An Immunogenic Region within Residues Val$^{1670}$-Glu$^{1684}$ of the Factor VIII Light Chain Induces Antibodies Which Inhibit Binding of Factor VIII to von Willebrand Factor*

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‡The abbreviations used are: FVIII, factor VIII; vWF, von Willebrand factor; ELISA, enzyme-linked immunosorbent assay.

We have identified a monoclonal anti-factor VIII (FVIII) antibody, C4, which inhibits the binding of purified human FVIII to purified human von Willebrand factor (vWF). Both whole immunoglobulin C4 and its Fab fragment demonstrated dose-dependent inhibition of FVIII binding to vWF immobilized on the surface of polystyrene beads. Synthetic peptides based on the amino acid sequence of FVIII were tested for the ability to block the binding of C4 to FVIII in an enzyme-linked immunosorbent assay system. A single synthetic FVIII pentadecapeptide, consisting of residues Val$^{1670}$-Glu$^{1684}$, was able to inhibit C4 binding to FVIII. Under the conditions used, the Val$^{1670}$-Glu$^{1684}$ peptide demonstrated total inhibition of C4 binding at a concentration of 1 μM. Synthetic FVIII peptides flanking and overlapping the Val$^{1670}$-Glu$^{1684}$ peptide had no significant inhibitory activity on C4 binding in concentrations up to 100 μM. A polyclonal antibody made to the Val$^{1670}$-Glu$^{1684}$ peptide also demonstrated inhibition of FVIII binding to vWF. Polyclonal antibodies made to synthetic FVIII peptides flanking and partially overlapping the Val$^{1670}$-Glu$^{1684}$ sequence did not demonstrate such inhibition. Localization of the binding region of the monoclonal anti-FVIII antibody C4 to residues Val$^{1670}$-Glu$^{1684}$ suggests that this site is at, or near, a major vWF binding domain of FVIII.

Factor VIII (FVIII)$^*$ and von Willebrand factor (vWF) circulate in plasma as a noncovalently linked protein complex. Each protein plays an important yet distinctly different role in the maintenance of hemostasis. FVIII functions as a non-enzymatic cofactor to accelerate the activation of factor X by factor IXa in the presence of calcium ions and phospholipids (1, 2). vWF is important in the mediation of platelet-vessel wall interactions at the site of vascular injury (3, 4). A major vWF binding domain for FVIII has been located within the first 272 amino acid residues of the mature vWF subunit (5).

The physicochemical nature of FVIII-vWF complex formation has been studied by several investigators. Owen and Wagner (6) demonstrated variable degrees of dissociation of FVIII from vWF in the presence of alkaline halides, detergents, and CaCl$_2$. Their data were consistent with contributions to binding by both electrostatic and hydrophobic interactions. Other investigators have demonstrated dissociation of the complex by 1–1.5 M NaCl (7, 8). 0.25 M CaCl$_2$ (9, 10), and by 10–20 mM EDTA (9, 11, 12).

Normal concentrations of circulating FVIII appear to be dependent on complex formation with vWF. This is most clearly demonstrated in severe von Willebrand disease in which complete deletion of the vWF gene is accompanied by markedly reduced concentrations of plasma FVIII (13). The importance of vWF in stabilization of FVIII has been suggested by the prolonged rise of FVIII seen in patients with von Willebrand disease after cryoprecipitate infusion. The FVIII levels reached could not be accounted for by the amount of FVIII infused (14). Stabilization of FVIII activity in plasma by vWF has been demonstrated in vitro (15). Infusion studies of partially purified FVIII in humans (16) and highly purified FVIII in dogs (17) have demonstrated a marked reduction in the circulating half-life of infused FVIII in the absence of endogenous vWF. vWF has also been reported to stabilize recombinant cDNA-produced FVIII in cell culture media (18, 19).

The mechanism(s) by which vWF stabilizes FVIII have not been established with certainty. In purified systems, vWF has been reported to protect FVIII from proteolysis by activated protein C (20) and by low concentrations of factor Xa (21). vWF has also been reported to protect recombinant cDNA-produced FVIII from proteolysis in cell culture media (18).

