The N-terminal Epidermal Growth Factor-like Domain of Coagulation Factor IX

PROBING ITS FUNCTIONS IN THE ACTIVATION OF FACTOR IX AND FACTOR X WITH A MONOCLONAL ANTIBODY

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The absence or reduced activity of coagulation factor IX (FIX) causes the severe bleeding disorder hemophilia B. FIX contains an N-terminal Gla domain followed by two epidermal growth factor-like (EGF) domains and a serine protease domain. In this study, the epitope of monoclonal antibody AW, which is directed against the C-terminal part of the first EGF domain in human FIX, was defined, and the antibody was used to study interactions between the EGF domain of FIX and other coagulation proteins. Antibody AW completely blocks activation of FIX by activated factor XI, but activation by activated factor FVII-tissue factor is inhibited only slightly. The antibody also causes a marginal reduction in the apparent $k_{cat}$ for factor X both in the presence and absence of activated factor VIII. Based on these results, we produced a preliminary model of the structure of the activated factor IX-activated factor VIII-AW complex on the surface of phospholipid. The model suggests that in the Xase complex, EGF1 of activated factor IX is not involved in direct binding to activated factor VIII. Studies of the interaction of antibody AW with a mutated FIX molecule (R94D) also suggest that the Glu78–Arg94 salt bridge is not important for maintaining the structure of FIX.

The severe bleeding disorder hemophilia B is caused by an absence or reduced activity of coagulation factor IX (FIX). FIX circulates in plasma as a zymogen that is converted to a serine protease either by activated factor VII (FVIIa) in complex with tissue factor (TF) or by activated factor XI (FXIa). Activation of FIX is a two-step process in which an activation peptide is

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1 The abbreviations used are: FIX, factor IX; FIXa, activated FIX; FVIIa, activated factor VII; TF, tissue factor; FXIa, activated factor XI; FVIII, factor VIII; FVIIa, activated factor VIII; FX, factor X; FXIa, activated factor X; Gla, γ-carboxyglutamic acid; EGF, epidermal growth factor-like; Xase, FIXa-FVIIIa complex; APTT, activated partial thromboplastin time; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; HPLC, high pressure liquid chromatography.

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main is the most important part of FIXa to mediate the interaction with FVIIIa. For example, the helix formed by residues 330–338 of the serine protease domain (residues 162–170 in the chymotrypsinogen numbering scheme) interacts with the A2 domain of FVIIIa (15, 16).

It is clear that EGF domains function as spacers to position the active site of FIX/FXa and related coagulation proteases at a suitable distance from the biological membrane for interaction with activators, cofactors, and substrates. EGF domains also contribute directly to certain protein interactions, as in the case of the interaction of EGF1 of FIX with FVIIa-TF (17). Attempts to establish the role of EGF1 of FIX in FX activation and, more specifically, whether there is a direct interaction between EGF1 and membrane-bound FVIIa have been inconclusive. For instance, recombinant mosaic FIXa, with EGF1 substituted for the corresponding domain of FX, has full biological activity (19). On the other hand, Ca²⁺ binding to EGF1 of FIX has been reported to promote binding of the FVIIIa light chain and enhance amidolytic activity and FX activation (18).

The effect on the amidolytic activity indicates that Ca²⁺-binding to EGF1 causes a reorientation of the Gla domain relative to EGF1 and that the ensuing conformational change is propagated by alterations to the inter-EGF1-EGF2 angle and thence the hydrophobic interface between EGF2 and the serine protease domain. Similar conformational transitions occur between the N-terminal EGF domain and the serine protease domain in FVII (20).

The G-terminal EGF domain and serine protease domains are linked during activation of FIX but only modestly reduces the FVIIa-TF-mediated activation of FIX (21). The mAb inhibits FXIa-mediated activation of FIX but only modestly reduces the FXa-TF-mediated activation. When FIXa is bound in the Xase complex, the mAb causes a marginal reduction in the apparent reaction. When FIXa was bound in the Xase complex, the mAb causes a marginal reduction in the apparent reaction. When FIXa was bound in the Xase complex, the mAb causes a marginal reduction in the apparent reaction.

Production of Fab Fragments—Proteolysis of intact mAb AW was performed according to Parham (26) with some modifications. mAb AW (in 0.1 M sodium citrate, pH 3.5) was digested with 0.5% (w/w) pepsin at 37 °C for 1 h, and then the pH was adjusted to 7.5 with 2 M Tris, and the solution was dialyzed against 0.1 M Tris-HCl, pH 7.5. Cysteine was added to a final concentration of 0.01 M, and the mixture was incubated at 37 °C for 2 h. After incubation at room temperature for 20 min, the sample was dialyzed against 0.02 M Tris-HCl, pH 8.5, and the Fab fragment was isolated by chromatography on Q-Sepharose.

