Role of Hirudin-like Factor Va Heavy Chain Sequences in Prothrombinase Function*

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Proexosite I on prothrombin has been implicated in providing a recognition site for factor Va within prothrombinase. To examine whether hirudin-like sequences (659–698) on the cofactor contribute to this interaction, we expressed and purified two-chain FVa derivatives that were intracellularly truncated at the C terminus of the heavy chain: FVa709 (des710–1545), FVa699 (des700–1545), FVa692 (des693–1545), FVa678 (des679–1545), and FVa658 (des659–1545). We found that FVa709, FVa699, FVa692, and FVa678 exhibited specific clotting activities that were comparable with plasmaspecified and recombinant FVa. Additionally, kinetic studies using prothrombin revealed that the $K_\text{m}$ and $k_{\text{cat}}$ values for these derivatives were unaltered. Fluorescent measurements and chromatography studies indicated that FVa709, FVa699, FVa692, and FVa678 bound to FXa membranes and thrombin-agarose in a manner that was comparable with the wild-type cofactors. In contrast, FVa658 had an ~1% clotting activity and reduced affinity for FXa membranes (20-fold) and did not bind to thrombin-agarose. Surprisingly, however, FVa658 exhibited essentially normal kinetic parameters for prothrombin when the variant was fully saturated with FXa membranes. Overall our results are consistent with the interpretation that any possible binding interactions between prothrombin and the C-terminal region of the FVa heavy chain do not contribute in a detectable way to the enhanced function of prothrombinase.

Blood coagulation factor Va (FVa) is a heterodimeric protein composed of a heavy chain (residues 1–709; 105 kDa) and a light chain (residues 1546–2196; 74 kDa) which arises from limited proteolysis of the pro-cofactor factor V (FV) (1). Factor Va reversibly associates with the serine protease factor Xa (FXa) on an appropriate membrane surface in the presence of calcium ions to form prothrombinase. This enzyme complex cleaves two peptide bonds in prothrombin, resulting in the generation of α-thrombin (IIa) (2). This reaction is also catalyzed by membrane-bound FXa; however, incorporation of FVa within prothrombinase has a profound effect on the rate of IIa generation. Additionally, prothrombinase has remarkable specificity, as its only known biological substrate is prothrombin (2).

Although the molecular basis underlying the rate-enhancing effect of FVa remains largely unknown, recent contributions have provided insight into how prothrombinase recognizes its protein substrate (3–5). These studies support a model in which binding specificity is determined by two resolvable steps involving an interaction at an exosite followed by active site docking. These exosites have yet to be fully defined, but there is some evidence that at least part of the enzyme exosite lies on the catalytic domain of FXa (6–8). Because it is well established that prothrombin binds the cofactor (9–16), this interaction may also play an important role in substrate recognition.

There is increasing evidence that catalytic domain structures on prothrombin provide an exosite docking surface for prothrombinase (3, 17, 18). A series of observations suggest that the proexosite I region, the precursor site to IIa exosite I (19), contributes either directly or indirectly to this substrate binding site. For example, reagents that block proexosite I such as hirugren and bothrojaracin inhibit macromolecular substrate cleavage by prothrombinase or the FXa-FVa complex (3, 20, 21). Additionally, proexosite I prothrombin-1 mutants and a naturally occurring prothrombin variant (Arg2 to His, chymotrypsin numbering 18) (22) are poor substrates for prothrombinase and FXa-FVa (23, 24).

Interestingly, these probes or mutations reduced the rate of IIa generation only in the presence of the cofactor, suggesting a link between FVa and proexosite I. Consistent with this, IIa exosite I mutants and exosite I probes inhibit FX activation (25–27). Additionally, detailed studies by Bock and co-workers have provided compelling evidence that the FVa heavy chain binds to exosite I of IIa (26, 28).

Taken together, these data are consistent with the idea that proexosite I plays an important role in macromolecular substrate recognition by facilitating productive interactions between FVa and prothrombin.

