The Sequence Glu$^{1811}$-Lys$^{1818}$ of Human Blood Coagulation Factor VIII Comprises a Binding Site for Activated Factor IX*

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In previous studies we have shown that the interaction between factors IXa and VIII involves the light chain of factor VIII and that this interaction is inhibited by the monoclonal antibody CLB-Cag A against the factor VIII region Glu$^{1778}$-Asp$^{1840}$ (Lenting, P. J., Donath, M. J. S. H., van Mourik, J. A., and Mertens, K. (1994) J. Biol. Chem. 269, 7150–7155). Employing distinct recombinant factor VIII fragments, we have now localized the epitope of this antibody more precisely between the A3 domain residues Glu$^{1801}$ and Met$^{1823}$. Hydropathy analysis indicated that this region is part of a major hydrophilic exosite within the A3 domain. The interaction of factor IXa with this exosite was studied by employing overlapping synthetic peptides encompassing the factor VIII region Tyr$^{1786}$-Ala$^{1834}$. Factor IXa binding was found to be particularly efficient to peptides corresponding to the factor VIII sequences Lys$^{1804}$-Lys$^{1818}$ and Glu$^{1811}$-Gln$^{1820}$. The same peptides proved effective in binding antibody CLB-Cag A. Further analysis revealed that peptides Lys$^{1804}$-Lys$^{1818}$ and Glu$^{1811}$-Gln$^{1820}$ interfere with binding of factor IXa to immobilized factor VIII light chain (K$\text{d} \approx 0.2$ mM and 0.3 mM, respectively). Moreover, these peptides inhibit factor X activation by factor IXa in the presence of factor VIIa (K$\text{d} \approx 0.2$ mM and 0.3 mM, respectively) but not in its absence. Equilibrium binding studies revealed that these two peptides bind to the factor IXA yozogly and its activated form, factor IXA, with the same affinity (apparent K$\text{d} \approx 0.2$ mM), whereas the complete factor VIII light chain displays preferential binding to factor IXA. In conclusion, our results demonstrate that peptides consisting of the factor VIII light chain residues Lys$^{1804}$-Lys$^{1818}$ and Glu$^{1811}$-Gln$^{1820}$ share a factor IXA binding site that is essential for the assembly of the factor X-activating factor IXA-factor VIIIa complex. We propose that the overlapping sequence Glu$^{1811}$-Lys$^{1818}$ comprises the minimal requirements for binding to activated factor IX.

Human blood coagulation factor VIII (FVIII) is an essential protein of the hemostatic system, which is evident from the severe bleeding disorder hemophilia A that is associated with FVIII deficiency or dysfunction (1). FVIII is synthesized as a single chain polypeptide containing a number of discrete domains arranged in the sequence A1-A2-B-A3-C1-C2 (2, 3). Examination of its primary structure reveals that FVIII shares considerable homology with the plasma proteins factor V (FV) and ceruloplasmin (4–6). Whereas ceruloplasmin comprises a triple A domain structure (A1-A2-A3), FV displays the same domain structure (7, 8). In contrast to FV, FVIII predominately circulates as a heterodimeric protein, consisting of a Me2$^+$-linked light and heavy chain (9–11). The heavy chain contains the A1-A2-B domains and is heterogeneous (M, 90,000–200,000) due to limited proteolysis at a number of positions within the B domain. The light chain of FVIII (M, 80,000) comprises the domains A3-C1-C2 (10, 12).

In the intrinsic pathway of blood coagulation, FVIII functions as a nonenzymatic cofactor in the factor X (FX)–activating complex (13). Within this complex, the serine protease factor IXa (FIXa) activates FX in the presence of calcium ions, phospholipids, and activated FVIII. In order to play its role in the generation of FXa, FVIII has to be activated (14, 15). Activation is achieved by limited proteolysis in both the FVIII heavy and light chain by FIXa or thrombin (12), which results in the formation of a heterotrimERIC product, FVIIIa (16, 17). The relatively labile FVIIIa heterotrimer is known to be stabilized by the enzyme FIXa in the presence of phospholipids (18). In addition, it has been reported that the phospholipid-FIXa complex enhances the reassociation of isolated FVIIIa subunits into the FVIIIa heterotrimer (19), indicating that FVIIIa is capable of directly interacting with FIXa.

