A Monoclonal Antibody to Factor VIII Inhibits von Willebrand Factor Binding and Thrombin Cleavage

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To study the interaction of human factor VIII (FVIII) with its various ligands, select regions of cDNA encoding FVIII light chain were cloned into the plasmid expression vector pET3B to overproduce FVIII protein fragments in the bacterium Escherichia coli. Partially purified FVIII protein fragments were used to produce monoclonal antibodies. One monoclonal antibody, 60-B, bound both an FVIII protein fragment (amino acid residues 1563 through 1909) and recombinant human FVIII, but not porcine FVIII. This antibody prevented FVIII-vWF binding and acted as an inhibitor in both the activated partial thromboplastin time (APTT) assay and a chromogenic substrate assay that measured factor Xa generation. The ability of the antibody to inhibit FVIII activity was diminished in a dose-dependent fashion by von Willebrand factor. This anti-FVIII monoclonal antibody bound to a synthetic peptide, KEFDIDYDEDE, equivalent to FVIII amino acid residues 1674 through 1684. The 60-B antibody did not react with a peptide in which the aspartic acid residue at 1681 (underlined) was changed to a glycine, which is the amino acid present at this position in porcine FVIII. Gel electrophoretic analysis of thrombin cleavage patterns of human FVIII showed that the 60-B antibody prevented thrombin cleavage at light chain residue 1689. The coagulant inhibitory activity of the 60-B antibody may be due, in part, to the prevention of thrombin activation of FVIII light chain.

MATERIALS AND METHODS

Select regions of the FVIII molecule were produced in E coli using a highly efficient expression vector." This expression system consists of three parts. The E coli strain, BL21(DE3), contains a lacUV5 controlled bacteriophage T7 RNA polymerase gene located in the chromosome. The T7 RNA polymerase recognizes only T7 promoters and is unaffected by rifampicin, an inhibitor of endogenous E coli RNA polymerase. A plasmid, (plys5), constitutively produces T7 lysozyme at low levels. The T7 lysozyme binds to and inactivates the small amount of T7 polymerase made constitutively in BL21(DE3). A second plasmid, pET3B, contains a truncated T7 coat protein gene under control of a T7 promoter. The cloned gene encoding the protein to be expressed is fused in frame to the 12th codon of the T7 coat protein gene. On induction with isopropyl thigalactoside (IPTG), sufficient T7 polymerase is made to overcome lysozyme inactivation and to transcribe the coat protein fusion construct at a very high level. The relative level of this transcript can be increased by shutting off all other transcription with rifampicin.

Constructs. Four FVIII light chain constructs were made in pET3B (Fig 1). An FVIII cDNA encoding nucleotides 4852 to 7379 on a BamHI fragment was ligated into BamHI-digested pET3B. Transformants of DH5-α were screened for correct orientation. The protein encoded in this construct, pET3B-8, contains FVIII amino acid residues 1563 through 2332, including 86 residues of the B domain and the complete FVIII light chain. Three additional clones were made by digesting the FVIII cDNA fragment with XhoI and ligating each of the three resultant fragments into BamHI digested pET3B. The pET3B-16 construct encoded FVIII residues 1563 through 1909 and terminated after 18 vector-derived residues. The smallest protein was encoded by pET3B-10, residues residues

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The samples were analyzed on these constructs were grown to an absorbance of 0.2 at 600 nm and transformed into BL21(DE-3) pLysS.

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The induced proteins was confirmed by protein sequencing.

