Prothrombin kringle 1 domain interacts with factor Va during the assembly of prothrombinase complex

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The kringle 2 domain of prothrombin has been shown to interact with factor Va during the activation of prothrombin by the prothrombinase complex composed of factor Xa, factor Va, negatively charged phospholipids and Ca^{2+} ions. However, contradictory results have been reported about the role of the kringle 1 domain of prothrombin during the assembly of the prothrombinase complex. In an attempt to clarify the role of the kringle 1 domain of prothrombin, its effect on the activation of prothrombin by the prothrombinase complex and its direct binding to human factor Va were assessed. Comparative evaluation with the effects caused by other prothrombin structural components [a fragment 1 (γ-carboxyglutamic acid and kringle 1 domains), a kringle 2 domain and a catalytic protease domain] was also performed. In the presence of factor Va, each kringle 1 and kringle 2 fragment significantly inhibited the factor Xa-catalysed prothrombin activation in the absence of phospholipids. However, in the absence of both factor Va and phospholipids, kringle 2 fragment, but not kringle 1 fragment, inhibited prothrombin activation. Evaluation of the molecular interaction of the kringle domains with factor Va in assays with solid-phase phospholipid vesicles showed that each kringle 1 and kringle 2 fragment inhibited the prothrombinase complex activity. Assessment of the direct binding of prothrombin and each kringle domain of prothrombin with factor Va by fluorescence polarization showed that prothrombin, kringle 1 and kringle 2 fragments bind directly to factor Va with dissociation constants of 1.9 ± 0.1, 2.3 ± 0.1 and 2.0 ± 0.4 μM (means $\pm$ S.D.) respectively. These findings suggest that both kringle 1 and 2 domains of prothrombin interact with factor Va during the assembly of the prothrombinase complex.

INTRODUCTION

Thrombin is the serine protease of the blood coagulation cascade that converts circulating fibrinogen to insoluble fibrin matrix, activates factors V, VIII and XIII and stimulates platelets and other cells having its specific receptor [1,2]. Thrombin is also crucial to the regulation of blood coagulation by activating protein C in the presence of thrombomodulin on endothelial cells [3]. Prothrombin is converted to thrombin on negatively charged phospholipids of platelet and endothelial cell membranes by the proteolytic activity of the prothrombinase complex, an enzymic complex composed of factor Xa, factor Va, phospholipids and Ca^{2+} ions [4]. Factor Va is important as a cofactor of factor Xa in the prothrombinase complex; its cofactor activity enhances 12000–22000-fold the factor Xa-catalysed prothrombin activation [5,6]. Kinetic analyses revealed that factor Va exerts its cofactor activity by binding directly to factor Xa [6,7] and prothrombin [7–10]. The molecular interaction of factor Va with factor Xa has been well characterized. A region (residues 263–274) of the heavy chain of factor Xa has been shown to bind to both the heavy and light chains of factor Va in the absence of phospholipids [11,12], with a dissociation constant (K_d) of 2.7 μM [7]. The binding of factor Va and factor Xa is enhanced and stabilized by negatively charged phospholipids by decreasing their K_d to 1 nM [13]. Unlike the molecular interaction between factor Xa and factor Va, that between prothrombin and factor Va is not completely understood. The molecule of prothrombin is composed of three structural regions: fragment 1, which contains the γ-carboxyglutamic acid (Gla) domain (residues 1–40) and kringle 1 domain (residues 41 to 155); fragment 2, which contains mainly the kringle 2 domain (residues 156–271); and a serine protease precursor domain (residues 272–579) [14]. Previous studies showed that the kringle 2 domain of prothrombin mediates the binding of prothrombin to factor Xa [15] and factor Va [9,16], and that the Gla domain binds to phospholipid surfaces and stabilizes the prothrombin molecule during the assembly of the prothrombinase complex [13,17,18]. However, contradictory results have been previously reported about the role of the prothrombin kringle 1 domain. Inhibition of the prothrombinase complex activity on the endothelial cell surface by monoclonal antibodies directed against the kringle 1 domain suggested that this domain participates in the formation of the prothrombinase complex [19]. In contrast, another recent study performed with mutants of the prothrombin molecule lacking the kringle 1 domain suggested that this domain is not involved in the interaction with factor Va during the activation of prothrombin by the prothrombinase complex [20]. In an attempt to clarify the role of this kringle 1 domain of prothrombin, we examined the effect of kringle 1 fragment, purified after proteolytic cleavage of prothrombin, on the coagulant activity of prothrombinase complex and the direct binding of this fragment with factor Va. Comparative evaluation of the effects induced by other fragments of prothrombin (fragment 1, kringle 1, kringle 2 and the catalytic thrombin domains) was also performed.

