The Role of Thrombin Exosites I and II in the Activation of Human Coagulation Factor V*

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Human blood coagulation Factor V (FV) is a plasma protein with little procoagulant activity. Limited proteolysis at Arg709, Arg1018, and Arg1545 by thrombin or Factor Xa (FXa) results in the generation of activated FV, which serves as a cofactor of FXa in prothrombin activation. Both thrombin exosites I and II have been reported to be involved in FV activation, but the relative importance of these regions in the individual cleavages remains unclear. To investigate the role of each exosite in FV activation, we have used recombinant FV molecules with only one of the three activation cleavage sites available, in combination with exosite I- or II-specific aptamers. In addition, structural requirements for exosite interactions located in the B-domain of FV were probed using FV B-domain deletion mutants and comparison with FV activating enzymes from the venom of Russell’s viper (RVV-V) and of Levant’s viper (LVV-V) known to activate FV by specific cleavage at Arg1545. Our results indicate that thrombin exosite II is not involved in cleavage at Arg709 and that both thrombin exosites are important for recognition and cleavage at Arg1545. Efficient thrombin-catalyzed FV activation requires both the N- and C-terminal regions of the B-domain, whereas only the latter is required by RVV-V and LVV-V. This indicates that proteolysis of FV by thrombin at Arg709, Arg1018, and Arg1545 show different cleavage requirements with respect to interactions mediated by thrombin exosites and areas that surround the respective cleavage sites. In addition, interactions between exosite I of thrombin and FV are primarily responsible for the different cleavage site specificity as compared with activation by RVV-V or LVV-V.

Human blood coagulation factor V (FV)2 is as a single-chain glycoprotein that consists of a mosaic domain structure composed of three homologous A-type domains, two smaller C-type domains, and a large B-domain (A1-A2-B-A3-C1-C2) (1). FV, which has little or no intrinsic procoagulant cofactor activity, is activated by thrombin or factor Xa (FXa) through limited proteolysis (2). Proteolytic activation of FV results in the removal of the FV B-domain and the exposure of regions in the FV molecule that are important for the expression of its procoagulant activity (3, 4). Activated FV (FVa) is a heterodimer that consists of a heavy chain (A3-A2 domains) and a light chain (A1-C1-C2 domains) that are noncovalently associated in a calcium-dependent manner (5, 6). FVa is the essential nonenzymatic cofactor of the prothrombinase complex, which upon complex formation with FXa accelerates FXa-catalyzed prothrombin activation in the presence of calcium ions and a phospholipid membrane surface by several orders of magnitude (2, 7).

Although FV can be activated by a number of proteases (8, 9), thrombin is the most potent physiological activator of FV. Activation of FV by thrombin is achieved through limited proteolysis at Arg709, Arg1018, and Arg1545, of which the cleavage after Arg1545 is required for full expression of FV activity (10). Thrombin-catalyzed activation of FV follows a kinetically preferred order of bond cleavage, in which cleavage at Arg709 occurs first, followed closely by cleavage at Arg1018 and slow cleavage at Arg1545, which results in formation of the FVa light chain (11, 12).

A characteristic feature of thrombin are two distinct electropositive surface regions, termed exosite I and exosite II, that contribute to the specificity of thrombin by mediating the recognition of its substrates, inhibitors and receptors (13, 14). Although both exosites have been implicated in FV activation (15–18), their role in the recognition of the individual thrombin cleavage sites is still unclear. In this study, we have used FV cleavage site mutants in combination with specific thrombin exosite I and II inhibitors to elucidate the importance of each exosite for the individual FV activation cleavages.

Furthermore, we have used FV B-domain deletion mutants to probe the structural requirements for thrombin exosite interactions located in the B-domain and compared them with the structural requirements for FV activation by RVV-V and LVV-V, which are snake venom proteases that are known to activate FV by a single cleavage at Arg1545 (19–21).
**EXPERIMENTAL PROCEDURES**