Several studies have been performed in order to characterize the assembly of the FIX:FVIII complex in more detail (19–22). The FVIII heavy chain regions Ser$^{559}$-Gln$^{565}$ and Arg$^{698}$. Ser$^{720}$ have been recognized to represent FIXa interactive sites (22, 23). Previously, we have shown that FIXa light chain comprises an exosite that binds FIXa with high affinity (21). In the same study, we found that the FIXa-FVIII light chain interaction was inhibited by the anti-FVIII antibody CLB-Cag A, which is known to bind to the FVIII A3 domain region Glu$^{1778}$-Asp$^{1840}$ (24). In the present study, we addressed the possibility that this region is involved in the assembly of the enzyme-cofactor complex. Therefore, we first located the binding site of antibody CLB-Cag A in more detail. Subsequently, a series of synthetic peptides was employed in order to define the FVIII region involved in FIXa binding. This approach allowed us to identify the FIXa light chain region Glu$^{1811}$-Lys$^{1818}$ as being involved in FIXa binding and in the assembly of the FX-activating FIXa-FVIIIa complex.

EXPERIMENTAL PROCEDURES

Materials—Protein A-Sepharose CL-4B was from Pharmacia Biotech Inc. Microtiter plates (Immulon) were from Dynatech (Plockingen, Germany). The in vitro transcription and translation kits employing the SP6-expression system as well as the plasmid pSP64 were from Promega. Restriction enzymes were obtained from Life Technologies, Inc. Goat anti-mouse antibodies, rabbit anti-mouse antibodies, and human
serum albumin (HSA) were from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands).

Antibodies and Other Proteins—The monoclonal anti-FVII antibodies CLB-CAG 12, CLB-CAG 69, and CLB-CAG A have been described previously (24, 25). The murine anti-FIX antibody CLB-FIX 14 (isotype IgG2a) was used as control reagent. Peptides in solution were transferred to immobilized peptides using the polymerase chain reaction products and plasmid pSP64 with the 5′-d(TTAGGATCC) restriction site at positions 2397 and 2408 of the FVIII gene (24). The enzymes used were BglII and HindIII. The products were ligated into the plasmid. The identities thereof (Fig. 1) were confirmed by mass spectrometry analysis (Eurosequence B.V., Groningen, The Netherlands). Mass spectrometry further confirmed that Cys-containing peptides did exist as monomers. Peptide Lys506-Lys518 was used from two separate preparations and produced identical results.

Constructions of Recombinant FVIII Fragments—Plasmid pSP-F8-98K1 (24) was used as template for the construction of truncated FVIII fragments employing the polymerase chain reaction. The DNA fragments were made by using the sense primer 5′-CTG-3′ and the antisense primer 3′-TTAGGATCC, and the identities of the resulting fragments were confirmed by automated sequencing. The construct FVIII fragments encompassing the residues Tyr1786–Glu1801, Gly1799–Lys1813, Lys1804–Lys1818, Gly1811–Gln1820, Tyr1815–Ala1834, and His1822–Ala1834 from human FVIII were synthesized using standard FMoc (N-(9-fluorenylmethoxycarbonyl) chemistry by the manual “T-bag” method (28, 29), or employing a 430A Applied Biosystems instrument (Pharmacia). Purity of the peptides was checked by HPLC reversed phase chromatography (29). Peptides Gly1799-Lys1813, Lys1804-Lys1818, and Gly1811–Gln1820 were more than 90% pure. Peptides Tyr1786-Glu1801, Tyr1805–Ala1834 and His1822–Ala1834 were further purified by reversed phase HPLC to the same degree of purity. The identities of the peptides Gly1799–Lys1813, Lys1804–Lys1818, Lys1820 were confirmed by mass spectrometry analysis (Eurosequence B.V., Groningen, The Netherlands). Mass spectrometry further confirmed that Cys-containing peptides did exist as monomers. Peptide Lys506-Lys518 was used from two separate preparations and produced identical results.

