Activation of Human Prothrombin by Arginine-specific Cysteine Proteinases (Gingipains R) from Porphyromonas gingivalis*

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The effect of 95-kDa (HRgpA) and 50-kDa gingipain R (RgpB), arginine-specific cysteine proteinases from periodontopathogenic bacterium Porphyromonas gingivalis on human prothrombin activation was investigated. Each enzyme released thrombin from prothrombin in a dose- and time-dependent manner with the former enzyme, containing adhesion domains, being 17-fold more efficient than the single chain RgpB. A close correlation between the generation of fibrinogen clotting activity and amidolytic activity indicated that α-thrombin was produced by gingipains R, and this was confirmed by SDS-polyacrylamide gel electrophoresis, thrombin active site labeling, and amino-terminal sequence analysis of prothrombin digestion fragments. Significantly, the catalytic efficiency of HRgpA to generate thrombin (k_{cat}/K_{m} = 1.2 \times 10^{5} \text{ M}^{-1} \text{s}^{-1}) was 100-fold higher than that of RgpB (k_{cat}/K_{m} = 1.2 \times 10^{3} \text{ M}^{-1} \text{s}^{-1}). The superior prothrombinase activity of HRgpA over RgpB correlates with the fact that only the former enzyme was able to clot plasma, and kinetic data indicate that prothrombin activation can occur in vivo. At P. gingivalis-infected periodontitis sites HRgpA may be involved in the direct production of thrombin and, therefore, in the generation of prostaglandins and interleukin-1, both have been found to be associated with the development and progression of the disease. Furthermore, by taking into account that the P. gingivalis bacterium has been immunolocalized in carotid atherosclerotic plaques at thrombus formation sites (Chiu, B. (1999) Am. Heart J. 138, S534–S536), our results indicate that bacterial proteinases may potentially participate in the pathogenesis of cardiovascular disease associated with periodontitis.

Blood coagulation is an important defense system, protecting the body against blood loss from injured vessels. The process is initiated by the binding of factor VII to tissue factor (1), present in tissues surrounding vessels (2), followed by proteolytic activation of plasma coagulation factors in a cascade pathway (3–5). Thrombin, the ultimate product of these reactions, is an extremely potent platelet activator (6, 7) and converts fibrinogen to a fibrin clot (8), thus plugging damaged vessels. Besides its central role in hemostasis, thrombin also enhances vascular permeability (9), induces leukocyte chemotaxis (10, 11), and potentiates lipopolysaccharide-stimulated interleukin-1 production by macrophages (12). These data, and the fact that prothrombin activation in vivo is known to be associated with inflammatory conditions, implicate thrombin as a major player in inflammation.

The deposition of fibrin is a common feature at the site of bacterial infection (13). Endotoxin can induce fibrin accumulation in vivo through the Shwartzman reaction (14), presumably by activating monocytes to express tissue factor (15). For this reason it is recognized as the component primarily responsible for blood coagulation associated with bacterial infections. Proteinases from such foreign sources are also thought to be virulence factors involved in various inflammatory events occurring at infected sites (16). For example, many of these enzymes present in snake venoms are known to activate prothrombin (17, 18); however, whereas bacterial proteinases may be able to convert prothrombin to thrombin, such a process has not been studied in detail.

A close relationship between Porphyromonas gingivalis (formerly Bacteroides gingivalis) and adult periodontitis has been reported (19–21), with proteolytic enzymes that are known to be produced in large quantity by this microorganism and have been shown to act as important pathogenic agents (22–24). From the culture medium of P. gingivalis HG606 we have purified previously two major forms of arginine-specific cysteine proteinases, HRgpA and RgpB, formerly referred to as high molecular mass gingipain R (95-kDa gingipain R1 or HRGP) and 50-kDa gingipain R2 (RGP-2), respectively (24, 26). Both of these enzymes are products of two distinct but related genes (27). rgpA encodes a polypeptide which, after post-translational processing/modifications, yields three different forms of the enzyme (28, 29); the major one is a non-covalent complex containing separate catalytic and adhesion/hemagglutinin domains (HRgpA). In contrast, the fragment encoding the latter domain(s) is missing in the rgpB gene structure, and its trans-