Based on these findings it is reasonable to hypothesize that the corresponding binding site on FVa for proexosite I is found within the hirudin-like C-terminal region of the FVa heavy chain (659–698; Fig. 1). Evidence to support this derives from recent experiments employing synthetic FVa peptides. One of these peptides (DYDYQ; 695–699, see Fig. 1), like hirugren, was found to bind specifically to IIa and inhibit prothrombin activation, suggesting this region of FVa provides a substrate binding site within prothrombinase (29, 30). In support of this, changing DYDY to KKF (FVa2K2F) significantly reduced FVa cofactor activity within prothrombinase (30). However, it is unlikely that these data provide a complete explanation since proteolysis within the C-terminal FVa heavy chain region by a variety of enzymes only modestly reduces cofactor function in certain assay systems (29, 31–34).

To evaluate the importance of this region of FVa to prothrombinase function in more detail, we have employed a novel strategy to express recombinant FVa derivatives with specific truncations within the C-ter-

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‡§1The abbreviations used are: FVa, activated factor V; FV, factor V; FXa, factor Xa; IIa, α-thrombin; BSA, bovine serum albumin; FPR, α-phenylalanyl-1-prolyl-1-arginine; PCPS, small unilamellar phospholipid vesicles composed of 75% (w/w) phosphatidylcholine and 25% (w/w) phosphatidylserine; rFV-DT, recombinant partial B-domainless (des811–1491) factor V; PD-FV, plasma-derived factor V; O\textsubscript{Gay}-FXa, factor Xa modified with Oregon Green\textsubscript{488}; rFVa709, recombinant FV with amino acids 710–1545 deleted; rFVa699, amino acids 700–1545 deleted; rFVa692, amino acids 693–1545 deleted; rFVa678, amino acids 679–1545 deleted; rFVa658, amino acids 659–1545 deleted; MOPS, 4-morpholinopropanesulfonic acid; PACE, paired basic amino acid cleaving enzyme; PT, prothrombin time.
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minus of the FVa heavy chain and used a series of physical and kinetic measurements to assess the contribution of this region to substrate binding within prothrombinase.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin (BSA) and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Sigma. Hippuryl-L-phenylalanine-L-pipeolcoyl-L-arginyl-p-nitroanilide (S2238) was purchased from Diapharma Group, Inc. (West Chester, OH), and its concentration was verified (in water) using \( E_{1%}^\text{nm} = 8270 \text{ M}^{-1} \text{ cm}^{-1} \) (35). Oregon Green\textsubscript{488} maleimide and succinimidyl acetothioacetate were from Molecular Probes (Eugene, OR).

Branch at Galveston.

N-terminal sequence analysis was performed in the laboratory of Dr. Dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide was purchased from HemeTools (Essex Junction, VT). All tissue culture reagents were from Roche Applied Science.

EXPERIMENTAL PROCEDURES

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Calbiochem. SulfoLink coupling gel was purchased from Pierce. Dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide was purchased from HemeTools (Essex Junction, VT). All tissue culture reagents were from Invitrogen except insulin-transferrin-sodium selenite, which was from Roche Applied Science.

—Human prothrombin, FX, and FV were isolated from human blood by established procedures (40). A recombinant B-domainless form of FV (rFV-DT) used to prepare rFVa was expressed and purified as previously described (41).

—Plasmids encoding each of the rFVa constructs were transfected into baby hamster kidney cells, and stable clones were established essentially as described (41). Protein expression levels varied from 1 to 4 μg/10⁶ cells/24 h. Each of the rFVa derivatives was expanded into triple flasks, and approximately 1 liter of conditioned media was harvested daily for 4 days and immediately loaded onto a SP-Sepharose (Amersham Biosciences) column equilibrated in 20 mM Hepes, 0.15 mM NaCl, 5 mM CaCl₂, pH 7.4. The column was washed with the same buffer and then eluted with 0.65 mM NaCl. Fractions containing FVa activity were pooled and concentrated by ultrafiltration (Millipore), and the buffer was exchanged to reduce the NaCl concentration. The protein was then loaded onto a Poros HS/20 (0.46 × 10 cm) column equilibrated with 20 mM Hepes, 0.15 mM NaCl, 5 mM CaCl₂, pH 7.4. Bound protein was eluted with a gradient of increasing NaCl (0.15–1.0 M). The final yield was ~0.5–2.0 mg of rFVa/liter of conditioned media, and the purified protein was stored at −80°C. Protein purity was assessed by SDS-PAGE using pre-cast 4–12% gradient gels (Invitrogen) under reducing conditions using the MOPS buffer system followed by staining with Coomassie Brilliant Blue R-250.