Clotting Assay—Plasma (100 μl) from healthy individuals or from a FIX-deficient patient (FIX <1%) was incubated for 15 min at room temperature with 2 μM mAb AW or its Fab fragment diluted in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and 1% (v/v) human serum albumin. The plasma was then mixed with 100 μl of thromboplastin (Sigma) or 100 μl of APTT reagent (Diagnostica Stago, Asnières-Sur-Seine, France). After incubation for 5 min at 37 °C, 100 μl of 0.025 M CaCl₂ was added, and the clotting time was recorded with a KC 10, Ameling (Germany). Each experiment was repeated twice.

Effect of mAb AW on the Amidolytic Activity of FIX and the Inhibitory Effect of Antithrombin—A 50-μl aliquot of FIXa (final concentration 2 μM) in 50 mM Tris-HCl, pH 8.4, containing 10 mM CaCl₂, 0.1 M NaCl, and 0.2% (w/v) bovine serum albumin was mixed with 50 μl of FIXa substrate CH₃SO₂-LGR-pNA (CBS 31.39; Diagnostica Stago) in a Sero-Wel 96-well plate (Bibby Sterilin Ltd., Stone, Staffs, UK). A₅₀₄ was recorded for 15 min. Antithrombin (2 μM) and heparin (800 IU/ml) were added to the assay, with or without 10 μM mAb AW.

Active Site Titration—FIXa and FXa (2 μM) were active site-labeled as described by Byrne et al. (27) in a Cary 4E spectrophotometer (Varian Pty. Ltd, Mulgrave, Australia) using a concentration of p-nitrophenyl-p'-guanidinobenzoate of 50 μM. The effect of mAb AW on the Activation of FIX by FIXa and FVIIIa—Human FIX (2 μM) was incubated at 37 °C with human FIXa (68 nm) in 20 mM Heps, 0.15 M NaCl, 5 mM CaCl₂, pH 7.4. In some assays, FIXa was preincubated for 5 min with 8 μM mAb HPC 11 or 8 μM mAb fragments of AW. Aliquots were withdrawn at intervals; diluted 1:5 in 0.05 M Tris-HCl, pH 8.4, containing 0.1 M NaCl, 10 mM CaCl₂, and 0.2% (w/v) human serum albumin; and then frozen on dry ice/ethanol and stored at −70 °C. Thawed samples were mixed with an equal volume of CH₃SO₂-LGR-pNA (Diagnostica Stago), and the A₅₀₄ was recorded for 15 min at 37 °C.

Human FIX (1.6 μM) was incubated at 37 °C with human FVIIIa (0.3 nm) in 0.1 M NaCl, 5 mM CaCl₂, 0.05 M Tris, pH 8.4. In some assays, FIXa was preincubated for 5 min with 8 μM mAb HPC 11 or 8 μM Fab fragments of mAb AW. The mixture was diluted 1:2 with Innovine

2 J. Stenflo, unpublished observations.
Effects of mAb AW on Activation of FX by FIXa—Phospholipid vesicles containing 70% l-α-phosphatidylcholine (Sigma) and 30% phosphatidyl-l-serine (ICN; Aurora, OH) were prepared as described (28) and added to a final concentration of 50 μM in a buffer consisting of 20 mM Hepes, 0.2% (w/v) human serum albumin, 0.15 μM NaCl, 10 mM CaCl₂, pH 7.5, and incubated for 10 min at 37 °C. Active site-titrated FIXa was then added to a concentration of 0.1 nM. In some assays 0.1 μM mAb AW or its Fab fragment was included, and the antibody and FIXa were preincubated for 5 min. Thrombin (6.4 nM; Kordia) and FVIIIa (9.4 nM; Recombinate, Baxter, Germany) were added, and the mixture was incubated at 37 °C for 2 min. FX was then added; aliquots were withdrawn after 0, 5, 10, and 15 s (when formation of FXa was linear); and the reaction was stopped by the addition of an equal amount of 20 mM Hepes, 0.15 μM NaCl, 25 mM EDTA, pH 7.4. A 100-μM aliquot of S-2222 (Chromogenix, Milano, Italy) was added to 100 μl of the reaction mixture, and A₄₀₅ was recorded for 15 min at 37 °C. An active site-titrated FXa preparation was used as a reference to convert absorbance values into molar concentrations. For assays performed in the absence of FVIIIa or phospholipid, 10 nM FIXa and 18.7 nM FVIIa were used, both with and without 0.1 μM mAb AW. In this case, the mixture was incubated for 11 min before the addition of FX. Aliquots were withdrawn after 0, 10, 15, and 30 s for assays without phospholipid and after 0, 1, 2, and 5 min for assays in the absence of FVIIIa (when formation of FXa was linear). In the absence of both FVIIIa and phospholipid, the concentration of FXa was increased to 400 nM (in the presence and absence of 4 μM mAb AW), and 1 μM FX was used. Aliquots were withdrawn after 5, 10, 20, 30, and 45 min.