Binding Assays—Synthetic peptides were immobilized onto microtiter wells in a volume of 100 μl, and remaining binding sites were blocked with 2% (w/v) BSA in 0.1% (v/v) Tween 20, 0.1 M NaCl, 25 mM Tris (pH 7.4). After washing, the immobilized peptides were incubated with antibody CLB-CAG A (625 nM) or FIXa (50 nM) in 2% (w/v) HSA, 0.1% (v/v) Tween 20, 0.1 M NaCl, 25 mM Tris (pH 7.4). The mixtures were incubated for 1 h at room temperature in order to reach equilibrium. Samples were subsequently incubated with immobilized peptide and antibody CLB-CAG A or FIXa to bind to the immobilized peptide. FIX or FIXa bound to the immobilized peptide was quantified employing the peroxidase-conjugated antibody CLB-FIX 14. The dissociation constants for the interaction with peptides in solution could be calculated as described (30).

FX Activation—FXa formation was determined as described (31). FX (0.2 μM) was activated in 3 mM CaCl2, 0.1 M NaCl, 0.2 mg/ml HSA, 0.05 mM Tris (pH 7.4) at 37 °C by FIXa (0.7 μM) in the presence of phospholipids (0.1 mM) and FXVIII (0.4 mM). FXVIII was preactivated in the same buffer for 5 min by thrombin prior to the addition. FX activation experiments in the absence of FXVIII were performed employing a FIXa concentration of 30 nM. FIXa formation was quantified employing the chromogenic substrate S-2222 (Chromogenix AB, Mölndal, Sweden). An active site titrated FXa preparation was used as a reference to convert absorbance values into molar FXa concentrations.

RESULTS

Interaction between Recombinant FVIII Fragments and Antibody CLB-CAG A—Binding of FIXa to FVIII light chain is inhibited by the monoclonal anti-FVII antibody CLB-CAG A (21). Since this antibody is known to bind to the FXVIII region Glu1786—Asp1842, Asp1562-Met1823, and carboxy-terminal truncations thereof (Fig. 1) were in vitro transcribed and translated, the latter of which in the presence of [35S]methionine. As determined by SDS-polyacrylamide gel electrophoresis, each radiolabeled polypeptide migrated as a single band within the expected M2, between 32,000 and 36,000 (not shown). These polypeptides were examined for their binding to antibodies CLB-CAG A and CLB-CAG 69 in immunoprecipitation studies. In these studies, antibody CLB-CAG 69 served as a control that should bind to all four polypeptides, as its epitope is known to encompass residues Lys1804–Lys1818 (24). Indeed, the antibody effectively bound the various polypeptides to the same extent (Fig. 1, lanes 1–4). With respect to antibody CLB-CAG A, the largest polypeptide (Asp1562–Met1823) was readily recognized by this antibody, whereas only a faint band was observed for the polypeptide Asp1562–Glu1801 (Fig. 1, lanes 7 and 8). In contrast, the two smaller polypeptides, respectively Glu1801–Glu1811 and Asp1562–Ser1791, did not bind to antibody CLB-CAG A (Fig. 1, lanes 5 and 6). From these results it appears that residues between Glu1801 and Met1823 are of particular importance for binding of antibody CLB-CAG A to the FXVIII polypeptide Asp1562–Met1823.