—Factor Va (200 nM) derivatives were prepared in assay buffer. Samples were then diluted to less than 1 nM in assay buffer with 0.1% albumin and specific clotting activity using FV-deficient plasma (George King Bio-medical Inc., Overland Park, KS) was performed as described (33).

—Samples (2.5 ml) in assay buffer were maintained at 25°C in 1 × 1 cm² stirred quartz cuvettes, and steady state fluorescence intensity was measured using λ\textsubscript{ex} = 480 nm and λ\textsubscript{em} = 520 nm with a long pass filter (KV500, Schott, Duryea, PA) in the emission beam. Measurements, including controls, were performed essentially as described (3, 6).

—Kinetics of Protein Substrate Cleavage—Steady state initial velocities of macromolecular substrate cleavage by prothrombinase were determined discontinuously at 25°C as described (17, 45). The kinetic parameters of prothrombinase-catalyzed prothrombin, prothrombin-2/fragment 1.2, or prothrombin-1 activation (\( k_\text{cat} \) and \( V_\text{max} \)) were determined in assay buffer by measuring the initial rate of Ila formation at increasing concentrations of macromolecular substrate. Assay mixtures contained PCPS (20 or 50 μM), FVa (20 nM), and various concentrations of prothrombin (0–1.5 μM), prothrombin-2/fragment 1.2 (0–1.5 μM; 1.5 molar excess of fragment 1.2), or prothrombin-1 (0–12 μM). The reaction was initiated with 0.1 nM FXa for prothrombin and prothrombin-2/fragment 1.2 or 0.5 nM FXa for prothrombin-1.

Initial rates of prothrombin activation in the absence of membranes were determined essentially as described (20). Reaction mixtures in assay buffer contained FVa (5 nM; 200 nM for rFVa\textsubscript{658}) and various

TTTCTCCTAATTCTCTATGCTG–3’ in which the first 18 bases correspond to cDNA sequence coding for a PACE/furin recognition sequence (RKRRKR), and the last 18 bases correspond to cDNA coding for residues 709–704; primer C, 5′-AGAAAGCTGTA-GAAACGGCAGCAATGGAACAGAAGA-3′ in which the first 18 bases correspond to cDNA sequence coding for RKRRKR and the last 21 bases correspond to cDNA sequence coding for residues 1546–1552; primer D, 5′-TCTGTCATGATGAAATGG-3′, which corresponds to the FV cdna sequence coding for residues 1877–1871. The resulting DNA fragment was digested with Bsu36I and SnaBl, gel-purified, and subcloned into pE-D-FV digested with the same enzymes. To ensure the absence of polymerase-induced errors, the entire modified cDNA was sequenced. The remaining constructs were prepared in the same way, except primer B was appropriately changed.

Expression and Purification of rFVa Derivatives—Plasmids encoding each of the rFVa constructs was transfected into baby hamster kidney cells, and stable clones were established essentially as described (41). Protein expression levels varied from 1 to 4 μg/10⁶ cells/24 h. Each of the rFVa derivatives was expanded into triple flasks, and approximately 1 liter of conditioned media was harvested daily for 4 days and immediately loaded onto a SP-Sepharose (Amersham Biosciences) column equilibrated in 20 mM Hepes, 0.15 mM NaCl, 5 mM CaCl₂, pH 7.4. The column was washed with the same buffer and then eluted with 0.65 mM NaCl. Fractions containing FVa activity were pooled and concentrated by ultrafiltration (Millipore), and the buffer was exchanged to reduce the NaCl concentration. The protein was then loaded onto a Poros HS/20 (0.46 × 10 cm) column equilibrated with 20 mM Hepes, 0.15 mM NaCl, 5 mM CaCl₂, pH 7.4. Bound protein was eluted with a gradient of increasing NaCl (0.15–1.0 M). The final yield was ~0.5–2.0 mg of rFVa/ liter of conditioned media, and the purified protein was stored at −80°C. Protein purity was assessed by SDS-PAGE using pre-cast 4–12% gradient gels (Invitrogen) under reducing conditions using the MOPS buffer system followed by staining with Coomassie Brilliant Blue R-250.

