Role of Proexosite I in Factor Va-dependent Substrate Interactions of Prothrombin Activation*

Patricia J. Anderson‡, Anna Nesset, Kumudini R. Dharmawardana, and Paul E. Bock§

From the Department of Pathology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

In the final zymogen activation step of blood coagulation, cleavage of two peptide bonds in prothrombin by factor Xa activates the prethrombin 2 domain and releases it as the blood-clotting proteinase, thrombin, along with prothrombin activation fragments 1 and 2 (1, 2). In addition to activation of the catalytic site, prothrombin activation results in expression of regulatory exosites I and II on thrombin (3–5). In the preceding paper, exosite I was shown to be present in a low affinity precursor state (proexosite I) on prothrombin, which binds hirudin54–65 peptide ligands specifically and undergoes an ~100-fold increase in affinity on thrombin formation (6). The increased affinity of exosite I for hirudin peptides is thought to be expressed on conversion of prothrombin to either of the activation intermediates, prethrombin 2 and meizothrombin (4). Once formed, exosites I and II play critical roles in regulating thrombin activity by mediating interactions with specific protein substrates, inhibitors, and regulatory proteins (3, 7, 8).

Calcium ion, phospholipid membranes, and the protein cofactor, factor Va, regulate the prothrombin activation reactions through the assembly on phospholipid surfaces of a factor Xa-factor Va-prothrombin ternary complex, mediated by protein-protein and protein-membrane interactions (1, 2, 9, 10). Compared with prothrombin activation by factor Xa alone, the rate is enhanced ~300,000-fold by these interactions (1, 2, 11, 12). Kinetic studies indicate that phospholipid interactions are primarily responsible for a ~100-fold decrease in the apparent kinetic constant for prothrombin activation, whereas the protein cofactor acts by increasing the apparent kinetic constant about 3,000-fold (11, 12). Studies of the reactions in the absence of phospholipid membranes show that the protein-protein interactions among factor Xa, factor Va, and prothrombin by themselves can account for the unique activity of factor Va to increase the apparent catalytic rate constant (13). The role of prothrombin-factor Va, substrate cofactor interactions in the mechanism of acceleration of prothrombin activation by factor Va is not completely understood, and the binding sites mediating these interactions have not been clearly established.

Binding sites distinct from the catalytic site of factor Xa have been shown to be involved in specific recognition of the activation intermediate, prethrombin 2, by the factor Xa-factor Va-phospholipid complex (14, 15), whereas prothrombin has not been similarly studied. Activation of prethrombin 2 is inhibited competitively by active site-blocked thrombin, indicating the presence of overlapping binding sites on the factor Xa-factor Va complex for this substrate and the product (14, 15). Exosite I is implicated in these interactions by the observation of selective inhibition of factor Va acceleration of prethrombin 2 activation by the exosite I-specific ligand, hirudin53–64 (4, 14), and reversal of the inhibitory effect of thrombin by the peptide (14). Binding of prethrombin 2 and thrombin to the factor Xa-factor Va complex is thought to occur through exosites on factor Xa rather than with sites on factor Va (14, 16). Recent studies characterizing thrombin binding to factor Va (17, 18), however, suggest the alternative explanation that the substrate and product interactions occur through shared sites on factor Va. The additional observations that (a) thrombin binding to factor Va is mediated by exosite I (b) thrombin (17, 18) and prothrombin (13, 19, 20) bind similarly to the factor Va heavy chain and

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§ To whom correspondence should be addressed: Dept. of Pathology, Vanderbilt University School of Medicine, C3321A Medical Center North, Nashville, TN 37232-2561. Tel.: 615-343-9863; Fax: 615-343-7023; E-mail: paul.bock@mcmail.vanderbilt.edu.

