R are the first-reported activators of factor IX of bacterial origin. By this effect they could be involved in the production of thrombin as well as the subsequent generation of prostaglandins and interleukin 1, all of which have been found to be associated with the development and progression of periodontitis.

Key words: pathogenesis, periodontitis, proteolysis, sepsis, zymogen activation.

INTRODUCTION

Factor IX, a precursor of the trypsin-like serine proteinase factor IXa, circulates in the blood as a single-chain glycoprotein [1,2]. In the course of coagulation this zymogen is activated and converted to the two-chain, proteolytically active, factor IXa by the factor VIIa–tissue factor complex or by factor XIa [3,4]. Factor IXa is known to assemble with factor VIIIa on phospholipid surfaces and to activate factor X in a Ca²⁺-dependent fashion, in this manner having a major role in the coagulation process [5]. The importance of factor IX in physiological blood clotting is apparent from the fact that any mutation that impairs either zymogen activation or factor IXa activity is associated with a severe bleeding tendency referred to as haemophilia B [6]. In contrast, inappropriate control of factor IX activation can give rise to thrombotic disorders.

Both pathogenic bacteria and snake venom can release activators of coagulation that are at least partly responsible for tissue damage either at a site of infection or a snake bite. Most of these are classified as proteinases that activate prothrombin [7,8], factor X [9,10] or protein C [11,12]. In addition, factor IX activation by a proteinase in the venom from Russell's viper has also been described [13]. Significantly, however, no results indicating activation of this coagulation factor by bacterial proteinase have yet been reported. This lack of knowledge is most probably due to technical difficulties in directly assaying factor IXa activity, because no suitable chromogenic or fluorogenic

substrates specific for this factor have been available. Instead, factor IXa activity has been determined indirectly by measuring factor X activation in the presence of factor VIIIa and phospholipids, with synthetic substrates specific for factor Xa. However, it has been found that alcohols markedly enhance the catalytic activity of factor IXa [14]; this, together with the availability of specific fluorogenic synthetic substrates, has enabled us to measure factor IXa activity directly and with high sensitivity.

Porphyromonas gingivalis is a well-known causative bacterium of adult periodontitis [15–17]; it produces proteolytic enzymes that act as important virulence factors [18–20]. From the culture medium of strain HG66 we have previously purified two arginine-specific cysteine proteinases, referred to as 50 kDa and 95 kDa gingipains R (RgpB and HRgpA respectively) [21,22]. These enzymes are products of two genes, *rgpB* and *rgpA* respectively, with each encoding a similar prepro-fragment and an identical catalytic domain but differing considerably in the translation of the 3'-downstream segment of each gene. In the *rgpA* gene this region encodes haemagglutinin/adhesion domains; however, this is absent from the *rgpB* gene [23,24]. Thus HRgpA occurs as a non-covalent complex of catalytic and haemagglutinin/adhesion domains that are generated through proteolytic processing of the nascent translation product of the *rgpA* gene [22,23]. In contrast, RgpB is a single-chain enzyme containing only a catalytic domain [21]. The crystal structure of RgpB has been solved recently, revealing a molecule that can be divided into two

Abbreviations used: AMC, 7-amino-4-methylcoumarin; APTT, activated partial thromboplastin time; gingipain R, arginine-specific gingipain; HRgpA, 95 kDa gingipain R; MS-D-CHG-Gly-Arg-MCA, methylsulphonyl-D-cyclohexylglycyl-L-glycyl-L-arginine-4-methyl-coumaryl-7-amide; RgpB, 50 kDa gingipain R; Tos-Lys-CH₂Cl, tosyl-lysylchloromethane ('TLCK').

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clearly separated segments, a catalytic subdomain with topological similarity to caspases and a C-terminal immunoglobulin-like domain [25]. On the basis of the virtual identity of the primary structures of the catalytic domains of RgpB and HRgpA, it can be assumed that these are identical in the crystal state. However, despite this likelihood both gingipains R show remarkable functional differences, which have not yet been fully elucidated [9,26].