Surface Plasmon Resonance—The interaction of FIX and FIXa with mAb AW or its Fab fragment was analyzed by a BIACORE 2000 biosensor (Biacore AB, Uppsala, Sweden). The mAb AW or its Fab fragment was immobilized on a CM5 sensor chip using an amine coupling kit (Biacore AB) according to the manufacturer’s instructions. Association and dissociation rates were determined at a flow rate of 30 μl/min at 25 °C. About 1500 response units of whole mAb and 600 response units of Fab fragment were immobilized on the chip. Proteins were diluted in a buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mg/ml bovine serum albumin, and 5 mM CaCl₂, and an aliquot of 90 μl was injected for 180 s, followed by the same buffer for 600 s. 2-Fold dilutions of protein over the range 50–1.6 nM were tested. Sensor chips were regenerated with a 5-μl pulse (5 μl/min) of 0.1 M glycine, 0.5 M NaCl, pH 2.7. One channel on the chip without coupled antibody was used for correction of the bulk sample effect. Data were evaluated with BIAlEvaluation 3.0 software, and dissociation constants were calculated from the association and dissociation rate constants. Each experiment was repeated twice.

Computer Modeling—A three-dimensional structural model of FVIII was obtained from the World Wide Web at eurepmm.csc.mrc.ac.uk (13). X-ray coordinates for full-length porcine FIXa (file 1pxd.pdb), the human FIXa EGF2-serine protease domain (file 1rnf.pdb), the FVIIa-TF complex (file 1dan.pdb), and a Fab fragment in complex with TF (file 1ahw.pdb) were obtained from the Protein Data Bank (available on the World Wide Web at www.rcsb.org/pdb) (5, 29–31). A model of full-length human FIXa was built using the coordinates for the human FIXa EGF2-serine protease domain and porcine FIXa. Certain side chains in the porcine Glα-EGF1 region were substituted for those corresponding to human FIX. Ca²⁺ ions were added according to the FVIIa x-ray structure. Angles between the different domains of porcine FIXa were kept in the human FIXa model. Possible variations in the Glα-EGF1 and EGF1-EGF2 interdomain angles were taken into account in subsequent structural analysis, whereas the EGF2-serine protease interface was assumed to be relatively rigid. Coordinates for the Fab fragment were extracted from file 1ahw, and the hypervariable loop regions were positioned on top of the expected epitope for mAb AW. Positioning of the Fab took into account only the overall shape complementarity between FIXa and the Fab structure; a full docking protocol could not be performed since the human FIXa sequence for mAb AW is not known. An approximate model for the FIXa-Fab complex was then positioned next to the FVIIa model using information previously reported for FVIIa, and further optimization of the system was performed interactively (13, 32). Modeling and structural analysis were performed on Silicon Graphics O₂ and FUEL workstations (Mountain View, CA) with the Accelrys package (available on the World Wide Web at www.accelrys.com; Accelrys, San Diego, CA). Visualization of the molecules during positioning was facilitated by the use of Stereo devices (Crystaleyes).

RESULTS

Characterization of Recombinant and Synthetic FIX Polypeptides—Wild-type FIX and four variants containing the point substitutions D49E or P55S (in EGF1), R94D (in EGF2), and E245K (in the serine protease domain; residue 80, chymotrypsinogen numbering) were produced in human kidney 293 cells and used in initial attempts to define the epitope of mAb AW (see below; Figs. 1 and 2) (21). The P55S, R94S, and E245K mutations have been found in hemophilia B patients (11, 33). The affinity-purified proteins were more than 95% homogeneous as judged by N-terminal sequencing and SDS-PAGE, and there were no signs of internal cleavages. Amino acid analysis of alkaline hydrolysates established that the FIX preparations were almost fully γ-carboxylated (12.4 mol of Gla/mol of FIX for plasma-derived FIX, 11.1 mol/mol for the recombinant wild-type protein, and 10.5 mol/mol for the mutated proteins). Clotting activities (mean of two or three measurements) were 100% for plasma-derived FIX and 96% for recombinant wild-type FIX. Those for the variants were D49E (18%), P55S (12%), R94D (100%), and E245K (<1%). The wild-type and P55S FIX proteins could be almost fully activated by FIXa at rates comparable with plasma-derived FIX, as analyzed by SDS-PAGE (not shown).

