

A Monoclonal Antibody to von Willebrand Factor (vWF) Inhibits Factor VIII Binding

Localization of its Antigenic Determinant to a Nonadecapeptide at the Amino Terminus of the Mature vWF Polypeptide

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Abstract

vWF is a multimeric glycoprotein that serves as the major carrier in plasma of Factor VIII (FVIII). We have used an anti-human vWF MAb W5-6A to investigate the FVIII binding site on vWF. W5-6A inhibited FVIII binding to vWF-coated polystyrene tubes in a concentration-dependent manner with 90% inhibition of FVIII binding at a concentration of 10 μ g/ml. The W5-6A epitope was identified by screening a vWF fragment library using the bacteriophage expression vector λ gt11. DNA sequence analysis of 29 immunoreactive phage clones localized the W5-6A epitope to a nonadecapeptide spanning amino acid residues threonine 78 to threonine 96 at the amino-terminus of the mature vWF polypeptide. Purified β -galactosidase/vWF fusion protein from one of these clones, vWF9, was incubated with radiolabeled W5-6A and caused near complete inhibition of W5-6A binding to vWF. Inhibitory activity was lost after vWF9 trypsinization or reduction and alkylation. These data indicate that (a) the antigenic determinant recognized by W5-6A localizes to a nonadecapeptide at the NH₂ terminus of the mature vWF polypeptide, (b) disulfide bonds within vWF9 may be necessary to maintain the structure required for immunoreactivity with W5-6A, and (c) W5-6A recognizes an immunogenic region on vWF that may be at (or near) the major FVIII binding domain.

Introduction

vWF and Factor VIII (FVIII)¹ are intimately related plasma glycoproteins with critical but distinct roles in the maintenance of hemostatic integrity. vWF mediates platelet adhesion to subendothelial collagen in areas of vessel wall injury (1), and stabilizes FVIII in plasma by forming a noncovalently linked protein complex that is maintained by hydrophobic and electrostatic interactions (2, 3). The ability of vWF to stabilize FVIII coagulant activity in plasma has been demonstrated in vitro (4), and in cell culture using the recombinant form of

FVIII (5). Clinical observations in humans confirm the importance of intact vWF in maintaining normal FVIII levels (6), and infusion studies in the dog using purified FVIII preparations demonstrate a marked loss of activity in the absence of endogenous vWF (7). Although the stoichiometry of the vWF-FVIII macromolecular complex has not been clearly defined in humans, studies using the porcine model suggest the potential availability of ~ 1 FVIII binding site per vWF monomer in steady-state plasma (8).

Previous studies have made use of large vWF proteolytic fragments (30–176 kD in size) to localize the vWF functional domains involved in binding to platelet glycoproteins Ib, IIB/IIIA, collagen, and heparin (reviewed in reference 9). Recently, a 272-amino acid proteolytic fragment from the amino-terminus of the mature vWF polypeptide has been demonstrated to contain the FVIII binding domain (10). This is consistent with, and refines two subsequent reports localizing this site to the NH₂-terminus of the mature vWF monomer (11, 12).

To further study the molecular interaction between vWF and FVIII, we report here the characterization of an anti-human vWF MAb (W5-6A) that inhibits FVIII binding to vWF. We have screened a library of small vWF peptide fragments expressed as fusion proteins in *Escherichia coli* using the expression vector λ gt11 to precisely localize the antigenic determinant on vWF recognized by W5-6A. These data localize the epitope to a nonadecapeptide spanning amino acids threonine 78 to threonine 96 on the mature vWF polypeptide.

