Amphipathic Helices Support Function of Blood Coagulation Factor IXa†

Mark D. Blostein,‡,§ Alan C. Rigby,‡ Barbara C. Furie,‡ Bruce Furie,‡ and Gary E. Gilbert*,‖

Center for Hemostasis and Thrombosis Research, Beth Israel Deaconess Medical Center and Department of Medicine, VA Boston Healthcare System, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02215

Received March 22, 2000

ABSTRACT: Blood coagulation factor IXa gains proteolytic efficiency upon binding to a phospholipid membrane. We have found that an amphipathic, membrane-binding peptide from the C2 domain of factor VIII, $fVIII_{2303-23}$, enhances proteolytic efficiency of factor IXa in the absence of phospholipid membranes. This enhancement is the result of a reduction in the K_M for the substrate, factor X, with little effect on the k_{cat}. Enhanced function requires interaction of the γ-carboxyglutamic acid (Gla) domains of factor IXa and factor X since (i) a synthetic peptide comprising the Gla domain of factor IXa and antibodies directed to the Gla domain of factor IXa inhibit this acceleration, (ii) the acceleration is Ca(II) dependent, and (iii) conversion of Gla-domainless factor X is not affected by the presence of $fVIII_{2303-23}$. The effect of $fVIII_{2303-23}$ on factor IXa parallels the enhanced function produced by phosphatidylserine-containing bilayers, and $fVIII_{2303-23}$ does not further enhance function of factor IXa when phospholipid vesicles are present. The critical feature of $fVIII_{2303-23}$ is apparently its amphipathic helix-forming structure [Gilbert, G. E., and Baleja, J. D. (1995) *Biochemistry 34*, 3022−3031] because other α-helical peptides such as a homologous peptide from the C2 domain of factor V and melittin have similar effects. Diastereomeric analogues of $fVIII_{2303-23}$ and melittin, which have reduced helical content, do not support factor IXa activity. A truncated peptide of $fVIII_{2303-23}$ with three C-terminal residues deleted retains α-helical content but loses capacity to enhance factor X cleavage, suggesting that a minimum length of α-helix is required. Although these results probably do not illuminate the physiologic function of the factor VIII peptide corresponding to $fVIII_{2303-23}$, they demonstrate a novel, membrane-mimetic role of amphipathic helical peptides in supporting function of factor IXa.

Blood coagulation results from a sequence of enzymatic reactions that ultimately produce thrombin and a fibrin clot. The tenase complex, composed of the serine protease, factor IXa, assembled with its cofactor, factor VIIIa, on a phospholipid surface in the presence of calcium, is necessary for normal blood coagulation. Factor IXa converts factor X to factor Xa. Factor Xa is the serine protease responsible for converting prothrombin to thrombin. The physiologic importance of the tenase complex is highlighted by the human coagulation disorders hemophilia A and hemophilia B, which result from deficiencies of factor VIII and factor IX, respectively [for review, see (*1*)].

Factor IXa and factor X are homologous proteins, both containing an N-terminal γ-carboxyglutamic acid (Gla)-rich domain, an aromatic amino acid stack domain, two epidermal growth factor domains, and a C-terminal serine protease domain (*2*). Both factor IXa and factor X bind to phospholipid membranes via their respective Gla domains. This interaction is calcium dependent as calcium stabilizes the tertiary structure of the Gla domain, facilitating the exposure of a phospholipid membrane binding site (*3*−*6*). Factor VIII has a domain structure of A1-A2-B-A3-C1-C2 (*7*) in which the C2 domain mediates the binding of factor VIII to phospholipid membranes (*8, 9*) and the A2 (*10*) and A3 domains (*11*) have binding sites for factor IXa.

Although factor IXa can convert factor X to factor Xa in solution, the rate is too slow to have physiologic relevance. Factor VIIIa and phospholipid membranes accelerate this reaction 200 000-fold through a combination of decreased K_M and increased k_{cat} (*12*). In the absence of factor VIIIa, phospholipid membranes decrease the K_M of factor IXa for factor X approximately 900-fold with little effect on k_{cat} (*12*, *13*). It is believed that membranes decrease the K_M of factor IXa for factor X, in part, by the simple addition of the binding affinities of factor IXa and the phospholipid membrane for factor X. However, in the presence of factor VIIIa, phospholipid membranes reduce the K_M and increase the k_{cat}, the latter increasing 1500-fold (*14*). Because only the combination of phospholipid membranes and factor VIIIa accelerates the k_{cat}, it is likely that phospholipid membranes cause a change in the conformation of the factor VIIIa−factor IXa complex near the catalytic site. Thus, phospholipid mem-

† This work was supported by NIH Grant PO1 HL 42443 and by a fellowship from the Medical Research Council of Canada (M.D.B.). G.E.G. is the recipient of Established Investigator Award 96001720 from the American Heart Association.

* Address correspondence to this author at VA Hospital, 1400 VFW Parkway, West Roxbury, MA 02132. E-mail: ggilbert@massmed.org. Phone: (617) 363-5686.

‡ Beth Israel Deaconess Medical Center, Harvard Medical School.

§ Current address: Lady Davis Institute for Medical Research, Sir Mortimer B. Davis−Jewish General Hospital, McGill University, Montreal, Quebec, Canada.

‖ Department of Medicine, Brockton−West Roxbury VA Medical Center, and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School.

10.1021/bi000655c CCC: $19.00
Published on Web 09/08/2000

branes apparently function both as condensation sites and as allosteric activators of the factor VIIIa−factor IXa complex.

A membrane binding peptide from the C2 module of factor VIII comprising residues 2303−2323 ($fVIII_{2303-23}$) competes with factor VIII for binding to phospholipid membranes and platelets (*15*, *16*). Antibodies directed against this sequence prevent binding of factor VIII to phospholipid vesicles, confirming that this peptide contains determinants of the membrane-binding motif of factor VIII (*17*). $FVIII_{2303-23}$ is predominantly a random coil in solution but adopts an amphipathic helical conformation in a membrane-mimetic environment of dodecyl phosphocholine micelles. A model of $fVIII_{2303-23}$ binding to phospholipid membranes proposes that the hydrophobic face of the helix interacts with the acyl chains of the individual phospholipids in a membrane such that the long molecular axis of the peptide lies parallel to the membrane interface.