Interaction between Synthetic Peptides and Antibody CLB-CAG A or FIXa—A hydrophy analysis of the FVIII A3 domain primary structure was performed in order to determine the hydrophilicity of the region Glu1801–Met1823. As can be seen in Fig. 2, this region is part of a markedly hydrophilic exosite encompassing the residues Arg1781–Met1823, which indicates that the region Glu1801–Met1823 may be exposed at the exterior of the FVIII light chain molecule. The possibility that the hydrophilic exosite comprises a FIXa binding region was addressed by employing a series of overlapping peptides that encompass the FVIII region between Tyr1789 and Ala1834 (Fig. 2). The interaction between these peptides and FIXa was as
sessed in binding studies employing immobilized peptides. FIXa displayed particular effective binding to peptide Lys^{1804–Lys^{1818}} and, to a lesser extent, to peptide Glu^{1811–Gln^{1820}} (Fig. 3). The same series of peptides were used to examine antibody CLB-CAg A binding (Fig. 3). Antibody CLB-CAg A displayed a similar pattern of specificity for these peptides as FIXa (Fig. 3), as most effective binding was observed for peptides Lys^{1804–Lys^{1818}} and Glu^{1811–Gln^{1820}}. Collectively, these data indicate that the A3 domain comprises a hydrophilic exosite that contributes to FIXa binding.

Effect of Synthetic Peptides on FIXa Binding to FVIII Light Chain—In order to investigate the interaction between FIXa and various synthetic peptides in solution, the effect of synthetic peptides on binding of FIXa to immobilized FVIII light chain was determined. In these experiments not all of the peptides of Fig. 2 could be evaluated, as the solubility of peptides Tyr^{1786–Glu^{1801}}, Tyr^{1815–Ala^{1834}} and His^{1822–Ala^{1834}} was limited to concentrations that are below the concentrations required for competition studies in solution. As shown in Fig. 4A, the peptide comprising the sequence Gly^{1799–Lys^{1813}} was incapable of interfering with binding of FIXa to immobilized FVIII light chain. In contrast, peptides Lys^{1804–Lys^{1818}} and Glu^{1811–Gln^{1820}} were found to inhibit binding of FIXa to immobilized FVIII light chain in a dose-dependent manner (Fig. 4A). By analyzing these data in a model for competitive inhibition (21), the inhibition constants were calculated to be 0.19 ± 0.01 mM (mean ± S.D.) and 0.27 ± 0.02 mM for peptide Lys^{1804–Lys^{1818}} and Glu^{1811–Gln^{1820}}, respectively. Thus, both peptides effectively compete with binding of FVIII light chain to FIXa. Therefore, these peptides should also interfere with FVIII-dependent activation of FX by FIXa.

Effect of Synthetic Peptides on FX Activation—The effect of peptides Glu^{1811–Gln^{1820}} and Lys^{1804–Lys^{1818}}, and Gly^{1799–Lys^{1813}} on FX activation by FIXa in the presence of phospholipids, calcium ions, and FVIII was determined. In the presence of peptide Gly^{1799–Lys^{1813}} little, if any, inhibition was observed (Fig. 4B). In contrast, FX activation was effectively inhibited in the presence of peptides Lys^{1804–Lys^{1818}} and Glu^{1811–Gln^{1820}} (Fig. 4B). Neither of the peptides inhibited FX activation in the absence of FVIII (not shown). Thus, peptides Lys^{1804–Lys^{1818}} and Glu^{1811–Gln^{1820}} indeed interfere with a FVIII-dependent step within the process of FX activation. The mechanism of inhibition was addressed by studying the effect of the inhibitory peptides on the kinetic parameters $K_m$ and $V_{max}$. In experiments employing peptide Lys^{1804–Lys^{1818}}, the apparent $K_m$ remained unchanged, whereas the apparent $V_{max}$ was dependent on the concentration of peptide (Fig. 5). Thus, peptide Lys^{1804–Lys^{1818}} inhibits FVIII-dependent FX activation by FIXa by a noncompetitive mechanism. The same results were obtained employing the peptide Gly^{1811–Gln^{1820}} (not shown). Using the normal model of noncompetitive inhibition, the inhibition constant was found to be 0.23 ± 0.05 mM and 0.25 ±

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**Fig. 1.** Immunoprecipitation of FVIII fragments with monoclonal antibodies. Recombinant FVIII fragments were obtained as outlined under “Experimental Procedures.” The fragments were labeled by in vitro translation employing [35S]methionine (24). Immunocomplexes were analyzed by 12% (w/v) SDS-polyacrylamide gel electrophoresis and subsequent autoradiography. Lanes represent immunocomplexes of the radiolabeled FVIII fragments from Asp^{1562} to Ser^{1791} (lanes 1 and 5), Gly^{1801} (lanes 2 and 6), Lys^{1811} (lanes 3 and 7) and Met^{1823} (lanes 4 and 8) with antibodies CLB-CAg 69 and CLB-CAg A, respectively. The residues Lys^{1673–Arg^{1689}} encompass the previously defined epitope for antibody CLB-CAg 69 (24).

