Inhibitory Sequences within the B-domain Stabilize Circulating Factor V in an Inactive State*

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Blood coagulation factor V circulates as a procofactor with little or no procoagulant activity. It is activated to factor Va by thrombin following proteolytic removal of a large central B-domain. Although this reaction is well studied, the mechanism by which bond cleavage and B-domain release facilitate the transition to the active cofactor state has not been defined. Here we show that deletion or substitution of specific B-domain sequences drives the expression of procoagulant function without the need for proteolytic processing. Conversion to the constitutively active cofactor state is related, at least in part, to a cluster of amino acids that is highly basic and well conserved across the vertebrate lineage. Our findings demonstrate that discrete sequences in the B-domain serve to stabilize the inactive procofactor state, with proteolysis primarily functioning to remove these inhibitory constraints. These unexpected results provide new insight into the mechanism of factor V activation.

Many proteins involved in hemostasis circulate in blood in a quiescent state and only express activity following proteolysis. One of these proteins, factor V (FV), is synthesized as a large multi-domain (A1-A2-B-A3-C1-C2) protein sharing homology with factor VIII (FVIII), except in the B-domain (1, 2). Factor V circulates as a procofactor and has minimal procoagulant activity (3, 4). Thrombin catalyzes the conversion of FV to FVa (FVa) following three cleavages (Arg709, Arg1018, and Arg1545), thereby releasing the heavily glycosylated B-domain that spans amino acids 710–1545 (5). Factor Va is a heterodimer consisting of a noncovalently associated heavy and light chain and functions as a cofactor within the prothrombinase complex. Because FV cannot function in prothrombinase, the proteolytic conversion of FV to FVa must result in structural changes necessary for function.

Most approaches aimed at understanding FV activation are principally based on correlating proteolysis within the B-domain with the expression of procoagulant activity (4, 6–17). These studies support the idea that bond cleavage at Arg1545 is required for the expression of full cofactor activity. However, how proteolysis directly contributes to the development of FVa cofactor activity is not well defined. An alternative way of looking at this problem is to evaluate how FV is preserved as an inactive procofactor. One possibility is that binding sites on the heavy and/or light chain that are important to cofactor function are in a conformational state that precludes factor Xa (FXa)/prothrombin binding. Proteolysis could then drive cofactor activation in a manner analogous to the activation strategy used by the chymotrypsin-like serine proteases, protease activated receptors, and fibrinogen (18–20). In this mechanism, bond cleavage is required to unmask new sequences, which then act to facilitate the necessary conformational change for activation (18). A second possibility is that B-domain sequences serve an inhibitory function by rendering binding sites on the heavy and/or light chain inaccessible to FXa or prothrombin. Proteolysis would then promote dissociation of inhibitory B-domain sequences effecting activation. Aspartic and cysteine proteases use this approach to control the inactivity of the zymogen (21). A signature feature of this strategy is the ability to constitutively activate the protein by removing inhibitory sequences in the absence of proteolysis. Evidence that FV may use this strategy comes from the expression of a FV derivative in which a large segment of the B-domain was deleted (FVΔ710–1545) or FV-810; see Scheme 1). This variant was shown to have significant procoagulant activity (13, 22), and more recent work indicates that FV-810 binds FXa membranes with high affinity and functions in an equivalent way to FVα in the absence of intentional proteolysis (23). These observations suggested that the B-domain must somehow interfere with binding interactions that govern the function of FVα. In the current study we used a series of activity and direct binding measurements to characterize a panel of progressively finer B-domain-truncated variants to explore this idea further and define regions within the B-domain that may be involved in stabilizing the FV procofactor state.