Previous studies have provided evidence that vWF (9, 20, 23) and phospholipid (24) binding domains reside on the FVIII light chain. In the present study, we have identified a monoclonal anti-FVIII antibody, C4, which blocks FVIII binding to vWF and have localized the binding region of this antibody to the amino-terminal portion of the light chain of FVIII. This study provides additional evidence that the vWF binding domain of FVIII resides on the light chain of FVIII and suggests that an area important for this binding is located at or near amino acid residues Val$^{1670}$-Glu$^{1684}$.

EXPERIMENTAL PROCEDURES

Purification of FVIII—The purification of FVIII from commercial factor VIII concentrates (Armour Pharmaceutical) by immunoabsorbent chromatography has previously been described (25–27). FVIII preparations prepared by this method had specific activities of 2900–3800 units/mg.

Purification of von Willebrand Factor—Purified vWF was obtained from commercial factor VIII concentrates (Armour Pharmaceutical) by immunoabsorbent chromatography as previously described (6, 25).

Monoclonal Antibodies—Murine monoclonal anti-human FVIII antibodies were produced, purified, and characterized as described (26).

Polyclonal Antibodies—Synthetic FVIII peptides were coupled to keyhole limpet hemocyanin with glutaraldehyde. One mg of coupled peptide in complete Freund's adjuvant was injected subcutaneously
Anti-FVIII Antibodies Which Inhibit FVIII Binding to vWF

into New Zealand White rabbits in multiple injection sites. Three booster immunizations of 1 mg of coupled protein in incomplete Freund's adjuvant were given at weekly intervals in the same manner. The animal was bled 2 weeks after the last injection and every 2 weeks thereafter. Serum collected from the rabbits was tested for reactivity with FVIII in both a standard ELISA assay and by Western blotting. Prior to use in the polystyrene bead competitive inhibition assays, monoclonal antibodies were partially purified by protein A affinity chromatography.

FVIII ELISA—Purified human FVIII was diluted to approximately 3 units/ml in 20 mM Tris-Cl, 0.02% NaN₃, pH 9.6, and 100 µl added to each well (1.0 × 0.6 cm) of a multi well tissue culture plate (Linbro, Flow Laboratories) and incubated at room temperature for 2 h. The plates were then stored in this solution at 4°C until use. Prior to use, the solution was removed and the wells were blocked with 200 µl of 20 mM Tris-Cl, 0.02% NaN₃, 1% bovine serum albumin for 1 h at room temperature. After blocking, the solution was removed, and the wells were washed 3X with 250 µl of a buffer containing 0.14 M NaCl, 0.0015 M KH₂PO₄, 0.0084 M Na₂HPO₄, 0.05% Nonidet P-40 (washing buffer). Alternatively, purified FVIII was diluted in 0.01 M NaHCO₃ and 100 µl was added to each well. The plates were then desiccated at room temperature, and the plates were stored at 4°C until use. Prior to incubation with polystyrene beads coated with purified vWF, the plates were blocked and washed as described above. One hundred µl of sample solution containing monoclonal anti-FVIII antibody C4 and synthetic FVIII peptide was then added to each well. C4 at a final concentration of 0.1 to 1 µg/ml, and FVIII peptide at a final concentration of 1 nm to 100 µM, were preincubated in washing buffer with 1% bovine serum albumin for 2 h at 37°C or overnight (16-24 h) prior to addition to the wells. The wells were washed 4X with 250 µl of washing buffer. One hundred µl of peroxidase-goat anti-mouse IgG (H+L) (Zymed Laboratories) diluted 1:2000 with washing buffer with 1% bovine serum albumin was added to each well and incubated for 1 h at room temperature. The solution was then removed, and the wells were washed 4X with 250 µl of washing buffer. One hundred µl of o-phenylenediamine substrate (Zymed Laboratories) dissolved in 0.024 M citric acid, 0.051 M Na₂HPO₄, 0.05% hydrogen peroxide, was added to each well and incubated at room temperature for 5 to 10 min before being quenched with 100 µl of 2 M H₂SO₄. The color change in each well was read on an MR 600 Microplate Reader (Dynatech). Results of the assay are reported as percent of inhibition of C4 binding. Percent of inhibition of C4 binding is defined as 100% - 100X (the absorbance reading of C4 binding in the presence of synthetic FVIII peptide divided by the absorbance of C4 binding in the absence of synthetic FVIII peptide). Each sample was assayed in duplicate or triplicate, and the results were averaged.

