Identification of a Factor Xa-interactive Site within Residues 337–372 of the Factor VIII Heavy Chain*

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We recently demonstrated that the residues 337–372, comprising the acidic C-terminal region in A1 subunit, interact with factor Xa during the proteolytic inactivation of factor VIIa (Nogami, K., Wakabayashi, H., and Fay, P. J. (2003) J. Biol. Chem. 278, 16502–16509). We now show this sequence is important for factor Xa-catalyzed activation of factor VIII. Peptide 337–372 markedly inhibited cofactor activation, consistent with a delay in the rate of cleavage at the A1-A2 junction. Studies using the isolated factor VIII heavy chain indicated that the peptide completely blocked cleavage at the A1-A2 junction (IC50 = 11 μM) and partially blocked cleavage at the A2-B junction (IC50 = 100 μM). Covalent cross-linking was observed between the 337–372 peptide and factor Xa following reaction with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, and the peptide quenched the fluorescence of dansyl-Glu-Gly-Arg active site-modified factor Xa, suggesting that residues 337–372 directly interact with factor Xa. Studies using a monoclonal antibody recognizing residues 351–365 as well as the peptide to this sequence further restricted the interactive region. Mutant factor VIII molecules in which clustered acidic residues in the 337–372 segment were converted to alanine were evaluated for activation by factor Xa. Of the mutants tested, only factor Xa-catalyzed activation of the D361A/D362A/D363A mutant was inhibited with 50% and an activation rate constant of 30% of the wild type values. These results indicate that the 337–372 acidic region separating A1 and A2 domains and, in particular, a cluster of acidic residues at position 361–363 contribute to a unique factor Xa-interactive site within the factor VIII heavy chain that promotes factor Xa docking during cofactor activation.

Factor VIII, a plasma protein deficient of defective in individuals with hemophilia A, functions as a cofactor for the serine protease, factor IXa, in the anionic phospholipid surface-dependent conversion of factor X to Xa (1). Factor VIII is synthesized as a multi-domain single chain molecule (A1-A2-B-A3-C1-C2) consisting of 2332 amino acid residues with a molecular mass of ~300 kDa (2, 3). Factor VIII is processed to a series of metal ion-dependent heterodimers by cleavage at the B-A3 junction, generating a heavy chain consisting of the A1 and A2 domains, plus heterogeneous fragments of a partially proteolyzed B-domain linked to a light chain consisting of the A3, C1, and C2 domains (2–4).

Factor VIII is converted into an active form, factor VIIIa, following limited proteolysis catalyzed by either thrombin or factor Xa (5). Cleavages at Arg372 and Arg740 of the heavy chain produce the 50-kDa A1 and 40-kDa A2 subunits. Cleavage of the 80-kDa light chain at Arg1689 produces a 72-kDa A3-C1-C2 subunit. Additionally, cleavage by factor Xa at Arg721 produces a 67-kDa A3-C1-C2 subunit. Proteolysis at Arg372 and Arg1689 is essential for generating factor VIIIa cofactor activity (see for review see Ref. 6). Cleavage at the former site exposes a functional factor IXa-interactive site within the A2 domain that is cryptic in the inactivated molecule (7). Cleavage at the latter site liberates the cofactor from its carrier protein, von Willebrand factor (8), as well as contributes to overall specific activity of the cofactor (9, 10). Factor Xa also inactivates factor VIII(a) following cleavages at Arg356 (5) and Lys36 (11) within the A1 subunit. Inactivation following cleavage at Arg356 probably occurs by altered interaction of the A2 subunit with the truncated A1 (12) coupled with increase in the Km for substrate factor X (13), the latter reflecting a loss of a factor X-interactive site within an acidic residue-rich region defined by residues 337–372 (14). Other proteases including activated protein C (5) and factor IXa (15) have been shown to also attack this site. Furthermore, cleavage at Lys36 by factor Xa has been suggested to alter the conformation of A1, limiting productive interaction with the A2 subunit (13). Thus, factor Xa represents a unique enzyme relative to factor VIII in that its catalytic mechanisms include both the up- and down-regulation of cofactor function.