The synthetic wild-type and variant FIX EGF1 domains each produced a single peak during reversed-phase HPLC on a C8 column and were judged to be folded to a native conformation; an ELISA using a polyclonal rabbit anti-human FIX antiserum was positive with the folded EGF1 domains but negative after reduction of disulfide bonds (not shown; the antiserum does not react with reduced FIX). Moreover, synthetic FIX has been shown to have the same affinity as FIXa for mAb AW (21).

Mapping the Epitope of mAb AW—Recombinant FIX variants and synthetic FIX EGF1 domains containing amino acid substitutions were used to define the epitope of mAb AW. Synthetic human FIX EGF1 and FIXa bound to mAb AW with the same affinity, whereas bovine FIX did not, although within EGF1 the bovine protein differs at only four positions (61, 74, 75 and 80; Fig. 1 and Ref. 21). We therefore synthesized human FIX with the following substitutions: S61M, F75A, and K80T. The substitution L57Q was also made to probe the surface of the face of EGF1 that is opposite to Lys⁸⁰ (see below; Fig. 2). Notably, FX and FVII have a Gln residue in the corresponding position. When tested in an ELISA, the L57Q and S61M substitutions bound both mAb AW and polyclonal anti-FIX antibodies with the same affinity as the wild-type domain. Domains with F75A and K80T substitutions did not bind the...
mAb (Fig. 3, A and B). Two full-length recombinant FIX variants (R94D and E245K) were also tested by ELISA (Fig. 3, C and D). The E245K mutation, which is located in the Ca\(^{2+}\)/H11001-binding loop of the serine protease domain (34), was chosen because we wanted to investigate whether the mAb could interact with a region close to the activation peptide. The R94D substitution was selected, since it could give an indication of the importance of the inter-EGF1-EGF2 area for binding of mAb AW. Both mAb AW and the polyclonal antiserum bound to FIX E245K as strongly as to wild-type FIX, whereas the affinities of the P55S and R94D proteins were reduced 8- and 2-fold, respectively, suggesting that residues 55 and 94 are located near to the epitope of the antibody but do not define the key binding surface for this antibody. However, with respect to the isolated EGF1, residues 72, 75, and 80 are key elements of the epitope, locating the epitope to the C-terminal part of EGF1, which is consistent with the fact that binding of the mAb is not Ca\(^{2+}\)-dependent (21). Overall, the results are fully consistent with a binding site for the antibody centered on residues Trp\(^{72}\)-Lys\(^{80}\), with an additional contribution from the region displaying residue 55 and a small contribution from the region of residue 94. Phe\(^{75}\) was found to be important for the interaction between the isolated EGF1 and mAb AW; it is located on the same side of the EGF domain as residues 49, 57, and 61 (Ca\(^{2+}\)-binding area) but on a different face than residues 55, 72, 80, and 94 (see Fig. 2). The reduced binding of EGF1 F75A could be due to the fact that Phe\(^{75}\) is fully exposed.
Functions of EGF1 of FIX

Table I
Interaction of mAb AW and its Fab fragment with mutated FIX proteins

| Protein   | $k_a$ (M$^{-1}$ s$^{-1}$) | $k_d$ (s$^{-1}$) | $K_D$ (M$^{-1}$) |
|-----------|--------------------------|-----------------|-----------------|
| FIX       | 1.2 $\pm$ 0.1 $\times$ 10$^5$ | 5.1 $\pm$ 0.3 $\times$ 10$^{-4}$ | 4.3 $\times$ 10$^{-9}$ |
| FIX p-d, Fab | 1.3 $\pm$ 0.3 $\times$ 10$^5$ | 7.2 $\pm$ 0.4 $\times$ 10$^{-4}$ | 5.6 $\times$ 10$^{-9}$ |
| FIX wild type | 1.2 $\pm$ 0.1 $\times$ 10$^6$ | 7.0 $\pm$ 0.1 $\times$ 10$^{-4}$ | 5.6 $\times$ 10$^{-9}$ |
| FIX D49E | 1.4 $\pm$ 0.1 $\times$ 10$^6$ | 7.0 $\pm$ 0.1 $\times$ 10$^{-4}$ | 5.2 $\times$ 10$^{-9}$ |
| FIX P55S | 1.6 $\pm$ 0.1 $\times$ 10$^4$ | 7.3 $\pm$ 0.1 $\times$ 10$^{-4}$ | 4.5 $\times$ 10$^{-8}$ |
| FIX R94D | 5.2 $\pm$ 0.2 $\times$ 10$^4$ | 5.6 $\pm$ 0.2 $\times$ 10$^{-4}$ | 1.1 $\times$ 10$^{-9}$ |
| FIXa p-d | 4.6 $\pm$ 0.1 $\times$ 10$^6$ | 4.9 $\pm$ 0.1 $\times$ 10$^{-4}$ | 4.6 $\times$ 10$^{-10}$ |
| FIXa, p-d, Fab | 1.3 $\pm$ 0.1 $\times$ 10$^6$ | 7.3 $\pm$ 0.3 $\times$ 10$^{-4}$ | 5.8 $\times$ 10$^{-10}$ |
| FIXa P55S | 9.9 $\pm$ 0.2 $\times$ 10$^4$ | 7.5 $\pm$ 0.1 $\times$ 10$^{-4}$ | 7.6 $\times$ 10$^{-9}$ |