1 The abbreviations used are: hirugen, Tyr63-sulfated N-acetylhirudin54–65; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-sn-glycero-3-phosphoserine; Hir54–65, Gly-Asp-Glu-Glu-Ile-Pro-Glu-Glu-Tyr-Leu-Gln; Hir54–65(SO3)2, Tyr56-sulfated hirudin54–65.
the isolated heavy subunit in calcium-independent interactions, and (c) proexosite I is present on prothrombin in a previously unrecognized functional state (6) suggest a common mode of prothrombin and thrombin binding to sites on the heavy chain of factor Va through proexosite I and exosite I (18). However, the fragment 2 domain of prothrombin has also been implicated in binding to factor Va from its accelerating effects on prothrombin 2 activation (21). Deletion of the fragment 2 domain of human prothrombin inhibits prothrombin activation in the presence of factor Va only partially, suggesting a role for fragment 2-independent interactions (22). In support of this, activation of prothrombin 2 was shown recently to be accelerated greatly by factor Va in the absence of fragment 2, demonstrating that sites within the prothrombin catalytic domain and independent of fragment 2 contribute to factor Va acceleration (16). Together, these observations suggest that in addition to a possible role for fragment 2 in productive prothrombin-factor Va interactions, substrate recognition by the factor Xa-factor Va complex may involve interactions of factor Va with proexosite I and exosite I on prothrombin, the activation intermediates, and the reaction product.

The novel characterization of proexosite I on prothrombin as a specific binding site for hirudin peptides prompted the present investigation of the role of this site in factor Va activity (6). Kinetic studies described here of the effect of Tyr65-sulfated hirudin (Hir54−65(SO3)) and the nonsulfated peptide (Hir54−65) as probes of proexosite I demonstrated that acceleration of prothrombin activation by factor Va was selectively inhibited, with no significant effect on the peptides on prothrombin activation catalyzed by factor Xa alone. Comparison of human and bovine prothrombin as substrates demonstrated that a common mechanism involving proexosite I regulates the reactions. The effect of Hir54−65(SO3) on the kinetics of prothrombin activation followed a mechanism in which the peptide bound exclusively to proexosite I of prothrombin, and the prothrombin-Hir54−65(SO3) complex did not interact productively with the factor Xa-factor Va complex but was an equivalent substrate of factor Xa alone. Assembly of the factor Xa-factor Va-prothrombin complex on phospholipid membrane vesicles counteracted inhibition by Hir54−65(SO3). The results show that proexosite I plays a major role in macromolecular substrate recognition by the factor Xa-factor Va complex, possibly by directly mediating productive protein-protein interactions between factor Va and prothrombin.

**EXPERIMENTAL PROCEDURES**

**Protein and Peptide Purification and Characterization—**Human prothrombin, thrombin, and hirudin peptides were obtained and characterized as described in the preceding paper (6). Human prothrombin 1, factor Xa, and factor Va were purchased from Hematologic Technologies. Human prothrombin 2, thrombin, and hirudin peptides were obtained and characterized as described in the previous paper (6). Factor Xa was inactivated by incubation with a 2.7-fold excess of D-Phe-Pro-Arg-CH2Cl, and excess inhibitor was removed by dialysis. Factor Xa (4.8 μM) and factor Va (1.0 μM) were preincubated with 1 μM D-Phe-Pro-Arg-CH2Cl at 25 °C for 30 min before chromatography of 300-μl samples on 2.7-ml affinity columns. The elution profiles of the proteins were measured as described before for the affinity matrix in the absence and presence of 1–2 mM NaCl and for the control matrix lacking the peptide (6).