Methods

Immunoscreening the vWF recombinant library

A vWF cDNA (13–16) fragment library in λ gt11 was constructed as described by Mehra and Young (17). Briefly, full-length vWF cDNA (13) was fragmented with DNAase I (Boehringer-Mannheim Biochemical, Indianapolis, IN), blunt-ended with T4 DNA polymerase (Boehringer-Mannheim Biochemical) and ligated to synthetic Eco RI linkers. vWF fragments in the size range of 200–500 bp were then ligated into the Eco RI site of the bacteriophage vector λ gt11. The library, containing $\sim 250,000$ independent clones, should be sufficient to represent any given portion of the vWF protein 100–1,000 times (Ginsburg, D., and A. Yang, manuscript in preparation). Immunoscreeing of recombinant phage was performed by the method of Young and Davis (18), except that preincubation and antibody treatment of filters was performed in BLOTTO (BLOTTO is 5% Carnation nonfat powdered milk, 10 mM Tris, pH 7.5, 140 mM NaCl) (19). W5-6A diluted 1:500 in BLOTTO was used to screen $\sim 200,000$ clones. After washing, filters were incubated for 2 h in a 1:500 dilution of an affinity-purified, peroxidase-tagged goat anti-mouse IgG (Cappel Laboratories, Malvern, PA), and developed by immersion in a substrate solution (10 mM Tris, pH 7.5), containing 20% methanol vol/vol, 0.06% 4-chloro-1-naphthol wt/vol, and 0.03% H₂O₂ vol/vol (20). Positive clones were plaque purified through additional rescreening steps using standard techniques (21).

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1. Abbreviations used in this paper: FVIII, factor VIII.

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DNA sequencing and fusion protein (vWF9) preparation

DNA from immunoreactive phage clones was purified from minilysates by standard methods (21), or the vWF cDNA inserts amplified directly from single phage plaques or crude lysates via the polymerase chain reaction (PCR) (22). Synthetic oligonucleotide primers beginning 69 bp upstream from the Eco RI cloning site (forward primer TGGCTGAATATCGACGGTTTCCAT) of λ gt11, and 79 bp downstream from the Eco RI site (reverse primer CAGACATGGCCTGCCCGTTATTA), were used for the PCR reaction. PCR conditions included a 1'15" denaturation step at 94°C, a 1' 55°C annealing step, and a 3' primer extension step at 72°C. Amplifications consisted of 30 cycles using a DNA thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT). Amplified cDNA insert sizes were determined by electrophoresis in an ethidium-stained 1.5% agarose gel, and PCR products were directly sequenced from both ends (across the Eco RI site) by a modification of the dideoxy chain termination method (23) using Sequenase (U.S. Biochemical Corporation, Cleveland, OH). The same oligonucleotides end-labeled with γ -³²P ATP (Amersham Corp., Arlington Heights, IL) (21) were used as sequencing primers. Sequences were localized within vWF by comparison to the full-length cDNA sequence (24) using the IBI-Pustell software package (International Biotechnologies, Inc., New Haven, CT) (25). β -Galactosidase-vWF fusion proteins from antibody-positive clones were prepared as previously described (19), and purified from crude *E. coli* lysates by passage over an anti- β -galactosidase affinity column (Cappel Laboratories). Analysis by SDS-polyacrylamide gels (SDS-PAGE) (26) and Western blotting were performed using standard methods.

Competitive binding assays

Preparation of vWF. Purified human vWF was prepared from cryoprecipitate by gel filtration chromatography (27). Fractions containing ristocetin cofactor activity were isolated, pooled, and extensively dialyzed against 20 mM histidine, 100 mM sodium chloride, 5 mM calcium chloride, and 0.02% sodium azide. vWF was separated from FVIII by dissolving the complex in 0.05 M MES (4-morpholinoethane sulfonic acid), 0.25 M CaCl₂, pH 7.5 and 5% (wt/vol) glycerol, followed by gel filtration in 4% agarose equilibrated with the same buffer. vWF containing fractions were then diluted to 1 U/ml (one unit is defined as ristocetin cofactor activity standardized against a pool of normal donors [28]) in TBS-azide (10 mM Tris, pH 7.5, 140 mM NaCl, 0.02% sodium azide) and stored in 1-cm³ aliquots at -20°C.

Factor VIII. Recombinant Factor VIII (0.38 mg/ml, 1,780 U/ml) was the gift of Genetics Institute, Cambridge, MA.