*Reagents—* Serum-free cell culture media (OptiMEM Glutamax) were from Invitrogen. Hepes, sodium chloride, calcium chloride, bovine brain phosphatidylserine, egg yolk phosphatidylcholine, heparin-Sepharose, ovalbumin, bovine serum albumin, and unfractionated heparin were from Sigma. 1 unit/ml unfractionated heparin contains ~5.7 μg/ml unfractionated heparin/ml (22). Pefabloc TH was a product of Pentapharm Ltd (Basel, Switzerland). The chromogenic substrate S2238 was supplied by Chromogenix (Milan, Italy). The Micro BCA protein assay kit for protein concentration determination was from Pierce Biotechnology (Essex Junction, VT). The monoclonal antibody 3B1 was biotinylated using the Pierce EZ-link sulfo-NHS-LC-Biotin kit according to the manufacturer's procedures. Monoclonal antibody 3B1 specific for the human FV C2 domain was from Sigma and was biotinylated using the Pierce EZ-link sulfo-NHS-LC-Biotin kit. Monoclonal antibody 3B1 was purified as described previously (20). Bovine FX was purified according to Fujikawa et al. (25). FXa was prepared from FX after activation by RVV-X (26). Human prothrombin was purified according to the method of Di Scipio et al. (27). The monoclonal antibody HV1 specific for the human FV C2 domain was from Sigma and was biotinylated using the Pierce EZ-link sulfo-NHS-LC-Biotin kit which was supplied by Pierce Biotechnology (Essex Junction, VT). The FV activator from the venom of Daboia russelli was supplied by Hematologic Technologies (Essex Junction, VT). The FV activator of Daboia lebetina turanica was supplied by Chromogenix (Milan, Italy). The Micro BCA protein assay kit (Pierce) using bovine serum albumin as a standard was supplied by Invitrogen (Carlsbad, CA). Sulfuric acid and 2-mercaptoethanol were from Merck (Darmstadt, Germany). Deoxyoligonucleotides GTTGGTAGGGCAGGTTGGGGTGACT (thrombin aptamer I) and AGTC-CGTTGGTAGGCGAGGTGTTGGACT (thrombin aptamer II) were supplied by Chromogenix (Milan, Italy). The Pierce EZ-link sulfo-NHS-LC-Biotin kit was supplied by Pierce Biotechnology (Essex Junction, VT). Human thrombin was from ERL (Swansea, United Kingdom). Human FV was purified essentially according to the method of Dahlbäck (23), modified as described (24). Human thrombin was from ERL (Swansea, United Kingdom). The FV activator from the venom of Daboia russelli was supplied by Hematologic Technologies (Essex Junction, VT). The FV activator of Daboia lebetina turanica was purified as described previously (20). Bovine FX was purified according to Fujikawa et al. (25). FXa was prepared from FX after activation by RVV-X (26). Human prothrombin was purified according to the method of Di Scipio et al. (27). The monoclonal antibody HV1 specific for the human FV C2 domain was from Sigma and was biotinylated using the Pierce EZ-link sulfo-NHS-LC-Biotin kit which was supplied by the manufacturer’s procedures. Monoclonal antibody 3B1 was directed against the heavy chain of human FV was a kind gift from Prof. B. N. Bouma.

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*Protein Concentration Determination—* Thrombin concentrations were determined using the chromogenic substrate S2238 (7). The concentration of the snake venom FV activators RVV-V and LVV-V was measured using the Micro BCA protein assay kit (Pierce) using bovine serum albumin as a standard. FVα was quantified as described below.

*Factor Va Assay—* FVα cofactor activity was measured via its ability to stimulate FXα-catalyzed thrombin generation as described (24). Unless noted otherwise, prothrombin activation was started by adding prewarmed FV/FVa (~20 pm) to a reaction mixture containing 25 mM Hepes, pH 7.5, 150 mM NaCl, 2 mM CaCl₂, 0.5 μM prothrombin, 5 mM FXa, 40 μM phospholipid vesicles (10% brain phosphatidylserine, 90% egg yolk phosphatidylcholine; mol/mol) and 0.5 mg/ml ovalbumin. The reversible thrombin inhibitor Pefabloc TH (1 μM) was present to avoid feedback activation of FV during the assay. Prothrombin activation was stopped after 1 min by dilution in ice-cold EDTA buffer (50 mM Tris, 175 mM NaCl, 40 mM EDTA, 0.5 mg/ml ovalbumin, pH 7.9). Thrombin was quantified using the chromogenic substrate S2238 (7). The molar FVα concentra-
were developed for 10 min with peroxidase substrate solution (3,3′,5,5′-tetramethyl-benzidine substrate kit; Pierce). The reaction was stopped by the addition of 5% (v/v) H₂SO₄, and the absorbance was measured at 490 nm. To rule out the possibility that the cell culture medium interfered with the ELISA assay, plasma-purified FV was diluted in mock medium or in buffer. No difference was detected between these diluted samples, indicating that the cell culture medium did not interfere with the FV quantitation.

**Thrombin Activation of FV Cleavage Site Mutants**—To determine the thrombin concentration needed to fully activate the FV cleavage site mutants, each of the recombinant FV variants (250 pm in 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mg/ml BSA, 5 mM CaCl₂) was incubated with increasing concentrations of thrombin (0–50 nM) for 15 min at 37 °C and then assayed for FVa as described above.

**Inhibition of Thrombin-catalyzed FV Activation by Thrombin Exosite I and II Aptamers**—To determine the aptamer concentrations needed for saturation of the inhibitory effect on thrombin-catalyzed activation of FV, 250 pm recombinant wild type FV (in 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mg/ml BSA, 5 mM CaCl₂) was activated with 10 nM thrombin in the presence of increasing concentrations aptamer I, II, or both. At different time points (0, 1, 3, 5, 7, 10, and 15 min), FVa concentrations were measured using the assay described above.

**Role of Thrombin Exosites I and II in FV Activation**—To investigate the importance of each of the two exosomes of thrombin for the recognition of the individual FV activation cleavage sites, activation of the recombinant FV cleavage site mutants RIQ, QRQ, and QIR (250 pm in 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mg/ml BSA, 5 mM CaCl₂) by thrombin (10 nM) was followed in the absence and presence of aptamer I (19 μM), aptamer II (5 μM), or both (8.2 μM aptamer I and 5 μM aptamer II). At different time points (0, 1, 3, 5, 7, 10, and 15 min), the concentration of FVa generated was measured as described above. Factor V activation rates were determined under initial steady-state second order rate conditions in which the initial rate of FV activation was linear with time and linearly proportional to the concentration of FV between 0 and 0.5 nM, as well as the concentration of thrombin present from 0.05 to 20 nM.

