Identification of a Binding Site for Blood Coagulation Factor IXa on the Light Chain of Human Factor VIII*

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The interaction between human factor IXa and factor VIII or its constituent units was investigated. Equilibrium binding studies were performed employing factor VIII light chain that was immobilized on a monoclonal antibody. Factor VIII light chain was observed to bind factor IXa with high affinity ($K_d = 14.8 \pm 3.2 \text{ nm}$) and approximately 1:1 stoichiometry. Optimal interaction required NaCl concentrations below 0.2 M and the presence of Ca$^{2+}$ ions. Factor VIII light chain in solution effectively inhibited binding of factor IXa to the immobilized light chain ($K_d = 10.9 \pm 1.9 \text{ nm}$). The isolated factor VIII light chain and the factor VIII heterodimer were equally effective in factor IXa binding, demonstrating that this interaction did not require the factor VIII heavy chain. Factor IXa and activated Protein C were found to be inefficient ($K_d \geq 1.2 \mu M$) in competing with factor IXa, indicating that the high affinity for factor VIII light chain was unique for factor IXa. The factor IXa-factor VIII light chain interaction was inhibited by von Willebrand factor, but this effect was abolished by cleavage of the factor VIII light chain by thrombin. An antibody that inhibits von Willebrand factor-factor IXa complex formation did not compete for factor IXa binding. In contrast, association of factor IXa with the factor VIII light chain was inhibited by an antibody directed against the factor VIII region Gln1778-Asp1840. We propose that this sequence provides a factor IXa binding site and that its exposure requires dissociation of the factor VIII-von Willebrand factor complex.

In the intrinsic pathway of blood coagulation, factor X (FX) is activated by a complex consisting of the serine protease factor IXa (FIXa), factor VIII (FVIII), calcium ions, and phospholipids. Within this complex, FVIII functions as a nonenzymatic cofactor. The fact that FVIII deficiency or dysfunction is associated with the severe bleeding disorder known as hemophilia A underscores that this cofactor is indispensable for appropriate blood coagulation. FVIII is synthesized as a single chain polypeptide with the domain structure A1-A2-B-A3-C1-C2 (3, 4). Due to endoproteolytic processing, FVIII circulates in plasma predominantly as a heterodimeric protein, consisting of a Me$^{2+}$-linked heavy and light chain (5-7). The light chain ($M_r = 80,000$) contains the A3-C1-C2 domains. The heavy chain is represented by the domain structure A1-A2-B, and is heterogeneous ($M_r = 90,000-200,000$) as the result of limited proteolysis at a number of positions within the B-domain (6, 8).

In plasma, FVIII is present as an inactive precursor, which is tightly associated with its carrier, von Willebrand factor (vWF) (2). FVIII activation is required for appropriate cofactor function in the FX activating complex (9, 10). Activation is achieved by limited proteolysis in both the heavy and light chain by FXa or thrombin (8), and results in a labile heterotrimetric activation product, FVIIa (11, 12). FVIII activity is subject to down-regulation by the serine protease activated Protein C (13, 14).

This serine protease, which binds to a site located at the C terminus of the A3 domain in the FVIII light chain (FVIII-LC) (13, 15), inactivates FVIII by at least two cleavages in the factor VIII heavy chain (FVIII-HC) (14).

Although less effective than activated Protein C, FIXa is also capable of inactivating FVIII by limited proteolysis (16, 17). Simultaneously, when bound to phospholipids, FXa stabilizes FVIIa (18) and reduces inactivation of FVIII by activated Protein C (19, 20). In addition, the phospholipid-FXa complex also enhances the reassociation of isolated FVIIa subunits (21). The involvement of FIXa in both stabilization and inactivation of FVIII seems to imply that intrinsic FIXa formation is regulated by interactions between FIXa and FVIII.