It is well known that the activation of zymogens in the fibrinolysis, coagulation and kallikrein–kinin cascades occurs by limited proteolysis after a specific arginine residue in each zymogen [27]. We have previously shown that, *in vitro* and *ex vivo* directly in human plasma, both gingipains R efficiently activate prekallikrein and protein C [11,28]. Although these proteinases decreased the activated partial thromboplastin time (APTT) for normal plasma, RgpB did not affect the APTT of factor X-deficient plasma [9]. This observation, although indicating that the contribution of RgpB to direct prothrombin activation is minimal, does not exclude the possibility of uncontrolled activation of coagulation factors upstream of factor X. Such events might also result in the significant production of thrombin through the cascade reaction. Moreover, the fact that RgpB decreased the APTT of factor XI-deficient plasma less efficiently than that of factor IX-deficient plasma [9] suggests the involvement of factor IX, which is activated just before factor X. This observation prompted us to investigate in more detail the ability of gingipains R to activate factor IX.

MATERIALS AND METHODS

Materials

Tosyl-lysylchloromethane (Tos-Lys-CH₂Cl, 'TLCK') was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.); purified human factor IXa β was from Enzyme Research Laboratories (South Bend, IN, U.S.A.). Platelin® (rabbit brain phospholipids) was purchased from Organon Teknika Corp. (Durham, NC, U.S.A.). Leupeptin and a standard 7-amino-4-methylcoumarin (AMC) were obtained from the Peptide Institute (Minoh, Japan). Methylsulphonyl-D-cyclohexylglycyl-L-glycyl-L-arginine-4-methyl-coumaryl-7-amide (MS-D-CHG-Gly-Arg-MCA) was purchased from Pentapharm Ltd. (Basel, Switzerland). Normal human plasma was obtained by centrifugation of a mixture of 9 vol. of freshly drawn blood from healthy volunteers and 1 vol. of 3.8% (w/v) sodium citrate. Other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Proteinase purification

HRgpA and RgpB were isolated by the method of Potempa et al. [21]. The amount of active enzyme in each purified proteinase was determined by active-site titration with D-phenylalanyl-prolylarginylchloromethyl ketone (D-Phe-Pro-Arg-CH₂Cl, 'FPRCK') [29]. The concentration of active gingipain R was calculated from the amount of inhibitor needed for complete inactivation of the proteinase.

Purification of factor IX

Factor IX was purified from human plasma by a combination of barium citrate adsorption, Q-Sepharose Fast Flow column chromatography and dextran-sulphate–agarose column chromatography as described previously [30]. The final purification was achieved by immunoaffinity chromatography with

Ca²⁺-dependent murine monoclonal antibodies [31]. Factor IX purified in this way exhibited a single band on SDS/PAGE under reducing conditions with an apparent molecular mass of 59 kDa (see Figure 2, lane b).

Activation of gingipains R

Each form of gingipain was activated at 37 °C for 10 min with 10 mM cysteine in 0.2 M Hepes buffer, pH 8.0, containing 5 mM CaCl₂. Before use, the activated proteinase (1 μ M) was diluted with 50 mM Tris/HCl, pH 7.4, containing 0.1 M NaCl and 5 mM CaCl₂.

Kinetic analysis of factor IX activation

To enhance the catalytic activity of factor IXa, assays were performed in the presence of ethylene glycol [14]. Factor IX, dissolved in 45 μ l of 50 mM Tris/HCl, pH 7.4, containing 0.1 M NaCl and 5 mM CaCl₂ was incubated with 5 μ l of either of the gingipains (5 nM final concentration) at 37 °C for 30, 60, 90, 120 or 150 s. Of this solution, 40 μ l was then transferred to 40 μ l of leupeptin [4 μ M in the same buffer supplemented with 45% (v/v) ethylene glycol], to inhibit cysteine proteinase activity completely. At this concentration leupeptin does not affect the amidolytic activity of factor IXa. This was followed by the addition of 40 μ l of a factor IXa-specific substrate, MS-D-CHG-Gly-Arg-MCA (1.25 mM) in the same buffer supplemented with 52.5% (v/v) ethylene glycol. Substrate cleavage and the release of AMC by factor IXa was monitored by the relative increase in fluorescence at 460 $\pm$ 20 nm after excitation at 360 $\pm$ 20 nm, with a microplate fluorescence spectrophotometer (CytoFluor Series 4000; PerSeptive Biosystems). The concentration of factor IXa generated by either of the gingipains was calculated by using as a standard the amidolytic activity of purified factor IXa that had been active-site titrated with *p*-nitro-*p*'-guanidinobenzoate [32]. The initial velocity of factor IXa production at various concentrations was determined by the best-fit line for the five incubation periods mentioned above. Several factor IX concentrations in the range 0.2–10 μ M were used for the kinetic study.