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Initial rates of prothrombin activation in the absence of membranes were determined essentially as described (20). Reaction mixtures in assay buffer contained FVa (5 nM; 200 nM for rFVa\textsubscript{658}) and various
Concentrations of prothrombin (0–15 μM). The reaction was initiated with 10 nM FXa, and the rate of thrombin generation was measured as described (17, 45).

Characterization of Heavy Chain Truncated FVa Variants—To directly assess the contribution of hirudin-like FVa sequences (659–698) to cofactor function, the entire B-domain and specific heavy chain sequences were removed, and a PACE-furin cleavage site was introduced between these deleted sequences and the light chain generating rFVa658 (des659–1545), the hirudin-like FVa sequences (659–698) to cofactor function, the

Characterization of Heavy Chain Truncated FVa Variants—Western blot analysis of condition media derived from stable cell lines revealed that each variant was intracellularly processed and secreted in the two chain form (data not shown). These derivatives were subsequently purified to homogeneity by ion exchange chromatography. SDS-PAGE analyses (Fig. 2) indicated that the proteins migrated according to the expected molecular weights of the standards are indicated on the left. The gel was run for an extended period of time to maximize separation of the heavy chains.

RESULTS

Construction of rFVa Variants—Directly assess the contribution of hirudin-like FVa sequences (659–698) to cofactor function, the entire B-domain and specific heavy chain sequences were removed, and a PACE-furin cleavage site was introduced between these deleted sequences and the light chain generating rFVa658 (des659–1545), rFVa678 (des679–1545), rFVa692 (des693–1545), rFVa699 (des700–1545), and rFVa709 (des710–1545; see Fig. 1). Recombinant FVa709 served as a control as it should be equivalent to PD-FVa and rFVa. This system takes advantage of the endogenous intracellular proteolytic processing machinery allowing for internally cleaved two-chain FVa variants to be secreted into the cell culture media. We used this strategy to overcome potential problems in the activation of the deletion variants by IIa or RVV-V (factor V, cleaving protease isolated from Russell’s viper venom) because of the elimination of possible enzyme binding sites contributed by heavy chain and B-domain sequences.

Characterization of rFVa Variants—Western blot analysis of condition media derived from stable cell lines revealed that each variant was intracellularly processed and secreted in the two chain form (data not shown). These derivatives were subsequently purified to homogeneity by ion exchange chromatography. SDS-PAGE analyses (Fig. 2) indicated that the proteins migrated according to the expected molecular weight. N-terminal sequence analysis of the light chains for each variant showed that the PACE-furin cleavage site was completely...
Characterization of Heavy Chain Truncated FvAs

<table>
<thead>
<tr>
<th>Cofactor species</th>
<th>Specific activity ± S.D.*</th>
<th>Initial velocity*</th>
<th>Amino-terminal sequence†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-FVa</td>
<td>1960 ± 380</td>
<td>145</td>
<td>Not determined</td>
</tr>
<tr>
<td>rFVa709</td>
<td>1950 ± 350</td>
<td>153</td>
<td>SNNGNRRNYY</td>
</tr>
<tr>
<td>rFVa699</td>
<td>1920 ± 330</td>
<td>158</td>
<td>SNNGNR–NYY</td>
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<tr>
<td>rFVa678</td>
<td>1530 ± 210</td>
<td>171</td>
<td>SNNGN—NYY</td>
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<tr>
<td>rFVa658</td>
<td>1650 ± 240</td>
<td>191</td>
<td>SNNG—NYY</td>
</tr>
<tr>
<td>rFVa658</td>
<td>2230 ± 450</td>
<td>198</td>
<td>SNNGN—N–Y</td>
</tr>
<tr>
<td>rFVa658</td>
<td>20 ± 6</td>
<td>33</td>
<td>SNNGNRRNYY</td>
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</table>

* Specific clotting activity was determined by a FV-specific PT-based clotting assay as described under “Experimental Procedures.” The data represent the average of three measurements, and the error represents the S.D.