**Fig. 2.** Synthetic peptides of the FVIII A3 domain. Kyte-Doolittle hydrophathy analysis (32) of the FVIII A3 domain sequence using a sliding window of 19 amino acids is shown on top. The most hydrophilic region comprises the residues Arg^{1781–Asp^{1842}}. Peptides overlapping the FVIII region Tyr^{1786–Ala^{1834}} that are used in this study are shown below. The primary sequence of the FVIII region Tyr^{1786–Ala^{1834}} is represented using the single-letter code. The lines below the amino acid sequence denote the inclusive amino acid residues of the peptides synthesized.
Uncleaved and Cleaved Forms of FIX—The FIX zymogen is dissimilar to the fully activated FIXa (13, 27). To examine whether FIX and FIXa also differ in cofactor binding, the interaction with FVIII light chain and, more specifically, with peptide Lys1804–Lys1818 was addressed. In line with our previous finding (27), FIX was less efficient than FIXa in binding FVIII light chain (Fig. 6). In contrast, when FIX and FIXa were compared for binding to the immobilized peptide Lys1804–Lys1818, this peptide bound the FIX zymogen and the FIXa enzyme to the same extent (Fig. 6, inset). The same observation was made employing peptide Glu1811–Gln1820 (not shown). However, studies employing immobilized peptides do not provide quantitative binding parameters. Therefore, the interaction between peptide Lys1804–Lys1818 and FIX or FIXa was further studied in solution under equilibrium conditions (see “Experimental Procedures”). These experiments revealed an apparent dissociation constant of $0.20 \pm 0.02 \text{mM}$ (mean $\pm$ S.D.) for the peptide-FIX interaction. This value is similar to the inhibition constants found for the inhibition of the FIXa-FVIII light chain interaction and FVIII-dependent activation of FX (see Fig. 4). The same value (0.23 $\pm$ 0.02 mM) was also found for the peptide-FIX interaction, demonstrating that indeed peptide Lys1804–Lys1818 is equally effective in binding FIX or FIXa. Thus, peptide Lys1804–Lys1818 does not distinguish between uncleaved and cleaved forms of FIX, whereas FVIII light chain does. This finding was confirmed by using FVIII light chain as competitor for the interaction between immobilized peptide Lys1804–Lys1818 and FIX or FIXa. Binding of FIXa to the immobilized peptide was readily inhibited in the presence of FVIII light chain (Fig. 7). In contrast, FVIII light chain proved to be inefficient in interfering with binding of FIX to the immobilized peptide. These data demonstrate that peptide Lys1804–Lys1818 and FVIII light chain compete for binding to FIXa but not to the FIX zymogen.