The aim of the present study was to identify the binding sites involved in assembly of the binary enzyme-cofactor complex. For this purpose, the interaction between FVIII and FIXa was assessed in equilibrium binding experiments using immobilized FVIII-LC. The same technique was used for competition studies employing other FVIII-binding proteins, including activated Protein C, FXa, vWF, and monoclonal antibodies with known epitopes. This approach allowed the identification of a FVIII-LC region associated with FIXa binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Protein A-Sepharose CL4B, Q-Sepharose FF, CNBr-Sepharose CL4B, S-Sepharose FF, and Sephacryl S-300 were from Pharmacia LKB Biotechnology Inc. Microparticles were from Dynatech (Plochingen, Germany). Albumin (HSA) was from Sigma. Glu-Gly-Arg-chloromethyl ketone (EGR-CK) was from Calbiochem.

**Antibodies**—The monoclonal antibodies CLB-Cag A, CLB-Cag 12, CLB-Cag 69, and CLB-Cag 117 against FVIII-LC and CLB-Cag 9 against FVIII-HC have been described previously (22-24). The antibody CLB-FIX 2 is a Ca$^{2+}$-dependent murine antibody against human FIX. Hybridomas were prepared using standard procedures. Cell supernatants were screened for binding to immobilized FIX in the presence of 20 mM CaCl$_2$, and positive clones were rescreened with EDTA replacing CaCl$_2$. One cell line, designated CLB-FIX 2, was found to produce an IgG$_1$, antibody displaying FIX binding that was strictly Ca$^{2+}$-dependent. All monoclonal antibodies used were purified from culture medium, employing Protein A-Sepharose as recommended by the manufacturer.
Polyclonal antibodies against human FIX were raised in rabbits and purified employing immobilized FIX. Antibodies were conjugated with horseradish peroxidase as described (25).

**FVIII and FVIII Subunits**—Human FVIII was obtained from immuno-purified FVIII concentrate, kindly provided by Dr. J. Over institute. Albumin was isolated from the concentrate by rechromatography on an anti-FVIII-LC affinity column (24) or by the same immunoaffinity step as employed in the production process. If present, residual albumin was removed by Q-Sepharose FF chromatography in 0.1 M NaCl, 5 mM CaCl₂, 10% (v/v) glycerol, and 20 mM Tris (pH 7.2) using a linear NaCl gradient for elution. FVIII was stored at -20 °C in 0.1 M NaCl, 5 mM CaCl₂, 55% (v/v) glycerol, 20 mM Tris (pH 7.2). The specific activity of these FVIII preparations ranged between 3,500 and 7,000 unit/mg. FVIII subunits were isolated from EDTA-dissociated FVIII. After extensive dialysis against 0.15 M NaCl, 25 mM EDTA, 10 mM benzamidine, and 20 mM Tris (pH 7.4), FVIII-HC was removed by CLB-CAg 9 affinity chromatography (24). Nonbound FVIII-LC was isolated by immunoaffinity chromatography using the antibody CLB-CAG 117 (24). If present, traces of vWF were removed by gel filtration using a Bio-Sil TSK-250 column (Bio-Rad) equilibrated in 0.5 M NaCl, 20 mM EDTA, 50 mM MOPS (pH 6.9). FVIII-LC preparations were stored at -20 °C in 0.1 M NaCl, 55% (v/v) glycerol, 20 mM histidine (pH 6.8). Cleared FVIII-LC was prepared by incubating FVIII-LC (0.25 μM) with thrombin (20 nM) for 20 h at 37 °C in 0.1 M NaCl, 1 mM CaCl₂, 50 mM Tris (pH 7.4). Cleaved FVIII-LC was separated from thrombin and the N-terminal fragment by anti-FVIII-LC affinity chromatography as described above. FVIII-HC was isolated in parallel with FVIII-LC by eluting the CLB-CAg 9 column with the same buffer as described for FVIII-LC. Residual FVIII-LC was removed by additional EDTA dissociation and anti-FVIII-LC chromatography. After rechromatography on the CLB-CAg 9 column, FVIII-HC was stored at -20 °C in 0.1 M NaCl, 55% (v/v) glycerol, 20 mM Tris (pH 7.4).

