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Cleavage requirements for activation of factor V by factor Xa

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Coagulation factor V circulates in plasma as a single chain protein which expresses little procoagulant activity. After its activation by limited proteolysis by thrombin or factor Xa, factor Va functions as cofactor to factor Xa in the activation of prothrombin. Thrombin cleaves human factor V at Arg709, Arg1018 and Arg1545 and factor Va is formed by the heavy and light chains, which correspond to the N-terminal and C-terminal fragments, respectively. Factor Xa has been shown to cleave factor V at Arg1018 and at a second undefined position close to Arg709. The factor-Xa-mediated cleavage at Arg1018 has been proposed to be sufficient for expression of full factor Va activity. To study the activation of factor V by factor Xa, site-directed mutagenesis was used to convert Arg709 to Gln, Arg1018 to Ile, and Arg1545 to Gln. Constructs containing all possible combinations of native and mutated residues in these positions were expressed transiently in COS 1 cells. The various factor-V mutants were incubated with factor Xa or thrombin. The proteolytic cleavage pattern was analyzed by Western blotting, and the specific factor-Va activities determined in a prothrombinase assay. Control experiments using thrombin gave results which were in agreement with those on record, i.e. cleavages at both Arg709 and Arg1545 were required for expression of full factor-Va activity, whereas the cleavage at Arg1018 enhanced the rate of cleavage at Arg1545. Factor Xa was found to cleave factor V at all three thrombin cleavage sites, i.e. at Arg709, Arg1018 and Arg1545. An additional factor-Xa-cleavage site was found in the light chain region at Arg1765. Cleavage at Arg1018 by factor Xa was not sufficient for expression of full factor-Va activity. Full factor-Va activity was only obtained after cleavage at both Arg709 and Arg1545. The factor-Xa-mediated cleavage at Arg709 was kinetically favourable over that at Arg1545. Factor V which was mutated at all three sites (at positions 709, 1018 and 1545) was resistant to activation by thrombin. However, treatment with factor Xa yielded an increased factor-Va activity which was associated with the cleavage at Arg1765. Our study extends previously results on thrombin activation of factor V and elucidates the relative importance of the different cleavage sites for activation of factor V by factor Xa.

Keywords: recombinant; factor V; activation; thrombin; factor Xa.

Coagulation factor V is a single chain glycoprotein (M_r 330000) circulating in human plasma at a concentration of 7–10 mg/ml (reviewed in [1, 2]). Like the homologous factor VIII molecule, factor V is composed of multiple A and C domains and a single B region (schematic model of factor V is shown in Fig. 4). Factor Va plays an important role as a phospholipid-bound cofactor to factor Xa in the activation of prothrombin. However, intact single chain factor V has little procoagulant activity and it is only after limited proteolysis by thrombin or factor Xa that factor V expresses full factor-Xa cofactor activity [3].

Activation of factor V by thrombin is the result of three proteolytic cleavages, at Arg709, Arg1018 and Arg1545 [4]. The activation process is associated with a major molecular rearrangement and only 50% of the factor-V mass forms the active species. Thus, factor Va is composed of the 105-kDa heavy chain (A1 and A2 domains) and the 71/74-kDa light chain (A3, C1 and C2 domains), which are derived from the N-terminal and C-terminal parts, respectively of factor V. The two chains of factor Va are held together by non-covalent calcium-dependent bonds. The B region is released from factor Va as two activation fragments of 71 kDa and 150 kDa [5–7].

Factor Xa is the enzyme that cleaves and activates prothrombin, and factor Va serves to enhance the catalytic efficiency of the factor-Xa-mediated cleavage [1, 2]. The light chain of factor Va binds the phospholipid membrane [8–11], whereas the heavy chain interacts with prothrombin [12]. Once factor Va is bound to the membrane, both the heavy and the light chains contribute to the binding of factor Xa [13]. Factor Va lacking the heavy-chain region Asp683–Arg709 has reduced factor Xa cofactor activity and demonstrates impaired binding of factor Xa and prothrombin, suggesting that the C-terminal part of the heavy chain is required for optimal interaction with factor Xa and prothrombin [14].

To elucidate the relative importance of the three thrombin-cleavage sites, Keller and coworkers [15] replaced the arginine residues at these sites with isoleucine residues and concluded that cleavage at Arg1545 was crucial for maximal activation of factor V. This is consistent with the finding that a factor-V acti-

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Abbreviations. RVV, Russell's viper venom; Dns-Glu-Gly-Arg-CH_2Cl, dansyl glutamylglycylargininechloromethane.

Enzymes. Thrombin (EC 3.4.21.5); coagulation factor Xa (EC 3.4.21.22).

vator isolated from Russell's viper venom (RVV) cleaves factor V at Arg1545, which leads to expression of full factor-Va activity [1, 15, 16].

The molecular events occurring upon activation of factor V by factor Xa which lead to increased expression of factor-Va procoagulant activity, are still incompletely understood. Moncovic and Tracy [17] demonstrated by N-terminal sequencing that factor Xa cleaves human plasma-derived factor V after Arg 1018, and proposed that factor Xa cleaved at a site adjacent to or at Arg709. These authors did not detect factor-Xa-mediated formation of the light chain and they suggested that cleavage at Arg1018 was sufficient to fully activate factor V. In contrast to results obtained with plasma-derived factor V, the light chain was found to be formed upon activation of platelet factor V by factor Xa [18]. Moreover, plasma-derived bovine factor V has been shown to be cleaved by factor Xa at two sites in the light chain region, giving rise to light chain fragments of 30 kDa and 46/48 kDa [19].

This site-directed mutagenesis study was performed to elucidate which sites in human factor V are cleaved by factor Xa and to investigate the relative importance of the three cleavage sites, Arg709, Arg1018 and Arg1545 for activation by factor Xa. The activation of the factor-V mutants by thrombin was studied in parallel for comparison and as a positive control. Factor Xa was found to cleave at the same three sites as thrombin and, in addition, at a site within the light chain, the latter cleavage being sufficient for partial activation of factor V. In contrast to previous reports, our results show that the factor Xa cleavage at Arg1545 was necessary for full activation of factor V.

EXPERIMENTAL PROCEDURES

Reagents. Restriction enzymes, PWO polymerase and T4 DNA ligase were purchased from Boehringer Mannheim. Double-stranded DNA sequencing kit was from Perkin Elmer. Cell culture media (Optimem, glutamax) were from Gibco, BRL. An amplification kit for development of Western-blot membranes with fluorescence was from Amersham. The molecular-mass standards were from Bio-Rad. Phospholipid vesicles composed of 25% (by mass) L-α-phosphatidyl-L-serine (P7769 from bovine brain; Sigma), 37.5% (by mass) L-α-phosphatidylcholine (P5763 from egg yolk; Sigma) and 37.5% (by mass) L-α-phosphatidylethanolamine (P7693 from bovine brain; Sigma) were prepared as described previously [20]. The chromogenic substrate S-2238 was obtained from Chromogenix. Rabbit polyclonal anti-human factor V serum (A299) and rabbit anti-sheep IgG coupled to horse radish peroxidase were purchased from Dakopatts. A rabbit polyclonal anti-human factor Va light chain serum (8138) was raised as described previously [21]. Sheep anti-human factor V polyclonal serum were from binding site.