EXPERIMENTAL

Materials

The synthetic substrate of thrombin, D-Phe-pipecolyl-Arg-*p*-nitroanilide (S-2238), was purchased from Chromogenix

Abbreviations used: aPMSF; 4-amidinophenylmethanesulphonyl fluoride; aPMS-thrombin; 4-amidinophenylmethanesulphonyl thrombin; Gla, γ-carboxyglutamic acid; S-2238, D-Phe-pipecolyl-Arg-*p*-nitroanilide; TBS, Tris-buffered saline.

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(Mölndal, Sweden). Snake venoms from Russell's-viper and rabbit brain cephalin composed of 35–40 % (w/w) phosphatidylethanolamine, 30–35 % (w/w) phosphatidylcholine and 10–15 % (w/w) phosphatidylserine [21] were from Sigma Chemicals (St. Louis, MO, U.S.A.). BSA was from Seikagaku (Tokyo, Japan). DEAE-Sephacel, PD-10 column, CNBr-activated Sepharose 4B and sulphated Sephadex G-50 were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Iodo-beads® were from Pierce Chemicals (Rockford, IL, U.S.A.). $Na^{125}I$ was from Du Pont–New England Nuclear (Billerica, MA, U.S.A.). Diisopropylfluorophosphate, 4-amidinophenylmethanesulphonyl fluoride (aPMSF) and bovine pancreas chymotrypsin were from Wako Pure Chemicals (Osaka, Japan). Maxisorp II microwell plates were from Nunc (Roskilde, Denmark). All other chemicals and reagents used were of the best quality commercially available. Citrated human plasma was obtained from healthy volunteers.

Proteins

Prothrombin and factor X were purified from human plasma as previously described [22]. Factor V was purified from human plasma by a modification of a method previously described [23]. Briefly, a pellet of the 8–12 % (w/v) poly(ethylene glycol) (6000 Da) fraction of the supernatant of barium citrate precipitation was dissolved in Tris-buffered saline (TBS) [50 mM Tris/HCl (pH 7.4)/150 mM NaCl] containing 5 mM $CaCl_2$ and 20 mM benzamidine. This solution was applied to a CNBr-activated Sepharose 4B column coupled with monoclonal mouse anti-human factor V IgG at 4 °C. The column was washed with the same buffer, and bound factor V was eluted with a solution containing 50 mM triethylamine and 1 M NaCl, pH 11.5. Factor X and factor V were respectively activated by factor X- and factor V-activating enzyme purified from Russell's viper venom, as described [24,25]. Thrombin (1800 units/mg), fragment 1 and kringle 2 fragment were prepared as described [26]. Kringle 1 fragment was prepared from fragment 1 by chymotrypsin digestion as follows: fragment 1 dissolved in TBS was incubated with chymotrypsin at room temperature for 50 min. The ratio of fragment 1 to chymotrypsin was 20:1 (w/w). Kringle 1 fragment was then purified by chromatography with DEAE-Sepharose CL 6B (Pharmacia LKB). Protein and fragment preparations were homogeneous (more than 95 % purity), as determined by SDS/PAGE. 4-Amidinophenylmethanesulphonyl thrombin (aPMS-thrombin) was prepared by incubating thrombin (1 mg/ml) with 1 mM aPMSF in TBS for 30 min, followed by dialysis in the same buffer. Residual thrombin activity was not detected in the purified prothrombin derivatives, but when present it was inactivated with 1 mM aPMSF, followed by dialysis against the assay buffer. Control buffer used in the same experiment was also incubated with 1 mM aPMSF and dialysed. The concentration of each protein was determined by measuring the absorbance at 280 nm, with the following specific absorption coefficients ($A^{1\%}_{1cm,280}$): prothrombin, 14.7 [15]; fragment 1, 11.9 [15]; fragment 2, 11.5 [16]; thrombin, 18.3 [16]; factor Xa, 11.6 [15]; Russell's viper venom-activated factor Va, 9.6 [27].

Labelling of proteins and fragments

Prothrombin was labelled with $Na^{125}I$ using Iodo-beads®, in accordance with the manufacturer's instructions. The N-termini of prothrombin, prothrombin kringle 1 fragment and prothrombin kringle 2 fragment were labelled with fluorescein-C^6-succinimidyl ester with a fluorescein-C_6 amine labelling kit (Pan Vera, Madison, WI, U.S.A.) in accordance with the manufacturer's instructions. Labelling was performed at neutral pH in PBS to obtain selective labelling of the N-terminal amino group. Thereafter, excess fluorescein was removed by gel-filtration chromatography, with a PD-10 column previously equilibrated with 50 mM Tris/HCl, pH 7.4.

