The C-terminal Region of the Factor V B-domain Is Crucial for the Anticoagulant Activity of Factor V*

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Factor V (FV) is recently shown to express anticoagulant activity. It functions as a synergistic cofactor with protein S to activated protein C (APC) in the degradation of factor VIIIa (FVIIIa). FV is composed of multiple domains, A1-A2-B-A3-C1-C2. Thrombin cleaves FV at Arg-709, Arg-1018, and Arg-1545 that leads to the generation of a procoagulant FV species which functions as a cofactor to factor Xa (FXa) in the activation of prothrombin to thrombin. During the activation process, the B-domain is released from the heavy (A1-A2) and light chains (A3-C1-C2) which constitute the active FV (FVa). To elucidate which effect the different thrombin cleavages in FV have on the ability of FV to express APC cofactor activity, seven recombinant FV mutants containing all possible combinations of mutated and native thrombin cleavage sites were tested in a FVIIIa degradation assay. Thrombin cleavage at Arg-709 and/or Arg-1018 yielded FV molecules that were still able to function as APC cofactors, whereas cleavage at Arg-1545 led to a complete loss in APC cofactor function. This suggests that the APC cofactor function of FV depends on the B-domain remaining attached to the A3 domain. The importance of the FV B-domain for expression of APC cofactor activity was further investigated using two B-domain deleted FV molecules, FV des-709–1545 (with the whole B-domain deleted) and FV des-709–1476 (with amino acids 710–1476 of the B-domain being removed). FV des-709–1476 expressed APC cofactor activity, whereas the FV des-709–1545 was completely devoid of such activity. Thus, the C-terminal part of the B-domain (residues 1477–1545) was crucial for the APC cofactor function. FV and factor VIII (FVIII) are homologous proteins having similar domain organization. A FV/FVIII chimera, harboring the B-domain from FVIII (FVIII) instead of the FV B-domain did not work as an APC cofactor, further illustrating the importance of the FV B-domain for the APC cofactor function.

The protein C pathway is a natural anticoagulant system that regulates the coagulation process through proteolytic cleavage and inactivation of factor VIIIa (FVIIIa) and factor Va (FVa) (1–3). FVIIIa and FVa bind to negatively charged phospholipids and serve as receptors/cofactors for the proteolytic enzymes, factor IXa (FIXa) and factor Xa (FXa), respectively (4). FIXa activates FX, whereas FXa activates prothrombin to thrombin. Amplification of the reactions mediated through thrombin activation of FVIII and FV results in explosive thrombin generation which results in coagulation of blood. In intact vessels, thrombin is an important component of the protein C anticoagulant pathway. It binds to thrombomodulin on the surface of endothelial cells, loses its procoagulant abilities, and becomes a potent activator of protein C (5). The anticoagulant properties of activated protein C (APC) is potentiated by a plasma protein denoted protein S. The high affinity binding of protein S to negatively charged phospholipid surfaces increases the affinity of APC for the surface (6, 7), and interactions between protein S and APC on the membrane surface leads to relocation of the active site of APC (8). Recently, we have shown that FV, apart from being a precursor to the procoagulant FVa, also has anticoagulant properties because it functions in synergy with protein S as a cofactor to APC in the degradation of FVIIIa (9, 10).

FV and FVIII are homologous, high molecular weight glycoproteins. The plasma concentration of FV is 10 μg/ml and that of FVIII is 0.2 μg/ml. Both FV and FVIII are synthesized as precursor proteins with molecular masses of approximately 300,000 Da (4). The two proteins share the domain organization A1-A2-B-A3-C1-C2 (11–13). The three A-domains of FV and FVIII are homologues to the three A-domains of ceruloplasmin, a copper-binding plasma protein (14). The two C-domains are members of the discoid-like family, several members of which express phospholipid binding properties (15). Whereas the A- and C-domains are 40% identical between FV and FVIII, there is no significant sequence similarity between the B-domain of FV and FVIII even though both B-domains have high carbohydrate contents. FV is activated by thrombin (11, 16) or FXa (17, 18) through cleavages at Arg-709, Arg-1018, and Arg-1545. Upon activation, the B-domain is released and the active factor V species (FVa) is formed by a noncovalent calcium-dependent complex between the heavy (A1-A2) and the light chain (A3-C1-C2) (19, 20).