**SDS-PAGE Analysis of FV Activation in the Absence and Presence of Thrombin Exosite Aptamers**—Time courses of activation of plasma-purified FV (75 nM in 50 mM Hepes, 150 mM NaCl, 5 mM CaCl₂, 1 mg/ml polyethylene glycol, pH 7.5) by thrombin were monitored in the absence and presence of thrombin exosite aptamer I (10 μM), aptamer II (5 μM), or both (10 and 5 μM, respectively). Various thrombin concentrations were used such that the effect of aptamers on FV cleavage could be visualized: 0.5 nM (without aptamer and in the presence of aptamer II), 2.5 nM (aptamer I), or 20 nM (in the presence of both aptamers). At various time points, aliquots were taken from the reaction mixtures and subjected to SDS-polyacrylamide gel electrophoresis (4–12%) under reducing conditions. Simultaneously, FVa activities were determined as described above. FV fragments were visualized by colloidal blue staining.

**Characterization of the FV B-domain Deletion Mutants**—The recombinant FV B-domain deletion mutants and wild type FV (150 pm in 25 mM Hepes, pH 7.4, 150 mM NaCl, 5 mg/ml BSA, 5 mM CaCl₂) were activated with 0.5 nM thrombin, RVV-V, or LVV-V. At various time points (0, 1, 3, 5, 7, and 10 min) aliquots were withdrawn, and FVa generated was measured. We chose to assay the FVa cofactor activity in assays at 0.5 nM FXa to minimize the intrinsic procoagulant activity reported for recombinant B-domain deleted FV forms (4, 33, 34). Under our conditions, activities of unactivated FV were typically <5% for wild type FV and 17–21% for variants lacking the B-domain. Mock conditioned medium gave no measurable FVa activity.

**Binding of Thrombin, RVV-V and LVV-V to Heparin-Sepharose**—Thrombin, RVV-V, or LVV-V (1 ml of 5 nM) in 25 mM Hepes, pH 7.5, 50 mM NaCl, 0.1 mg/ml BSA) were applied to a 1-ml heparin-Sepharose column that was pre-equilibrated with the same buffer. The column was washed with 4 ml of the same buffer followed by elution with a 10-mL linear gradient of 50–1000 mM NaCl. Elution fractions (1 ml) were diluted twice in compensating buffer (25 mM Hepes, pH 7.5, 0.1 mg/ml BSA, and an appropriate amount of NaCl) to fix the final NaCl concentration in each elution fraction to 500 mM. The presence of FV activator was monitored by the ability of aliquots from the fractions to activate bovine FV. To this end, 50 μl of 20 nM bovine FV in 25 mM Hepes, pH 7.5, 50 mM NaCl, 0.1 mg/ml BSA, and 20 mM CaCl₂ was added to 150 μl of elution fraction. After 10 min of incubation at 37 °C, the FVa generated was measured. During this 10-min incubation period, FV generation was linear in time for each of the three activators, verifying a linear dependence between FVa activity generated and the amount of FV activator present. Elution profiles were represented as percentages of the amount of FVa generated in the peak fraction.

**FV Activation in the Presence of Heparin**—To investigate the effect of heparin on the activation of 5 nM FV by either thrombin, RVV-V, or LVV-V, FV was incubated with 0.1 nM of each activator in the presence of increasing concentrations of heparin (0–1000 units/ml) in 25 mM Hepes, pH 7.5, 150 mM NaCl, 5 mg/ml BSA, and 5 mM CaCl₂. Samples were taken at different time points during the first 2 min of activation, and the amount of FVa generated was measured. From the resulting progress curves, the initial FV activation rates were calculated (nm FVa/min) and plotted as a function of the heparin concentration.

**SDS-PAGE Analysis of FV Activation in the Presence of Heparin**—To identify fragment generation during FV activation in the presence of heparin, 75 nM plasma-purified human FV (in 50 mM Hepes, 150 mM NaCl, 5 mM CaCl₂, 1 mg/ml polyethylene glycol, with or without 10 units/ml heparin, pH 7.5) was activated with 1 nM thrombin or 5 nM snake venom activator. At various time points, aliquots were removed and subjected to SDS-polyacrylamide gel electrophoresis (4–12%) under reducing conditions. Simultaneously, FVa activities were determined as described above. FV fragments were visualized by colloidal blue staining.

**RESULTS**

**Role of Thrombin Exosites I and II in FV Activation**—To examine the role of thrombin exosites in FV activation, a panel of FV cleavage site mutants was expressed in COS1 cells. The expression levels of the FV variants, as determined by ELISA,
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were between 0.6 and 1 nM. Thrombin titration was performed to determine the concentration required for full activation of the different mutants (Fig. 1). All of the mutants, except the QIR mutant, reached a maximal and stable level of cofactor activity after 15 min of activation with 5 nM thrombin. A control experiment, with excess thrombin (50 nM) or RVV-V (25 nM), verified that the QIR variant could be completely activated to a stable FVa activity level, corresponding to an activity:antigen ratio that was similar to that of wild type FV (not shown). As expected, incubation of the QI and QIQQ mutants did not result in an increase in FVa cofactor activity, which illustrates that both FV variants are completely resistant to thrombin activation. Full cofactor activity of wild type FV and the RIR variant were observed at low thrombin concentration (typically 1–2 nM). Furthermore, cleavage at Arg709 was more sensitive to thrombin than cleavage at Arg1545 as illustrated by the fact that the QIQ mutant reached maximum activity with 1 nM thrombin, whereas the QIR mutant required 50 nM for full activation within 15 min. Finally, complete activation of the QRQ mutant was observed at 2.5 nM thrombin, but only a slight increase (~2-fold) in FVa cofactor activity was observed between the fully activated and nonactivated forms.