**EXPERIMENTAL PROCEDURES**

**Materials**—The peptidyl substrate H-D-phenylalanyl-L-pipe- colyl-L-arginyl-p-nitroanilide (S2238) was from Diapharma Group, Inc. (West Chester, OH). Benzamidine, bovine serum...
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albumin, and 4-amidinophenylmethanesulfonyl fluoride hydrochloride were purchased from Sigma. Dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide (DAPA) was from Hematologic Technologies (Essex Junction, VT). All of the tissue culture reagents were from Invitrogen except insulin-transferrin-sodium selenite, which was from Roche Applied Science. Small unilamellar phospholipid vesicles (PCPS) composed of 75% (w/w) hen egg l-α-phosphatidylcholine and 25% (w/w) porcine brain t-α-phosphatidylerine (Avanti Polar Lipids, Alabaster, AL) were prepared and characterized as described (24, 25). Oregon Green488 maleimide and succinimidyl acetothiogalactose were from Amersham Biosciences. Simplastin Excel was from BioMerieux (Durham, NC), and FV-deficient plasma was from George King Bio-medical Inc. (Overland Park, KS). Unless otherwise noted, all of the functional assays were performed at 25 °C in 20 mM HEPES, 0.15M NaCl, 2 mM CaCl2, 0.1% polyethyleneglycol 8000, pH 7.5 (assay buffer).

Proteins—Human prothrombin, FX, and FV were isolated from plasma as described previously (26–28). Thrombin, prethrombin-2, and FXa were prepared and purified by established procedures (29–31). Human plasma derived FV (PD-FV) and thrombin-2, and FXa were prepared and purified by established procedures (29–31). Oregon Green488-procFVa and rFVa as described (23, 32). Human plasma derived FV (PD-FV) and thrombin-2, and FXa were prepared and purified by established procedures (29–31). Human plasma derived FV (PD-FV) and thrombin-2, and FXa were prepared and purified by established procedures (29–31). Oregon Green488-procFVa and rFVa as described (23, 32). Human plasma derived FV (PD-FV) and thrombin-2, and FXa were prepared and purified by established procedures (29–31).

Molecular weights and extinction coefficients (ε280 nm) of the various proteins used were: prothrombin, 72,000 and 1.47 (29); prethrombin-2, 37,500 and 1.95 (29); thrombin, 37,500 and 1.94 (33); FXa, 45,300 and 1.16 (34); PD-FVa, 173,000 and 1.78 (23); rFXa, 175,000 and 1.78 (23); and FV-810, 216,000 and 1.54 (23), respectively. The molecular measured weights and extinction coefficients for the rFV derivatives were: FV-902, 229,000 and 1.55; FV-956, 232,000 and 1.59; FV-1033, 239,000 and 1.52; FV-1053, 241,000 and 1.50; FV-1106, 258,000 and 1.49; and FV-1152, 264,000 and 1.46, respectively. The molecular weights and extinction coefficients of FVBB-131, FVBB-104, and FVBB-46 were assumed to be the same as FV-1033.

Construction of Recombinant FV Derivatives—The Construction of FV-810 (see Scheme 1) has been previously described (23). Specific oligonucleotides used to generate FV-902 were as follows: primer A, 5’-GAAGAGGTGGAATCTCTG-3’ corresponds to the cDNA sequence encoding for amino acid residues 319 to 325; primer B, 5’-CTCAATGTAATCCTGTATCACTA-GAGGGTC-3’ in which the first 15 bases encode for residues 1877–1881. Primer C, 5’-CACCTCTCCAGTATACAGATATT-GAG-3’, in which the first 15 bases encode for residues 898–902 and the last 15 bases encode for residues 1492–1496; and primer D, 5’-TCTGTCATAGTAAAGATG-3’ corresponds to the FV cDNA sequence encoding for residues 1877–1871. The resulting DNA fragment was TOPO cloned (Invitrogen), then digested with Bsu36I and SnaBI, gel-purified, and subcloned into pED-FV digested with the same enzymes (23). To ensure the absence of polymerase-induced errors, the entire modified cDNA was sequenced. The remaining constructs outlined in Scheme 1 were prepared in the same way, except primers B and C were appropriately changed.

Expression and Purification of rFV Derivatives—Plasmids encoding each of the FV constructs were transfected into baby hamster kidney cells, and stable clones were established as described (23, 35). Protein expression levels varied from 0.5 to 4 μg/106 cells/24 h. Each of the FV derivatives was purified essentially as described (23) with a final yield of ~0.5–2 mg of protein/liter of conditioned medium. Protein purity was assessed by SDS-PAGE under reducing conditions followed by staining with Coomassie Brilliant Blue R-250.