Amphipathic helical peptides, a common structural motif found in membrane-binding peptides and proteins (*18*), have polar residues aligned on one side of the helix and hydrophobic residues along the other. Examples include melittin, the active component in bee venom (*19*, *20*), pardaxin, a neurotoxin purified from the Red Sea Moses sole, *Pardachus marmoratus (21*), and magainin, a toxin found in frogs (*22*). Their cytolytic, hemolytic, and antibacterial properties are due to their ability to disrupt eukaryotic and prokaryotic membranes (*23*−*25*). These peptides are typically unstructured in solution but adopt an amphipathic helical structure upon association with lipid bilayers. Melittin is a prototypical amphipathic helix that has been studied extensively by both X-ray crystallography and 2D NMR spectroscopy (*26*−*29*). In solution, melittin exists as an unstructured monomer, but in the presence of a membrane-mimetic environment, the secondary structure of melittin is almost completely α-helical (*23*). It is composed of two helical segments connected by a hinge that creates a bent α-helical rod with hydrophobic residues on one face and hydrophilic residues on the other. The angle formed by the hinge region differs depending on the surrounding environment (*27*).

In this report, we describe enhanced function of factor IXa caused by $fVIII_{2303-23}$ in the absence of phospholipid membranes. Contrary to our expectations, the enhanced function appears to rely on the amphipathic helical properties of $fVIII_{2303-23}$, rather than the specific amino acid sequence. Therefore, the results probably do not demonstrate a physiologic function of $fVIII_{2303-23}$. Rather they suggest the surprising result that, for factor IXa, an amphipathic helix can mimic a phospholipid bilayer for enhancing substrate affinity.

EXPERIMENTAL PROCEDURES

Materials. Human factor X, Gla-domainless human factor X, human factor IXa, and human factor Xa were purchased from Hematologic Technologies Inc. (Burlington, VT). Chromogenic substrate S-2765 was purchased from Diapharma Group Inc. Melittin was purchased from Sigma. Antibodies to the Gla domain of factor IX were prepared as previously described (*30*).

Peptide Synthesis. $fVIII_{2303-23}$ was synthesized using an Applied Biosystems 430A Peptide Synthesizer and 9-fluorenylmethoxycarbonyl (FMOC) chemistry on a Rink amide HBHA resin (NovaBiochem). Following cleavage of the FMOC groups with 20% piperidine in DMF, the free N-terminal amide was acetylated using 10% acetic aldehyde in *N*-methylpyrrolidone. The peptide was purified as described previously (*15*).

A peptide comprising the γ-carboxyglutamic domain of factor IX (fIX_{1-47}) (*31*) and a peptide comprising the propeptide region of proprothrombin (proPT18) (*32*) were synthesized as previously described. The $fVIII_{2146-66}$, $fV_{2170-90}$, D^4-melittin, D^4-$fVIII_{2303-23}$, and $fVIII_{2303-20}$ peptides were synthesized on an Applied Biosystems 430A Peptide Synthesizer using FMOC chemistry and purified using reverse-phase high-performance liquid chromatography. Purity was greater than 95%, and the amino acid composition of the collected peak was correct as judged by mass spectrometry.

Preparation of Phospholipid Vesicles. Phospholipid vesicles were prepared by extrusion through two stacked polycarbonate membranes of 0.1 μm pore size (Nucleopore Corp.) under argon as described previously (*33*). Phospholipid concentration was determined by phosphorus assay (*34*).

Enzymatic Assays. The activation of factor X by factor IXa in the presence of different peptides was measured with a two-step amidolytic substrate assay (*14*) with the following modifications. Factor IXa (20 nM) was incubated with the specified concentrations of peptide and varying concentrations of factor X in 150 mM NaCl, 50 mM Tris, pH 7.4, 1.5 mM $CaCl_2$, and 0.1% BSA for 30 min at 25 °C. The reaction was then stopped with 150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA. The amount of factor Xa generated was determined immediately using the chromogenic substrate S-2765 (0.31 mg/mL) on a Molecular Devices ELISA plate reader. For studies with Gla-domainless factor X, factor IXa (20 nM) was incubated with varying concentrations of Gla-domainless factor X in the presence and absence of 20 μM $fVIII_{2303-23}$ prior to the addition of EDTA and S-2765. For studies with varying concentrations of calcium, factor IXa (20 nM) was incubated with factor X (500 nM) and the indicated concentrations of calcium in the presence and absence of 20 μM $fVIII_{2303-23}$ prior to the addition of EDTA and S-2765. For studies in 2% TFE, factor IXa (20 nM) was incubated with varying concentrations of factor X in the presence and absence of 20μM $fVIII_{2303-23}$ in 150 mM NaCl, 50 mM Tris, pH 7.4, 1.5 mM $CaCl_2$, 0.1% BSA, and 2% TFE for 30 min at 25 °C. For studies with factor VIIIa, factor IXa (40 nM), factor X(500 nM), factor VIIIa (40 nM), and varying concentrations of $fVIII_{2303-23}$ were incubated for 40 min in 150 mM NaCl, 50 mM Tris, pH 7.4, 1.5 mM $CaCl_2$, and 0.1% BSA at 37 °C. For inhibition studies with the anti-factor IXa Ca(II)-dependent antibodies or with fIX_{1-47}, factor IXa (20 nM) was incubated with factor X (1000 nM) and the indicated concentration of antibody or fIX_{1-47} for 30 min prior to the addition of EDTA and chromogenic substrate.