Assay of prothrombin activation by factor Xa in the absence of phospholipids

Prothrombin was activated by factor Xa alone or factor Xa plus factor Va, in the absence of phospholipids. The assay permitted the evaluation of the effect of prothrombin derivatives on thrombin generation. All reagents were dissolved in TBS containing 0.1 % BSA and 5 mM $CaCl_2$. In the assay with factor Xa and factor Va, 50 μl of various concentrations of prothrombin derivatives were incubated with 50 μl of each of factor Va (25 nM), factor Xa (1.7 nM) and prothrombin (300 nM) at room temperature for 1 h. In a separate experiment, various concentrations of prothrombin were added to the reaction mixture in the presence or absence of kringle 1 fragment (1 μM). Thereafter, 25 μl of S-2238 in 100 mM EDTA was added to the mixture and the preparation was further incubated at room temperature. Absorbance at 405 nm was then monitored with a microplate reader (EAR 340; SLT-Lab Instruments, Salzburg, Austria). From the change in A_{405} the amount of thrombin was calculated from a calibration curve made with known amounts of thrombin. The calibration curve was determined under the assay conditions described above. The rate of thrombin formation in the reaction mixture was calculated from the amount of thrombin generated during different time intervals. For the construction of the Lineweaver–Burk plots, the mean value of the thrombin formation rate of three determinations was used. In the assay with factor Xa alone as a catalyst, 50 μl of various concentrations of prothrombin derivatives were incubated with 50 μl of each of prothrombin (300 nM) and factor Xa (13.6 nM) at room temperature for 1 h. Then 25 μl of S-2238 in 100 mM EDTA was added and the mixture was further incubated at room temperature for 2 h. Absorbance at 405 nm was then measured with a microplate reader.

Assay of prothrombinase complex activity in a solid-phase system

Rabbit brain cephalin (13 mg/ml) suspended in 100 μl of 100 mM sodium carbonate buffer, pH 9.3, was coated on microplate wells by an overnight incubation at room temperature. The microwells were then washed twice with TBS containing 5 mM $CaCl_2$ and 0.1 % BSA (used as washing and incubation buffer). Non-specific bindings were blocked by incubating with TBS containing 5 mM $CaCl_2$ and 5 % (w/v) BSA at 37 °C for 2 h. To bind factor Va to phospholipids, after the wells had been washed three times, 100 μl of factor Va (25 nM) was added to the wells and incubated at room temperature for 20 min. The wells were then washed three times to remove unbound factor Va, and incubated with 100 μl of a mixture of various concentrations of prothrombin derivatives and 300 nM prothrombin at room temperature for 1 h. After three washes, 100 μl of factor Xa (0.7 nM) were added to the wells and incubated at room temperature for 2 h. Then 50 μl of S-2238 in 100 mM EDTA was added to the wells and the preparation was further incubated at room temperature for 1 h. Absorbance at 405 nm was measured with a microplate reader.

Assay of ^{125}I-prothrombin binding to solid-phase phospholipids

Prothrombin binding to immobilized phospholipids was assayed with ^{125}I-prothrombin. A mixture (100 μl) of ^{125}I-prothrombin

(300 nM) and various concentrations of prothrombin derivatives were added to microwells coated with rabbit brain cephalin, followed by incubation at room temperature for 1 h. The wells were then washed three times with TBS containing 5 mM $CaCl_2$ and 0.1 % BSA; the remaining radioactivity was measured with a γ-counter (Auto-well gamma system, ARC-6000; Aloka, Tokyo, Japan).

Fluorescence polarization studies

A fluorescence polarization study was performed to evaluate the direct interaction of factor Va with prothrombin, kringle 1 fragment or kringle 2 fragment by using the Beacon® fluorescein polarization system (Pan Vera, Madison, WI, U.S.A.). The Beacon® system is based on a static mode with excitation at 490 nm and emission at 520 nm. Background fluorescence was determined with a tube containing only TBS with 5 mM $CaCl_2$. After fluorescein labelling, 10 nM prothrombin, kringle 1 fragment or kringle 2 fragment was added to a tube containing TBS with 5 mM $CaCl_2$ and an initial reading was performed. Reading was done in 12 mm × 75 mm borosilicate glass tubes containing a total volume of 1 ml. Fluorescein-labelled protein (10 nM) was also placed into tubes containing various concentrations of factor Va or BSA diluted in TBS containing 5 mM $CaCl_2$. After gentle mixing, the mixture was incubated for 30 min at room temperature and the fluorescence was measured with the Beacon® system. Data were plotted as the change in fluorescence anisotropy between the interacting proteins and the blank, and expressed as millipolarization units (mP). The change in polarization of each fluorescein-labelled compound (prothrombin, kringle 1 fragment or kringle 2 fragment) induced by factor Va binding was obtained by subtracting the non-specific polarization induced by BSA. The resulting concentration-dependent increase in anisotropy (ΔA) was fitted to the following equation:

$$\Delta A = \Delta A_{max}[L]/K_d + [L]$$

where [L] is the concentration of free ligand. ΔA_{max} was treated as a fitting parameter because the amount of protein added was not enough to achieve saturation. Because the concentration of labelled protein was small compared with K_d, the concentration of free protein (factor Va) was summed up to reach the same value of the total concentration of factor Va.