Proteins. The factor V activator from RVV was purified as described [22]. Human thrombin and factor Xa were purchased from Haematologic Technologies Inc. Human factor V was purified as described previously [7, 23] and human prothrombin as described by Stenflo [24].

Mutagenesis. The Arg1018 to Ile mutation has been previously described [25]. At positions 709 and 1545 we chose to make the more conservative replacement of Arg to Gln. The mutations were created by PCR using an expression vector containing the full length cDNA of human factor V, PMT2-V [26] as template. For construction of the Arg 709 to Gln mutation, oligonucleotides 5'GCATTAGGAATTCAATCATTCCGAAAC-3' (1; nucleotides 2287−2313) and 5'-GTTTTAAGAGTAACAGATCACTAG-3' (2; nucleotides 2896−2873) were used in the first PCR. The second PCR used oligonucleotides 5'-GTTTCGGAATGATTGAATTCCTAATGC-3' (3; nucleotides 2313−2287) and 5'-GTGTTTGATGAGAACAAAAGC-3' (4; nucleotides 1738−1758). In the third PCR, the products from PCR reactions 1 and 2 were used as templates together with oligonucleotides 2 and 4. The 900-bp PCR fragment resulting from the third PCR reaction was cut with *Blp*I and *Kpn*I, then ligated to two other fragments, a 9-kb PMT2-V vector-containing fragment (PMT2-V cut with *Kpn*I to yield a 9-kb fragment) and a 2.1-kb factor-V cDNA fragment (derived from PMT2-V when cut with *Blp*I and *Kpn*I). Positive colonies containing full length factor V were sequenced to confirm the mutation.

The Arg1545 to Gln mutation was done in one PCR reaction using PMT2-V as template and oligonucleotides 5'-GCATGGTACCTCCAAAGCAACAATGGA-3' (5; nucleotides 4797−4821) and 5'-CCCTGATCGCTCAGTGGCATG-3' (6; nucleotides 5241−5221). The resulting 400-bp PCR fragment was cut with *Sna*BI and *Kpn*I and ligated to two other fragments, an 8.6-kb PMT2-V vector-containing fragment (PMT2-V was cut with *Sna*BI and *Kpn*I to yield the 8.6-kb fragment) and a 3.0-kb *Kpn*I factor V cDNA fragment (derived from *Kpn*I-cleaved PMT2-V). Positive colonies containing full length factor V were subjected to automatic DNA sequencing to confirm the mutation. Once the single mutants had been created, factor-V mutants containing all the possible combinations of mutations were constructed using restriction enzyme digestions and ligations of appropriate fragments. The three single-site mutants were called [Gln709]factorV, [Ile1018]factorV and [Gln1545]factorV, the double mutants [Gln709,Ile1018]factorV, [Ile1018,Gln1545]-factorV, and [Gln709,Gln1545]factorV, and the triple mutant [Gln709,Ile1018,Gln1545]factorV. The wild-type recombinant factor-V molecule contained arginine residues at the three positions.

Expression and quantification of recombinant factor V. The recombinant proteins were expressed in COS 1 cells as described previously [26] and the proteins were collected in a serum-free medium (Optimem Glutamax) 65 hours after transfection. The expression level was determined with ELISA. Microtiter wells were coated overnight at 4 °C with 10 mg/ml polyclonal rabbit-anti human factor V serum (8138). The plates were quenched for 30 min with 50 mM Tris, 150 mM NaCl, 0.1% (mass/vol.) BSA, pH 7.4 (Tris/NaCl/BSA), then washed three times with 0.1% (by vol.) Tween-20 in Tris/NaCl. Samples were added at different dilutions and, after 2 hours incubation, the plates were washed three times and polyclonal sheep anti-human factor V serum (PC 105) was added. After washing, bound PC 105 was detected with a horseradish-peroxidase-coupled rabbit anti-sheep serum. The plates were developed for 10 min with 1,2-phenylenediamine (Dakopatts) and H_2O_2 diluted in 0.1 M sodium citrate, pH 5.0. The reaction was stopped by the addition of 1.0 M H_2SO_4 and the absorbance measured at 490 nm. Standard curves were constructed using purified plasma-derived human factor V. Samples and antibodies were diluted in Tris/NaCl/BSA.

Thrombin-catalyzed and factor-Xa catalyzed activation of recombinant factor V. Recombinant factor V from conditioned media was diluted to 1.5 nM in Tris/NaCl/BSA and incubated for 30 min at 37 °C with different concentrations of thrombin (0−6.4 nM) in the presence of $CaCl_2$ (2.0 mM), or with factor Xa (0−1.5 nM) in the presence of phospholipids (20 μM) and $CaCl_2$ (2.0 mM). To follow the time course of the reaction, the recombinant factor-V preparations, diluted to 1.5 nM in Tris/NaCl/BSA, were incubated at 37 °C with thrombin (3.2 nM) in the presence of $CaCl_2$ (2.0 mM) or with factor Xa (1.5 nM) in the presence of phospholipids (20 μM) and $CaCl_2$ (2.0 mM). At

different time points (0–30 min), samples were drawn and assayed for factor-Va activity.