The appropriates were cut out with a razor blade. The protein was electro-eluted from the gel and dialyzed into phosphate-buffered saline essentially as described by Goding.15 Alternatively, induced cells were lysed by sonication, and the cell pellets containing the insoluble FVIII protein were collected and pooled. Only the B-16 fusion protein remained soluble after dialysis into TBS. The gel-purified protein was then added to the reaction mixture to 0.34 μmol/L. After 3 minutes the generation of factor Xa was stopped by the addition of EDTA to 0.13 mol/L. The amount of factor Xa present was determined by adding N-benzyloxycarbonyl-Ile-L-Glu-Gly-L-Arg-p-nitroanilide HCl and its methyl ester in equal amounts (S-2222) (concentration 0.4 mol/L) to the above reaction mixture and, following the change in absorbance at 405 nm, using a Vimax kinetic microplate reader (Molecular Devices Corporation, Palo Alto, CA). The relative amount of factor Xa present was determined using SOFTmax, the software supplied with the plate reader.

The primary screen of cell culture supernatants was performed by radioimmunoassay.16 Supernatants from hybridoma cells were incubated in rabbit antiserum coated polystyrene tubes, and the capture of iodinated recombinant FVIII or iodinated fusion protein B-8 was measured.

Reagents. Recombinant human FVIII and the FVIII cDNA were obtained from Genetics Institute (Boston, MA). Iodination of protein was performed by the iodine transfer method of Dewanjee et al.17 Human plasma vWF was isolated from frozen plasma prepared from blood anticoagulated with citrate-phosphate-dextrose (Fenwal, Deerfield, IL). The vWF was prepared as described by Thorell and Blombäck18 up to collection of the Type II precipitate. This precipitate was then dissolved in 0.05 mol/L 2-[N-Morpholino]ethanesulfonic acid, 0.25 mol/L CaCl₂, pH 7.4. The dissolved proteins were fractionated on a column of 4% agarose equilibrated in the same buffer. The fractions containing vWF, as determined by the ristocetin-induced platelet agglutination assay,20 were pooled and dialyzed versus TBS. Porcine factors IX, II, X, IXa, and Ila were prepared as described by Lollar et al.15 Fab fragments were produced using the Immunopure Fab Preparation Kit from Pierce (Rockford, IL).

Assays. FVIII coagulant assays were performed as a modified activated partial thromboplastin time test.21 One unit of FVIII is defined as that amount of FVIII procoagulant activity found in 1 mL of pooled frozen normal human male plasma taken from 30 individuals.

The Bethesda assay22 was used to determine anti-FVIII inhibitor activity of the murine antibodies. An assay measuring the binding of labeled FVIII on vWF-coated tubes was used as previously described.23 Western transfers onto PVDF paper24 were probed with MoAbs and developed with rabbit antiserum IgGalkaline phosphatase conjugates. Fluorescence polarization (FP) determinations were made on an On Line Systems (Jefferson, GA) modified SLM spectrophotometer.25 The assay program was used with excitation at 340 nm and emission at 540 nm. Samples were assayed at 22°C in TBS within 5 minutes of mixing. Extended incubations did not alter the results. The fluorescence polarization value determined in each assay is an average of 7200 samplings taken over a 2-minute period.

Chromogenic assay. Factor Xa production by the enzymatic complex consisting of factor IXa, phosphatidyl-choline/phosphatidyl-serine (75%/25%) (PCPS) vesicles, calcium, and thrombin-activated FVIII (FVIIIa) was measured in a manner similar to that described by Lollar et al.26 Briefly, factor IXa (1 mmol/L), PCPS vesicles (15 μmol/L, phosphorous), factor VIII (0.14 mmol/L), and calcium (5 mmol/L) were incubated with thrombin (4 mmol/L) for 1 minute in TBS containing 0.01% polyethylene glycol-8000, in a microtiter plate (Immulon 1 Removawell Strip; Dynatech Laboratories, Inc, Chantilly, VA). Factor X was then added to the reaction mixture to 0.34 μmol/L. After 3 minutes the generation of factor Xa was stopped by the addition of EDTA to 0.13 mol/L. The amount of factor Xa present was determined by adding N-benzyloxycarbonyl-Ile-L-Glu-Gly-L-Arg-p-nitroanilide HCl and its methyl ester in equal amounts (S-2222) (concentration 0.4 mmol/L) to the above reaction mixture and, following the change in absorbance at 405 nm, using a Vimax kinetic microplate reader (Molecular Devices Corporation, Palo Alto, CA). The relative amount of factor Xa present was determined using SOFTmax, the software supplied with the plate reader.