† Initial steady state rates were determined in assay buffer using the indicated cofactors (0.1 nM) plus 1.4 μM prothrombin and 20 μM PCPS. The reaction was initiated with 1.0 nM FXa. Aliquots of the reaction mixture were removed at 0.5, 1.0, 1.5, 2.0, and 3.0 min, and IIa generation was monitored as described under “Experimental Procedures.”

‡ N-terminal sequence analysis was determined on the light chain. The expected N-terminal sequence is SNNGNRRNYY. The dash (–) indicates that the yield was too low to accurately assign an amino acid.

removed, as the N terminus was found to be SNNGNRRN (Table 1). Because it has been previously shown that baby hamster kidney cells process C-terminal basic amino acids from the light chain of protein C (52) and rFVa709 appears functionally equivalent to plasma-derived rFVa (see Table 1), we speculate that the RKRRKR sequence is removed from the C-terminal region of the heavy chain, most likely by an Arg/Lys carboxypeptidase (53–55).

Assessment of Specific Activity—Initial characterization of the FVa derivatives using a one-stage PT-based clotting assay with FV-deficient plasma indicated that rFVa709, rFVa699, rFVa692, and rFVa678 had specific clotting activities that were comparable with wild-type FVa (Table 1). These data show that removal of amino acids 679–709 has little if any impact on cofactor function in this assay system. These results deviate somewhat from previous reports which found a ~50–60% reduction in clotting activity using FVa cleaved by proteases within the C-terminal region of the heavy chain (29, 33). Interestingly, further truncation to position 658 resulted in a substantial reduction in activity. To verify the results of the clotting assay, a prothrombinase assay employing purified reagents was established. Progress curves of the conversion of prothrombin to IIa using limiting amounts of cofactor showed that rFVa658 had reduced cofactor activity, whereas the other variants had activity profiles similar to wild-type FVa (Table 1). These data indicate that 1) acidic region 2 (679–709) does not influence in an obvious way FVa cofactor function within prothrombinase, and 2) deletion of residues 659–709 impairs assembly of FvAs in prothrombinase or otherwise alters cofactor activity.

FVa Binding to IIa-agarose—Studies with immobilized IIa and kinetic inhibition experiments with (pro)exosite I probes indicate that IIa and prothrombin likely share a common mode of binding to FVa (20, 28). Affinity chromatography studies were used to qualitatively assess whether the truncated FVa variants bind IIa. As shown in Fig. 3, with the exception of rFVa658, each derivative was retained by the IIa-agarose column and eluted after the addition of 2 M NaCl. Electrophoretic analysis of the samples after chromatography did not reveal any significant proteolytic degradation (data not shown). None of the FvA constructs bound to a BSA-agarose column used to test nonspecific binding (data not shown). The data demonstrate that acidic region 2 (679–709) does not provide a binding site for IIa, a surprising contrast to conclusions obtained from peptide inhibition studies (29, 30). Although the results with rFVa658 indicate that acidic region 1 (659–678) may provide a binding site for IIa, we cannot rule out the possibility that this derivative is altered in some other fashion precluding its binding to IIa-agarose. Overall the chromatography data correlate well with the functional measurements (Table 1) in terms of which variant is altered. However, close inspection of the functional data reveals that there is a discrepancy in the magnitude of the reduction between the two assay systems for rFVa658. This discrepancy suggests that the underlying nature of the reduced cofactor activity of this variant is complex and likely reflects alterations to multiple aspects of cofactor function.