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1. The abbreviations used are: HRgpA and RgpB, arginine-specific gingipains, products of rgpA and rgpB genes, respectively; PT, prothrombin time; TLCK, (S)-2-chloro-(2-methyl-propyl)-amino-4-phenylsulfonyl fluoride; PAG, polyacrylamide gel electrophoresis; APPT, activated partial thromboplastin time; Boc-Val-Pro-Arg-MCA, t-butylxoycarbonyl-L-Val-L-Pro-L-Arg-4-methylcoumaryl-7-amide.
lation product is a single protein with a primary structure essentially identical to the catalytic domain of HRGpA (30, 31). Despite both a structural similarity and a specificity restricted to Arg-Xaa peptide bonds, HRGpA and RgpB show considerable differences in catalytic potency (26) which is most profoundly manifested in their ability to activate factor X (32) and protein C (33). In addition to activation of these members of the coagulation cascade pathway, it was also shown that RgpB was capable of generating kalikrein from plasma prekallikrein (16). Thus, it may be anticipated that gingipains R could activate other coagulation cascade proenzymes in this pathway, since each of these processes requires cleavage of peptide bonds at the carboxy-terminal side of specific arginine residues (34).

In the present study, we describe the results of experiments designed to investigate the ability of two forms of gingipains R to convert prothrombin to thrombin, an enzyme known to have multiple functions in both coagulation and pro-inflammatory processes.

EXPERIMENTAL PROCEDURES

Materials—Benzoyl-L-arginine-p-nitroanilide, tosyl-L-lysine chloromethyl ketone (TFLK) leupeptin, and PMSF were purchased from Sigma. Factor X-deficient plasma was obtained from George King Bio-Medical, Inc. (Overland Park, KS). Purified human prothrombin, α-, β- and γ-thrombins, and bimonthylated Phe-Pro-Arg-chloromethyl ketone were purchased from Hematologic Technologies, Inc. (Essex Junction, VT). Z-Butyloxy-carbonyl-L-Val-L-Pro-L-Arg-4-methylcoumaryl-7-amide (Boc-Val-Pro-Arg-MCA) was obtained from the Peptide Institute (Minoh, Japan); p-nitrophenyl-p'-guanidobenzoate was a product from Nacalai Tesque (Kyoto, Japan) and DX-9065a, a specific factor Xa inhibitor, was obtained from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). The purified human factor X was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN). Factor X-, IX-, and XI-deficient plasmas and Platelet® (rabbit brain phospholipids) were obtained from George King Biomedical (Overland Park, KS), and from Organon Teknika (Durham, NC), respectively. Normal human plasma was prepared 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—RgpB and HRGpA were isolated according to the method described by Puttema et al. (26). The amount of active enzyme in each purified proteinase was determined by active site titration using Phe-Pro-Arg-chloromethyl ketone (35), with the concentration of active gingipain R being calculated from the amount of inhibitor needed for complete inactivation of the proteinase.

Activation of Proteinases—Each P. gingivalis proteinase 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 diluted to 0.1 M Tris-HCl, pH 7.3, containing 150 mM NaCl (TBS) and 5 mM CaCl₂ prior to use.

Determination of Prothrombin Concentration—The molar concentration of purified prothrombin was calculated using \( A_{280}^{nm} = 13.8 \) and a molecular mass of 72 kDa (36).

Clotting Assay—The fibrinogen clotting activity of released thrombin was measured by incubating 90 μl of warfarin (90 μg/ml) with 10 μl of a given proteinase at 37 °C for 3 min. One hundred μl of fibrinogen (3 mg/ml), prewarmed to 37 °C, was then added to the mixture, and the clotting time was measured with a Coagulometer KC 1A (Amelung, Lemgo, Germany). For plasma clotting time assays, 90 μl of factor X-deficient plasma supplemented with 4 μM factor X-specific inhibitor (Roche Molecular Biochemicals) and preheated to 37 °C in a water bath was added to 162 μl of a proteinase at 37 °C for 3 min. One hundred μl of a proteinase was added and the clotting time was measured with a Coagulometer KC 1A (Amelung, Lemgo, Germany).