The values for K_m and V_{max} in the Michaelis–Menten equation were obtained by using three different plots: $[S]_0/v$ against $[S]_0$, $1/v$ against $1/[S]_0$ and v against $v/[S]_0$, where v and $[S]_0$ denote the catalytic rate and the initial substrate concentration respectively. Best-fit values were determined by the method of least squares with a Taylor expansion by using software described by Sakoda and Hiromi [33]. Of the three plots, the best-fit values obtained from each plot of $[S]_0/v$ against $[S]_0$ were closest to those determined by the method of least squares with a Taylor expansion; plots of $[S]_0/v$ against $[S]_0$ are therefore shown in the insets to Figure 4. Each experiment was performed three times and the deviations of the observed values were within 10% error. Therefore the average K_m is presented in Table 1.

Relative activation velocities of HRgpA for factor IX, factor X and prothrombin were calculated from the best-fit line equations of plots of $[S]_0/v$ against $[S]_0$ with each zymogen plasma concentration as $[S]_0$ [27]. The values shown in Table 2 are the initial velocities of each activated factor produced by HRgpA when that factor was at a concentration typical of normal human plasma.

SDS/PAGE

Analysis by SDS/PAGE was performed with a 15% (v/v) slab gel by the method of Laemmli [34]; 0.8% Coomassie Brilliant Blue R-250 was used for protein staining.

N-terminal sequence analysis

Automatic sequence analysis was performed with a pulsed-liquid-phase sequencer (model 477A Protein Sequencer; Perkin-Elmer/Applied Biosystems). To determine the N-terminal sequence of factor IX-derived fragments, each protein fragment was separated by SDS/PAGE and transferred to an ImmobilonTM PVDF transfer membrane (Millipore Co., Bedford, MA, U.S.A.). The proteins transferred to the PVDF membrane were detected by staining with Coomassie Brilliant Blue R-250. Bands were excised and placed on a Polybrene-treated glass filter; sequence analysis was then performed.

Determination of factor IX concentration

The molar concentration of purified factor IX was calculated by using $A_{280}^{1\%} = 13.2$ and a molecular mass of 57 kDa [2].

RESULTS

Activation of factor IX by gingipains R

To investigate factor IX activation by gingipains, we incubated HRgpA or RgpB with purified zymogen and measured the activity of factor IXa released. Starting at as low as a 1 nM final concentration, both gingipains released factor IXa linearly in a dose-dependent and time-dependent manner (Figure 1). Because Tos-Lys-CH₂Cl-treated proteinases did not induce any factor IX activation (Figure 1, upper panel) it is apparent that this process is dependent on the proteolytic activity of gingipains R. Interestingly, at 1 μ M factor IX, activation by HRgpA was approx. 2.4-fold more efficient than by RgpB.