Aptamer titrations were performed to determine the concentration required to inhibit thrombin-catalyzed activation of wild type FV (Fig. 2). The aptamers used are single-stranded DNA oligonucleotides, which have been shown to be specific for thrombin exosite I (aptamer HD1 (35–39)) or exosite II (aptamer HD22 (40)). Aptamer I almost completely inhibited FV activation at a concentration of 19 μM. Maximal inhibitory activity of thrombin exosite II aptamer was observed at 5 μM, but this aptamer inhibited the activation of wild type FV only partially.

The importance of thrombin exosites I and II for the recognition of the individual FV activation cleavage sites was investigated by monitoring the time course of activation of FV cleavage site mutants with only one thrombin cleavage site available. These time courses were recorded in the absence and presence of aptamer I (19 μM), aptamer II (5 μM), or both (8.2 μM aptamer I and 5 μM aptamer II). Thrombin-catalyzed cleavage at Arg709 (mutant RIQ) was almost completely blocked in the presence of exosite I aptamer, whereas thrombin exosite II aptamer showed a minor effect (Fig. 3, left panel). Although activation of FV mutant QRQ by thrombin-catalyzed cleavage at Arg1018 resulted in only a 2-fold increase of FVa cofactor activity in prothrombin activation, inhibition of activation of this mutant was observed by exosite I aptamer with only a marginal effect by thrombin exosite II aptamer (Fig. 3, middle panel). Activation of FV variant QIR, which can only be cleaved by thrombin at Arg1545, was significantly inhibited by both aptamer I and II, and with both aptamers simultaneously present, thrombin-catalyzed activation was virtually completely inhibited (Fig. 3, right panel).

Because the kinetics of the individual cleavage sites are not independent of one another (12, 29, 45) and therefore the results with the mutants cannot be interpreted with certainty as reflecting the kinetics of cleavage of wild-type FV, we employed SDS-PAGE analysis and compared cleavage reactions of plasma-purified FV by thrombin in the absence and presence of thrombin aptamers (Fig. 4). Thrombin aptamer I (Fig. 4B, upper right panel) decreased the rate of disappearance of single chain FV (330 kDa) and delayed the appearance of the 280-kDa intermediate and the heavy chain (105 kDa), which result from cleavage of single chain FV at Arg1545. In addition, the formation
of the FVa light chain (71/74 kDa), which is formed after cleavage at Arg^{1545}, was considerably delayed in the presence of aptamer I. Inhibition of cleavage at Arg^{709} and Arg^{1545} resulted in the accumulation of 220- and 150-kDa intermediates originating from single cleavage at Arg^{1018} in single chain FV.

Thrombin aptamer II (Fig. 4B, lower left panel) decreased the rate of disappearance of single chain FV and formation of the 280-kDa intermediate but had no major effect on the rate of formation of the 280-kDa intermediate and the heavy chain. In the presence of both aptamers (Fig. 4B, lower right panel), FV cleavage was almost completely blocked, and under the conditions chosen, only the 220- and 150-kDa intermediates were formed during the first 45 min of activation. In summary, the data obtained with wild-type FV also show that thrombin exosite I is essential for cleavage at Arg^{709} and Arg^{1545}, whereas exosite II is particularly important for cleavage at Arg^{1545}.

**FV Cleavage Requirements for Activation by Thrombin, RVV-V, and LVV-V**—To evaluate the structural elements in the substrate FV B-domain that are important for FV activation, we expressed a number of FV B-domain deletion mutants in COS1 cells. FV expression levels were determined using a quantitative FV ELISA and a functional assay (Table 1).

The activity:antigen ratio for B-domain deleted mutant FV des^{827–1498} was the same as the ratio obtained for the wild type construct, whereas the activity of FV des^{709–1545} and FV des^{709–1476} were somewhat decreased. The FV cleavage requirements for thrombin, RVV-V, and LVV-V (0.5 nM) were investigated by following the time course of activation of wild type FV and the B-domain deletion mutants (150 pM). Although all of the B-domain deletion constructs could be activated by incubation with excess activator (Table 1) no increase in cofactor activity was observed for the FV des^{709–1545} and FV des^{709–1476}...
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mutants upon incubation with 0.5 nM thrombin, whereas FV des 827–1498 was activated by thrombin at a rate similar to that observed for wild type FV (Fig. 5, left panel).

Upon incubation with RVV-V or LVV-V, no increase in cofactor activity was observed for the FV des 709–1545 mutant, indicating that this mutant could also not be activated by the snake venom FV activators (Fig. 5, middle and right panels). FV des 709–1476 and FV des 827–1498 were rapidly activated by RVV-V and LVV-V and appear to be even more rapidly activated than wild type.

The C-terminal region of the FV B-domain is enriched in acidic amino acids and also contains several sulfated tyrosine residues that have been proposed to be important for interaction with the positively charged exosites of thrombin (29, 44). The observation that RVV-V and LVV-V require the C-terminal region of the B-domain for efficient activation of FV indicates that this acidic region is also of vital importance for the recognition of the Arg\textsuperscript{1545} cleavage site by the venom FV activators.