Binding of the Cofactors to FXa Membranes—To examine this further we evaluated the ability of each of the FvA variants to bind FXa membranes. Equilibrium fluorescence measurements were used to establish binding parameters describing the interaction between the cofactor and membrane-bound FXa. Using a fixed concentration of OG488-FXa, subsequent titration with incremental additions of PD-FvAs, rFva709, rFva699, rFva692, or rFva678 (Fig. 4) yielded a saturable increase in fluorescence intensity with comparable dissociation constants ($K_d$) and stoichiometries (n) (Table 2). These data indicate that complete removal of acidic region 2 (see Fig. 1) does not detectably influence binding interactions with FXa membranes. Similar results were obtained from kinetic assessment of cofactor binding to FXa membranes (data not shown). In contrast, rFva658 bound with a reduced affinity (~20-fold) to FXa membranes. This finding implies that amino acids within acidic region 1 (659–678) somehow play a role in the high affinity interaction with FXa membranes. The simplest interpretation is that these residues provide a primary binding site. An alternative expla-
nation is that structural changes that accompany deletion of these residues adversely impact FXa binding. It is also possible that these same structural changes influence Ila binding (Fig. 3). The decreased affinity of rFVa658 for FXa membranes explains at least in part the decreased structural changes influence IIa binding (Fig. 3). The decreased affinity of the fragment 1 or fragment 1.2 domains revealed differences in the hirudin-like sequences on the FVa heavy chain did not significantly influence the kinetics of prothrombin activation, initial velocity studies were conducted with prothrombin in the absence of membranes (FXa-FVa) or with prothrombin-1 and prothrombinase. Consistent with the results presented above, in the absence of anionic membranes each of the recombinant variants, including rFVa699, had a Km for prothrombin that was comparable with wild-type FVa (Table 3). Because the conditions chosen for this experiment do not permit reliable calculation of the reaction, suggesting that the fractional saturation of FXa with each of the FVa species was similar. The reduced Vmax, obtained with rFVa658 likely reflects its reduced affinity for FXa (Fig. 4). Similar to results obtained with prothrombinase (Table 3), the data indicate that removal of hirugen-like sequences from the C terminus of the FVa heavy chain does not alter prothrombin binding to the FXa-FVa complex. In contrast to these results, differences in the initial rates of Ila generation were observed with each of the truncated FVas derivatives when using prethrombin-1. Measurements of the conversion of varying concentrations of prethrombin-1 to Ila were conducted using fixed, saturating amounts of membranes and cofactor. The deviations in rate principally arose from an increased Kcat value (~5–10-fold) for rFVa699, rFVa678, and rFVa658 (Table 3). Factor Va658 had a 15-fold increase in Km and a ~4.5-fold reduced kcat. Similar results were obtained with prethrombin-2 (data not shown). Interestingly, saturation of prethrombin-2 with fragment 1.2 completely restored the Km values to that seen with wild-type FVas (data not shown). Thus, elimination of the fragment 1 or fragment 1.2 domains revealed differences in cofactor function after removal of even a small portion of the heavy chain (rFVa699). These differences in the Km, however, are relatively small considering that sequences within the C terminus of the FVa heavy chain were completely removed.

DISCUSSION

It has been previously demonstrated that hirugen inhibits Ila generation only when using FXa-FVa or when using a non-membrane-bound protein substrate such as prethrombin-1 (3, 20). Because eliminating hirudin-like sequences on the FVa heavy chain did not significantly influence the kinetics of prothrombin activation, initial velocity studies were conducted with prothrombin in the absence of membranes (FXa-FVa) or with prothrombin-1 and prothrombinase. Consistent with the results presented above, in the absence of anionic membranes each of the recombinant variants, including rFVa699, had a Km for prothrombin that was comparable with wild-type FVa (Table 3). Because the conditions chosen for this experiment do not permit reliable calculation of the rate constant of prethrombin with fragment 1.2 completely restored the Km values to that seen with wild-type FVas (data not shown). Interestingly, saturation of prethrombin-2 with fragment 1.2 completely restored the Km values to that seen with wild-type FVas (data not shown). Thus, elimination of the fragment 1 or fragment 1.2 domains revealed differences in cofactor function after removal of even a small portion of the heavy chain (rFVa699). These differences in the Km, however, are relatively small considering that sequences within the C terminus of the FVa heavy chain were completely removed.