Preparation of W5-6A. A panel of anti-human vWF MAb was prepared using standard hybridoma technology (29). Commercial FVIII/vWF concentrate (Armour Pharmaceuticals, Kankakee, IL) was gel purified, and the vWF-containing fraction isolated by passage over a triethylaminoethyl cellulose column (Schleicher & Schuell, Keene, NH). This fraction was absorbed onto aluminum hydroxide, and used as the immunogen. Anti-vWF MAb were identified using 10% human plasma, and an affinity-purified ¹²⁵I-radiolabeled rabbit anti-human vWF antibody. They were subsequently purified from murine ascitic fluid by protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) chromatography (30).

W5-6A was radiolabeled with ¹²⁵I-sodium iodide by the method of Hunter and Greenwood (31), and isolated by gel filtration to a specific activity of 7 μ Ci ¹²⁵I/ μ g of IgG.

Radiolabeling FVIII. Recombinant FVIII was labeled with ¹²⁵I-sodium iodide by iodogen transfer (32), and purified by gel filtration. Its specific activity was 1.5 μ Ci of ¹²⁵I/mg of recombinant FVIII.

Fab preparation. W5-6A Fab was prepared from a commercial preparation kit, as outlined by the manufacturer (Pierce Chemical Co., Rockford, IL). Protein concentrations were determined from bicinchoninic acid (BSA) protein reagents according to the manufacturer's specifications (Pierce Chemical Co.). (a) **vWF-FVIII competition assays.** Polystyrene tubes were incubated in a buffer containing human vWF (0.05 U/ml in 50 mM sodium carbonate buffer, pH 9.5) at 4°C

overnight, washed twice with TBS-azide, and incubated at room temperature for 1 h in BLOTTO.

Competitive binding assays of FVIII to vWF-coated polystyrene tubes were performed as follows: vWF fusion protein or W5-6A (along with the appropriate controls, see below) were diluted to various concentrations in BLOTTO/0.01% azide. 200- μ l aliquots of the resulting mixture were incubated overnight (at 4°C) with ¹²⁵I-labeled recombinant FVIII in the vWF-coated polystyrene tubes. The tubes were then washed three times with TBS-azide, and assayed in duplicate for bound ¹²⁵I. Results are reported as the percent inhibition of FVIII binding after the subtraction of background counts. Zero percent inhibition is defined as bound ¹²⁵I in the absence of competitive ligands.

(b) **W5-6A-vWF9 competition assays.** The vWF/ β -galactosidase fusion protein (modified and/or enzymatically digested, see below), was preincubated with ¹²⁵I-W5-6A at 24°C in a solution containing 20 mM Tris, pH 7.3, 200 mM sodium chloride, 0.02% sodium azide, and 1% Carnation powdered milk. After 4 h, the solution was transferred to vWF-coated polystyrene tubes and incubated for 18 h at 4°C. Tubes were then washed three times with TBS and counted in duplicate for bound radiolabeled ¹²⁵I. Percent binding was calculated as stated above.

Modification of vWF9

Reduction and alkylation. The vWF9 fusion protein was diluted in 6M guanidinium chloride and 100 mM Tris, pH 8.5, and reduced under nitrogen at 37°C for 4.5 h with 32.5 mM DTT or under nitrogen at 24°C for 2 h with 12.8 mM 2-mercaptoethanol. Alkylation of the dithiothreitol-reduced sample was achieved with 65 mM iodoacetamide; the reaction was allowed to proceed for 20 min at 24°C in the dark before being quenched with 2-mercaptoethanol. Alternatively, the reduced sample was alkylated at 24°C for 2 h with 74 mM 4-vinyl pyridine, and both reaction mixtures were dialyzed against water and lyophilized.

Enzymatic modifications. Enzymatic cleavage of vWF9 was performed by dilution into or dialysis into 5% ammonium bicarbonate (250–500 μ l total volume). Enzyme was then added (10 μ g of *N*-tosyl-L-phenylalanine chloromethyl ketone- [TPCK] treated trypsin or *N*-tosyl-L-lysine-chloromethyl ketone- [TLCK] treated chymotrypsin) in 10 μ l of 0.001 N HCl; the reaction mixture was flushed with nitrogen and kept at 37°C for 4 h. At that time an additional 10- μ l aliquot of enzyme was added and the digest was allowed to proceed for 16 h under nitrogen at 37°C. The enzyme was then inactivated with a 100-fold molar excess of the appropriate inhibitor (TLCK for trypsin, TPCK for chymotrypsin), and the reaction mixture lyophilized. Proper digestion was confirmed by HPLC.