Kinetic Analysis of Prothrombin Activation—Prothrombin, dissolved in 50 μl of 0.1 M Tris-HCl, pH 7.6, containing 0.15 M NaCl and 5 mM CaCl₂, was incubated with the same volume of either gingipain R (0.1 nm HRGpA or 0.4 nm RgpB final concentration) dissolved in the same buffer supplemented with 80 μM phospholipids at 37 °C for 30, 60, 90, or 120 s. Then, 50 μl of 6 mM leupeptin in the same buffer was added, to inhibit completely the cysteine proteinase activity. At this concentration leupeptin does not affect the amidolytic activity of thrombin. To this mixture 50 μl of a thrombin-specific substrate, Boc-Val-Pro-Arg-MCA (0.4 mM), in the same buffer was added. Substrate cleavage and the release of AMC by thrombin was monitored by the relative fluorescence increase at 440 ± 20 nm after excitation at 380 ± 20 nm, using a microplate fluorescence spectrophotometer (CytoFluor Series 4000, Perspective Biosystems). To calculate concentrations of thrombin produced by either gingipain R, the amidolytic activity of purified α-thrombin, which had been titrated with p-nitrophenyl-p'-guanidobenzoate (33), was used as a standard. The initial velocity of thrombin purification at various prothrombin concentrations (final concentrations: 50, 100, 150, 200, 300, 400, 600, and 1000 nm for HRGpA and 1, 2, 3, 4, 5, 7, and 10 μM for RgpB) was determined by the best fit line after incubation for various periods. The values for \( k_{cat} \) and \( V_{max} \) were extracted by direct fit of the Michaelis-Menten equation to experimental data using nonlinear curve fitting employing the method of least squares with Taylor expansion (38). Moreover, because the values generated in this way were very similar to the ones obtained by three transformations of the Michaelis-Menten equation \((S)_l/v \ versus \ (S)_o, \ v \ versus \ 1/(S)_o, \ 1/v \ versus \ u/(S)_o\) where \(v\) and \(S_o\) denote the catalytic rate and the initial substrate concentration, respectively, the means ± S.D. derived from four independent experiments and four different transformations of the Michaelis-Menten equation were calculated and presented in Table IV.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—Eighteen microliters of activated HRGpA or RgpB (3.6 pmol) were added to 162 μl of prothrombin (3.68 nmol in 0.1 M Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM CaCl₂, and 0.5 mM benzamidine), and the mixture (20 mM and 20 μM final concentration of HRGpA or RgpB and thrombin, respectively) was incubated at 37 °C. At 0.5 mM concentration, benzamidine inhibits thrombin activity but not that of gingipains, and it was included into the assay buffer to avoid autocatalytic cleavage. At specific time intervals, aliquots were withdrawn, and 1.5 μl of 0.5 M-α- and p-phenylmethyl chloromethyl ketone (10 mM) was added to terminate the reaction. Samples were boiled in reducing treatment buffer and applied for SDS-PAGE with 10% slab gels, according to the method of Laemmli (39).

Immunological detection of the Western blots of the lysates or the digestion products of gingipain incubation mixture were transferred to 8 μl of HEPES, pH 7.6, containing 10 μM biotinylated Phe-Pro-Arg-chloromethyl ketone, incubated for 10 min at room temperature, and boiled in reducing treatment buffer. After SDS-PAGE the separated protein fragments were electroblotted onto a polyvinylidine difluoride membrane (Hybond-P membrane from Amersham Pharmacia Biotech). The membrane was incubated with streptavidin-horseradish peroxidase conjugate, and bands were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Amino-terminal Sequence Analysis—Automatic sequence analysis was performed with a pulse liquid-phase sequencer (model 477A Protein Sequencer, PerkinElmer Life Sciences/Applied Biosystems Inc.). To determine the amino-terminal sequence of prothrombin-derived fragments, the mixture was separated by SDS-PAGE and transferred to polyvinylidene difluoride transfer membrane (Millipore Co., Ltd., Bedford, MA). The transferred proteins were visualized by staining with Coomassie Brilliant Blue R-250. Excised bands were placed on a Polybrene-treated glass fiber prior to sequence analysis.

RESULTS

Activation of Human Prothrombin by Gingipains R—In order to determine whether gingipains R activate prothrombin, each bacterial proteinase was incubated with the human zymogen, and the release of thrombin activity was measured. Both proteinases caused prothrombin activation in a dose- and incubation time-dependent manner (Fig. 1, A and B), with HRGpA being nearly 17 times more potent. At conditions used for SDS-PAGE analysis of the prothrombin degradation pattern (Fig. 3), the thrombin activity released from 20 μM prothrombin by 20 nm of either gingipain transiently reached a peak after 10 min of incubation and then slowly disappeared during prolonged enzyme exposure (Fig. 2). Significantly, no generation of thrombin activity was observed by the proenzyme with HRGpA incubated with TLCK-treated gingipains R (Fig. 1). Due to autoproteolytic cleavage or fragmentation by other proteolytic enzymes, thrombin in vitro can occur in three major forms referred to as α-, β-, and γ-thrombin. All of these enzymes have amidolytic activity, but only α-thrombin is capable
of clotting fibrinogen. Therefore, to determine if amidolytic activity released by gingipains from the zymogen is at least partially due to the presence of α-thrombin, which is the more important form of this proteinase, the samples of prothrombin incubated with gingipains were examined for clotting activity. As summarized in Table I both gingipains induced fibrinogen clotting activity from prothrombin in a time- and concentration-dependent manner. However, due to the apparent progressive cleavage of the α-thrombin B-chain and the creation of β- and/or γ-thrombin, a correlation between clotting and amidolytic activity was observed only at short preincubation times. Indeed, after prolonged incubation the former activity decreased much faster than the latter activity (data not shown).