RESULTS

Effect of prothrombin derivatives on factor Xa-catalysed prothrombin activation in the absence of phospholipids

Incubation of prothrombin (300 nM) with factor Xa (13.6 nM) in the absence of both factor Va and phospholipids for 1 h at room temperature led to little activation of prothrombin. In this assay, prothrombin activation increased in a dose-dependent fashion after the addition of factor Va (results not shown), confirming the stimulatory effect of factor Va on the factor Xa-catalysed prothrombin activation even in the absence of phospholipids [6,9]. In the presence of factor Va (25 nM) but the absence of phospholipids, each prothrombin fragment 1 and kringle 2 fragment markedly inhibited the factor Xa-catalysed activation of prothrombin (Figure 1). The IC_{50} values (means ± S.D.) of fragment 1 and kringle 2 fragment were 0.18 ± 0.05 and 0.41 ± 0.01 μM respectively. Kringle 1 fragment also inhibited prothrombin activation in a dose-dependent manner with an IC_{50} of 1.50 ± 0.49 μM, but this inhibitory effect was weak compared with that induced by fragment 1.

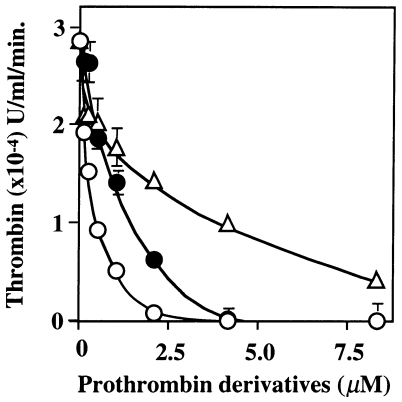

Figure 1 Inhibition of factor Xa-catalysed prothrombin activation by prothrombin derivatives in the presence of factor Va but the absence of phospholipids

Various concentrations of prothrombin derivatives [fragment 1 (○), kringle 1 fragment (△) or kringle 2 fragment (●)] were added to 50 μl of TBS with 0.1% BSA and 5 mM $CaCl_2$ containing factor Va (25 nM), factor Xa (1.7 nM) and prothrombin (300 nM), and the reaction mixture was incubated at room temperature for 1 h. The thrombin generated was determined with the chromogenic substrate S-2238. Each value represents the mean ± S.D. of triplicate determinations and the data are expressed as the concentrations of thrombin units (1800 units/mg) generated per min.

In contrast, in the absence of both factor Va and phospholipids, kringle 2 fragment induced a dose-dependent inhibition of factor Xa-catalysed prothrombin activation with an IC_{50} of 3.2 ± 0.12 μM (Figure 2). However, the inhibitory activities of fragment 1 and kringle 1 fragment were not significant. Approx. 70 % of the prothrombin activation induced by factor Xa was still observed even at a concentration of 5 μM fragment 1, and approx. 90 % at a concentration of 5 μM kringle 1 fragment. These findings suggest that the inhibitory activity of the kringle 2 fragment on prothrombin activation by factor Xa is significant

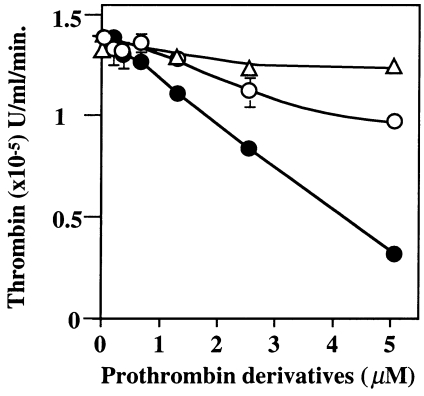

Figure 2 Inhibition of factor Xa-catalysed prothrombin activation by prothrombin derivatives in the absence of both factor Va and phospholipids

Various concentrations of prothrombin derivatives [fragment 1 (○), kringle 1 fragment (△) or kringle 2 fragment (●)] were added to 50 μl of TBS with 0.1% BSA and 5 mM $CaCl_2$ containing factor Xa (13.6 nM) and prothrombin (300 nM), and the reaction mixture was incubated at room temperature for 1 h. The thrombin generated was determined with the chromogenic substrate S-2238. Each value represents the mean ± S.D. of triplicate determinations and the data are expressed as the concentrations of thrombin units (1800 units/mg) generated per min.