To measure antibody inhibition of factor Xa generation, FVIII (0.28 mmol/L) was incubated with varying concentrations of 60-B IgG or Fab fragment in TBS containing 0.2 mg/mL bovine serum albumin for 30 minutes at room temperature. Factor IXa, Ca²⁺, PCPS, and thrombin were added and the assay performed as described previously.

Peptide synthesis. Synthetic peptides were prepared using RAMPS multiple peptide synthesis system (DuPont, Wilmington, DE). Rapid Amide resin, and fluorenylmethoxycarbonyl (FMOC)-amino acid pentafluorophenyl esters were used. After cleavage from the resin and deprotection of side chain groups with trifluoroacetic acid (TFA)-phenol, the peptides were purified by reversed-phase high performance liquid chromatography (HPLC) (Vydac C₈ column, aqueous TFA-acetonitrile gradient; Vydac, Hesperia, CA) and characterized by Edman degradation (Applied Biosys-
Peptides were dansylated before treatment with TFA-phenol. Approximately one third of each of the resin-bound peptides was removed and later treated with 0.5 mmol/L of dansyl chloride in 0.1 mL of diisopropylethylamine and 3.0 mL of dimethylformamide. The resulting peptides were cleaved and deprotected with TFA-phenol and purified by HPLC.

Approximately 100 pg of the synthetic peptide, corresponding to residues 1670 to 1684 of human FVIII, was digested with 4 pg of tosylphenylalanine-chloromethylketone-treated trypsin in 5% ammonium bicarbonate for 4 hours at 37°C. The acidified reaction mixture was fractionated by HPLC, and the larger of the resulting peptides was isolated and shown to have the NH2-terminal sequence KEFD. The mass of peptides used in the inhibition of binding experiments was estimated from the area of the absorbance peak at 220 nm from HPLC chromatograms. The FP studies were performed with peptides whose mass was determined gravimetrically on lyophilized samples.

RESULTS

Production of MoAbs. Fusion proteins, purified from SDS-polyacrylamide gels, were used to inject mice for the production of MoAbs. Two monoclonals were selected by reactivity with recombinant human FVIII and the fusion protein B-8. The antibody, 1-b, was isolated from a mouse injected with the B-8 fusion protein. The antibody, 60-B, was isolated from a mouse presumably injected with fusion protein B-10. Using Western blot analysis, the anti-FVIII MoAb 60-B was found to react with recombinant FVIII and fusion proteins B-8 and B-16, but not B-10 or B-15 (Fig 2). Faint bands appearing on the Western blots in positions corresponding to albumin, B-10, or B-15 are considered artifacts as they are present in the absence of primary antibody (data not shown). This and subsequent work described below suggest that B-8 or B-16 may have been the immunogen for the antibody 60-B.

Western blots. Each MoAb reacted with both recombinant human FVIII light chain and purified fusion proteins B-8 and B-16 on Western blots (Fig 2A and data not shown). On some Western blots, the 60-B antibody also reacts with material that is of higher molecular weight than intact FVIII light chain. Similar bands have been reported to be amino extensions of light chain present in recombi-
nant factor VIII.29 When thrombin-treated FVIII was probed, neither of the antibodies bound to the large FVIII light-chain fragment, residues 1690 through 2332. This result was consistent with antibody binding to the fragment (residues 1649 through 1689) released from FVIII light chain by thrombin cleavage. The released 41-amino acid residue fragment was not seen on the gels. It was also possible that the antibody-binding site spanned the thrombin-cleavage site. This question was resolved when a Western blot of thrombin-cleaved fusion protein B-16 was probed with the antibodies. This fusion protein is cleaved by thrombin into fragments of 15 Kd (FVIII residues, 1563 through 1689) and 25 Kd (FVIII residues, 1690 through 1909). The MoAbs reacted with the 15-Kd fragment on the Western blots (Fig 2B, lanes 5 and 6, and data not shown). Thus, the antibody-binding site for 60-B and 1-b was localized to residues 1649 through 1689. These antibodies were found not to react with porcine FVIII spotted onto nitrocellulose (data not shown).