A factor Xa-interactive site required for cofactor activation has been mapped within the residues 2253–2270 of the C2 domain of factor VIII (16). Furthermore, factor VIII alloantibody inhibitors recognizing this epitope within the C2 domain inhibit factor Xa-catalyzed factor VIII activation by directly blocking the interaction between the factor VIII and factor Xa (17). Thus, the interaction of factor Xa with the light chain of the cofactor is a requisite step for catalysis. However, we recently presented kinetic evidence suggesting that residues 337–372, which comprise the C-terminal acidic region in A1 subunit, may directly interact with factor Xa (11), possibly via the heparin-binding exosite of the proteinase (18). In the current study, we identify a factor Xa-interactive site within the 337–372 acidic region that separates the A1 and A2 domains using a combination of approaches employing synthetic peptides, antibodies, and recombinant factor VIII mutants in both functional and physical assays. Our results indicate that the
337–372 region and, in particular, a cluster of acidic residues at position 361–363 contribute to a unique factor Xa-interactive site within the factor VIII heavy chain that promotes factor Xa docking during co-factor activation.

**MATERIALS AND METHODS**

**Reagents**—Purified recombinant factor VIII preparations were generous gifts from Bayer (Berkeley, CA). The factor VIII heavy chain was isolated from EDTA-treated factor VIII following chromatography on SP- and Q-Sepharose columns (Amersham Biosciences) as described previously (7). The monoclonal antibodies 58.12 (19) and C5 (20) recognizing the N-terminal and C-terminal ends of the factor VIII A1 domain were gifts from Drs. James Brown and Zaverio Ruggeri, respectively. The monoclonal antibody R8B12 and the polyclonal antibody GMA-7 recognizing the factor VIII A2 domain were obtained from Green Mountain Antibodies (Burlington, VT). IgG was purified following protein A-Sepharose chromatography. Human α-thrombin and factor Xa (Enzyme Research Laboratories, South Bend, IN), DEGR-factor Xa (Hematologic Technologies Inc., Essex Junction, VT), peroxidase-conjugated streptavidin (Calbiochem), EDC, 1 and sulfo-N-hydroxysuccinimide-biotin (Pierce) were purchased from the indicated vendors. Phospholipid Factor VIII molecules bearing cluster mutation of 341–372 region and, in particular, a cluster of acidic residues at position 361–372 contribute to a unique factor Xa-interactive site within the factor VIII heavy chain that promotes factor Xa

**RESULTS**

\[
F(t) = 100 - h\left(D_0 + P_0 - D_k + \frac{K_a}{k_d} - 4D_kP_k\right) \quad (Eq. 2)
\]

where \( F \) is the relative fluorescence (%), \( D_0 \) is the factor VIIIa concentration, \( P_0 \) is the peptide concentration, \( K_a \) is the dissociation constant, and \( k_d \) is a constant.

Cross-linking with EDC—Cross-linking reactions contained the indicated reactant concentrations and were run in buffer containing 20 mM HEPES, pH 7.2, 5 mM CaCl\(_2\), and 0.01% Tween 20 at 4 °C for 1 h. Reactions were terminated by addition of SDS electrophoresis sample buffer and boiling.

**Electrophoresis and Western Blotting**—SDS-PAGE was performed on 8 or 15% gels using the procedure of Laemmli (25). Electrophoresis was carried out using a Bio-Rad mini-gel apparatus at 150 V for 1 h. For Western blotting, the protein was transferred to a polyvinylidene difluoride membrane and probed using the indicated monoclonal or polyclonal antibody followed by anti-mouse or anti-rabbit alkaline phosphatase-linked secondary antibody, respectively. The signal was detected using the enhanced chemiluminescence system (Amersham Biosciences), and the blots were scanned at 570 nm using Storm 860 (Molecular Devices). Densitometric scans were quantitated using ImageQuant software (Molecular Devices).