Binding of Thrombin, RVV-V, and LVV-V to Heparin-Sepharose—Structural models of RVV-V and LVV-V show the presence of two positively charged surface regions that may play a role in FV recognition and activation (20). By analogy to thrombin, these positively charged surface regions in RVV-V and LVV-V may represent potential exosites for the binding to macromolecular ligands other than FV (13, 14). One such potential ligand is the thrombin exosite II ligand heparin.

| TABLE 1 |
| FV concentrations |
| --- |
| FV concentrations were determined by an ELISA as described under “Experimental Procedures.” FVa activities were determined using the FVa assay as described under “Experimental Procedures” using the kinetic parameters as determined for plasma-purified FV (28). The values are the averages of three experiments, and the standard deviations were <5% for all of the FV variants. As a parameter of specific activity, the ratio of FV activity to antigen was calculated. |
| Construct | Activity | Antigen | Activity:antigen ratio |
| --- | --- | --- | --- |
| WT-FV | 0.517 | 0.53 | 0.98 |
| FV des 709–1476 | 0.331 | 0.86 | 0.38 |
| FV des 709–1545 | 0.463 | 0.78 | 0.59 |
| FV des 827–1498 | 0.491 | 0.54 | 0.91 |
| WT | 0.60 | 0.65 | 0.92 |
| QIQ | 0.07 | 0.69 | 0.11 |
| QIQQQ | 0.05 | 0.97 | 0.06 |
| QIR | 0.47 | 0.57 | 0.81 |
| RIQ | 0.20 | 0.58 | 0.33 |
| QIQ | 0.06 | 0.66 | 0.09 |
| RIR | 0.65 | 0.74 | 0.88 |

To determine whether the snake venom activators bound heparin, both activators were subjected to heparin-Sepharose affinity chromatography. Both RVV-V and LVV-V bound to heparin-Sepharose and eluted at ~500 mM NaCl, whereas thrombin eluted at ~600 mM NaCl (Fig. 6). This indicated that RVV-V and LVV-V can bind heparin, although with a somewhat lower affinity than thrombin.

**FV Activation in the Presence of Heparin**—To further assess the importance of thrombin exosite II for FV activation, we compared the effect of heparin on thrombin-catalyzed activation of FV with RVV-V and LVV-V-catalyzed activation. Fig. 7 shows initial rates of FV activation in the presence of increasing concentrations of heparin. Low concentrations of heparin (<5 units/ml) accelerated FV activation by approximately 3-fold (RVV-V and thrombin) to 4-fold (LVV-V) with an optimum enhancement at 5 units/ml. At higher heparin concentrations (up to 1000 units/ml), the rates of FV activation gradually declined, demonstrating an inhibitory effect on FV activation by all three activators. The observation that heparin has an optimal concentration for acceleration of FV activation, with the activities decreasing at concentrations above and below this value, suggests that heparin serves as a template to which both enzyme and FV bind.

**SDS-PAGE Analysis of FV Activation in the Presence of Heparin**—To assess whether heparin alters the cleavage pattern of FV activation and/or influences cleavages other than the cleavage at Arg\textsuperscript{1545} by thrombin, RVV-V, or LVV-V, time courses of activation were followed in the absence and presence of heparin (Fig. 8A).

At various times, aliquots from the activation mixture were taken and subjected to SDS-PAGE to characterize the FV fragments formed during FV activation (Fig. 8B). For the thrombin-catalyzed reaction, cleavage of the 330- and 280-kDa bands and corresponding formation of the light (71/74 kDa) and heavy chain (105 kDa) were accelerated ~2-fold by the presence of heparin. In case of RVV-V and LVV-V, cleavage of the 330-kDa band in the presence of heparin was accelerated 5–6-fold and coincides with a faster formation of the 290-kDa heavy chain and the 71/74-kDa light chain typically observed upon cleavage of FV at Arg\textsuperscript{1545}. Notably, upon prolonged activation with excess snake venom factor V activator in the presence of heparin, the FVα heavy chain (290 kDa) was further cleaved into fragments with apparent masses of 150 and 175 kDa (Fig. 8B,
through its binding to thrombin and not to FV. Thrombin catalyzed cleavage at position 1545 is mediated by a function of increasing concentrations of heparin (0–1000 units/ml). One unit of heparin was defined as 5.7 μg/ml (22). The initial rates of FV activation were measured as described under “Experimental Procedures” and plotted as a function of the heparin concentration. The symbols are as follows: thrombin (○), RVV-V (△), and LVV-V (□). The inset shows the first part of the curve.

Role of the Sodium-binding Loop of Thrombin in FV Activation—We have investigated the role of the sodium-binding site of thrombin in the recognition of the individual thrombin cleavage sites at positions 709 and 1545. The initial activation rates of the FV cleavage site mutants RIQ and QIR by thrombin were increased (2–4-fold) in the sodium concentration range tested (20–200 mM Na⁺; ionic strength was kept constant with choline chloride (see supplemental materials)). In a control experiment in which the FV variant QIR was activated with the snake venom FV activator RVV-V (which does not contain the Na⁺-binding site), the FV activation rate remained unchanged over the entire Na⁺ concentration range tested. This demonstrates that the effect of sodium on the thrombin catalyzed cleavage at position 1545 is mediated through its binding to thrombin and not to FV.