During coagulation, FV activation intermediates, which are cleaved at one or more of the three cleavage sites, are formed. The sites at Arg-709 and Arg-1018 are kinetically favored over the cleavage at Arg-1545, which leads to formation of FV intermediates that are not cleaved at Arg-1545 (16, 18, 21). The partially cleaved FV activation intermediates express different levels of procoagulant activities (18, 21). The purpose of this study was to use recombinant FV, in which one or more of the

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‡ The abbreviations used are: FVIIIa, activated factor VIII; FVa, activated factor V; FIXa, activated factor IX; FXa, activated factor X; APC, activated protein C; RVV-V, purified factor V activator from Russell’s viper venom; ELISA, enzyme-linked immunosorbent assay; WT, wild type.
thrombin cleavage sites was eliminated through mutagenesis, to investigate which FV activation intermediates express the anticoagulant APC-cofactor activity. Moreover, B-domain deletions and FV/FVIII chimeric molecules were used to identify important regions in the FV B-domain for expression of the APC-cofactor function.

**EXPERIMENTAL PROCEDURES**

Reagents—Purified bovine FIXa, bovine FX, and the chromogenic substrate S-2222 were from Chromogenix (Mölndal, Sweden). Hirudin and Russell’s viper venom (RVV) were obtained from Sigma. Human α-thrombin and human FV were purchased from Hematologic Technologies, Essex Junction, VT. Octanotide M (Pharmacia) was used as the source of FVIII. The factor V activator from RVV (RVV-V) was purified as described (22). Human protein S and human activated protein C were purified according to previously described methods (23). Phospholipid vesicles composed of 25% phosphatidylserine (w/w), 37.5% (w/w) phosphatidylcholine, and 37.5% (w/w) phosphatidylcholine were prepared as described previously (24). The phospholipid components were purchased from Sigma. Rabbit polyclonal anti-human factor V serum (A299) and swine anti-rabbit IgG coupled to fluorescein were purchased from Dako. Centricron 30 used to concentrate the conditioned media was from Amicon. Nitrocellulose transfer membranes were from Micron Separations. Serum-free cell media (Optimem Glutamax) was purchased from Life Technologies, Inc.

**Recombinant FV Variants**—The FV cDNA constructs containing different combinations of mutated and native amino acids at the three thrombin cleavage sites have been described previously (18). The FV mutants were named according to which amino acid was present at each thrombin cleavage site, *i.e.* WT FV (Arg-709, Arg-1018, Arg-1545), QRQ (Gln-709, Arg-1018, Arg-1545), RRQ (Arg-709, Arg-1018, Gln-1545), and QRR (Gln-709, Arg-1018, Arg-1545). The deleted factor V constructs lacking the whole B-domain (FV des-709–1545) or the B-domain region between 710 to 1477 (FV des-709–1476) were previously described (25). A FV/FVIII chimeric molecule containing FV A1-A3, C1 and C2, and residues 741–1484 from the FVIII B-domain was described in Ref. 26.

**DNA Transfection and Analysis of Conditioned Media**—Plasmid DNA containing various FV cDNA constructs were transfected into COS-1 cells by the diethylaminoethyl (DEAE) dextran procedure (27). Serum-free conditioned medium was harvested at 66 h post-transfection. The FV antigen levels were quantitated using an ELISA specific for FV as described (18). The ELISA could not be used to quantitate the constructs lacking the whole B-domain (FV des-709–1545), the construct containing the C-terminal part of the B-domain (FV des-709–1476) or the construct with the FV B-domain exchanged for the FVIII B-domain (FVVBVIII). These constructs were quantitated by Western blotting. Conditioned media was concentrated in a Centricron 30 concentrator and was then added at different dilutions on SDS-PAGE 7.5% gels. The different FV species were transferred to polyvinylidene difluoride membranes using a semidry blotting technique. The protein bands were detected using rabbit polyclonal anti-human factor V serum (A299) as primary antibody and a swine anti-rabbit antibody coupled to fluorescein as secondary antibody. The blots were allowed to dry and then scanned in a FluorImager (Molecular Dynamics). The intensities of the bands were quantitated in the ImageQuant software program (Molecular Dynamics). A standard curve was prepared using serial dilutions of human FV.