**Data Analysis**—To evaluate the catalytic efficacy of factor Xa for factor VIII, we calculated the activation rate constants based on the values of generated factor VIIIa activity. Assuming that the cleavage event and release of products are rapid, the concentration of free factor Xa should be constant. Therefore, the rate constants correlate with the concentration of substrates as shown in Scheme 1.

\[
k_1 \quad k_2
\]

**Scheme I**

Factor VIIIa and factor VIIIi represent the activated factor VIII and inactivated factor VIIIa, respectively. The apparent rate constant values (\(k_1\) and \(k_2\)) in Scheme 1 are based on a series reactions for factor VIII activation by factor Xa and were estimated by nonlinear least-squares regression using Equation 3.

\[
[factor VIII]_{a} = [factor VIII]_{0} \times e^{-k_{f}(t-k_{i})} 
\]

where \([factor VIII]_{a}\) is the concentration at time point \(t\) of factor VIIIa and \([factor VIII]_{0}\) is the initial concentration of factor VIII. All of the experiments were performed at least three separate times, and the average values are shown.

**RESULTS**

Inhibition of Factor Xa-catalyzed Factor VIII Activation by Synthetic Peptide 337–372—We recently demonstrated that...
factor Xa interacted with residues 337–372 that form the C-terminal acidic region in the A1 subunit of factor VIIIa, leading to the proteolytic inactivation of the cofactor (11). To investigate whether this same region, which separates the A1 and A2 domains in the heavy chain of the factor VIII procofactor, represents a factor Xa-interactive site utilized in the activation of factor VIII, factor Xa-catalyzed activation of factor VIII was performed in the absence and presence of the 337–372 peptide. Factor VIIIa activity was followed using a one-stage clotting assay as described under “Materials and Methods.” Control experiments showed the presence of either peptide or factor Xa did not affect this assay, probably the result of the 5000-fold dilution of the reaction mixture prior to factor VIIIa activity determination. Using reaction conditions of 100 nm factor VIII and 1 nm factor Xa, maximal factor VIIIa activity was observed at the 1-min time point and reflected an ~2.5-fold increase (Fig. 1A). The 337–372 peptide demonstrated a dose-dependent inhibition of factor Xa-catalyzed activation of factor VIII activation with up to ~70% inhibition observed (Fig. 1B). In the presence of 337–372 peptide (400 μM), the peak value of factor VIIIa activity was reduced by ~50% and the time increment to reach this value was delayed compared with that of control.

These observations in activity were supported by evaluation of extents of factor VIII proteolysis during the time course. Western blotting using the anti-A1 monoclonal antibody C5. This antibody binds an epitope defined by residues 351–365 in the A1 subunit, Fig. 2B and product (A2 subunit, Fig. 2C). The 337–372 peptide (125 μM) completely blocked the cleavage at the A1-A2 junction. The inhibition was observed in a dose-dependent manner with an IC50 value of 11 ± 5 μM. In addition, cleavage at the A2-B junction was partially blocked (~70%) with the IC50 value of 100 ± 20 μM. In a control experiment, a peptide to the adjacent residues (373–395) representing the N-terminal region of the A2 domain showed marginal (~10%) inhibition of heavy chain cleavage at the maximal peptide concentration employed (Fig. 2C). Interestingly, the 337–372 peptide demonstrated no effect on the cleavage of isolated heavy chain by thrombin (data not shown). These data indicate that the 337–372 region in A1 subunit contributes to a specific interactive site for factor Xa necessary to facilitate cleavage of the heavy chain.

Localization of a Factor Xa Site within the 337–372 Region.—To validate the data obtained using the synthetic peptides, we examined factor Xa-catalyzed factor VIII activation and cleavage of the isolated heavy chain cleavage in the presence of the anti-A1 monoclonal antibody C5. This antibody binds an epitope defined by residues 351–365 in A1 subunit (20). Factor VIII (100 nm) preincubated with the various concentrations of C5 IgG for 1 h was reacted with factor Xa (1 nm), and factor VIIIa activity was determined using a one-stage clotting assay. Control experiments showed that the antibody did not affect the procoagulant activity in this assay over the range of concentrations employed. C5 IgG resulted in a dose-dependent decrease in the peak levels of factor VIIIa activity activated by factor Xa (data not shown). In contrast, the an-
ti-A2 antibody R8B12 used in a control experiment to exclude any nonspecific inhibition possessed little inhibitory activity.

