RESEARCH COMMUNICATION

Regions 301–303 and 333–339 in the catalytic domain of blood coagulation Factor IX are Factor VIII-interactive sites involved in stimulation of enzyme activity

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The contribution of the Factor IX catalytic domain to Factor VIIIa binding has been evaluated by functional analysis of Factor IX variants with substitutions in α-helix region 333–339 and region 301–303. These regions were found to play a prominent role in Factor VIIIa-dependent stimulation of Factor X activation, but do not contribute to the high-affinity interaction with Factor VIIIa light chain. We propose that complex assembly between Factor IXa and Factor VIIIa involves multiple inter active sites that are located on different domains of these proteins.

Key words: haemophilia B, mutagenesis, serine proteinase.

INTRODUCTION

The coagulation cascade comprises a series of enzymic conversions that are driven by the assembly of activated serine proteases with membrane-bound cofactors [1,2]. Cofactor-dependent haemostatic enzymes include the activated forms of Factor VII (FVIIa), Factor IX (FIXa) and Factor X (FXa), and their respective cofactors are tissue factor, activated Factor VIII (FVIIIa) and activated Factor V (FVa). The three-dimensional structures of FIXa, FVa and FVIIIa are very similar [3]. As for the cofactors, FVIIa and FVa are large (260 kDa) homologous plasma proteins, whereas tissue factor is a transmembrane protein that is much smaller (37 kDa), lacking any significant homology with FVIIIa and FV [1]. In addition to their structural similarity, FIXa, FIXa and FVa share slow or negligible activity towards their natural substrates, unless in complex with their physiological cofactors. Enzyme–cofactor complex assembly is associated with a dramatic increase in catalytic efficiency [1] by a mechanism that remains poorly understood. The crystal structure of FVIIa in complex with tissue factor [4] has identified molecular sites within FVIIa that are in contact with tissue factor, and thus contribute to cofactor-dependent stimulation of enzymic activity.

Unlike the FIXa–tissue factor interaction, no details are available concerning the molecular sites involved in FVIIIa-dependent stimulation of FIXa activity. Like FVIIa and FVa, FIXa consists of two disulphide-linked polypeptide chains: a light chain comprising the phospholipid-binding region and two epidermal growth factor (EGF)-like domains, and a heavy chain corresponding to the catalytic domain [3]. In the cofactor FIXa, FIXa-interactive sites are present in the A2-domain of the FIXa heavy chain and the A3-domain of the FIXa light chain (for a review see [5]). With respect to the enzyme FIXa, the light chain has been implicated in binding to the FIXa A3-domain [6], whereas the catalytic domain presumably interacts with the FIXa A2-domain [7,8]. The factor VIII-interactive site on the FIXa light chain has not been identified, but is known to involve Ca²⁺-binding to the first EGF-like domain [9] and a salt bridge between residue Glu⁷⁸ in the EGF-1 domain and Arg⁴¹ in the EGF-2 domain [10]. The FVIIIa-catalytic domain has been proposed to involve residues 181–310 [11], but its precise location has remained unidentified so far.

The aim of the present study was to identify FVIII-interactive site(s) within the catalytic domain of human FIX. We considered the hypothesis that, in spite of the lack of sequence similarity between tissue factor and FVIIIa, the catalytic domains of FVIIa and FIXa share similar cofactor-binding regions. Accordingly, we constructed FIX variants with mutations in two distinct sites, region 301–303 and α-helix region 333–339, which are the counterparts of the cofactor-binding regions 275–277 and 306–312 in FVIIIa [4,12]. These mutants were expressed in mammalian cells and the purified activated forms thereof were functionally characterized, with particular reference to the effect of FVIIIa on enzymic activity.