**Proexosite I in Prothrombin Activation—**Initial rates of prothrombin activation by factor Xa in the presence and absence of factor Va and phospholipid were measured by discontinuous assay of the thrombin formed, determined from the increase in the initial rate of hydrolysis of 100 μM p-Phe-Pip-Arg-g-nitroanilide (where Pip is piperidic acid) at 405 nm. Reaction mixtures containing prothrombin, factor Va, and various concentrations of hirudin peptides in 50 mM Hepes, 0.11 mM NaCl, 5 mM CaCl2, 1 mg/ml polyethylene glycol 8000, 1 mg/ml soybean trypsin inhibitor (type IS), Sigma, pH 8.2, to give a final pH of 7.4. Quenched samples were assayed in 96-well microtiter plates that had been coated with polyethylene glycol 20,000 to minimize protein adsorption. Control experiments demonstrated that quenching of the reactions was instantaneous. The addition of factor Xa to a mixture of prothrombin and factor Va in an EDTA and soybean trypsin inhibitor-quenched buffer mixture did not produce any measurable activity. Thrombin chromogenic substrate activity in the quenched samples was stable for at least 2 h. Thrombin concentrations were determined by comparison of the measured initial rate to the rates for known concentrations of thrombin under identical solution conditions, including matched concentrations of the hirudin peptides. This was necessary to correct the rates for the effect of the peptides on thrombin activity, which amounted to an increase of 30–8% by Hir54−65(SO3) and 15–12% by Hir54−65 at 100 μM chromogenic substrate. Plots of the concentration of thrombin formed with time for at least two reactions in which <8% prothrombin was consumed were fit by a straight line to obtain the initial rate. Reactions in the presence of 95% DOPC, 5% DOPS phospholipid vesicles were performed similarly at 1 or 10 mM factor Xa, 50 μM phospholipid in the absence and presence of 10 μM or 5 μM factor Va and various concentrations of Hir54−65(SO3), as indicated. The mixtures were preincubated at 25 °C for 10 min before initiation of the reactions by the addition of 100 mM prothrombin. The observed initial rate of prothrombin activation (vobs) by factor Xa in the presence and absence of factor Va was represented by the sum of the factor Va-accelerated (vVa) and unaccelerated (vU) rates as shown in Equation 1.

\[
 v_{\text{obs}} = v_{\text{Va}} + v_{\text{U}} \quad (\text{Eq. 1})
\]

Previous studies of the mechanism of bovine prothrombin activation in the absence of phospholipid support a mechanism in which factor Xa, factor Va, and prothrombin form a highly productive, factor Xa-factor Va-factor thrombin ternary complex through factor Xa-factor Va and prothrombin-factor Xa interactions (13). In those studies, the uncatalyzed activation of prothrombin by factor Xa alone was concluded to not contribute significantly to the interactions of ternary complex formation. The present studies were analyzed according to similar simplifying cases derived from the general ternary complex mechanism in which all three possible binary complexes and one ternary complex can be formed, and the uncatalyzed reaction is included as shown in Scheme 1. This mechanism contains the model concluded in previous studies to describe the kinetics of bovine prothrombin activation. The initial velocity of thrombin (T) formation for the ternary complex model in terms of the total factor Xa concentration (Xa), the free concentrations of prothrombin ([Pro]) and factor Va ([Va]), and the dissociation constants for the factor Xa ([X]), factor Va (V), and prothrombin (P) binary and ternary complexes is given by Equation 2 (28).

\[
 v_{\text{obs}} = \frac{\beta [\text{Xa}] [\text{Pro}]}{K_{\text{XP}}} + \frac{\gamma [\text{Va}]}{K_{\text{VP}}} \quad (\text{Eq. 2})
\]
The first term represents the factor Va-accelerated reaction ($v_{Va}$), and the second term represents the uncatalyzed reaction ($v_{un}$), and the second term represents the uncatalyzed reaction ($v_{un}$), and

$$[\text{Pro}] = [\text{Pro}]_0 - [\text{Pro-Hir}]$$  \hspace{1cm} (Eq. 7)

where [Pro-Hir] represents the prothrombin-hirudin peptide complex concentration. The concentration of the complex was calculated with the quadratic binding equation (Equation 8), assuming that the concentrations of prothrombin in complexes with factor Xa and factor Va were small enough to be neglected under the experimental conditions where $[\text{Pro}]_0 \gg [\text{Va}]$, $[\text{Va}]$

$$[\text{Pro} \cdot \text{Hir}] = \frac{(K_P + n[\text{Pro}]) + [\text{Hir}]}{2} - \frac{(K_P + n[\text{Pro}] + [\text{Hir}])^2 - 4n[\text{Pro}][\text{Hir}]}{2}$$  \hspace{1cm} (Eq. 8)

The rate of thrombin formation as a function of prothrombin concentration in the absence and presence of fixed levels of Hir(54–65)(SO₃⁻) and factor Va was analyzed by fitting of Equations 6–8 with $K_{m,app}$, $k_V$, $k_s$, and the dissociation constant for hirudin peptide binding to prothrombin ($K_d$) as the fitted constants. The stoichiometric factor ($n$) for peptide binding was fixed at one.