Results

Inhibition of FVIII/vWF binding by W5-6A. Competitive binding assays between ¹²⁵I-radiolabeled FVIII and vWF-coated polystyrene tubes were carried out for a number of MAbs, and results are conveniently reported as percent inhibition, as calculated above. W5-6A inhibited binding in a concentration-dependent manner, resulting in 52% reduction of FVIII binding at 1 μ g/ml and 90% reduction at 10 μ g/ml (Fig. 1). Maximal ¹²⁵I-radiolabeled FVIII binding (no ligand) was 2,140 \pm 32 cpm and background ¹²⁵I-radiolabeled FVIII (no vWF) was 72 \pm 12 cpm. Specific counts for W5-6A were 1,702 \pm 70 (0.1 μ g), 993 \pm 18 (1 μ g), and 218 \pm 23 cpm (10 μ g). In the same competition assay a second anti-human vWF MAb (W5-5) and an anti-human Factor IX antibody (40D11) both showed minimal inhibitory activity at concentrations up to 10 μ g/ml. Fab fragments were prepared from W5-6A and evaluated in the same competition assay. A similar pattern of concentration-dependent inhibition was seen.

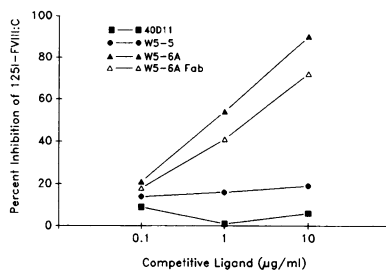


Figure 1. vWF/FVIII competition binding assay. ^{125}I -radiolabeled FVIII was incubated with various ligands and binding to vWF-coated polystyrene tubes measured as counts per minute bound ^{125}I . Percent inhibition is calculated as stated in text. W5-6A (anti-vWF MAb). W5-5 (anti-vWF MAb). 40D11 (anti-factor IX MAb).

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Immunological screening and W5-6A epitope localization. To precisely localize the W5-6A epitope, ~200,000 clones from the vWF fragment library were screened with W5-6A. Immunoreactive clones were detected at a frequency of ~1/1,000, 29 of which were plaque purified. The sequence at both ends of the cDNA inserts was determined, with 16 out of 29 inserts representing unique sequences. vWF inserts ranged from 152 to 458 bp in size and all overlapped in a small region at the amino-terminal portion of the mature vWF polypeptide (Fig. 2). The antigenic site recognized by W5-6A could be mapped to a 19-amino acid segment of vWF spanning residues threonine 78 to threonine 96. Of 20 anti-vWF MAb screened to date, none have localized to the NH_2 terminus of vWF.

vWF- β -galactosidase fusion proteins. Lysogens for two vWF phage clones immunoreactive with W5-6A, vWF7 and vWF9 (see Fig. 2B), were isolated in *E. coli* host strain Y1089, and fusion proteins prepared as previously described (19). Fig.