Factor-Va activity assay. Eppendorf tubes containing 97 µl of a mixture of prothrombin (1.0 µM), phospholipids (20 µM) and $CaCl_2$ (2.0 mM) in Tris/NaCl/BSA (mixture A) were prepared and kept on ice. Factor V/Va (1.5 µl drawn from the thrombin-catalyzed or factor-Xa-catalyzed activation mixtures) was added to mixture A to give a final concentration of 22.5 pM. Immediately thereafter, the prothrombin activation was initiated by the addition of factor Xa (1.5 µl 4.0 nM stock solution giving a final concentration of 60 pM) and the tubes were transferred to 37 °C. To measure thrombin generation, aliquots of the reaction mixtures were drawn after 5 min and diluted 1:5 in a buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.1% (mass/vol.) poly(ethylene glycol)-6000, pH 7.5), and stored on ice until all the samples were collected. 5–10 µl of the solutions were added to microtiter wells containing 85–90 µl buffer (50 mM Tris, 150 mM NaCl, 20 mM EDTA, 1% (mass/vol.) poly(ethylene glycol)-6000, pH 7.9) and 5 µl 4.0 mM S-2238, warmed to 37 °C. After 10 min, the reactions were stopped by the addition of 60 µl glacial acetic acid and the absorbance was read at 405 nm. When factor V was activated by factor Xa in the presence of phospholipid, thrombin generation was linear with time after an initial short lag phase of approximately 30 s. When factor V was activated by thrombin, the rate of thrombin generation gradually increased during the first 1–1.5 min before the thrombin generation was linear with time. At low concentrations of factor Xa and factor Va, it is probable that establishment of the factor-Xa–factor–Va complex formation equilibrium may take some time. Possibly, in the experiments in which factor V is activated by factor Xa in the presence of phospholipid, this equilibrium was already established in the activation mixture, which resulted in faster initial thrombin generation. This may result in a slight overestimation of the specific activity after factor-Xa activation, as compared to thrombin activation. However, this overestimation of factor-Va activity was relatively small and the difference did not significantly affect the interpretation of the results. Samples without factor Va (blanks) did not show any activity in the prothrombinase assay. Standard curves were prepared using pooled barium-adsorbed (using $BaCl_2$) human plasma. Diluted plasma was activated with the RVV factor-V activator (1.65 µg/ml) for 10 min at 37 °C and assayed at different concentrations in the prothrombinase assay. The RVV factor-V activator was used rather than thrombin to activate plasma factor V because thrombin induced clotting of fibrinogen. One unit of factor Va was defined as the activity present in 1 ml fully activated human plasma.

Western-blot analysis of factor-V activation by thrombin or Xa. Prior to activation, conditioned media was concentrated in a Centricon 30 concentrator (Amicon). Recombinant factor V (25–50 nM) was activated with thrombin (24 nM), or with factor Xa (factor V/factor Xa; 4:1 and 1:1 molar ratio) for 10 min at 37 °C in Tris/NaCl. When recombinants were activated with factor Xa, 2.0 mM $CaCl_2$ and 50 µM phospholipids were added. Samples were reduced with dithiothreitol and heated at 90 °C for 5 min. The proteins were run on SDS/PAGE using 5–15% gradients, then transferred to poly(vinylidene difluoride) membranes using a semidry transfer blotting technique. The proteins were detected using A299, a polyclonal antibody against human factor V. Two Western-blot developing techniques were used to visualize the protein bands. (A) An alkaline-phosphatase-conjugated swine anti-rabbit serum was used as secondary antibody. After development of the bands, the membranes were scanned in a densitometer (Molecular Dynamics). (B) A secondary antibody, swine anti-rabbit coupled to fluorescein was used from a Western blotting kit (Amersham). The membranes were scanned in a FluorImager (Molecular Dynamics) at 530 nm. When the intensity of the bands was low, the membranes were incubated with a third antibody, an alkaline-phosphatase-coupled anti-fluorescein antibody, and the signal was amplified by the addition of Attophos and the membranes were scanned at 570 nm.

N-terminal sequence analysis. Factor V purified from plasma was activated with factor Xa at a 1:1 molar ratio for 20 min at 37 °C in the presence of phospholipids and $CaCl_2$. The proteins were run on reduced SDS/PAGE using 5–15% gradient gels, then transferred to ProBlot membrane (Applied Biosystems). Following transfer, the membrane was stained with Coomassie brilliant blue and the 46/48-kDa fragment derived from the C-terminal part of the light chain excised from the membrane and sequenced as described previously [27].

RESULTS

Expression and characterization of recombinant factor V. In order to study the activation of factor V by thrombin and factor Xa, site-directed mutagenesis and eukaryotic expression was used to prepare recombinant factor V having either arginine or glutamine residues at positions 709 and/or 1545, and arginine or isoleucine residues at position 1018. Wild-type factor V and seven mutants containing one, two or three of the mutated residues were expressed in COS 1 cells, and conditioned media was collected 65 hours after transfection. As estimated by ELISA, the expression level ranged over 1.0–1.5 mg/ml. As judged by Western blotting, wild-type and mutant recombinant factor V preparations all contained the 330-kDa single-chain form of factor V. The presence of additional bands with molecular masses of approximately 220–240000 Da (doublet) and 140000 Da indicated that the recombinant protein was partially proteolyzed (Fig. 1). The 140000 band was more readily observed with the detection system (Fig. 1B), than that used otherwise (Fig. 1A). Similar bands have also been observed in purified plasma factor-V preparations [7, 23], in platelets [18, 28] and in recombinant factor V preparations from other laboratories [15]. These bands were also present in the mutant factor V containing Ile at position 1018, suggesting that the proteolysis was not mediated by thrombin or factor Xa. It is likely to be the result of an intracellular cleavage at a position slightly N-terminal of residue 1018. As previously shown for platelet factor V, the 140-kDa and 220/240-kDa bands represented the N-terminal and C-terminal parts of factor V, respectively [28]. The ELISA was equally efficient in quantifying single-chain factor V as proteolyzed factor V, which was important since the recombinant factor-V preparations contained partially proteolyzed products. This was assured by the essentially identical results obtained when testing the [Gln709,Gln1545]factorV mutant with the ELISA before and after thrombin cleavage (results not shown).

Proteolysis of wild-type and mutant factor V by thrombin. Incubation of wild-type factor V with thrombin yielded the heavy (105 kDa) and light (71/74 kDa) chains of factor Va, the 150-kDa B fragment as well as bands of 220 kDa and 280 kDa, representing partially proteolyzed factor V (Fig. 1). The 280-kDa band is presumably the result of cleavage at Arg709, whereas the 220-kDa band was formed by cleavage at Arg1018 as shown elsewhere [7]. In our Western-blot system, the 71/74-kDa light chain and its typical doublet pattern were difficult to visualize. This may be due to bad transfer of the light chain or to insufficient reactivity of the factor-V serum. In order to facilitate