The reactions were also studied as a function of peptide concentration at levels of factor Xa, factor Va, and prothrombin all below saturation. Under these further simplifying conditions, where $[\text{Pro}]_0 \ll K_{m,app}$, Equation 6 reduced to two linear terms, as follows.

$$\frac{v_{Va}}{[\text{Xa}]_0} = \frac{k_V[\text{Pro}]}{K_{m,app} + [\text{Pro}]} + \frac{k_s[\text{Pro}]}{[\text{Va}]_0 + [\text{Va}]}$$  \hspace{1cm} (Eq. 9)

The rate was verified experimentally to be a linear function of factor Xa, factor Va, and prothrombin concentration under these conditions. Inhibition by the peptides was analyzed using Equations 7–9, with the two apparent bimolecular rate constants ($k_V$ and $k_{ Va}$) and the parameters for hirudin peptide binding to prothrombin as the fitted constants. Nonlinear least squares analysis was performed with Scienist software. All estimates of error represent ± 2 S.D.

### RESULTS

#### Affinity Chromatography of Factor Xa and Factor Va on Hir(54–65)(SO₃⁻)-Agarose—To test the specificity of hirudin peptide binding for prothrombin, the possibility of interactions of the peptide with factor Xa or factor Va was first tested by affinity chromatography on Hir(54–65)(SO₃⁻)-agarose. As shown previously (6), prothrombin eluted from the immobilized peptide matrix in $f$ 0.15 m, 5 mM CaCl₂, pH 7.4, buffer as a broad peak at high elution volume (Fig. 1A). By contrast, factor Va and factor Xa both eluted in essentially the same volume as the void volume of either the peptide matrix or the control matrix lacking the peptide (Fig. 1, B and C). Elution of factor Xa and factor Va from the peptide matrix was not significantly affected by buffer containing 2 mM NaCl. Recovery of the proteins in these experiments was 73–104%, indicating that the proteins behaved homogeneously. These results indicated that Hir(54–65) (SO₃⁻) bound to human prothrombin with highest affinity, whereas the peptide interacted with factor Va or factor Xa more weakly, if at all. In support of this specificity, the fluorescence of fluorescein-labeled Hir(54–65)(SO₃⁻) changed by ≤5% in titrations with up to 4 μM factor Xa or factor Va and was unaffected by the addition of an excess of the unlabeled peptide (results not shown).

#### Inhibition of Prothrombin Activation by Hir(54–65)(SO₃⁻) and Hir(54–65)—The effect of Hir(54–65)(SO₃⁻) on prothrombin activation was studied primarily in the absence of phospholipid vesicles to simplify the reactions to the dependence of the rate on protein-protein interactions among factor Xa, factor Va, and prothrombin. The observed initial rate of thrombin formation was described by the sum of the factor Xa-accelerated reaction and the unaccelerated activation of prothrombin by factor Va alone (see “Experimental Procedures”). The reactions were studied initially under first-order conditions of low factor Xa, factor Va, and prothrombin concentrations, as a function of hirudin peptide concentration. That these conditions were
achieved was verified experimentally by the linear dependence of the rate on the concentrations of factor Xa (2-20 nM), factor Va (0.5–10 nM), and prothrombin (0.1–2 μM) in the absence and presence of hirudin peptides (results not shown). As shown in Fig. 2A, Hir54–65(SO32)-agarose inhibited the factor Va-accelerated rate hyperbolically while having no effect (±13%) on the slower reactions in the absence of factor Va (Fig. 2A). To estimate the dissociation constant for the inhibitory interaction and the limiting rate at saturating peptide concentration (vlim), the dependence of the observed velocity (vobs) on inhibitor concentration was analyzed by assuming that fractional inhibition was due to Hir54–65(SO32) binding to proexosite I, as shown by Equation 10.