The same process of excessive α-thrombin cleavage in the presence of increased concentrations of gingipains most likely skewed a concentration-dependent release of fibrinogen clotting activity from prothrombin. This is particularly apparent in the case of RgpB where the doubling of enzyme concentration resulted only in the moderate shortening of the fibrinogen clotting time (Table I). In comparison to HRgpA, an ~5-fold higher concentration of RgpB was necessary to induce clotting activity from prothrombin, and significantly, clotting times determined after the same preincubation time were 3–4 times longer. Taken together, these results indicate that HRgpA is about 20 times more efficient than RgpB in α-thrombin generation. This is in keeping with zymogen activation as measured with an amidolytic substrate in which HRgpA was shown to be 17-fold more efficient than RgpB (Fig. 1).

The prothrombin activation assays in vitro based on generation of amidolytic and/or fibrinogen clotting activities do not reflect the complexity of reactions in blood plasma where a multitude of other proteins could hinder the interaction of gingipains R with prothrombin. Therefore, to determine if gingipains R can produce a significant amount of α-thrombin in plasma, we measured the clotting time of factor X-deficient plasma incubated with gingipains. In order to evaluate interference from any residual factor X, which may still exist in deficient plasma, the assay was performed in the presence of a factor Xa-specific inhibitor in comparison to the deficient plasma reconstituted with the physiological concentration of factor X. The results obtained are summarized in Table II and show that only HRgpA was able to clot factor X-deficient plasma in a dose-dependent manner, clearly indicating the generation of α-thrombin. The clotting time of the plasma reconstituted with factor X and incubated with HRgpA was basically the same, apparently due to the fact that in the absence of phospholipids and Ca²⁺ factor Xa is a very poor activator of prothrombin. This result indicates that the presence of factor X does not affect prothrombin activation by HRgpA. Significantly, in this assay HRgpA was shown to be at least 10-fold more efficient than RgpB. These results correlate very well with the ability of both gingipains R to induce fibrinogen clotting by prothrombin (Table I) and confirm that out of two gingipains R, HRgpA is the predominant activator of this zymogen.

In addition to the ability to generate directly α-thrombin from prothrombin, gingipains R are capable of efficiently activating factors X and IX and accelerating in this way plasma coagulation through the cascade reaction of the clotting factors in the presence of phospholipids and Ca²⁺ (32, 40). In order to understand the relative importance of these three activities, we have directly compared the ability of HRgpA to promote the clotting of normal plasma versus factor X-, IX-, and XI-deficient plasmas by measuring APTT. This assay using phospholipids,
and Ca\(^{2+}\) enables us to study the plasma clotting via the cascade reaction. From the data summarized in Table III, it is also apparent that in this assay HRgpA, in concentrations much lower than those required in the plasma clotting assay, significantly decreased APTT in a dose-dependent manner not only in the normal plasma but also in plasmas deficient in factors X, IX, and XI. Although shortening of the clotting time of factor IX-deficient plasma is most likely due to the additive effect of activation of factor X and prothrombin, the clotting of factor X-deficient plasma must be predominantly triggered by generation of \(\alpha\)-thrombin directly from the zymogen of this key clotting factor. Taken together these results further confirm that prothrombin is, indeed, an important pathological target for HRgpA-mediated blood coagulation and indicate that this process can occur \textit{in vivo}.

\textbf{Cleavage of Prothrombin by Gingipains R—}To elucidate further the mechanism of prothrombin activation, thezymogen was incubated with gingipains R for various times, and the products obtained were analyzed by SDS-PAGE. The major polypeptide bands were then subjected to amino-terminal amino acid sequence analysis. From both the electrophoretic mobility and amino-terminal sequence of each prothrombin-derived fragment, bands of apparent molecular mass of 55, 45, 38.5, 34, 33, 32, and 15 kDa were found and indicated as being prethrombin 1 (fragment 2/A-chain/B-chain), fragment 1-2, prethrombin 2 (A-chain/B-chain), \(\alpha\)-thrombin, fragment 1, B-2-chain of \(\beta\)-thrombin, and fragment 2, respectively (Fig. 3A).