Circular Dichroism Measurements. The CD spectra of $fVIII_{2303-23}$, $fVIII_{2303-20}$, D^4-$fVIII_{2303-23}$, mellitin, and D^4-melittin were collected on an Aviv model 62 DS spectropolarimeter equipped with a thermoelectric temperature control unit regulated to 0.1 °C. Spectra were recorded between 195 and 250 nm using a path length of 0.01 cm and a peptide concentration of 20 μM in 40% TFE with signal averaging of 16 s/nm. All spectra represent the signal average of 3 scans. The spectropolarimeter was routinely calibrated at

Table 1: Peptides Evaluated for Effect on Function of Factor IXa

$fVIII_{2303-23}$	TRYLRIHPQSWVHQIALRMEV
D^4-$fVIII_{2303-23}$[a]	TRYLRIHPQSWVHQIALRMEV
$fVIII_{2303-20}$	TRYLRIHPQSWVHQIALR
$fVIII_{2146-66}$	ARYIRLHPTHYSIRSTLRMEL
melittin	GIGAVLKVLTTGLPALISWIKRKRQQ
D^4-melittin	GIGAVLKVLTTGLPALISWIKRKRQQ
$fV_{2170-90}$	SRFIRVIPKTWNQSIALRLFL
ϵ-TxIX/24	LKRTIRTRLDDRECCEDGWCCTAA
proPT18	HVFLAPQQRRSLLQRVRR

[a] Diasteromeric amino acids are underlined.

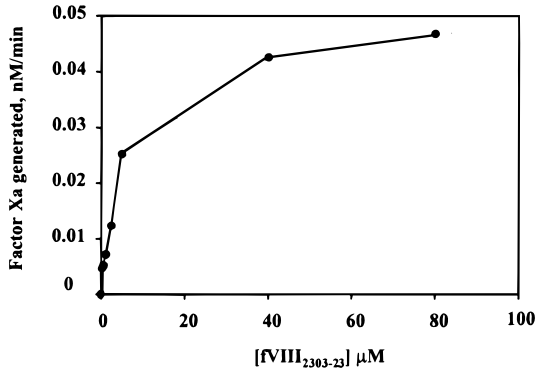

FIGURE 1: Activation of factor X by factor IXa in the presence of $fVIII_{2303-23}$. Factor IXa (20 nM) and factor X (80 nM) were incubated for 30 min with varying concentrations of $fVIII_{2303-23}$, and the concentration of factor Xa generated was measured by hydrolysis of the peptide substrate, S2765.

Table 2: Effects of Peptides on Kinetic Parameters of Cleavage of Factor X by Factor IXa

peptide	K_M (μM)[a]	k_{cat} ($\times 10^{-3}$ min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
$fVIII_{2303-23}$	0.17 ± 0.007[b]	15 ± 3.1	88
melittin	0.13 ± 0.05[c]	14 ± 1.7	108
D^4-$fVIII_{2303-23}$	9.2 ± 1.7[d]	3.2 ± 0.5	0.35
no peptide	95 ± 50[e]	9.5 ± 4	0.1

[a] The rate of factor Xa formation over 30 min at 37 °C was measured as a function of factor X concentration in the presence of 20 nM factor IXa and the peptide. [b] 20 μM $fVIII_{2303-23}$. Mean ± SEM for five experiments, each performed in duplicate. [c] 20 μM melittin. Mean ± SEM for four experiments, each performed in duplicate. [d] 20 μM D^4-$fVIII_{2303-23}$. Mean ± SEM for three experiments, each performed in duplicate. [e] Mean ± SEM for three experiments, each performed in duplicate.

192.5 and 290.5 nm with *d*-10-camphorsulfonic acid (CSA) before data collection. The percentage of α-helical content in melittin was estimated as previously described (*35*, *36*). The CD spectra generated in 2% TFE were performed on a JASCO spectropolarimeter.

RESULTS

A membrane-binding peptide from the C2 domain of factor VIII, $fVIII_{2303-23}$, has been previously characterized (*15*, *16;* Table 1). The peptide is now acetylated at the amino terminus and amidated at the carboxy terminus to improve solubility (*37*). $FVIII_{2303-23}$ greatly enhanced cleavage of factor X by factor IXa in the absence of factor VIIIa and phospholipid (Figure 1, Table 2). Experiments performed with varying concentrations of factor X with and without $fVIII_{2303-23}$ indicated that the acceleration occurred primarily by reducing the K_M of the reaction 600-fold from 95 μM to 173 nM, while affecting the k_{cat} by less than 2-fold (Table 2).