**RESULTS**

**Expression of FV Derivatives**—We have previously shown that FV-810 is functionally equivalent to FVa (23). We hypothesized that reintroducing portions of the B-domain would yield a molecule with procofactor-like properties. Because the C-terminal half of the B-domain (i.e. residues 1019–1545) varies considerably in length or is absent among different vertebrates (41–43), we chose to extend the length of FV-810 by ~50–100-amino acid increments from the N-terminal side (Scheme 1). SDS-PAGE analyses indicated that each of the proteins...
migrated with the expected mobility (Fig. 1). Plasma-derived FV, PD-FVa, and rFVa used as controls are included on the gel. Following treatment with thrombin, the proteins migrated in the expected way on the gel yielding FVa. Additional control experiments revealed no significant difference in the rate of proteolysis of the variants compared with FV when using thrombin or FXa (data not shown). In both the clotting and purified component assays, pretreatment of each of these rFV derivatives with thrombin resulted in full activation (Tables 1 and 2).

**Characterization of FV Variants**—Initial experiments focused on the clotting activity of the B-domain variants prior to and following treatment with thrombin (Table 1). In this assay system, PD-FV has a low specific activity, which increases ~10-fold following thrombin activation. This specific activity is comparable with the levels observed for PD-FV, rFVa, and FV-810. Extending the B-domain length by adding amino acids 811–902 to FV-810 (resulting in FV-902) did not significantly change the specific clotting activity. A further increase in B-domain length to 378 amino acids (FV-1033) decreased the specific activity to levels seen for the procofactor, PD-FV. Extension beyond 378 amino acids (FV-1053, FV-1106, and FV-1152) was without functional consequence, because each of these variants had a procofactor-like specific activity. Factor V-956 had an intermediate specific activity (Table 1).

To verify the results of the clotting assay, a purified component assay was employed. Progress curves of the conversion of prethrombin-2 to thrombin using equimolar concentrations of (pro)cofactor (3 nM) and FXa (3 nM) yielded results that were consistent with the clotting assay. Thrombin increased linearly over time when using PD-FVa, rFVa, FV-810, and FV-902, and the initial rates of thrombin generation were within a factor of two (Table 2 and Fig. 2). Once again, FV-956 had reduced activity compared with FVa. In contrast, PD-FV, FV-1152, FV-1106, FV-1053, and FV-1033 had very little activity (Table 2 and Fig. 2). Furthermore, the progress curves were characterized by an obvious lag in thrombin generation, which is expected when the product further activates the procofactor (Fig. 2). Similar results were obtained when using prothrombin as the macromolecular substrate (data not shown). In both the clotting and purified component assays, pretreatment of each of these rFV derivatives with thrombin resulted in full activation (Tables 1 and 2).

**Binding of (Pro)Cofactors to FXa Membranes**—Based on the functional measurements, the B-domain-truncated FV derivatives could be grouped into two categories: cofactor-like (FV-810, FV-902, and FV-956) and procofactor-like (FV-1033, FV-1053, FV-1106, and FV-1152). Because FV binds poorly to active site-blocked FXa (23), we evaluated the ability of each variant 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 OG-FXa, subsequent titration with incremental additions of rFVa, FV-810, and FV-902 (Fig. 3) yielded a saturable increase in fluorescence intensity with comparable dissociation constants ($K_d$) and stoichiometries ($n$) (Table 1). Recombinant FV-956 bound with a 5–10-fold reduced affinity, which possibly accounts for the decreased cofactor activity observed in the functional measurements (Table 1), because both of these assays use limiting concentrations of cofactor. In contrast, no significant increase in fluorescence intensity was observed when using PD-FV or FV-1033 (Fig. 3). Similar results were obtained with FV-1053, FV-1106, and FV-1152 (data not shown). Treatment of PD-FV, FV-1033, FV-1053, FV-1106, and FV-1152 with thrombin followed by assessment of direct binding yielded dissociation constants consistent with FVa (data not shown).
TABLE 1
Characterization of B-domain-truncated FV variants