**FVIII binding assay.** These two anti-FVIII MoAbs were then assayed for binding to native FVIII. Each antibody was added to a rabbit antimouse-Ig-coated tube, and the subsequent capture of iodinated FVIII was measured (see Materials and Methods). Only 60-B reacted with native FVIII in this assay (data not shown).

**vWF binding assay.** The amino terminus of the FVIII light chain is involved in vWF binding.71,12,30 Having determined that our MoAbs bound FVIII in this region, we tested them for their ability to inhibit FVIII-vWF interactions. The assay used to determine if 60-B blocked vWF: FVIII interaction was based on the amount of labeled FVIII that bound to a vWF coated tube. When the antibodies were included in the incubation, only 60-B was found to inhibit FVIII-vWF interactions. As seen in Fig 3A, FVIII-vWF binding was inhibited in a dose-dependent manner. In this assay 50% inhibition of FVIII binding required about 1.0 nmol/L antibody. Fab fragments also inhibited FVIII-vWF binding in a dose-dependent manner and at concentrations of combining sites essentially identical to whole IgG (Fig 3A).

**Peptide binding, FVIII assay.** In a previous report, the anti-FVIII MoAb C4 was characterized as inhibiting the interaction of FVIII and vWF.7 Other monoclonal anti-FVIII antibodies that inhibit FVIII-vWF binding have also been described: CLA:CAg-58,30 CLB-CAg 65, and CLB: CAg-69.9 Using synthetic peptides, the binding site on FVIII of the C4 and CLB:CAg-69 antibodies was localized to residues 1670 through 1684 and 1673 through 1689, respectively. Because the antibody described here, 60-B, also inhibited FVIII-vWF interactions, we synthesized a "human" peptide, encoding FVIII residues 1670 through 1684 (Fig 4). We used this human peptide to test the possibility that the 60-B antibody bound to the same peptide as the C4 antibody. Because the antibody 60-B did not react with porcine FVIII, a second peptide, called the "hybrid" peptide, was made. The hybrid peptide contained a single amino acid substitution, glycine for aspartic acid, at residue 1681. This represents a change from the residue found in the human sequence to that found in the porcine sequence. This residue was chosen because it is well isolated from the other nonconserved residues.

The anti-FVIII MoAb 60-B was tested for reactivity to both the human and hybrid peptides. The binding of iodinated FVIII to antibody 60-B-coated tubes was inhibited by the addition of the human peptide. This inhibition...
FACTOR VIII LIGHT CHAIN

![Diagram](image_url)

**Fig 4.** Comparison of human and porcine FVIII light-chain activation peptide amino acid sequences. The amino acid sequence of human FVIII activation peptide residues 1649 through 1689 is shown as single-letter abbreviations. Directly below the human residues are the corresponding nonconserved residues from porcine FVIII. The "human" peptide, 1670 through 1684, is underlined. The "hybrid" peptide is identical to the "human" peptide except for a glycine for aspartic acid substitution at residue 1681. The "tryptic" peptide is the trypsin digestion product of the "human" peptide.

was dose dependent with 50% inhibition at 0.09 μmol/L peptide. The hybrid peptide showed no inhibitory activity at 4.5 μmol/L (data not shown).

**FVIII-vWF binding.** Neither peptide, when present up to 4.5 μmol/L, could alter FVIII binding to vWF-coated tubes (data not shown). However, the human peptide reversed 60-B inhibition of FVIII binding to vWF in a dose-dependent manner (Fig 3B). Total reversal of the inhibitory activity of 3 nmol/L of antibody required about 2 μmol/L of human peptide. Human peptide at 0.09 μmol/L was required to reverse 50% of the inhibitory activity. The hybrid peptide, at concentrations up to 2.5 μmol/L, did not influence the inhibitory effect of the antibody (Fig 3B). These results indicated that the aspartic acid at residue 1681 makes an important contribution to the 60-B antibody-binding site on FVIII.