**DISCUSSION**

Anion-binding exozites I and II of thrombin have been demonstrated to be involved in the activation of factor V (15–18). However, the importance of each exosite in the recognition of the individual thrombin cleavage sites in FV is still unclear. Using recombinant FV variants that harbor only one of the three thrombin cleavage sites in combination with aptamers that specifically bind to either thrombin exosite I or II, we have studied the role of thrombin exosite I and II in FV activation. The data obtained with the RIQ mutant in combination with aptamers specific for thrombin exosite I and/or II show that thrombin exosite I, but not exosite II, plays a critical role in the recognition of the Arg⁷⁰⁹ cleavage site and consequently in the initiation of FV activation.

This observation is supported further by SDS-PAGE analysis of cleavage of plasma-purified FV by thrombin in the absence and presence of thrombin aptamers. Cleavage at Arg⁷⁰⁹ and formation of the FVa heavy chain were significantly delayed in the presence of aptamer I, but not in the presence of aptamer II. These data are in agreement with results reported by others who have shown that residues Arg⁷³ and Tyr⁶⁶ (chymotrypsin numbering) in exosite I are essential for the recognition and cleavage of FV at Arg⁷⁰⁹ (41). Furthermore, fluorescence binding studies using active site labeled thrombin have demonstrated that exosite I is predominantly responsible for the interaction with a site on the FV/FVa heavy chain preceding Arg⁷⁰⁹ (42).

Our results also yield information concerning the other cleavages associated with FV activation. We found that thrombin exosite I and II are both involved in the recognition and cleavage at Arg²⁰⁴⁵. The observation by Dharmawandana and colleagues (16) that FV activation by thrombin in the presence of the exosite-I specific peptide hirudin-(54–65) showed the accumulation of activation intermediates resulting from cleavage at Arg¹⁰¹⁸ supports our finding that exosite I is important for cleavage at Arg¹⁵⁴⁵.

Although it is widely accepted that thrombin exosite I is involved in FV activation, the role of exosite II in this process has been less clear. Because thrombin exosite II ligands (prothrombin fragment 2 and heparin) did not inhibit FV activation, even when thrombin exosite I was not functional, Arocas et al. (17) suggested that thrombin exosite II is not directly involved in FV recognition by thrombin. Others, however, have proposed exosite II to be involved in FV activation because mutations in exosite II residues showed a reduced capacity to cleave and activate FV (15, 41). Here, we demonstrate using recombinant FV cleavage site mutants that thrombin exosite II is involved in the cleavage at Arg²⁰⁴⁵, but not in cleavage at Arg⁷⁰⁹ and Arg¹⁰¹⁸ (Fig. 3, left and right panels). This result is confirmed by SDS-PAGE analysis of cleavage of plasma-purified FV by thrombin in the presence of aptamers. We observed that blocking of exosite II has a major effect on the formation of the FVa light chain but not on formation of the heavy chain (Fig. 4B, lower left panel).

Because it has been demonstrated that thrombin activation of FV requires an extensive interaction interface (41), we also investigated whether in addition to thrombin exosites I and II, the sodium-binding site of thrombin is also involved in FV activation. The initial rates of FV activation of the cleavage site mutants RIQ and QIR by thrombin appeared to be increased (2–4-fold) between 20 and 200 mM Na⁺ (see also online sup-
These results confirm and extend earlier mutagenesis data reported by Myles et al. (41) showing that the Na\(^+\)/H\(^+\) binding loop of thrombin is involved in FV activation. From our results, it can be concluded that the effect of Na\(^+\)/H\(^+\) binding is similar for all cleavages involved. Thus, in contrast to the finding that exosites I and II differentially stimulate the different FV cleavages, the thrombin Na\(^+\)/H\(^+\) binding loop is involved to a similar extent in all FV cleavages. Discrete regions in the B-domain have been reported to maintain FV in an inactive procofactor state (34). These inhibitory constraints are removed after limited proteolysis in the B-domain, a process that is mediated by electrostatic interactions between highly acidic regions in the FV molecule and positively charged regions (exosites) in the FV-activating enzyme (1, 29, 43). To investigate the structural requirements for exosite interactions located in the B-domain of FV, we expressed FV variants in COS1 cells that lack the complete B-domain (FV des 709–1545), the region preceding the Arg\(^{1545}\) cleavage site (FV des 709–1476), or a region between the Arg\(^{709}\) and Arg\(^{1545}\) cleavage site (FV des 827–1498). Although FV des 709–1545 and FV des 709–1476 could be fully activated after prolonged incubation with excess thrombin (as described under “Experimental Procedures”; data not shown), no substantial activation of these mutants was observed at low levels of thrombin (Fig. 4A). On the other hand, activation of FV des 827–1498 was comparable with wild type FV. This indicates that both the N- and C-terminal region of the B-domain are required for efficient activation by thrombin and thus that the sequences 710–826 and 1499–1544 contain sites that contribute to optimal activation of FV by thrombin. These results seem to contradict earlier studies showing the importance of an acidic region preceding Arg\(^{709}\) for thrombin-catalyzed FV activation. This region is not present in the B-domain but resides within the FVa heavy chain. A likely explanation is that the region between residues 709–827 in the B-domain contains sequences that are part of the thrombin recognition site. We can, however, not exclude the possibility that the deletion of a large part of the B-domain introduces local structural changes in the acidic region preceding the Arg\(^{709}\) cleavage site that hamper the interaction between thrombin and this part of the FV molecule.