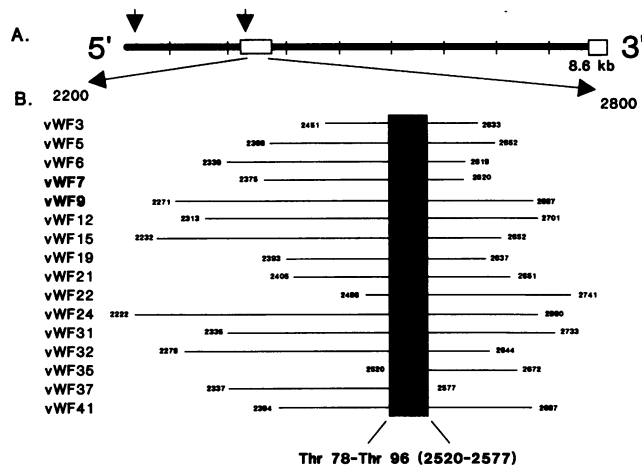


Figure 2. W5-6A epitope localization schema. (A) Full-length vWF cDNA spans ~8.6 kb with a cleavable 22-amino acid signal peptide (first arrow) and a large propolypeptide fragment identical to vWFagII (45) cleaved at nucleotide 2,289 (second arrow). The poly-A tail is represented by the square at the 3' end, and the rectangle represents the magnified portion of vWF represented in B. (B) W5-6A was used to screen the recombinant vWF fragment library, and cDNA inserts from immunoreactive phage sequenced at both ends. All 16 unique phage clones localized to the same region of vWF, with the overlap restricted to nucleotides 2,520–2,577 of full-length vWF cDNA, corresponding to the nonadecapeptide threonine 78–threonine 96 of the mature vWF polypeptide. Two immunoreactive phage clones, vWF7 and vWF9, were used for the preparation of fusion proteins (see text and Fig. 4).

3A shows an 8% SDS-polyacrylamide gel of the crude bacterial lysates stained with Coomassie blue. β -Galactosidase-vWF fusion proteins of the expected size were observed when compared with wild-type β -galactosidase. Western blot analysis with W5-6A (Fig. 3B) confirms immunoreactivity only with the vWF fusion proteins. By similar analysis, W5-5 does not react with vWF fusion proteins vWF7, vWF9, or vWF19.

Characterization of vWF9. To further characterize the antigenic site recognized by W5-6A, the vWF9 fusion protein was incubated with ^{125}I -W5-6A and binding to vWF-coated polystyrene tubes was assayed. As determined by competition assays, undiluted extracts of vWF9 inhibited binding 13-fold when compared with pooled normal plasma (data not shown). However, vWF9 maintained >90% inhibition at a 1,000-fold dilution of the fusion protein (Fig. 4). A control β -galactosidase fusion protein (vWF-C) from another region of vWF (amino acids 859–934) demonstrated no inhibition of binding at an equimolar concentration to undiluted vWF9. Treatment of vWF9 with 0.05% trypsin in two additions for 4 and 16 h completely obliterated its immunoreactivity. In contrast, proteolysis with 2% chymotrypsin had only minimal effect, demonstrating persistent inhibitory activity parallel to that of unmodified vWF9. Reduction and alkylation of vWF9 eliminated inhibition of W5-6A binding at all concentrations tested. Competitive and direct binding assays similar to those described above were performed to assess the ability of vWF9 to either bind directly to FVIII or to competitively inhibit FVIII/vWF binding. The fusion protein showed no activity in either assay (data not shown).

Discussion

vWF is known to be synthesized only in endothelial cells and megakaryocytes (1). In culture, the endothelial cell constitutively secretes predominantly dimeric vWF, although, with appropriate stimuli, an alternative pathway is available for the secretion of high molecular weight multimers (33). Less is known about the regulation of FVIII secretion, although recent evidence suggests that the primary site of synthesis is the hepatocyte (34). Both in vivo and in vitro evidence confirm the necessary role of vWF in maintaining FVIII stability (4–7).

An anti-human FVIII MAb has been described that inhibits binding of FVIII to vWF (35). By performing competitive binding assays with a series of small synthetic polypeptide fragments, the binding site for this antibody was localized to a pentadecapeptide on the FVIII light chain. This region was postulated to be at or near the region on FVIII required for its binding interaction with vWF.

Two prior reports have documented that the amino-terminal portion of mature vWF is involved in FVIII binding. Jorrieux and colleagues (12) and Takahashi and colleagues (11) both confirmed that V-8 protease fragment SPIII (36) (amino acids 1–1,365) retained direct functional FVIII binding activity, whereas no binding was seen with SPI (residues 911–1,365) (12) or SPII (residues 1,366–2,050). Evidence by Foster and colleagues (10) has further localized this binding region to a 35-kD tryptic fragment containing the first 272 amino acids of the mature vWF polypeptide. This monomeric fragment, T4, was able to competitively inhibit FVIII binding in a dose-dependent manner with no inhibition seen by the heterodimeric peptide fragment T2 located toward the carboxyl end of SPIII.