FVIII Binding Activity Titer—The FVIII binding activity titer of the monoclonal anti-FVIII antibodies C4 and J31B3 was determined in the FVIII ELISA assay as described above. The absorbance levels produced by each monoclonal antibody at concentrations ranging from 0.005 µg/ml to 1 µg/ml were measured. The relative titer of J31B3 to C4 was calculated by dividing the minimal concentration of J31B3 required to produce a specific absorbance level by the minimal concentration of C4 to produce the same specific absorbance level. The absorbance level was chosen from an area of the absorbance binding activity curve which was linear. The FVIII binding activity titer of the polyclonal antibodies was performed as described for the monoclonal antibodies except peroxidase-goat anti-rabbit IgG (H+L) (Zymed Laboratories) was substituted for peroxidase-goat anti-mouse IgG (H+L). The titers were calculated relative to polyclonal antibody 2845 as described for the monoclonal antibodies.

FVIII-vWF Binding Assay—The polystyrene bead competitive inhibition assay has been previously described (5). In brief, polystyrene beads were incubated in a solution containing 2-10 µg of whole unreduced vWF and then blocked with 3% human serum albumin. After washing, the beads were incubated for 1 h at room temperature with 1-2 units of purified FVIII, and 0-200 µg of monoclonal anti-FVIII antibody. The beads were then washed and incubated for 1 h at room temperature with 1.2-2.4 × 10⁶ cpm of ¹²⁵I-monoclonal anti-FVIII light chain antibody (specific activity 8.3-9.9 × 10⁶ cpm/mg) or 2.4-4.9 × 10⁶ cpm of ¹²⁵I-monoclonal anti-FVIII heavy chain antibody (specific activity 0.8-3.2 × 10⁶ cpm/mg). After washing, the beads were separately counted for bound radioactivity. Results are reported as percent of inhibition of FVIII binding, with 0% inhibition defined as the counts/min bound in the absence of competing ligand. Plotted data represent the mean of three to six determinations.

Protein Concentrations—Protein concentrations were determined by the method of Bradford (28) or the method of Lowry et al. as modified by Peterson (29).

Radiolabeling of Monoclonal Antibodies—Monoclonal anti-FVIII antibodies were radiolabeled with ¹²⁵I by the method of Fraker and Stuck (90) of a specific activity of 3.2 × 10⁹ cpm/mg.

Peptide Synthesis—Synthetic FVIII peptides were produced as previously described by Houghten (31).

Fab Preparation—Fab fragments were prepared from C4 by digestion with immobilized papain (Pierce) according to the instructions of the manufacturer and separated from undigested C4 by protein A affinity chromatography.

RESULTS

Monoclonal anti-FVIII antibodies were screened for the ability to block the binding of purified human FVIII to purified human vWF immobilized on the surface of polystyrene beads. Binding of FVIII to vWF has been demonstrated to be specific and reversible in this system (5). The binding of FVIII was detected by the binding of ¹²⁵I-labeled monoclonal anti-FVIII antibody to the FVIII bound to the immobilized vWF. Monoclonal anti-FVIII antibody, C4, demonstrated dose-dependent competitive inhibition of FVIII binding to vWF. Under the conditions used in this experiment, 90% inhibition of FVIII binding occurred at a concentration of 50 µg/ml (Fig. 1). Three other monoclonal anti-FVIII antibodies, one specific for light chain, one specific for B domain, and one specific for heavy chain, as determined by immunoblotting, failed to produce any significant inhibition of FVIII binding to vWF when tested at concentrations up to 200 µg/ml (data not shown). The effect of C4 on FVIII binding was the same whether the radiolabeled detecting antibody used was anti-heavy chain or anti-light chain. This indicated that the reduction in bound radioactivity produced by C4 was due to reduced FVIII binding and not due to interference of the binding of the radiolabeled indicator anti-FVIII antibody by C4.