Effects of C5 IgG on the cleavage of the isolated heavy chain by factor Xa were further examined by Western blotting using a polyclonal anti-A2 antibody for fragment detection. Preincubation of heavy chain (50 nM) with C5 IgG (250 nM) for 2 h completely inhibited cleavage at the A1-A2 junction and partially inhibited cleavage of A2-B junction by factor Xa (5 nM) (Fig. 3A). Calculated IC$_{50}$ values obtained by densitometry scanning were 45 ± 10 and 410 ± 81 nM for inhibition of cleavage at the A1-A2 and A2-B sites, respectively (Fig. 3B). Because the epitope for C5 is well defined (20), we performed a similar series of experiments using synthetic peptide 351–365. The 351–365 peptide inhibited maximal generation of activated factor VIIIa activity catalyzed by factor Xa in a dose-dependent manner, and this inhibitory activity was similar to that of the 337–372 peptide (Fig. 1B). Furthermore, the 351–365 peptide (500 μM) blocked factor Xa-catalyzed cleavages at A1-A2 and A2-B junction sites in the isolated heavy chain by ~80 and ~50%, respectively (Fig. 2, B and C), although this level of inhibition was somewhat weaker compared with that of the 337–372 peptide. Taken together, these results based upon peptide and antibody-dependent inhibition assays suggest that residues 351–365 within the A1 C-terminal acidic region contribute to an interactive site for factor Xa.

**Fig. 2. Effects of the 337–372 peptide on cleavage of isolated heavy chain by factor Xa.** A, the isolated heavy chain (50 nM) was reacted with factor Xa (5 nM) and phospholipid vesicles (10 μM) in the presence of various concentration of the 337–372 peptide for 2 h. Samples were run on 8% gels followed by Western blotting using an anti-A2 monoclonal antibody. Lane 1 shows the intact heavy chain. Lanes 2–11 show the cleavage of heavy chain in the presence of 337–372 peptide (0, 2, 4, 8, 16, 31, 62, 125, 250, and 500 μM, respectively). Panels B and C show quantitative densitometry of intact heavy chain and A2 subunit, respectively. Density values of intact heavy chain without factor Xa or A2 subunit generated by factor Xa cleavage in the absence of peptide were used to represent the 100% level. The symbols used are as follows: open circles, 337–372 peptide; closed circles, 351–365 peptide; squares, 373–395 peptide.
treatment with the factor X-activator in Russell’s viper venom to liberate the activation peptide (27). These observations suggested the potential for the formation of a cross-linked product using factor Xa. Thus, the zero-length cross-linking reagent EDC was used to monitor the interaction between the 337–372 peptide and factor Xa. Variable concentrations of biotinylated peptides were combined with a fixed concentration of factor Xa (430 nM), and the mixtures were reacted with 300 μM EDC at pH 7.2 at 4 °C for 1 h. Following this cross-linking protocol, the samples were subjected to SDS-PAGE and immunoblotting (Fig. 5). Results using the 337–372 peptide showed the formation of a cross-linked product of ~40 kDa that increased in intensity with increasing amounts of the peptide (Fig. 5, A and B). Mass of the adduct (~40 kDa) was consistent with a 1:1 stoichiometry of 337–372 peptide (~4 kDa) and factor Xa heavy chain (~36 kDa). The failure to observe the biotinylated 337–372 peptide at the dye front in Western blot is not known. One possibility is that the high concentration of acidic residues in this reagent may affect its binding and/or retention to the polyvinylidene difluoride membrane. A product comprised of the peptide and factor Xa light chain (~17 kDa) was not evident, consistent with our earlier observations indicating the absence of a peptide-factor X light chain cross-linked product (27). Control experiments indicated no cross-linked product comprised of a biotinylated 373–395 peptide and factor Xa (data not shown). Furthermore, the 337–372 peptide did not form an adduct with thrombin (430 nM) following treatment with EDC under the same conditions (Fig. 5A). These data suggest that the association of the 337–372 and factor Xa occurs from a salt linkage(s) formed between this region in the A1 subunit and the heavy chain of factor Xa.