**Other Proteins**—Human FIXαβ and FXα were prepared as described previously (26). Human activated Protein C was obtained essentially as described (27). Recombinant human vWF was expressed in AtT-20 cells using an excess of FVIII-LC. In each individual series of experiments, the amount of antibody-bound FVIII-LC was determined. The isolated FVIII-LC, with known concentration was incubated with thrombin (20 nM) for 20 h at 37 °C in 0.1 M NaCl, 1% (w/v) HSA, 10 mM Tris (pH 7.4). Cleaved FVIII-LC was detected using peroxidase-conjugated CLB-CAg 12 or 117 in 1:15 dilution. The antibody CLB-CAg A was used instead of CLB-CAG 12. Nonspecific binding was determined for each separate incubation by performing the experimental procedure in the absence of FVIII-LC and found to be 3–6% of the concentration of FIXαβ added. All data were corrected for nonspecific binding.

**Calculations**—Dissociation constants (Kd) and the maximum number of bindings sites (Bmax) were estimated from equilibrium binding assays. Binding data obtained in the presence of a varying concentrations of competitor were analyzed using the following equation (32).

\[
\beta = 1 - \left(1 + \frac{K_d}{[\text{nonbound}]_{\text{max}}} + \frac{K_d}{[\text{nonbound}]_{\text{max}}} \right) \frac{[\text{bound}]}{[\text{nonbound}]_{\text{max}} + [\text{bound}]} - \frac{1}{2} \frac{[\text{Il}]}{[\text{Il}]} + \frac{1}{2} \frac{[\text{Il}]}{[\text{Il}]}
\]

where Kd and Bmax are obtained from equation (1). The inhibition constant Ki reflects the dissociation constant Kd of the interaction between inhibitor and FVIII or immobilized FVIII-LC.

**RESULTS**

**Design of Equilibrium Binding Studies**—The interaction between FIXα and FVIII has been studied employing an experimental approach based on the immobilization of the FVIII-LC by monoclonal antibodies. Previous studies from our laboratory have identified several monoclonal antibodies that are particularly effective in immobilizing FVIII (22) and resistant to elution under moderately chaotropic conditions (23). Two of these antibodies, called CLB-CAG A and CLB-CAG 12, were further evaluated in the present study. The antibody CLB-CAG A is known to be a strong inhibitor of FVIII activity and has its epitope within the A3 domain on the FVIII-LC (24). The antibody CLB-CAG 12 differs from CLB-CAG A in that it is noninhibitory and binds to another, so far unidentified epitope of FVIII (22).

**FVIII Binding Properties of Monoclonal Antibodies**—Antibody CLB-CAG 12 or CLB-CAG A (both 1 μg/ml) was adsorbed to microtiter wells. After blocking with HSA-containing buffer, the immobilized antibodies were incubated with various concentrations of FIXαβ for 16 hours at 4 °C in a 1:15 dilution with 0.1% (v/v) HSA, 20 mM Tris histidine (pH 6.2). Concentrations of total and nonbound FIXαβ were determined, and Equation 1 (see above) was used to estimate the Kd and Bmax. The Kd was calculated from dissociation experiments in which dissociation of FVIII-LC was monitored by analyzing subsamples from the supernatant during a 4-h incubation in a buffer containing 0.15 M NaCl, 5 mM CaCl₂, 0.1% (v/v) Tween-20, 1% (v/v) HSA, 20 mM histidine (pH 6.2).

**Equilibrium Binding Assays**—Monoclonal antibody CLB-CAG 12 (1 μg/ml) was immobilized to microtiter wells in a volume of 100 μl.
checked and found to be consistently close to the calculated maximum number of binding sites (cf. \( B_{\text{obs}} \) and \( B_{\text{max}} \) in Table I). Since dissociation of the FVIII-antibody complex was negligible under these conditions, the amounts of immobilized FVIII-LC were precisely known and constant over time. This provided the basis for equilibrium binding studies to assess the parameters of FIXa binding to the immobilized FVIII-LC.