\[
v_{\text{obs}} = v_{\text{lim}} - v_{\text{lim}} \frac{[\text{ProHir}]}{[\text{ProHir}]} + v_{o} \quad \text{(Eq. 10)}
\]

The initial rate in the absence of the inhibitor was represented by v0, and [ProHir] was calculated from the conservation and quadratic binding equations (Equations 7 and 8). Analysis of the results in Fig. 2A in this way gave a dissociation constant for hirudin peptide binding of 1.6 ± 0.6 μM and a limiting rate of 0.10 ± 0.09 nm thrombin/min at saturating peptide concentration. The latter value was indistinguishable from the average rate measured in the absence of factor Va of 0.027 ± 0.001 nm thrombin/min, consistent with complete inhibition of the factor Va-dependent reaction (Table I). The dissociation constant was indistinguishable from the value of 2.6 ± 0.6 μM, determined for Hir54–65(SO32) binding to proexosite I of human prothrombin (6), supporting the specific involvement of this interaction. To better estimate the maximum extent of inhibition, the residual factor Va activity in stimulating prothrombin activation was measured at 100 μM Hir54–65(SO32) as a function of factor Va concentration (results not shown). The 4 ± 2% residual activity level was experimentally indistinguishable from the 2.5% level predicted at this peptide concentration for the complete inhibition mechanism described below. This ∼2% difference implied that the peptide inhibited factor Va activity by ≥50-fold, approximating complete inhibition.

To further evaluate the role of proexosite I in the mechanism of inhibition, the effect of the nonsulfated hirudin peptide, Hir54–65, which has a 17-fold reduced affinity for proexosite I (6), was compared with the sulfated analog. Selective inhibition of the factor Va-accelerated reaction was again observed, with a negligible limiting rate of −0.01 ± 0.07 nm thrombin/min at saturating peptide concentration (Fig. 2B, Table I). The dissociation constant for Hir54–65 derived from analysis of these results was the substantially higher value of 39 ± 9 μM, in good agreement with the value of 45 ± 7 μM, determined for the binding of this peptide to proexosite I (Ref. 6; Table I). The quantitative correlation between the inhibition constants and binding affinities of structurally different hirudin peptides was consistent with a critical role for access to proexosite I in the mechanism of factor Va cofactor activity.

**Inhibition of Bovine Prothrombin Activation by Hir54–65(SO32) and Hir54–65**—Bovine prothrombin was shown previously to
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Kinetic constants are listed from analysis of the inhibition of human and bovine prothrombin activation by Hir54–65(SO3)2 and Hir54–65. For human prothrombin, parameters were obtained from analysis of the peptide concentration dependence (Fig. 2), prothrombin concentration dependence (Fig. 4), and global analysis of these data. The rates, $v_0$, $v_{lim}$, and $v_u$ represent the measured initial rates from the peptide concentration dependence experiments and the rates calculated for the same conditions of 5 nM factor Va, 10 nM factor Xa, and 500 nM prothrombin with the best fit parameters from analysis of the prothrombin concentration dependence and the global fit. Dissociation constants listed for binding of the peptides to human and bovine prothrombin were from Anderson et al. (6). Experiments were performed, and the kinetics were analyzed as described under “Experimental Procedures” and in the text.

| Interaction          | Method                          | Kinetic constants | Binding constants, $K_D$ |
|----------------------|--------------------------------|-------------------|------------------------|
| Human prothrombin    |                                 | $v_0$          | $v_{lim}$          | $v_u$         | $K_D$          |
| Hir54–65(SO3)2       | Peptide concentration dependence| 1.6 ± 0.6       | 1.6 ± 0.1          | 0.10 ± 0.09   | 0.027 ± 0.001  | 2.6 ± 0.6      |
|                      | Prothrombin concentration       | 2.4 ± 0.6       | 1.8 ± 0.1          | 0.018 ± 0.008 | 0.026 ± 0.001  |                |
|                      | dependence                      |                  |                      |               |                |                |
|                      | Global analysis                 | 2.4 ± 0.4       | 1.8 ± 0.2          | 0.02 ± 0.01   | 0.02 ± 0.01    |                |
|                      |                                  | 39 ± 9           | 1.7 ± 0.1          | -0.01 ± 0.07  | 0.028 ± 0.003  | 45 ± 7         |
| Bovine prothrombin   |                                 |                  |                      |               |                |                |
| Hir54–65(SO3)2       | Peptide concentration dependence| 7 ± 7            | 2.0 ± 0.2          | 0.4 ± 0.4     | 0.067 ± 0.003  | 8 ± 1          |
|                      |                                  | 72 ± 32          | 2.2 ± 0.2          | -0.01 ± 0.3   | 0.17 ± 0.02    | 79 ± 11        |