| Cofactor species | Specific activity\(a\) | Specific activity\(b\) | \(K_d\) \(b\) | \(n^c\) |
|------------------|--------------------------|------------------------|----------------|-------|
|                  | units/mmol               | units/mmol             | nM             | nM/mol|
| PD-FVa           | 235 ± 40                 | NA\(d\)                | 0.50 ± 0.08    | 1.2 ± 0.04 |
| rFVa             | 215 ± 26                 | NA                     | 0.90 ± 0.13    | 1.0 ± 0.03 |
| FV-810           | 220 ± 17                 | 213 ± 6.5              | 0.96 ± 0.17    | 1.1 ± 0.05 |
| FV-902           | 210 ± 9.0                | 206 ± 23               | 0.81 ± 0.13    | 1.1 ± 0.04 |
| FV-956           | 74.1 ± 6.7               | 187 ± 6.1              | 5.2 ± 1.0      | 0.98 ± 0.09 |
| FV-1033          | 17.5 ± 1.9               | 233 ± 33               | –              | –     |
| FV-1053          | 13.0 ± 1.1               | 210 ± 11               | –              | –     |
| FV-1106          | 13.6 ± 1.3               | 206 ± 37               | –              | –     |
| FV-1152          | 14.9 ± 3.2               | 165 ± 16               | –              | –     |
| PD-FV            | 19.4 ± 2.5               | 172 ± 20               | –              | –     |

\(a\) The mean values ± S.D. are presented from three determinations. The data are representative of two to three similar experiments.
\(b\) Activated with thrombin prior to the addition in the prothrombinase assay.
\(c\) Reported errors represent ± 2 S.D. The data are representative of two to three similar experiments.
\(d\) NA, not applicable.

\(n\), not applicable.

shown), indicating that once activated each of the variants can assemble within prothrombinase. Consistent with our previous findings (23), control experiments indicated that each of the FV variants was not cleaved during the binding measurements (data not shown; see Fig. 5B for FV-810 and FV-1033).

**Contribution of Specific B-domain Sequences to Preserving the Procofactor State**—The findings suggest that either specific sequences (residues 902–1033) or a specific length (≈378 residues) of the B-domain somehow plays a role in the mechanism by which FV is maintained as a procofactor. To discriminate between these possibilities, we constructed additional FV variants that retained a B-domain length of 378 amino acids but that had portions of residues 900–1030 exchanged with FVIII B-domain sequences that share no homology with sequences within the FV B-domain.

Using the procofactor-like FV-1033 as a scaffold, three derivatives were prepared with B-domain lengths of 378 amino acids: FVB8–131, FVB8–104, and FVB8–46, representing 131, 104, and 46 amino acids from the FV B-domain exchanged with FVIII B-domain (Fig. 4). We chose regions of the FVIII B-domain that did not contain known thrombin or intracellular furin cleavage sites (44). As expected, SDS-PAGE revealed that FVB8–131, FVB8–104, and FVB8–46 have apparent molecular weights equivalent to FV-1033 (Fig. 4). Following incubation with thrombin, each of the derivatives was processed to yield the expected heavy and light chains, and the rate of proteolysis was comparable with FV (data not shown).

By using either a purified component assay (data not shown) or a one-stage prothrombin time-based clotting assay or by monitoring direct binding to FXa membranes, we found that each of these variants had properties most consistent with the cofactor-like form (Table 3 and Fig. 5A). Control experiments revealed that the variants were not cleaved during the course of the binding measurements (Fig. 5B). This indicates that proteolysis of the variants is not a requirement for high affinity binding to FXa membranes. When the variants were incubated with thrombin, their specific activities increased only 1.5-fold...
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FIGURE 4. Schematic representation and SDS-PAGE analysis of FV-1033 and derivatives. Left panel, schematic representation. The gray bar represents the B-domain, which is 378 amino acids for each variant. The blue bar represents amino acids 900–1030 of the B-domain with the entire sequence shown above. The hatched bars represent FVIII sequences that have been exchanged with FV B-domain sequences. Sequences removed and inserted are shown on the right. Right panel, SDS-PAGE analysis. Purified proteins (4 μg/lane) were subjected to SDS-PAGE and visualized by staining with Coomassie Blue R-250. Lane 1, PD-FV; lane 2, FV-1033; lane 3, FVB8–131; lane 4, FVB8–104; lane 5, FVB8–46; lane 6, FV-810; lane 7, rFVa; lane 8, PD-FVa. The apparent molecular weights of the standards are indicated on the left.