**Binding of FIXa to Immobilized FVIII-LC**—The analysis of the interaction between FIXa and FVIII may be complicated by the notion that FIXa may cleave both the heavy and light chain of FVIII under particular conditions (16, 17). To exclude any interference by limited proteolysis, all binding studies were performed using FIXa that had been inactivated by the irreversible serine protease inhibitor, EGR-CK. In preliminary experiments, the time dependence of the association between inactivated FIXa and FVIII was examined using FVIII-LC that had been immobilized on the antibody CLB-CAg 12. FIXa binding was observed to increase until a maximum was reached after 2 h (results not shown). In all further experiments, 4-h incubation periods were employed to ensure that equilibrium had been attained. This interaction was explored in more detail by varying the concentration of FIXa. As shown in Fig. 1, a saturable, dose-dependent binding was observed. These data were in agreement with a model describing the interaction of FIXa with one single class of binding sites (see Fig. 1). The dissociation constant was calculated to be 14.8 ± 3.2 nM (average ± S.D.), while the maximum number of FIXa binding sites was found to be 0.9 ± 0.1 pmol/well. Similar values could be derived from the Scatchard analysis of the same data (Fig. 1, inset). Using the given amount of immobilized FVIII-LC (Table I, \( B_{\text{obs}} \)), the stoichiometry was estimated to be 0.8 ± 0.2 mol of FIXa/mol of FVIII-LC.

As shown in Table I, the antibody CLB-CAg A is equally effective in immobilizing the FVIII-LC as the antibody CLB-CAg 12. However, the same binding experiments failed to reveal any FIXa binding to FVIII-LC immobilized on this antibody (Fig. 1). Apparently, interaction of the antibody CLB-CAg A with the FVIII-LC is incompatible with FIXa binding. As this may be due to the inhibitory nature of this particular antibody (see below), the noninhibitory antibody CLB-CAg 12 was used for further studies.

**Competition Studies with FIXa-related Serine Proteases**—The interaction between the FVIII-LC and FIXa was further studied by competition studies using related vitamin K-dependent proteases. For this purpose, human FIXa and activated Protein C were inactivated using EGR-CK and subsequently tested for inhibition of FIXa binding to the immobilized FVIII-LC (Fig. 2). With regard to FIXa, no competition was observed, even at concentrations as high as 1.2 μM, representing a 40-fold molar excess over FIXa. Similar experiments using activated Protein C displayed no inhibition at concentrations up to 0.5 μM. At higher concentrations, a slight inhibition became apparent, which corresponds with a \( K_i \) in the μM range.

**TABLE I**

| Monoclonal antibody | \( K_d \) (nM) | \( K_d \) (μM) | \( B_{\text{obs}} \) (pmol/well) | \( B_{\text{max}} \) (pmol/well) |
|---------------------|---------------|---------------|-----------------|-----------------|
| CLB-CAg 12          | 3.3 ± 0.8     | 3.3 ± 0.8     | 1.6 ± 0.1       | 1.6 ± 0.1       |
| CLB-CAg A           | 0.6 ± 0.2     | 0.6 ± 0.2     | 1.3 ± 0.2       | 1.3 ± 0.2       |

These data demonstrate that among the vitamin K-dependent enzymes tested, FIXa displays a unique interaction with the FVIII-LC.

**Effect of Ionic Strength and Ca\(^{2+}\) Ions on FIXa Binding to the FVIII-LC**—To further characterize the interaction of FIXa with FVIII-LC, the effect of ionic strength was examined. A constant amount of FIXa was incubated with immobilized FVIII-LC (1.2 ± 0.3 pmol/well) in the presence of various concentrations of NaCl. Between 50 and 150 mM NaCl (Fig. 3), the number of FIXa binding sites was constant under the conditions of Fig. 3. The observed effect of ionic strength, therefore, directly reflects the binding of FIXa to the FVIII-LC. The complex NaCl dependence suggests that both hydrophobic and electrostatic interactions contribute to the association between FVIII and FIXa.

Since virtually all biological functions of vitamin K-dependent proteins require Ca\(^{2+}\) ions, it was of further interest to examine the effect of these divalent cations on the interaction...
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Effect of NaCl and CaCl2 concentration on FIXa-FVIII-LC interaction. EGR-CK-inactivated FIXa (40 nm) was incubated with immobilized FVIII-LC in a buffer containing 25 mM histidine (pH 6.2), 5 mM CaCl2, 0.1% (v/v) Tween-20, 1% (w/v) HSA, and varying concentrations of NaCl. FIXa binding was assessed as described under "Experimental Procedures." The inset shows binding experiments under similar conditions, except that the NaCl concentration was fixed at 0.15 M, while the CaCl2 concentration was varied. Binding of FIXa in the absence of CaCl2 refers to experiments in the presence of 10 mM EDTA.