MATERIALS AND METHODS

Materials

CNBr-Sepharose CL4B and Q-Sepharose FF were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). t-α-Phosphatidyl-l-serine and t-α-phosphatidylcholine were obtained from Sigma (St. Louis, MO, U.S.A.). Oligonucleotide primers were purchased from Gibco-BRL (Breda, The Netherlands). The Thermo Sequenase cycle sequencing kit was obtained from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). Pfu-polymerase was obtained from Stratagene (Cambridge, U.K.). CH₃SO₂-L-leucyl-L-glycyl-L-arginine p-nitroanilide (CH₃SO₂-LGR-pNA), product name CBS 31.39, was purchased from Diagnostica Stago (Asnières, France).

Proteins

The monoclonal anti-FIX antibody CLB-FIX 14 has been described previously [13]. Plasma-derived FIX was prepared

Abbreviations used: FV, FVII, FVIII, IX, FX and FXI, Factors V, VII, VIII, IX, X and XI respectively; CH₃SO₂-LGR-pNA, CH₃SO₂-o-leucyl-L-glycyl-L-arginine p-nitroanilide; HSA, human serum albumin; EGF, epidermal growth factor.

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as described elsewhere [6]. FXIa was obtained from Enzyme Research Laboratories (South Bend, IN, U.S.A.). FVIII was purified as outlined previously [14]. FX was purified as described in [15]. Purified antithrombin and human serum albumin (HSA) were from the Division of Products of CLB, Amsterdam, The Netherlands.

Protein concentrations

Protein was quantified by the method of Bradford [16], using HSA as a standard. The concentration of active FIXa was determined by active-site titration with antithrombin [6]. FVIII activity was measured by a spectrophotometric assay using bovine coagulation factors (Coatest FVIII; Chromogenix AB, Mölndal, Sweden). The amount of FVIII in 1 ml of human plasma (1 unit/ml) was assumed to correspond to 0.35 nM.

Expression and purification of recombinant FIX

The mammalian expression plasmid pKG5 containing human FIX cDNA [17] was used as a template for standard PCR mutagenesis. The mutated full-length FIX cDNAs were verified by DNA sequence analysis. Stable cell lines producing FIX variants were prepared as described in [9,10]. Purification of recombinant FIX from concentrated medium was performed using the same immunoaffinity step as outlined previously [9]. As established previously, recombinant FIX produced by this expression system displays normal calcium-dependent properties and similar activities for recombinant wild-type FIX and plasma-derived FIX [9,17]. Recombinant and plasma-derived FIX were converted into FIXa by FXIa as described in [6]. FIXa was purified from the activation mixture employing anion-exchange chromatography as outlined previously [6].

Amidolytic activity

Amidolytic activity was assessed by measuring CH$_2$SO$_4$-LGR-pNA hydrolysis as outlined previously [6] in 0.2 mg/ml HSA/0.1 M NaCl/5 mM CaCl$_2$/0.05 M Tris, pH 7.4. Catalytic efficiency of substrate hydrolysis was determined using substrate concentrations between 0 and 5 mM.

FX activation

FX activation was assayed essentially as described in [10]. Briefly, FIXa was incubated with various concentrations of FX in the presence of 0.1 mM phospholipid vesicles (phosphatidylserine/phosphatidylcholine, 1:1, mol/mol) and calcium ions. FIXa formation was stopped by the addition of EDTA (10 mM) and subsequently quantified employing the chromogenic substrate S2222 (Chromogenix). In experiments using various FVIIIa concentrations, unactivated FVIII was added to the reaction mixture containing phospholipid vesicles, FIXa and thrombin (5 nM). After 1 min of incubation, FX activation was initiated by the addition of FX (0.2 μM). FIXa formation was quantified as described above and initial rates were calculated from FIXa concentrations after 2 min of incubation.