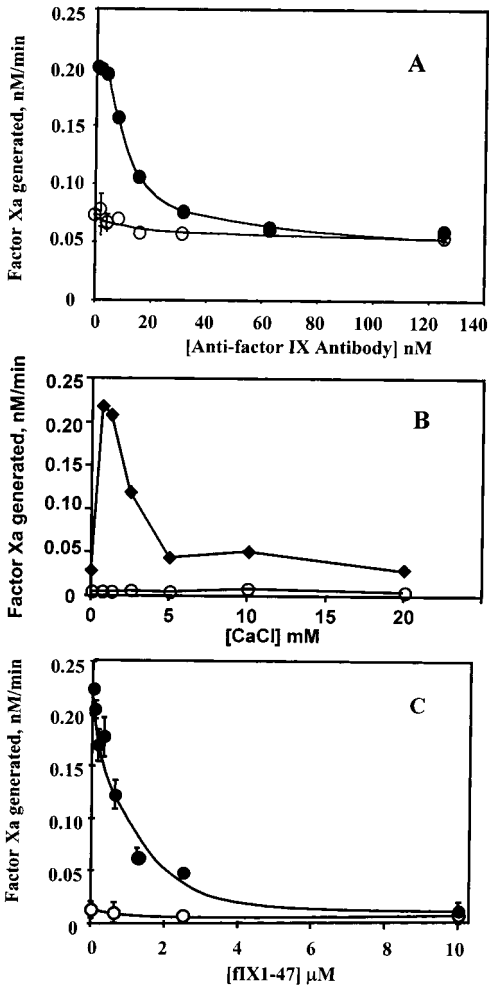

FIGURE 2: Importance of the factor IXa Gla domain for $fVIII_{2303-23}$ enhancement of factor IXa activity. (A) Factor IXa (20 nM) was incubated with factor X and the indicated concentrations of anti-factor IX-Ca(II)-specific antibodies for 30 min at 37 °C in the presence (●) and absence (○) of 50 μM $fVIII_{2303-23}$. Results are a mean of three separate experiments performed in duplicate. (B) Factor IXa (20 nM) and factor X (500 nM) were incubated for 30 min at the indicated concentrations of $CaCl_2$ in the presence (◆) and absence (○) of 20 μM $fVIII_{2303-23}$. (C) Factor IXa (20 nM) was incubated with factor X and the indicated concentrations of fIX_{1-47} for 30 min in the presence (●) and absence (○) of 20 μM $fVIII_{2303-23}$. Results are a mean of three separate experiments performed in duplicate.

The Gla Domains of Factor IXa and Factor X Participate in the Catalytic Enhancement. To determine whether the factor IXa Gla domain participated in this catalytic enhancement, we performed experiments with conformation-specific polyclonal antibodies, anti-factor IX-Ca(II)-specific antibodies (*30*), that bind to the Gla domain of factor IXa only in the presence of Ca(II) ions. These antibodies inhibited $fVIII_{2303-23}$-mediated enhancement of factor IXa function. The specificity of the inhibition by the anti-factor IX-Ca(II)-specific antibodies for peptide-dependent catalysis was confirmed by the observation that the antibodies did not inhibit factor IXa-mediated activation of factor X in the absence of $fVIII_{2303-23}$ (Figure 2A). We evaluated the effect of Ca(II) ions, which are required for correct folding of the Gla domains (Figure 2B). We found that the effect of $fVIII_{2303-23}$ is largely Ca(II)-dependent and is optimal at 0.5−1.0 mM Ca(II). This concentration is close to the physiologic concentration of free Ca(II) but is slightly lower than the

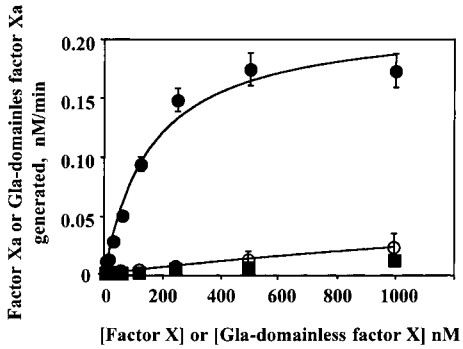

FIGURE 3: Importance of the factor X Gla domain for $fVIII_{2303-23}$ enhancement of factor IXa activation of factor X. Factor IXa (20 nM) and the indicated concentrations of Gla-domainless factor X were incubated for 30 min in the presence (■) and absence (○) of 20 μM $fVIII_{2303-23}$. Factor IXa and the indicated concentrations of factor X were incubated in the presence of 20 μM $fVIII_{2303-23}$ (●). Results are a mean of three separate experiments performed in duplicate.

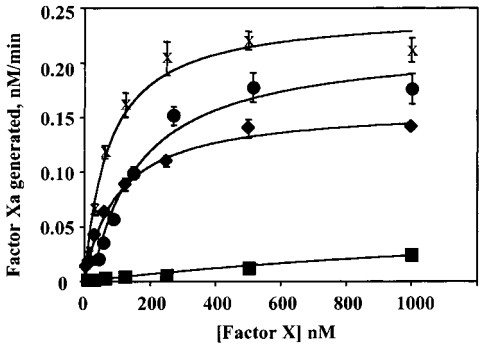

FIGURE 4: Specificity of $fVIII_{2303-23}$ enhancement of factor X activation of factor IXa. Factor IXa (20 nM) and varying concentrations of factor X were incubated for 30 min at 37 °C with 20 μM $fV_{2170-90}$ (×), 20 μM $fVIII_{2146-66}$ (◆), and 20 μM ϵ-TxIX/24 (■). For comparison, results with 20 μM $fVIII_{2303-23}$ (●) from Figure 3 are plotted. Results are a mean of three separate experiments performed in duplicate.

approximately 5 mM optimal concentration for cleavage of factor X by factor IXa on phospholipid membranes (*12*). $fVIII_{2303-23}$-supported activity, like phospholipid-supported activity, decreases at Ca(II) concentrations above the optimal level, but the effect is more pronounced. We also determined the effect of a synthetic peptide with the sequence of the factor IX Gla domain, fIX_{1-47} (*31*), on $fVIII_{2303-23}$ enhancement of factor X activation. FIX_{1-47} also inhibited enhanced turnover of factor X but did not affect factor IXa conversion of factor X in the absence of $fVIII_{2303-23}$. At 10 μM, fIX_{1-47} inhibited factor Xa generation to the level observed in the absence of $fVIII_{2303-23}$ (Figure 2C). These results are consistent with involvement of the Gla domain of factor IXa.

To determine whether the Gla domain of factor X was also necessary for peptide-enhanced factor IXa activation of factor X, we performed experiments with factor X from which the Gla domain had been proteolytically removed (Figure 3). In the absence of $fVIII_{2303-23}$, Gla-domainless factor X was activated by factor IXa with a K_M and k_{cat} similar to those for activation of native factor X by factor IXa. This demonstrates that the Gla domain of factor X is not critical for activation by factor IXa in the absence of phospholipid. Gla-domainless factor X cleavage by factor IXa was not accelerated in the presence of $fVIII_{2303-23}$. These data indicate either that $fVIII_{2303-23}$ binds to both Gla domains or that it indirectly mediates the binding of the Gla domain of one protein to that of the other.