Despite large differences in the efficiency of prothrombin activation by HRgpA and RgpB, the zymogen digestion pattern was similar as determined by laser densitometry analysis (data not shown) of the gels shown in Fig. 3A. With HRgpA digestion it is clear that the first major cleavages occurred specifically at the Arg\(^{271}\)-Thr\(^{272}\) and/or Arg\(^{320}\)-Ile\(^{321}\) peptide bonds, releasing simultaneously fragment 1-2, prethrombin 2, and \(\alpha\)-thrombin, followed by hydrolysis of fragment 1-2 at the Arg\(^{155}\)-Ser\(^{156}\) peptide bond (Fig. 3B). In contrast, during the initial phases of prothrombin degradation by RgpB two major cleavages were found to take place at the Arg\(^{155}\)-Ser\(^{156}\) and Arg\(^{271}\)-Thr\(^{272}\) peptide bonds, leading to the initial accumulation of prethrombin 1, prethrombin 2, fragment 1 (Fig. 3A, lanes i and j), and only a minor amount of \(\alpha\)-thrombin. In this regard the non-effective cleavage of the Arg\(^{270}\)-Ile\(^{321}\) peptide bond in prothrombin explains why RgpB is about 20-fold slower in \(\alpha\)-thrombin generation than HRgpA. Taken together, differences in the pattern of prothrombin-derived products generated by HRgpA and RgpB can be observed only at the initial incubation times with qualitative difference limited to release of B-chain of \(\alpha\)-thrombin only by HRgpA. The variations in the rate of cleavage of the Arg\(^{155}\)-Ser\(^{156}\) peptide bond contributed only to qualitative difference that was later annihilated by further cleavage to the same molecular mass fragments or digestion to small molecular peptides poorly visible in the gels.

The initial accumulation of prethrombin 2 during 15 min of incubation (Fig. 3A, lanes b–d and i–k) indicates that the peptide bond (Arg\(^{270}\)-Ile\(^{321}\)) at the junction between the A- and B-chains in prethrombin 2 is relatively refractory to cleavage by gingipains. The lack of conversion of prethrombin 2 into \(\alpha\)-thrombin, as shown by both SDS-PAGE (Fig. 3A, lanes e and l) and the time course for the generation of thrombin activity after prethrombin 2 incubation (Fig. 2), argues that this form of zymogen is only a minor intermediate source of active enzyme that undergoes exhaustive degradation by gingipains. Such an interpretation is supported by the appearance of peptide fragments of lower molecular mass than prethrombin 2 but bearing the same amino-terminal sequence (Fig. 3, lanes d and k, bands between B-2-chain and fragment 2).

Significantly, RgpB was able to convert \(\alpha\)-thrombin to \(\beta\)-thrombin by cleavage at the Arg\(^{383}\)-Asn\(^{394}\) peptide bond, with release of both the B-1- and B-2-chains (Fig. 3A, lanes p–r). Furthermore, the B-2-chain was slowly degraded by this gingipain, resulting in a gradual loss of enzymatic activity. In the case of HRgpA interacting with \(\alpha\)-thrombin a similar pattern was observed, but the substrate cleavage occurred at higher rate, and after 60 min all \(\alpha\)-thrombin was totally de-

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**TABLE I**

**Fibrinogen clotting by prothrombin incubated with gingipains R**

| Gingipain R | Concentration | Clotting time | s | s | s |
|-------------|---------------|---------------|---|---|---|
|             |               | 1 min\(^a\)   | 2 min\(^b\) | 3 min\(^c\) |
| HRgpA       | 5             | ND            | 41.1 ± 1.5 | ND            |
|             | 10            | 42.3 ± 1.2    | 25.7 ± 1.4 | 22.4 ± 1.4    |
|             | 15            | 20.5 ± 1.1    | ND          | ND            |
| HRgpA-TLCK  | 15            | ND            | ND          | 116.3 ± 6.1   |
| RgpB        | 50            | 137.5 ± 6.1   | 111.3 ± 8.4 | 93.7 ± 5.2    |
| RgpB-TLCK   | 50            | ND            | ND          | >300\(^c\)    |

\(^{a}\) Concentration during incubation with prothrombin.

\(^{b}\) Preincubation time of gingipain with prothrombin.

\(^{c}\) Not determined.

\(^{d}\) Treated with TLCK.

\(^{e}\) Not clotted after a 300-s incubation.

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**TABLE II**

**Effect of gingipains R on factor X-deficient plasma clotting time**

| Plasma          | Proteinase | Concentration\(^a\) | Clotting time | s | s | s |
|-----------------|------------|----------------------|---------------|---|---|---|
| FX-def. + 5 \(\mu\)M DX-9065a (−) | HRgpA 1000 | 1000 >1,000\(^b\) | 41.4 ± 6.1 |
|                 | RgpB 1000 | 1000 >1,000\(^b\) | 41.4 ± 6.1 |
|                 | 300 114.5 ± 2.8 | 300 114.5 ± 2.8 | 41.4 ± 6.1 |
|                 | 1000 42.1 ± 1.1 | 1000 42.1 ± 1.1 | 41.4 ± 6.1 |
| FX-def. + FX rec. (−) | HRgpA 1000 | 1000 >1,000\(^b\) | 41.4 ± 6.1 |
|                 | RgpB 1000 | 1000 >1,000\(^b\) | 41.4 ± 6.1 |
|                 | 300 113.8 ± 4.1 | 300 113.8 ± 4.1 | 41.4 ± 6.1 |
|                 | 1000 41.4 ± 0.3 | 1000 41.4 ± 0.3 | 41.4 ± 6.1 |

\(^{a}\) Concentration during an incubation with plasma.