- A: Effect of Hir54–65(SO3)2 on the Kinetics of Human Prothrombin Activation
- B: Effect of Hir54–65(SO3)2 and Hir54–65 on bovine prothrombin activation in the presence and absence of factor Va

**Figure 3.** Effect of Hir54–65(SO3)2 and Hir54–65 on bovine prothrombin activation in the presence and absence of factor Va. A, the observed initial rate of 1 μM bovine prothrombin activation ($v_{obs}$) by 20 nM human factor Xa in the presence (●) and absence (○) of 15 nM human factor Va is plotted as a function of total, sulfated hirudin peptide concentration ([Hir54–65(SO3)2]). B, the observed initial rate of prothrombin activation as in A in the presence (●) and absence (○) of factor Va, plotted as a function of total Hir54–65 concentration ([Hir54–65]). The lines for the data with factor Va represent the nonlinear least squares fit with Equations 7, 8, and 10 under “Experimental Procedures.” The lines for the data without factor Va represent the fit by a straight line. Initial rates were measured, and the data were analyzed as described under “Experimental Procedures.”

- A: Effect of Hir54–65(SO3)2 on the Kinetics of Human Prothrombin Activation
- B: Effect of Hir54–65(SO3)2 and Hir54–65 on bovine prothrombin activation in the presence and absence of factor Va

- A: Effect of Hir54–65(SO3)2 on the Kinetics of Human Prothrombin Activation
- B: Effect of Hir54–65(SO3)2 and Hir54–65 on bovine prothrombin activation in the presence and absence of factor Va
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The observed initial rate of prothrombin activation (v_{obs}) by 10 nm human factor Xa is plotted as a function of total prothrombin concentration ([Pro]_o) for reactions in the presence (closed symbols) and absence (open symbols) of 5 nm factor Va and concentrations of Hir^{54–65}(SO_3)^- of 0 μM (○, ◯), 10 μM (●, □), and 20 μM (▲, △). The lines represent the nonlinear least squares fit of the equations for the model described under “Experimental Procedures” to the data with the parameters given in the text. Initial rates were measured and analyzed as described under “Experimental Procedures.”

Figure 4. Effect of Hir^{54–65}(SO_3)^- on the kinetics of human prothrombin activation in the presence and absence of factor Va.

The results of these studies demonstrate that specific binding of hirudin peptides to proexosite I on human and bovine prothrombin selectively inhibits the cofactor activity of factor Va in regulating prothrombin activation. The simplest mechanism that can account for the results is that proexosite I directly mediates productive binding of prothrombin to factor Va within the factor Xa-factor Va catalytic complex and that the peptides bind competitively to the proexosite. Evidence in support of this conclusion includes (a) identification of prothrombin as the component responsible for selective inhibition of factor Va activity, (b) conformity of the kinetics as a function of prothrombin and peptide concentrations to the competitive binding mechanism, and (c) the correspondence between the inhibition and binding constants for both the sulfated and nonsulfated peptides to both human and bovine prothrombin.