Specificity of $fVIII_{2303-23}$. Our initial hypothesis was that $fVIII_{2303-23}$ bound specifically to the Gla domain(s) of factor IXa and/or factor X, mimicking factor VIII. To test the specificity of the interaction, other peptides were investigated for their ability to enhance factor IXa cleavage of factor X. In particular, two homologous peptides, one from the factor V C2 domain ($fV_{2170-90}$) and another from the C1 domain of factor VIII ($fVIII_{2146-66}$), were employed (Table 1). Both peptides (20 μM) cause a similar acceleration of factor X activation as observed with $fVIII_{2303-23}$ (Figure 4). These results indicate that enhancement of factor IXa function is not strictly dependent upon the amino acid sequence of $fVIII_{2303-23}$. A nonhomologous peptide of similar size from the cone snail, *Conus textile* (ϵ-TxIX/24) (Table 1), did not accelerate factor X activation, indicating that there are specific features of the three homologous peptides required to produce the rate enhancement (Figure 4).

Since $fVIII_{2303-23}$ adopts an amphipathic helical conformation in membrane-mimetic environments, we explored the helical content of $fV_{2170-90}$ and $fVIII_{2146-66}$ in a similar environment. Circular dichroism indicated that $fV_{2170-90}$ and $fVIII_{2146-66}$, like $fVIII_{2303-23}$, are α-helical in 40% TFE/water, a membrane-mimetic environment known to induce helical structure in peptides with a propensity to be helical (*39*). The amino acid sequences predict that the helices of $fV_{2170-90}$ and $fVIII_{2146-66}$ are amphipathic, similar to $fVIII_{2303-23}$, suggesting a common functional motif. We hypothesize that $fVIII_{2303-23}$ and other amphipathic helices enhance the activity of factor IXa by binding to the membrane-binding motif(s) of factor IXa and/or factor X and simulating a membrane.

The membrane-mimetic hypothesis suggests that $fVIII_{2303-23}$ should also accelerate factor IXa activity in the presence of the cofactor, factor VIIIa. This is indeed the case (Figure 5A), with $fVIII_{2303-23}$ concentrations of 40 μM resulting in a greater than 3-fold enhancement. Additional experiments indicated that the K_M for the factor VIIIa−factor IXa complex is reduced 10-fold in the presence of $fVIII_{2303-23}$. However, k_{cat} is not enhanced. The membrane-mimetic hypothesis also predicts that $fVIII_{2303-23}$ will not enhance the function of factor IXa when a phospholipid membrane is present. To test this prediction, the effect of $fVIII_{2303-23}$ was evaluated in the presence of phospholipid membranes. As shown in Figure 5B, factor Xa generation increased with increasing concentrations of $fVIII_{2303-23}$ in the absence but not in the presence of phospholipid membranes as predicted by the membrane-mimetic hypothesis.

Structural Basis for the $fVIII_{2303-23}$-Catalyzed Acceleration of Factor X Turnover. To confirm that $fVIII_{2303-23}$ enhances factor IXa activity while in an α-helical conformation, we performed enzymatic assays in the presence of trifluoroethanol. CD spectra of 20 μM $fVIII_{2303-23}$ indicated that the α-helical conformation was induced by as little as 2% TFE, with characteristic minima at 208 and 222 nm. In the presence of 2% TFE, $fVIII_{2303-23}$ enhanced factor IXa function in a similar fashion, and factor X was cleaved with a K_M of 0.39 μM. As observed without TFE, the k_{cat} was not altered more than 2-fold.

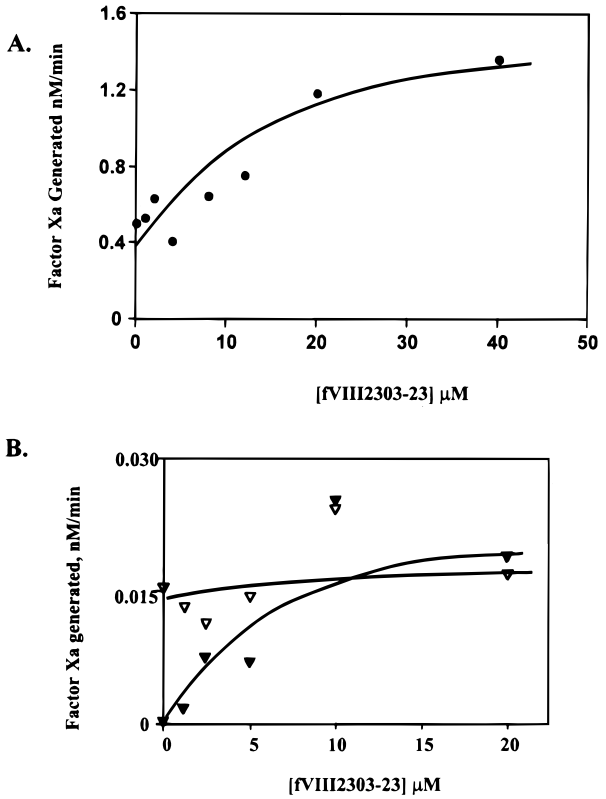

FIGURE 5: Effect of $fVIII_{2303-23}$ on the activation of factor X by factor IXa in the presence of phospholipid or factor VIIIa. (A) Factor IXa (40 nM), factor VIIIa (40 nM), and factor X (400 nM) were incubated for 40 min in the presence of the indicated concentrations of $fVIII_{2303-23}$. (B) Factor IXa, factor X (65 nM), and varying concentrations of $fVIII_{2303-23}$ were incubated in the presence (▽) and absence (▼) of 50 μM phospholipid vesicles (PS:PC 25:75). The lower rate of product accumulation compared to Figure 2 correlates to the lower concentration of the substrate utilized. Displayed results are representative of three such experiments.