Similar to the Arg\(^{709}\) cleavage site, the Arg\(^{1545}\) cleavage site is preceded by an acidic region that shows sequence homology to hirudin-(54–65) and that contains three putative sulfated tyrosine residues (1, 43). In agreement with our findings, this region has been proposed as a possible thrombin-binding site important for cleavage at Arg\(^{1545}\) (29, 43). However, the acidic region...
preceding the Arg\textsuperscript{1545} cleavage site seems not to be solely responsible for efficient cleavage by thrombin at Arg\textsuperscript{1545}, as indicated by the observation that mutant FV des 709–1476 was not substantially activated by thrombin. Local structural changes in the region preceding the Arg\textsuperscript{1545} cleavage site that might be introduced by the deletion can be excluded, because mutant FV des 709–1476 was efficiently activated by RVV-V and LVV-V. This again illustrates that the initial cleavage at Arg\textsuperscript{709}, which coincides with the liberation of the FVa heavy chain, facilitates cleavage at Arg\textsuperscript{1545}. The facilitating effect of thrombin cleavage at Arg\textsuperscript{709} (and also at Arg\textsuperscript{1018}) on cleavage at Arg\textsuperscript{1545} has been demonstrated earlier using FV cleavage site mutants (12, 29, 45).

In contrast to thrombin, the presence of the region preceding the Arg\textsuperscript{1545} cleavage site seems to be sufficient for efficient FV activation by RVV-V and LVV-V. Remarkably, FV variants FV des 709–1476 and FV des 827–1498 were more rapidly activated by RVV-V and LVV-V than wild type FV. It is possible that access to the Arg\textsuperscript{1545} cleavage site in these mutants is less restricted than in WT FV because of the absence of a large part of the FV B-domain. Alternatively, deletion of the central part of the B-domain may cause conformational changes in the region around Arg\textsuperscript{1545} that result in an increased affinity of RVV-V and LVV-V for this cleavage site. It is likely that FV recognition by RVV-V and LVV-V, like thrombin, is also mediated by exosite-driven interactions, given the requirement of the acidic region that precedes the Arg\textsuperscript{1545} cleavage site for efficient activation by both proteases. Three-dimensional structural models of RVV-V and LVV-V reveal the presence of two positively charged surface regions on opposite sides of their active site that may be involved in the recognition of the Arg\textsuperscript{1545} cleavage site (20). Nonetheless, the thrombin exosite aptamers did not inhibit FV activation by RVV-V and LVV-V (data not shown). This not only suggests that the thrombin exosites are distinctly different from the positively charged surface regions in RVV-V and LVV-V, but it also indicates that the inhibition of thrombin-dependent FV activation by the aptamers is due to the binding to thrombin and not due to interaction with FV.

The high degree of overall sequence identity (30%) of RVV-V and LVV-V with thrombin indicates evolutionary conservation of structure and function. In search of a suitable anionic ligand for the positively charged surface regions of RVV-V and LVV-V, we subjected both enzymes to heparin affinity chromatography. Both enzymes bound with high affinity to heparin, which is a thrombin exosite II ligand (46, 47) that is of clinical importance both because of its common use as an antithrombotic drug but also because heparin resembles the endogenous glycosaminoglycans that line the inner wall of our vasculature. Because exosite II is also involved in FV activation, we have studied the effect of heparin on FV activation by thrombin, RVV-V, and LVV-V. Heparin was found to stimulate FV activation by thrombin and the two snake venom FV activators 3–4-fold at the optimal concentration (5 units/ml). Our results confirm and extend earlier reports concerning the effects of heparin on thrombin-catalyzed FV activation (15, 17).

The observation that heparin does not inhibit FV activation by thrombin was reported earlier by Esmon and Lollar (15). However, the fact that a stimulating effect of heparin on FV activation was not observed can be explained by the higher FV/heparin ratio used in this study (15). A similar accelerating effect of heparin on FV activation by thrombin was found by Aracosa et al. (17), who suggested that the effect of heparin could result from an allosteric modification of the active site of thrombin promoting FV cleavage at Arg\textsuperscript{709} and Arg\textsuperscript{1018}. However, we have found that heparin did not accelerate chromogenic substrate hydrolysis by thrombin.\textsuperscript{3} This likely suggests that together with the observation that heparin has an optimal concentration for acceleration of FV activation and the fact that this stimulating effect is not observed at higher concentrations, heparin serves as a template to which both enzyme and substrate bind. Moreover, neither low molecular mass heparin (∼6000 Da) nor pentasaccharide accelerated FV activation (data not shown), which supports further our idea that heparin accelerates FV activation by acting as a template.

Time courses of FV cleavage show that in the thrombin-catalyzed reaction, cleavage of the 330- and 220-kDa FV fragments was accelerated by heparin. The accelerated cleavage of the 330-kDa fragment indicates that heparin facilitates the initial cleavage at Arg\textsuperscript{709}, whereas the accelerated cleavage of the 220-kDa intermediate and the simultaneous generation of the 71/74-kDa FVa light chain point to an enhancement of the cleavage at Arg\textsuperscript{1545}. Furthermore, activation of FV by RVV-V and LVV-V in the presence of heparin resulted in the accelerated cleavage at Arg\textsuperscript{1545} and the formation of the heavy (290 kDa) and light chain (71/74 kDa).