The specificity of the 337–372 peptide and factor Xa interaction as monitored by cross-linking was examined using unlabeled peptide to block formation of the biotinylated peptide-factor Xa adduct. Variable concentrations of unlabeled peptides were combined with fixed concentrations of factor Xa (430 nM) and biotinylated 337–372 peptide (40 μM), and the mixtures were reacted with 300 μM EDC. Fig. 5C illustrates the loss of biotinylated product following titration with the unlabeled 337–372 peptide, and this response is quantitated following densitometry scanning (Fig. 5D). Unlabeled 337–372 peptide (160 μM) completely inhibited the formation of EDC cross-linking product between the biotinylated 337–372 peptide and factor Xa. This observation indicated that the unlabeled peptide possessed a somewhat greater affinity for factor Xa than that observed for the biotinylated reagent, suggestive of the biotin label partially inhibiting the intermolecular interaction. The IC50 value calculated for this inhibition was ~20 μM. Compared with the 337–372 peptide, unlabeled 351–365 peptide showed moderate inhibition of complex formation between the biotinylated 337–372 peptide and factor Xa (IC50 ~90 μM). A control peptide (373–395 peptide) showed little inhibitory effect (~20%) on formation of the product. These data support the specificity in the interaction between the 337–372 region and factor Xa as well as further restricting of a portion of the interactive region to A1 residues 351–365.

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the C-terminal region of A1 subunit is the high content of acidic residues (13/36 residues, either Glu or Asp). Thus, this region could define an interactive region for an anion-binding site on the protease. In fact, a heparin-binding exosite on factor Xa was described recently (18). Furthermore, we have presented suggestive evidence that the A1 subunit may interact with factor Xa via the heparin-binding exosite of this protease (11). To investigate the role of acidic residues in this region in contributing to a factor Xa-interactive site, we focused on three sequences of clustered acidic residues, 341–345 (Glu-Glu-Ala-Glu-Asp), 347–349 (Asp-Asp-Asp), and 361–363 (Asp-Asp-Asp).

These factor VIII reagents (100 nM) were evaluated for activation by factor Xa (1 nM) (Fig. 7). Extents of peak activities and rates of factor VIIIa formation and decay were evaluated as described under “Materials and Methods.” We observed that factor Xa-catalyzed activation of the 361–363Ala mutant was significantly inhibited with peak activity of −54% of factor VIII.
Factor VIII residues 337–372, which constitute a highly acidic spacer region separating the A1 and A2 domains of the heavy chain and form the C-terminal region of the A1 subunit in the activated cofactor, participate in at least three protein-protein interactions. Earlier results from our laboratory indicated that this region contributed to a unique factor Xa-interactive site within the factor VIII heavy chain.

**DISCUSSION**

Factor VIII residues 337–372, which constitute a highly acidic spacer region separating the A1 and A2 domains of the heavy chain and form the C-terminal region of the A1 subunit in the activated cofactor, participate in at least three protein-protein interactions. Earlier results from our laboratory indicated that this region contributed to an A2-interactive site (28). More recently, the nature of this contribution was shown to involve the orientation of A2 subunit within factor Xase, yielding maximal stimulation of factor IXa catalytic activity (12). This region is also implicated in the binding of factor X (14), and recent evidence suggests a functional contribution to the $K_m$ for factor X interaction with factor Xase (13). The acidic region is bracketed by Arg$^{336}$ and Arg$^{372}$. Cleavage of the heavy chain at the latter site by thrombin or factor Xa (5) contributes to the activation of the procofactor, whereas cleavage at the former site by enzymes including activated protein C (5), factor IXa (15), and factor Xa (5) correlates with factor VIIIa inactivation and thus represents a mechanism for the dampening of factor Xase activity (29). Results presented in the current study employing a combination of functional and physical approaches now demonstrate that this acidic region constitutes an interactive site for factor Xa.