TABLE 3
Characterization of FV-1033 derivatives

| Cofactor species | Specific activitya | Specific activityb | Ka-c | n° |
|------------------|-------------------|-------------------|------|----|
| FV-1033a | 17.5 ± 1.9 | 222 ± 33 | – | – |
| FVB8–131 | 155 ± 16 | 226 ± 35 | 2.1 ± 0.7 | 92 ± 0.9 |
| FVB8–104 | 158 ± 29 | 236 ± 40 | 1.9 ± 0.5 | 10 ± 0.1 |
| FVB8–46 | 161 ± 14 | 235 ± 42 | 2.5 ± 0.5 | 99 ± 0.9 |

a The mean values ± S.D. are presented from three determinations. The data are representative of two to three similar experiments.
b Activated with thrombin prior to the addition to the clotting assay.
c The reported errors represent ± 2 S.D. The data are representative of two to three similar experiments.
d The data for FV-1033 are for reference and are from TABLE 1.
e “–” indicates that we were not able to accurately determine a value.

DISCUSSION

Previous approaches aimed at better understanding FV activation have primarily relied on activity measurements using proteolytic fragments or FV variants that were subjected to limited proteolysis (4, 6–17, 45–47). The inherent complexity in preparing well-defined products using proteolysis and associated problems with activity measurements (i.e., preventing feed-back activation) impose limitations on this approach. To circumvent this, we used FV B-domain-truncated variants in conjunction with activity and direct binding measurements. The strengths of this approach are: 1) well-defined purified products were used; 2) proteolytic processing was not necessary to assess the functional state of the variants; 3) direct binding measurements were used to confirm activity assays; and 4) the variants analyzed were not loss of function, because they either had constitutive procoagulant activity or could be fully activated to FVa. Thus, by using this strategy, we were able to directly evaluate the role of specific regions of the FV B-domain in preserving the procofactor state.

The results obtained by employing this approach support the conclusion that discrete regions of the FV B-domain play an important role in stabilizing the procofactor state. Our findings are consistent with a model in which the B-domain contributes steric and/or conformational constraints that interfere with structural determinants governing the function of the active cofactor species. Because of its size, it is unlikely that a single region of the B-domain provides this inhibition. Rather, it is likely that other components also play a substantial role; however, the variants analyzed in the current study only revealed one of the major contributors to this process. Our data suggest that the role of proteolysis in FV activation is to facilitate removal of these inhibitory B-domain sequences in a release from inhibition mechanism. Interestingly, these results appear to deviate from the mechanism by which FVIII is maintained as a procofactor. For example, FVIII remains a procofactor even in the absence of the B-domain. Full activation is accomplished following cleavage between the A1 and A2 domains (48).

A striking feature of a portion of the B-domain sequences (residues 963–1008) identified is that this cluster of amino acids is unusually basic, with 18 of 46 residues being Arg or Lys (Fig. 4). This short basic motif is conserved among mammals and is even present in pufferfish and chicken (43). This observation is surprising considering that the FV B-domain has weak conservation among mammals (<50% identity) and is poorly con-

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served among other vertebrates (<20% identity). Although it is unknown whether this sequence motif functions the same way in other species, the observation that this basic region is one of only a few portions of the B-domain that is conserved among most vertebrates suggests it plays an important functional role. Interestingly, this sequence is not found in FV derived from certain Australian elapids (*Pseudonaja textilis*, *Oxyuranus scutellatus*, and *Oxyuranus microlepidotus*). These snakes have two genes for FV, one expressed in the liver and the other in the venom gland (49−51). The FV expressed in the venom is in complex with a FXa-like enzyme and is a powerful prothrombin activator. Remarkably, the FV sequence from these genes is predicted to have a B-domain of −50 amino acids. Thus, either FV derived from these snakes uses a different mechanism to maintain the procofactor state, or the proteins are synthesized as “active” cofactors. If this is the case, it would be the first example in nature of a FV derivative that does not require processing to become activated.