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DISCUSSION

It is now well established that exosite interactions play a principal role in macromolecular substrate recognition by prothrombinase (5).
Characterization of Heavy Chain Truncated FVas

Although extended surfaces on FXa are at least partly involved (6–8, 56), there is increasing evidence that productive binding interactions between prothrombin and FVa also play a role. Recent work suggests that the proexosite I region on the zymogen contributes to this recognition surface for FVa within prothrombinase (25–28). Furthermore, peptide inhibition studies have shown that sequences (695–699) within the hirudin-like C-terminal region of the FVa heavy chain may provide a binding site for prothrombin (29, 30).

Drawing on these ideas, we directly examined the importance of these cofactor sequences by engineering a series of variants lacking portions of the C terminus of the heavy chain. The current work provides strong evidence that any possible binding interaction between these cofactor sequences and prothrombin do not contribute in a detectable way to the enhanced function of prothrombinase. This is not to say that removal of amino acids 659–709 had no influence on cofactor function, since rFVa658 had reduced affinity for FXa membranes. This finding likely explains the reduction in cofactor activity for rFVa658 noted in Table 1 since both assays employed limiting amounts of FVa and were very sensitive to changes in the FXa-FVa interaction (1). Additionally, we were also able to show that whereas removal of residues 679–709 had no obvious influence on the FVa-IIa interaction, further truncation to position 658 abrogated binding to IIa-agarose. At present we cannot no obvious influence on the FVa-IIa interaction, further truncation to position 658 abrogated binding to IIa-agarose. At present we cannot

<table>
<thead>
<tr>
<th>Cofactor species</th>
<th>Enzyme/Substrate</th>
<th>Kₘ ± S.D.</th>
<th>kₐ₉ ± S.D.</th>
<th>Kₖ₉ ± S.D.</th>
<th>Vₘ₉ ± S.D.</th>
<th>Kₘ ± S.D.</th>
<th>Kₚ₉ ± S.D.</th>
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<tbody>
<tr>
<td>PD-FVa</td>
<td>Prothrombinase/Prothrombin</td>
<td>0.36 ± 0.02</td>
<td>2550 ± 55</td>
<td>2.0 ± 0.32</td>
<td>37 ± 1.3</td>
<td>5.9 ± 0.43</td>
<td>1080 ± 71</td>
</tr>
<tr>
<td></td>
<td>Prothrombinase/FXa</td>
<td>0.30 ± 0.02</td>
<td>2100 ± 44</td>
<td>2.0 ± 0.32</td>
<td>37 ± 1.3</td>
<td>5.9 ± 0.43</td>
<td>1080 ± 71</td>
</tr>
<tr>
<td>rFVa</td>
<td>Prothrombinase/Prothrombin</td>
<td>0.35 ± 0.02</td>
<td>2610 ± 52</td>
<td>3.5 ± 0.50</td>
<td>90 ± 3.7</td>
<td>6.3 ± 0.58</td>
<td>710 ± 32</td>
</tr>
<tr>
<td>rFVa699</td>
<td>Prothrombinase/Prothrombin</td>
<td>0.58 ± 0.02</td>
<td>3040 ± 55</td>
<td>1.5 ± 0.20</td>
<td>33 ± 1.0</td>
<td>40 ± 7.7</td>
<td>610 ± 95</td>
</tr>
<tr>
<td>rFVa692</td>
<td>Prothrombinase/Prothrombin</td>
<td>0.44 ± 0.01</td>
<td>2830 ± 33</td>
<td>1.2 ± 0.22</td>
<td>34 ± 1.3</td>
<td>61 ± 18</td>
<td>940 ± 250</td>
</tr>
<tr>
<td>rFVa692</td>
<td>Prothrombinase/Prothrombin</td>
<td>0.40 ± 0.02</td>
<td>3300 ± 74</td>
<td>1.6 ± 0.30</td>
<td>45 ± 1.8</td>
<td>28 ± 2.9</td>
<td>1000 ± 82</td>
</tr>
<tr>
<td>rFVa658</td>
<td>Prothrombinase/Prothrombin</td>
<td>0.33 ± 0.02</td>
<td>1650 ± 35</td>
<td>1.8 ± 0.40</td>
<td>10 ± 0.6</td>
<td>87 ± 33</td>
<td>200 ± 71</td>
</tr>
</tbody>
</table>