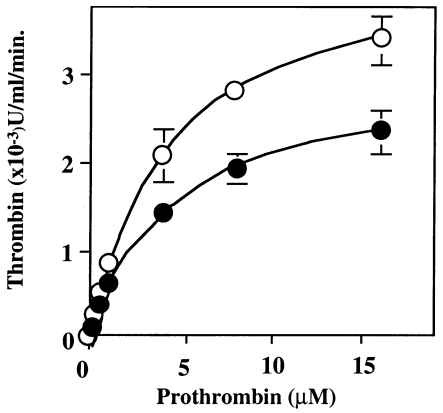

Figure 3 Effect of kringle 1 fragment on kinetic constant of factor Xa-catalysed prothrombin activation in the absence of phospholipids

Various concentrations of prothrombin were added to 50 μl of TBS with 0.1% BSA and 5 mM $CaCl_2$ containing factor Va (25 nM) and factor Xa (1.7 nM) in the presence (●) or absence (○) of kringle 1 fragment (1 μM), and the reaction mixture was incubated at room temperature for 1 h. The thrombin generated was determined with the chromogenic substrate S-2238. Each value represents the mean ± S.D. of triplicate determinations and the data are expressed as the concentrations of thrombin units (1800 units/mg) generated per min.

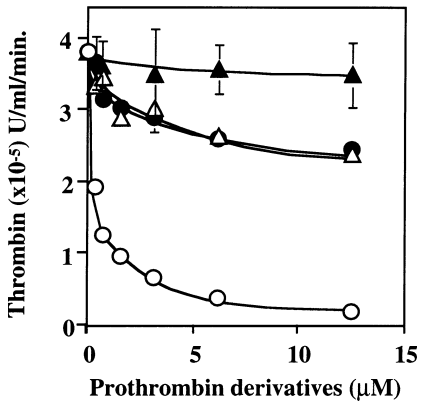

Figure 5 Effect of prothrombin derivatives on the factor Xa-catalysed activation of prothrombin in the presence of factor Va bound to solid-phase phospholipids

Prothrombin (300 nM) was incubated with factor Va (25 nM) bound to phospholipid-coated microwells in the presence of various concentrations of prothrombin derivatives [fragment 1 (○), kringle 1 fragment (△), kringle 2 fragment (●) or aPMS-thrombin (▲)] at room temperature for 1 h. Prothrombin bound to factor Va in the wells was activated by factor Xa (0.7 nM), and the thrombin generated was determined with the chromogenic substrate S-2238. Each value represents the mean ± S.D. of triplicate determinations and the data are expressed as the concentrations of thrombin units (1800 units/mg) generated per min.

even in the absence of factor Va, whereas that of fragment 1 and kringle 1 fragment is significant only in the presence of factor Va.

To confirm whether the effect of kringle 1 fragment was dependent on factor Va, we determined the kinetic constants of the reaction (means ± S.D.) (Figure 3). Kringle 1 fragment decreased the V_{max} from 4.24 ± 0.06 to 2.98 ± 0.55 m-units/min per ml, but had little effect on the K_m of the reaction (4.1 ± 1.0 μM in the absence of kringle 1 fragment; 4.5 ± 1.5 μM in its presence).

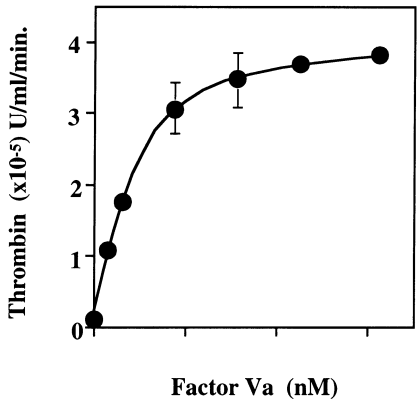

Figure 4 Factor Xa-catalysed activation of prothrombin bound to solid-phase phospholipids

Activation of prothrombin by factor Xa depended on the amount of factor Va bound to phospholipids coated on microwells. Various concentrations of factor Va (100 μl) were added to phospholipid-coated microwells, and unbound factor Va was then washed out. After adding 300 nM prothrombin (100 μl) to the microwells, the reaction mixture was incubated for 2 h, followed by washing of the wells. Then 0.7 nM factor Xa (100 μl) was added and the thrombin generated was determined with S-2238. Each value represents the mean ± S.D. of triplicate determinations and the data are expressed as the concentrations of thrombin units (1800 units/mg) generated per min.