Phe$^{75}$, which is also on this face, cannot be a direct binding site for mAb AW in full-length FIX. Our results for FIX R94D also shed some light on the possible importance of the salt bridge formed between Glu$^{78}$ and Arg$^{94}$. Because the epitope of mAb AW is located next to this region and is influenced by residue 94, we expected the FIX-mAb AW interaction to be largely impaired by the R94D mutation. However, the mutation caused only a 2-fold decrease in the binding affinity of mAb AW, suggesting that the salt bridge is not critical for the proper orientation of the EGF domains. Based on these results, an approximate three-dimensional model for the FIXa-mAb AW (Fab fragment) complex was constructed (Fig. 2). The hypervariable regions of the Fab used to mimic Alexa were centered on residues 72 and 80 of FIX. Only one overall orientation of the Fab fragment was found to be consistent with the three-dimensional structures of FIX and the Fab and our experimental results. In this orientation, the active site of FIXa is remote from the bound Fab (see below), whereas the Fab is adjacent to, but does not seem to interfere with, the activation peptide region, the area around residue 245, or the EGF1 Ca$^{2+}$-binding region.

Affinity of mAb AW for FIX and FIXa—The affinities of intact mAb AW and the corresponding Fab fragment for plasma-derived FIX and FIXa were determined by surface plasmon resonance. The antibody was immobilized on a sensor chip, and FIX and FIXa were in the mobile phase. We have previously observed a 10-fold higher affinity of intact mAb AW for FIXa than for FIX (21). This was also observed for the Fab fragment (Table I). To investigate whether the activation-induced conformational transition in EGF1 (as manifested by the difference in affinity of zymogen and active enzyme for the mAb) also occurred in FIX with a mutated EGF1 domain, we compared the affinities of the zymogen and activated forms of FIX P55S for mAb AW. The results were similar to those observed for the wild-type protein (i.e. FIXa P55S had a 6-fold higher affinity for mAb AW than the corresponding zymogen).

Effect of mAb AW on the Amidolytic Activity of FIXa and on Activation of FIX by FVIIa-TF and by FIXa—mAb AW or its Fab fragment did not inhibit the amidolytic activity of FIXa using the synthetic substrate CH$_3$SO$_2$-LGR-pNA; nor did it affect the ability of antithrombin to inhibit FIXa (data not shown). Therefore, mAb AW could be used to study the activation of FIX and the interaction of FIXa with its cofactor and substrate in plasma clotting systems. Clotting was initiated both by the intrinsic pathway (APTT reagent) and by the extrinsic pathway (thromboplastin reagent (i.e. TF)). When the APTT reagent was used to induce coagulation, mAb AW (or its Fab fragment) prolonged the clotting time about 3-fold (from 38.1 to 98.0 s (mean of triplicate assay)). However, if coagulation was induced with the thromboplastin reagent, the mAb

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in the isolated domain, whereas this residue is partially covered by EGF2 in full-length FIX. Along this line of reasoning, it should be noted that Ser$^{53}$ and Ser$^{61}$ are O-glycosylated in FIX, whereas the synthetic EGF1 is not (35, 36). Since mAb AW has the same affinity for synthetic EGF1 and FIXa, the glycosylated residues cannot be part of the epitope (21). The glycosylated residues are located on the same face as residues 49 and 57, which are not part of the epitope of mAb AW. Therefore,

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**Fig. 3.** Epitope mapping mAb AW by ELISA. The antigenic specificity of mAb AW was studied in an ELISA by testing the binding of polyclonal rabbit anti-FIX antiserum (A, C) or mAb AW (B, D) to synthetic EGF domains (A, B) or recombinant FIX (C, D), which had been coated at different concentrations on the plate. A and B show wild-type ( ), S61M ( ), L57Q ( ), K50T ( ), and R75A ( ) EGF domains. C and D, wild-type ( ), E245K ( ), and R94D ( ) FIX proteins.