Cleavage of factor IX by HRgpA

To investigate the mechanism of factor IX activation in more detail we examined the pattern of zymogen cleavage during its incubation with HRgpA, by using SDS/PAGE and N-terminal sequence analysis. After incubation with HRgpA, factor IX was cleaved into four fragments with apparent molecular masses of 41, 30, 22 and 11 kDa (Figure 2) and N-terminal sequences of AETVFPDVD, VVGGEDAKP, YNSGKLEEF and AETVPDVD (single-letter amino acid codes) respectively. On the basis of both molecular masses and N-terminal sequences, the bands were identified as the heavy chain of factor IX α (41 kDa), the heavy chain of factor IX $\alpha\beta$ (30 kDa), the factor IXa light chain (22 kDa) and the activation peptide of factor IX (11 kDa). The generation of the factor IXa light chain (22 kDa) reached a plateau after only 3 min of incubation. Accumulation of the heavy chain of factor IX α (41 kDa) peaked in 3 min and subsequently decreased. In contrast, bands corresponding to both the heavy chain of factor IX $\alpha\beta$ (30 kDa) and the activation peptide (11 kDa) increased continuously during incubation. These results clearly indicate that zymogen cleavage by HRgpA occurred first between Arg¹⁴⁵ and Ala¹⁴⁶, leading to the formation of the intermediate factor IX α . This was followed by hydrolysis between Arg¹⁸⁰ and Val¹⁸¹ to release the activation peptide and generate factor IX $\alpha\beta$.

Effect of phospholipids on factor IX activation by gingipains R

Phospholipids are important physiological cofactors in blood coagulation. Their appearance signals damage to the endothelium and locally accelerates the clotting reaction. We therefore studied the effect of phospholipids on factor IX activation by gingipains

Figure 1 Activation of factor IX by HRgpA or RgpB

Factor IX dissolved in 45 μ l of 50 mM Tris/HCl, pH 7.4, containing 0.1 M NaCl and 5 mM CaCl₂ (1.1 μ M final concentration) was incubated with 5 μ l of either of the gingipains R at 37 °C for various periods. Of this solution, 40 μ l was added to 40 μ l of leupeptin [4 μ M in the same buffer supplemented with 45% (v/v) ethylene glycol]. This was followed by the addition of 40 μ l of a factor IXa-specific substrate, MS-D-CHG-Gly-Arg-MCA [1.25 mM in the same buffer supplemented with 52.5% (v/v) ethylene glycol]. Upper panel: proteinases of various concentrations were incubated with factor IX for 2 min. The final concentrations of each proteinase are shown. Symbols: Δ , HRgpA; $\circ$, RgpB; $\blacktriangle$, Tos-Lys-CH₂Cl-inactivated HRgpA; $\bullet$, Tos-Lys-CH₂Cl-inactivated RgpB. Lower panel: a proteinase (2 nM final concentration) was incubated with factor IX at 37 °C for various periods. Symbols: Δ , HRgpA; $\circ$, RgpB; $\square$, Tris-buffered saline instead of a proteinase solution.

R. In the presence of Ca²⁺ ions the rate of production of factor IXa catalysed by HRgpA increased in a concentration-dependent manner, reaching a plateau at a phospholipid concentration above 60 μ g/ml. At this concentration, factor IX activation by HRgpA was amplified approx. 2.3-fold in comparison with controls (Figure 3). For this effect to occur, the presence of Ca²⁺ ions was absolutely necessary, although this cation on its own was without effect on HRgpA activity. Interestingly, regardless of the presence or absence of Ca²⁺ ions, phospholipids did not affect zymogen activation by RgpB (Figure 3), suggesting a role in this process for the haemagglutinin/adhesion domains that are present only in HRgpA.

Kinetics of factor IX activation by gingipains R

To investigate the kinetics of factor IX activation by gingipains, the k_{cat} and K_m were determined with both enzymes (Figure 4). The K_m of HRgpA was one-third of that of RgpB, although their k_{cat} values were equal (Table 1), the catalytic efficiency (k_{cat}/K_m)

Figure 2 Cleavage of factor IX incubated with HRgpA

Factor IX (40 μ l; 10 μ M), dissolved in 0.1 M Tris/HCl (pH 7.6)/150 mM NaCl/5 mM CaCl_2 , was incubated with 10 μ l of HRgpA (0.05 μ M) at 37 $^\circ\text{C}$ for various periods. Of this solution, 10 μ l was subjected to SDS/PAGE in the presence of 2 mM 2-mercaptoethanol. Lane a, molecular mass markers (bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; α -lactalbumin, 13 kDa); lane b, factor IX only; lanes c–f, factor IX incubated with HRgpA for 1, 3, 10 and 30 min respectively; lane g, factor IXa β (1 μ M). Molecular masses are indicated (in kDa) on the right.