The hypothesis that amphipathic helices may mimic a membrane's effect on factor IXa was further tested using melittin, a prototypical amphipathic helix (Table 1). Melittin enhanced function of factor IXa, similar to $fVIII_{2303-23}$ (Figure 4, Table 2). To evaluate the importance of the α-helical motif, we synthesized a diastereomeric melittin peptide with Val 5, Val 8, Ile 17, and Lys 21 replaced with the respective D-amino acids (Table 1). This diastereomeric analogue of melittin retains little α-helical structure or hemolytic activity as demonstrated previously. Circular dichroism spectra demonstrate loss of α-helix even in the presence of 40% TFE. Using previously published methods for calculating the α-helical content in melittin, the substitution of these four L-amino acids with their D-amino acid forms resulted in a reduction of predicted α-helical content from 78% to 15% as previously reported (*35*, *36*). At concentrations up to 60 μM, D^4-melittin did not enhance cleavage of factor X appreciably (Figure 6), confirming that the secondary structure of melittin affects its capacity to enhance the function of factor IXa.

Similar experiments were performed to determine whether α-helix-forming capacity is also necessary for enhancement of factor IXa function by $fVIII_{2303-23}$. As previously determined using NMR spectroscopy, $fVIII_{2303-23}$ is an amphipathic helical peptide with two helical segments separated by proline residue 8, which kinks the peptide backbone (*15*).

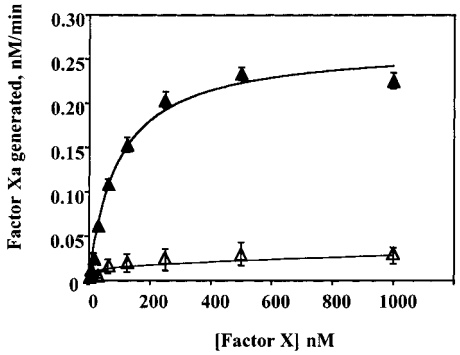

FIGURE 6: Effect of diastereomers of melittin on factor X activation by factor IXa. Factor IXa (20 nM) and the indicated concentrations of factor X were incubated in the presence of 20 μM melittin (▲) and 20 μM D^4-melittin (△). Results are a mean of three separate experiments performed in duplicate.

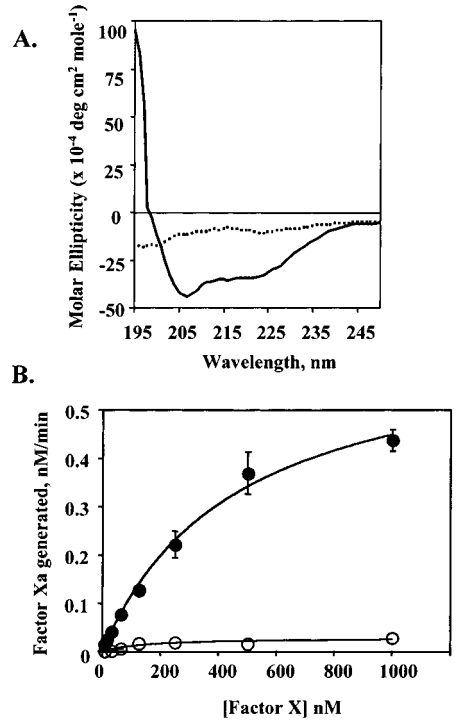

FIGURE 7: Effect of D^4-$fVIII_{2303-23}$ on factor X activation and the circular dichroism spectrum of $fVIII_{2303-23}$. (A) The CD spectrum of $fVIII_{2303-23}$ shows minima at 207 and 223 nm consistent with a predominantly helical structure. The CD spectrum of D^4-$fVIII_{2303-23}$ shows a minimum at 200 nm consistent with a random coil structure. $fVIII_{2303-23}$ and D^4-$fVIII_{2303-23}$ are at a concentration of 20 μM in 40% TFE/water. Solid line, $fVIII_{2303-23}$; dashed line, D^4-$fVIII_{2303-23}$. (B) Factor IXa (20 nM) and the indicated concentrations of factor X were incubated for 30 min at 37 °C in the presence of 20 μM $fVIII_{2303-23}$ (●) and 20 μM D^4-$fVIII_{2303-23}$ (○). Results are a mean of three separate experiments performed in duplicate.

To disrupt the α-helical content of the peptide, Tyr 3, Ile 6, Val 12, and Ile 15 were replaced with D-amino acids (Table 1). These substitutions resulted in almost complete loss of α-helical content, as evidenced in the CD spectra in 40% TFE as shown in Figure 7A, and also resulted in complete loss of $fVIII_{2303-23}$ stimulatory activity at concentrations of 0−60 μM (Figure 7B, Table 2).

To determine whether there is a minimum length of amphipathic peptide required to support the interaction with factor IXa and/or factor X, a truncated form of $fVIII_{2303-23}$ was synthesized with the three C-terminal residues deleted

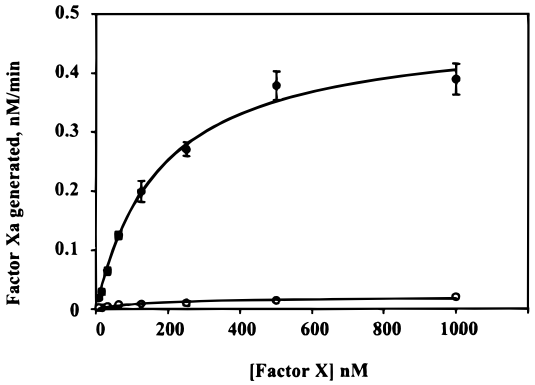

FIGURE 8: Effect of $fVIII_{2303-20}$ on factor X activation. Factor IXa (20 nM) and the indicated concentrations of factor X were incubated for 30 min at 37 °C in the presence of 20 μM $fVIII_{2303-23}$ (●) and 20 μM $fVIII_{2303-20}$ (○). Results are a mean of three separate experiments performed in duplicate.