**Tryptic digests.** The human synthetic peptide was subjected to tryptic digestion to further characterize the anti-FVIII MoAb-binding site. The digestion products were isolated by HPLC and identified by sequence analysis. The peptide was cleaved between the lysines at residues 1673 and 1674 (Fig 4). The binding of iodinated FVIII to antibody 60-B-coated tubes was inhibited in a dose-dependent manner by the trypsin digest fragment ("tryptic") of the human peptide containing only residues 1674 through 1684 (data not shown).

**Fluorescence polarization.** The interactions of the MoAbs with FVIII and the synthetic peptides were studied in the fluid phase by observing changes in the FP of dansylated peptides as a result of binding to an antibody. Fluorescence polarization values of 2 μmol/L dansylated human or hybrid peptides remained at their baseline levels of 0.05 after addition of 2 μmol/L of control antibody W3-3 directed at porcine FVIII. When 60-B or 1-b was added in increasing amounts (0.1 to 2 μmol/L) to dansylated human peptide (2 μmol/L), the FP values increased from baseline and plateaued at 0.10 and 0.12, respectively (data not shown). The dansylated human peptide (2 μmol/L) could be displaced from 1 μmol/L of 60-B by addition of either the human peptide or the tryptic peptide, but not by addition of the hybrid peptide (Fig 5A). The 1-b antibody (1 μmol/L) reacted with the dansylated human peptide, and the human peptide, hybrid peptide, or tryptic peptides were effective competitors (Fig 5B). The FP value of 2 μmol/L dansylated hybrid peptide was unchanged on addition of either antibody W3-3 or 60-B, but was reactive in a dose-dependent manner with 1-b (data not shown).

**Anticoagulant activity of 60-B.** We found that the 60-B antibody had an FVIII inhibitory activity of 9 Bethesda Units (BU)/mg IgG in the clotting assay. The inhibitory effect of 60-B on FVIII coagulant activity was further characterized by use of a defined multicomponent chro-
mogenic substrate assay (see Materials and Methods). This assay indirectly measures the amount of activated FVIII by monitoring the generation of factor Xa in the presence of factor IXa, factor X, PCPS vesicles, and calcium. The factor Xa produced releases p-nitroaniline (pNA) from the factor Xa substrate S-2222. The amount of pNA generated is proportional to the amount of thrombin-activated FVIII present. The MoAb 60-B was preincubated with 0.28 nmol/L FVIII for 30 minutes before thrombin activation. The inhibition of factor Xa generation by 60-B and its Fab is shown in Fig 6A. Complete inhibition of factor Xa activity occurred when antibody concentrations were over 100 nmol/L. Fifty-percent inhibition of factor Xa generation was seen near 1.0 nmol/L 60-B. The amount of Fab required for this degree of factor Xa inhibitory activity was approximately 10-fold higher. Addition of 1 μmol/L of antibody 60-B after the thrombin incubation had no effect on factor Xa generation (data not shown).

Relief of inhibition by vWF. The antibody 60-B and vWF both appear to bind to FVIII in a mutually exclusive manner and could be expected to compete for binding. The amount of 60-B that inhibited factor Xa generation by 60% (3 nmol/L) was preincubated with FVIII in the presence of varying amounts of vWF (0.1 to 2.2 U/mL). As can be seen in Fig 6B, 0.5 U/mL of vWF reversed the inhibition by 50%, and 2 U/mL completely reversed the antibody-induced inhibition of FVIII coagulant activity.