Figure 3 Effect of phospholipids on factor IX activation by HRgpA or RgpB

A proteinase (5 μ l; 0.02 μ M) was added to a mixture of 40 μ l of factor IX [1.25 μ M dissolved in 50 mM Tris/HCl (pH 7.4)/0.1 M NaCl supplemented with (○, Δ) or without (●, $\blacktriangle$) 5 mM CaCl_2] and 5 μ l of phospholipids and incubated for 2 min at 37 $^\circ\text{C}$. To 40 μ l of the reaction mixture were added 40 μ l of leupeptin [4 μ M in the above buffer supplemented with 45% (v/v) ethylene glycol], then 40 μ l of MS-D-CHG-Gly-Arg-MCA [1.25 mM in the same buffer supplemented with 52.5% (v/v) ethylene glycol]; AMC release was then measured. Symbols: Δ , $\blacktriangle$, HRgpA; ○, ●, RgpB.

of the former gingipain therefore being 3-fold that of RgpB. This result seems to be consistent with the fact that HRgpA activated factor IX 2.4-fold more efficiently than RgpB at a concentration of 1 μ M (Figure 1). However, phospholipids in the presence of Ca^{2+} ions decreased the K_m 2.2-fold and increased the k_{cat} value 1.3-fold for the HRgpA-catalysed reaction of factor IX activation. Thus, in the presence of these physiological cofactors, which are known to be used in the coagulation cascade, the catalytic potency of HRgpA was 8.5-fold that of RgpB.

The kinetic constants for factor IX activation by gingipains R were compared with those of either activated factor VII (VIIa) complexed with tissue factor [4] or factor XIa [35]. The K_m values for gingipains were higher than those of physiological factor IX

Figure 4 Kinetic study of factor IX activation by HRgpA or RgpB

The initial velocities of factor IX activation by HRgpA (upper panel) or RgpB (lower panel) were determined under the conditions described in the Materials and methods section. The concentrations of proteinase were 2 nM. Plots of the data as $[S]_0/v$ against $[S]_0$ are shown in each inset. Symbols: ○, values in the presence of phospholipids (60 μ g/ml); ●, values in the absence of phospholipids.

Table 1 Kinetic constants for the activation of factor IX

Where phospholipids were added, their concentration was 60 μ g/ml.

Enzyme	K_m (μ M)	k_{cat} (s^{-1})	$10^{-6} \times k_{\text{cat}}/K_m$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
HRgpA	1.9	1.3	0.68
HRgpA + phospholipids	0.88	1.7	1.9
RgpB	5.7	1.3	0.23
Factor VIIa–tissue factor + phospholipids*	0.54	0.55	1.0
Factor XIa†	0.49	7.7	16

* Data obtained from [4].

† Data obtained from [35].

activators, whereas k_{cat} values were lower than the k_{cat} of factor XIa but higher than the k_{cat} of the factor VIIa–tissue factor complex (Table 1). Taken together, the k_{cat}/K_m of HRgpA was approximately double that of the factor VIIa–tissue factor complex but 8-fold lower than that of factor XIa. These results suggest that HRgpA is a more potent factor IX activator than RgpB and is comparable with the factor VIIa–tissue factor complex in its ability to activate factor IX. The k_{cat} of coagulation

Table 2 Comparison of the relative activation velocities of factor IX, factor X and prothrombin by HRgpA in plasma

K_m and k_{cat} were measured in the presence of phospholipids and Ca^{2+} ions. Results for factor X are from [9], those for prothrombin are from unpublished work (T. Imamura, A. Banbula, P. J. B. Pereira, J. Travis and J. Potempa) and those for plasma concentrations are from [27]. Relative activation velocities were calculated from best-fit line equations of plots of $[S]_0/v$ against $[S]_0$, with each zymogen plasma concentration as $[S]_0$.