**DISCUSSION**

During the process of FX activation, the enzyme FIXa assembles with the nonenzymatic cofactor FVIIIa into a lipid-bound complex. In previous studies we have shown that FVIII light chain contains a site that binds FIXa with high affinity (K$_d$ $\approx$ 15 nM) and that FIXa binding is inhibited by the FVIII light chain-directed antibody CLB-CAg A (21). Here we show that this antibody is directed against an exoside on the A3 domain (Figs. 2 and 3). Because such hydrophilic regions are likely to be exposed at the exterior of the protein (32), we addressed the possibility that this exosite comprises a FIXa binding site. Indeed, FIXa binds to synthetic peptides that consist of FVIII sequences that are part of the hydrophilic exoside Arg1781-Asp1842 (Fig. 3). Competition studies demonstrated that peptides corresponding to the exosite regions Lys1804–Lys1818 and Glu1811–Gln1820 effectively inhibit binding of FIXa to immobilized FVIII light chain (Fig. 4A). The same peptides also interfere with FVIII-dependent activation of FX by FIXa (Fig. 4B). Inhibition of FX activation is noncompetitive (Fig. 5), which strongly suggests that the peptides inhibit the enzyme FIXa by binding at a site distinct from the substrate binding pocket. Collectively, our data demonstrate that peptides consisting of the FVIII amino acid residues Lys1804–Lys1818 and Glu1811–Gln1820 represent a FIXa binding
Factor IXa Binding to the Factor VIII Sequence Glu$^{1811}$–Lys$^{1818}$

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Fig. 5. Kinetic analysis of FX activation in the presence of peptide Lys$^{1804}$–Lys$^{1818}$. FXa generation experiments were performed in the absence (●) or presence of 0.2 mM (●), 0.4 mM (●), or 0.6 mM (●) of the peptide Lys$^{1804}$–Lys$^{1818}$ as described under “Experimental Procedures,” except that variable FX concentrations (2.5–50 nM) were used. Initial rates of FXa formation are plotted as a function of the substrate concentration. Data represent the mean ± S.D. of three experiments. The calculated apparent $V_{max}$ values in the absence or presence of 0.2 mM, 0.4 mM, or 0.6 mM peptide were 6.0 ± 0.3, 4.5 ± 0.2, 3.8 ± 0.2, and 2.8 ± 0.1 nM FXa/min, respectively (mean ± S.D.). The apparent $K_{m}$ values were 5.3 ± 1.0, 5.0 ± 0.7, 6.1 ± 0.9, and 4.6 ± 0.8 nM FX, respectively.

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Fig. 6. Binding of FIX or FIXa to immobilized peptide Lys$^{1804}$–Lys$^{1818}$ or FVIII light chain. Various concentrations of Glu–Gly–Arg chloromethyl ketone-treated FIXa (●) or FIX (○) were incubated with immobilized FVIII light chain (0.7 pmol/well added) as described under “Experimental Procedures.” Association between FVIII light chain and FIXa was assessed as described (21). Inset, various concentrations of Glu–Gly–Arg chloromethyl ketone-treated FIXa (●) or FIX (○) were incubated with immobilized peptide Lys$^{1804}$–Lys$^{1818}$ (0.8 nM/well added) as described under “Experimental Procedures.” Binding was detected employing the peroxidase-labeled anti-FIX antibody CLB-FIX 14. Absorbance was measured at 450 nm using 540 nm as reference. Plotted is the absorbance versus the concentration of FIX or FIXa. Data represent mean values ± S.D. of three to six experiments.

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site. It is of importance to note that the $K_{d}$ for the binary FIXa–FVIII light chain interaction is similar to the $K_{d}$ found for FX activation in the complete FX activating complex, thus including the entire FVIIIa heterotrimer (Figs. 4 and 5). Assembly of the functional FIXa–FVIII complex apparently is directly related to binding of FIXa to the FVIII A3 domain exosite. In this respect it should be mentioned that FIXa binding is not an exclusive property of the FVIII A3 domain. FIXa recognition sites have been identified within the FVIII A2 domain regions Ser$^{558}$–Gln$^{565}$ (22) and Arg$^{698}$, Ser$^{710}$ (23). As synthetic peptides corresponding to these A2 domain regions also interfere with FVIIIa cofactor function, it seems reasonable to assume that both FVIII heavy chain and light chain regions participate in FIXa–FVIII complex formation.