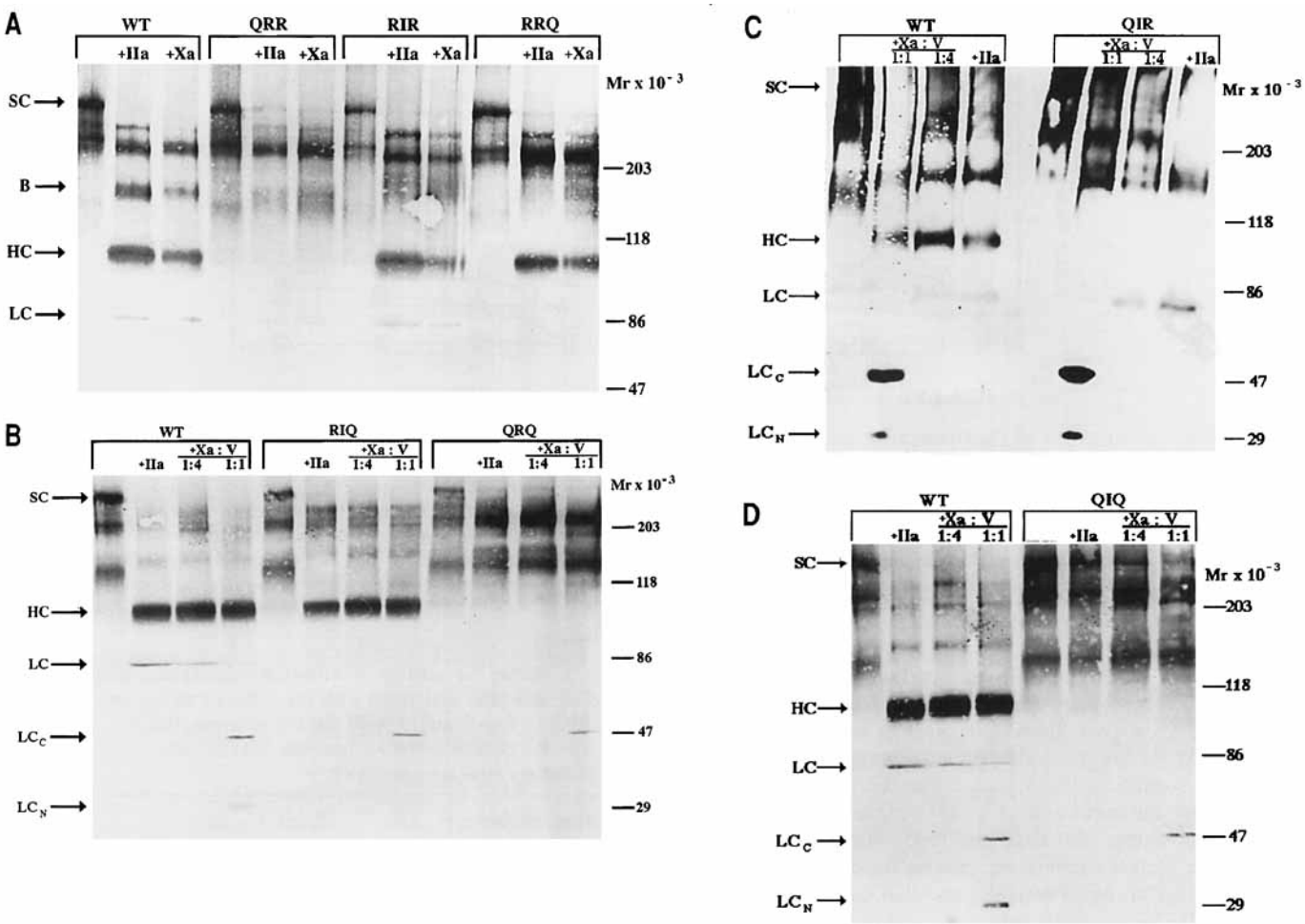

Fig. 1. Western blotting of wild-type factor V and factor V mutants before and after cleavage by thrombin and factor Xa. Samples of conditioned medium from transfected COS cells (continaing 25−50 nM factor V) were incubated with thrombin (24 nM) or with factor Xa (factor V (V)/factor Xa (Xa) at 4:1 and 1:1 molar ratios), phospholipids (50 μM) and 2.0 mM $CaCl_2$ for 10 min at 37 °C and analyzed by Western blotting using 5−15% polyacrylamide gel electrophoresis run in the presence of SDS. Samples (40 μl giving 0.3−0.7 μg/lane) were reduced with dithiothreitol and heated at 90 °C for 5 min. The positions of the 330-kDa single chain (SC), 150-kDa B fragment (B), the 105-kDa heavy chain (HC), the 71/74-kDa light chain (LC), the 48-kDa C-terminal light chain fragment (LC_C) and the 30-kDa N-terminal light chain fragment (LC_N) are indicated. Positions of molecular-mass markers are shown to the right. (A) Wild-type factor V (WT), [Gln709]factorV (QRR), [Ile1018]factorV (RIR) and [Gln1545]factorV (RRQ) were detected using a polyclonal rabbit anti-human factor V serum (A299) and alkaline-phosphatase-coupled swine anti-rabbit serum. After development, the gel was scanned in a densitometer (Molecular Dynamics). (B) Western blotting of wild-type (WT), [Ile1018, Gln1545]factorV (RIQ) and [Gln709,Gln1545]factorV (QRQ). A polyclonal rabbit anti-human factor V serum (A299) was used as primary antibody and a fluorescein-coupled swine anti-rabbit serum as secondary antibody. The membrane was scanned in a Molecular dynamics FluorImager SI. (C) Western blotting of wild-type factor V(WT) and [Gln709,Ile1018]factorV (QIR). The blot was first incubated with A299, then with a fluorescein-labeled swine anti-rabbit serum, followed by the addition of an alkaline-phosphatase-linked anti-fluorescein serum. The blot was developed by the addition of the substrate Attophos, which is converted into a highly fluorescent product that adheres to the blotting membrane. The membrane was scanned in a Molecular dynamics FluorImager SI. (D) Western blotting of wild-type factor V (WT) and [Gln709,Ile1018,Gln1545]factorV (QIQ) was performed as described in (B).

demonstration of the light chain, an amplification system was used (Fig. 1C). In some experiments, the doublet pattern of the light chain was observed, whereas in others the light chain appeared as a single chain.

Mutations of the three thrombin-cleavage sites yielded the expected proteolytic pattern on Western blotting. Thus, as [Gln709]factorV was resistant to cleavage at position 709, no 105-kDa heavy chain was formed. After proteolysis at position 1018, the heavy chain remained connected to the 71-kDa B-domain activation fragment, and co-migrated with the 150-kDa B fragment (Fig. 1A). The mutant [Ile1018]factorV was resistant to thrombin cleavage at position 1018 but the heavy and the light chains were formed. No light chain appeared upon incubation of [Gln1545]factorV with thrombin but cleavages at positions 709 and 1018 yielded the heavy chain and a 220-kDa fragment which contained the light chain linked to the B region (Fig. 1A).

The recombinant factor V molecules containing combinations of the three mutations also yielded the expected cleavage pattern (Fig. 1B). Thrombin cleavage of [Ile1018,Gln1545]factorV yielded the heavy chain band but no light chain. The 220/240-kDa band present in the uncleaved recombinant was not cleaved further, while the 140-kDa band disappeared due to cleavage at Arg709. [Gln709,Gln1545]factorV was only cleaved at Arg1018, as demonstrated by the appearance of 150-kDa and 220-kDa bands. Only the light chain was released by thrombin cleavage of [Gln709,Ile1018]factorV (Fig. 1C). [Gln709, Ile1018,Gln1545]factorV was resistant to thrombin cleavage and the Western-blot pattern remained unchanged upon incubation with thrombin (Fig. 1D).