Coagulation factor	K_m (nM)	k_{cat} (s^{-1})	Plasma concentration (nM)	Relative activation velocity
Factor IX	880	1.7	80	0.13
Factor X	98	0.4	170	0.25
Prothrombin	270	0.34	1100	0.33

factor activation by HRgpA was the highest for factor IX; however, the K_m was also the highest in comparison with those values for factor X [9] or prothrombin (T. Imamura, A. Banbula, P. J. B. Pereira, J. Travis and J. Potempa, unpublished work) (Table 2). Taking into account the plasma concentrations of these three zymogens [27], the ratio of the activation velocities of factor IX, factor X and prothrombin by HRgpA was approx. 1:2:2.5 (Table 2).

DISCUSSION

Various techniques are routinely employed to measure factor IXa activity directly: the esterase activity assay with benzoyl-L-arginine [3H]ethyl ester or tosyl-L-arginine [3H]methyl ester [13] or the activation peptide release assay with 3H -labelled factor IX [35]. The first two assays are not sensitive and require a large amount of factor IXa. However, the last method, although sensitive, is not only cumbersome but also unsuitable for measuring factor IX activation unless a proteinase precisely cleaves the activation peptide. In contrast, the direct assay method described here used a fluorogenic oligopeptide substrate, specific for measuring factor IXa activity, in the presence of 33 % (v/v) ethylene glycol in the assay buffer, which allowed us to measure this active enzyme with high sensitivity. Indeed, the application of this assay facilitated the kinetic analysis of factor IX activation by gingipains R. Although a protease in the venom from Russell's viper has also been shown to activate factor IX [3], no kinetic study has yet been reported. Furthermore, in this regard our report not only is the first to note factor IX activation by bacterial proteinases but also provides an initial kinetic analysis of this process and permits a comparison of its efficiency with other physiological activators of factor IX.

The mechanism of factor IX activation by a protease from Russell's viper venom is different from that by factor XIa or the factor VIIa–tissue factor complex. Factor XIa first cleaves the Arg¹⁴⁵–Ala¹⁴⁶ bond, converting factor IX to a two-chain intermediate without catalytic activity, referred to as factor IX α . In a subsequent step the Arg¹⁸⁰–Val¹⁸¹ peptide bond is hydrolysed, resulting in the formation of factor IXa β and the release of the activation peptide [3,4]. In contrast, the venom proteinase cleaves the Arg¹⁸⁰–Val¹⁸¹ bond only, producing factor IXa γ with clotting activities similar to those of factor IXa β , as measured in human [3] and bovine [13] systems. The results presented with gingipains for factor IX activation seem to support an activation process identical with the physiological pathway (i.e. the activation of factor IX by factor XIa).

Phospholipids are known to augment the HRgpA-induced, but not the RgpB-induced, activation of factor X [9], prothrombin (T. Imamura, A. Banbula, P. J. B. Pereira, J. Travis and J. Potempa, unpublished work) and protein C [11] in the presence of Ca^{2+} ions. All of these coagulation factors, as well as factor IX, contain γ -carboxyglutamic acid residues whose bind-

ing to phospholipids is mediated by Ca^{2+} ions [27]. In addition, HRgpA, but not RgpB, binds to phospholipids in a Ca^{2+} -dependent manner [37]. Thus it is likely that the Ca^{2+} -mediated activation of factor IX by HRgpA occurs through independent or shared binding of phospholipids. In the presence of phospholipids the K_m decreased and the k_{cat} increased for the HRgpA-catalysed reaction of factor IX activation (Figure 4, upper panel, and Table 1), thus enhancing the zymogen activation 2.3-fold (Figure 3). The rate of factor IX activation by HRgpA in the presence of phospholipid was significantly higher than that for the activation of prothrombin (1.5-fold) and protein C (1.4-fold) but lower than that for factor X activation (7–8-fold). The structure of factor IX is similar to that of factor X and protein C but different from that of prothrombin [27]; therefore the differences in phospholipid augmentation rates for factor IX activation by HRgpA cannot be explained by structural differences between the zymogens.