In order to minimize the possible steric blocking of the vWF binding domain by C4, C4 Fab fragment was produced by papain digestion. This fragment was tested under identical conditions as C4. C4 Fab fragment also demonstrated dose-dependent inhibition of FVIII binding but at higher concent-

FIG. 1. Inhibition of binding of FVIII to vWF by monoclonal anti-FVIII antibody C4 and C4 Fab fragment. One unit of highly purified FVIII was incubated with 0-200 µg of C4 or C4 Fab fragment prior to incubation with polystyrene beads coated with purified vWF as described under "Experimental Procedures." Data are plotted as the percent of inhibition of FVIII binding produced by C4 or C4 Fab fragment. 0% inhibition represents the amount of FVIII binding in the absence of C4 or C4 Fab. Error bars represent the standard deviation of the mean of three to six determinations. Standard deviations of 1% or less are not indicated by error bars.
trations than that required for whole C4 (Fig. 1).

Localization of the C4 binding region was achieved in an ELISA system, in which synthetic FVIII peptides were evaluated for the ability to block the binding of C4 to purified human FVIII adsorbed to the surface of microtiter wells. The binding region of C4 had previously been localized to the 80-kDa light chain of FVIII (26). Failure of C4 to bind to the 72-kDa thrombin-generated fragment of the light chain suggested the C4 binding region was in the amino-terminal portion of the 80-kDa light chain which precedes the thrombin cleavage site at Arg<sup>1606</sup>. Overlapping peptides were synthesized based on the amino acid residue sequence of FVIII in this area. Fig. 2 depicts the amino acid sequence of the amino-terminal portion of the light chain of FVIII and the sequences of the peptides tested for C4 binding inhibition in the ELISA system.

Only one synthetic FVIII peptide, Val<sup>1670</sup>-Glu<sup>1684</sup>, inhibited C4 binding to FVIII. Approximately 50% inhibition was seen at a concentration of 10 nM and total inhibition at a concentration of 1 μM (Fig. 3). The partially overlapping peptides Asp<sup>1666</sup>-Ile<sup>1679</sup>, Thr<sup>1680</sup>-Lys<sup>1694</sup>, and Glu<sup>1696</sup>-Lys<sup>1700</sup> produced no inhibition of C4 binding at concentrations up to 100 μM (data for 0–10 μM shown in Fig. 3). The flanking peptides Ile<sup>1659</sup>-Tyr<sup>1668</sup> and Arg<sup>1645</sup>-Glu<sup>1659</sup> had no effect on C4 binding at concentrations up to 100 μM (data not shown). Three synthetic FVIII peptides from the heavy chain of FVIII, Glu<sup>2642</sup>, Asp<sup>2656</sup>, Asn<sup>2660</sup>-Glu<sup>2654</sup>, and Thr<sup>2651</sup>-Ser<sup>2655</sup> were also tested. These peptides contain amino acid sequence homology with the area of interest in the light chain. None of these peptides demonstrated any significant inhibition of C4 binding to FVIII in concentrations up to 100 μM. The data for the Thr<sup>2651</sup>-Ser<sup>2655</sup> peptide are shown in Fig. 3.

The synthetic FVIII peptides used in the ELISA assay were also used to immunize rabbits to produce polyclonal antisynthetic FVIII peptide antibodies. These antibodies were partially purified by means of protein A affinity chromatography and then tested in the polystyrene bead assay system for the ability to block FVIII binding to vWF. A polyclonal antibody, 2645, made to the Val<sup>1670</sup>-Glu<sup>1684</sup> peptide demonstrated dose-dependent inhibition of FVIII binding (Fig. 4). Polyclonal antibodies to the partially overlapping and flanking peptides and the heavy chain control peptides did not produce an effect on FVIII binding when tested at a concentration of 400 μg/ml (Fig. 5).