The present study revealed that the 337–372 peptide inhibited factor Xa-catalyzed factor VIII activation. The observed reduction in activation rate as judged by cleavage of the factor VIII heavy chain resulted in a reduction in peak factor VIIIa activity by ~50%. This effect on activity is probably the result of effectively reducing the concentration of the activator, because at any point in the time course, the observed activity reflects a dynamic condition of unactivated factor VIII, activated cofactor molecules, and inactivated cofactor due to A2 subunit dissociation and/or proteolytic attack. Because, under these reaction conditions, A2 dissociation is the dominant mechanism for cofactor inactivation, this effect is dependent upon the concentration of factor VIIIa, which in turn is limited by peptide-dependent inhibition of factor Xa-catalyzed activation.

Recently, a factor Xa-interactive site has been identified in the C2 domain by analyses including antibody inhibition and anhydro-factor Xa binding assays (16). Results presented in that study showed the C2 domain-specific antibody completely blocked factor Xa-catalyzed cleavages in the factor VIII light chain (at Arg$^{348}$ and Arg$^{372}$) while only partially blocking cleavage of the heavy chain (at Arg$^{372}$). These observations were made independent of the presence of a phospholipid surface and suggested a C2-mediated regulation relative to light chain cleavage as well as this domain exerting some influence on cleavage of the heavy chain. Thus, one may hypothesize the presence of an independent factor Xa-interactive region within the heavy chain that would serve a role for the regulation of proteolysis at these sites.

Because of the prominent contribution of factor VIII light chain to factor Xa-catalyzed mechanisms, we evaluated interactions of the proteinase with factor VIII heavy chain independent of the light chain. Indeed, while we observed that factor Xa cleavage of the A1-A2 junction in factor VIII was

**Table I**

| Factor VIII | Ac/Ag ratio$^a$ | Peak activity$^b$ | Rate constant$^c$ |
|------------|----------------|-------------------|------------------|
|            | % WT           |                   | $k_1$           | $k_2$           |
| Wild type  | 1              | 100               | 0.501 ± 0.048    | 0.020 ± 0.002   |
| 341–345Ala | 0.45           | 90.3 ± 20.3$^d$   | 0.366 ± 0.032    | 0.020 ± 0.002   |
| 347–349Ala | 0.35           | 166.1 ± 20.7$^d$  | 0.361 ± 0.035    | 0.023 ± 0.003   |
| 361–363Ala | 0.78           | 54.1 ± 10.8$^d$   | 0.186 ± 0.042    | 0.032 ± 0.009   |

$^a$ Ac/Ag ratio was calculated based on the assumption that one unit of native factor VIII is equivalent to 300 ng of protein. The value for wild type was set to unity.

$^b$ Factor VIII (100 nM) was reacted with factor Xa (1 nM) in the presence of phospholipid (10 µM) as described under “Materials and Methods.”

$^c$ Rate constant values were calculated from the fitted data shown in Fig. 7 using the formula presented under “Materials and Methods.”

$^d$ No significant difference ($p > 0.05$) compared with wild type.

$p < 0.01$ (compared with wild type).
partially inhibited in the presence of the 337–372 peptide, this cleavage was completely inhibited in isolated heavy chain. Therefore, these findings strongly suggest that the 337–372 acidic region of the A1 domain as well as the C2 domain contribute to the factor Xa-interactive site during the proteinase-catalyzed factor VIII activation.

Furthermore, the factor Xa site within the 337–372 segment could be further restricted based upon peptide and antibody-dependent inhibition and site-directed mutagenesis. The former analyses suggested a critical region of the interactive site is defined by epitope for the C5 antibody (residues 351–365) (20), because both the purified IgG and a synthetic peptide to this region effectively blocked factor Xa-catalyzed proteolysis on the factor VIII substrates. In addition, evaluation of the cluster mutants showed that only the acidic cluster comprising residues 361–363 appeared to modulate factor Xa activity. Thus, it appears that residues in and around this cluster of aspartic acid residues make a primary contribution to the binding of factor Xa.