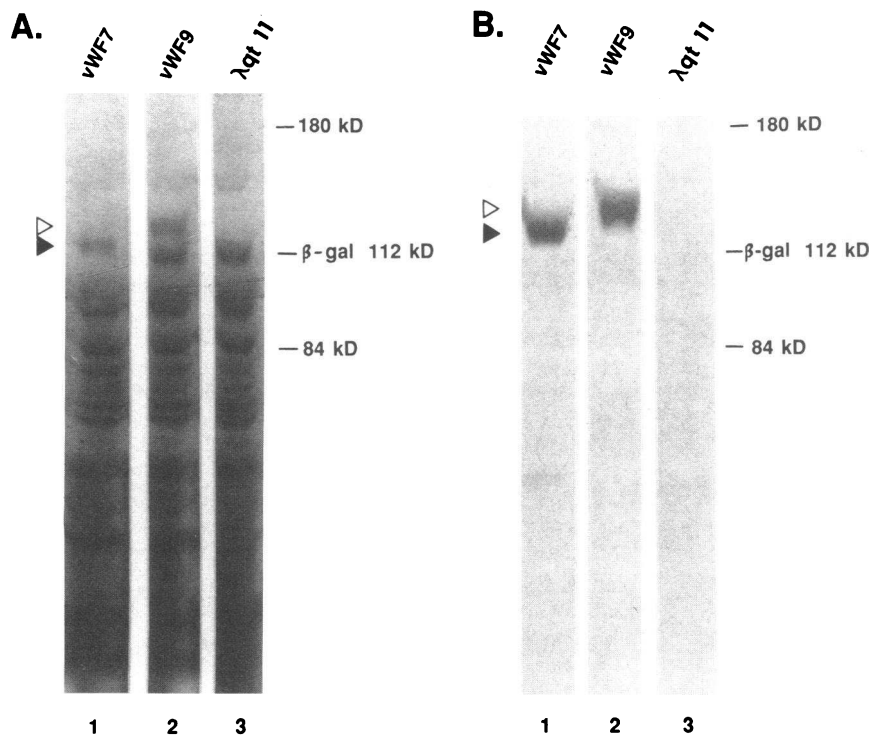


Figure 3. Characterization of vWF- β -galactosidase fusion proteins. (A) Lysogens of two immunoreactive phage clones vWF7 and vWF9 (see Fig. 2) were prepared, and total protein from crude bacterial lysates was electrophoresed on an 8% SDS-PAGE gel and stained with Coomassie blue: vWF7 (lane 1), vWF9 (lane 2), and wild-type λ gt11 (lane 3). Fusion proteins of the expected sizes (vWF7, 130 kD, dark arrow; vWF9, 140 kD, clear arrow) were observed. (B) Immunoblot of a duplicate gel with W5-6A reveals reactivity only with vWF7 and vWF9 fusion proteins and not with β -galactosidase from wild-type λ gt11.

To date, one anti-vWF MAb (C3) has been described that inhibits binding of FVIII to vWF (10). C3 reacted with monomeric T4 on immunoblots, and incompletely inhibited FVIII binding to vWF. Although its epitope was not localized, the partial inhibition of FVIII binding suggested that its antigenic determinant was in proximity to, but did not coincide with the FVIII binding domain on vWF.

To further study the vWF/FVIII interaction, we have identified an anti-human vWF MAb W5-6A that inhibits binding of FVIII to vWF in a solid-phase assay. By screening a recombinant vWF fragment library with W5-6A, we have localized its immune epitope to a short peptide sequence (threo-

nine 78 to threonine 96) at the amino-terminus of the mature vWF polypeptide. Chymotryptic digests of vWF9 should generate a peptide spanning amino acid residues 74–93 (37). These digests maintained the inhibition of 125 I-radiolabeled W5-6A to vWF, thereby possibly refining the antigenic determinant to the hexadecapeptide threonine 78-tryptophan 93. The W5-6A epitope is within SPIII (11, 12) and T4 (10), and could represent a portion of the vWF binding domain responsible for maintenance of the vWF/FVIII interactive site. However, we cannot exclude the possibility that W5-6A is exerting its effect on this binding interaction via remote steric effects or by inducing distant conformational changes within vWF.