This mechanism may also extend to interactions of prothrombin activation intermediates and thrombin. Previous studies of bovine prethrombin 2 as the substrate are compatible with this mechanism in showing inhibition of its factor Va-accelerated activation by hirudin (4, 14). A shared binding site for prethrombin 2 and thrombin on the factor Xa-factor Va-phospholipid complex was demonstrated by the finding that active site-blocked thrombin acts as an exosite I-dependent competi-
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TABLE II
Effect of phospholipid vesicles on inhibition of prothrombin activation by Hir54–65(SO3m)

| Enzyme components | Hir54–65(SO3m) | vobs/[Xa]o | Relative rate | Inhibition |
|-------------------|----------------|------------|--------------|------------|
| Factor Xa         | 0              | 0.00059 ± 0.00004 | 1.0 ± 0.1 | 6 ± 8 |
|                   | 10             | 0.00055 ± 0.000003 | 0.94 ± 0.08 | 17 ± 10 |
| Factor Xa, phospholipid | 0        | 0.015 ± 0.003 | 26 ± 6 |
|                   | 10             | 0.013 ± 0.004 | 23 ± 6 | 11 ± 31 |
| Factor Xa, factor Va | 0            | 0.012 ± 0.001 | 21 ± 2 | 19 ± 22 |
|                   | 10             | 0.009 ± 0.001 | 16 ± 2 | 80 ± 8 |
| Factor Xa, factor Va, phospholipid | 0        | 0.0026 ± 0.0006 | 4 ± 1 | 94 ± 8 |
|                   | 10             | 3.9 ± 0.2 | 6560 ± 610 |
|                   | 100            | 2.8 ± 0.3 | 4740 ± 600 |
|                   | 1000           | 2.2 ± 0.1 | 3670 ± 340 |

The characteristics of thrombin (17, 18) and prothrombin (13, 14) binding to factor Va are similar, with both interactions occurring through the heavy chain of the factor Va heterodimer and to the isolated heavy subunit. The site of exosite I-mediated thrombin binding has not been further defined but is thought to involve factor V sequences homologous to hirudin peptides and containing sulfated tyrosine residues within residues 659–698, near the carboxyl terminus of the human factor Va heavy chain (32–34). Suppression of the sulfation of these sequences in recombinant factor V decreases the rate of thrombin activation of factor V (34), consistent with their postulated role in thrombin binding. Moreover, suppression of the sulfation of residues in the heavy chain of factor Va reduces its cofactor activity in prothrombin activation (34). Together, the above observations support the possibility that prothrombin, the prothrombin activation intermediates, prethrombin 2 and mezothrombin, and the product, thrombin, share a common mode of binding to hirudin-like sequences in the factor Va heavy chain through proexosite I and exosite I that plays a critical role in substrate recognition by the factor Xa-factor Va complex. A similar involvement of proexosite I was found for both human and bovine prothrombin activation, with quantitative differences that were accounted for by species-specific differences in proexosite I affinity. Interestingly, factor Va was 3–5-fold less effective in accelerating bovine prothrombin activation compared with human prothrombin. Although the correlation may be fortuitous, this factor Va-dependent decreased specificity is speculated to be related to the 2–5-fold lower affinity of bovine proexosite I for hirudin peptides.

Other studies of the mechanism of bovine prothrombin 2 activation by the factor Xa-factor Va-phospholipid complex indicate that the substrate and product interactions may be more complex and support mechanisms alternative to the competitive binding model (14, 15). Analysis of the accessibility of the active site of factor Xa in the membrane-bound factor Xa-factor Va complex to substrates and inhibitors revealed a two-step mechanism in which prethrombin 2 binds initially to the factor Xa-factor Va complex through an exosite, followed by engagement of the catalytic site of factor Xa by prethrombin 2 in an isomerization of the ternary complex that precedes bond cleavage (15). The exosite for prethrombin 2 binding is thought to be expressed on factor Xa as a result of factor Va binding, rather than to represent a binding site on factor Va (14, 16). This is based in part on the fact that prethrombin 2 lacks the fragment 2 domain, which is thought to mediate binding of prothrombin to factor Va. In this model, inhibition of prethrombin 2 activation by hirugen and the exosite I dependence of inhibition by active site-blocked thrombin were concluded to be due to an indirect role of exosite I. This was supported by studies of product inhibition of prethrombin 2 activation by proteolytic derivatives and fragments of thrombin, which concluded that thrombin binding was not mediated by exosites I or II (14). The effects of hirugen have been explained instead by allosteric linkage between binding of the peptide to exosite I of prethrombin 2 and thrombin and a decrease in affinity of interactions occurring through a separate site that mediates binding of these proteins to factor Xa in the factor Xa-factor Va complex (14). As is generally the case, direct binding to exosite I and
binding through a different site that is extremely linked to exosite I cannot be readily distinguished. Thus, an alternative model in which prothrombin, prethrombin 2, and thrombin bind to the factor Xa-factor Va complex through exosites on factor Xa and in which the affinities of these interactions are negatively linked to binding of hirugen to proexosite I cannot be excluded by the present results. A concern with this mechanism, however, is that binding of prothrombin substrate species and thrombin to factor Va plays no apparent role in substrate recognition by the factor Xa-factor Va complex, whereas the formation of these complexes with factor Va have been established (13, 17–20).