As peptides Lys$^{1804}$–Lys$^{1818}$ and Glu$^{1811}$–Gln$^{1820}$ proved more efficient in their interaction with FIXa than the other peptides tested (Figs. 3 and 4), we propose that the minimal requirements for FIXa binding are met by the overlapping residues Lys$^{1811}$–Lys$^{1818}$. This region, including its direct environment (residues Gly$^{1799}$–Gln$^{1820}$), is strikingly rich in basic Lys residues, which are located at positions 1804, 1808, 1813, and 1818 (Fig. 8). These Lys residues appear to be unique for the FVIII A3 domain, as they are not only lacking in the FIXa A1- and A2 domains but also in the A3 domains of the structurally related proteins FV and ceruloplasmin (Fig. 8). The same Lys residues are conserved in the FVIII A3 domain of a rodent species (Fig. 8), which would be compatible with the involvement of these residues in a FVIII A3 domain-specific event such as FIXa binding. However, peptide Gly$^{1799}$–Lys$^{1813}$ with Lys residues at 1804, 1808, and 1813 proved considerably less efficient in its interaction with FIXa than peptide Glu$^{1811}$–Gln$^{1820}$ with Lys residues at 1813 and 1818 (Figs. 3 and 4). Apparently, the presence of the Lys residues alone is not sufficient for FIXa binding. It should be mentioned that peptide Glu$^{1811}$–Gln$^{1820}$ contains a triplet of aromatic residues (Tyr$^{1815}$, Phe$^{1816}$, Trp$^{1817}$), which is also present in the inhibitory peptide Lys$^{1804}$–Lys$^{1818}$ but lacking in the noninhibitory peptide Gly$^{1799}$–Lys$^{1813}$. Because part of this sequence is conserved in other A domains (Fig. 8), it is unclear how these residues may be involved in a FVIII A3 domain-specific function. To what extent individual amino acids in the FIXa binding region contribute to FIXa–FVIII light chain complex formation, therefore, remains to be investigated. It seems of interest to note that mutations at positions Ser$^{784}$, Leu$^{1789}$, Met$^{1823}$, Pro$^{1825}$, and Thr$^{1826}$ have been determined to be associated with moderately severe hemophilia A (33). As these mutations are in close proximity to the FIXa binding region, it is tempting to speculate that the bleeding tendency that is associated with these mutations is due to a suboptimal assembly of the FIXa–FVIII light chain complex.

Recently, we demonstrated that uncleaved FVIII light chain is similar to FVIII light chain derivatives that have been cleaved by the activators thrombin or FXa in that they display similar affinity for FIXa (31). Apparently, the FIXa recognition site is fully exposed in the intact FVIII light chain. In agreement with previous observations, we have found that FVIII light chain is more efficient in binding the fully activated FIXa than the uncleaved FIX zymogen (Figs. 6 and 7; Ref. 27). These
data indicate that FVIII light chain displays preferential binding to the enzyme FIXa rather than to the nonactivated FIX zymogen. In this regard, FVIII light chain seems similar to FVa and thrombomodulin, because these cofactors are more efficient in binding to their respective enzymes than to the uncleaved proenzymes (34, 35). Surprisingly, this seems to be untrue for the FIXa-binding peptides Lys1804–Lys1818 and Glu1811–Gln1820, because these peptides do not distinguish between the enzyme FIXa and the FIX zymogen (Figs. 6 and 7).

Several possibilities may be considered that may explain these observations. First, it is possible that the relative size of the FVIII light chain prevents binding to the intact FIX zymogen, while this restriction is overcome by limited proteolysis of the zymogen at its activation sites Arg1845 or Arg180 (27). It should be noted here that cleavage at Arg1845 is sufficient for full exposure of the FVIII light chain binding site, whereas cleavage at Arg180 results in a suboptimal exposure (27). Alternatively, the conformation of the FIXa-binding motif in synthetic peptides may differ from its conformation in the complete FVIII light chain. This may be due to other portions of the light chain that provide the region Glu1811–Lys1818 with its specificity for binding to the activated form of FIX. In this respect it is of importance to note that the Asn residue at position 1810 is a potential site for N-linked glycosylation in FVIII (2, 3). As this site is located adjacent to the FIX-binding motif Glu1811–Lys1818, it seems conceivable that glycosylation of this site contributes to the specificity for binding of FIXa to its binding sequence Glu1811–Lys1818.

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