Effect of prothrombin derivatives on the prothrombinase complex activity in a solid-phase system

To assess the direct interaction of prothrombin with factor Va during the assembly of the prothrombinase complex, we developed an assay system in which factor Va was bound to solid-phase phospholipids. First, various preliminary experiments were performed to confirm the suitability of this assay system for evaluating the cofactor activity of factor Va, as described in the Experimental section. In this assay, as shown in Figure 4, factor Xa-catalysed prothrombin activation depended on the amount of factor Va bound to phospholipids in the solid phase. This binding of factor Va was maximum at a concentration of 25 nM, and the apparent K_d between factor Va and phospholipids was 3.1 nM. These values are in accord with previously reported results (K_d 2.7–56 nM) [4,27,28]. With these preliminary results we confirmed the suitability of this novel assay for evaluating the interaction between prothrombin and factor Va bound to phospholipids. Further, to avoid the influence of factor Xa on the thrombin generation in this assay, factor Xa was added in the final step of the reaction after appropriate washing.

To identify which segment of the prothrombin molecule interacted with factor Va in the presence of phospholipids, the effect of prothrombin derivatives on the binding of prothrombin to phospholipid-bound factor Va was assessed by using the solid-phase system described above. Various concentrations of fragment 1, kringle 1 fragment, kringle 2 fragment or aPMS-thrombin were incubated in microwells with the same concentration of prothrombin, and prothrombin activation by factor Xa was monitored. As shown in Figure 5, although aPMS-thrombin inhibited the activation of prothrombin only weakly, significant inhibition of prothrombin activation was observed in the presence of fragment 1 with an IC_{50} (mean ± S.D.) of 0.69 ± 0.08 μM. Each kringle 1 and kringle 2 fragment also inhibited (40%) the prothrombin activation at a concentration of 12.5 μM. These findings suggest that besides fragment 1 (Gla domain plus kringle 1 domain), kringle 1 fragment and kringle 2 fragment also inhibit

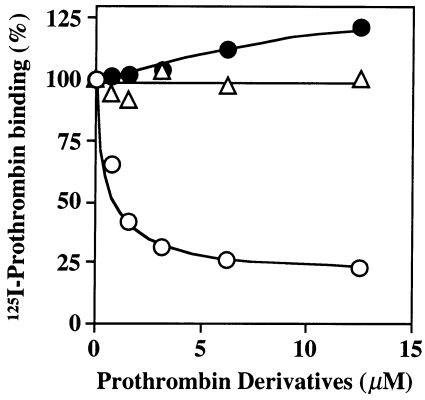

Figure 6 Effect of prothrombin derivatives on the binding of ^{125}I-prothrombin to fixed phospholipids

^{125}I-Prothrombin (300 nM) was incubated with various concentrations of prothrombin derivatives [fragment 1 (○), kringle 1 fragment (△) or kringle 2 fragment (●)] in phospholipid-coated microwells at room temperature for 1 h. The amount of ^{125}I-prothrombin bound to solid-phase phospholipids was determined by measuring the remaining radioactivity. Each value represents the mean ± S.D. of triplicate determinations and the data are expressed as the percentages of the control (binding in the absence of prothrombin derivatives).

the prothrombin activation by interfering with the interaction of prothrombin with factor Va bound to solid-phase phospholipids.

Effect of prothrombin derivatives on the binding of ^{125}I-prothrombin to phospholipids

To evaluate whether the above-described inhibitory activity of fragment 1, kringle 1 fragment or kringle 2 fragment on the prothrombinase complex activity occurs because of their suppressive effect on the interaction between prothrombin and phospholipids, the effect of each prothrombin derivative on the binding of ^{125}I-prothrombin to solid-phase phospholipids was examined. As depicted in Figure 6, fragment 1 significantly inhibited the ^{125}I-prothrombin binding to phospholipids, whereas kringle 1 fragment did not affect this prothrombin binding. In contrast, the kringle 2 fragment slightly enhanced the prothrombin binding to phospholipids. These findings indicate that inhibition of the prothrombinase complex activity by each kringle 1 and kringle 2 fragment, shown in Figure 5, is not caused by the inhibition of the prothrombin binding to phospholipids but by the direct inhibition of the prothrombin binding to factor Va.