An unexpected finding from our work is that removal of the heavily glycosylated C-terminal half of the B-domain (residues 1034−1491) was without functional consequence because the procofactor state was maintained. One interpretation of these data is that these sequences are not involved in maintaining the procofactor state. An alternative explanation is that this region of the B-domain (or associated carbohydrate) complements the inhibitory basic region (possibly in a steric fashion) but is not absolutely required to keep FV inactive. Whether this region alone (in the absence of the basic region) can maintain FV in an inactive state is not clear, and future work will need to address this point. Interestingly, these sequences encompass >50% of the B-domain and contain an unusual series of tandem repeats (5). Recent comparative sequence analysis of FV sequences from mammals to fish to snakes has revealed that the B-domains vary considerably in length (*Homo sapiens*, 836 residues; *Takifugu rubripes*, 465 residues; *P. textilis*, 46 residues). This major difference in length is due to the C-terminal half of the B-domain (i.e. from residues 1019 to 1545), which is significantly shortened or absent compared with the human FV sequence (41, 42).3 This possibly suggests that these sequences were acquired later in the evolutionary process and may function in some other capacity such as in clearance mechanisms or may be involved in the FV secretory process.

Our findings do not indicate whether the identified inhibitory region of the B-domain directly preserves the procofactor state or if changing this region had an indirect effect on protein function. A direct effect would be envisioned as intramolecular binding of these B-domain sequences to a site on the heavy and/or light chain, thereby concealing critical binding interactions (i.e. FXa-binding site). Alternatively, an indirect effect would be seen as the inhibitory region positioning other portions of the B-domain in such a way to block cofactor function. In either scenario, disruption of these sequences by proteolysis or recombinant truncation would have the same outcome. During the normal activation process, cleavage at three sites liberates the B-domain as two fragments (N-terminal, 71-kDa fragment; C-terminal, 150-kDa fragments; Scheme 1) (5, 8). The inhibitory region identified in the current study is located within the N-terminal 71-kDa fragment. Thus, removal of this fragment via cleavage at Arg709 and Arg1018 should yield a FV(a) derivative with significant procoagulant activity. There is some support for this (6, 10, 13, 15); however, in both the human and bovine systems, full procoagulant activity is typically observed following cleavage at Arg1545. This has been interpreted to suggest that release of the B-domain from the light chain is required for maximal activation. Based on our current findings, we speculate that cleavage at Arg1545 may function to facilitate removal of one or more inhibitory regions of the B-domain from the rest of the FV(a) structure. Detailed structural information that would provide insight into this process is currently not available. What is known is that the B-domain is responsible for the high asymmetry of FV (52, 53), because it resembles a long tail projecting from the globular heavy and light chains (53−56). These groups of studies also support the idea that the heavy and light chains remain closely

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**FIGURE 5. Direct binding measurements with FV-1033 derivatives.**

A, reaction mixtures containing 10 nM OG488-FA and 50 μM PCPS in assay buffer were titrated with increasing concentrations of PD-FVα ([□]), FVB8-131 (●), FVB8-104 (■), FVB8-46 (▲), and FV-1033 (∇). The data for FV-1033 are provided as a comparison and are the same as those in Fig. 3. Fluorescence (F) intensity was measured at 25 °C. The lines are drawn following analysis to independent, noninteracting sites, and the fitted values (Kd and n) are given in Tables 1 and 3. The data are representative of two to three similar experiments. B, SDS-PAGE analysis. Purified proteins (4 μg/lane) prior to the start of the experiment (Start) and taken from the cuvette at the end of the experiment (End) were subjected to SDS-PAGE. Lane 1, FV-810; lane 2, FVB8-131; lane 3, FVB8-104; lane 4, FVB8-46; lane 5, FV-1033. FV-1033 was run on a separate gel. The apparent molecular weights of the standards are indicated on the left.
associated in the transition from FV to FVa, with no major structural rearrangement noted. Based on the proposed structure of FVa (57), it is possible that specific portions of the B-domain could block regions of the heavy and/or light chain. Additional evidence for this comes from monoclonal antibody studies. In one study, two antibodies were identified that were selective for FVAs but not FV (58). It was suggested that the new epitope(s) expressed on FVAs could arise from conformational transitions or simply from loss of B-domain fragments with subsequent exposure of binding sites. These studies and the work presented herein illustrate in a general way how the B-domain could obscure important binding sites on the heavy and/or light chain.

In summary, the current study indicates that discrete B-domain sequences are involved in the mechanism by which FV activates and lays the groundwork for defining the pre- and future work will be aimed at better defining these sequence constraints. Our data nonetheless provide new insights about FV activation and lay the groundwork for defining the precise mechanism by which the B-domain regulates the FV procoagulant to cofactor transition.

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