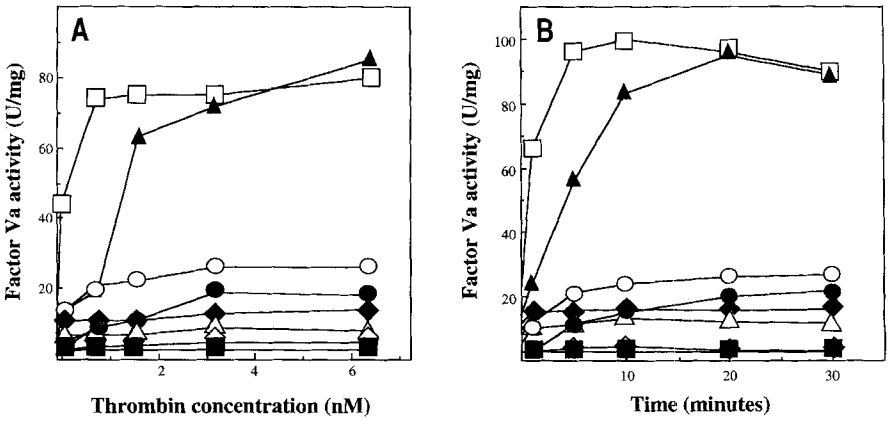

Fig. 2. Thrombin-activation of recombinant factor V mutants. The measured factor-Va activities before and after activation by thrombin are plotted for wild-type factor V (□), [Ile1018]factorV (▲), [Gln709]factorV (○), [Gln1545]factorV (◆), [Gln709,Ile1018]factorV (●), [Ile1018, Gln1545]factorV (△), [Gln709,Gln1545]factorV(◇) and [Gln709,Ile1018,Gln1545]factorV (■). In (A), the recombinant factor-V molecules were incubated with increasing concentrations of thrombin in buffer containing 2.0 mM $CaCl_2$. After 30 min at 37°C, the factor-Va activity was measured in a prothrombinase assay. The plotted values represent means of two experiments. In (B) the different recombinant factor-V preparations (1.5 nM) were incubated with 3.2 nM thrombin in the presence of 2.0 mM $CaCl_2$ at 37°C. At intervals, samples were drawn and factor-Va activity measured in a prothrombinase assay. The plotted values represents means of three experiments performed with conditioned medium from different transfections.

Proteolysis of wild-type and mutant factor V by factor Xa. The various recombinant factor V preparations were incubated with factor Xa at two different enzyme to substrate ratios (1:4 and 1:1). At the lower enzyme/substrate ratio, the cleavage patterns were similar to those seen when the recombinants were cleaved with thrombin (Fig. 1A–D) indicating that factor Xa cleaves at positions 709, 1018 and 1545. Thus, the heavy chain appeared in mutants having an arginine residue at position 709 (wild-type factor V, [Ile1018]factorV, [Gln1545]factorV and [Ile1018,Gln1545]factorV), whereas it did not appear in mutants containing glutamine at this position. Factor Xa is known to be able to cleave at position 1018 [17], which is consistent with the now observed resistance to cleavage at this position in mutants having Arg1018 replaced with an Ile ([Ile1018]factorV, [Ile1018,Gln1545]factorV, [Gln709,Ile1018]factorV and [Gln709,Ile1018,Gln1545]factorV). Factor Xa was found to cleave at Arg1545 and when this position was occupied by a glutamine residue, no light chain was cleaved off ([Gln1545]factorV, [Ile1018,Gln1545]factorV, [Gln709,Gln1545]-factorV and [Gln709,Ile1018,Gln1545]factorV). The factor Xa cleavage pattern was not affected by the addition of hirudin (results not shown), demonstrating that the cleavages were not due to contaminating thrombin.

Factor Xa was also able to cleave at a position located within the light chain, at Arg1765. This cleavage was distinctly evident from incubations containing the higher factor Xa concentration but also noticeable in some of the incubations containing the lower enzyme/substrate ratio (results not shown). The smaller of the two fragments, LC_N, was derived from the N-terminal part of the light chain and, therefore, not seen in the recombinant proteins containing glutamine at position 1545, while the 48-kDa C-terminal light chain fragment, LC_C, was cleaved off from all the mutants (Fig. 1B–D). Although not evident from the presented pictures, in some gels, the 48-kDa fragment appeared as a doublet, consistent with the findings that the molecular basis of the light-chain doublet is located within the C-terminal region of the C2 domain in the light chain [11]. Plasmin has been reported to cleave the bovine light chain into two fragments of M_r = 30000 and M_r = 48000 [29]. To rule out the possibility that the light-chain cleavage was due to trace amounts of plasmin, the plasmin inhibitor aprotinin and the factor-Xa-inhibitor dansyl glutamylglycylarginine-chloromethane (Dns-Glu-Gly-Arg-CH_2Cl)were used to inhibit the cleavage. No inhibition of light-chain cleavage by aprotinin was observed. In contrast, the cleavage was inhibited by Dns-Glu-Gly-Arg-CH_2Cl (results not shown) which showed that the cleavage within the light chain was indeed the result of proteolysis by factor Xa.

Table 1. Factor Va activity of wild-type protein and mutant factor V before and after activation with thrombin or factor Xa. The curves presented in Figs 2 and 3 were used to estimate the EC_{50}. The EC_{50} indicates the concentration of thrombin (IIa) or factor Xa (Xa) giving approximately 50% dose response.

Recombinant factor V	Basal factor Va activity	EC_{50}		Maximal factor Va activity	
		IIa	Xa	IIa	Xa
	U/mg	nM		U/mg	
Wild-type factor V	20	0.1	0.05	100	98
[Gln709]factor V	5	0.3	0.3	27	49
[Ile1018]factor V	15	1.1	0.15	95	97
[Gln1545]factor V	12	–	–	16	25
[Ile1018,Gln1545]factor V	8	–	–	13	22
[Gln709,Ile1018]factor V	3	1.2	0.3	22	30
[Gln709,Gln1545]factor V	3	–	–	5	19
[Gln709,Ile1018,Gln1545]factor V	3	–	–	3	14

Identification of the factor Xa cleavage site within the light chain. Human plasma-derived factor V was digested with factor Xa (1:1 molar ratio) in the presence of phospholipids and $CaCl_2$ and the C-terminal-derived 46/48-kDa light-chain fragment was isolated by SDS/PAGE and sequenced as described in Materials and Methods. The following amino acid sequence was obtained: Leu, Thr, Ser, Ser, Glu, Met, Lys, Lys, and Ser, which showed that factor Xa cleaved at Arg1765.