Evaluation of the interaction between prothrombin, kringle 1 fragment or kringle 2 fragment and factor Va by fluorescence polarization

To determine the direct interaction between prothrombin, kringle 1 fragment or kringle 2 fragment and factor Va, changes in fluorescence anisotropy of each fluorescein-labelled prothrombin derivative after incubation with various concentrations of factor Va were examined. In the absence of factor Va or BSA, polarization values of prothrombin, prothrombin kringle 1 fragment and kringle 2 fragment were 89.5, 137 and 127 mP respectively. These polarization values of prothrombin, kringle 1 fragment and kringle 2 fragment increased saturably in a dose-dependent manner after the addition of factor Va; the polarization change of prothrombin reached values up to 116 mP at 1.7 μM factor Va, and that of kringle 1 and kringle 2 fragments reached 154 and 145 mP respectively, at 4 μM factor Va (Figure 7). The

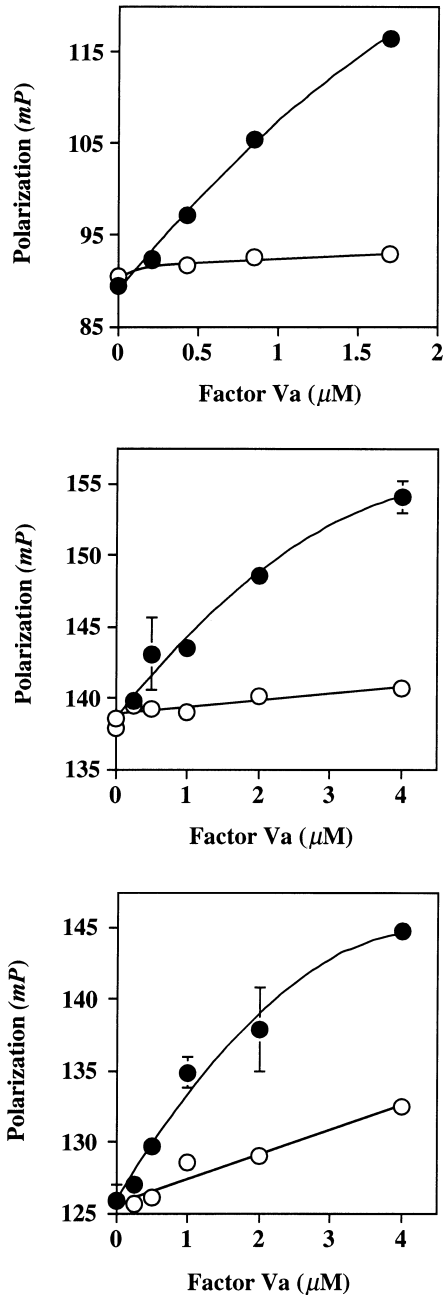

Figure 7 Polarization changes during the binding of each prothrombin, kringle 1 and kringle 2 fragment to factor Va

Fluorescence polarization was measured with 1 ml of a mixture containing various concentrations of factor Va (●) or BSA (○) (non-specific binding) and 10 nM fluorescein–prothrombin, kringle 1 fragment or kringle 2 fragment. Changes in fluorescence anisotropy are expressed as mP, as described in the text. The results show the polarization changes of fluorescein–prothrombin (top panel), fluorescein–kringle 1 fragment (middle panel) or fluorescein–kringle 2 fragment (bottom panel). Curves represent the best fit for the equation described in the Experimental section for calculating the K_d values.

apparent K_d values (means ± S.D.) of factor Va binding to each prothrombin, kringle 1 or kringle 2 fragment were 1.9 ± 0.1, 2.3 ± 0.1 and 2.0 ± 0.4 μM respectively. BSA provoked little increase in polarization without reaching saturation, indicating that the interaction between BSA and each fragment was non-

specific. These findings suggest that both kringle 1 and kringle 2 fragments bind directly to factor Va.

DISCUSSION

The formation of prothrombinase, a complex consisting of factor Xa, factor Va, phospholipid vesicles and Ca^{2+} ions, is required for the efficient activation of prothrombin [4]. Although prothrombin can also be activated by factor Xa alone, the activation rate is approx. one-thousandth of that observed in the presence of the prothrombinase complex [5,6]. Factor Va, a component of the prothrombinase complex, is an essential cofactor for the full activation of prothrombin [5,6]. During the assembly of the prothrombinase complex, factor Va interacts not only with factor Xa but also with prothrombin [6,7,13]. This latter interaction is believed to promote the rate of factor Xa-catalysed proteolysis of prothrombin by inducing a conformational change in the molecule of prothrombin suitable for proteolysis by factor Xa [7,10]. In addition, prothrombin was suggested to contribute to the assembly of factor Xa and factor Va on the surface of phosphatidylserine-containing phospholipids [29].