In conclusion, this work provides new information on the role of the exosites of thrombin for FV recognition and activation and on the regions in the FV B-domain that are important for the interaction with thrombin. We propose that exosite I of thrombin plays a dominant role in the activation of FV; cleavage at Arg\textsuperscript{709} precedes those at Arg\textsuperscript{1018} and Arg\textsuperscript{1545} and requires the interaction between exosite I of thrombin and regions surrounding the scissile bond at Arg\textsuperscript{709} in FV. Exosites I and II appear to be equally important for the proteolytic cleavage at Arg\textsuperscript{1545} in FV.

REFERENCES

1. Jenny, R. J., Pittman, D. D., Toole, J. J., Kriz, R. W., Aldape, R. A., Hewick, R. M., Kaufman, R. J., and Mann, K. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4846–4850
2. Nesheim, M. E., Taswell, J. B., and Mann, K. G. (1979) J. Biol. Chem. 254, 10952–10962
3. Steen, M., and Dahlbäck, B. (2002) J. Biol. Chem. 277, 38424–38430
4. Tosio, R., and Camire, R. M. (2004) J. Biol. Chem. 279, 21643–21650
5. Esmen, C. T. (1979) J. Biol. Chem. 254, 964–973
6. Hibbard, L. S., and Mann, K. G. (1980) J. Biol. Chem. 255, 638–645
7. Rosing, J., Tans, G., Govers-Rijnsdorp, J. W., Zwaal, R. F., and Hemker, H. C. (1980) J. Biol. Chem. 255, 274–283
8. Monkovic, D. D., and Tracy, P. B. (1990) Biochemistry 29, 1118–1128
9. Tans, G., Nicolaes, G. A., Thomassen, M. C., Hemker, H. C., van Zonneveld, A. J., Pannekoek, H., and Rosing, J. (1994) J. Biol. Chem. 269, 15969–15972
10. Thorell, E., Kaufman, R. J., and Dahlbäck, B. (1998) Thromb. Haemostasis 80, 92–98
11. Suzuki, K., Dahlbäck, B., and Stenflo, J. (1982) J. Biol. Chem. 257, 6556–6564

\textsuperscript{3} G. A. F. Nicolaes, unpublished observation.
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12. Thorelli, E., Kaufman, R. J., and Dahlbäck, B. (1997) Eur. J. Biochem. 247, 12–20
13. Huntington, J. A. (2005) J. Thromb. Haemostasis 3, 1861–1872
14. Bode, W. (2006) Semin. Thromb. Hemostasis 32, (Suppl. 1) 16–31
15. Esmon, C. T., and Lollar, P. (1996) J. Biol. Chem. 271, 13882–13887
16. Dharmawardana, K. R., and Bock, P. E. (1998) Biochemistry 37, 13143–13152
17. Arocas, V., Lemaire, C., Bouton, M. C., Bezeaud, A., Bon, C., Guillin, M. C., and Jandrot-Perrus, M. (2006) J. Thromb. Haemostasis 3, 1861–1872
18. Bode, W. (2006) Semin. Thromb. Hemostasis 32, (Suppl. 1) 16–31
19. Esmon, C. T., and Lollar, P. (1996) J. Biol. Chem. 271, 13882–13887
20. Dharmawardana, K. R., and Bock, P. E. (1998) Biochemistry 37, 13143–13152
21. Arocas, V., Lemaire, C., Bouton, M. C., Bezeaud, A., Bon, C., Guillin, M. C., and Jandrot-Perrus, M. (2006) J. Thromb. Haemostasis 3, 1861–1872
22. Bukys, M. A., Orban, T., Kim, P. Y., Beck, D. O., Nesheim, M. E., and Kalafatis, M. (2006) J. Biol. Chem. 281, 18569–18580
23. Kisiel, W. (1979) J. Biol. Chem. 254, 12230–12234
24. Segers, K., Rosing, J., and Nicolaes, G. A. (2006) Proteins 64, 968–984
25. Siigur, E., Aaspollu, A., and Siigur, J. (1999) Biochem. Biophys. Res. Commun. 262, 328–332
26. Fernandez, J. A., Petaja, J., and Griffin, J. H. (1999) J. Thromb. Haemostasis 3, 1861–1872
27. Dahlbäck, B. (1980) J. Clin. Investig. 66, 583–591
28. Dharmawardana, K. R., Olson, S. T., and Bock, P. E. (1999) J. Biol. Chem. 274, 18635–18643
29. Tsiang, M., Jain, A. K., Dunn, K. E., Rojas, M. E., Leung, L. L., and Gibbs, C. S. (1995) J. Biol. Chem. 270, 16854–16863
30. Tasset, D. M., Kubik, M. F., and Steinier, W. (1997) J. Mol. Biol. 272, 688–698
31. Myles, T., Yun, T. H., Hall, S. W., and Leung, L. L. (2001) J. Biol. Chem. 276, 25143–25149
32. Dharmawardana, K. R., Olson, S. T., and Bock, P. E. (1999) J. Biol. Chem. 274, 18635–18643
33. Hortin, G. L. (1990) Blood 76, 946–952
34. Sheehan, J. F., and Sadler, J. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5518–5522
35. Carter, W. J., Cama, E., and Huntington, J. A. (2005) J. Biol. Chem. 280, 2745–2749