Specific activities of mutant factor V expressed after full thrombin activation and relative sensitivity of different cleavage sites for thrombin. The ability of the recombinant factor V molecules to function as cofactors to factor Xa was

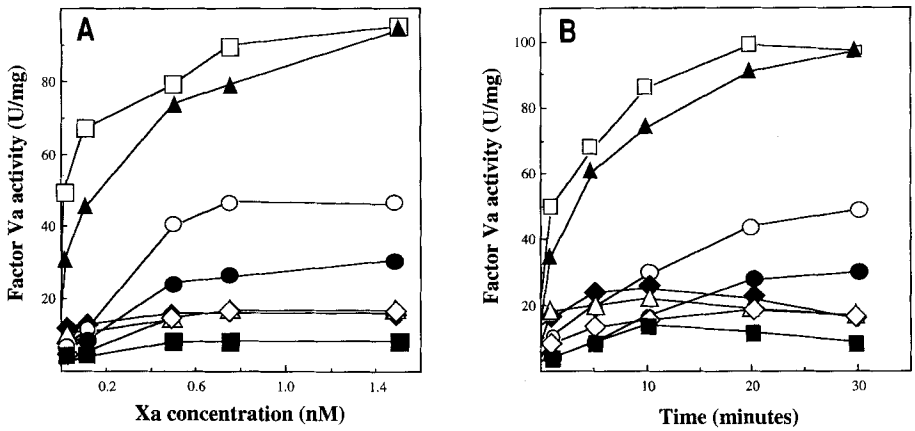

Fig. 3. Factor Xa activation of recombinant factor V mutants. The measured factor-Va activities before and after activation by factor Xa are plotted for wild-type factor V (□) and mutants [Ile1018]factorV (▲), [Gln709]factorV (○), [Gln1545]factorV (◆), [Gln709,Ile1018]factorV (●), [Ile1018,Gln1545]factorV (△), [Gln709,Gln1545]factorV (◇) and [Gln709,Ile1018,Gln1545]factorV (■). In (A), the recombinant factor-V molecules (1.5 nM) were incubated with increasing concentrations of factor Xa in buffer containing 2.0 mM $CaCl_2$ and 20 μM phospholipids. After 30 min at 37 °C, factor-Va activity was measured in a prothrombinase assay. The plotted values represent means of two experiments. In (B), different recombinant factor-V preparations (1.5 nM) were incubated with 1.5 nM factor Xa in the presence of 2.0 mM $CaCl_2$ and 20 μM phospholipids at 37 °C. At intervals, samples were drawn and the factor-Va activity measured in a prothrombinase assay. The plotted values represents means of three experiments performed with conditioned medium from different transfections.

measured in a prothrombinase assay before and after incubation of 1.5 nM recombinant protein with increasing concentrations of thrombin (0–6.4 nM; Fig. 2A). The concentration causing 50% effect for wild-type factor V was 0.1 nM thrombin (Table 1), whereas tenfold more thrombin (concentration causing 50% effect = 1.1 nM) was required for [Ile1018]factor V to express the same specific activity. This is in agreement with the suggestion made by Marquette et al. [25] that cleavage at position 1018 results in a conformational change at the cleavage site at position 1545, which makes it more susceptible to thrombin. The concentration causing 50% effect for [Gln709]factorV (0.3 nM) was threefold higher than that of the wild-type, indicating that cleavage at Arg709 may have a similar effect. When both positions 709 and 1018 were mutated ([Gln709,Ile1018]factorV), the concentration causing 50% effect was 1.2 nM, i.e. Arg1545 was less sensitive to thrombin digestion than when Arg709 and Arg1018 were individually mutated. The concentration causing 50% effect could not be estimated for the other mutants tested, but the activity of [Gln709,Gln1545]factorV increased slightly, whereas the activity of [Gln709,Ile1018,Gln1545]factorV did not change upon thrombin activation (Fig. 2A).

Wild-type factor V was rapidly activated by thrombin and reached its maximum activity (100 U/mg) after 5–10 min incubation. Activation of [Ile1018]factorV was slower and the peak activity (95 U/mg) occurred after 20 min. [Gln709]factorV was maximally activated after 10–20 min, while [Gln709,Ile1018]-factorV had to be activated for 20–30 min with thrombin in order to reach its peak activation (Fig. 2B).

Comparison of the specific activities of the fully activated recombinant factor-V mutants demonstrated the importance of cleavage at both positions 709 and 1545 for expression of full factor-Va activity (Fig. 2B, Table 1). When either of these two sites were mutated, as in [Gln709]factorV and [Gln1545]factorV, the specific activities were less than 30% of those expressed by wild-type factor Va or fully activated [Ile1018]factorV. Mutants having arginine at position 709 all expressed higher basal activities as compared to those having glutamine at this position. The cleavage at position 709 is very sensitive to both thrombin and to factor Xa; this is possibly due to cleavage at Arg709 during the prothrombinase assay.

Specific activities of mutant factor V expressed after full factor-Xa activation and relative sensitivity of different cleavage sites for factor Xa. Factor V recombinants (1.5 nM) were incubated with increasing concentrations of factor Xa (0–1.5 nM) in the presence of phospholipids (20 μM) and $CaCl_2$ (2.0 mM) for 30 min at 37 °C and the factor-Va activity measured (Fig. 3A and Table 1). The specific activity of factor Xa-cleaved wild-type factor V (99 U/mg) was similar to that observed after thrombin activation (100 U/mg). Cleavages at both positions 709 and 1545 were found to be required for full activation of factor V by factor Xa, whereas the cleavage at position 1018 was of less significance. This conclusion was based on the following observations. (A) The very low concentration causing 50% effect for wild-type factor V (0.05 nM) as compared to that of [Gln709]factorV (0.3 nM) demonstrated the site at Arg709 to be very sensitive to cleavage by factor Xa and that this cleavage is important for further proteolysis of the factor-V molecule. (B) The low specific activity of fully activated [Gln1545]factorV demonstrated the cleavage at position 1545 is essential for expression of full activity even though it was less sensitive to factor Xa than that at position 709 (as demostrated by a concentration causing 50% effect of 0.3 nM for [Gln709,Ile1018]factorV). (C) The sensitivity of [Ile1018]factorV (concentration causing 50% effect of 0.15 nM) was only slightly lower than that of wild-type factor V (concentration causing 50% effect of 0.05 nM), suggesting the cleavage site at position 1018 is of relatively minor importance.

Several of the mutants, e.g. [Gln709]factorV, [Ile1018, Gln1545]factorV, [Gln709,Gln1545]factorV, [Gln709,Ile1018]-factorV and [Gln709,Ile1018,Gln1545]factorV, expressed higher specific activity after incubation with factor Xa than after thrombin incubation (Fig. 3B, Table 1), e.g. the activity of [Gln709,Gln1545]factorV increased from 3 U/mg before activation to 19 U/mg after 20 min activation. The activity after thrombin activation of this mutant was only 5 U/mg. Likewise, upon incubation with factor Xa, the activity of [Gln709,Ile1018, Gln1545]factorV increased to 14 U/mg, whereas no increased activity was observed after incubation with thrombin. The increased activity observed after factor-Xa activation was presumably the result of the factor Xa-mediated cleavage at Arg1765.