Interaction between FIXa and FVIII in Solution—Our experimental approach also provided the possibility to examine the interaction between FIXa and FVIII in solution. For this purpose, known amounts of immobilized FVIII-LC (see Table I) were incubated with a mixture of FIXa and FVIII or its purified subunits in varying concentrations. As shown in Fig. 4, the isolated FVIII-LC was found to effectively inhibit the binding of FIXa to the immobilized FVIII-LC in a dose-dependent manner. The experimental data were in agreement with the model of free FIXa-LC competing with its immobilized counterpart for FIXa binding (Fig. 4). The calculated inhibition constant (10.9 ± 1.9 nM) was similar to the $K_i$ of FIXa binding to the immobilized FVIII-LC (cf. Fig. 1). This finding demonstrates that free and immobilized light chain are equivalent with respect to FIXa binding. In contrast, the isolated heavy chain did not affect FIXa binding to the FVIII-LC (Fig. 4). Furthermore, the heterodimeric complex comprising light and heavy chain was found to compete for FIXa binding with the same effectiveness as the isolated light chain alone ($K_i = 13.4 ± 2.0$ nM) (see Fig. 4). Neither as the free subunit nor as part of the heterodimer does the FVIII-HC seem to support FIXa binding to the FVIII-LC.

Effect of vWF on the Interaction between FIXa and the FVIII-LC—The FVIII-LC is known to contain binding sites for a number of components involved in FIXa function, including phospholipids (33) and activated Protein C (15). As these interactions are regulated by complex formation between FVIII and its physiological carrier vWF (34, 35), we examined whether vWF affects FIXa binding. For this purpose, immobilized FVIII-LC was allowed to interact with varying amounts of purified recombinant vWF. After incubation, residual nonbound vWF was quantified, and the amount of vWF bound was then calculated. At the highest concentration tested (45 nM) (see Fig. 5), about 0.6 μg of vWF was adsorbed, which is equivalent to 2 pmol of vWF monomers. These data demonstrate that a significant proportion of the immobilized FVIII-LC (−1.2 pmol/well) (see Table I) was indeed complexed to vWF. Subsequently, FIXa was added, and binding to FVIII-LC was assessed. As shown in Fig. 5, the association between FIXa and the FVIII-LC was effectively inhibited by the presence of vWF in a dose-dependent manner. The inhibitory effect of vWF was also evaluated using thrombin-cleaved FVIII-LC, which lacks the acidic region involved in high affinity vWF binding (36–38). As shown in Fig. 5, FIXa binding to thrombin-cleaved FVIII-LC was retained but no longer subject to inhibition by vWF. This demonstrates that vWF and FIXa have distinct requirements for binding to the FVIII-LC.

Effect of Monoclonal Antibodies against the FVIII-LC on FIXa binding—Two monoclonal antibodies against the FVIII-LC were considered to be of particular interest with regard to FIXa binding. The first antibody, called CLB-CAg 12 in the presence of variable concentrations of recombinant vWF (0–44 nM). Then binding of EGR-CK-inactivated FIXa (40 nM) was assessed (see "Experimental Procedures") in the presence of similar concentrations of vWF as used in FVIII-LC immobilization.

Effect of vWF on the FIXa-FVIII-LC interaction. FVIII-LC (○) or its thrombin-cleaved derivative (●) were adsorbed onto the monoclonal antibody CLB-CAg 12 in the presence of variable concentrations of recombinant vWF (0–44 nM). Then binding of EGR-CK-inactivated FIXa (40 nM) was assessed (see "Experimental Procedures") in the presence of similar concentrations of vWF as used in FVIII-LC immobilization.