The β -galactosidase/vWF fusion protein, vWF9, contains 138 amino acid residues of human vWF, including a number of cysteine residues, four of which are located within the nonadecapeptide (37). Reduction and alkylation of the fusion protein abolishes antigenic reactivity, suggesting that cysteines within this region are necessary in maintaining the W5-6A epitope. This would imply that the epitope is not a simple, linear determinant, but rather includes a conformational component. A number of immune epitopes have been shown to be composed of discrete, noncontinuous peptide segments that are contiguous on the surface as a result of protein folding (38).

Our inability to demonstrate direct or indirect interaction between vWF9 and FVIII may be based on several factors. It is possible that vWF9 does not contain the entire FVIII binding domain. Secondly, steric hindrance by β -galactosidase may interfere with the tertiary folding of vWF9, or physically hinder interaction with the large FVIII protein. Finally, vWF undergoes extensive posttranslational modifications (39) which may be inadequately carried out in this bacterial expression system. The estimation of vWF9 concentration in the extract is ~ 1 mg/ml, as judged by comparison to a known β -galactosidase standard (Sigma Chemical Co., St. Louis, MO) (data not shown). This would be ~ 100 -fold the concentration

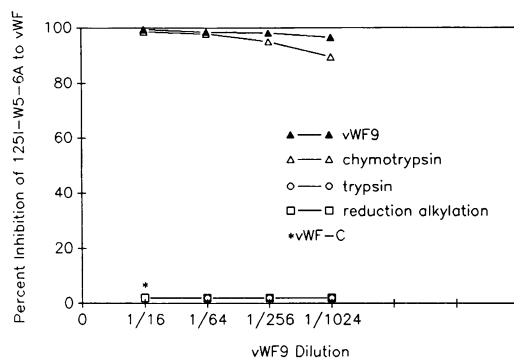


Figure 4. vWF/W5-6A competition assay with vWF9 (untreated or enzymatically modified). Untreated vWF9, chymotrypsin-modified, reduced, and alkylated or trypsinized vWF9 was incubated with 125 I-radiolabeled W5-6A and binding to vWF-coated polystyrene tubes measured as counts per minute bound 125 I. Both untreated vWF9 and chymotrypsin-treated peptide inhibit W5-6A, whereas trypsinization or reduction and alkylation eliminate inhibition of W5-6A binding. A control fusion protein, vWF-C, not from the NH₂-terminus, shows no inhibitory effect.

of plasmatic vWF (10 µg/ml), further suggesting that the above interactive limitations may affect the functional efficacy of the fusion protein.

Acquired or congenital defects in the vWF/FVIII interaction may be implicated in the pathogenesis of several clinical disorders. Montgomery and colleagues (40) have described a pedigree with an autosomal dominant disease manifest as a mild bleeding disorder with abnormal FVIII coagulant activity. They have suggested that this would be consistent with a vWF variant caused by defective binding of FVIII. It would be of interest to determine if the molecular defect in such patients was due to an abnormality in the region of our nonadecapeptide. In addition to the frequent development of FVIII alloantibodies in patients with severe hemophilia (41), spontaneously occurring autoantibodies to FVIII may also occur, leading to a severe hemorrhagic disorder with potentially grave sequelae (42). Gawryl and Hoyer have suggested that a subclass of such antibodies may inactivate FVIII by interfering with the vWF/FVIII interactive site (43). Although the development of allo- or autoantibodies to vWF is a rare occurrence (44), no such antibodies have been described with similar characteristics. Further refinement of the structural requirements involved in the vWF/FVIII interaction may improve our understanding of these clinical disorders, and have implications for the biology of both vWF and FVIII function. Finally, by studying reagents designed to interfere with this binding interaction, clinically useful drugs may eventually be developed for their potential use as anticoagulant agents.

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