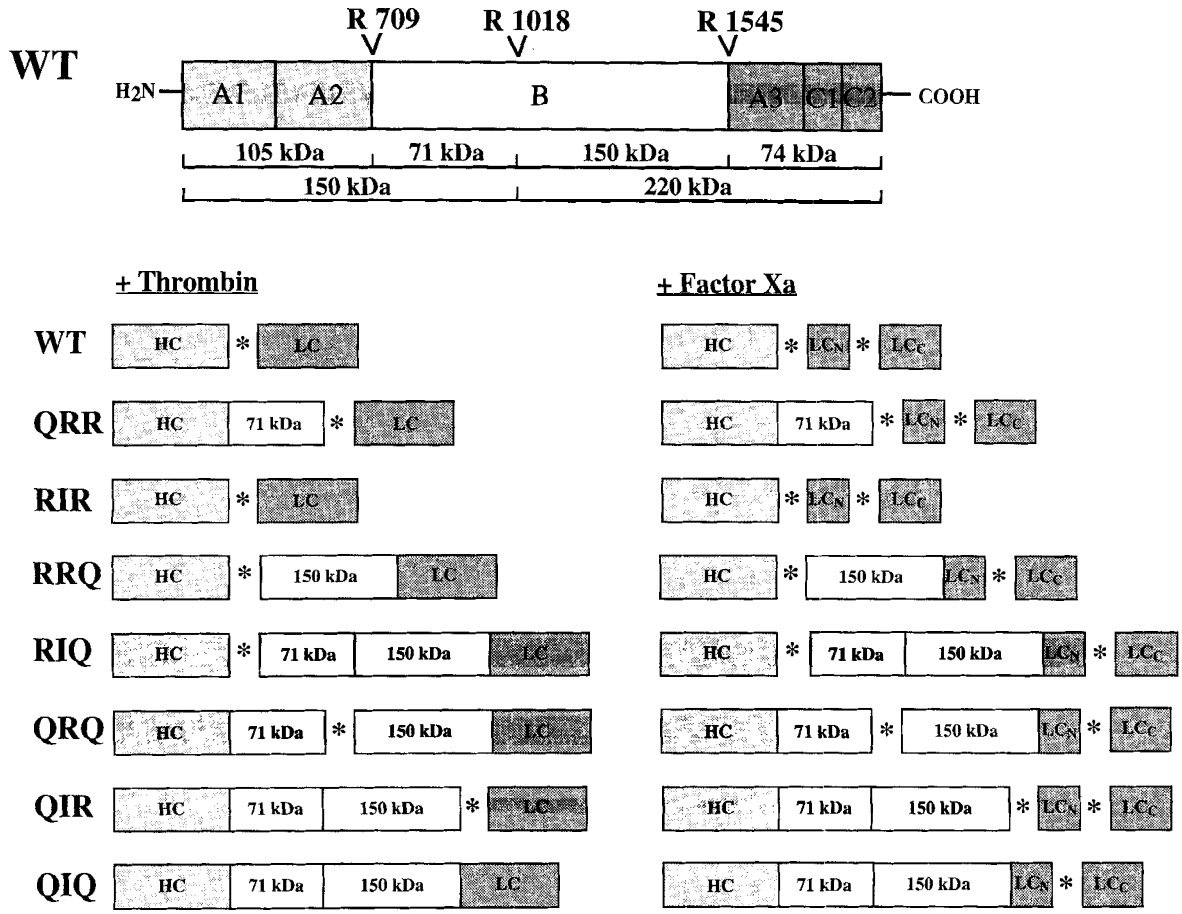

Fig. 4. Schematic model of wild-type factor V (WT) and factor V mutants [Gln709]factorV (QRR), [Ile1018]factorV (RIR), [Gln1545]factorV (RRQ), [Ile1018,Gln1545]factorV (RIQ), [Gln709,Gln1545]factorV (QRQ), [Gln709,Ile1018]factorV (QIR) and [Gln709,Ile1018,Gln1545]-factorV (QIQ) after thrombin and factor Xa cleavage. (A) Single-chain factor V (M_r 330) is comprised of multiple regions which are arranged in the order A1-A2-B-A3-C1-C2. Cleavage at Arg709, Arg1018 and Arg1545 leads to a molecular rearrangement and the formation of factor Va, which functions as cofactor to factor Xa. Factor Va is formed by the heavy chain (HC) and the light chain (LC) which are held together by non-covalent calcium-dependent bonds. The heavy chain is composed of A1 and A2 modules, whereas the light chain contains A3, C1 and C2 modules. (B) Schematic representation of the active factor-Va molecules which are formed upon incubation with thrombin or FXa. Asterisks denote an interaction between the fragments. Shaded boxes represent the heavy and light chains.

The factor-Va activity expressed after cleavage of [Gln709, Ile1018,Gln1545]factorV with factor Xa was labile (Fig. 3). Maximum factor-Va activity for this mutant was expressed after 10 min incubation with factor Xa, then the activity declined during the following 20 min. This decline in activity explains why (Fig. 3A) increasing concentrations of factor Xa did not appear to lead to increased factor-Va activity as the activity in this experiment was measured after 30-min incubation. That incubation of [Gln709,Ile1018,Gln1545]factorV with factor Xa indeed led to increased factor-Va activity was further demonstrated in an experiment in which [Gln709,Ile1018,Gln1545]factorV (1.5 nM final concentration) was mixed with factor Xa (1.5 nM) and phospholipid (20 μM) in Tris/NaCl/BSA with 2.0 mM $CaCl_2$. Prothrombin (1.4 μM) was either added immediately or after 10 min incubation at 37 °C, and the rate of thrombin generation was followed during 5 min. When prothrombin was added immediately, no thrombin generation was observed. In contrast, 20 U/ml thrombin was formed during the first minute in the incubated factor-Xa/[Gln709,Ile1018,Gln1545]factorV mixture. This was most likely due to the factor-Xa-mediated cleavage at Arg1765 by factor Xa.

The relative rates by which the four factor-Xa-cleavage sites were cleaved were estimated from the time course of the activation reactions (Fig. 3B, Table 1). It is noteworthy that the activities of wild-type factor V, [Ile1018]factorV,[Gln709]factorV and [Gln709,Ile1018]factorV, which all were cleaved at Arg1545 and within the light chain, continued to increase throughout the 30 min of incubation with factor Xa, suggesting the Arg1545 cleavage to be relatively slow. In contrast, the increase in activity of [Gln1545]factorV and [Ile1018,Gln1545]factorV observed after addition of factor Xa was very fast and maximum activity was already expressed after 5 min. Thereafter, the activities of these preparations slowly declined. The similar activation patterns of [Ile1018,Gln1545]factorV and [Gln1545]factorV support the notion that cleavage at position 1018 is of minor importance for factor Xa activation and that the site at position 709 is efficiently and very rapidly cleaved by factor Xa.