**Preparation of FVIII Reagent I**—Thrombin-activated FVIII (FVIIIa) was prepared by incubating a solution containing FVIII (50 milliunits/ml), FIXa (25 milliunits/ml), and phospholipid vesicles (20 µM) with α-thrombin (5 milliunits/ml). The buffer was 50 mM Tris, 150 mM NaCl (TBS), 10.0 mM CaCl2, 0.2% bovine serum albumin (BSA), pH 7.5. After 3 min, the reaction was stopped by the addition of hirudin to a final concentration of 8 milliunits/ml. Due to the labile nature of FVIIIa, this FVIIIa degradation assay was then performed as described above.

**RESULTS**

**APC-cofactor Function of Recombinant Factor V and Effect of Thrombin Cleavage**—Recombinant FV variants having one or mixed with increasing concentrations of recombinant FV that had or had not been incubated with the RVV-V-thrombin mixture. The final volume was 25 µl, and medium from cells that had not received DNA (control medium) was used as diluent. Reagent I (80 µl) was added, and after 2.5 min, 20 µl of bovine FX (1.2 units/ml) was added. After 6 min of incubation at 37 °C, 50 µl of chromogenic substrate S-2222 was added. After 10 min, the hydrolysis of the substrate by FXa was stopped by the addition of 50 µl of 50% acetic acid, and the absorbance at 405 nm was measured.

To check whether the thrombin/RVV-V mixture influenced the FVIIIa activity, control medium was incubated with the RVV-V-thrombin mixture for 1 h at 37 °C, and hirudin was then added. APC and protein S as well as reagent I were added to the sample as described above. The FVIIIa activity was found to be the same as in control medium with APC, PS, and reagent I but without RVV-V-thrombin. From this we concluded that the FV activation mixture did not influence the FVIIIa activity.

The potential influence of the control medium on the FVIIIa degradation was evaluated by performing the assay by adding FV purified from plasma together with APC and protein S either to TBS/BSA buffer or to control medium. Results obtained in TBS/BSA buffer were the same as those obtained in control medium. This taken together with the observation that WT FV is an equally good APC cofactor as FV purified from plasma validate the use of recombinant FV in conditioned medium to evaluate the APC-cofactor function of FV.

**Inhibition of FVIIIa Degradation by Monoclonal Antibodies Against FV**—Monoclonal antibody HV4 reacts with the 150-kDa B-domain fragment and is known to inhibit the anticoagulant activity of human FV in normal plasma (28) and in a purified system (9). Monoclonal HV4 (20 µg/ml) was incubated with the recombinants WT FV or FV des-709–1476 (45 nm) at room temperature for 10 min. APC (final concentration of 5 nm) and protein S (final concentration of 5 nm) were mixed with increasing concentrations of recombinant FV (final concentrations of 0–9 nm) that had been incubated with HV4, and the FVIIIa degradation assay was then performed as described above.
more of the three thrombin cleavage sites mutated were expressed in COS-1 cells. In a FVIIIa degradation assay, WT recombinant FV was found to work as an APC cofactor essentially as described previously for plasma-derived FV (Fig. 1). Maximum FVIIIa inhibition was reached at approximately 8–10 nM FV. The APC-cofactor activity was lost after incubation of the FV with an RVV-V/thrombin mixture which yielded complete cleavages at all three thrombin cleavage sites, as judged by Western blotting (not shown). The influence of individual thrombin cleavages on the ability of FV to express APC-cofactor activity was investigated using recombinant FV in which one, two, or all three thrombin cleavage sites had been eliminated through mutagenesis. The RIQ mutant, a recombinant which could only be cleaved at position 709, expressed full APC-cofactor activity after incubation with the thrombin/RVV-V mixture (Fig. 2A). This suggests that the Arg-709 cleavage does not affect the APC-cofactor activity of FV. Similar results were obtained for the Arg-1545 site because the RRQ mutant demonstrated similar APC-cofactor activity before and after incubation with the activation mixture (Fig. 2B). The APC-cofactor activity of the QIR mutant was unaffected by the incubation with thrombin/RVV-V (Fig. 2D). This was in contrast to results obtained with the QIR mutant that completely lost APC-cofactor activity after incubation with the activation mixture (Fig. 2C). This mutant was fully cleaved at Arg-1545 according to results of Western blotting. The remaining FV mutants which were tested further supported the conclusion that only the Arg-1545 cleavage led to loss of APC-cofactor activity. Thus, both the QRR (mutated at Arg-709) and RIR (mutated at Arg-1018) lost APC-cofactor activity after thrombin/RVV-V proteolysis, whereas QRQ (mutated at Arg-709 and Arg-1545) retained full APC-cofactor activity (results not shown).