A role for fragment 2 in the mechanism of substrate recognition is additionally still possible. Evidence that fragment 2 mediates binding of prothrombin to factor Va is mostly indirect, based initially on the factor Va-dependent accelerating effect of fragment 2 on prothrombin 2 activation (21). A recent analysis of the kinetics, however, concluded that interactions of prethrombin 2 with the factor Xa-factor Va-phospholipid complex alone are sufficient for acceleration by factor Va and are independent of fragment 2 and interactions it may mediate (16). However, this does not account for the substantial accelerating effects of fragment 2 on factor Va-dependent prethrombin 2 activation in the absence of phospholipid membranes (16, 21). On the basis of the information presently available, it is uncertain whether fragment 2 mediates factor Va binding directly and whether this interaction contributes to productive substrate recognition. The possibility remains that prothrombin interactions with factor Va involving proexosite I may also be influenced by fragment 2 on prothrombin, and exosites on factor Xa in addition to the catalytic site may also be involved in formation of the productive factor Xa-factor Va-prothrombin ternary complex.

Assembly of the factor Xa-factor Va-prothrombin complex on phospholipid vesicles counteracted inhibition by the hirudin peptides through membrane interactions of prothrombin. In the presence of phospholipid vesicles containing 5% phosphatidylserine, factor Va-accelerated prothrombin activation was inhibited to a limited but significant extent by HgI₂, 65% (SO₄), whereas no inhibition was observed with 25% phosphatidylserine membranes. The source of this difference is thought to be the lower affinities of the proteins for membranes containing lower phosphatidylserine levels (2) and a greater dependence of the rate on the protein-protein interactions that are inhibited by the peptide. The results of similar experiments with prethrombin 1 showed that elimination of membrane binding of prothrombin restored inhibition by the peptides, indicating similar protein-protein interactions in solution and between prethrombin 1 and the factor Xa-factor Va complex bound to membranes. These results are consistent with maintenance of the proexosite I-dependent interaction of prothrombin with factor Va on membrane surfaces but indicate that prothrombin-membrane binding can counteract competitive inhibition by hirudin peptides and dominate the assembly of the productive ternary complex. On the basis of these observations, it is uncertain whether prothrombin activation would be inhibited by proexosite I ligands under physiological conditions. Although the activated platelet membranes that constitute the physiological surfaces for prothrombin activation contain relatively low levels (10%) of phosphatidylserine (35), these membranes are much more complex. Flow conditions in vivo may also affect the assembly of the prothrombin activation complex, however, as indicated by the reported dependence of factor Xa-factor Va-membrane complex formation on prothrombin interactions in a model flow system (36). Provided that the prothrombin-factor Va interactions that are inhibited by hirudin peptides have a controlling influence on the rate of prothrombin activation under physiological conditions, the results suggest that proexosite I represents a potentially important new target for anticoagulant drug design. Selective inhibition of factor Va-dependent thrombin formation by proexosite I ligands would presumably have advantages over anticoagulants that inhibit thrombin activity directly and thereby inhibit both procoagulant and anticoagulant functions of the proteinase.

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Patricia J. Anderson, Anna Nesset, Kumudini R. Dharmawardana and Paul E. Bock

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