RESULTS AND DISCUSSION

FIX mutagenesis strategy

To investigate the contribution of the catalytic domain of FIX to FVIIIa binding, we took advantage of the crystal structure of the FVIIIa–tissue factor complex and the interactive sites located therein. Within the FVIIIa catalytic domain, these comprise region 275–277 and the α-helix region 306–312 [4,12]. Figure 1 shows the three-dimensional structure of the FVIIIa catalytic domain and that of FIXa. FVIIIa region 275–277 has its counterpart in residues 301–303 in FIXa. These residues (Lys-Arg-Gly) form the connection between an α-helix and a β-strand [18]. This region is well conserved in FIX from nine different species [19], but differs in FVII (Phe-Val-Arg). To address the role of this region, we constructed a FIX variant in which Lys$^{301}$ and Phe$^{302}$ were replaced by Ala. Figure 1 further shows the second tissue-factor-binding site, α-helix region 306–312, which has its structural counterpart in FIX region 333–339. The sequence of this α-helix (Arg-Ala-Thr-Cys-Leu-Arg-Ser) is completely conserved in FIX from other species [19], but different in the related enzymes FVIIa and FXa, which do not bind to FVIIIa. To assess the functional role of this α-helix, we introduced the corresponding sequence of FX (Arg-Asn-Ser-Cys-Lys-Leu-Ser). We further made two point mutations in this region (Arg$^{323}$ → Gln and Leu$^{327}$ → Phe), which are known to be associated with severe haemophilia B [20], and as such are candidates for contributing to cofactor-interactive sites. Functional characterization of these mutants focused on the effect of FVIIIa on FIXa enzymic activity.

FIXa activity in the absence of FVIIIa

First, we examined the FVIIIa-independent properties of the activated FIX mutants and normal FIXa. Therefore we measured amidolytic activity and enzymic activity towards FX in the absence of FVIIIa. As summarized in Table 1, the catalytic efficiency for CH$_2$SO$_4$-LGR-pNA hydrolysis of FIXa-Lys$^{301}$Ala/Phe$^{302}$Ala was indistinguishable from normal FIXa whereas the activity towards FX was slightly reduced. (Figure 2A and Table 1). The three FIX variants with substitutions in region 333–339 displayed a 2–4-fold reduction in $k_{cat}$/$K_m$ for CH$_2$SO$_4$-LGR-pNA hydrolysis compared with normal FIXa. Reduced amidolytic activity was accompanied by a decrease in FX activation (Figure 2A and Table 1). FIXa-Leu$^{327}$Phe displayed the greatest decrease in catalytic efficiency of FX activation (8-fold), whereas $k_{cat}$/$K_m$ was approx. 1.5-fold decreased for the other two FIX variants. Apparently, mutations in α-helix region 333–339 interfere directly or indirectly with substrate binding. This observation is in agreement with a previous report concerning the role of residue 338 in FX binding [21]. In this respect it is noteworthy that α-helix region 333–339 is linked to region 340–347, which is part of the substrate-binding groove in FIXa. Mutations in the α-helix might change the conformation of this substrate-binding region and thereby affect the enzymic activity of FIXa. However, the moderate reduction in FX activation observed for FIXa-Arg$^{323}$Gln and FIXa-Leu$^{327}$Phe in the absence of FVIIIa (Table 1) does not fully explain the low biological activity (< 1% of normal FIXa) found in haemophilia B patients with these mutations.

FIXa activity in the presence of FVIIIa

The effect of substitutions in regions 301–303 and 333–339 was further explored by measuring FX activation in the presence of various concentrations of FVIIIa. As shown in Figure 2B, FXa generation by normal FIXa was enhanced by FVIIIa in a saturable and dose-dependent manner. However, FVIIIa-dependent activity toward FX was strongly reduced for all FIX mutants. Apparently, mutations in regions 301–303 and 333–339 result in FIXa molecules that display a dramatically reduced response to FVIIIa. The kinetic data were used for calculation of the apparent dissociation constant ($K_a$) for FVIIIa binding. As
Factor VIII binding to the Factor IX catalytic domain

Table 1. CH$_3$SO$_2$-LGR-pNA hydrolysis and FX conversion by mutant and normal FIXa