Direct binding between the acidic region and factor Xa in the fluid phase was confirmed using two independent methods, changes in the dansyl fluorescence of DEGR-factor Xa, and visualization of a peptide-factor Xa adduct following zero-length cross-linking with EDC. Earlier studies indicated that this acidic region constitutes a low affinity ($K_a \sim 1–3 \mu M$) factor Xa-interactive site (14). Cross-linking studies using EDC identified a salt bridge(s) between residues 349–372 of acidic region and the serine protease-forming domain of the factor X heavy chain exclusive of the activation peptide (27). This observation was similar to results of the current cross-linking study using the active proteinase form. Thus, bothzymogen and active enzyme appear to share an overlapping interactive site within this acidic region. Furthermore, the results obtained with the cluster mutations suggest that (an) acidic residue(s) in the 361–363 segment may participate in the formation of the salt bridge(s) stabilized by the cross-linking reaction.

We suggest that the basic residue component to the salt bridge is contained within factor Xa. A recent study identified a cluster of basic residues that comprise a heparin-binding exosite in the proteinases (18). Results from that report indicated that selected residues contributed to the interaction of factor Xa with the factor Va cofactor as well as in defining substrate recognition by prothrombinase. We recently presented evidence that the C-terminal acidic region in A1 subunit may interact with factor Xa via this heparin-binding exosite (11). This speculation was based upon the observations that heparin blocked factor Xa-catalyzed cleavage of A1 subunit and that an A1 subunit truncated at Arg$^{386}$ (designated A1$^{386}$) was a poor substrate for factor Xa-catalyzed cleavage at Lys$^{386}$ compared with an intact A1 subunit. Thus, we propose that the 361–363 segment contains a unique interactive region in the factor VIII heavy chain for the heparin-binding exosite of factor Xa.

Factor VIII and factor V share homologous structures and functions (30). Although factor V shows no acidic region separating the A1 and A2 domains, a functionally important acidic-rich residue region was recently localized to N-terminal region of the A2 domain of factor Va (residues 323–331) (31). Specifically, residues Glu$^{329}$, Val$^{330}$, Glu$^{331}$, and Tyr$^{332}$ of factor Va heavy chain were suggested to comprise a factor Xa-binding site required for the expression of cofactor activity (32) based on peptide inhibition and site-directed mutagenesis studies. Although factor Xa interactions with factor VIII and V are functionally distinct, reflecting primarily enzyme-substrate and enzyme-cofactor interactions, respectively, these observations taken together suggest a common docking mechanism utilized by the proteinase.

Factor Xa has been shown to attack factor VIII at identical sites as those cleaved by thrombin. Although both proteases bind the C2 domain, an interaction that appears to mediate cleavage of light chain at Arg$^{668}$, the binding sites are not identical (33). Functional differences for these interactions are also observed. Interaction of the C2 domain with factor Xa regulates in part the cleavage of heavy chain, whereas C2 interaction with thrombin shows no influence on this cleavage (34). Furthermore, Y346F mutants are efficiently cleaved by factor Xa, indicating that tyrosine sulfation at this site does not influence the binding of factor Xa (35). We have observed additional function differences in proteolytic mechanisms in this study. Thrombin-catalyzed cleavage of the heavy chain was neither inhibited by the 337–372 peptide nor was it affected by the cluster mutation converting the Asp residues at positions 361–363 to Ala. Furthermore, thrombin yielded no apparent cross-link with the 337–372 peptide in the presence of EDC. These observations were in contrast to those obtained with factor Xa and suggest significant mechanistic differences in the interactions of the two proteinases during cofactor activation.

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Identification of a Factor Xa-interactive Site within Residues 337–372 of the Factor VIII Heavy Chain

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