The kinetic analysis (Table 1) supported the result that HRgpA was more potent than RgpB in factor IX activation (Figure 1), which seems to be a common theme in the activation of coagulation factors by the two gingipains R ([9,11], and T. Imamura, A. Banbula, P. J. B. Pereira, J. Travis and J. Potempa, unpublished work). However, the k_{cat}/K_m for HRgpA was only 8.5-fold that of RgpB in factor IX activation (Table 1), which is a relatively small difference in comparison with the 46-fold or 118-fold difference in the activation of factor X [9] and prothrombin (T. Imamura, A. Banbula, P. J. B. Pereira, J. Travis and J. Potempa, unpublished work), respectively. The fact that the K_m for HRgpA activation was one-third of that of RgpB but the k_{cat} values were the same (Table 1) implies that the haemagglutinin/adhesion domains of HRgpA are responsible for the increased activity of this gingipain for factor IX activation. This interaction is further specifically enhanced in the presence of phospholipids. Although factor XIa is the best factor IX activator (Table 1), it is surprising that HRgpA, augmented with phospholipids, was more potent than the factor VIIa–tissue factor complex, the physiological activator of factor IX in the extrinsic pathway of coagulation.

Of the three coagulation factors investigated so far, the relative velocity of HRgpA-mediated prothrombin activation in human plasma is the fastest (Table 2), indicating that prothrombin is the primary target of HRgpA (T. Imamura, A. Banbula, P. J. B. Pereira, J. Travis and J. Potempa, unpublished work). In fact, HRgpA induced the clotting of factor X-deficient plasma and also that of the same deficient plasma reconstituted with the missing factor (T. Imamura, A. Banbula, P. J. B. Pereira, J. Travis and J. Potempa, unpublished work). Although HRgpA activates factor IX in plasma 2-fold or 2.5-fold more slowly than factor X and prothrombin respectively in plasma (Table 2), one must keep in mind the fact that factor IX is located further upstream in the coagulation cascade pathway than either of these other two factors. Factor IXa and factor Xa, in the presence of

each cofactor, activated factor X and prothrombin respectively faster than did HRgpA [9], and T. Imamura, A. Banbula, P. J. B. Pereira, J. Travis and J. Potempa, unpublished work). In the cascade reaction, factor IXa initially activated by HRgpA would generate factor Xa more efficiently than HRgpA alone, finally leading to the release of much more thrombin than could be produced by the direct activation of factor X by HRgpA. It is therefore likely that factor IX activation by HRgpA contributes significantly to the bacterial proteinase-induced coagulation through triggering of the amplifying system.

Gingipains R generate bradykinin [28], a potent vascular-permeability-enhancing peptide, which ensures a local influx of plasma coagulation factors to the site of *P. gingivalis* infection. At such a site, α -thrombin is produced through the activation of factor IX, factor X or prothrombin. In addition, to cause further vascular permeability enhancement [38] and leukocyte chemotaxis [39,40], α -thrombin induces the production of prostaglandins [41,42] and interleukin 1 [43]. Hence the elevated levels of these two inflammatory mediators in the gingival crevicular fluid of patients with adult periodontitis [44,45] might be connected to thrombin production by the proteinases from *P. gingivalis*. Thrombin stimulates bone resorption by osteoclasts through a prostaglandin-dependent pathway [46] and could therefore be associated with the alveolar bone resorption seen at periodontitis sites. In severe periodontitis, *P. gingivalis*-derived products, including gingipains R, can easily access the circulation through the enlarged surface of the pocket epithelium (more than 72 cm²) [47–49]. Our findings that gingipains R, and especially HRgpA, can readily activate factor IX, factor X and/or prothrombin leading ultimately to the release of thrombin suggest the possibility of bacterial proteinase-induced disseminated intravascular coagulation (DIC), which occurs frequently in septic patients [50–53]. This possibility might be supported by the fact that a patient with dentoalveolar abscess developed both sepsis of *Bacteroides melaninogenicus*, a bacterium similar to *P. gingivalis*, and DIC [54].

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