\(^{b}\) Not clotted after a 1,000-s incubation.
Prothrombin-activating Proteinases from *P. gingivalis*

**Effect of HRgpA on APTT of plasmas deficient in a factor in the intrinsic coagulation pathway**

Ninety μl of citrated plasma was mixed with 90 μl of PTF-LT® (1.2 mg/ml cephalin, 1 mg/ml silica) and preheated to 37 °C in a plastic cell for 1 min. Then 20 μl of HRgpA was added, and the mixture was incubated at 37 °C for 2 min. After adding 100 μl of 25 mM CaCl₂, the clotting time was measured. Each value denotes the mean ± S.D. in triplicate assays.

| Plasma                      | Clotting time, HRgpA concentration* | 3 nM       | 10 nM       | 30 nM       |
|-----------------------------|--------------------------------------|------------|------------|------------|
| Normal                      | 37.9 ± 0.5                           | 26.5 ± 0.6 | 21.1 ± 0.2 | 15.4 ± 0.3 |
| Factor X-deficient          | 154.8 ± 0.7                          | 142.7 ± 1.2| 108.2 ± 0.4| 70.9 ± 0.6 |
| Factor IX-deficient         | 111.0 ± 1.2                          | 82.9 ± 0.7 | 46.5 ± 0.4 | 25.6 ± 0.6 |
| Factor XI-deficient         | 119.4 ± 1.5                          | 63.6 ± 1.2 | 32.0 ± 0.4 | 17.9 ± 0.2 |

*a* The concentration in the plasma.

**TABLE III**

**FIG. 3. Cleavage of prothrombin by gingipains R.** A, prothrombin (20 μM) and gingipains R (20 nM) were incubated together as described in Fig. 2 legend in the buffer supplemented with 0.5 mM benzamidine. At specific time intervals, aliquots were withdrawn, and 1.5 μl of o-Phe-Phe-Arg-chloromethyl ketone (10 mM) was added to terminate the reaction. Samples were boiled in sample treatment buffer and analyzed by SDS-PAGE. Lane a, prothrombin alone (3.5 μl loaded); lanes b–f, prothrombin incubated with HRgpA for 0, 1, 2, 5, 15, and 60 min, respectively; lanes g–m, prothrombin incubated with RgpB for 0, 1, 2, 5, 15, and 60 min, respectively; lanes n–r, α-thrombin (20 μM) incubated with RgpB (20 μM) for 0, 1, 2, 5, 15, and 60 min, respectively (2 μg loaded); lanes s and t, pure β- and γ-thrombin, respectively (1.3 μg loaded). The positions of molecular mass standard markers are indicated to the right of the gel. The major fragments of prothrombin indicated by arrows were subjected to amino-terminal sequence analysis and identified as follows: f, prothrombin 1–2; fragment 1–2; β, prothrombin 2–4; B-chain of α-thrombin; fragment 1; 6, B-chain of β-thrombin; and 7, fragment 2, B, schematic diagram of prothrombin fragment with major cleavage sites for HRgpA and RgpB. The size of arrowheads indicates the relative efficiency of cleavage of specific peptide bonds in the prothrombin polypeptide chain by the two proteinases tested. β- and γ-Thrombin is generated by cleavage at the Arg142–Tyr143 or Arg142–Asn143 peptide bonds in the α-thrombin B-chain, giving rise to the B1 and B2 peptides. An additional cleavage at Lys154–Gly155 of the B2-chain generates γ-thrombin.

**FIG. 4. Western blot analysis of different forms of active-site biotinylated thrombin generated during prothrombin incubation with gingipains R.** Prothrombin was incubated with HRgpA (A) or RgpB (B) as described in the legend for Fig. 2. At specific time intervals, 2-μl aliquots were transferred to 8 μl of 0.1 M HEPES, pH 7.6, containing 10 μM biotinylated Phe-Pro-Arg-chloromethyl ketone. After 10 min of incubation at room temperature, samples were boiled in reducing treatment buffer and subjected to SDS-PAGE, followed by protein electrotransfer onto polyvinylidene difluoride membranes. The blotted membranes were incubated with streptavidin-horseradish peroxidase conjugate, and bands were developed by enhanced chemiluminescence for about 6 and 90 s for HRgpA and RgpB digestion products, respectively. Lanes a–g, prothrombin preincubated with each gingipain for 0, 0.5, 1, 2, 5, 15, and 60 min, respectively; lane h, a mixture of α-, β-, and γ-thrombin; lane i, prothrombin preincubated with TLCK-treated gingipain for 60 min.