The relative titer of FVIII binding activity of each of the polyclonal antibody preparations was determined by measuring the minimal concentration required to produce a specific absorbance level in the FVIII ELISA assay. The minimal concentrations were normalized relative to the minimal concentration of polyclonal antibody 2645 required to produce the desired absorbance level. The values ranged from 0.54 for 2293 (indicating an approximately 2-fold greater titer of FVIII binding activity relative to 2645) to 1.19 for 3237 (indicating 84% of the titer of FVIII binding activity relative to 2645). A concentration of 200 μg/ml of 2645 produced approximately 85% inhibition of FVIII binding to von Willebrand factor (Fig. 4). The various polyclonal antibodies were tested at concentrations of 400 μg/ml. Since the lowest titer polyclonal antibody had a titer of 84% that of 2645, its effective concentration was 1.7 times greater than the concentration of 2645 necessary to produce 85% inhibition of FVIII binding. Yet this antibody, as well as those with higher titer, had no effect on binding. Thus, the results illustrated in Fig. 5 are due to the inability of these polyclonal antibodies to interfere with FVIII binding and are not due to differences in titer of FVIII binding activity.

![Fig. 2. Amino acid sequence of the amino-terminal area of FVIII light chain](image)

![Fig. 3. Inhibition of monoclonal anti-FVIII antibody C4 binding to FVIII by synthetic FVIII peptide Val<sup>1670</sup>-Glu<sup>1684</sup>](image)

![Fig. 4. Inhibition of FVIII binding to vWF by polyclonal anti-Val<sup>1670</sup>-Glu<sup>1684</sup> peptide antibody 2645. Polyclonal anti-Val<sup>1670</sup>-Glu<sup>1684</sup> peptide antibody (0–400 μg) was incubated with FVIII (1.5 units) as described under "Experimental Procedures." Data are plotted as in Fig. 1.](image)
The antibody J31B3 raised to the Glu<sup>1660</sup>-Lys<sup>1674</sup> peptide was a monoclonal antibody. Although its FVIII binding activity titer cannot be directly compared to the polyclonal antibody 2645, it does have approximately 3.5 times the titer of FVIII binding activity as that of the monoclonal antibody C4. C4, at a concentration of 50 µg/ml, blocked FVIII binding by 95% (Fig. 1). The inability of monoclonal antibody J31B3, at a concentration of 200 µg/ml, to block FVIII binding to vWF (Fig. 5) is therefore not related to differences in titer of FVIII binding activity. The results presented in Fig. 5 provide further evidence that the segment of FVIII encompassed by the sequence Val<sup>1670</sup>-Glu<sup>1684</sup> is immunogenic for antibodies which inhibit FVIII-vWF interactions.

We were unable to raise a polyclonal antibody to the Asp<sup>1660</sup>lle<sup>1679</sup> peptide. Since this overlaps the Val<sup>1670</sup>-Glu<sup>1684</sup> peptide, it is possible that an antibody to this peptide may also have some FVIII binding inhibitory activity.

**DISCUSSION**

This study identifies an immunogenic region between residues Val<sup>1670</sup>-Glu<sup>1684</sup> of the FVIII light chain. Intact antibodies and a Fab fragment to this region inhibited binding of FVIII to vWF in a purified system. This suggests that the sequence Val<sup>1670</sup>-Glu<sup>1684</sup> may be located at or very near a major vWF binding domain. It is also possible that antibodies binding to this region may induce conformational changes in FVIII which disrupt a distant vWF binding domain.

The localization of the C4 binding region to the same area of FVIII has also been demonstrated by an entirely different method. Recombinant cDNA techniques have been used to generate a series of clones expressing FVIII fragments. These clones were immunologically screened with C4 and the overlapping DNA sequences were determined. The C4 binding region was restricted to a region of FVIII between residues 1675 and 1688 by this method.<sup>2</sup>

The light chain of FVIII has been previously reported as containing the vWF binding domain. Fass et al. (32) described a monoclonal anti-porcine FVIII antibody which had light chain specificity and which had no anti-FVIII inhibitory activity in plasma. This antibody reacted strongly with purified FVIII, but reacted only weakly with FVIII in plasma. It was suggested that the binding region of this antibody was not accessible when FVIII was complexed with vWF, indicating that FVIII light chain may be involved with vWF binding.

More direct evidence of light chain involvement with vWF binding has been described by other investigators. Hamer et al. (9) demonstrated the binding of <sup>125</sup>I-labeled FVIII and <sup>125</sup>I-labeled isolated FVIII light chain to vWF immobilized to the surface of microtiter wells. In addition, this binding could be completely inhibited by a monoclonal anti-FVIII light chain antibody. That monoclonal antibody, unlike that reported in this study, demonstrated anti-FVIII inhibitory activity in plasma. Sewerin et al. (22) and Ezban et al. (23) both demonstrated the binding of isolated FVIII light chain to vWF coupled to Sepharose. None of these three investigators, nor Eaton and Vehar (33), was able to demonstrate isolated heavy chain binding to vWF.