($fVIII_{2303-20}$). Despite the loss of these residues, $fVIII_{2303-20}$ was still predominantly an α-helix. The CD spectrum of $fVIII_{2303-20}$ at a concentration of 20 μM in 40% TFE/water shows minima at 207 and 223 nm, consistent with a predominantly helical structure. However, at concentrations up to 80 μM, this peptide no longer supported factor IXa conversion of factor X to factor Xa (Figure 8). Similarly, another shorter amphipathic peptide similar in size to $fVIII_{2303-20}$ and comprising the 18 amino acid propeptide region of prothrombin (Table 1) did not enhance factor IXa function (data not shown). These data suggest that a minimum of three adjacent helical turns is required to support enhancement of factor IXa activation of factor X.

DISCUSSION

We report that $fVIII_{2303-23}$ enhances factor IXa-mediated activation of factor X. This enhancement results from a decrease in the K_M of the reaction with little effect on the k_{cat}. The effect is strikingly similar to that of phospholipid membranes in that enhanced function results from a similar reduction of K_M (*12*) and relies upon engagement of the Gla domains of factor IXa and factor X. The membrane-mimetic effect of this peptide is related to both the potential for helical secondary structure and the length of the helix.

The Gla domains of the vitamin K-dependent proteins mediate binding to phospholipid membranes (*3*). The Gla domain of factor IXa may also bind to factor VIIIa when both proteins are bound to a phospholipid membrane (*40*, *41*). In the present study, antibodies to the membrane-binding motif of the factor IX Gla domain and a peptide analogue of the Gla domain of factor IX (fIX_{1-47}) both inhibited $fVIII_{2303-23}$-mediated function of factor IXa. These data indicate that the Gla domain of factor IX interacts with $fVIII_{2303-23}$. Furthermore, $fVIII_{2303-23}$'s enhancement of factor IXa function requires the presence of the Gla domain of factor X since removal of the Gla domain of factor X results in loss of enhancement by $fVIII_{2303-23}$. The failure of $fVIII_{2303-23}$ to enhance cleavage of Gla-domainless factor X indicates either that $fVIII_{2303-23}$ interacts with the Gla domain of factor X or that $fVIII_{2303-23}$ causes an interaction between factor IXa and the Gla domain of factor X. Our working hypothesis is that, like phospholipid membranes, an amphipathic helix provides a site to which the Gla domains of factor IXa and factor X bind.

To our knowledge, this is the first detailed report of an amphipathic helical peptide mimicking a membrane-supported blood coagulation process. Others have demonstrated that polylysine is able to accelerate prothrombin activation by factor Xa (*42*). However, polylysine's mechanism of activation appears to be different as prethrombin I (prothrombin lacking a Gla domain) is activated by factor Xa in the presence of polylysine whereas in our system Gla domainless factor X is not activated by factor IXa in the presence of $fVIII_{2303-23}$. Furthermore, calcium inhibits polylysine activation of prothrombin whereas calcium is required for factor IXa activation of factor X in the presence of $fVIII_{2303-23}$ (Figure 2B). In the presence of factor IXa and factor X, soluble short-chain phosphatidylserine (C6PS) behaves similarly to these amphipathic helices. Gilbert and Arena demonstrated a 900-fold reduction in K_M with little effect on k_{cat}. In the presence of 0.25 mM C6PS, the K_M of factor IXa turnover of factor X is 200 nM, similar to the K_M obtained with $fVIII_{2303-23}$ (*43*).

We propose that the amphipathic helices provide a similar chemical environment to that of a membrane which allows factor IXa and factor X to interact. The juxtaposition of these two proteins on a short helical segment facilitates binding to one another, thereby reducing the K_M of the reaction. The length of the helix is critical as illustrated by the inability of a truncated form of $fVIII_{2303-23}$, $fVIII_{2303-20}$, to support this enhancement even though the α-helical nature of the residual peptide is preserved. We hypothesize that a minimum length of a hydrophobic face is required to allow binding of both factor IXa and factor X.

The amphipathic helices utilized in this report require detergent micelles, TFE, or another change in environment to stabilize formation of the helix. This raises the question of what mechanism induces α-helix formation in the presence of factor IXa and factor X. The Gla domain of factor IX binds calcium ions and undergoes a calcium-dependent conformational alteration in its tertiary structure exposing a hydrophobic patch that is believed to be responsible for binding the hydrophobic acyl chains in phospholipid membranes (*4*). Therefore, it is possible that the hydrophobic patch of the Gla domain induces the formation of the amphipathic helix and the hydrophobic face of the peptide.

Our results suggest that amphipathic helical peptides will be useful to elicit details in the enzymology and structure−function relationships of factor IXa and factor X. For example, helical peptides may prove more suitable for development of cocrystals of the factor IXa and factor X Gla domains than phospholipid vesicles or micelles. Helical peptides may be used in comparison to phospholipid vesicles to investigate the acceleration of enzymatic activity due to condensation of multiple enzyme and substrate molecules on a phospholipid surface vs acceleration due to a conformational change induced by engagement of the phospholipid-binding motif. They may also prove suitable for fluorescence anisotropy studies which would allow distinction between a conformational change in the factor IXa catalytic domain caused by engagement of a membrane-binding (or amphipathic peptide binding) site vs immobilization of factor IXa on a large vesicle. The interaction of $fVIII_{2303-23}$ with factor IXa and factor X is probably not representative of the interaction of factor VIIIa with factor IXa and factor X for two reasons. First, the interaction has limited specificity for

amino acid sequence of the helical peptide. Second, in the recently published crystal structure of the factor VIII C2 domain (*44*), $fVIII_{2303-23}$ has a predominantly β-sheet conformation and is apparently constrained from assuming a helical conformation. Thus, the interaction that we describe does not appear to be a physiologic model. However, our report highlights the possibility that currently unidentified amphipathic helical peptides or protein motifs could serve as activators of factor IXa through the membrane-binding motif.

ACKNOWLEDGMENT

We are grateful to Andrew Arena and Ameet Brahmavar for excellent technical assistance.