**Fig. 4.** Epitope mapping mAb AW by a displacement assay. The binding of mAb AW to mutated EGF domains was studied in a competition displacement assay. A plate was coated with mAb AW, and increasing concentrations of wild-type ( ) or W72F ( ) EGF domains were added in competition with a constant amount of Eu$^{153}$-labeled FIXa.
and the amidolytic activity was measured as described under “Experimental Procedures.”

The addition of mAb AW to FIXa-mediated activation of FX (Fig. 5). The mAb AW Fab fragment only partially inhibited the activation of FIX by FXIa, whereas activation by FXIa was almost completely blocked, results that concur with the plasma clotting assays. Identical results were obtained with the intact antibody (not shown). These findings support the overall orientation of the Fab shown in Fig. 2, in which the activation peptide area is not in direct contact with the mAb.

**Effect of mAb AW on Activation of FX**—To investigate the effect of mAb AW on FXIa-mediated activation of FX, particularly the interaction of FIXa with FXIa, activation was performed in the presence and absence of phospholipid and/or rate-limiting concentrations of FXIa. Titration with increasing concentrations of Fab AW was performed in the presence of phospholipid, FIXa, and FXIa (Fig. 6). The Fab fragment maximally inhibited the activation of FX when present at a 1000-fold molar excess over FIXa (when 99.8% of FIXa was calculated to be in complex with mAb AW, assuming that the Fab binding to FIXa is unperturbed by FXIa and phospholipid). Under these conditions, activation of FX appeared to be inhibited in a noncompetitive manner. A ~15-fold decrease in $k_{\text{cat(app)}}$ was observed, whereas only a minor change in apparent $K_m$ ($K_{\text{m(app)}}$) was seen (Fig. 7A, Table II).

In the absence of FXIa, a 100-fold molar excess of mAb AW (where 100% of FIXa was calculated to be in complex with mAb AW) produced maximal inhibition of FX activation (not shown). In this case, $k_{\text{cat(app)}}$ was reduced ~8-fold by the mAb, whereas $K_m$ (app) was reduced 5-fold (Fig. 7B, Table II), although the value for $K_{\text{m(app)}}$ is uncertain due to the low absorbances measured. The addition of FXIa increased $k_{\text{cat(app)}}$ 13,000-fold in the absence of mAb AW; in the presence of antibody, the increase was 6000-fold. Moreover, in the presence of FXIa and mAb AW, $k_{\text{cat(app)}}$ was 800-fold greater than when FXIa and mAb AW were absent. It is therefore apparent that mAb AW causes only a modest inhibition of the intact Xase complex.

The addition of the mAb in the presence of FXIa but without phospholipid completely blocked the activation of FX (Fig. 7C). In the absence of both phospholipid and FXIa, at a FXIa concentration of 400 nM, there was a major inhibitory effect when the mAb AW was added at a 10-fold molar excess over FIXa, but due to the low absorbances, the extent of inhibition could not be accurately determined (Fig. 8). In all cases, the
functions of EGF1 of FIX

We investigated the role of the EGF1 domain of FIX in activation of FIX by FXIa and FVIIa and also clarified some of the roles of this domain within the Xase complex. The mAb AW, which has its epitope in the C-terminal part of EGF1 was used to probe the functions of EGF1. The epitope of the mAb was defined by means of site-directed mutagenesis of FIX, tests with synthetic EGF1 domains, and structural analysis. The two mutant forms of EGF1 were probed as well as a residue adjacent to the activation peptide area. The EGF1 region displaying residues Thr^{22}, Phe^{76}, and Lys^{80} was found to be of major importance for the interaction of synthetic EGF1 with mAb AW, with some contribution from residues Pro^{55} and Arg^{94}. The lower affinity of the mAb for F75A synthetic EGF1 could be due to the fact that in the absence of EGF2, the hypervariable regions of the mAb can contact Phe^{75} while still having a main interaction area in the region of residues 72 and 80. For reasons outlined under "Results," the epitope of mAb AW is predicted to be centered on residues 72 and 80. In such an orientation, the mAb leaves the active site of FIXa fully accessible to substrate. Our experimental results concur with the orientation of the mAb shown in Fig. 2, since the addition of the mAb AW did not affect either the amidolytic activity of FIXa or the inhibitory effect of antithrombin on FIXa.