The C-terminal Region of the FV B-domain Is Crucial for Expression of APC-cofactor Function—The C-terminal part of the B-domain is rich in acidic amino acid residues and contains tyrosines that can be sulfated (29, 30). This region has been suggested to be important for interaction with thrombin, possibly through binding to one of the exosites in thrombin (25). The C-terminal region of FVIII also contains many negatively charged residues and sulfated tyrosines (31). This part of the FVIII B-domain region is found to be crucial for von Willebrand factor binding (31, 32).

To elucidate the importance of the C-terminal part of the B-domain for expression of APC-cofactor activity, constructs with either the whole B-domain deleted (FV des-709–1545) or parts of the B-domain deleted (FV des-709–1476) were tested for their ability to function as APC cofactors. The involvement of the B-domain was further studied using a chimeric FV/FVIII molecule composed of A- and C-domains from FV and residues 740–1648 from the FVIII B-domain (FVBVIII). The FV des-709–1476 molecule expressed clear APC-cofactor activity,
whereas FV des-709–1545 and FVBVIII were completely inactive as APC cofactors (Fig. 3). Consistently, the FV des-709–1476 construct was less active as APC-cofactor than WT FV. Moreover, the FV des-709–1476 behaved differently to incubation with the thrombin/RVV-V mixture than WT FV because it expressed partial APC-cofactor activity also after incubation with the activator mixture (Fig. 4A). This may be due to inefficient cleavage by thrombin at Arg-709, which has been reported (25) and was also seen on Western blotting in this study (not shown), or to close spatial proximity of the A3-domain to the region containing amino acid residues 1477–1545, also after cleavage. Thus, the local structural changes taking place after activation with thrombin/RVV-V may not have been as important in the deletion molecule as in the full-length WT FV.

We have previously found that the APC-cofactor activity of plasma FV was partially inhibited by a monoclonal antibody (HFV-4) directed against an epitope of the B-domain (9). In this study, HFV-4 was found to inhibit the APC-cofactor activity of both WT FV and FV des-709–1476 (Fig. 4B), suggesting that the epitope was located somewhere in the C-terminal region of the B-domain.

DISCUSSION

Recently, new insights have been gained into the mode of action of protein S in degradation of FVa (33). Thus, protein S has been found to specifically stimulate APC-mediated cleavage at Arg-306, whereas the cleavage rate at Arg-506 is unaffected by the presence of protein S. This specific action of protein S on APC-cleavage at Arg-306 may be related to the ability of protein S to relocate the active site of APC closer to the phospholipid membrane (8). Whether such a mechanism is also involved in the degradation of FXIIa is not known. In this latter reaction, FV has been found to work as an APC cofactor in synergy with protein S (9, 10, 34–36).

We have previously reported that the APC-cofactor activity of plasma FV was lost upon activation of FV by thrombin or RVV-V (9). Results presented by Varadi et al. (34, 35) support this conclusion, whereas Lu et al. (36) reported that the APC-cofactor activity of their FV preparation was retained after incubation with thrombin. The reason for this discrepancy is not obvious. Based on specific inhibition of the APC-cofactor activity by a B-domain monoclonal antibody (9), we proposed that the FV B-domain was important for the APC-cofactor function, a conclusion that was further supported by Lu et al. (36). In this study, we found that cleavage at Arg-709, and/or Arg-1018, did not lead to loss of the APC-cofactor function, whereas cleavage...
at Arg-1545 resulted in complete loss of APC-cofactor activity. This observation may provide a possible explanation for the discrepancy of results on record. Lu et al. (36) found that FV after incubation with thrombin retained APC-cofactor activity, whereas purified FVAs (lacking the B-domain) had no APC-cofactor activity. Possibly, their FV may not have been fully cleaved at Arg-1545 by the thrombin used, because this site is relatively resistant to thrombin cleavage even after prolonged incubation with thrombin. In our experience, full cleavage at Arg-1545 of both recombinant and plasma-derived FV was only obtained by the thrombin/RVV-V mixture.