REFERENCES

1. Furie, B., and Furie, B. C. (1992) *N. Engl. J. Med. 326*, 800−806.
2. Furie, B., and Furie, B. C. (1988) *Cell 53*, 505−518.
3. Furie, B., Bouchard, B. A., and Furie, B. C. (1999) *Blood 93*, 1798−1808.
4. Freedman, S. J., Blostein, M. D., Baleja, J. D., Jacobs, M., Furie, B. C., and Furie, B. (1996) *J. Biol. Chem. 271*, 16227−16236.
5. Sunnerhagen, M., Forsen, S., Hoffren, A. M., Drakenberg, T., Teleman, O., and Stenflo, J. (1995) *Nat. Struct. Biol. 2*, 504−509.
6. Sunnerhagen, M., Drakenberg, T., Forsen, S., and Stenflo, J. (1996) *Haemostasis 26*, 45−53.
7. Lenting, P. J., van Mourik, J. A., and Mertens, K. (1998) *Blood 92*, 3983−3996.
8. Foster, P. A., Fulcher, C. A., Houghten, R. A., and Zimmerman, T. S. (1990) *Blood 75*, 1999−2004.
9. Arai, M., Scandella, D., and Hoyer, L. W. (1989) *J. Clin. Invest. 83*, 1978−1984.
10. Fay, P. J., Beattie, T., Huggins, C. F., and Regan, L. M. (1994) *J. Biol. Chem. 269*, 20522−20527.
11. Lenting, P. J., Van de Loo, J.-W. H. P., Donath, M.-J. S. H., van Moorik, J. A., and Mertens, K. (1996) *J. Biol. Chem. 271*, 1935−1940.
12. van Dieijen, G., Tans, G., Rosing, J., and Hemker, H. C. (1981) *J. Biol. Chem. 256*, 3433−3442.
13. Rawala-Sheikh, R., Ahmad, S. S., Ashby, B., and Walsh, P. N. (1990) *Biochemistry 29*, 2606−2611.
14. Gilbert, G. E., and Arena, A. A. (1996) *J. Biol. Chem. 271*, 11120−11125.
15. Gilbert, G. E., and Baleja, J. D. (1995) *Biochemistry 34*, 3022−3031.
16. Veeraraghavan, S., Baleja, J. D., and Gilbert, G. E. (1998) *Biochem. J. 332*, 549−555.
17. Scandella, D., Gilbert, G. E., Shima, M., Nakai, H., Eagleson, C., Felch, M., Prescott, R., Rajalakshmi, K. J., Hoyer, L. W., and Saenko, E. (1995) *Blood 86*, 1811−1819.
18. Shai, Y. (1995) *Trends Biochem. Sci. 20*, 460−464.
19. Habermann, E. (1972) *Science 177*, 314−322.
20. Habermann, E., and Jentsch, J. (1967) *Hoppe Seyler's Z. Physiol. Chem. 348*, 37−50.
21. Lazarovici, P., Primor, N., and Loew, L. M. (1986) *J. Biol. Chem. 261*, 16704−16713.
22. Zasloff, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5449−5453.
23. Dempsey, C. E. (1990) *Biochim. Biophys. Acta 1031*, 143−161.
24. Fletcher, J. E., and Jiang, M. S. (1993) *Toxicon 31*, 669−695.
25. Bechinger, B. (1997) *J. Membr. Biol. 156*, 197−211.
26. Ikura, T., Go, N., and Inagaki, F. (1991) *Proteins: Struct., Funct., Genet. 9*, 81−89.
27. Okada, A., Wakamatsu, K., Miyazawa, T., and Higashijima, T. (1994) *Biochemistry 33*, 9438−9446.
28. Terwilliger, T. C., and Eisenberg, D. (1982) *J. Biol. Chem. 257*, 6010−6015.
29. Terwilliger, T. C., and Eisenberg, D. (1982) *J. Biol. Chem. 257*, 6016−6022.
30. Liebman, H. A., Furie, B. C., and Furie, B. (1987) *J. Biol. Chem. 262*, 7605−7612.
31. Jacobs, M., Freedman, S. J., Furie, B. C., and Furie, B. (1994) *J. Biol. Chem. 269*, 25494−25501.
32. Sanford, D. G., Kanagy, C., Sudmeier, J. L., Furie, B. C., Furie, B., and Bachovchin, W. W. (1991) *Biochemistry 30*, 9835−9841.
33. Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55−65.
34. Chen, P. S., Toribara, T. Y., and Warner, H. (1956) *Anal. Chem. 28*, 1756−1758.
35. Oren, Z., and Shai, Y. (1997) *Biochemistry 36*, 1826−1835.
36. Wimley, W. C., and White, S. H. (1996) *Nat. Struct. Biol. 3*, 842−848.
37. Venkatachalapathi, Y. V., Phillips, M. C., Epand, R. M., Epand, R. F., Tytler, E. M., Segrest, J. P., and Anentharamaiah, G. M. (1993) *Proteins 15*, 349−359.
38. Morita, T., Fukudome, K., Miyata, T., and Iwanaga, S. (1991) *J. Biochem. (Tokyo) 110*, 990−996.
39. Rothemund, S., Beyermann, M., Krause, E., Krause, G., Bienert, M., Hodges, R. S., Sykes, B. D., and Sonnichsen, F. D. (1995) *Biochemistry 34*, 12954−12962.
40. Larson, P. J., Stanfield-Oakley, S. A., VanDusen, W. J., Kasper, C. K., Smith, K. J., Monroe, D. M., and High, K. A. (1996) *J. Biol. Chem. 271*, 3869−3876.
41. Astermark, J., Hogg, P. J., Bjork, I., and Stenflo, J. (1992) *J. Biol. Chem. 267*, 3249−3256.
42. Vogel, C. N., Butkowski, R. J., Mann, K. G., and Lundblad, R. L. (1976) *Biochemistry 15*, 3265−3269.
43. Gilbert, G. E., and Arena, A. A. (1997) *Biochemistry 36*, 10768−10776.
44. Pratt, K. P., Shen, B. W., Takeshima, K., Davie, E. W., Fujikawa, K., and Stoddard, B. L. (1999) *Nature 402*, 439−442.

BI000655C