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A and FIXa share common binding sites on the FVIII-LC. The inhibition fitted well into the model of competitive inhibition as antibody CLB-CAg A effectively inhibited FIXa binding to the FVIII-LC, at Arg1719 (16, 17). As FMa inhibits inactivation by activated Protein C proved inefficient in competing with FIXa for FVIII-LC binding (Fig. 4). We considered the possibility that this could be due to denaturation of the isolated FVIII-HC. However, this seems unlikely, as reconstitution experiments (not shown) demonstrated that the isolated FVIII-HC and FVIII-LC could be effectively assembled into biologically active heterodimers. The findings do not necessarily mean that the FVIII-HC is not involved in FIXa-FVIII interaction. It is conceivable that the FVIII-LC is responsible for complex assembly with FIXa, while FVIII-HC might induce repositioning of the FIXa active site.

In order to identify the FVIII-LC region involved in FIXa binding, we have performed competition studies using ligands with previously established binding sites. One possibility that we addressed was that the observed FIXa binding was associated with its known cleavage site within the N-terminal portion of the FVIII-LC, at Arg1719 (16, 17). To exclude that this enzyme substrate binding would compromise our studies on enzyme cofactor association, active-site blocked FIXa has been employed in our experiments. Therefore, proteolytic interactions are not likely to contribute to the observed binding of FIXa to FVIII. Moreover, FIXa was not capable of competing with FIXa for FVIII binding, although it cleaves the light chain at almost the same position, Arg1719 (8). This supports our view that the high affinity interaction observed in our studies originated from enzyme cofactor assembly. Another component that we studied was activated Protein C, which binds to the C-terminal part of the FVIII A3 domain, within the sequence His2009-Val2018 (15). As FIXa inhibits inactivation by activated Protein C (19, 20), it would have been conceivable that these two enzymes have overlapping binding sites. However, activated Protein C proved inefficient in competing with FIXa for FVIII-LC binding (Fig. 2), indicating that FIXa primarily binds to another region in the FVIII-LC. A positive identification of the FIXa binding site on the FVIII-LC was achieved using the monoclonal antibody CLB-CAg A (Fig. 6). In previous studies, we have shown that this antibody is a strong inhibitor of FVIII activity and has its epitope within the light chain A3 domain (24). Several other antibodies have been described that have their epitope on the FVIII-LC and also strongly interfere in FVIII function. In a number of cases, studies using such inhibitory antibodies as functional probes have contributed to our current understanding of FVIII function. For instance, antibodies against the C2 domain inhibit FVIII function by interfering in the interaction between FVIII and phospholipids (45). Furthermore, an antibody against the N-terminal acidic region has been described to interfere in the proteolytic activation of FVIII at Arg1689 by thrombin (46). Our monoclonal antibody CLB-CAg A has its

**DISCUSSION**

Within the blood coagulation cascade, FVIII and factor V (FV) serve as nonenzymatic cofactors that assemble into phospholipid-bound complexes with the enzymes FIXa and FXa, respectively (1). As FVIII and FV exhibit considerable homology, it is generally assumed that these cofactors support coagulation by essentially the same mechanism (1, 39). With respect to FV, it has been shown that the FVa heterodimer is more effective than its isolated heavy or light chain in FIXa binding (40) and that both the light chain (41) and the heavy chain (42) contain FIXa binding sites. Moreover, both subunits are capable of interacting with FXa (43), although the precise sites of interaction remain unidentified. In view of these observations, it is not unexpected that the isolated light chain of FVIII displays FIXa binding (Fig. 1). A further similarity between FVIII and FV is that FIXa binding to FVIII has the same Ca2+ dependence as reported for the interaction between FVa and FXa (Fig. 3) (cf. Ref. 43). However, FVIII seems different from FV in that it displays a higher affinity ($K_d = 13.4 \text{nM}$) (see Fig. 4) than FVa for enzyme binding (0.8 µM) (see Ref. 43). We have considered the possibility that high affinity FIXa binding could have been induced by FVIII-LC being adsorbed to an antibody in our studies. However, competition studies have established that FVIII-LC in solution has the same affinity as FIXa binding (Fig. 4). The observation that the intact FVIII heterodimer has similar affinity as the isolated light chain (Fig. 4) suggests that FIXa binding may be predominantly due to the FVIII-LC.