Gel analysis of thrombin cleavage. PAGE was used to analyze the thrombin cleavage patterns of recombinant FVIII in the presence and absence of the antibody 60-B, or its Fab derivative (Fig 7). Analysis of the gels indicated that both 60-B and its Fab derivative protect FVIII light chain from thrombin cleavage at residue 1689. This is evidenced by the absence of the 70-Kd activated FVIII light-chain band.

DISCUSSION

Selected regions of human FVIII light chain were expressed as fusion proteins in E. coli. These FVIII fusion proteins were used to produce anti-FVIII light-chain MoAbs. One antibody, 60-B, was shown to inhibit FVIII binding to vWF, to inhibit FVIII procoagulant activity, and to prevent thrombin cleavage of FVIII at residue 1689. The 60-B and 1-b antibody-binding sites on FVIII were localized by taking advantage of the unique amino terminal extension on the FVIII light-chain fusion protein B-16. Synthetic peptides were used in both fluorescence polarization studies and solid-phase binding assays to identify FVIII light-chain residues 1674 through 1684 as the binding site for both 60-B and 1-b. Another peptide indicated that 60-B but not 1-b binding was sensitive to a substitution for aspartic acid at FVIII residue 1681.

Immunologic evidence suggests that the amino terminus of FVIII light chain may be involved in vWF binding to FVIII. In addition to the antibody described in this work, other laboratories have produced monoclonal anti-FVIII light-chain antibodies to this region of FVIII that inhibit FVIII-vWF interaction. These studies suggest that the activation peptide of FVIII contains a highly antigenic region that contributes to the structures required for vWF binding. Physical studies using analytical sucrose gradient centrifugation demonstrated that intact FVIII light chain is required for vWF binding. This same type of analysis indicated that porcine FVIII association with vWF was destroyed by thrombin treatment and that FVIII heavy chain did not influence the stoichiometry of binding.
with the antibody data which indicate that antibody binding because such mutants fail to bind vWF. This is in agreement binding' (and this report). The failure of peptides spanning to FVIII residues 1674 through 1684 inhibits FVIII-vWF through 1689 directly influence the vWF binding site. The FVIII from a severe hemophilia A patient has been characterized as having a cysteine substituted for the arginine at residue 1689, and this amino acid substitution also results in the loss of thrombin cleavage of FVIII light chain and activity. Although this mutant FVIII bound vWF, neither its procoagulant activity nor its resistance to thrombin cleavage was altered by the presence of vWF.

It has also been suggested that the cleavage of FVIII light-chain residue 1689 is not essential for procoagulant activity, but may serve only to dissociate FVIII from the vWF. Cleavage of porcine FVIII by a snake venom at heavy-chain residues 372 and 740, but not at light-chain residue 1689, were sufficient to produce active FVIII in the absence of vWF. No active FVIII was produced if the snake venom cleavages were made in the presence of vWF. If thrombin cleavage at 1689 is not required for activity, then perhaps the cleavage product of the FVIII:60-B complex is analogous to the snake venom-activated FVIII inhibited by vWF; as the antibody would remain bound at or near the vWF binding site.

Any ligand bound near the a2:A3 junction may prevent the assembly of a productive factor X activating complex. The observation that 60-B binds to both synthetic peptides and to the smaller thrombin cleavage product of B-16 but does not inhibit FVIIIs after thrombin activation supports the observation that the a2 fragment does not participate in cofactor activity. The reported lack of procoagulant activity in the patient and site-directed mutant FVIIIs would then be attributable to the effect of a nonconservative amino acid substitution (I or C for R at residue 1689) on the structure of FVIII and not due solely to the lack of thrombin cleavage.

We have shown that the 60-B inhibition of FVIII procoagulant activity can be blocked by the addition of physiologic amounts of vWF, and that 60-B blocks vWF binding of FVIII. However, the binding sites on FVIII for vWF and antibody 60-B appear not to be identical, because the FVIII associated with vWF is readily cleaved by thrombin, whereas MoAb 60-B protects FVIII from thrombin cleavage.

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A monoclonal antibody to factor VIII inhibits von Willebrand factor binding and thrombin cleavage

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