We have shown previously that mAb AW has a 10-fold higher affinity for FIXa than for FIX (21). This difference in affinity is also observed for the Fab fragment (Table I). For the intact mAb, the possibility cannot be excluded that the difference in affinity between FIX and FIXa is due to steric hindrance caused by the activation peptide (36 residues), but in the molecular model of the Fab fragment bound to FIXa (Fig. 2), it appears as if the Fab can bind without interference from the activation peptide. The affinity of mAb AW for FIX E245K was the same as for wild-type protein, which also favors a model where the mAb binds FIX without direct contact with the activation peptide. The difference in affinity between wild-type FIX and FIXa was also observed for the P55S variant. Our experiments lend support to the notion that there is an intramolecular communication between the serine protease domain and EGF1. Similar results have been reported by Leonard et al. (20), who characterized a mAb with a 3-fold higher affinity for the N-terminal EGF domain in zymogen FVII than in the activated form. In addition, Lenting et al. (18) showed that Ca^{2+} binding to EGF1 affected the amidolytic activity of FIXa. It has also been found that activated FIX P55L is much more rapidly cleaved at Arg^{94} in the serine protease domain than the wild-type counterpart. Altogether, this points toward a model where a conformational change in the N-terminal part of the molecule can be propagated to the C-terminal part and vice versa. Such a view is further supported by molecular dynamics studies in which it was noticed that activation led to major orientational changes in the EGF2-catalytic domain alignment against the remainder of the molecule, while the overall length of the molecule changed only slightly (40). This kind of conformational change seems to be recognized by mAb AW.

It has been reported that Arg^{94} in FIX forms a functionally important salt bridge with Glu^{78}, which stabilizes the molecule and promotes interaction with FVIIa (5, 9). Since mAb AW binds in the area of residues 78 and 94, we constructed the FIX R94D protein to evaluate the effect of removal of the salt bridge on binding of the mAb. We chose not to make R94S (which is an existing patient mutation), since it is known that the Ser residue becomes O-glycosylated. Surprisingly, mAb AW had almost as high an affinity for FIX R94D as it had for the wild-type protein. We also noted that clotting was not affected by insertion of the R94D mutation. Our results are in line with those demonstrating that R94D is not important for assembly of the Xase complex on phospholipid vesicles (41). We believe that the results from our experiments show that the Glu^{78}–Arg^{94} salt bridge is not essential for maintenance of the FIX structure but that other interactions in this area are sufficient to maintain proper alignment of the light chain domains. Notably, two negatively charged residues are found close in space at equivalent positions in FXa (42). Moreover, our data rule out direct contacts between residue 94 or 78 and FVIIa, since this surface is covered by mAb AW, and antibody-bound FIXa still functions in the Xase complex, albeit with a moderately reduced $k_{\text{cat(app)}}$.

Results in the literature have been contradictory concerning...
the participation of EGF1 of FIX in activation by FXIa or FVIIa-TF. For example, FIX Q50P has been studied by two different groups (43, 44). Zhong et al. (43) found that activation of this variant by FXIa was normal, whereas Lozier et al. (44) reported that activation was markedly delayed. We have now used mAb AW to study the activation of FIX. We find here that activation of FIX by FXIa is completely blocked by the addition of mAb AW, and we also find a prolonged clotting time (APTT). Presumably, the FIX-bound mAb or its Fab fragment sterically interferes with the bulky FXIa molecule to preclude activation. The mAb may also prevent a putative direct interaction of FIX-EGF1 and FXIa. The rate of activation of FIX by FVIIa-TF was only moderately reduced when mAb AW was added, and in the thromboplastin-activated clotting assay, no prolongation of the clotting time was observed. In a recent study, it was shown that EGF1 in FIX is important for binding to TF (17). We conclude that the binding site in FIX EGF1 for FVIIa-TF is not located in the C-terminal part of EGF1 around residues 72 and 80, since this region embodies the binding site for mAb AW.

Whether EGF1 of FIXa interacts directly with FVIIIa in the Xase complex or if it merely positions the serine protease domain at the correct distance above the membrane surface has been a matter of debate (10, 15, 18). We have shown that the part of EGF1 that embodies the binding site for the mAb AW (around FIXa residues 55, 72, 80, and 94) is not involved in direct binding to FVIIIa, since the major increase in $k_{cat(app)}$ caused by the addition of FVIIIa is also observed in the presence of the mAb. The affinity of FIXa for mAb AW is higher than the affinity between FIXa and FVIIIa, even in the presence of phospholipid, and with the experimental conditions used, more than 99% of FIXa would be expected to be in complex with the mAb. If FVIIIa had been in direct contact with FIXa residues 72 and 80 (and surrounding areas of the same face of EGF1), the effect of mAb AW on FX activation in the presence of phospholipid ought to be much larger than the apparently noncompetitive 15-fold reduction that was observed.