DISCUSSION

Activation of human and bovine plasma-derived factor V by thrombin has been extensively studied by several investigators [5–7]. Keller and coworkers [15] used *in vitro* mutagenesis and expression of mutated recombinant factor V to elucidate which of the thrombin cleavage sites were necessary for the activation of human factor V by thrombin. From their work they concluded that cleavage at Arg709 resulted in little increase in cofactor

activity, but that this cleavage was important for rapid thrombin cleavage at Arg1545 and maximal activation by thrombin. They concluded that cleavage of Arg1545 was most important for expression of full factor-Va activity. Our results obtained on the thrombin-mediated activation of factor V confirm those of Keller et al. [15] and further show the importance of the cleavage at Arg709. This cleavage is very rapid, which presumably explains the high basal factor-Va activity expressed by recombinant factor V mutants having Arg at position 709, and it is needed for expression of maximal factor-Va activity. Cleavage at Arg1018 by thrombin or factor Xa did not lead to increased factor-Va activity, suggesting that cleavage of this site may have another function. Marquette et al. [25] reported that cleavage at position 1018 facilitated further cleavage at position 1545 and our results were in agreement with this conclusion. We found the cleavage site at position 709 to have a similar function. In contrast, we obtained no indication to suggest that the cleavage site at position 1545 or that mutation at this site affects the cleavages at positions 709 or 1018. This also agrees with observations made in previous time-course studies demonstrating the individual sensitivities of the various sites for thrombin. In these experiments, it was shown that the site at position 1545 was less readily cleaved than the other two sites by thrombin and that the cleavage at position 1545 is the last site to be cleaved during thrombin activation [7].

It was noteworthy that mutants with arginine at position 1545 ([Gln709]factorV and [Gln709,Ile1018]factorV) did not give full activity after activation with thrombin or factor Xa. This is surprising as these recombinant molecules, after activation, yield a form of factor V similar to factor V cleaved by the RVV factor-V activator, which has been reported to have the same activity as thrombin-activated factor V. It is possible that mutation at positions 709 and 1018 might cause secondary changes in the conformation of factor V such that the cleavage at position 1545 is significantly influenced. As a result, thrombin would be less efficient in cleaving this site in mutant factor V compared to wild-type factor V. For this reason, we also tried to activate the mutant factor V with a combination of thrombin and the RVV factor-V activator. However, the activities of the [Gln709]factorV and [Gln709,Ile1018]factorV mutants were found not to increase further after combined activation with thrombin and RVV factor-V activator (results not shown), which indicate that the low activities were not related to partial cleavage at position 1545, but that they were inherent properties of the two mutants. Moreover, on Western-blot analysis, we found that the mutant factor-V species were fully cleaved after thrombin digestion. Possibly, the low specific activity of fully activated [Gln709]factorV and [Gln709,Ile1018]factorV is caused by secondary conformational changes in factor V induced by the mutation. Alternatively, the specific activity of the wild-type activated by RVV factor V activator may not represent the activity of factor V which is only cleaved at position 1545, but rather factor V which is cleaved at both positions 709 and 1545, because the site at position 709 probably is rapidly cleaved by factor Xa during the prothrombinase assay which is used to quantify factor-Va activity. This could also explain the observed higher basal activities expressed by mutants having arginine at position 709 as compared to those having glutamine at this position.

Moncovic et al. [17] reported that factor Xa did not cleave plasma-derived factor V at position 1545. Our results demonstrate that factor Xa is able to cleave factor V at the same three sites as thrombin, i.e. at positions 709, 1018 and 1545. The most striking difference between our results and those on record is that we found the cleavage at Arg1545 to be crucially important for expression of full factor-Va activity after factor Xa cleavage, whereas Moncovic and Tracy [17, 18] reported cleavage at Arg1018 to be sufficient. In our hands, cleavage at position 1545 was not only observed for recombinant factor V but also for factor V purified from human plasma, which rules out the possibility that the specific factor-Xa cleavage pattern now observed was only valid for recombinant factor V. In our experimental system, cleavage at Arg1018 by factor Xa did not result in increased factor-Va activity, which was most clearly demonstrated by the observation that [Gln709,Gln1545]factorV and [Gln709,Ile1018,Gln1545]factorV after incubation with factor Xa expressed similar specific activities. It is likely that the low biological activity of cleaved [Gln709,Gln1545]factorV is due to resistance to cleavage at the mutated positions (positions 709 and 1545) even though the possibility that the loss of biological activity was due to secondary-structural changes caused by the mutations cannot be completely ruled out.

Factor Xa was also found to cleave human recombinant and plasma-derived factor V at Arg1765, a site located within the A3 domain of the light chain. The cleavage yielded two fragments of 30 kDa and 48 kDa, the latter being derived from the C-terminal half of the light chain. In this context, it is noteworthy that bovine factor V [19] and bovine factor Va [30] have been shown to be cleaved by factor Xa at a site located within the light chain between Arg1753 and Ala 1754 [31]. Moreover, factor Xa cleaves factor VIII at the same sites as thrombin and, in addition, at a site within the light chain, at Arg1721 [32]. Recently, it was demonstrated that this latter factor Xa cleavage was associated with factor VIII activation [33]. As judged from our studies, human factor V and factor VIII are similar in the respect that both molecules can be activated by factor Xa cleavage in the A3 domain.

The different molecular structures of the factor Va species formed upon thrombin or factor Xa cleavage of the recombinant factor V mutants (Fig. 4) may explain the different specific activities observed. Factor Va formed after thrombin cleavage of wild-type factor V and [Ile1018]factorV are identical and express similar specific activities. The same holds true for the factor-Xa-activated forms. It is known that the heavy chain of factor Va interacts with prothrombin [12] and that both the light chain and the heavy chain bind to factor Xa [13, 34]. The lower specific activities expressed of the mutants which were not cleaved at either positions 709 or 1545 were probably caused by the B-region fragments which remained attached to either the heavy or the light chains. It remains to be elucidated whether these attached fragments affect binding of factor Xa, the factor Xa catalytic activity or the interaction of prothrombin with the respective prothrombinase complex.

Our study provides new information on the activation of factor V by factor Xa and an idea about the specific activities of the fully activated factor-V mutants. These latter results are of biological interest because some of the activated factor-V mutants correspond to activation intermediates which are formed during physiological activation of factor V. Thus, fully activated [Ile1018, Gln1545]factorV and [Gln1545]factorV correlates to such activation intermediates, i.e. factor V cleaved at position 709 or at position 709 plus position 1018, respectively. The higher activity of [Ile1018,Gln1545]factorV as compared to [Gln709,Ile1018,Gln1545]factorV suggests that the rapid initial cleavage at position 709 is associated with increased factor-V activity which may be crucial for early positive feed-back amplification of the coagulation system.

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