<table>
<thead>
<tr>
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<th>Amidolytic activity ([k_{cat}/K_m}) (M$^{-1}$·s$^{-1}$)</th>
<th>FX activation ([10^{-3} \times \text{apparent } k_{cat}/k_m}) (M$^{-1}$·s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal FIXa</td>
<td>180 ± 3</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td>FIXa-Lys$^{301}$Ala/Phe$^{302}$Ala</td>
<td>172 ± 10</td>
<td>3.0 ± 0.8</td>
</tr>
<tr>
<td>FIXa-Arg$^{333}$Gln</td>
<td>90 ± 0.2</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>FIXa-Leu$^{333}$Phe</td>
<td>54 ± 1.1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>FIXa-helix$^{333-339}$/FX</td>
<td>76 ± 4</td>
<td>4.8 ± 1.2</td>
</tr>
</tbody>
</table>

listed in Table 2, the apparent \(K_m\) for FVIIIa binding to normal FIXa was 0.8 nM, whereas this constant was 3–8-fold increased for the activated FIX mutants. It should be noted that the FVIIIa concentrations used (0–1.75 nM) are below the apparent \(K_m\) for each of the FIXa mutants, resulting in relatively large S.D. values for the \(K_m\) estimates. Nevertheless, these data support the conclusion that substitutions in regions 301–303 and 333–339 are associated with a decreased affinity for FVIIIa. The relatively small increase in apparent \(K_m\), however, suggests that both regions play a limited role in the FIXa–FVIIIa interaction. Our observation that mutations in the FIXa catalytic domain are associated with a mild defect in complex formation but a major decrease in FVIIIa-dependent stimulation of FX activation would be compatible with complex assembly and stimulation being mediated by different molecular sites.

Implications for FIXa–FVIIIa complex assembly

As summarized in Table 2, we identified two regions in the FIXa catalytic domain that are involved in FVIIIa-dependent enzymic activity and, as such, may represent FVIIIa-binding sites. One of these sites, \(\alpha\)-helix region 333–339, has previously been implicated as being part of an extended exosite involved in binding of FIXa to its substrate, FX [21]. The same helix has further been proposed to be of major importance for the interaction of FIXa

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Figure 2 Activity of mutant and normal FIXa toward FX in the presence or absence of FVIIIa

(A) FX activation in the absence of FVIIIa was assessed by incubation of various concentrations of FX (0–0.5 μM) in 5 mM CaCl2/0.1 M NaCl/0.2 mg/ml HSA/0.05 M Tris, pH 7.4, with 30 nM of normal FIXa (○), FIXa-Lys301Ala/Phe302Ala (▲), FIXa-Arg333Gln (▲), FIX-Leu337Phe (●) and FIXa-helix333−339/FX (■) in the presence of 0.1 mM phospholipid vesicles. (B) FVIII-dependent activation of FX by mutant or normal FIXa was evaluated by incubation of 0.1 nM of normal FIXa (○), FIXa-Lys301Ala/Phe302Ala (▲), FIXa-Arg333Gln (▲), FIX-Leu337Phe (●) and FIXa-helix333−339/FX (■) in the same buffer with various concentrations of FVIIIa (0–1.75 nM), FX (0.2 μM) and 0.1 mM phospholipid vesicles. Mean values for three experiments are presented.

Table 2 FVIIIa-dependent properties of normal and mutant FIXa

<table>
<thead>
<tr>
<th></th>
<th>Apparent Kd (nM)</th>
<th>Fxa formation (mol of Fxa/min per mol of IXa)</th>
<th>FVIII-light-chain binding (mol of FIXa/mol of FVIII)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal FIXa</td>
<td>0.8 ± 0.1</td>
<td>35.4 (100%)</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>FIXa-Lys301Ala/Phe302Ala</td>
<td>2.2 ± 0.4</td>
<td>4.6 (13%)</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>FIXa-Arg333Gln</td>
<td>6.4 ± 1.5</td>
<td>1.5 (4%)</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>FIX-Leu337Phe</td>
<td>N. D.</td>
<td>1.0 (3%)</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>FIXa-helix333−339/FX</td>
<td>2.2 ± 0.3</td>
<td>1.9 (3%)</td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