**Effect of Phospholipids and Ca²⁺ on Prothrombin Activation by Gingipains R**—Phospholipids and Ca²⁺ are important cofactors accelerating the proteolytic cascade reaction of coagulation factors. Therefore, we studied their effect on prothrombin activation by gingipains R. Thrombin production by HRgpA increased in phospholipid in a concentration-dependent manner, with the effect reaching a plateau at concentrations above 40 μg/ml. At this point phospholipids augmented prothrombin activation by HRgpA about 1.5-fold over the control. Interestingly, phospholipids did not affect prothrombin activation by RgpB. Phospholipids also did not increase prothrombin activation by HRgpA in the absence of Ca²⁺, and the prothrombin activation by the two proteinases was not affected by Ca²⁺ in
the absence of phospholipids (data not shown).

**Kinetics of Prothrombin Activation by Gingipains R**—To investigate the kinetics of prothrombin activation by gingipains R, the values of $K_m$ and $k_{cat}$ were measured in the presence of phospholipids (40 μg/ml) and determined as 0.26 ± 0.01 μM and 0.32 ± 0.02 s$^{-1}$ for HRgpA and 6.6 ± 0.4 μM and 0.076 ± 0.005 s$^{-1}$ for RgpB, the catalytic efficiency ($k_{cat}/K_m$) of the former enzyme was 100-fold higher than that of RgpB. The kinetic constants of gingipains R for prothrombin conversion to thrombin were compared with those for activated factor X (Xa) in the presence of activated factor V (Va) (41), as well as with snake venom prothrombin activators from *Oxyuranus scutellatus* (OSV-PTA) and *Notechis scutulus scutulus* (NSSV-PTA), again in the presence of factor Va (7, 8). The $K_m$ value of HRgpA was comparable to the values of the physiological prothrombin activator and venom prothrombin activators but much lower than the $K_m$ value of RgpB (Table IV). In addition, the $k_{cat}$ values of gingipains R were also less than that of factor Xa and OSV-PTA, although higher than the value of NSSV-PTA (Table IV). The $k_{cat}/K_m$ values of the bacterial proteinases were substantially lower than those of factor Xa and OSV-PTA, but the value for HRgpA was higher than that of NSSV-PTA (Table IV). Significantly, the $k_{cat}/K_m$ value of RgpB, which contains no adhesin domain, was the lowest of the five compared. These data suggest that HRgpA is a more potent prothrombin activator than RgpB and NSSV-PTA, although it is less potent than either factor Xa or OSV-PTA.

**DISCUSSION**

With the exception of staphylocoagulase which causes human plasma coagulation through formation of an active molecular complex with prothrombin (42), surprisingly little is known as to how bacteria-derived proteins interact with thiszymogen. Indeed, until now the only bacterial proteinase that had been shown to activate prothrombin by limited proteolysis was a metalloproteinase from *Staphylococcus aureus* (43). Our data indicate, however, that the activity of this proteinase was several thousand-fold lower than that of either of the gingipains R (data not shown). The present work, therefore, demonstrates that gingipains R of *P. gingivalis* are the most potent proteolytic human prothrombin activators of bacterial origin yet described.

Although the catalytic domains of both gingipains R are essentially identical (30), these proteinases activated prothrombin with distinctly different kinetics. Taking into account that during prothrombin incubation with either gingipain the first active product was α-thrombin-released by simultaneous cleavage of prothrombin at Arg$^{271}$–Thr$^{272}$ and Arg$^{320}$–Ile$^{321}$ peptide bonds (Fig. 3), it is apparent that the data describe the direct generation of the physiologically active form of thrombin. The difference in the rate of the Arg$^{271}$–Ile$^{321}$ peptide bond cleavage is most likely to be responsible for the 17-fold less efficient prothrombin activation by RgpB than HRgpA, because it directly contributes to the $k_{cat}$ value of prothrombin activation that is 5 times lower for the former gingipain R. On the other hand, the presence of the hemagglutinin/adhesin domain in the HRgpA molecule may be responsible for the increased affinity of HRgpA to prothrombin in comparison to RgpB.