The $K_d$ for binding of FIXa to the FVIII-LC (Figs. 1 and 4) is about 5-fold higher than reported recently for the complete FVIII heterodimer (44). In this regard, it should be noted that these studies have addressed the interaction between FIXa and FVIII in the presence of phospholipids, whereas we have studied phospholipid-independent binding. As phospholipids contribute to the assembly of the FVa-FXa complex (1, 43), it seems reasonable to assume that a similar effect on the interaction between FVIII and FIXa explains the lower affinity of the phospholipid-independent binding as revealed by our studies. It is further relevant to note that we have evaluated FIXa-FVIII interaction by direct binding, whereas Duffy et al. (44) have monitored complex formation by FVIII-induced conformational changes in the FIXa active site. Employing similar techniques, it has been observed that the A2 domain of the FVIII-HC is required for maximal changes within the FIXa active site (21). This might seem in conflict with our observation that the FVIII-HC does not contribute to the affinity of FIXa for the FVIII-LC (Fig. 4). We considered the possibility that this could be due to denaturation of the isolated FVIII-HC. However, this seems unlikely, as reconstitution experiments (not shown) demonstrated that the isolated FVIII-HC and FVIII-LC could be effectively assembled into biologically active heterodimers. The findings do not necessarily mean that the FVIII-HC is not involved in FIXa-FVIII interaction. It is conceivable that the FVIII-LC is responsible for complex assembly with FIXa, while FVIII-HC might induce repositioning of the FIXa active site.

**Fig. 6. Effect of monoclonal anti-FVIII-LC antibodies on the interaction of FVIII-LC with FIXa.** FVIII-LC was immobilized on monoclonal antibody CLB-CAg A (see Table 1). Binding of EGR-CK inactivated FIXa (40 nm) was determined as described under "Experimental Procedures" in the presence of varying concentrations of antibody CLB-CAg A or CLB-CAg G (3). The drawn lines were obtained from a model describing competitive inhibition (see "Experimental Procedures"). The calculated inhibition constant for antibody CLB-CAg A was 0.5 ± 0.2 nm.

absent in the thrombin-cleaved FVIII-LC (Fig. 5). The second antibody, CLB-CAg A, was of interest, because it is a strong inhibitor of FVIII activity (24). Moreover, as is apparent from Fig. 1, FIXa did not bind to FVIII-LC that was immobilized on this antibody instead of antibody CLB-CAg A. Indeed, antibody CLB-CAg A effectively inhibited FIXa binding to the FVIII-LC (Fig. 6). The observed dose dependence of this inhibition fitted well into the model of competitive inhibition as described under "Experimental Procedures." The calculated inhibition constant was 0.51 ± 0.22 nm, which is similar to the $K_d$ for the binding of the FVIII-LC to the same antibody (see Table 1). These data suggest that the monoclonal antibody CLB-CAg A and FIXa share common binding sites on the FVIII-LC.

Our monoclonal antibody CLB-CAg A has its epitope on the FVIII-LC and also strongly interfere in FVIII function. In a number of cases, studies using such inhibitory antibodies as functional probes have contributed to our current understanding of FVIII function. For instance, antibodies against the C2 domain inhibit FVIII function by interfering in the interaction between FVIII and phospholipids (45). Furthermore, an antibody against the N-terminal acidic region has been described to interfere in the proteolytic activation of FVIII at Arg1689 by thrombin (46). Our monoclonal antibody CLB-CAg A has its...
epitope within the A3 domain sequence Gln1778-Asp1840 provides a FIXa binding site. The notion that binding to this region strongly interferes with epitope within the FIXa binding site is not likely to be accessible when the nonactivated heterodimer also displays high affinity for FIXa binding (Fig. 4), as a consequence, FIXa binding would be among the very few processes within the coagulation mechanism that might escape proteolytic control. However, the affinity for FIXa by vWF is about 0.1 nM (37), which exceeds the affinity for FIXa by two orders of magnitude. Therefore, the FIXa binding site is not likely to be accessible when vWF is simultaneously present. The exposure of the FIXa binding site then requires dissociation of the FIXa-vWF complex. As this process is a consequence of proteolytic cleavage of the FIXa LC at position Arg1689 (36) (see also Fig. 5), the same proteolytic event may result in the exposure of the FIXa binding site.

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