* The Vₘ₉ of the reaction is given rather than the Kₘ₉ since limiting amounts of FVa were used, and thus, the total enzyme concentration is not known with certainty. The solution phase dissociation constant for FXa-FVa is ~10⁻⁶ (59). The reaction was performed at 10 nM FXa and 5 nM FVa, with the exception of rFVa658, which was used at 200 nM. Asp 697→Lys, Tyr 699→Phe) rFVa62K2F had markedly reduced activity (>20-fold) in conditioned media compared with wild-type FVa (30). It should be pointed out, however, that the molecular basis underlying this reduction was not determined. In support of these data, the hirudin-like peptide DYDYQ was found to inhibit prothrombin activation when using prothrombinase as the enzyme. This observation, however, deviates from results obtained with bothrojaracin and hirugen. These proexosite I probes were only effective inhibitors if substrate-membrane binding was eliminated or if negatively charged phospholipids were omitted from the reaction (20, 21). It was suggested that assembly of FXa-FVa-prothrombin on phospholipid membranes somehow counteracted the effectiveness of the inhibitors. This implies that data obtained with DYDYQ cannot be interpreted in a way that is equivalent to these proexosite I probes. These observations together with the results of the current study cast doubt on the importance of FVa residues 659–709 in providing a productive binding site for prothrombin within prothrombinase.

Although removal of the FVa hirudin-like heavy chain region had little influence on prothrombin activation, this was not the case when using prethrombin-1. A significant difference in the rate of IIa generation was found with each of the truncated FVa variants fully assembled into prothrombinase. Surprisingly, the effect on the Kₘ₉ (4–14-fold) was similar for each variant with no obvious relationship between the magnitude of the change and the length of the heavy chain remaining. The significance and nature of this change in substrate recognition and how it relates to the mechanism of prothrombin recognition by prothrombinase is not clear. The data are consistent with the conclusion that the C terminus of the FVa heavy chain somehow plays a role in prothrombin-1 substrate recognition. These deviations in the Kₘ₉, however, were not related to the absence of substrate membrane binding as indistinguishable Kₘ₉ values for prothrombin were obtained with each of the variants using the FXa-FVa complex. This result was somewhat surprising considering that hirugen is known to effectively inhibit prethrombin-1 activation using prothrombinase and prothrombin activation in the absence of anionic membranes (20). Interestingly, recent data indicate that removal of fragment 1 or fragment 1.2 from prothrombin alters the environment of the protease domain, in particular the (pro)exosite I region (57, 58). It was suggested that modulation of (pro)exosite I affinity by the fragment 1 domain may affect interactions of prothrombin activation species with FVa (58). Whether our observations with the truncated FVa variants and prethrombin-1 are related to this is not clear, and more studies are needed to help better understand these observations.
In summary, the results of the current study challenge existing conclusions that hirudin-like sequences within the C-terminal heavy chain region of FVa provide a primary binding site for prothrombin. The deletion approach employed offered a direct assessment of FVa cofactor function and IIa binding. Analyzing loss of function data using either mutant proteins or peptides can be problematic because accounting for indirect effects is often difficult. On the other hand, maintenance of cofactor function has obvious interpretative advantages. We cannot rule out the possibility that removal of these heavy chain sequences was accompanied by favorable compensating effects. This scenario, however, requires invoking a secondary mechanism by which prothrombinase recognizes its macromolecular substrate. Our findings do not exclude a dominant role for proexosite I in macromolecular substrate recognition mediated by FVa within prothrombinase. Rather, the data show that this productive binding site on FVa cannot be attributed to the C terminus of the FVa heavy chain.

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