In the presence of FVIIIa but in the absence of phospholipid, the mAb AW blocked FX activation completely. In solution, the molecules are not preoriented for optimal interaction, and during formation of an encounter complex, the contacts between the molecules are weak. Any direct or indirect disturbance created by the mAb would inhibit evolution toward the formation of a normal stable complex. The mAb could also slightly

![FIG.9. Model of the FIXa-FVIIIa-mAb AW Fab fragment complex. Top panel, FIXa is shown as a ribbon diagram in yellow (Gla domain), white (EGF1), orange (EGF2), and white (serine protease domain). The active site residues and segments important for interaction with FVIIIa are shown in red. A peptide segment (residues 68–94 of EGF1), which is expected to interact with the A3 domain of FVIIIa, is also shown in red. The key residues involved in binding to AW are shown as yellow and blue spheres, whereas amino acids that are not involved in the interaction with the antibody are shown as magenta spheres. To simplify the figure, residues 75 and 245 of FIXa are not shown. Ca$^{2+}$ ions that could be positioned in our human FIXa model are shown as small blue spheres (some additional Ca$^{2+}$ ions could be present within the Gla domain area). In the FVIIIa model, regions important for interacting with FIXa are colored red (around residues 558 and 1804; see “Results”). Bottom panel, a Fab fragment (green and blue) mimicking AW is shown as a solid surface and has been positioned on its epitope as in Fig. 2. In this orientation, the key binding area in the serine protease domain of FIXa could contact the A2 domain of FVIIIa without interference from the mAb. FIXa and FVIIIa are separated in the figure for the sake of clarity.](image-url)
modify the positioning of the FIXa active site with respect to the interaction of FX in such a way that the catalytic machinery does not function properly. This is also consistent with our results (Fig. 8) with only FIXa and FX in the assay, where the mAb still has an inhibitory effect.

Concerning the contribution of EGFI of FIX in FX activation, it has been shown that the entire EGFI domain of FIXa can be replaced by that of FX or protein C (in the absence of phospholipid), without major functional implications. Because EGFI of activated protein C has a major loop inserted at a position corresponding to FIXa residue 54, it seems unlikely that this part of EGFI in FIXa makes a direct contact with FVIIIa (15, 19). There is evidence that residues 68–94 of FIXa bind to FVIIIa, specifically to residues 1804–1818, and also that the FIXa segment 84–91 can be involved in FVIII binding (18, 39). Our data, in conjunction with data from others, are not consistent with a direct contact between EGFI of FIXa and FVIIIa. However, a region around residues 85–90 in the linker area between EGFI and EGFP2 might contact the FVIIIa A3 domain. Flexibility in the linker region could facilitate docking with target proteins via effective conformational coupling between the different domains.

Based on our results and the large body of data for the Xase complex, we propose a preliminary computer model of the FIXa-FVIIIa-mAb AW complex (Fig. 9). In this model, the key interacting regions of FIXa and FVIIIa can be aligned as previously reported with only minor reorientations (13, 32). A Fab fragment mimicking AW can be centered on the epitope defined within EGFI of FIXa, and the mAb-FIXa complex can accommodate the expected contacts with FVIIIa without interference from the mAb. Reorientation of the Gla and EGFI domains of the FIXa light chain, according to the x-ray structures of FVIIIa and FIXa, was attempted in order to probe alternative conformations for this part of FIXa (30, 43). In all situations, the Fab fragment would point away from FVIIIa and away from the FIXa active site, and the only key difference seen between the models was that the Gla domain of the modified FIXa could be closer to the C2 domain of FVIIIa than it is in the orientation shown in Fig. 9. In all of the models, only the region of FIXa encompassing residues 85–90 (but not 78 or 94) could have direct contact with the A3 domain of FVIIIa, whereas the serine protease domain of FIXa could have interactions with the A2 domain. The region around residue 54 of FIXa is not in direct contact with FIXa in our model, in agreement with the fact that mosaic FIXa, in which EGFI was replaced by the corresponding domain of protein C, was still fully functional in the Xase complex in the absence of phospholipid. In conclusion, we propose a model where EGFI of FIXa is not in direct contact with FVIIIa in the Xase complex.

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