Table 2 suggests that FVIIIa binding of normal and mutant FIXa were calculated from the data presented in Figure 2(B). The FX activation rate was determined at a FVIIIa concentration of 1 unit/ml. FVIII-light-chain binding of normal and mutant FIXa was performed as described in [14]. The FIXa concentration in these experiments was fixed at 15 nM, i.e. the Kd for FVIII light-chain binding of normal FIXa. Abbreviation: N. D., could not be quantified.

with its cofactor FVIIIa, as reported in two recent abstracts [22,23]. In both studies, kinetics of FXa formation were used to calculate binding parameters of FIXa–FVIIIa interaction. The complexity of such kinetic systems may contribute to the disparity of data obtained. For instance, a 100-fold increase in Kd has been reported for an Arg$$\rightarrow$$!Leu substitution [22], but only a 5–10-fold increase for an Arg$$\rightarrow$$!Ala substitution [23]. Using a similar kinetic approach, we observed an 8-fold increase of apparent Kd for the Arg$$\rightarrow$$!Gln substitution (Table 2), while FX activation was 25-fold decreased (Figure 2B and Table 2). Also other mutants display a dissimilar decrease in FX activation versus decrease in apparent affinity for FVIIIa (Table 2). Therefore the underlying assumption that FX activation is directly proportional to the concentration of the FIXa–FVIIIa complex [21,22,23] may not be generalized.

We have previously reported that FIXa binds with high affinity (Kd ≈ 15 nM) to the FVIII light chain in direct binding studies [14]. Similar equilibrium binding studies were performed using the various FIXa variants with decreased FVIIIa-dependent activity. The data reveal little or no effect on the interaction with FVIII light chain (see Table 2). We therefore conclude that mutation of either region 301–303 or α-helix 333–339 affects FVIIIa-dependent FIXa activity, but has limited impact on FIXa-FVIIIa complex assembly per se. This apparently paradoxical view is in full agreement with the notion that the FIXa light chain binds to the A3-domain of FVIII with high affinity [6,25], whereas the FIXa catalytic domain interacts with the FVIII A2-domain with much lower affinity [7]. It should be noted that the light chains of FIXa and FVIII also comprise the phospholipid-binding domains of both proteins. Therefore it seems reasonable to suppose that this interaction provides the driving force for FIXa–FVIIIa complex assembly. Once assembled on the phospholipid membrane, a low-affinity, secondary interaction may be sufficient to effectively bring the FVIII A2-domain in contact with the FIXa catalytic domain, which finally changes the FIXa active site and increases its capability to activate FX.

Our finding that the FIXa catalytic domain comprises two distinct FVIII-interactive sites, none of which binds to the FVIII light chain, raises the possibility that these sites interact with the FVIII A2-domain. As extensively reviewed elsewhere [5], indeed two FIXa binding sequences have been identified in the FVIII A2-domain, comprising residues 558–565 and 698–710. The current FVIII model demonstrates that these sites are exposed at
the same side of the protein, at a distance of about 2.0–2.5 nm (20–25 Å) [24]. It is striking that the FIXa crystal structure [18] predicts a similar distance for the side chains of region 301–303 and the centre of α-helix region 333–339 are in contact with FVIII region 558–565, while residues in region 301–303 interact with FVIII region 698–710. Although fully compatible with currently available data, the crystal structure of the FIXa–FVIIIa complex will be needed to verify this view.

Our study demonstrates that the catalytic domains of FVIIa and FIXa, in spite of being stimulated by totally dissimilar cofactors, share the same cofactor-interactive sites. The same structural elements are also present in other, related serine proteases from the blood coagulation system, such as FXa, activated protein C and thrombin [3]. This raises the possibility that, in these enzymes, the corresponding regions are also involved in the stimulation of proteolytic activity.

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