In pathophysiological conditions in vivo where the substrate concentration is set, the affinity of RgpA for prothrombin ($K_m$) may be a factor limiting the ability of gingipains R to activate efficiently this zymogen. The $K_m$ value of HRgpA (0.26 μM) for prothrombin is 4-fold lower than the normal plasma concentration of this protein (around 80 μg/ml, 1.1 μM) (34) and comparable to the $K_m$ value of the prothrombinase complex (Table IV), supporting the likelihood that this gingipain may activate prothrombin in vivo. In contrast, the high $K_m$ value of 6.6 μM for RgpB interaction with prothrombin does not favor activation of this coagulation factor. In addition, RgpB activates prothrombin about 20 times slower than HRgpA explaining why only the later gingipain R can clot plasma. Significantly, however, in comparison to fibrinogen, plasma clotting occurred at much higher gingipain concentrations. This discrepancy is predominantly due to the fact that in fibrinogen clotting experiments designed to prove that α-thrombin was generated, prothrombin was first preincubated with gingipains R before being mixed with fibrinogen, whereas in the plasma clotting test there was no preincubation step. Second, interference from other plasma proteins in gingipain R prothrombin activation and/or by degradation of fibrinogen by gingipains R (44) could also add to slower plasma clotting. In contrast, the absence of calcium in the plasma clotting assay should have no contribution to the observed difference since it was shown that Ca$^{2+}$ affects neither prothrombin activation by gingipains R nor conversion of fibrinogen to fibrin by α-thrombin (34).

The superior ability of HRgpA in comparison to RgpB to activate prothrombin directly is most likely related to the presence of the hemagglutinin/adhesin domain in the former proteinase. It is conceivable that this domain participates in the initial binding of prothrombin to HRgpA, enforcing the proper orientation of the zymogen for proteolytic attack at the Arg$^{271}$–Thr$^{272}$ and Arg$^{320}$–Ile$^{321}$ peptide bonds, which is required for α-thrombin production. Because the hemagglutinin/adhesin domain is involved in high affinity binding of fibronectin and fibrinogen to HRgpA (44), these proteins, which occur in

**Table IV**

Kinetic constants for the activation of prothrombin

| Enzymes | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (μM s$^{-1}$) |
|---------|------------|---------------------|--------------------------|
| HRgpA   | 2.6 ± 0.1 × 10$^{-7}$ | 3.2 ± 0.2 × 10$^{-1}$ | 1.2 × 10$^6$ |
| RgpB    | 6.6 ± 0.4 × 10$^{-6}$ | 7.6 ± 0.5 × 10$^{-2}$ | 1.2 × 10$^4$ |
| Factor Xa + factor Va$^b$ | 1.2 ± 10$^{-7}$ | 11 | 9.2 × 10$^{-7}$ |
| OSV-PTA | 3.4 ± 10$^{-7}$ | 36 | 1.0 × 10$^{8}$ |
| NSSV-PTA + factor Va$^d$ | 1.6 ± 10$^{-7}$ | 2.5 × 10$^{-2}$ | 1.6 × 10$^{5}$ |

$^a$ Data in parentheses for factor X activation were taken from Ref. 34. All values were measured in the presence of calcium ions and phospholipids. Factor Xa + factor Va indicates activated factor X in the presence of activated factor V. OSV-PTA indicates proteinase from *O. scutellatus* venom. NSSV-PTA + factor Va indicates prothrombin activator from *N. scutatus scutatus* in the presence of activated factor V.

$^b$ Data were obtained from Ref. 44.

$^d$ Data were obtained from Ref. 19.

$^d$ Data were obtained from Ref. 20.
plasma at relatively high concentrations, may compete with prothrombin for binding to this bacterial proteinase. It is tempting to hypothesize that when fibrinogen or fibronec tin occupies the hemagglutinin/adhesion domain of HRgpA; this complex behaves more like RgpB in its ability to activate prothrombin.

The significant difference between prothrombin activation by prothrombinase complex or OSV-PTA and gingipains R is the ability of HRgpA to multiply cleavage at the Arg 52–Ile53 in the heavy chain of this vascular complications because factors aggravating and/or sustaining chronic inflammatory disease (51, 52). First, gingipains would have an indirect role as factors aggravating and/or sustaining chronic inflammation. Second, they may contribute more directly to cardiovascular complications because P. gingivalis has been immunohistochemically in atherosclerotic plaque shoulders and macrophage-rich infiltrate has been associated with ulcer and thrombus formation (53). If these bacterial cells still express gingipains, it is conceivable that these proteinases could affect local homeostasis through effective and uncontrolled activation of both proteinase-activated receptors2 and coagulation factors.

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Activation of Human Prothrombin by Arginine-specific Cysteine Proteinases (Gingipains R) from Porphyromonas gingivalis
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