Our present results suggest that a region surrounding the Arg-1545 cleavage site in FV is important for the expression of APC-cofactor function. Thus, FV completely lacking the B-domain (FV des-709–1545 and FVBVIII) had no APC-cofactor activity, whereas the FV mutant containing the 69 amino acids just prior to the Arg-1545 site (FV des-709–1476) expressed significant APC-cofactor activity. Moreover, the APC-cofactor activity of WT FV and FV des-709–1476 could be inhibited by a monoclonal antibody that recognizes the B-domain of factor V. In order to investigate our data from a structural standpoint, sequence analysis of FV was carried out in conjunction with inspection of a three-dimensional model for the A-domains of FV (37) and of previously reported electron microscopy studies (38–42). Secondary structure and solvent accessibility predictions were performed using the Profile fed neural network systems from HeiDellberg (PHD) (43, 44) and the human FV sequence was provided as query input (data not shown). The results indicated that the B-domain of FV contained few secondary structure elements. The region surrounding residue 1545 was predicted as a solvent-exposed loop structure. Furthermore, it is known that in this region, tyrosine residues can be sulfated (29, 30), supporting the overall solvent accessibility of this area.

Molecular models of the A-domains of FV/FVa can be built unambiguously using the x-ray structure of ceruloplasmin (45). These three domains are arranged in a triangular fashion and their structural organization should remain essentially the same before and after activation. This suggests that the B-domain protrudes between the A2 and A3-domains. It is known that FV and FVAs have the same affinity for the appropriate membrane surface, suggesting that the B-domain does not interact with the membrane. The results from the electron microscopy studies of Stoylova et al. (42) and Lampe et al. (38) show that the light chain component (A3-C1-C2) of FVAs bound to a membrane appears to be largely external to the phospholipids.

Before activation by thrombin and RVV-V, the C-terminal part of the B-domain of FV may display a surface-exposed loop important for the interaction with APC. This is consistent with the fact that protein-protein interactions involve essential contacts through surface-exposed loops. After activation, the B-domain is removed and the region surrounding Arg-1545 could undergo conformational changes. During this process, this region loses some key residues that should be important for proper interaction with APC. In FV des-709–1476, where residues 1477–1545 from the B-domain are present between the A2- and A3-domains, the negatively charged loop may adopt a conformation similar to the one expected in intact FV, explaining why this construct expresses APC-cofactor activity.

FV mutants which are cleaved at one or more of the thrombin cleavage sites are equivalent to naturally occurring activation intermediates. The procoagulant properties of these FV activation intermediates have been measured in a prothrombinase assay using purified components (18). The same factor V species are now characterized with respect to their procoagulant activities. Mutants expressing the highest procoagulant activities are those having intact B-A3 junction, and they lose their anticoagulant activities upon cleavage at Arg-1545. Cleavage at Arg-1545 converts FV from an anticoagulant to a predominantly procoagulant species. This is consistent with the burst of thrombin seen in plasma concomitant with the Arg-1545 cleavage (46).

FV and FVAs bind to negatively charged phospholipids (47). Both forms of FV have been shown to interact with APC on the membrane surface, and the presence of FVVs increases the affinity of APC for the membrane 10-fold (48). Protein S interacts with both APC and FVAs on the membrane, and it is reasonable to suggest that the FVIIIA-degrading activity is associated with a multimeric phospholipid-bound complex formed by APC, protein S, and FV. In this report we have taken the first steps on a path leading to the elucidation of structure-function relationships for the anticoagulant activity of FV by demonstrating that an intact C-terminal region of the B-domain is crucial for expression of APC-cofactor activity.

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