Supporting evidence for the assignment of a major vWF binding domain to the amino-terminal portion of the light chain, as reported in this study, is indirect. Thrombin activation is believed to cause release of active FVIII from vWF. This has been inferred from the observation that vWF and FVIII coagulant antigen are associated in plasma, but dissociated in serum (34), as well as by the inability of thrombin-activated FVIII, or thrombin-activated FVIII light chain, to reassociate with vWF (35, 36). The release of FVIII peptides from vWF by thrombin cleavage has been reported. Hamer et al. (35) reported that thrombin activation of FVIII bound to immobilized vWF produced cleavage of the 80-kDa light chain with release of the 72-kDa subunit peptide. They also reported release of the 54-kDa heavy chain subunit peptide, but no release of the 44-kDa heavy chain subunit peptide. Sewerin et al. (37) reported that thrombin cleavage of FVIII bound to vWF-Sepharose produced release of the 72-kDa light chain subunit and the release of the intact 92-kDa heavy chain as well as 54-kDa and 44-kDa heavy chain subunit peptides.

Although there is disagreement as to whether the entire heavy chain is released, both of these observations support the hypothesis that FVIII light chain binds to vWF via an area within the acidic region of the light chain (residues 1649-1689), and that activation of FVIII by thrombin cleavage at Arg<sup>1660</sup> causes release of the 72-kDa FVIII light chain fragment from vWF. Our description of the location (residues Val<sup>1670</sup>-Glu<sup>1684</sup>) of the binding region of monoclonal and polyclonal anti-FVIII antibodies which block FVIII binding to vWF also supports this hypothesis.

Recombinant cDNA data also indirectly support this area of FVIII as being critical for vWF binding. The stabilization of recombinant cDNA-produced FVIII by vWF in the cell culture media or by the co-expression of vWF by the transfected cells has been reported (18, 19). A recombinant cDNA FVIII molecule with a 947-residue deletion (residues 741-1689 inclusive) extending from the end of the heavy chain past the acidic region of the light chain has been expressed. This molecule has been demonstrated to be thrombin-activatable, but could not be stabilized in the cell culture media by added vWF (38). Based on these data and the previously described
fact that the entire B domain of FVIII is not necessary for vWF binding (39), the authors inferred that the acidic region of the light chain is required for vWF interaction.

The data presented in this report and those of Hamer et al. (9) have demonstrated that FVIII binding to vWF can be totally inhibited by monoclonal anti-FVIII light chain antibody. The data of Sewerin et al. (37) demonstrating release of the 92/72-kDa FVIII heterodimer from vWF by thrombin is consistent with a single vWF binding domain on the acidic region of FVIII light chain. Although isolated heavy chain has not been demonstrated to directly bind to vWF (9, 22, 23, 33), there may be some association of heavy chain with vWF other than via its calcium linkage with light chain. The data of Hamer et al. (35), which demonstrate thrombin release of the 72-kDa light chain subunit, but only the 54-kDa heavy chain subunit, would suggest that a secondary association between vWF and FVIII may occur with the 44-kDa subunit of heavy chain. This was also suggested by their data demonstrating that treatment of FVIII with anti-FVIII heavy chain antibody before binding to vWF resulted in increased dissociation of FVIII from vWF by EDTA, CaCl2, and NaCl. The authors felt that since this apparent secondary site was not disrupted by reagents of high ionic strength, that a hydrophobic interaction may occur. The same investigators however have reported release of vWF from both the 54-kDa and 44-kDa heavy chain subunit peptides by factor Xa (21). Both factor Xa and thrombin cleave FVIII at Arg272 to produce these subunit peptides (40). Since the subunit peptides produced by factor Xa and thrombin cleavage appear identical, it is difficult to explain this apparent discrepancy in vWF binding to the 44-kDa heavy chain subunit peptide (44, 45) and with data that suggest that active FVIII does not remain bound to vWF (34-36).

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