The kringle 2 domain of prothrombin has been shown to play a role in the interaction of prothrombin with factor Va [9,16] and factor Xa [15]. However, it is not clear whether the kringle 1 domain of prothrombin also participates in the interaction with factor Va. Sugo et al. [19] suggested that the prothrombin kringle 1 domain participates in the assembly of the prothrombinase complex, on the basis of the finding that a monoclonal antibody directed against the kringle 1 domain inhibited the prothrombinase activity of endothelial cells. In contrast, Kotkow et al. [20] suggested that the kringle 1 domain does not take part in the prothrombin activation induced by the prothrombinase complex, on the basis of results of experiments performed with mutants of the prothrombin molecule lacking either the kringle 1 or the kringle 2 domain. However, they could not completely explain the binding of prothrombin to factor Va solely on the basis of kringle 2 domain-mediated binding, because a prothrombin mutant lacking the kringle 2 segment was also found to bind to factor Va according to their kinetic studies. Their results pose the possibility that another molecular segment of prothrombin participates in the interaction of prothrombin with factor Va. To clarify this controversial point, in the present study we evaluated the role of each prothrombin segment in the interaction of prothrombin with factor Va.

In a phospholipid-free solution phase, either kringle fragment inhibited prothrombin activation in the presence of factor Va, whereas only the kringle 2 fragment showed inhibitory activity in the absence of factor Va. This latter finding might occur as a result of direct binding of the kringle 2 domain of prothrombin to factor Xa, as suggested previously by Taneda et al. [15]. The inhibitory effect of the kringle 1 fragment on the prothrombinase complex activity was observed only in the presence of factor Va; in this reaction the K_m but not the V_{max} was affected by the kringle 1 fragment. These findings suggest that the kringle 1 domain binds to factor Va, resulting in the inhibition of the molecular interaction between prothrombin and factor Va [30]. Moreover, in the assay performed in the presence of phospholipids, each kringle 1 and kringle 2 fragment inhibited the activation of prothrombin, suggesting that both of these domains of prothrombin bind to factor Va. In the absence of phospholipids, direct binding of each kringle domain of prothrombin to factor Va was also confirmed by results obtained with the fluorescence polarization method. Overall these findings suggest that in addition to kringle 2, the kringle 1 domain of prothrombin also binds to factor Va. Our present results might explain the interaction of prothrombin lacking the kringle 2 domain with factor Va, as reported by Kotkow et al. [20]. Kringle 1 fragment might be the candidate for this additional interaction site between prothrombin and factor Va during the assembly of the prothrombinase complex. Furthermore, besides the potential function of the kringle regions of prothrombin during the assembly of the prothrombinase complex, the release of these proteolytic fragments of prothrombin might inhibit by themselves the formation of the prothrombinase complex on negatively charged phospholipids of platelets or endothelial cells at sites of inflammation *in vivo*. The abrogation of the lethal effect of tissue factor and thrombin by fragments 1 and 2 of prothrombin reported in experimental animals [31] supports this hypothesis.

A direct binding study with the fluorescence polarization method demonstrated that the K_d of prothrombin with human factor Va (1.9 μM) was less than those found with bovine factor Va (7.8–10 μM) [7,20,32]. The discrepancy between our results and those of Kotkow et al. might depend on different experimental conditions, such as the protein employed for labelling and the different species of factor Va. Species-related differences cannot be fully excluded, because there has been no kinetic study of the interaction between prothrombin and factor Va purified from human plasma. In support of the latter possibility, we found that the K_m of prothrombin with the human factor Va–factor Xa complex (4.1 ± 1.0 μM) is less than that with the bovine factor Va–factor Xa complex (9.0 ± 0.4 μM).

The binding affinity of each kringle 1 and kringle 2 fragment for factor Va observed in our assays was similar to that for the entire molecule of prothrombin for factor Va (Figure 7). The proteolytic dissociation of the structural components of the prothrombin molecule performed in the present investigation might influence the binding property of these clotting factors. However, the purified kringle 2 fragment used in this study bound to factor Va with a binding affinity comparable to that reported previously, and significantly inhibited prothrombin binding to factor Va, suggesting that proteolytic fragmentation does not significantly alter the structure of each prothrombin segment. In contrast, besides the kringle regions, the Gla domain of prothrombin has been also ascribed an important function in the molecular interaction of prothrombin with other clotting factors [4,17,33,34]. For instance, Gla domain has been reported to mediate the self-association of other prothrombin derivatives forming fragment 1–prothrombin or fragment 1–fragment 1 complexes [35,36]. Fragment 1 is also known to bind to factor X through the Gla domain region [34]. The Gla domain might explain the different inhibitory activities of fragment 1 and the kringle 1 fragment towards the factor Xa-catalysed prothrombin activation in the presence of factor Va but in the absence of phospholipids, shown in the present study. Further studies are required to clarify the role of the prothrombin Gla domain in the molecular interaction occurring during clotting activation.

In summary, the results of the present study suggest that both the kringle 1 and kringle 2 domains of prothrombin interact with factor Va during the assembly of the prothrombinase complex.

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